FEBS Letters 355 (1994) 157–162

FEBS 14785

Okadaic acid stimulates ouabain-sensitive $^{86}Rb^+$-uptake and phosphorylation of the Na^+/K^+-ATPase α-subunit in rat hepatocytes

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Received 10 October 1994

Abstract Ca^{2+}-mobilizing and cAMP-dependent hormones rapidly increase sodium, potassium-dependent adenosine triphosphatase (Na^+/K^+-ATPase)-mediated transport in rat hepatocytes. To explore the possible role of protein phosphatases in these responses we used a protein phosphatase inhibitor, okadaic acid. Okadaic acid stimulation of ouabain-sensitive $^{86}Rb^+$-uptake was maximal between two and three minutes and displayed an EC_{50} of 41 ± 1 nM. Inhibition of Na^+/H^+ exchange with an amiloride analog abolished the response to insulin, but had no effect on okadaic acid-mediated stimulation of Na^+/K^+-ATPase transport. In hepatocytes metabolically-radiolabeled with $^{32}P_i$, okadaic acid stimulated the incorporation of radioactivity into several 95 kDa peptides, one of which reacted with anti-LEAVE peptide antisera, that recognizes Na^+/K^+-ATPase α-subunits. In other experiments Na^+/K^+-ATPase was immunoprecipitated from detergent-solubilized membrane fractions of metabolically-radiolabeled cells with an antisera to purified rat kidney Na^+/K^+-ATPase. A 95 kDa phosphoprotein was immunoprecipitated using anti-Na^+/K^+-ATPase antisera, but not by preimmune serum. Okadaic acid stimulated incorporation of radioactivity into this band by 220 ± 28%. These findings provide support for the hypothesis that rapid stimulation of hepatic Na^+/K^+-ATPase by hormones may be related to protein kinase/phosphatase-mediated changes in the phosphorylation state of the Na^+/K^+-ATPase α-subunit.

Key words: Na^+/K^+-ATPase; Protein phosphatase; Insulin; Liver; Okadaic acid

1. Introduction

Several hormones stimulate the transport activity of Na^+/K^+-ATPase in liver cells. Some of these induce Na^+/K^+-ATPase synthesis over a period of days or weeks [1]. Others, like insulin and epidermal growth factor stimulate amiloride-sensitive Na^+/H^+ exchange [2,3]. The subsequent change in intracellular [Na^+] increases Na^+/K^+-ATPase activity secondarily [2–5]. In contrast, glucagon rapidly stimulates Na^+/K^+-ATPase activity in hepatocytes without stimulating Na^+ influx and the calcium mobilizing hormones similarly do not stimulate Na^+/H^+ exchange [6–8,12]. This effect is in contrast to that observed in kidney where such hormones inhibit the enzyme [9–15]. However, stimulatory effects of hormones on Na^+/K^+-ATPase are not limited to liver cells. Indeed, the cAMP-coupled hormones, like glucagon, rapidly increase Na^+/K^+-ATPase activity in several other cells and tissues including vascular smooth muscle [16,17], cardiac myocytes [18], blood platelets [19], skeletal muscle [20], pancreatic acinar cells (see [21] for review), renal medulla [22,23] and elasmobranch rectal gland [24]. Cellular mechanisms underlying these stimulatory effects are not clear. One possibility is that protein kinase/phosphatases may be involved. Several serine/threonine kinase phosphorylation consensus sequences can be found on the α-subunit of Na^+/K^+-ATPase and in vitro phosphorylation studies have demonstrated that the Na^+/K^+-ATPase α-subunit isoforms are indeed substrates for several serine/threonine protein kinases [25–28] and at least one of these sites has been identified as the site for cAMP-dependent inhibition observed in the kidney ([29] c.f. [30]). While co-mixture of activated protein kinase A with vascular smooth muscle (one of the tissues which exhibits the stimulatory response) plasma membranes increases Na^+/K^+-ATPase activity in the membranes [16], there has yet to be a demonstration in intact cells of protein kinase-mediated phosphorylation of the enzyme under conditions in which the enzyme is rapidly stimulated by hormones. In addition, we hypothesize that if a phosphorylation mechanism is involved in the stimulatory Na^+/K^+-ATPase response, a protein phosphatase(s) may play an important role.

