Glucagon stimulation of hepatic Na⁺-pump activity and α -subunit phosphorylation in rat hepatocytes

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In this study the possible role of Na⁺ influx, arachidonate mediators and α -subunit phosphorylation in the stimulatory response of hepatic Na⁺/K⁺-ATPase to glucagon was examined. Glucagon stimulation of ouabain-sensitive ⁸⁶Rb⁺ uptake in freshly isolated rat hepatocytes reached maximal levels in less than 1 min after hormone addition and was half-maximal (EC₅₀) at a concentration of $2.4(\pm 1.3) \times 10^{-10}$ M. Analysis of the K⁺-dependence of this response indicates an effect on the apparent V_{max} for K⁺ with no significant change in the apparent $K_{0.5}$. Unlike monensin, glucagon stimulation of Na⁺/K⁺-ATPase-mediated transport activity was not associated with an increase in ²²Na⁺ influx. This indicates that the stimulation of Na⁺/K⁺-ATPase by glucagon is not secondary to an increase in Na⁺ influx. A role for arachidonate mediators in this effect also appears unlikely because neither basal nor glucagon-stimulated ouabain-sensitive ⁸⁶Rb⁺ uptake was significantly affected by supramaximal concentrations of cyclo-oxygenase, lipoxigenase, cytochrome P-450 or phospholipase A₂ inhibitors. To study the possible role of protein kinase-mediated phosphorylation in the stimulation of ouabain-sensitive ⁸⁶Rb⁺ uptake, hepatocytes were metabolically radiolabelled with [³²P]P_i. Glucagon stimulated

incorporation of ³²P into a 95 kDa phosphoprotein that co-migrates with Na⁺/K⁺-ATPase α -subunit immunoreactivity in two-dimensional gel electrophoresis. The α -subunit could be immunoprecipitated from detergent-solubilized particulate fractions of hepatocytes using an anti-(rat kidney Na⁺/K⁺-ATPase) serum. When hepatocytes were metabolically radiolabelled with [³²P]P_i, the immunoprecipitated α -subunit contained ³²P. Glucagon increased the incorporation of ³²P into the immunoprecipitated subunit by $197 \pm 21\%$ ($n = 6$). Similar results were observed with a rabbit anti-peptide serum ('anti-LEAVE' serum) prepared against an amino acid sequence in the α -subunit. The EC₅₀ for glucagon-stimulated phosphorylation of the α -subunit ($\sim 1 \times 10^{-10}$ M) was very close to that for glucagon stimulation of ouabain-sensitive ⁸⁶Rb⁺ uptake. In conclusion, it appears that glucagon stimulation of hepatic Na⁺/K⁺-ATPase-mediated transport activity is not secondary to increases in Na⁺ influx or changes in the levels of an arachidonate mediator. The data provide support for the hypothesis that glucagon stimulation of Na⁺-pump activity in hepatocytes may be related to protein kinase-mediated changes in the phosphorylation state of the α -subunit.

INTRODUCTION

Many agents that alter the metabolic or secretory activity of the liver also rapidly stimulate the transport activity of Na⁺/K⁺-ATPase. In this study three possible mechanisms for glucagon stimulation of hepatic Na⁺/K⁺-ATPase activity were evaluated. The first hypothesis was that stimulation of Na⁺-pump activity is Na⁺-dependent and results from an increase in hormone-stimulated Na⁺ influx. Examples of hormones that work by this mechanism include insulin and epidermal growth factor. These hormones stimulate amiloride-sensitive Na⁺/H⁺ exchange in liver and the resulting increase in intracellular [Na⁺] quickly increases Na⁺/K⁺-ATPase activity [1–3]. The second hypothesis was that glucagon stimulation of Na⁺-pump activity arises from the production of a stimulatory arachidonate metabolite or loss of an inhibitory arachidonate metabolite. Several arachidonate metabolites that potentially inhibit Na⁺/K⁺-ATPase have been described [4–9] (for a review see ref. [10]) and at least one of these may be responsible for dopamine inhibition of renal Na⁺/K⁺-ATPase. The third hypothesis examined was that changes in the phosphorylation state of the α -subunit of the enzyme may be associated with changes in enzyme activity. Several serine/threonine kinase phosphorylation consensus sequences can be

found on the $\alpha 1$ -subunit [11] and *in vitro* phosphorylation studies have demonstrated that α -subunit isoforms are substrates for several serine/threonine protein kinases [12–16]. In addition, agonists of protein kinase C and protein kinase A that inhibit renal Na⁺/K⁺-ATPase have been reported to stimulate the phosphorylation of the α -subunit of Na⁺/K⁺-ATPase [15,17]. The addition of protein kinase A to Na⁺/K⁺-ATPase from several sources has been reported to either stimulate [18] or inhibit [12,15] Na⁺/K⁺-ATPase enzyme activity. Lastly, okadaic acid, a protein phosphatase inhibitor, stimulates ouabain-sensitive ⁸⁶Rb⁺ uptake and phosphorylation of the α -subunit in rat hepatocytes [19].

