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EFFECT OF BUFALIN AND PREGNANES ON VASOREACTIVITY OF HUMAN RESISTANCE ARTERIES

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Endogenous Na/K ATPase inhibitory activity has been implicated in salt and water homeostasis in mammals and amphibians. Recent interest has focused on endogenous cardiac glycosides, some progesterone derivatives (pregnanes) and the amphibian bufodienolides. This study has examined the effects of non-planar and planar pregnanes and the bufodienolide bufalin on vasoreactivity of human resistance arteries. Bufalin and a non-planar pregnane caused concentration-dependent potentiation of the tone of submaximally pre-contracted arteries and inhibited endothelium-dependent relaxation, whereas a planar pregnane affected neither response. The relative potency of the compounds studied suggest the results do not simply reflect degrees of Na/K ATPase inhibition. The active compounds may be important in the regulation of vascular tone.

The presence of a highly specific receptor for cardiac glycosides, the ubiquitous enzyme Na/K ATPase, has for many years stimulated the quest for a naturally occurring ligand with cardiac-glycoside like activity (1). Considerable evidence from bioassays has suggested the presence of an endogenous ligand which has been implicated in the regulation of the extracellular fluid volume and has thus been ascribed the role of a natriuretic hormone (2). Moreover, this endogenous cardiac glycoside-like compound has also been implicated in the aetiology of essential hypertension (3) and pregnancy-induced hypertension (4), since inhibition of the sodium pump by reportedly high glycoside-like activity in the serum could theoretically lead to vasoconstriction (5). Until recently no single compound had been successfully characterised, but the remarkable discovery in human serum of a compound structurally indistinguishable from ouabain now strongly supports the suggestion that cardiac glycosides or similar compounds may play a role as physiological modulators of Na/K ATPase activity (6).

The discovery of endogenous cardiotonic steroids is not unique in the animal kingdom since compounds with cardiac glycoside-like activity and structure (the bufodienolides) have been

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isolated from the skin, venom and plasma of the *bufonid* toad. These compounds play an important role in sodium and water homeostasis in this species which alternate between a fresh water and sea *habitus*. The bufodienolides have also been shown to have pressor activity in mammals (7).

In recent years, derivatives of progesterone (pregnanes) bearing structural and functional similarities to cardiac glycosides have attracted much interest (8). Whilst some pregnanes are synthetic compounds, others are likely to be present *in vivo* and they are therefore strong candidates for endogenous modulators of Na/K ATPase activity. Structurally, pregnanes may be either planar (*trans* configuration e.g. chlormadinone acetate, CMA) or non-planar (*cis* configuration e.g. 3β-((L-rhamnosyl)oxy)-2Oβ-14β-diOH pregnane, JT-153) whereas, in contrast, the steroid nucleus of the plant derived cardiac glycosides, is non-planar.

We have previously shown that ouabain potentiates noradrenaline-induced tension and inhibits endothelium-dependent relaxation of isolated human resistance arteries (9,10). In this study we have determined whether these effects of ouabain are shared by the bufodienolide, bufalin, and by two structurally different (one planar, one non-planar) pregnanes.

MATERIALS AND METHODS

Preparation of arterial rings: Human subcutaneous resistance arteries (n=69), mean internal diameter 236 ± 8 μm were obtained from biopsies of anterior abdominal wall fat taken during routine abdominal surgery on 41 normotensive patients receiving no medication (26 females, mean age 42 ± 2 years, mean blood pressure 121/78 ± 1/1 mmHg). The arteries were dissected free from connective tissue and were mounted as a ring preparation on a Mulvany-Halpern myograph capable of measuring isometric tension (11). The arteries were bathed in physiological salt solution (PSS) at pH 7.4 at 37°C and bubbled with 5% CO_2 in O_2. The study was approved by the Ethical Committee of St Thomas' Hospital and informed consent was given by each patient from whom subcutaneous fat was harvested.

Following five stretches the passive tension-internal circumference characteristics of the arteries were determined (11). The arteries were stretched to achieve an internal circumference equivalent to 90% of that which they would have had when relaxed *in situ* under a transmural pressure of 100 mmHg (determined by Laplace's relationship). The maximum active tension for the minimum resting tension is developed at approximately this circumference (11). To assess their contractile response, the arteries were then contracted for 2 minutes every 10 minutes on 5 occasions using the following protocol. The first, second and fifth contractions were produced with noradrenaline 5 μM in 125 mM potassium solution (NAK); the third contraction with noradrenaline (NA) 5 μM in PSS and the fourth with 125 mM potassium solution. Any artery failing to produce a maximum active tension equivalent to a pressure of 100 mmHg on the final NAK response (see later) was rejected.

