Effects of direct renal arterial infusion of bufalin and ouabain in conscious sheep

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1 The presence of an endogenous digitalis-like factor (EDLF) in the plasma of both normal and volume expanded animals is well documented. In this study we have used ouabain and bufalin as pharmacological analogues to mimic the renal effects of EDLF and to investigate whether any interaction occurs between atrial natriuretic factor (ANF) and EDLF.

2 Conscious Na replete sheep with chronically indwelling catheters in the renal artery received renal arterial infusion of ouabain (1000 $\mu\text{g h}^{-1}$) or bufalin (500 $\mu\text{g h}^{-1}$) for 60 min. Renal arterial infusion of bufalin increased sodium excretion ($U_{\text{Na}}V$) from 120 ± 13 to $596 \pm 161 \mu\text{mol min}^{-1}$ after 45 min. Bufalin infusion did not alter glomerular filtration rate (GFR), effective renal plasma flow (ERPF), or lithium clearance. Ouabain infusion increased $U_{\text{Na}}V$ from 124 ± 57 to $764 \pm 123 \mu\text{mol min}^{-1}$ in the first hour after infusion.

3 ANF infusion increased $U_{\text{Na}}V$ from 159 ± 34 to $583 \pm 134 \mu\text{mol min}^{-1}$. When renal arterial bufalin infusion was followed by renal arterial ANF infusion (50 $\mu\text{g h}^{-1}$) $U_{\text{Na}}V$ was increased from 155 ± 31 to $795 \pm 96 \mu\text{mol min}^{-1}$. This increase in $U_{\text{Na}}V$ is approximately equal to the sum of the separate effects of bufalin and ANF.

4 The natriuretic effects of ouabain at pharmacological doses in sheep are confirmed by this study. The data presented here do not support the hypothesis that EDLF sensitizes the kidney to the natriuretic effects of ANF.

Keywords: Natriuretic hormone; natriuretic peptides; atrial; ouabain; bufanolides; sheep kidney

Introduction

The discovery of atrial natriuretic factor (ANF) by de Bold *et al.* (1981) and the subsequent explosion of research on ANF has provided new insight into the physiology of sodium homeostasis. However, many findings remain unexplained and many workers would still argue that a second natriuretic endocrine system must exist (reviewed by de Wardener & Clarkson, 1985; Kramer *et al.*, 1990). Plasma from volume-expanded animals contains a substance which cross reacts with digoxin antibodies and which inhibits (Na^+K^+)-ATPase (Gruber *et al.*, 1980). Although a number of factors with natriuretic activity have been isolated, few have been chemically identified. Exceptions to this are the identification of linoleic (18:2) and oleic (18:1) acids (Tamura *et al.*, 1985) and lysophosphatidylcholine γ -stearol (Tamura *et al.*, 1987) and the recent isolation by Hamlyn *et al.* (1991) of a substance chemically indistinguishable from ouabain in human plasma.

Ouabain has been demonstrated to inhibit renal (Na^+K^+)-ATPase when administered *in vivo* (Toretti *et al.*, 1972), and the inhibition of renal (Na^+K^+)-ATPase has been shown to be correlated with natriuretic responses to ouabain (Hook, 1969). Present evidence suggests that it is highly likely that the endogenous digoxin-like substance (EDLF) is ouabain or a chemically similar substance (Hamlyn *et al.*, 1991).

Bufalin is a cardiodienolide isolated from toad skin (although not from any mammalian source) (Lichstein *et al.*, 1986) which has been demonstrated to have similar effects on the cardiovascular system to ouabain and other cardiac glycosides (Yamahara *et al.*, 1986). Recently Pamnani *et al.* (1991) have reported the renal effects of intravenous infusion of bufalin in the rat. Pamnani *et al.* (1991) found bufalin to be more potent than ouabain.