In this study, we have found that glucagon does not stimulate Na⁺ uptake. Furthermore, inhibitors of arachidonate metabolism have no significant effect on either basal or glucagon-stimulated Na⁺/K⁺-ATPase transport activity. This would seem to argue against a role for Na⁺ influx or arachidonate cell-signalling cascades in this response. In contrast, the action of glucagon on liver cells is associated with phosphorylation of the Na⁺/K⁺-ATPase α -subunit. These findings provide support for the hypothesis that protein kinase-mediated α -subunit phosphorylation may be involved in the rapid regulation of hepatic Na⁺-pump activity by glucagon.

Abbreviations used: IEF, isoelectric focusing.

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EXPERIMENTAL

Materials

$^{86}\text{RbCl}$, $^{22}\text{NaCl}$, $^{32}\text{P}\text{P}_i$ and ^{125}I -labelled secondary antibodies were obtained from New England Nuclear. Bacto-Gelatin (gelatin) was from Difco Laboratories (Detroit, MI, U.S.A.). Immobilized Protein A (immunoprecipitin) was from Gibco/BRL (Gaithersburg, MD, U.S.A.). Glucagon was purchased from Sigma. Collagenase D (lot no. DHA-142) was from Boehringer-Mannheim Corp. (Indianapolis, IN, U.S.A.). Rat kidney Na^+/K^+ -ATPase was purified with SDS as described by Jorgensen [20], and partially purified rat brain Na^+/K^+ -ATPase preparations were obtained by the method of Akera et al. [21] by deoxycholate and NaI treatment of microsomal fractions. Anti-serum to purified rat kidney Na^+/K^+ -ATPase [rabbit anti-(rat Na^+/K^+ -ATPase) serum] was produced by inoculating rabbits with 0.3 mg of protein per injection using the injection protocol of Green et al. [22]. A rabbit anti-peptide serum was prepared against the peptide KNCLVKNLEAVE [23] which was cross-linked to the hapten-carrier protein keyhole limpet haemocyanin (Pierce) using the water-soluble sulpho- analogue of the hetero-bifunctional cross-linker, succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate. Peptide antiserum (designated rabbit anti-LEAVE serum according to the nomenclature of Pressley [23]) was then raised as described by Green et al. [22].

Hepatocyte isolation

Hepatocytes were prepared from Sprague-Dawley rats as previously described [19,22,24,25] using a Vanderbilt perfusion apparatus with minor modifications. Rat liver was perfused *in situ* at 37 °C with perfusion buffer (120 mM NaCl, 4.5 mM KCl, 1.2 mM NaH_2PO_4 , 1.2 mM MgSO_4 , 25 mM NaHCO_3 , 11 mM glucose, 5 mM sodium pyruvate, 5 mM sodium glutamate, pH adjusted to 7.4 by constant gassing with humidified 95% O_2 /5% CO_2). Once the perfusion was established, collagenase D was added to the perfusate which continued to recirculate (30 mg/150 ml of recirculating perfusate) for approx. 20 min. At this time the partially digested liver was removed, gently minced in perfusion buffer containing 1.5% gelatin and shaken for 10 min at 37 °C with constant gassing (95% O_2 /5% CO_2). The resulting suspension was filtered through a 250 μm nylon mesh. Hepatocytes were collected by centrifugation at 50 *g* for 2.5 min. The cell pellet was then washed three times with gentle resuspension in physiological buffer (120 mM NaCl, 4.5 mM KCl, 1.2 mM NaH_2PO_4 , 1.2 mM MgSO_4 , 25 mM NaHCO_3 , 11 mM glucose, 5 mM sodium pyruvate, 5 mM sodium glutamate, 1.5% gelatin, 1.5 mM CaCl_2 , pH adjusted to 7.4 by constant gassing with humidified 95% O_2 /5% CO_2).

$^{22}\text{Na}^+$ uptake

Temperature-sensitive $^{22}\text{Na}^+$ uptake was measured in 5 ml aliquots of cell suspension (15–20% cytocrit in 25 ml Erlenmeyer flasks) that had been allowed to equilibrate for 30 min at 37 °C in physiological buffer containing BSA instead of gelatin with constant gassing (95% O_2 /5% CO_2). Uptake reactions were initiated by adding $^{22}\text{NaCl}$ (0.9×10^9 – 1.0×10^9 c.p.m./mol) to the cells along with drugs or vehicle. The reaction was stopped by centrifuging 1 ml aliquots of cells through 10 ml of ice-cold 10% sucrose in normal saline at speeds of up to 800 *g* for 30 s. Centrifugation was rapidly terminated with a manual hand brake and the tubes were inverted to drain the sucrose solution and placed inverted into a test-tube rack with paper towels underneath to absorb additional liquid. At the conclusion of the

experiment, the sides of the inverted tubes were rinsed with normal saline taking care to avoid the pellet, and the contents were transferred with 6 ml of BCS scintillant (Amersham) to 20 ml scintillation vials for determination of radioactive content by liquid-scintillation counting. The uptake of $^{22}\text{Na}^+$ at 37 °C was linear for up to 3 min and then levelled off (results not shown). Therefore subsequent uptake experiments were carried out for 2 min. Cell-pellet-associated radioactivity observed in cells chilled to 2 °C under otherwise identical conditions was taken as a blank and subtracted from that observed at 37 °C. Neither monensin nor glucagon affected the amount of cell-associated radioactivity at 2 °C.