In this communication we show that a protein phosphatase inhibitor, okadaic acid [31], can rapidly stimulate hepatic Na^+/K^+-ATPase activity without increasing Na^+/H^+ exchange activity. We also show that okadaic acid stimulation of Na^+/K^+-ATPase is associated with increased phosphorylation of the 95 kDa α-subunit of the enzyme isolated from metabolically radiolabelled cells. These findings provide support for the hypothesis that protein kinase-mediated α-subunit phosphorylation may be involved in the rapid stimulatory regulation of Na^+/K^+- ATPase by hormones in liver cells.

2. Materials and methods

2.1. Materials

Anti-LEAVE antibody, an antibody directed against a peptide corresponding to a sequence found in Na^+/K^+-ATPase α-subunits, was a generous gift of Dr. Thomas Pressley (University of Texas Medical School, Houston, TX). 5-(*N,N*-hexamethylene)-amiloride (HM-Amiloride) a potent amiloride analog with very high specificity towards the Na^+/H^+ exchanger was obtained from Research Biochemicals Inc. (Natick, MA). Bacto-Gelatin (Gelatin) was from Difco Laboratories (Detroit, MI). Immobilized protein A (Immuno-precipitin) and okadaic acid were from Gibco, BRL (Gaithersburg, MD). Collagenase D (Lot No. DHA-142) was from Boehringer Mannheim Corp. (Indianapolis, IN). Rat kidney Na^+/K^+-ATPase was purified with sodium dodecyl sulfate (SDS) as described by Jorgensen [32]. Antiserum to purified rat kidney Na^+/K^+-ATPase (rabbit anti-rat Na^+/K^+-ATPase) was pro-

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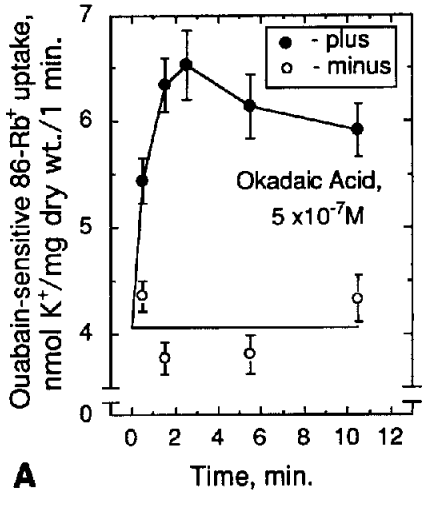
duced by inoculating rabbits with 0.3 mg of protein per injection using the injection protocol of Green et al. [33].

2.2. Hepatocyte isolation and Na^+/K^+-ATPase transport measurements

Hepatocytes were prepared from Sprague–Dawley rats as previously described [7] using a Vanderbilt Perfusion apparatus. Na^+/K^+-ATPase-mediated transport activity was measured in 5 ml aliquots of cell suspension (15–20% cytocrit) which had been allowed to equilibrate for 30 min at 37°C with constant gassing (95% O_2/5% CO_2) as described by Lynch et al. [7]. Centrifugation was rapidly terminated with a manual hand brake. Concentration–response data were computer fitted using the non-linearizing curve fitting routines provided with the SigmaPlot program to estimate Na^+/K^+-ATPase-mediated transport parameters.

2.3. Metabolic radiolabeling of hepatocytes with $^{32}P_i$

Hepatocytes were metabolically radiolabelled with $^{32}P_i$ (0.4–0.5 mCi/ml) according to the procedure of Garrison [34]. Labeling reactions were terminated by centrifuging 1 ml aliquots of cell suspension through 10 ml of ice-cold 10% sucrose/4.5 mM KCl for 30 s at speeds accelerating to 800 × g_{max}. Centrifugation was rapidly terminated with a manual hand brake and the resulting cell pellets were frozen in liquid nitrogen.