ANF has been demonstrated to have potent effects on the kidney and cardiovascular system and is generally believed to be an important part of the homeostatic system regulating

sodium and fluid balance. It has become apparent however, that there is a discrepancy between the observed concentration of ANF in plasma and the level of exogenous ANF which is required to elicit a natriuretic response in the isovolaemic animal (Richards *et al.*, 1988; McDougall *et al.*, 1988). In circumstances of volume (McDougall *et al.*, 1991) or sodium loading (Yates *et al.*, 1990), the responsiveness of the kidney to ANF is observed to increase and in sodium depletion to decrease (Yates *et al.*, 1990). It is possible that the presence of EDLF in the plasma of volume-expanded subjects is responsible for the increased responsiveness to ANF during volume expansion. The natriuretic response to ANF may be synergistically enhanced by the presence of an endogenous inhibitor of (Na^+K^+)-ATPase during volume expansion. In addition, Morise *et al.* (1991) have reported a stimulation of ANF secretion and synthesis by ouabain and digoxin, indicating interaction between these substances.

This study examines the renal effects of ouabain and bufalin in conscious sheep. In addition, we examined the natriuretic effect of serial renal arterial infusions of bufalin and ANF to investigate any possible interaction between ANF and an EDLF.

Methods

Mature cross-bred merino ewes (body weight 28–42 kg) were prepared under general anaesthesia with a chronic indwelling cannula in the left renal artery, contralateral nephrectomy, left carotid artery loop and bilateral oophorectomy (Yates *et al.*, 1988).

On the day prior to experiment two polyethylene cannulae were inserted into the right jugular vein, while the bladder was catheterized with a Foley self-retaining catheter.

Animals were offered 800 g lucerne-oaten chaff daily ([Na] 60–100 mmol kg^{-1}) and water *ad libitum*. On the day of experiment an 18 gauge needle was inserted into the exteriorised left carotid artery for direct measurement and

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on-line monitoring of blood pressure via a transducer (Scoggins *et al.*, 1985). Glomerular filtration rate (GFR), effective renal plasma flow (ERPF) and proximal tubular function were measured by established clearance procedures (Whitworth *et al.*, 1978; Yates *et al.*, 1988).

Each animal received an initial injection of [125 I]-pAH ($4 \mu\text{Ci}$), [51 Cr]-EDTA ($20 \mu\text{Ci}$) and Li (3 mmol) followed by continuous infusion of [125 I]-pAH ($4 \mu\text{Ci h}^{-1}$), [51 Cr]-EDTA ($20 \mu\text{Ci h}^{-1}$) and Li (1 mmol h^{-1}). After 60 min equilibration, a renal arterial infusion of 0.15 mol l^{-1} NaCl at 12 ml h^{-1} was started. At this time serial 15 min urine collections were commenced. A 5 ml blood sample was taken at the mid-point of each collection period. After 4 collection periods (60 min) the renal arterial infusion was changed to either bufalin (Sigma; $500 \mu\text{g h}^{-1}$; $n = 5$) or ouabain (Sigma; $1000 \mu\text{g h}^{-1}$; $n = 5$). After a further 4 collection periods (60 min) the bufalin or ouabain infusion was stopped and infusion of 0.15 mol l^{-1} NaCl only continued for 120 min. Urine and plasma samples were analysed for ^{125}I , ^{51}Cr (Cobra 5010 gamma counter, Packard), Na, K (System E2A Beckman), Ca (Autoanalyser IOI), and Li (SpectrAA Varian Techtron).

In a separate series of experiments a 60 min renal arterial bufalin infusion was followed by renal arterial infusion of synthetic human atrial natriuretic factor (99–126) (Peninsula) at $50 \mu\text{g h}^{-1}$ for 60 min ($n = 6$). Alternatively ANF only was infused after 120 min of 0.15 mol l^{-1} NaCl infusion ($n = 5$). ANF and bufalin were infused sequentially in an attempt to allow for the difference in time required for onset of effects, bufalin having no effect for 45 min, ANF having an observable effect within 15 min.

This study has not attempted to produce dose-response curves for the agents examined. It is impractical to administer multiple doses, of agents such as ouabain which have slow onset and turnoff of effect, on a single day. Also other agents such as ANF can produce tachyphylactic responses even after a short period of administration (Yates *et al.*, 1988).