One- and two-dimensional PAGE and Western blotting

One-dimensional SDS/PAGE was performed as described by Laemmli [26]. Two-dimensional isoelectric focusing (IEF)/SDS/PAGE was performed as detailed previously [19,27]. Western blotting of proteins in SDS/polyacrylamide gels to Nitroplus membranes (Micron Separations Inc., Westboro, MA, U.S.A.) was performed overnight at 30–35 V in a Bio-Rad transfer apparatus. Blots were temporarily stained with Ponceau S and destained with water. Immunoblots were processed as previously described [27,28] with ^{125}I -labelled goat anti-rabbit IgG Fab₂' fragments (2.5×10^5 c.p.m./ml of blotting buffer) as the secondary antibody.

Other methods

Na^+/K^+ -ATPase-mediated transport activity was measured as ouabain-sensitive $^{86}\text{Rb}^+$ uptake in 5 ml aliquots of cell suspension (15–20% cytocrit in 25 ml Erlenmeyer flasks) that had been allowed to equilibrate for 30 min at 37 °C with constant gassing (95% O_2 /5% CO_2) as described previously by Lynch et al. [19,24,29]. In initial experiments, collagenase preparations from several commercial suppliers were tested for their ability to produce viable hepatocytes that had a low rate of Na^+ leakage as determined by basal Na^+/K^+ -ATPase, measured after a 30 min equilibration period. Those collagenase lots that produced cells with basal ouabain-sensitive K^+ -uptake rates between 8 and 30 nmol/5 min per mg dry wt. were considered to be suitable for these studies. To estimate Na^+/K^+ -ATPase-mediated transport parameters, concentration–response data were computer-fitted to the equation for the Law of Mass Action using the non-linearizing curve-fitting routines provided with the SigmaPlot program.

Metabolic radiolabelling with $^{32}\text{P}\text{P}_i$ was performed by the method of Garrison [30] as previously described in detail [19]. Solubilization of particulate proteins from metabolically $^{32}\text{P}\text{P}_i$ -radiolabelled hepatocytes and immunoprecipitation of solubilized particulate proteins were also performed as described previously [19] using immunoprecipitin and the indicated dilutions of rabbit anti-(rat Na^+/K^+ -ATPase) serum or anti-LEAVE serum. Antibody complexes were released by brief sonication in Laemmli SDS/PAGE sample buffer [26], and solubilized proteins were separated by SDS/PAGE. Autoradiographs of the resulting gels were prepared at –84 °C after Coomassie Blue staining, using Kodak X-AR5 film with enhancing screens.

Each of the experiments shown are representative of replicate studies. Statistical differences between control and experimental samples were determined using Student's *t* test.

RESULTS

Effect of glucagon on Na^+/K^+ -ATPase activity

Ouabain-sensitive (2 mM ouabain) $^{86}\text{Rb}^+$ uptake was measured

Figure 1 Effect of glucagon on Na⁺/K⁺-ATPase-mediated transport activity

(a) The time-dependent effect of glucagon on ouabain-sensitive ⁸⁶Rb⁺ uptake was measured in 5 ml liver cell suspensions for 1 min periods (0–1, 1–2, 2–3, 5–6 and 10–11 min) after no addition (△) or after the addition of 100 nM glucagon (▲). The data are means ± S.E.M. from triplicate determinations from a single experiment that is representative of two such studies. (b) The concentration-dependent effect of glucagon on ouabain-sensitive ⁸⁶Rb⁺ uptake into isolated hepatocytes was measured over a 5 min period. The line is computer-fitted to the data as described in the Experimental section. The results are means ± S.E.M. from a single experiment representing two such studies.

in isolated hepatocytes to assay Na⁺/K⁺-ATPase-mediated transport activity. Figure 1(a) shows that glucagon rapidly stimulated Na⁺-pump activity in isolated liver cells. In agreement with previous studies, the stimulation reaches maximal levels at the first measurable time point [1,3,31,32]. By measuring Na⁺-pump transport activity in 1 min intervals at various times after hormone addition, it can be shown that glucagon-mediated increases are biphasic and activity remains elevated within the measured time. The half-maximal (EC₅₀) stimulation of Na⁺/K⁺-ATPase activity

Figure 2 Effect of K⁺ on glucagon stimulation of ⁸⁶Rb⁺ uptake

Flasks containing isolated hepatocytes were incubated for 30 min with increasing concentrations of K⁺ in the absence (△, ▲) or presence (○, ●) of 2 mM ouabain. ⁸⁶Rb⁺ uptake was subsequently measured over a 5 min period. Closed symbols indicate the presence of 0.1 μM glucagon added with the ⁸⁶Rb⁺. Results are the means ± S.E.M. from a single experiment representing four such studies.

in isolated liver cells by glucagon was $2.4(\pm 1.3) \times 10^{-10}$ M. Figure 2 shows that, when the extracellular K⁺ concentration is varied, the glucagon effect is mainly on the apparent $V_{\max}$ for K⁺ rather than the apparent $K_{0.5}$. Apparent $K_{0.5}$ values were averaged from four separate experiments [control, 1.14 ± 0.4 mM K⁺; plus glucagon, 1.7 ± 0.4 mM K⁺ (not significantly different)] and apparent $V_{\max}$ values were for the control, 28 ± 2.5 nmol of K⁺/5 min per mg dry wt. and plus glucagon, 40 ± 3.1 nmol of K⁺/5 min per mg dry wt. ($P < 0.05$). At higher concentrations of K⁺ a slight effect on the ouabain-insensitive uptake was noted.