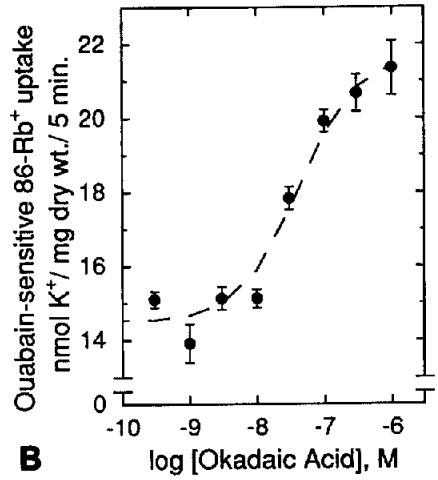


Fig. 1. Effect of okadaic acid on ouabain-sensitive $^{86}Rb^+$-uptake. (A) Ouabain-sensitive $^{86}Rb^+$-uptake was measured in 1 min increments at different times following the addition of vehicle (open circles) or 500 nM okadaic acid (closed circles). The data are means from triplicate determinations from a single experiment which is representative of two such studies. (B) The concentration-dependent effect of okadaic acid on Na^+/K^+-ATPase-mediated $^{86}Rb^+$-uptake was measured over five min. The symbols are the means and error bars are the standard error of the mean from triplicate determinations representative of duplicate experiments.

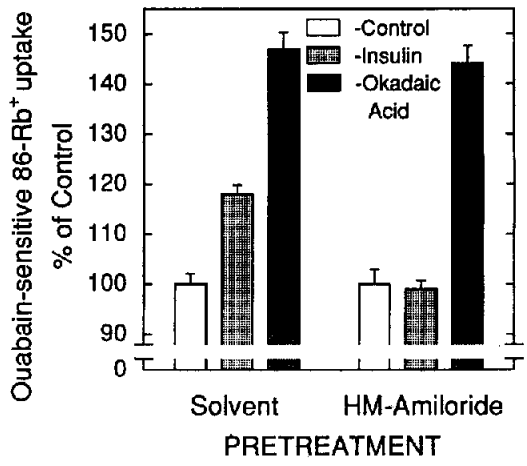


Fig. 2. Effect of HM-amiloride on insulin and okadaic acid stimulation of ouabain-sensitive $^{86}Rb^+$-uptake. Cells were incubated in the presence of 50 μM HM-amiloride or drug vehicle (i.e. DMSO, bars labeled 'Solvent') for 10 min prior to the measurement of basal, 100 nM insulin- or 500 nM okadaic acid-stimulated ouabain-sensitive $^{86}Rb^+$-uptake for five min. Basal Na^+/K^+-ATPase transport activity was 15.1 ± 0.25 in the absence and 12.1 ± 0.35 nmol K^+/mg dry wt./5 min in the presence of HM-amiloride. The vertical bars are means from triplicate determinations and error bars indicate the standard error of the mean from a single experiment representative of duplicate experiments.

2.4. SDS-PAGE and immunoblotting

One dimensional (SDS-PAGE) and two dimensional (IEF/SDS-PAGE) gel electrophoresis and Western blotting was performed as previously described [35]. ^{125}I-goat anti-rabbit IgG $F'Ab_2$ fragments (2.5×10^5 cpm/ml blotto; from New England Nuclear, Boston, MA) was used as the second antibody prior to autoradiography with Dupont enhancing screens at −84°C [35].

