Br. J. Pharmac. (1985), **86**, 151–161

Effects of noradrenaline, vasopressin and angiotensin on the Na-K pump in rat isolated liver cells

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1 The effects of noradenaline (via α_1-adrenoceptors) and of the peptidic hormones vasopressin and angiotensin on the Na-K pump have been studied in rat isolated liver cells.

2 The three hormones increased the cytosolic Ca concentration, stimulated the Na-K pump and decreased the internal Na concentration of the cells. The effects were dose-dependent and were blocked by the corresponding antagonists.

3 The simultaneous addition of maximal doses of noradrenaline and angiotensin or vasopressin were not additive suggesting that the hormones use a common mechanism to stimulate the carrier.

4 Incubating the cells in Ca-free medium for long periods (Ca-depletion) increased the Na-K pump activity and reduced the stimulatory action of vasopressin, angiotensin and noradrenaline.

5 The effect of the Ca indicator quin2, used as an intracellular Ca chelator, was also studied. The cells were loaded with a maximal concentration of [^{3}H]-quin2 acetoxymethyl ester in the presence of external Ca for 6 min. The final cell content was 3.1 nmol quin2 mg^{-1} cell dry wt. In these cells the cytosolic Ca, as monitored from the fluorescence emission of the indicator, was about 200 nM and Na-K pump activity was normal and the cells remained responsive to the three hormones.

6 Loading the cells with quin2 in the absence of external Ca reduced the $[Ca]_i$ from 200 nM to about 40 nM and increased the Na-K pump activity but not as a result of a rise in internal Na concentration. In addition, the rat hepatocytes were no longer sensitive to the hormones.

7 It is proposed that Ca inhibits the Na-K pump by binding the internal sites and that vasopressin, angiotensin and noradrenaline stimulate the carrier by interfering with the inhibitory Ca sites.

Introduction

Noradrenaline (via α_1-adrenoceptors) stimulates glycogenolysis in mammalian liver by increasing the concentration of cytosolic Ca (Williamson *et al.*, 1981; Exton, 1981; Claret & Binet, 1984). In a number of species the increased $[Ca]_i$ promotes a net loss of K and a hyperpolarization, resulting from activation of Ca-dependent K channels (see the review by Jenkinson *et al.*, 1983) occurs. In contrast, in the perfused liver of the rat (Northrop, 1968; Friedmann & Park, 1968; Jakob & Diem, 1975; Becker & Jakob, 1982; Reinhart *et al.*, 1984; Storm *et al.*, 1984) and in rat isolated hepatocytes, noradrenaline initiates a transient net uptake of K (Burgess *et al.*, 1981; Berthon *et al.*, 1983) and a net loss of intracellular Na (Capiod *et al.*, 1982; Berthon *et al.*, 1983). This action has been ascribed to a primary α_1-adrenoceptor mediated stimulation of the Na-K pump.

The first aim of the present work was to extend our study of the action of noradrenaline on the Na-K pump and to examine the effects of other hormones, such as vasopressin and angiotensin, which in common with noradrenaline are known to promote glycogenolysis by increasing $[Ca]_i$ in rat isolated liver cells (Charest *et al.*, 1983; Berthon *et al.*, 1984). The second aim was to investigate the role of Ca in the cell response to these three hormones. This was prompted by the previous finding that the stimulatory action of noradrenaline is altered in rat liver cells depleted of their Ca by long incubation periods in low-Ca media (Capiod *et al.*, 1982; Berthon *et al.*, 1983). External Ca was reduced by EGTA and cytosolic Ca either by long incubation periods in low-Ca media or by using the intracellular Ca chelator quin2 (Tsien *et al.*, 1982). We will show that vasopressin and angiotensin stimulate

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the Na-K pump by using the same coupling mechanism as noradrenaline. Lowering $[Ca]_i$ stimulated the carrier to a level close to that initiated by the three hormones and abolished their stimulatory action. It is proposed that: (1) resting $[Ca]_i$ may serve as a regulator of the Na-K pump by binding to inhibitory sites located on the internal part of the protein; (2) noradrenaline, vasopressin and angiotensin may decrease the affinity (and/or number) of inhibitory sites for Ca.

Methods

Preparation of dispersed liver cells of the rat

Parenchymal cells were isolated from the liver of fed female Wistar rats using the collagenase method as described previously (Berthon *et al.*, 1983) and resuspended in Eagle's solution containing (mM): NaCl 116, KCl 5.4, $CaCl_2$ 1.8, $MgSO_4$ 0.8, NaH_2PO_4 0.96, $NaHCO_3$ 25 and (mg l^{-1}): L-glutamine 292, phenol red 10, glucose 1000. The medium was supplemented with vitamins, aminoacids and bovine albumin (2%). The pH was maintained by passing 5% CO_2 in O_2 over the surface of the cell suspension and the flask was shaken at 120 strokes min^{-1} at 37°C. After equilibration (30 min), the cells were resuspended in a fresh identical solution supplemented with 5 μM propranolol (to block β-adrenoceptors) for the duration of the experiments (cell concentration, ca.10 mg dry wt.ml^{-1}).

