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Vasopressin stimulation of vanadate-sensitive Na^+ transport by liver plasma membrane vesicles. Evidence for regulation via phospholipase C and protein kinase C activities

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The rate of vanadate-sensitive $^{22}Na^+$ uptake by isolated liver membrane vesicles, reflecting transport by Na^+/K^+-ATPase, was measured to study the role played by phospholipase C and protein kinase C in the regulation of this process by vasopressin. Na^+ uptake was enhanced 2–3-fold by 100 nM [Arg^8]vasopressin and the hormone effect was mimicked by 0.1 μM inositol 1,4,5-trisphosphate as well as by 1.0 μM *myo*-inositol. The stimulation by vasopressin was potentiated by phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis* (5–10 mU/ml). No effect of the bacterial enzyme was observed in the absence of the hormone. Phorbol myristate acetate (0.5–1 μM) suppressed the stimulation by vasopressin but had no effect in the absence of the hormone. High concentrations of bacterial phosphatidylinositol-specific phospholipase C (50–100 mU/ml) also antagonized the hormone stimulation. Staurosporine (50–100 nM) prevented the antagonistic effect of bacterial phospholipase C (50 mU/ml) and EGTA (1 mM) partially protected the hormonal stimulation in the presence of phorbol myristate acetate. Our results suggest that the stimulatory effect of vasopressin on Na^+ transport is mediated by phospholipase C and products derived from the inositol moiety of membrane phospholipids. Membrane-associated protein kinase C appears to be at least partially responsible for the desensitization to stimulation by vasopressin.

Introduction

Metabolic responses to several hormones are characteristically linked to ion redistribution processes in rat liver. Transport of Na^+ and K^+ across the plasma membrane by Na^+/K^+-ATPase is influenced by insulin, glucagon, vasopressin, angiotensin II and α_1-adrenergic agonists. Phenylephrine enhances ouabain-sensitive K^+ uptake by perfused rat livers [1] whereas glucagon and insulin [2] as well as vasopressin, angiotensin II and norepinephrine [3–5] stimulate $^{86}Rb^+$ uptake in isolated hepatocytes. Different mechanisms appear to be involved: Insulin results in a stimulation of ouabain-sensitive $^{86}Rb^+$ uptake by enhancing Na^+ influx via Na^+/H^+ exchange [2]. In contrast, glucagon stimulates the hepatic Na^+ pump by a different, presumably cyclic AMP-dependent mechanism which is not secondary to Na^+ influx [6,7]. Ca^{2+}-mobilizing hormones seem to act by yet another mechanism because pretreatment of hepatocytes with glucagon causes no desensitization to subsequent stimulation by vasopressin, whereas other Ca^{2+}-mobilizing hormones result in heterologous desensitization [5]. In the case of vasopressin it is unlikely that Na^+ influx into intact cells is responsible for the stimulation of the Na^+ pump because cell integrity is not required for this effect; the hormone is able to stimulate vanadate-sensitive Na^+ transport into isolated hepatic plasma membrane vesicles [8]. Whether vasopressin receptors couple to the Na^+ pump by G protein-mediated activation of phospholipase C and standard signal mechanisms involving Ins(1,4,5)P_3 and Ca^{2+} or diacylglycerol and protein kinase C or by more direct interactions is not clear. In perfused rat livers addition of exogenous Ca^{2+} results in enhanced K^+ uptake in the presence of

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Abbreviations: Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; PMA, 4β-phorbol 12β-myristate 13α-acetate; PI-PLC, phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis*; PC-PLC, phosphatidylcholine-specific phospholipase C from *Bacillus cereus*.

phenylephrine or after prior Ca^{2+}-depletion with EGTA [9] and it is possible that an increase in cytosolic Ca^{2+} stimulates the Na^+ pump in hepatocytes [10]. However, the stimulatory effect of Ca^{2+} on hepatic Na^+/K^+-ATPase remains controversial [3,9,11]. Alternatively, evidence for the stimulation by exogenous phospholipase C, diacylglycerol and phorbol myristate acetate has suggested that the effect of Ca^{2+}-mobilizing hormones on Na^+/K^+-ATPase-mediated transport may involve diacylglycerol formation and protein kinase C in isolated hepatocytes [4]. Depressed Na^+/K^+-ATPase activity in diabetic nerve has been normalized by phorbol myristate acetate and diacylglycerol [12] and these observations further support the conclusion that protein kinase C may be involved in the stimulation of the Na^+ pump.