Role of Na⁺ influx

Temperature-sensitive ²²Na⁺ uptake was measured in isolated hepatocytes in order to examine the role of Na⁺ influx in the response of the Na⁺ pump to glucagon. The sodium ionophore monensin, which stimulates ouabain-sensitive ⁸⁶Rb⁺ uptake in hepatocytes [25] significantly increased temperature-sensitive ²²Na⁺ uptake (Table 1). In contrast, glucagon did not stimulate Na⁺ influx (Table 1) in agreement with a previous report [2].

Role of soluble mediators derived from arachidonate

In several systems arachidonate metabolites have been shown to acutely alter Na⁺/K⁺-ATPase activity [4–9,33–35] (for a review see ref. [10]) by some as yet unknown mechanism. Therefore various inhibitors of the arachidonic acid cascade were employed to help determine whether products of cyclo-oxygenase, lipoxygenase, cytochrome P-450 or auto-oxidation might be involved in regulating hepatic Na⁺-pump activity or glucagon stimulation of pump activity. Table 2 shows that two well-characterized inhibitors of cyclo-oxygenase, indomethacin and meclofenamate, had no effect on either basal or glucagon-stimulated Na⁺/K⁺-ATPase-mediated transport activity. Furthermore two inhibitors of lipoxygenase, 5, 8, 11, 14-eicosotetraynoic acid and nordihydro-

Table 1 Effect of glucagon and monensin on $^{22}\text{Na}^+$ uptake

The results shown are means $\pm$ S.E.M. ($n = 6$ per condition) from a single experiment that is representative of two. *Significantly different from control as determined by Student's t test.

Condition	Concentration (μM)	$^{22}\text{Na}^+$ uptake (nmol/2 min per mg of protein)
Control	—	16.6 ± 0.8
Glucagon	0.1	15.4 ± 0.5
Monensin	50	$54.4 \pm 2.7^*$

guairesic acid, as well as the non-specific cytochrome *P*-450 inhibitor, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF-525A) and the isoenzyme-specific cytochrome *P*-450-dependent mono-oxygenase-specific inhibitor [32], ethoxyresorufin, had no effect on either basal or glucagon-stimulated Na^+/K^+ -ATPase-mediated transport activity in hepatocytes. Lastly, *P*-bromophenacyl bromide quinacrine (in the form of mepacrine) and 7,7-dimethyleicosadienoic acid, all common phospholipase A_2 inhibitors, had no statistically significant effect on either basal or glucagon-stimulated Na^+ -pump activity.

Role of phosphorylation of the α -subunit

To explore the possibility that changes in the phosphorylation state of the α -subunit of Na^+/K^+ -ATPase might be associated with the response of the enzyme to the glucagon, liver cells were metabolically radiolabelled with $[^{32}\text{P}]\text{P}_i$ as described by Garrison [30]. Radioequilibrated cells were then incubated in the presence or absence of glucagon. Denatured proteins from these cells were subjected to two-dimensional IEF/SDS/PAGE and autoradiography. A comparison of the two gels in Figure 3 shows that glucagon increased incorporation of $[^{32}\text{P}]\text{P}_i$ into a number of proteins whereas other phosphoproteins were unaffected. Close

Table 2 Effect of inhibitors on control and glucagon-stimulated ouabain-sensitive $^{86}\text{Rb}^+$ uptake

Results are means $\pm$ S.E.M. and are from individual experiments representative of two. Inhibitors were added 15 min before the measurement of ouabain-sensitive $^{86}\text{Rb}^+$ uptake for 5 min. Glucagon concentrations in different experiments were 50 or 100 nM. BPB, *f*-Bromophenacyl bromide; DEDA, 7,7-dimethyleicosadienoic acid; ETYA, 5, 8, 11, 14-eicosatetraynoic acid; NDGA, nordihydroguairesic acid. *Significantly different from control basal ouabain-sensitive $^{86}\text{Rb}^+$ uptake as determined by Student's t test.

Experiment no	Condition	Concn. (μM)	Ouabain-sensitive $^{86}\text{Rb}^+$ uptake (% of basal control)	
			Basal	Glucagon-stimulated
1	Control	—	100 ± 6	$158 \pm 10^*$
	BPB	30	100 ± 17	$161 \pm 11^*$
	Mepacrine	100	97 ± 5	$147 \pm 4^*$
	DEDA	100	96 ± 9	$154 \pm 13^*$
2	Control	—	100 ± 8	$161 \pm 12^*$
	Indomethacin	20	102 ± 4	$171 \pm 10^*$
	Meclofenamate	10	89 ± 7	$151 \pm 15^*$
3	Control	—	100 ± 7	$150 \pm 6^*$
	ETYA	50	95 ± 2	$143 \pm 8^*$
	NDGA	10	102 ± 4	$149 \pm 3^*$
	Ethoxyresorufin	20	96 ± 9	$165 \pm 14^*$
	SKF-525A	50	95 ± 6	$142 \pm 13^*$

Figure 3 Effect of glucagon on the phosphorylation of hepatocyte proteins separated by two-dimensional PAGE