Internal Na concentration

Four millilitres from the incubation flask were spun down (50 g for 2 min) and resuspended in 10 ml of Eagle's solution (cell concentration ca.4 mg dry wt.ml^{-1}) containing ^{22}Na (1 μCi l^{-1}). The suspension was stirred and pH maintained by circulating 5% CO_2 in O_2 at 37°C. After 15–20 min ^{22}Na uptake reached a maximal and constant (over 90 min) value. The time constant of ^{22}Na uptake was 4.50 ± 0.32 min ($n = 5$) in agreement with that found in perfused liver (Claret & Mazet, 1972). Hence an incubation period of 30 min with ^{22}Na was routinely used as it was sufficient for complete Na exchange. At the times indicated in Figure 4, 1 ml samples of the cell suspension were centrifuged 3 times at 50 *g* for 90 s with 5 ml of the ice-cold 'washing solution' containing 150 mM NaCl and 2 mM EGTA (pH 7.4). All the steps were performed at 1°C. The supernatants were discarded and the pellets dispersed in 1 ml of deionized water. Two samples (100 μl) were counted for ^{22}Na and K measured by flame spectrophotometry and one sample (700 μl) was used for estimating cell dry weight. Ion concentrations were calculated from the cell water content determined from the difference between the wet and dry weights of the cells as indicated in Berthon *et al.* (1980). Samples (1 ml) of cell suspension containing [^{3}H]-inulin for estimating the extracellular water trapped in the pellet were centrifuged at 5000 *g* for 1 min. The cell water content was 2.31 ± 0.11 μl mg^{-1} dry wt.($n = 24$).

Measurement of unidirectional ^{86}Rb influx

The Na-K pump activity was determined from the unidirectional influx of ^{86}Rb sensitive to ouabain (1 mM). In rat isolated liver cells, it has been shown that Rb behaves in a very similar way to K ions and can be used as a convenient substitue for K ions (Weiss & Putney, 1978; Ihlenfedt, 1981; Fehlmann & Freychet, 1981; Berthon *et al.*, 1983). Routinely, a 1 ml sample (ca.10 mg dry wt.ml^{-1}) of the flask cell suspension was centrifuged and the pellet resuspended in 1 ml of Eagle's medium (control cells) or in 1 ml of Eagle's solution containing ouabain (1 mM). The tubes were equilibrated at 37°C with 5% CO_2 and 95% O_2. Six minutes later, ^{86}Rb was added for 90 s: at that time, 100 μl samples were removed, then centrifuged in Eppendorf tubes containing 200 μl of di-*n*-butylphthalate (density: 1.045 g ml^{-1}) and 1 ml of 'washing solution', all at 1°C. The pellet was dispersed in 100 μl distilled water and counted for ^{86}Rb.

Cell Ca depletion

The hepatocytes (ca.4 mg ml^{-1}) were incubated either in the same supplemented Eagle's medium (control cells) or in a medium containing no added Ca and 200 μM EGTA (depleted cells) for 90 to 150 min. The unidirectional ^{86}Rb influx, ion contents and cell dry weight were then determined as indicated above.

Quin2 cell loading

The method used to load the cells was based on that described by Berthon *et al.* (1984). The cells (2 mg dry wt.ml^{-1}) were incubated in a supplemented Eagle's medium containing [^{3}H]-quin2-tetra (acetoxymethyl) ester ([^{3}H]-quin2/AM; 75 μM) for 6 min, either in the presence of Ca (1.8 mM) or in the absence of added Ca and supplemented with 200 μM EGTA. The cell samples (100 μl) were washed three times and [^{3}H]-quin2/AM uptake determined by scintillation spectrophotometry. The ouabain-sensitive ^{86}Rb uptake, Na and K contents and cell dry weight were measured as indicated above.

$[Ca]_i$ detection

The cells (2 mg ml^{-1}) were preloaded with quin2/AM (75 μM) for 6 min, then washed twice in Eagle's medium containing no vitamins, amino acids or albumin, centrifuged at 50 *g* for 1 min and transferred

Table 1 The effect of Ca-mobilizing hormones and (−)-isoprenaline on the total and ouabain-sensitive ^{86}Rb influx in rat isolated hepatocytes

	Total influx (nmol $mg^{-1}min^{-1}$)	*Ouabain-resistant influx* (nmol $mg^{-1}min^{-1}$)	*Ouabain-sensitive influx* (nmol $mg^{-1}min^{-1}$)
Controls	6.56 ± 0.17	1.49 ± 0.05	5.09 ± 0.16
NA	9.04 ± 0.17**		7.56 ± 0.17**
Controls	7.23 ± 0.59	1.65 ± 0.08	5.58 ± 0.52
VP	10.51 ± 0.84**		8.87 ± 0.77**
Controls	6.45 ± 0.43	1.40 ± 0.03	5.04 ± 0.42
AII	9.02 ± 0.45**		7.62 ± 0.44**
Controls	8.71 ± 0.49	1.82 ± 0.09	6.88 ± 0.37
Iso	10.08 ± 0.69		8.24 ± 0.64

The influx was measured from the ^{86}Rb uptake of cells incubated with the tracer and (−)-noradrenaline (NA; 1μM, in the presence of 5 μM propranolol), angiotensin (AII; 1nM), vasopressin (VP; 10nM) or (−)-isoprenaline (Iso; 1 M) for 90 s. When used, ouabain (1mM) was added 6 min before the hormones. Means ± s.e.mean of 14 to 27 experiments are shown. **$P<0.01$, significantly different from paired controls (t test).

into the cuvette of a Jobin-Yvon JY3D spectrofluorimeter under continuous magnetic agitation and aeration at 37°C. Fluorescence (excitation at 342 nm and emission at 492 nm) was recorded on a potentiometric recorder. At the end of each experiment, $[Ca]_i$ was calibrated by the successive additions of 4 μM digitonin to equilibrate external Ca and quin2 (free tetraanion of quin2/AM) and addition of Ca or of excess EGTA to obtain maximal and minimal fluorescences, respectively (for other details, see Berthon *et al.*, 1984).

Materials

Radioisotopes ^{86}Rb was supplied by I.E.R., ^{22}Na, [^{3}H]-inulin and [^{3}H]-quin2 acetoxymethyl ester by Amersham (England).