Solutions and Chemicals: The arteries were bathed in a physiological salt solution (PSS) containing, NaCl 119 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.17 mM, $NaHCO_3$ 25 mm, KH_2PO_4 1.18 mM, EDTA 0.026 mM, glucose 5.5 mM, of pH 7.4 at 37°C, bubbled with 5% CO_2 in O_2. The potassium solution was made by equimolar substitution of KCl for NaCl in PSS, resulting in a final K^+ concentration of 125 mM. Noradrenaline (Winthrop, Guildford, UK),

acetylcholine (Sigma, Poole, UK), chlormadinone acetate (CMA, Sigma, Poole, UK), JT-153 (483 Daltons, prepared by JF Templeton), bufalin (Sigma, Poole, UK). Bufalin and both pregnanes tested were dissolved first in absolute alcohol before being diluted with water. The maximum concentration of ethanol in the final solution was 0.1%.

Statistics: Results are expressed as means ± standard error (SEM). Potentiation of contractions are expressed as a percentage of the response to the final NAK response in order to take into account variation in arterial diameter. Relaxation is expressed as a percentage of the pre-contraction with a figure of 100% corresponding to complete relaxation.

To analyse sensitivity of the response, a Hill plot was constructed from the concentration response for the individual arteries and the EC_{50} was calculated. This value is expressed as the pEC_{50}, where $pEC_{50} = -\log[EC_{50}\ (M)]$. Differences between means were assessed by ANOVA or two-tailed unpaired t-tests. The criterion of statistical significance was taken as $p<0.05$.

Effect of compounds on pre-contracted resistance arteries: Noradrenaline (NA) concentration response curves ([NA] from 0.02 to 10 μM) were constructed for the arteries used and the concentration producing approximately 50% of maximum contraction was calculated. The arteries were then submaximally contracted with this concentration of NA in the presence of 3 μM cocaine (to exclude any effect due to the inhibition of pre-synaptic uptake of NA resulting from inhibition of Na/K ATPase). The initial contraction reached a plateau after 4 minutes and subsequent changes in tension were recorded every 4 minutes for a further 16 minutes. The experiment was then repeated but on this occasion after 4 minutes the arteries were sequentially exposed to CMA (1 nM to 10 μM) or JT-153 (1 nM to 1 μM) or bufalin (1 nM to 10 μM) for 4 minutes at each concentration.

Effect of compounds on endothelium-dependent relaxation: Acetylcholine (ACh) concentration responses were constructed by the cumulative addition of increasing concentrations of ACh (1 nM - 0.1 mM) in PSS at 2 minute intervals to resistance arteries pre-contracted with 3 μM NA for 3 minutes.

Five sequential ACh concentration responses were performed with a 15 minute washout period between each response. Following the first of these responses, arteries were exposed to CMA (1 μM), JT-153 (10 nM or 1 μM), bufalin (1 nM or 10 nM) or PSS (control) thereafter. The test substance was included with the second NA stimulation and was present in the organ bath thereafter.

RESULTS

Effect of compounds on pre-contracted resistance arteries

CMA (from 1 nm to 10 μM) did not potentiate the tone of submaximally pre-contracted arteries (n=6). In contrast, JT-153 caused a concentration-dependent increase in tension with a maximum response of 27.1 ± 3.7% and a pEC_{50} of 6.56 ± 0.19 (n=7). Bufalin produced still greater potentiation with a maximum response of 57.1 ± 5.2% (vs. JT-153: $p<0.01$) and pEC_{50} of 7.45 ± 0.21 (vs. JT-153: $p<0.01$), n=8. See Figure 1.

Effect of compounds on endothelium-dependent relaxation

Prolonged incubation with 1 μM CMA (n=6) did not inhibit ACh-induced relaxation whereas similar exposure to 1 μM JT-153 (n=6) caused marked and rapid inhibition of this

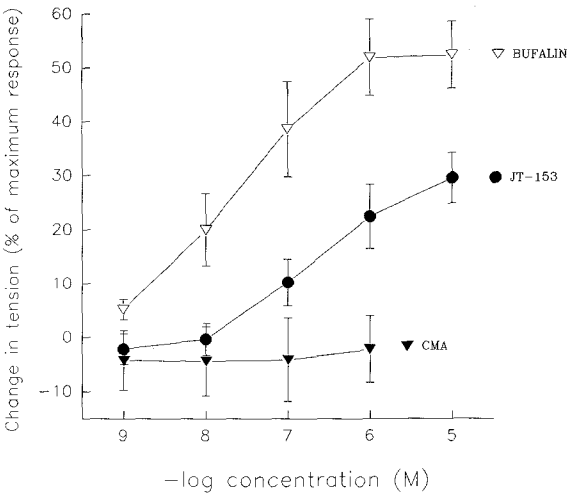

Figure 1
Cumulative concentration responses to bufalin, JT-153 and CMA for human resistance arteries submaximally pre-contracted with noradrenaline.

response. See Figure 2. Absence of inhibition following incubation with 10 nM JT-153 (n=7) suggests that this is a concentration-dependent response.

Prolonged incubation in 1 nM bufalin (n=6) resulted in delayed inhibition of ACh-induced relaxation, whereas 10 nM bufalin (n=11) caused rapid and profound inhibition of this response. See Figure 2.