In the present report we describe experiments performed to study the regulation of vanadate-sensitive uptake of Na^+ into membrane vesicles isolated from rat liver. In particular, effects of bacterial phospholipases C and of several agents influencing the transduction of hormonal signals have been investigated. The results indicate that the stimulatory effect of vasopressin on Na^+ transport may be mediated by an uncharacterized product derived from the inositol moiety of membrane phospholipids. In contrast, protein kinase C appears to antagonize the hormonal stimulation. Some results have been reported in preliminary form [13].

Materials and Methods

Preparation of plasma membrane vesicles

Homogenates were obtained from livers of male Wistar rats (200–230 g) by homogenization with 5 vol. of cold buffer (0.25 M sucrose, 0.2 mM $CaCl_2$, 10 mM Hepes/KOH, pH 7.5) in a Dounce homogenizer (loose-fitting pestle). Plasma membrane vesicles were prepared by centrifugation in a self-forming Percoll gradient [14]. The final membrane fraction (approx. 20 mg protein) was suspended in 5 ml medium containing 250 mM sucrose and 10 mM Tris/Hepes (pH 7.5), and vesiculated by syringe with 15 passages through a 25-gauge needle. Protein was assayed according to Lowry et al. [15], with bovine serum albumin as standard. The enrichment of ouabain-sensitive Na^+/K^+-ATPase [16], 5'-nucleotidase [17], alkaline phosphatase [18] and glucose 6-phosphatase [19] was determined and was 17-, 15-, 23-, and 1.5-fold, respectively. Since these values and the rates of vanadate-sensitive uptake of $^{22}Na^+$ per mg protein were close to those previously reported for a membrane preparation obtained by another Percoll gradient centrifugation procedure, we assumed that the fraction of tightly sealed vesicles with inside-out orientation was also approx. 20% as reported before [8].

Uptake of $^{22}Na^+$ into membrane vesicles

Transport activities of Na^+/K^+-ATPase were estimated by measuring the vanadate-sensitive uptake of $^{22}Na^+$ into inside-out membrane vesicles. For the next steps the vesicles were diluted 10-fold with intravesicular medium. The composition of the intravesicular medium varied according to the experiment, but always contained 90 mM tetramethylammonium and Cl^- (obtained by neutralization of tetramethylammonium hydroxide with HCl), 60 mM KCl and 5 mM Tris/Hepes (pH 7.5). The vesicles were loaded with this medium by vesiculation followed by preincubation at 37°C for 1 h. After deposition of ATP (in 20 μl H_2O) on the wall of the tube, transport assays were started by the addition of extravesicular medium and by mixing. The membranes suspended in intravesicular medium were diluted 10-fold by this procedure. Extravesicular medium was kept at 37°C and contained NaCl, KCl, $MgCl_2$, valinomycin, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone and vanadate if necessary, to give final concentrations of 90 mM, 60 mM, 1 mM, 5 μM, 5 μM and 700 μM, respectively. $^{22}Na^+$ was also added to the extravesicular medium (0.22 μCi/ml). ATP was used in a final concentration of 1 mM. In some experiments EGTA (added to intra- and extravesicular medium) was present in a final concentration of 1 mM. In this case the concentration of ATP and $MgCl_2$ were increased to 4 mM and 3.5 mM, respectively. Each measurement was performed at 37°C in the absence and presence of vanadate by incubating 100–200 μg of membrane protein in a total volume of 1 ml.