Other materials We are most grateful to Smith, Kline and French for dibenzyline (phenoxybenzamine). The following were obtained from Sigma: (−)-noradrenaline bitartrate; (±)-propranolol hydrochloride; arginine vasopressin; isoleucine angiotensin II; albumin (fraction V); (−)-isoprenaline. Collagenase and strophantin-G (ouabain) were supplied by Boerhinger and BME vitamin solution and aminoacid solution with L-glutamine by Gibco Europe. Quin2/AM was purchased from Lancaster Synthesis (Morecambe). We thank S. Jard and G. Guillon for providing us with the angiotensin and vasopressin antagonists. All chemicals and drugs were of analytical reagent grade.

Table 2 Lack of effect of (A) (−)-noradrenaline, vasopressin, angiotensin, (B) long-term incubation in low-Ca media and (C) intracellular quin2 on the ouabain-resistant ^{86}Rb influx in rat hepatocytes

		Ouabain-resistant ^{86}Rb influx (nmol $mg^{-1}min^{-1}$)
A	Control	1.45 ± 0.05
	NA	1.53 ± 0.06
	VP	1.28 ± 0.06
	AII	1.51 ± 0.06
B	Control	1.71 ± 0.10
	EGTA	2.10 ± 0.17
C	Control	1.36 ± 0.14
	Quin2/AM	1.82 ± 0.16

(A) The effects of (−)-noradrenaline (NA; 1μM in the presence of 5μM propranolol), vasopressin (VP; 10nM) or angiotensin (AII; 1nM) on ^{86}Rb influx were measured in the presence of ouabain (1mM).
(B) Rat isolated hepatocytes were incubated in control medium (Ca, 1.8 mM) or in Ca-free medium supplemented with EGTA (200 μM) for 90 to 150 min and ^{86}Rb influx measured as in (A).
(C) The cells were equilibrated either in control medium containing the solvent DMSO (5 μl) or in Ca-free medium containing quin2/AM 875 μM in 5 μl DMSO) and EGTA (200 μM) for 6 min; ^{86}Rb influx was measured as in (A).
Data shown are means ± s.e.mean of 5 to 6 experiments.

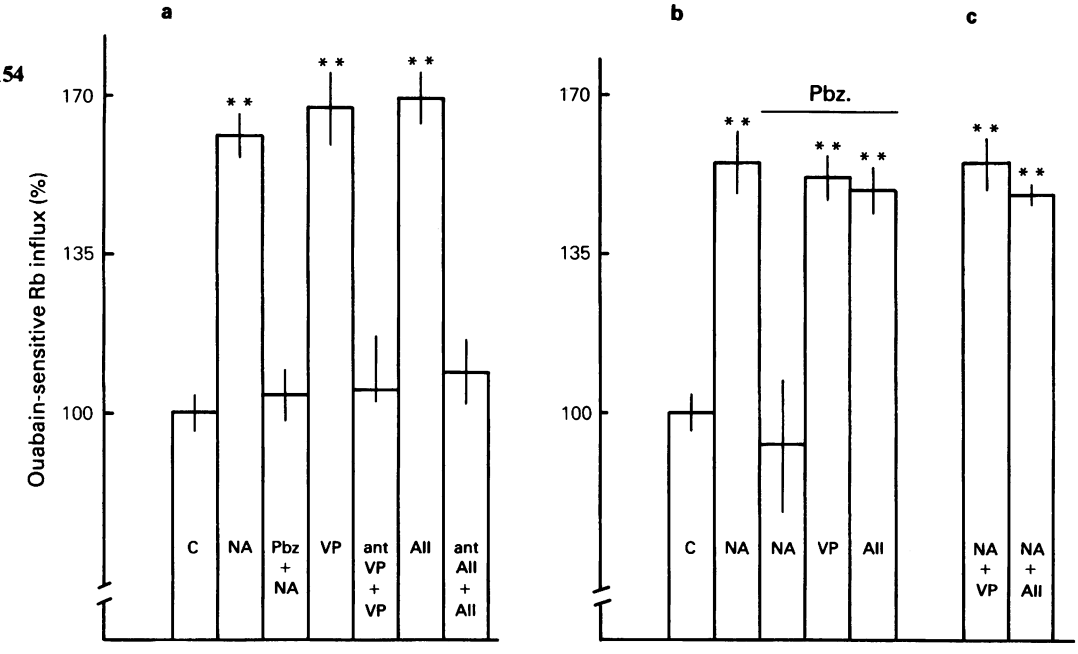

Figure 1 (a) Application of (−)-noradrenaline (NA; 1 μM, in the presence of 5 μM propranolol), angiotensin (AII; 1 nM) and vasopressin (VP; 10 nM) stimulates the ouabain-sensitive ^{86}Rb influx in rat isolated liver cells (C, controls). The antagonists phenoxybenzamine (Pbz; 5μM), Sar-Ile-angiotensin (ant AII; 50 nM) and cyclo-(tyr)-eth-vasopressin (ant VP; 50 nM) applied 6 min beforehand abolished the action of the corresponding hormone. (b) Lack of effect of the α-adrenoceptor antagonist phenoxybenzamine (5 μM) on the response to vasopressin (10 nM) and angiotensin (1 nM). When used phenoxybenzamine was added 6 min before the hormones. (c) Shows that simultaneous addition of maximal concentrations of noradrenaline (1 μM) and vasopressin (10 nM) or angiotensin (1 nM) did not produce an additive response. The stimulation of the Na-K pump is expressed as percentage of the control value (control (a) = 6.03 ± 0.21; control (b) = 5.73 ± 0.17 nmol ^{86}Rb mg^{-1}min^{-1}). Each column shows the mean and vertical lines s.e.mean of 5 to 14 experiments. $^{**}P < 0.01$, significantly different from paired controls (*t* test).