2.5. Immunoprecipitation of solubilized particulate proteins

Particulate proteins were prepared by sonicating frozen radiolabelled cell pellets in 1 ml of pH 7.4 ice-cold immunoprecipitation buffer (IPB) containing 90 mM NaCl, 25 mM sodium pyrophosphate, 25 mM sodium HEPES, 3 mM KCl, 2.5 mM EDTA, 10 mM ATP and 50 μM sodium orthovanadate, diluting to 8 ml and centrifugation at 200,000 × g for 30 min. The resulting supernatant was discarded and the particulate proteins in the pellet were solubilized on ice by stepwise addition of 133 μl of ice-cold IPB containing 2% SDS, 667 μl of ice-cold IPB containing 2% NP-40 followed by 533 μl more of ice-cold IPB containing 2% NP-40. Samples were sonicated on ice for 5–10 s between each addition. The suspension was centrifuged at 100,000 × g for 1 h at 2°C and 1 ml of the resulting supernatant was transferred to an ice-cold 15 ml conical centrifuge/culture tube and the remainder discarded. The detergent concentration was then reduced 10-fold by adding 9 ml of ice-cold buffer IPB in 3 ml increments with brief mixing between each addition. Solubilized liver proteins were immunoprecipitated using Immuno-precipitin. Immuno-precipitin suspensions were washed by centrifugation at 4000 × g for 20 min in immunoprecipitation buffer containing 0.2% NP-40 resuspended to the original volume prior to use in these experiments. To remove hepatic protein A binding proteins, 0.5 ml of ice-cold Immuno-precipitin suspension was added to each of the 15 ml tubes containing 10 ml of ^{32}P-labeled solubilized liver particulate proteins. This mixture was rocked for 1 h at 2–4°C and then centrifuged for 20 min at 4000 × g. The supernatant was transferred to another ice-cold tube containing 100 μl of rabbit pre-immune serum or antiserum to purified rat kidney Na^+/K^+-ATPase. This mixture was then rocked overnight at 2–4°C. Precipitation of antibody–