Hepatocytes were metabolically radiolabelled with $[^{32}\text{P}]\text{P}_i$ [19] and then incubated for 5 min without further addition (top) or with 100 nM glucagon (bottom). The reaction was terminated by centrifuging 1 ml aliquots of cell suspension through 10 ml of ice-cold 10% sucrose/4.5 mM KCl. The supernatant was discarded and the cell pellets were frozen in liquid nitrogen. The cell-pellet proteins were then solubilized and separated by two-dimensional IEF/SDS/PAGE. Autoradiographs of the dried gels were made at -84°C with Dupont enhancing screens. The range of the pH gradient and the positions of the molecular-mass markers (kDa) are shown. The position of a 95 kDa peptide (2) which was previously identified immunologically as the α -subunit of Na^+/K^+ -ATPase is also shown [19].

examination of the autoradiographs shows that glucagon increased radiolabelling of several distinct regions in the 95 kDa portion of these gels, i.e. where the α -subunit migrates. We have shown that one of these phosphopeptides (no. 2 in Figure 3) co-migrates with a Coomassie Blue-stained spot that has α -subunit immunoreactivity [19]. Laser densitometry indicated that glucagon increased the radioactivity in this spot approximately twofold compared with the control (Figure 3 and results not shown). These findings provide support for the hypothesis that the α -subunit of Na^+/K^+ -ATPase is partially phosphorylated in unstimulated hepatocytes and that the level of phosphorylation is increased by glucagon.

In order to provide further evidence in support of these findings, immunoprecipitation experiments were performed. Two antibodies produced in our laboratory, a rabbit anti-(rat kidney Na^+/K^+ -ATPase) serum and a so-called anti-LEAVE antibody also produced in rabbits, were able to immunoprecipitate

Figure 4 Detection of Na⁺/K⁺-ATPase subunits in immunoblots with two rabbit antisera

Partially purified rat brain Na⁺/K⁺-ATPase (8 µg of protein) was solubilized in Laemmli sample buffer [26] and separated by SDS/PAGE (7.5% acrylamide). Immunoblotting was performed as described in the Experimental section. Left, rabbit anti-rat kidney Na⁺/K⁺-ATPase serum was used at a dilution of 1:200. (The lower-molecular mass portion of this blot is not shown but contained no detectable radioactivity.) Right, rabbit anti-LEAVE peptide serum was used at a dilution of 1:200.

detergent-solubilized Na⁺/K⁺-ATPase (results not shown). Figure 4 shows that the rabbit anti-(rat kidney Na⁺/K⁺-ATPase) serum recognized both the rat α- and β-subunits of the enzyme, whereas the anti-LEAVE serum recognized the α-subunit only, as expected. The ability of an anti-LEAVE serum to immunoprecipitate the enzyme is in agreement with the findings of Middleton et al. [17].

Metabolically ³²P-radiolabelled hepatocytes were incubated for 5 min in the presence or absence of glucagon (as in Figure 3). Particulate fractions of the liver cells were prepared, solubilized in detergent at 2 °C and immunoprecipitated in the cold as detailed previously [19] with immune serum in the presence of immunoprecipitin. Rabbit antisera to purified rat kidney Na⁺/K⁺-ATPase precipitated a 95 kDa phosphoprotein (Figure 5). Laser densitometry of autoradiographs from six immunoprecipitations were performed to quantify the changes in [³²P]P_i incorporation into the 95 kDa band. Glucagon increased the absorbance of the 95 kDa band by 197 ± 21 % in replicate experiments. Similar results were obtained when anti-LEAVE serum was used instead of the anti-(rat kidney Na⁺/K⁺-ATPase) serum (Figure 6). The ability of glucagon to increase the phosphorylation of the α-subunit was concentration-dependent with an EC₅₀ (~ 1 × 10⁻¹⁰ M) close to that for glucagon stimulation of ouabain-sensitive ⁸⁶Rb⁺ uptake (Figure 6).

Figure 5 Effect of glucagon on the phosphorylation of the 95 kDa α-subunit of Na⁺/K⁺-ATPase immunoprecipitated with rabbit anti-rat kidney Na⁺/K⁺-ATPase serum

Metabolically [³²P]P_i-radiolabelled hepatocytes were incubated for 5 min without further addition (i.e. control, lane C) or with 100 nM glucagon (lane G) for 5 min. Immunoprecipitation from the soluble portion of the detergent extracts was performed as previously described [19] using antiserum to purified rat kidney Na⁺/K⁺-ATPase (1:500 or 1:200 dilution as indicated). The results shown are from a single experiment representative of six such studies. The positions of the molecular-mass markers (kDa) are shown on the right-hand side.

DISCUSSION

In hepatocytes, glucagon rapidly stimulates Na⁺/K⁺-ATPase activity ([2,25,31,32] and Figure 1a). The time course of this early response is too rapid (seconds) to be accounted for by changes in protein turnover (Figure 1b). In this report several possible mechanisms based on how other hormones bring about rapid changes in Na⁺/K⁺-ATPase activity are examined. Support is provided for the hypothesis that phosphorylation of the α-subunit may be involved in the regulatory pathway.