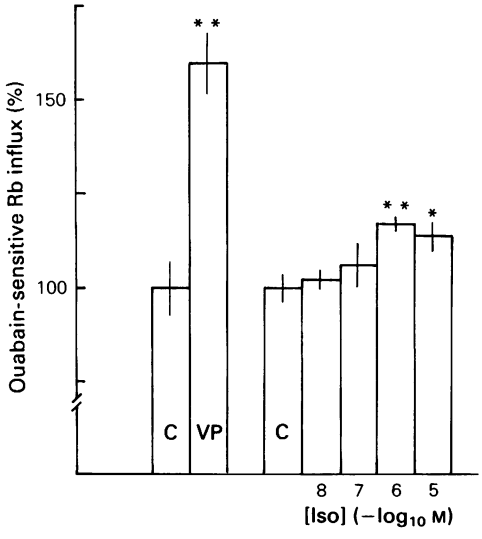

Figure 2 Dose-response relationship for the action of (−)-isoprenaline (Iso in the presence of 5 μM phentolamine) in comparison with vasopressin (VP; 10 nM). Results are expressed as percentage of the control value (control (C) VP = 6.57 ± 0.47); control (C) Iso = 7.04 ± 0.28 nmol, ^{86}Rb mg^{-1}min^{-1}. Each column shows the mean and vertical lines s.e.mean of 20 to 22 experiments. $^{*}P < 0.05$; $^{**}P < 0.01$, significantly different from paired controls (*t* test).

Results

Effect of noradrenaline, angiotensin and vasopressin on ^{86}Rb influx

Earlier work (Berthon *et al.*, 1983) has shown that noradrenaline, via α_1-adrenoceptors, causes a rapid and transient increase in the Na-K pump activity in rat isolated liver cells. The aim of the first experiments was to test whether the stimulation could be initiated by other Ca-mobilizing hormones such as angiotensin and vasopressin, which are thought to have the same action as α_1-receptor agonists on glycogen phosphorylase and net Ca fluxes in the liver (Exton, 1981; Claret & Binet, 1984). Rat hepatocytes were incubated with the hormones and the ^{86}Rb influx marker (^{86}Rb) for 90 s. Table 1 shows that angiotensin (1 nM) and vasopressin (10 nM), as well as noradrenaline (1 μM), significantly increased both the total ^{86}Rb influx and the ouabain-sensitive uptake indicating Na-K pump stimulation. The lack of effect of the hormones on the ouabain-resistant ^{86}Rb influx (Table 2) confirms earlier observations that the hormone-mediated $[Ca]_i$ increase (Charest *et al.*, 1983; Berthon *et al.*, 1984) does not increase K permeability in rat isolated liver cells (Burgess *et al.*, 1981; Berthon *et al.*, 1983; Jenkinson *et al.*, 1983).

Figure 1a shows that the α-adrenoceptor antagonist phenoxybenzamine (5 μM), and the peptide antagonists, 1-sarcosine, 8-isoleucine angiotensin (Sar-Ile-angiotensin; 50 nM) and d(CH_2)5Tyr(Et)Valinearginine-vasopressin (cyclo-(tyr)-vasopressin; 50 nM), which by themselves had no effect on the Na-K pump, totally abolished the cell response to their corresponding hormones. The Na-K pump was poorly sensitive to the α_2-adrenoceptor agonist clonidine (10 nM to 10 μM). Figure 2 shows that the β-adrenoceptor agonist isoprenaline, which increases the level of cyclic AMP (adenosine 3':5'-cyclic monophosphate) was able to stimulate the Na-K pump. However, the effect was substantially lower than that of Ca-mobilizing hormones (vasopressin in Figure 2).

Dose-responsive curves for the action of angiotensin and vasopressin on ^{86}Rb influx are shown in Figure 3; half-maximal stimulations were observed at about 0.1 nM and 1 nM, respectively.

The non-additive effects of the hormones

Figure 1b supports the view that angiotensin and vasopressin interact with the Na-K pump by activating membrane receptors different from α_1-adrenoreceptors. The stimulation of the ouabain-sensitive ^{86}Rb influx by noradrenaline (1 μM) was blocked by phenoxybenzamine (5 μM) at a concentration which did not affect responses to angiotensin and vasopressin. However, the effects of noradrenaline and the peptide hormones were not additive (Figure 1c). In these experiments, saturating concentrations of hormones were used to ensure that maximal stimulation was evoked by each hormone alone. Addition of angiotensin or vasopressin with noradrenaline resulted in Na-K pump activation equivalent to the individual responses to each hormone. The similarity of maximal responses and their lack of an additive effect suggest that the three hormones use a common coupling mechanism to control the Na-K pump.

Effect on the internal Na

Figure 4 compares the actions of angiotensin and vasopressin with that of noradrenaline on the internal Na concentration of the hepatocytes. The three hormones initiated similar rapid decreases in $[Na]_i$. The effects were detectable as early as 15 s. Calculation shows that the Na-K pump stimulation estimated from the ^{22}Na lost was quite similar to that calculated from the ouabain-sensitive ^{86}Rb influx. Figure 4 shows that the cell Na decreased by about 3.5 mM within 90 s which corresponds to a Na loss of 5.5 nmol mg^{-1} min^{-1} or (assuming the Na-K pump carried 3 Na per 2 Rb) to a stimulated ^{86}Rb influx of 3.6 nmol mg^{-1} min^{-1}; this value is close to the increment of ^{86}Rb influx induced by the three hormones (see Table 1). These results show that the Na-K pump stimulation by