Values are presented as mean $\pm$ standard error of mean (s.e.mean). Statistical analysis was performed using SPSS, by analysis of variance with the Student-Newman-Keuls procedure to identify changes from control at specific time points. Statistical significance was taken at $P < 0.05$.

Results

Sodium excretion ($U_{\text{Na}}V$) increased from a control level of 120 ± 13 to a mean level $485 \pm 157 \mu\text{mol min}^{-1}$ in the 60 min period after bufalin infusion was stopped ($P < 0.05$). $U_{\text{Na}}V$

was not different to control in the second hour following bufalin infusion, i.e. 120 min after the start of bufalin infusion. This contrasts with the effect of ouabain which increased $U_{\text{Na}}V$ from a control of 124 ± 57 to $764 \pm 123 \mu\text{mol min}^{-1}$ ($P < 0.05$) and remained elevated in the second hour post infusion ($647 \pm 135 \mu\text{mol min}^{-1}$) (Figure 1).

Renal arterial infusion of bufalin increased the fractional excretion of Na (FE Na) from $2.9 \pm 0.5\%$ to $11.2 \pm 3.4\%$ in the first hour after bufalin infusion ($P < 0.05$). Ouabain infusion increased the FE Na from $2.9 \pm 0.8\%$ to $26 \pm 4.4\%$ in the first hour post infusion and to $25 \pm 3.1\%$ in the second hour post infusion ($P < 0.05$). The fractional clearance of lithium (C_{Li}/GFR) was not significantly changed following renal arterial infusion of bufalin, although it was increased by ouabain infusion from $35 \pm 4\%$ during the control period to $56 \pm 5\%$ in the first hour post infusion ($P < 0.05$). This increase was sustained during the second hour post-infusion.

Mean arterial pressure (MAP), heart rate (HR) and potassium excretion ($U_{\text{K}}V$) were unchanged by infusion of either bufalin or ouabain. Urine flow (U_{V}) was significantly increased ($P < 0.05$) by both bufalin and ouabain infusions following cessation of infusion (Table 1). The effect of bufalin on $U_{\text{Ca}}V$ was variable between animals although there was no statistically significant effect observed overall.

Bufalin infusion had no effect on glomerular infiltration (GFR) (control $32 \pm 6 \text{ ml min}^{-1}$) or effective renal plasma flow (ERPF) (control: $248 \pm 10 \text{ ml min}^{-1}$). Ouabain infusion

Figure 1 Effect of renal arterial infusion of bufalin ($500 \mu\text{g h}^{-1}$; $n = 5$) ($\diamond$) and ouabain ($1000 \mu\text{g h}^{-1}$; $n = 5$) ($\blacksquare$) on sodium excretion in the conscious sheep. Infusion commenced at time 0 and ceased at 60 min.

Table 1 Effect of renal arterial infusion of ouabain ($1000 \mu\text{g h}^{-1}$) or bufalin ($500 \mu\text{g h}^{-1}$)

		Control	Infusion	1 h Post	2 h Post
$U_{\text{Na}}V$	B	120 (13)	187 (49)	485 (157)*	163 (39)
($\mu\text{mol min}^{-1}$)	O	124 (57)	208 (57)	764 (123)*	647 (135)*
$U_{\text{K}}V$	B	114 (23)	102 (55)	111 (24)	105 (24)
($\mu\text{mol min}^{-1}$)	O	146 (54)	122 (34)	128 (17)	125 (7)
$U_{\text{Ca}}V$	B	0.38 (0.06)	0.76 (0.30)	2.87 (1.41)	0.93 (0.41)
($\mu\text{mol min}^{-1}$)	O	0.85 (0.38)	1.91 (1.26)	7.92 (2.47)*	5.16 (1.65)
GFR	B	32 (6)	33 (7)	30 (4)	29 (4)
(ml min^{-1})	O	27 (4)	27 (2)	21 (1)	18 (2)
ERPF	B	248 (10)	257 (32)	263 (27)	253 (28)
(ml min^{-1})	O	250 (29)	220 (9)	165 (9)*	205 (10)
C_{Li}/GFR	B	30 (2)	29 (2)	35 (5)	28 (3)
(%)	O	35 (4)	32 (4)	56 (5)*	57 (5)*
FE Na	B	2.9 (0.5)	4.4 (1.1)	11.2 (3.4)*	4.2 (0.1)
(%)	O	2.9 (0.8)	6.2 (1.7)	25.9 (4.4)*	25.0 (3.1)*
U_{V}	B	0.9 (0.1)	1.2 (0.2)	3.1 (1.0)*	1.2 (0.3)
(ml min^{-1})	O	1.0 (0.3)	1.5 (0.4)	6.2 (0.9)*	4.6 (0.9)*