Effect of glucagon on Na⁺/K⁺-ATPase activity

The ability of glucagon to rapidly stimulate ouabain-sensitive ⁸⁶Rb⁺ uptake in a concentration-dependent fashion (Figure 1) is in agreement with previous reports, including one in which Na⁺/K⁺-ATPase activity was measured in membranes isolated from livers perfused with glucagon [31,32]. By making measurements at different K⁺ concentrations we observed that the glucagon appears to increase the apparent *V*_{max} for K⁺ without affecting the apparent *K*_{0.5} (Figure 2). Because of the complexity of the enzyme reaction, it is difficult to interpret these results mechanistically. However, similar experiments were performed by Middleton et al. [17] in studies of the mechanism underlying phorbol ester inhibition of kidney cell Na⁺/K⁺-ATPase activity. The effects of phorbol esters, even through inhibitory, also appear to affect the apparent *V*_{max} for K⁺ as opposed to the apparent *K*_{0.5}.

At higher concentrations of K⁺ a slight stimulatory effect on the ouabain-insensitive uptake was noted (Figure 2). This may be due to either ineffectiveness of ouabain at these higher K⁺ concentrations or the fact that glucagon, in addition to stimu-

Figure 6 Effect of glucagon on the phosphorylation of the 95 kDa α -subunit of Na^+/K^+ -ATPase immunoprecipitated with rabbit anti-LEAVE peptide serum

Metabolically [^{32}P] P_i -radiolabelled hepatocytes were incubated for 5 min without further addition (i.e. control lane and 0 bar) or with the indicated concentrations of glucagon. Proteins were immunoprecipitated from the soluble detergent extracts (prepared as in Figure 5 legend) using rabbit anti-LEAVE peptide serum (1:100 dilution) and immobilized Protein A. The results shown are from a single experiment representative of two preimmune serum and six antiserum studies.

lating Na^+/K^+ -ATPase, also increases K^+ permeability in hepatocytes [2], which may lead to increased rates of ion exchange through ion channels.

Role of Na^+ influx

The mechanisms by which agents acutely stimulate Na^+/K^+ -ATPase activity can be classified as Na^+ -dependent or Na^+ -independent. For example, insulin and epidermal growth factor, which stimulate Na^+/K^+ -ATPase-pump activity in hepatocytes, also stimulated amiloride-sensitive Na^+/H^+ exchange [1,2]. The resulting increase in intracellular $[\text{Na}^+]$ brought about by increased Na^+/H^+ exchange is thought to be responsible for the resulting increases in Na^+/K^+ -ATPase. Monensin, a Na^+ ionophore, also increases Na^+/K^+ -ATPase activity in hepatocytes [25] by increasing the influx of Na^+ (Table 1). In contrast, glucagon stimulation of Na^+/K^+ -ATPase-pump activity in hepatocytes (Figures 1 and 2) does not appear to be associated with a stimulation of Na^+ influx (Table 1), in agreement with a previous report [2]. Thus our data do not support a role for Na^+ in this response to glucagon.

Role of soluble mediators derived from arachidonate

In 1985 Schwartzman and co-workers [5] reported purification of a cytochrome *P*-450 metabolite, 11,12-dihydroxyeicosatrienoic acid, that potently inhibited renal Na^+/K^+ -ATPase. More recently Satoh et al. [34,35] reported that cyclic AMP-dependent

inhibition of the kidney enzyme by dopamine requires the activity of the enzyme cascade responsible for the formation of epoxyeicosatrienoic acids. No arachidonate metabolites have been reported to stimulate Na^+/K^+ -ATPase. However, to examine the hypothesis that the effects of glucagon on Na^+/K^+ -ATPase are the result of changes in the synthesis or breakdown of inhibitory arachidonate metabolites, the effects of inhibitors of arachidonate metabolism were investigated.

The enzyme cyclo-oxygenase is the first step in the conversion of arachidonate to various prostaglandins and thromboxanes [10]. Inhibitors of this enzyme include indomethacin and meclofenamate. Members of the cytochrome *P*-450 family of enzymes are involved in the conversion of arachidonate into epoxyeicosatrienoic acids. Inhibitors of these enzymes include SKF-525A and ethoxyresorufin. The reaction catalysed by lipoxygenase, which is inhibited by 5,8,11,14-eicosatetraenoic acid and nordihydroguaiaretic acid, is the starting point for the synthesis of leukotrienes, lipoxins and various hydroperoxy acids. The latter can also be formed non-enzymically from arachidonate by auto-oxidation [10]. Phospholipase A_2 inhibitors, such as *p*-bromophenacylbromide, quinacrine and 7,7-dimethyleicosadienoic acid block the production of these auto-oxidation metabolites (as well as the formation of arachidonate metabolites arising from cyclo-oxygenase, cytochrome *P*-450 and lipoxygenase activities). Our results show that none of these inhibitors had a significant effect on ouabain-sensitive $^{86}\text{Rb}^+$ uptake in hepatocytes at supramaximal concentrations (Table 2) that either alter Na^+/K^+ -ATPase activities in renal tubules or block the production of these metabolites in other cells [10,34,35]. These data do not support the involvement of arachidonate metabolites in regulating Na^+/K^+ -ATPase in hepatocytes.