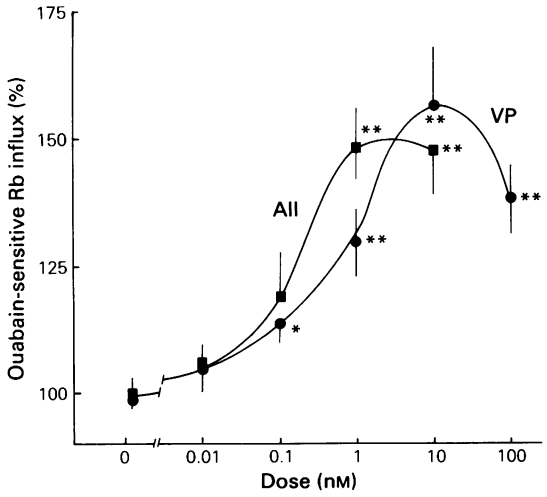

Figure 3 Dose-response curves for the action of angiotensin (AII; ■) and vasopressin (VP; ●) on the Na-K pumps activity. Influx was expressed as percentage of the control value (control AII, 5.44 ± 0.30; control VP, 4.12 ± 0.12 nmol, ^{86}Rb $mg^{-1}min^{-1}$). Each point shows the mean and vertical lines s.e.mean of 4 to 8 experiments. $*P<0.05$; $**P<0.01$, significantly different from paired controls (*t* test).

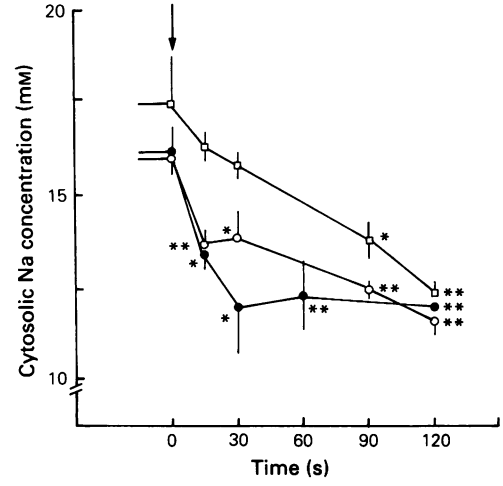

Figure 4 Time course of the action of (−)-noradrenaline (●), 1 μM) in the presence of 5 μM propranolol), vasopressin (○, 10 nM) and angiotensin (□, 1 nM) on the internal ^{22}Na concentration of the rat hepatocytes. The cells were equilibrated to isopotic equilibrium i.e. 30 min with the tracer. Each point shows the mean and vertical lines s.e.mean of 3 to 6 experiments. For other details, see Figure 1. $*P<0.05$; $**P<0.01$, significantly different from paired controls (*t* test).

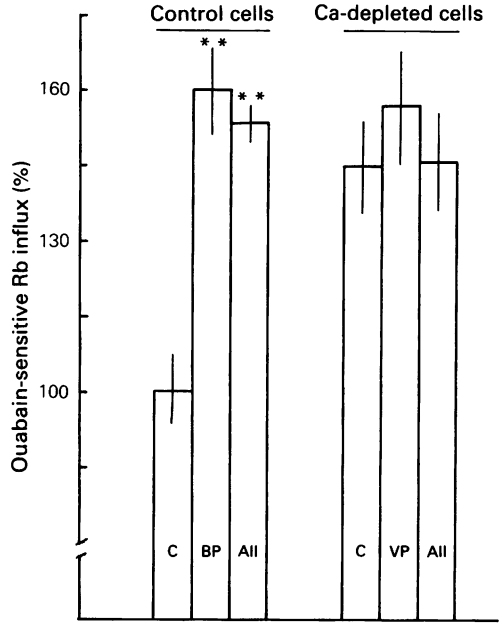

Figure 5 The effect of Ca-depletion on the stimulatory action of vasopressin (VP; 10 nM) and angiotensin (AII; 1 nM) on the Na-K pump activity (ouabain-sensitive ^{86}Rb influx). The cells were equilibrated in control medium containing either 1.8 mM Ca (control cells) or zero Ca and EGTA (200 μM) (Ca-depleted cells) for 90 to 120 min. The Na concentration of the cells as determined from ^{22}Na content was 17.1 ± 0.6 mM for control cells and 17.4 ± 1.3 mM for Ca-depleted cells. Results are expressed as percentage of control (7.35 ± 0.9 nmol $^{86}Rb\ mg^{-1}\ min^{-1}$). Each column represents the mean and vertical lines s.e.mean of 4 to 5 experiments. For other details see legend to Figure 1. $^{**}P < 0.01$, significantly different from paired controls (*t* test).

Ca-mobilizing hormones is not as a consequence of an increase in $[Na]_i$.

Role of Ca in the action of the hormones in stimulating the Na-K pump

Since noradrenaline, vasopressin and angiotensin promote a fast (< 5–15 s) increase in the cytosolic free Ca by stimulating Ca influx and releasing Ca stores in rat isolated liver cells (Charest *et al.*, 1983; Berthon *et al.*, 1984), the role of external and intracellular Ca in the cell response was studied. This was done by examining the action of the Ca chelating agents (EGTA and internal quin2 acting intracellularly) on the resting activity of the Na-K pump and that stimulated by vasopressin, angiotensin and noradrenaline.