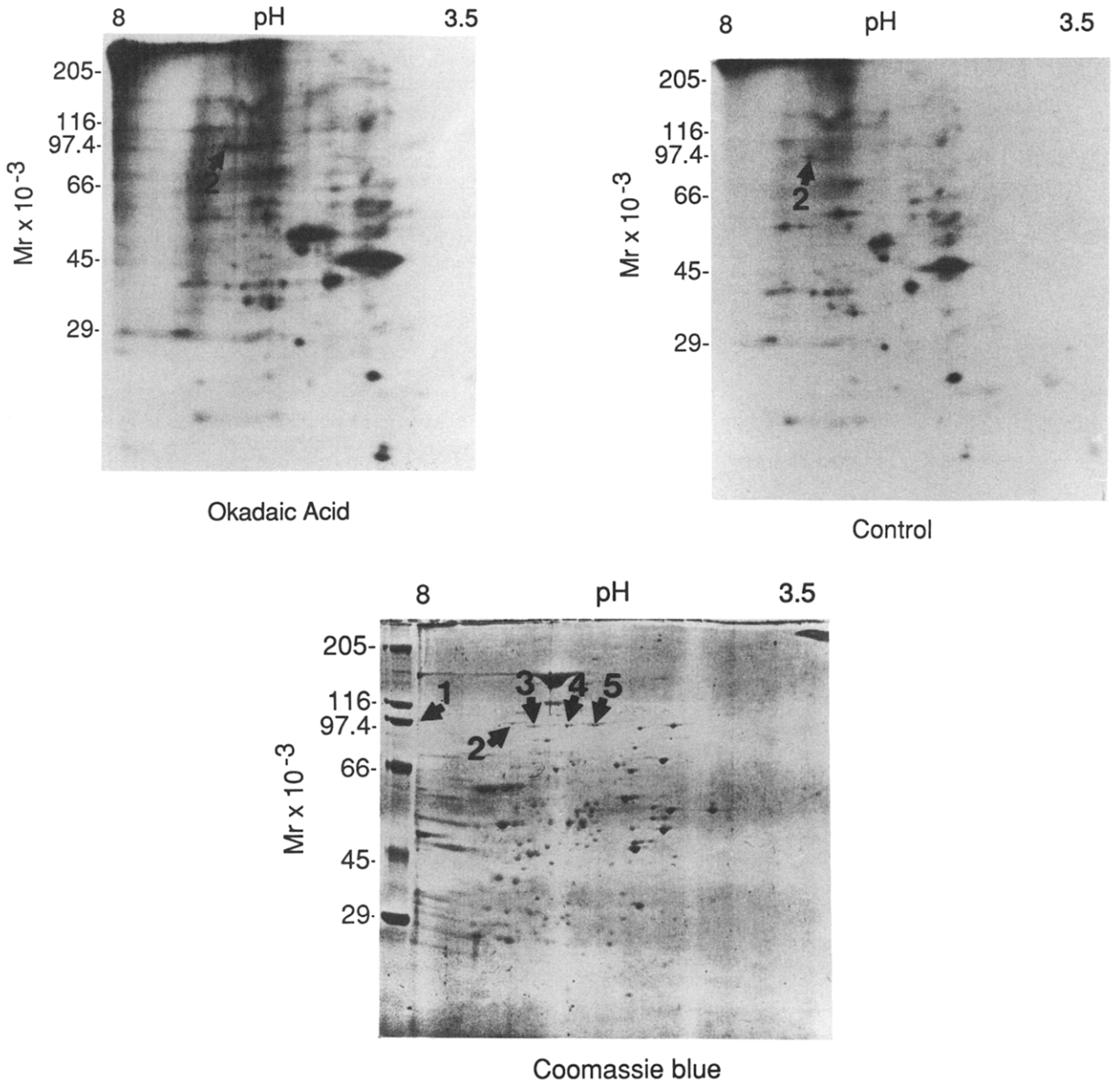


Fig. 3. Effect of okadaic acid on the phosphorylation of hepatocyte proteins separated by two-dimensional polyacrylamide gel electrophoresis. Metabolically radiolabelled hepatocytes were incubated in the presence or absence of 300 nM okadaic acid for 5 min. The reaction was terminated as in the $^{86}Rb^+$-uptake experiments and the cellular proteins were solubilized and separated by two dimensional IEF/SDS- PAGE as previously described [35]. The second dimension gels were Coomassie blue stained ('Coomassie blue') and autoradiographs of the dried gels were made at −84°C with Dupont enhancing screens ('Control' and 'Okadaic acid' panels). The range of the pH gradient and the position of molecular weight markers is shown. Several phosphoproteins migrating at the 95 kDa position were arbitrarily assigned numbers 1–5 ('Coomassie blue' panel), but only the position of phosphoprotein 2, which reacted with anti-LEAVE antisera is shown in the other panels.

protein complexes was then performed the next day by adding 0.7 ml of Immuno-precipitin suspension and incubating for 1 h with rocking at 2–4°C, followed by centrifugation (20 min at 4000 × *g*). The supernatant was discarded and Immuno-precipitin pellets were washed two times by centrifugation through 10 ml of immunoprecipitation buffer containing 0.2% NP-40 and the supernatants were discarded. A third centrifugation for 2 min was performed to remove excess buffer on the tube sides. Antibody complexes were released by briefly sonicating in SDS-PAGE sample buffer 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 in cassettes with enhancing screens.

3. Results

3.1. Effect okadaic acid on Na^+/K^+-ATPase mediated transport

Ouabain-sensitive $^{86}Rb^+$ uptake was measured in isolated hepatocytes to assay Na^+/K^+-ATPase activity. Okadaic acid stimulation of Na^+/K^+-ATPase activity was maximal by 1–3 min (Fig. 1, top panel). The EC_{50} for okadaic acid stimulation of Na^+/K^+-ATPase activity in isolated liver cells was 41 ± 0.4 nM (Fig. 1, bottom panel). The magnitude of the stimulation above basal (8.5 ± 0.5 nmol K^+/mg dry wt./5 min) in different