Role of phosphorylation of the α -subunit

Since several putative consensus sequences for serine/threonine protein kinases can be found on the α -subunit and in view of recent reports in which *in vitro* protein kinase-mediated phosphorylation of this subunit has been observed [12–18,36], we tested the hypothesis that glucagon-mediated increases in Na^+ -pump (Figure 1) and Na^+/K^+ -ATPase [31,32] activities might be associated with protein kinase-mediated phosphorylation of α -subunit. The following observations support the role of Protein kinase A-mediated phosphorylation of the α -subunit in the response of hepatic Na^+/K^+ -ATPase to glucagon. (1) The response is mimicked by cyclic AMP analogues and forskolin [25]. (2) Stimulation of hepatic Na^+/K^+ -ATPase is observed in response to the phosphoprotein phosphatase inhibitor, okadaic acid [19]. (3) Kraus-Friedmann et al. [31] have shown that this response to glucagon is preserved when hepatocytes are broken, and cell membranes isolated from the broken cells are assayed for Na^+/K^+ -dependent ouabain-sensitive ATPase activity. Thus the action of glucagon is not limited to transport activity alone and can be preserved after the cell membranes have been disrupted and washed by centrifugation, as would be expected for a kinase-mediated response. (4) A discrete 95 kDa region in two-dimensional electrophoresis gels, previously shown to co-migrate with Na^+/K^+ -ATPase α -subunit immunoreactivity, contained ^{32}P when proteins from hepatocytes metabolically radiolabelled with [^{32}P] P_i were separated (Figure 3). Glucagon increased the ^{32}P content of this spot (Figure 3). (5) A 95 kDa [^{32}P]phosphoprotein was immunoprecipitated from detergent extracts of metabolically radiolabelled hepatocytes with either a rabbit antiserum to purified rat kidney Na^+/K^+ -ATPase (Figure 5) or anti-LEAVE serum (Figure 6), both of which recognize the α -subunit of Na^+/K^+ -ATPase. No phosphorylation in the region

of the β -subunit was observed. (6) The EC₅₀ for glucagon stimulation of ouabain-sensitive ⁸⁶Rb⁺ uptake (Figure 1) was similar to the concentration-dependence for phosphorylation of the α -subunit (Figure 6).

Short-term hormonal regulation of Na⁺/K⁺-ATPase activity occurs in many tissues. Cyclic AMP-coupled hormones such as glucagon rapidly increase Na⁺/K⁺-ATPase activity in several other cells and tissues including vascular smooth muscle [18,33,37], cardiac myocytes [38], blood platelets [39], skeletal muscle [40], pancreatic acinar cells [41] (see ref. [42] for review), renal medulla [43–45] and the elasmobranch rectal gland [46] (compare with ref. [12]). Interestingly, in other tissues such as kidney tubules cyclic AMP-dependent agonists inhibit the enzyme [15,17,47–51]. Furthermore, *in vitro* additions of purified protein kinase A have been reported to either stimulate [18] or inhibit [12,15] the activity of the enzyme from different sources. These observations, i.e. that the same signalling pathway stimulates the enzyme in one tissue and inhibits it in another, should not be considered paradoxical. Such divergency may be related to the fact that Na⁺/K⁺-ATPase fulfils tissue-specific roles that must be regulated appropriately in different tissues according to different needs. The mechanisms involved in these tissue-specific responses have yet to be elucidated, but Moore and Fay [37] have speculated that a tissue-specific factor that allows phosphorylation to give rise to stimulation in some tissues and inhibition in others could explain the divergent regulation. Our data provide additional support for the hypothesis that, in tissues such as liver where cyclic AMP-dependent agonists give rise to stimulation rather than inhibition of Na⁺/K⁺-ATPase-mediated transport activity, phosphorylation of the α -subunit may also be involved.