The experiments confirmed a previous observation

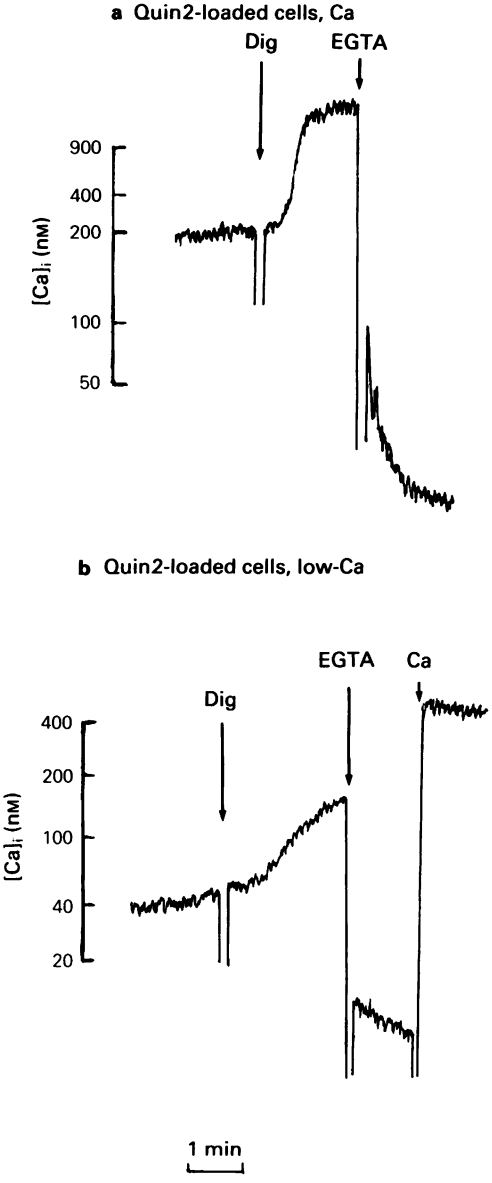

Figure 6 Effect of intracellular quin2 on the cytosolic Ca concentration, $[Ca]_i$, measured from the fluorescence emission of the indicator in cells loaded in the presence or in the absence of Ca. The hepatocytes were loaded with quin2/AM (75 μM, 5 μl DMSO) in the presence of either (a) 1.8 mM Ca (quin2-loaded cells, Ca) or (b) EGTA (200 μM) and zero Ca (quin2-loaded cells, low-Ca) for 6 min. The cells were washed and resuspended in 1.8 mM Ca or 22 μM Ca, respectively. Maximal (F_{max}) and minimal (F_{min}) fluorescence emissions were obtained by adding an excess of Ca or EGTA after lysis of the cells with 4 μM digitonin (Dig; see Methods).

(Berthon *et al.*, 1983) that incubating the cells in low-Ca-EGTA media for a short period (6 min) had no apparent effect on the Na-K pump or on the cell response to noradrenaline. This indicates that external Ca is not directly involved in the hormonal action. In contrast, long term incubation in low-Ca media (90–150 min), which progressively depletes the cells of their Ca, had two effects on the Na-K pump (illustrated in Figure 5). Firstly, the treatment increased the basal level of the Na-K pump activity suggesting that internal Ca is involved in the carrier activity. Secondly, it substantially reduced the stimulatory action of vasopressin, angiotensin and noradrenaline. This did not result from pump saturation since it has been found that in enriched-$[Na]_i$ cells the resulting 3 fold increase in ^{86}Rb influx was further increased by noradrenaline (Berthon *et al.*, 1983) It was found that the action of Ca-depletion was reversible. About 15–20 min incubation in 1.8 mM Ca were required to restore the basal activity of the Na-K pump and the response to noradrenaline in Ca-depleted liver cells (not shown).

The lack of effect of extracellular Ca and the effectiveness of Ca depletion in impairing the action of the Ca-mobilizing hormones made it interesting to examine the effect of an intracellular chelator that may, under certain conditions, directly alter cytosolic and bound Ca. The cells were loaded with a high concentration of quin2 by means of its permeant ester derivative quin2/AM which is hydrolyzed to the free chelator by intracellular esterases (see Tsien *et al.*, 1982; 1984). The experiments were carried out in the presence of Ca to allow cell homeostasis to restore $[Ca]_i$ or in the absence of Ca to promote a sustained drop in $[Ca]_i$ (Tsien *et al.*, 1984). The loading method was based on that developed by Berthon *et al.* (1984),