* $P < 0.05$. Values are mean (s.e.mean). B: bufalin; O: ouabain; $U_{\text{Na}}V$: sodium excretion; $U_{\text{K}}V$: potassium excretion; $U_{\text{Ca}}V$: calcium excretion; U_{V} : urine flow rate; GFR: glomerular filtration rate; ERPF: effective renal plasma flow; FE Na: fractional sodium excretion; C_{Li}/GFR : fractional lithium clearance.

Figure 2 Comparison of the effects of renal arterial infusion of bufalin ($n = 5$) ($\diamond$), atrial natriuretic factor (ANF, $n = 5$) ($\times$) and ANF + bufalin ($n = 6$) ($\blacksquare$) on sodium excretion. Bufalin was infused between 0 and 60 min, ANF was infused between 60 min and 120 min.

however decreased ERPF ($P < 0.05$) in the first hour post infusion from a control of $250 \pm 29 \text{ ml min}^{-1}$ to $165 \pm 9 \text{ ml min}^{-1}$. ERPF returned towards control levels in the second hour post infusion. GFR was not significantly altered by ouabain infusion.

Renal arterial infusion of bufalin did not produce any increase in $U_{\text{Na}}V$ in the first 45 min of infusion. In comparison, the natriuretic effects of ANF were observed within 15 min of infusion. The natriuretic effects of bufalin and ANF at the doses used in this study were of similar magnitude (maximum rate 596 ± 161 and $583 \pm 134 \text{ } \mu\text{mol min}^{-1}$ respectively). When bufalin and ANF infusions were, combined in the sequential manner described in the methods, a maximum rate of sodium excretion of $795 \pm 96 \text{ } \mu\text{mol min}^{-1}$ was reached (Figure 2).

Discussion

Comparison of the effects of approximately equimolar doses of bufalin and ouabain indicates that some differences may exist in their action on renal function and contrasts with the findings of Pamnani *et al.* (1991) who found bufalin to be the more potent agent. The difference in findings may reflect species variation, or perhaps be a consequence of the route of administration. Ouabain infusion produced larger and more sustained effects on $U_{\text{Na}}V$, $U_{\text{Ca}}V$, U_{v} , FE Na and C_{Li}/GFR than bufalin. In addition, ouabain infusion was associated with a significant decrease in ERPF which was not altered by bufalin infusion. Some similarities in effect were also apparent. In particular the time of onset before the renal effects were observed and the absence of any effect on potassium excretion. This suggests that both substances may be working through the same or similar mechanism(s), and that the difference in effect is primarily due to differences in potency. Alternatively, ouabain may be producing additional systemic effects which enhance the renal effects.

The long duration of action of ouabain is presumably due to the very slow rate of dissociation of ouabain from its binding sites on $(\text{Na}^+\text{K}^+)\text{ATPase}$ (half-time approximately 150 min, Wallick *et al.*, 1980; Forbush, 1983). The much reduced duration of effect of bufalin may be due to its lack of sugar group, since Yoda *et al.* (1975) report that the presence and number of sugar groups on cardiac glycoside molecules determines their dissociation rate. Cardiac aglycones (e.g. ouabagenin) all have high dissociation rates compared to the cardiac glycosides. The shorter duration of effect of bufalin may make it preferable to ouabain as a

pharmacological probe for investigation of the *in vivo* effects of EDLF.