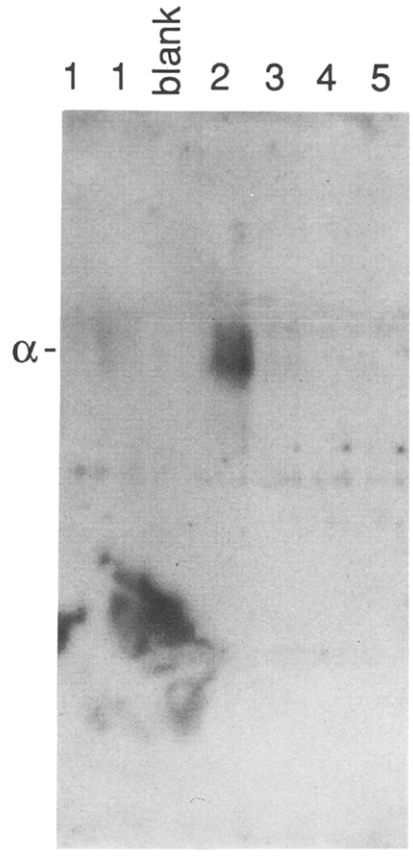


Fig. 4. Immunoreactivity of 95 kDa phosphoproteins with Anti-LEAVE antibody. Gels from experiments such as that in Fig. 4 were stored to allow for decay of $^{32}P_i$ radioactivity (the samples shown here were stored for over a year). Regions containing five ~95 kDa phosphoproteins were excised from six of these gels. The 95 kDa region corresponding to the 'basic streak' (No. 1) was punched out with a clean 6 mm diameter hole punch. The other regions (2–5) which corresponded to Coomassie blue-stained spots were excised with a clean razor blade (typically 2 × 3 to 4 mm rectangles). The samples were pooled in five microfuge tubes and rehydrated subjected to electrophoresis on a 7.5% acrylamide SDS-PAGE resolving gel with a 4% stacking gel. The larger No. 1 pieces had to be loaded in two lanes and were separated from samples 2–5 by a blank lane. The proteins were transferred to Nitroplus membranes for immunoblotting with anti-LEAVE antiserum (1:100 dilution).

experiments was not significantly different from that of glucagon (not shown), but was significantly greater than the size of the response observed with supra-maximal insulin concentrations (Fig. 2). Fig. 2 shows that okadaic acid stimulation of Na^+/K^+-ATPase transport activity in liver cells is not blocked by HM-amiloride. In contrast, insulin stimulation of Na^+/K^+-ATPase transport activity was blocked by this highly specific Na^+/H^+-exchange antagonist [2,7].

3.2. Studies on the phosphorylation of the α-subunit

Protein kinase C and protein kinase A agonists that inhibit renal Na^+/K^+-ATPase have been reported to stimulate the phosphorylation of the α-subunit of Na^+/K^+-ATPase [9–15,36]. 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 okadaic acid, liver cells were metabolically radiolabelled with $^{32}P_i$. Radioequilibrated cells were then incubated in the presence or absence of okadaic acid. Denatured proteins from these cells were subjected to 2D IEF/SDS-PAGE and autoradiography (Fig. 3). Comparison of the control and okadaic acid panels in Fig. 3 shows that okadaic acid increased the incorporation of $^{32}P_i$ into a number of proteins while other phosphoproteins were unaffected. Okadaic acid increased radiolabeling into several proteins around the 95 kDa regions (i.e. where the alpha subunit migrates) in these gels. The phosphoproteins corresponded to four Coomassie blue peptides and the region of the 'basic streak' in Coomassie blue-stained gels (Fig. 3). Recently Middleton et al. [36] reported that the phosphorylated Na^+/K^+-ATPase α1-subunit could be detected in 2D IEF/SDS-PAGE gels using anti-LEAVE peptide antibody. Therefore after the radioactivity in these gels decayed (the gels in the experiments shown were a year old) we removed five 95 kDa regions from the aged Coomassie blue-stained gels including a 6 mm portion of the basic streak region and four Coomassie-blue stained spots that had also co-migrated with ^{32}P radioactivity in Fig. 3. The gel pieces were re-hydrated, separated by SDS-PAGE and Western blotted. Autoradiographs of the Western blots were clear on overnight exposure, confirming complete ^{32}P decay (not shown). On subsequent immunoblotting with anti-LEAVE antibody only phosphopeptide No. 2 was recognized (Fig. 4). These findings suggest that phosphopeptide No. 2 is the α-subunit of Na^+/K^+-ATPase. Okadaic increased the radioactivity in this spot by approximately two-fold compared to control as determined by laser densitometry.

In order to provide further evidence in support of these findings, immunoprecipitation experiments were performed. Particulate fractions from $^{32}P_i$-labelled cells were detergent solubilized and immunoprecipitated with either pre-immune serum or immune serum in the presence of immunoprecipitin. Rabbit antisera to purified rat kidney Na^+/K^+-ATPase, but not pre-immune serum, precipitated a 95 kDa phosphoprotein corresponding to the 95 kDa α_1-subunit (Fig. 5). Okadaic acid increased the incorporation of radioactivity into this band. Laser densitometry of autoradiographs from six immunoprecipitations were performed to quantitate the changes in $^{32}P_i$ incorporation into the 95 kDa band. Okadaic increased the optical density of the 95 kDa band by 220 ± 28% in replicate experiments.