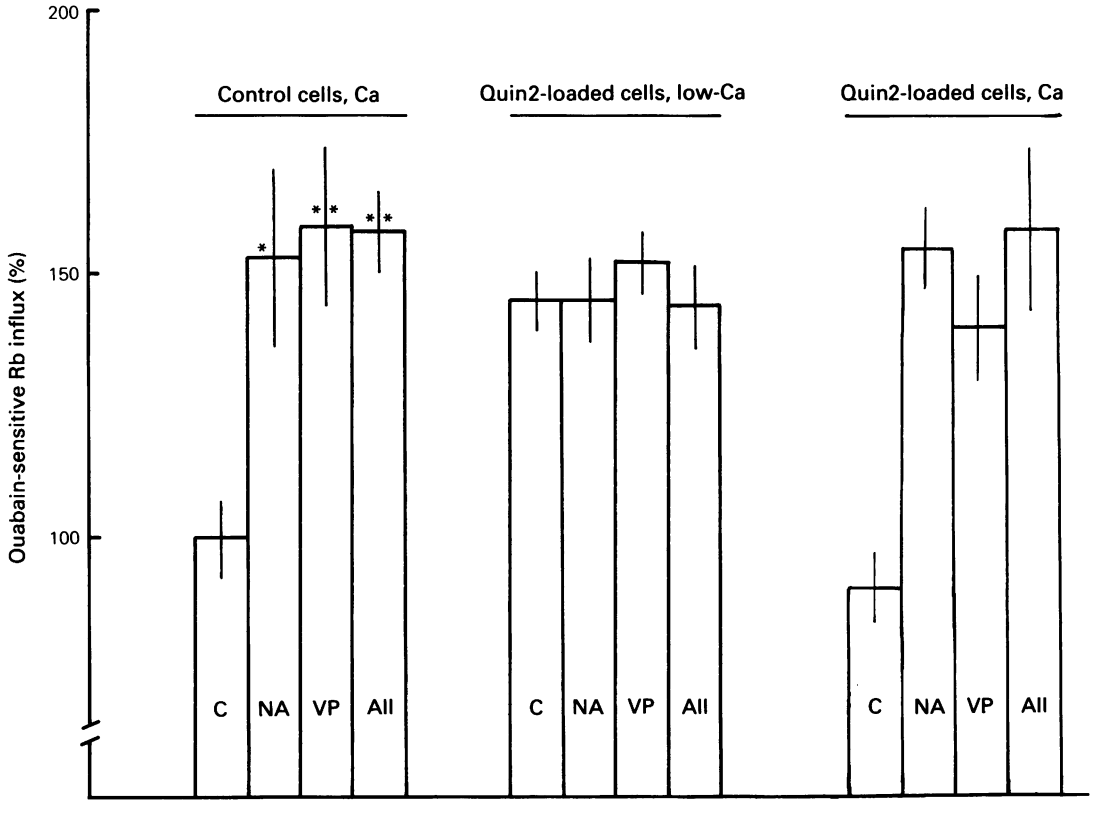

Figure 7 Effect of loading the cells with the internal Ca chelator quin2 on the Na-K pump and on its response to the three hormones. Rat isolated hepatocytes were incubated either in (a) control medium supplemented with DMSO (5 μl) (control cells, Ca) or (b) medium containing quin2/AM (75 μM, 5 μl DMSO) for 6 min, in the presence of either EGTA (200 μM) and zero Ca (quin2-loaded cells, low-Ca) or (c) 1.8 mM Ca (quin2-loaded cells, Ca). The influx was then measured from ^{86}Rb uptake in the presence of (−)-noradrenaline (NA; 1 μM, and 5 μM propranolol), vasopressing (VP; 10 nM) or angiotensin (AII; 1 nM) for 90 s as indicated in the legend to Figure 1. Each column represents the mean and vertical lines s.e.mean of 6 to 8 experiments. $*P < 0.05$; $**P < 0.01$, significantly different from paired controls (t test).

Table 3 Internal Na and K concentrations of control and quin2-loaded rat hepatocytes in the presence or absence of external Ca

Medium	*Intracellular concentration*	*Control cells* (mM)	*Quin2-loaded cells* (mM)
Ca (1.8mM)	$[K]_i$	139 ± 3.4	150 ± 13.1
	$[Na]_i$	19 ± 1.8	17.4 ± 1.4
Ca-free EGTA (1mM)	$[K]_i$	138 ± 7.2	146 ± 4.1
	$[Na]_i$	23.0 ± 0.7	20.2 ± 1.3

The cells were first equilibrated with ^{22}Na for 30 min then in media containing ^{22}Na and either Ca (1.8 mM) or Ca-free EGTA (200 μM) in the presence of quin2/AM (75 μM in 5 μM DMSO) or 5 μl DMSO for 6 min. Data shown are means ± s.e.mean of 5 to 6 experiments.

except that the rat hepatocytes were incubated for 6 min with 75 μM quin2/AM in the presence and in the absence of Ca. Internal cell Na and K were measured from ^{22}Na content and flame photometry, respectively, and quin2 content from the uptake of [^{3}H]-quin2/AM. The final quin2 cell content was 3.06 ± 0.17 nmol mg^{-1} dry wt.($n = 8$), which is twice the usual concentration used to measure $[Ca]_i$ in these cells (Berthon *et al.*, 1984).

In parallel experiments, the cytosolic Ca concentration was also determined from the fluorescence emission of the indicator in cells incubated under the same loading conditions (see Methods). Figure 6 illustrates the results. In the presence of extracellular Ca, $[Ca]_i$ amounted to about 200 nM, a value not different from that found in cells loaded with a lower quin2 concentration (Charest *et al.*, 1983; Berthon *et al.*, 1984). Loading the hepatocytes with the indicator in the absence of Ca caused $[Ca]_i$ to decrease to about 40 nM. Figure 7 shows that this $[Ca]_i$ reduction increased the pump activity to a level close to that promoted by noradrenaline, vasopressin and angiotensin in control cells. This effect did not result from an indirect stimulation of the Na-K pump by internal Na (see Table 3). In addition the ouabain-resistant ^{86}Rb influx was not altered. These results demonstrate that cytosolic Ca in the submicromolar concentration range may control the Na-K pump activity in rat liver, probably by binding to internal inhibitory sites, as recently described in human red blood cells (Yingst & Hoffman, 1984). Figure 7 also shows that in these low-$[Ca]_i$ cells, the stimulatory effect of the three hormones was abolished. Additional evidence for a role of Ca was the finding that a quin2 load in the presence of external Ca not only restored the resting $[Ca]_i$ (Figure 6) but also the basal level of the Na-K pump and its response to noradrenaline, vasopressin and angiotensin (Figure 7).

Discussion

The present results are the first indication that, in common with noradrenaline (Burgess *et al.*, 1981; Capiod *et al.*, 1982 Berthon *et al.*, 1983), angiotensin and vasopressin rapidly stimulate the Na-K pump in rat isolated liver cells. The action of each hormone was dose-dependent, blocked by preincubation with the corresponding antagonist and occured via activation of different types of receptors (see also Campanile *et al.*, 1982; Keppens *et al.*, 1982; Jard, 1983). Two different results seem to indicate that noradrenaline, angiotensin and vasopressin make use of a common mechanism to control Na-K pump activity in this tissue in keeping with their action on the Ca-dependent phosphorylase, the key enzyme for glycogen breakdown, and on Ca fluxes (see Williamson *et al.*, 1981; Exton, 1981; Claret & Binet, 1984). First, the individual responses to each hormone had similar maximal amplitudes. Second, the cell responses to maximal doses of the hormones were not additive

The β-adrenoceptor agonist, isoprenaline, stimulated the Na-K pump slightly, indicating that the carrier is not totally independent of intracellular cyclic-AMP. This is consistent with previous accounts of the involvement of both α and β-receptors in stimulation of the Na-K pump in muscle (Clausen & Flatman, 1980) or in other tissue (see Swann, 1984). However, it is clear that the effects observed here were essentially mediated via an α_1-adrenoceptor effect of noradrenaline, in agreement with the predominant number of α_1-adrenoceptors in rat liver (Exton 1981; Goodhart *et al.*, 1984).