The fractional clearance of Li is an indicator of proximal fractional fluid delivery (Koomans & Dorhout Mees, 1989; Leyssac, 1989). If bufalin or ouabain were inhibiting $(\text{Na}^+\text{K}^+)\text{ATPase}$ in the proximal tubule, they would be expected to cause an increase in the fractional clearance of Li. No significant effect of bufalin infusion was observed on this parameter despite a large increase in the fractional excretion of Na. Ouabain however did produce an increase in fractional Li clearance. Although bufalin had no significant effect on C_{Li}/GFR , when proximal Na reabsorption is calculated from the Li data (Fractional proximal Na reabsorption = $(\text{GFR} \cdot P_{\text{Li}} - U_{\text{Li}}V)/(\text{GFR} \cdot P_{\text{Li}})$) it is clear that the effect of bufalin on sodium excretion is mediated almost entirely through the proximal tubule. Similarly the natriuretic effect of ouabain is proximally mediated. Doucet & Barlet (1986) have reported binding sites for ouabain at two different sites on the renal tubule with different affinities. Binding sites on the collecting duct were found to have a higher affinity than those identified on the proximal tubule. The distal binding sites do not appear to contribute significantly to the natriuretic effect of ouabain or bufalin. The greater effect of ouabain on fractional Li clearance at a similar infusion rate suggests that either ouabain has a higher affinity for the proximal species of $(\text{Na}^+\text{K}^+)\text{ATPase}$ than does bufalin or else clearance of ouabain is sufficiently slow to allow a significantly higher plasma concentration of ouabain to accumulate. At higher rates of infusion bufalin might then be expected to cause a significant increase in fractional Li clearance.

Hamlyn *et al.* (1991) have reported the isolation of ouabain or an isomer of ouabain from human plasma. Further they have developed an immunoassay for ouabain with which this substance strongly cross reacts. Hamlyn *et al.* (1991) propose that EDLF is ouabain or an isomer of it. This finding is not easily reconcilable with the very low cross-reactivity of some digoxin antibodies which have detected 'EDLF' previously (Gruber *et al.*, 1980). The possibility does exist that Hamlyn *et al.* (1991) have isolated a substance consisting of ouabagenin and a sugar such as L-fucose. The cross-reactivity of the EDLF isolated by Hamlyn *et al.* (1991) with digoxin antibodies has not been reported. Using this ouabain immunoassay they have measured plasma EDLF concentrations of $138 \pm 43 \text{ pmol l}^{-1}$ in man, and $80 \pm 18 \text{ pmol l}^{-1}$ in rats. Rats with mineralocorticoid induced hypertension had an elevated plasma concentration of $975 \pm 79 \text{ pmol l}^{-1}$. The dose of ouabain used in the present study should produce a plasma concentration in the renal artery some 1000 times greater than the basal levels. Unpublished observations in the sheep revealed no natriuretic effect of ouabain at a dose 10 fold lower than the dose used here (Yates & McDougall, unpublished). It is unlikely that ouabain would circulate at sufficiently high concentrations to exert a direct natriuretic effect. If EDLF does have role in Na homeostasis, then the endogenous isomer must be significantly more active. Alternatively it may have an autocrine role within the kidney, or it is interacting with other physiological systems to increase greatly the magnitude of its effects. This does not mean that EDLF does not have direct effects on the vasculature or on other physiological systems and indeed de Wardener (1991) has suggested that EDLF is more important in cardiovascular control than in control of sodium homeostasis.

We have demonstrated unequivocally the alteration in renal response to ANF following alteration in sodium and/or volume status (Yates *et al.*, 1990; McDougall *et al.*, 1989; McDougall *et al.*, 1991) and by treatment with specific hormones (McDougall *et al.*, 1989). One possible mechanism to explain the alteration in renal sensitivity is that EDLF released during hypervolaemic or hypernatremic states was sensitizing the kidney to ANF. If this were the case it would have been expected that bufalin would markedly enhance the

natriuretic response to ANF. This was not observed and the additive nature of the combined response reinforces the different mechanisms of action of ANF and bufalin in the kidney.

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