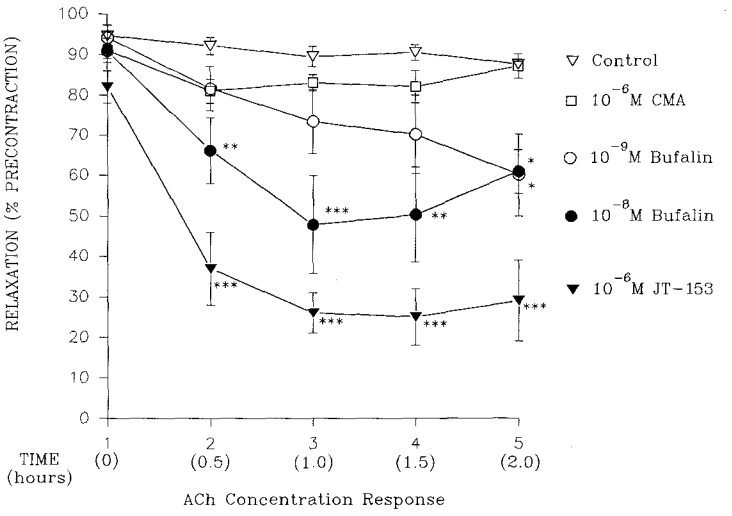

Figure 2
Following an initial acetylcholine concentration response, four further responses were performed at 30 minute intervals with arteries incubated in PSS, 1 μM CMA, 1 μM JT - 153, 10 nM bufalin or 1 nM bufalin. This figure illustrates maximum relaxation of each response. Asterisks refer to significant differences between treated arteries and control (PSS).
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The maximum relaxation achieved within the control group (n=8) did not change significantly during the course of the experiment. See Figure 2.

DISCUSSION

The principal observation of this study is that Na/K ATPase inhibitory activity due to non-planar compounds is associated with pressor activity *in vitro*. In particular, both the non-planar pregnane JT-153 and bufalin were shown to cause concentration-dependent potentiation of tone of submaximally pre-contracted human resistance arteries. In contrast, the planar pregnane CMA, which has previously been shown to inhibit Na/K ATPase at similar concentrations (12), failed to potentiate tone. Although potentiation may be due to depolarisation of vascular smooth muscle arising from inhibition of Na/K ATPase, an alternative mechanism could involve reverse mode Na/Ca exchange (i.e. Na^+ out, Ca^{2+} in) due to elevation of intracellular sodium (4,9). Substantial evidence exists to support an important role for the latter mechanism in vascular smooth muscle cells from conduit arteries (13).

Similarly, whereas CMA failed to inhibit ACh-induced relaxation, both JT-153 and bufalin caused concentration-dependent inhibition. A previous study from this laboratory has suggested that the loss of endothelium-dependent relaxation following Na/K ATPase inhibition is due to an effect on the synthesis or release of nitric oxide (NO) rather than on its effector pathway or indeed on the prostanoid pathway (10). Synthesis and release of NO are both calcium-dependent processes (14,15) but, in contrast to vascular smooth muscle cells, endothelial cell depolarisation (for example resulting from Na/K ATPase inhibition) leads to a fall in intracellular calcium and not to a rise (15).

The relative responses to JT-153 and bufalin are preserved in each set of experiments with CMA having no demonstrable effect. However, it is unlikely that these differences are a simple reflection of graded Na/K ATPase inhibition. This can be assumed from the similar potencies of CMA and JT-153 in effecting inhibition of Na/K ATPase (IC_{50}: CMA 300 nM [7], JT-153 75 nM [16]). In contrast the response to bufalin (IC_{50}: 10 nM [17]) compared with JT-153 may reflect the greater potency. There is no immediate explanation for the observed lack of effect of CMA and it must be deduced that the biological activity of the pregnanes does not depend entirely on inhibition of Na/K ATPase.

Our observations are consistent with earlier findings in which a difference in inotropic activity between planar and non-planar pregnanes was noted with only the latter demonstrating positive inotropism (12). We have previously hypothesised that progesterone and its pregnane derivatives may have two effector mechanisms (12,7). Firstly, inhibition of membrane-bound

Na/K ATPase may lead to reversal of the important Na/Ca exchanger with resultant accumulation of intracellular calcium leading to positive inotropism. Secondly, the planar pregnanes may occupy an as yet undefined intracellular site which prevents intracellular accumulation of calcium, and thus accounts for their negatively inotropic action. This hypothesis concludes by suggesting that planar pregnanes, such as CMA, have a substantially greater affinity than non-planar pregnanes for the intracellular site. The findings of this study are consistent with a bimodal mechanism of action being present in both vascular smooth muscle and endothelial cells.

These data may have relevance to the demonstration of potent Na/K ATPase inhibitory activity in a fraction of neonatal serum which appears close to progesterone after HPLC fractionation (18). The responsible compound is as yet unidentified but theoretically could be a pregnane.

In summary, this study describes two mechanisms by which Na/K ATPase inhibition *in vivo* might lead to raised peripheral vascular resistance. It emphasises the relevance of the recent identification of several endogenous inhibitors in both mammals and amphibians, in health and disease states.

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