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REFERENCES

- Fehlmann, M., Canivet, B. and Freychet, P. (1981) *Biochem. Biophys. Res. Commun.* **100**, 254–260
- Fehlmann, M. and Freychet, P. (1981) *J. Biol. Chem.* **256**, 7449–7453
- Pouyssegur, J. (1985) *Trends Biochem. Sci.* **10**, 453–455
- Schwartzman, M., Carroll, M. A., Abraham, N. G., Ferreri, N. R., Songu-Mize, E. and McGiff, J. C. (1985) *Hypertension* **7**, 1136–1144
- Schwartzman, M., Ferreri, N. R., Carroll, M. A., Songu-Mize, E. and McGiff, J. C. (1985) *Nature (London)* **314**, 620–622
- Schwartzman, M. L., Abraham, N. G., Masferrer, J., Dunn, M. W. and McGiff, J. C. (1987) *Adv. Prostaglandin Thromboxane Leukot. Res.* **17A**, 78–83
- Schwartzman, M. L., Balazy, M., Masferrer, J., Abraham, N. G., McGiff, J. C. and Murphy, R. C. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8125–8129
- Schwartzman, M. L. (1990) *Adv. Prostaglandin Thromboxane Leukot. Res.* **20**, 241–249
- Schwartzman, M. L., Davis, K. L., Nishimura, M., Abraham, N. G. and Murphy, R. C. (1991) *Adv. Prostaglandin Thromboxane Leukot. Res.* **21A**, 185–192
- Shimizu, T. and Wolfe, L. S. (1990) *J. Neurochem.* **55**, 1–15
- Bairoch, A. (1991) *Nucleic Acids Res.* **19**, 2241–2245
- Bertorello, A. M., Aperia, A., Walaas, S. I., Nairn, A. C. and Greengard, P. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11359–11362
- Chibalin, A. V., Vasilets, L. A., Hennekes, H., Pralong, D. and Geering, K. (1992) *J. Biol. Chem.* **267**, 22378–22384
- Chibalin, A. V., Lopina, O. D., Petukhov, S. P. and Vasilets, L. A. (1993) *J. Bioenerg. Biomembr.* **25**, 61–66
- Fisone, G., Cheng, S. X., Nairn, A. C., Czernik, A. J., Hemmings, H. C., Jr., Hoog, J. O., Bertorello, A. M., Kaiser, R., Bergman, T., Jornvall, H., Aperia, A. and Greengard, P. (1994) *J. Biol. Chem.* **269**, 9368–9373
- Lowndes, J. M., Hokin-Neaverson, M. and Bertics, P. J. (1990) *Biochim. Biophys. Acta* **1052**, 143–151
- Middleton, J. P., Khan, W. A., Collinsworth, G., Hannun, Y. A. and Medford, R. M. (1993) *J. Biol. Chem.* **268**, 15958–15964
- Scheid, C. R., Honeyman, T. W. and Fay, F. S. (1979) *Nature (London)* **277**, 32–36
- Lynch, C. J., Mader, A. C. L., McCall, K. M., Ng, Y.-C. and Hazen, S. A. (1994) *FEBS Lett.* **335**, 157–162
- Jorgensen, P. L. (1988) *Methods Enzymol.* **156**, 29–43
- Akera, T., Ku, D., Tobin, T. and Brody, T. M. (1976) *Mol. Pharmacol.* **12**, 101–114
- Green, N., Alexander, H., Olson, A., Alexander, S., Shinnick, M., Sutcliffe, J. G. and Lerner, R. A. (1982) *Cell* **28**, 477–487
- Pressley, T. A. (1992) *Am. J. Physiol.* **262**, C743–C751
- Lynch, C. J., Wilson, P. B., Blackmore, P. F. and Exton, J. H. (1986) *J. Biol. Chem.* **261**, 14551–14556
- Lynch, C. J., Bocchino, S. B., Blackmore, P. F. and Exton, J. H. (1987) *Biochem. J.* **248**, 807–813
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lynch, C. J., McCall, K. M., Billingsley, M. L., Bohlen, L. M., Hreniuk, S. P., Martin, L. F., Witters, L. A. and Vannucci, S. J. (1992) *Am. J. Physiol.* **262**, E608–E618
- Lynch, C. J., Blackmore, P. F., Johnson, E. H., Wange, R. L., Krone, P. K. and Exton, J. H. (1989) *J. Clin. Invest.* **83**, 2050–2062
- Lynch, C. J., Sobo, G. E. and Exton, J. H. (1986) *Biochim. Biophys. Acta* **885**, 110–120
- Garrison, J. C. (1983) *Methods Enzymol.* **99**, 20–36
- Kraus-Friedmann, N., Hummel, L., Radomska-Pyrek, A., Little, J. M. and Lester, R. (1982) *Mol. Cell. Biochem.* **44**, 173–180
- Radomska-Pyrek, A., Kraus-Friedmann, N., Lester, R., Little, J. M. and Denkins, Y. (1982) *FEBS Lett.* **141**, 56–58
- Lockette, W. E., Webb, R. C. and Bohr, D. F. (1980) *Cir. Res.* **46**, 714–720
- Satoh, T., Cohen, H. T. and Katz, A. I. (1993) *J. Clin. Invest.* **91**, 409–415
- Satoh, T., Cohen, H. T. and Katz, A. I. (1992) *J. Clin. Invest.* **89**, 1496–1500
- Yeh, L. A., Ling, L., English, L. and Cantley, L. (1983) *J. Biol. Chem.* **258**, 6567–6574
- Moore, E. D. and Fay, F. S. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8058–8062
- Desilets, M. and Baumgarten, C. M. (1986) *Am. J. Physiol.* **251**, H218–H225
- Turaihi, K., Khokher, M. A., Barradas, M. A., Mikhailidis, D. P. and Dandona, P. (1989) *Metabolism* **38**, 773–777
- Clausen, T. and Flatman, J. A. (1987) *Am. J. Physiol.* **252**, E492–E499
- Tung, P., Pai, G., Johnson, D. G., Punzalan, R. and Levin, S. R. (1990) *J. Biol. Chem.* **265**, 3936–3939
- Hootman, S. R. (1986) *Int. Rev. Cytol.* **105**, 129–181
- Charlton, J. A. and Baylis, P. H. (1990) *J. Endocrinol.* **127**, 213–216
- Pippard, C. and Baylis, P. H. (1984) *Clin. Sci.* **66**, 561–567
- Pippard, C. and Baylis, P. H. (1986) *J. Endocrinol.* **108**, 95–99
- Lear, S., Cohen, B. J., Silva, P., Lechene, C. and Epstein, F. H. (1992) *J. Am. Soc. Nephrol.* **2**, 1523–1528
- Bertorello, A. and Aperia, A. (1989) *Am. J. Physiol.* **256**, F57–F62
- Delamere, N. A. and King, K. L. (1992) *Invest. Ophthalmol. Vis. Sci.* **33**, 430–435
- Ibarra, F., Aperia, A., Svensson, L. B., Eklof, A. C. and Greengard, P. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 21–24
- Middleton, J. P., Raymond, J. R., Whorton, A. R. and Dennis, V. W. (1990) *J. Clin. Invest.* **86**, 1799–1805
- Takemoto, F., Cohen, H. T., Satoh, T. and Katz, A. I. (1992) *Pflügers Arch.* **421**, 302–306