4. Discussion

Cyclic-AMP analogs and/or cAMP-dependent hormones have been reported to either stimulate (e.g. [2,6–8,16–24]) or inhibit (e.g. [9–15]) Na^+/K^+-ATPase activity in a tissue-dependent fashion. Similar observations have been made with Ca^{2+}-mobilizing hormones and tumor promoters. This is not surprising since Na^+/K^+-ATPase fulfills different roles in different tissues. Although recent studies have provided clues concerning the mechanisms involved in the inhibitory responses, little is known about how the stimulatory responses are brought about. Our laboratory is interested in the stimulatory regulation of Na^+/K^+-ATPase seen in hepatocytes. Here glucagon and cAMP analogs as well as Ca^{2+}-mobilizing hormones and tumor-promoters rapidly stimulate ouabain-sensitive $^{86}Rb^+$ uptake [2,6,8]. The stimulation is not secondary to increased Na^+/H^+-exchange or Na^+ influx [2] and is too rapid (seconds) to be accounted for by changes in protein turnover [6,8]. Kraus-Friedmann et al. [8] have reported that glucagon stimulation is

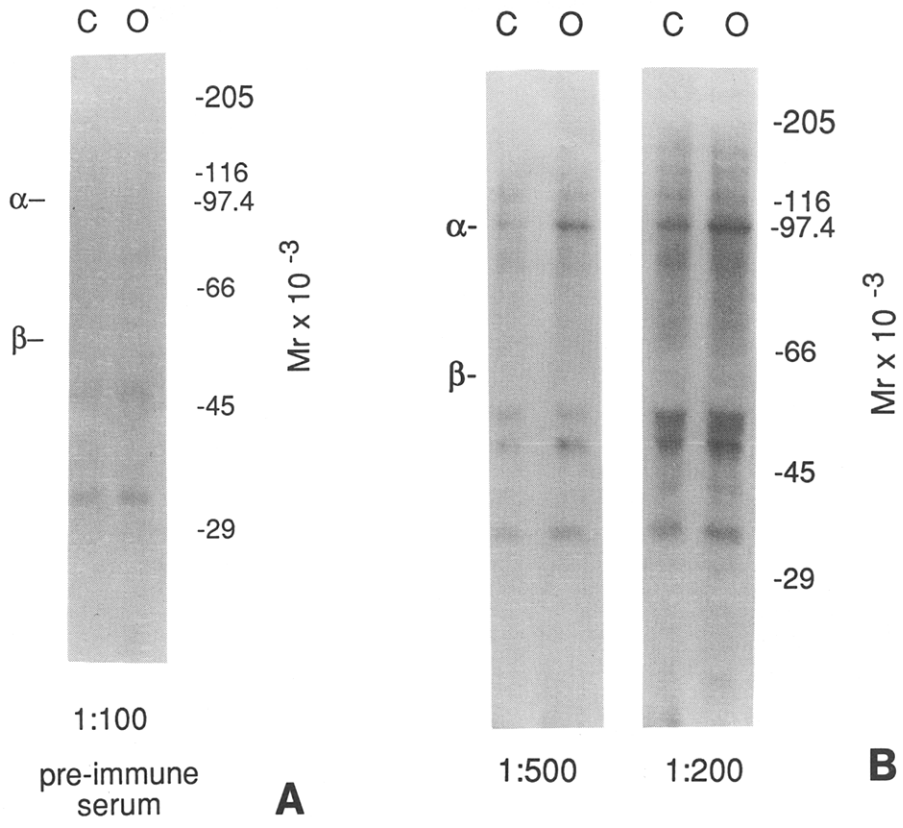


Fig. 5. Immunoprecipitation of Na^+/K^+-ATPase. Metabolically ($^{32}P_i$)- radiolabelled hepatocytes were incubated for 5 min without further addition (control, Lane C) or with 500 nM okadaic acid (Lane O) for 5 min. The reaction was terminated by centrifuging 1 ml aliquots of cell suspension through 10 ml of ice-cold 10% sucrose/4.5 mM KCl. The supernatant was discarded and the cell pellets were frozen in liquid nitrogen. Detergent soluble extracts were prepared from the 100,000 × *g* particulate fractions of these cells. Proteins were immunoprecipitated from the soluble portion of the detergent extracts using immobilized protein A and either rabbit pre-immune serum (Panel A, 1:100 dilution) or antiserum to purified rat kidney Na^+/K^+- ATPase (Panel B, 1:500 or 1:200 dilution as indicated) as described in section 2. The results shown are representative of a single experiment, two (pre-immune serum) or six (antiserum) such studies.

preserved following the isolation of plasma membranes from treated cells for measurement of Na^+/K^+-ATPase-mediated ATPase activity.

Recent studies in kidney and related cell lines have suggested that the observed inhibitory responses to cAMP-dependent hormones and tumor promoters may be brought about by phosphorylation of the α-subunit [11,12,29,36] (c.f. [10,15]). As a first step to study the possible role of phosphorylation in the liver response we have examined the effects of okadaic acid, a protein phosphatase inhibitor [31], on ouabain-sensitive $^{86}Rb^+$ uptake and phosphorylation of the Na^+/K^+-ATPase α-subunit. Okadaic acid brought about a time- and dose-dependent increase in Na^+/K^+-ATPase activity (Fig. 1). Consistent with its intracellular site of action, the peak response to okadaic acid occurs about a minute later than that