The finding that noradrenaline, vasopressin and angiotensin triggered an immediate decrease (< 15 s, see Figure 4) in the internal Na content rules out activation of the carrier by increase in cytosolic Na. This could have occurred if the hormones had in-

creased membrane permeability to Na or if the receptor-mediated increase in $[Ca]_i$ had promoted an influx of Na via, for example, activation of a Na-Ca exchange mechanism. Additional evidence is the failure of noradrenaline to increase Na influx in rat isolated liver cells (Burgess *et al.*, 1981; Berthon *et al.*, 1983). Since these hormones also promote a decrease in external K concentration in rat hepatocytes (Burgess *et al.*, 1981; Berthon *et al.*, 1983) and perfused liver (Northrop, 1968; Friedmann & Park, 1968; Becker & Jacob, 1982; Reinhart *et al.*, 1984; Storm *et al.*, 1984), it may be concluded that these net ion movements are a secondary consequence of Na-K pump stimulation by noradrenaline, vasopressin and angiotensin. In this context, it is interesting to note that the hormone-mediated decrease in $[Na]_i$ could play a part in the transport of non-electrolytes such as aminoacids, which is dependent on the electrochemical gradient of Na ions in rat liver (Kilberg, 1982; Moore, 1983).

The present results indicate that quin2 may be easily used as a Ca chelator in addition to its capacity for detecting cytosolic free Ca, as shown by Tsien *et al.* (1982), Kesteven (1982) and Lew *et al.* (1982). In a number of cell types, it has been found that the uptake of quin2/AM and its resulting hydrolysis to the free chelator, as monitored from [^{3}H]-quin2/AM uptake and from the shift of fluorescence emission, are rapidly compensated by a net gain of extracellular Ca (Tsien *et al.*, 1982, 1984). In rat isolated liver cells (Berthon *et al.*, 1984) this action is complete within 5–6 min. At that time, $[Ca]_i$ amounted to 200 nM which does not differ from the resting $[Ca]_i$ detected in rat isolated liver cells loaded with significantly lower quin2 concentrations (Berthon *et al.*, 1984). In these normal $[Ca]_i$ cells the basal activity of the Na-K pump was not increased and the cells were responsive to the three hormones. When loading was carried out in the absence of Ca, the chelating action of quin2 was not balanced by net influx of Ca and $[Ca]_i$ was decreased to very low levels. Within a few minutes $[Ca]_i$ was held at 40 nM resulting in increased Na-K pump activity. This indicates that internal $[Ca]_i$ inhibits the Na-K pump by binding to some internal part of the protein. It has long been recognized that Ca is a potent inhibitor of the Na-K pump as well as Na-K ATPase (Sarkadi & Tosteson, 1980; Trachtenberg *et al.*, 1981). However, this action has been most often observed at Ca concentrations significantly higher than those observed here in intact hepatocytes. A similar observation was made by Yingst & Hoffman (1984) on resealed ghosts of red blood cells. They demonstrated that $[Ca]_i$, as detected by entrapped arsenazo III, inhibits the Na-K pump in the range of micromolar and submicromolar Ca concentrations. Similar measurements of $[Ca]_i$ could not be performed in these cells incubated for 90–120 min in low Ca-medium because intracellular quin2 is not irreversibly trapped. The molecule leaves the cytosol with an apparent time constant of about 40 min (Berthon *et al.*, 1984). However, we have found that if rat hepatocytes were loaded with quin2 in the presence of Ca, the subsequent removal of external Ca promotes a very slow decrease in $[Ca]_i$ as detected by the cytosolic indicator. Within ten minutes, the $[Ca]_i$ decreased from 175 nM to 145 nM (not shown). It appears that lowering cytosolic Ca by external EGTA is a much slower process than that caused by quin2. This probably explains the observation that very long periods of incubation in Ca-free medium ($>$ 90 min) were required to alter significantly the basal activity of the Na-K pump in rat isolated liver cells (Berthon *et al.*, 1983; present results).

The finding that lowering cytosolic Ca both increases the Na-K pump activity and impairs the stimulation by the three hormones may suggest that the latter interfere with the inhibitory Ca site located on the Na-K pump (Berthon *et al.*, 1983). These hormones could decrease the affinity or the number of the inhibitory sites for Ca resulting in the observed effects. It has been found that catecholamines are able to stimulate Na K-ATPase in brain (for ref. see Vizi 1978; see also Lee & Phillis, 1977; Hexum 1977; Desaiah & Ho, 1977; Van der Kro & Belfroid, 1980; Sawas & Gilbert, 1981). The removal of Ca may promote a stimulation of the enzyme and abolish the activation by α-adrenoceptor agonists. There is little to indicate the molecular mechanism underlying the action of the hormones in rat isolated liver cells; it might be mediated by acceleration of phosphoinositide metabolism which is dependent on Ca-mobilizing hormones. (Michell *et al.*, 1981; Berridge & Irvine, 1984). However, this requires further investigation.

We thank R. Leuillet and J. Tansini for their technical assistance.

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(Received February 12, 1985.
Revised April 15, 1985.
Accepted May 1, 1985.)