of the Ca^{2+}-mobilizing hormones [7] and glucagon ([6] and data not shown) but about as fast as tumor promoters which also have an intracellular site of action [7,8]. The ability of okadaic acid to bring about this response alone supports the notion that protein phosphatase(s) may participate in the stimulatory response. Like Ca^{2+}-mobilizing hormones and glucagon, the okadaic acid response was not amiloride-sensitive and therefore is probably not secondary to an increase in Na^+-influx through the Na^+/H^+-exchanger as occurs with insulin ([2,7] and Fig. 2).

Since several putative consensus sequences for serine threonine protein kinases can be found on the α-subunit and in view of the recent reports in which in vitro protein kinase mediated phosphorylation of this subunit has been observed (e.g. [12,25–29,36]), we tested the hypothesis that okadaic acid-mediated increases in Na^+-pump activity might be associated with protein kinase-mediated phosphorylation of α-subunit (Figs. 3–5). Our findings support this hypothesis. Fig. 3 shows that okadaic acid stimulates the phosphorylation of several 95 kDa polypeptides from liver cell membranes. This observation supports the emerging notion that constitutive 'background' phosphorylation is continuously occurring, even in un-stimulated cells, and that phosphorylation is brought about by shifts in the balance between kinase and phosphatase activities. Fig. 4 shows that one of the 95 kDa polypeptides reacts with an anti-peptide antibody directed against a sequence common to the α1, α2 and α3 isoforms of the α-subunit [36]. Using another antibody that we produced to rat kidney Na^+/K^+-ATPase, which recognizes both the α- and β-subunits of the enzyme, it was possible to immunoprecipitate the detergent solubilized enzyme (Fig. 5) and demonstrate independently this effect of okadaic acid on the phosphorylation of the α-subunit. Phosphorylation of the β-subunit was not observed.

In conclusion, in several tissues Ca^{2+}-mobilizing and cAMP-dependent hormones rapidly stimulate Na^+/K^+-ATPase-mediated transport and/or ATPase activity in a Na^+-independent

fashion. Protein kinase mediated phosphorylation of the α-subunit in vitro has also been demonstrated by several groups. In this report we provide further evidence supporting the hypothesis that phosphorylation of the α-subunit and protein phosphatase(s) may be involved in the regulatory pathway involved in stimulation of the enzyme by demonstrating that under conditions in which okadaic acid stimulates hepatic Na^+/K^+-ATPase transport activity phosphorylation of the α-subunit occurs.

Acknowledgements: This work was supported by National Institutes of Health Grants DK43402 (CJL), HL 39723 (YCN) as well as grants and a fellowship from the American Heart Association, Pennsylvania Affiliate (CJL, YCN and SAH).

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