Na^+ transport was terminated after 25 s by addition of 3 ml of an ice-cold solution containing NaCl, KCl, $MgCl_2$ and Tris/Hepes in the same concentrations as present in the extravesicular medium. The suspensions were filtrated by suction through Millipore HAWP 0.45 μm filters. The membranes were washed three times with 3 ml of the same solution and counted in 10 ml Instagel. Na^+ uptake was calculated from the specific activities of $^{22}Na^+$ in the extravesicular medium, and reported as vanadate-sensitive transport obtained by subtracting the values measured in the presence of the inhibitor from those measured in its absence. All assays were performed in triplicate with freshly prepared membranes. Data show the means ± S.E. of three different membrane preparations.

Chemicals

[Arg^8]Vasopressin was from Bachem AG (Bubendorf, Switzerland). Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (Fluka) and valinomycin (Sigma) were dissolved in ethanol. Percoll was obtained from Pharmacia. 4β-phorbol 12β-myristate 13α-acetate from Sigma, 4β-phorbol 12,13-didecanoate from Fluka and staurosporine (a gift from Dr. J. Pfeilschifter, Ciba-Geigy, Basel, Switzerland) were dissolved in 10 mM

dimethyl sulfoxide GR from Merck. Phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis* was from Pharmacare (Brügg, Switzerland) and phosphatidylcholine-specific phospholipase C from *Bacillus cereus* from Boehringer. D-*myo*-Inositol 1,4,5-trisphosphate was also from Boehringer, *myo*-inositol from Calbiochem, ATP was obtained from Sigma, and ^{22}NaCl from New England Nuclear.

Results

Na^+ transport mediated by Na^+/K^+-ATPase was studied by measuring vanadate-sensitive uptake of $^{22}Na^+$ into liver membrane vesicles [8]. The time course of Na^+ uptake (Fig. 1) showed that a transient maximum was reached after approx. 30 s. Longer incubation at 37°C presumably resulted in an equilibration of intra- and extravesicular Na^+ concentrations and in Na^+ uptake values approaching 50–100 nmol/mg. Previous results showed that Na^+ uptake measured at 25°C was about 50% lower and in these experiments no decrease of the Na^+ content was observed during an incubation for 120 s [8]. Loading the vesicles with medium containing 100 nM vasopressin for 1 h enhanced the transport of Na^+ (Fig. 1). During the first 25 s full stimulation by approx. 70% was reached and the stimulation did not fall below this value during the later course of the experiment. Na^+ uptake measured after 25 s was 144.7 ± 6.0 in the absence and 290.9 ± 28.7 nmol/mg in the presence of the hormone (n = 3).

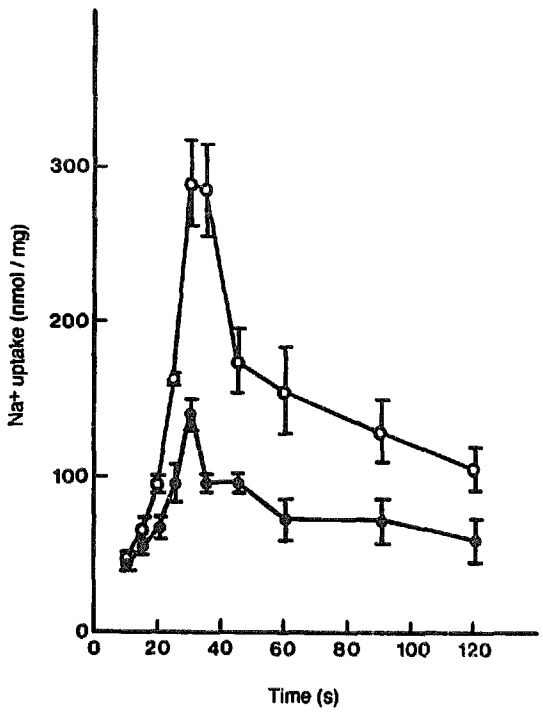

Fig. 1. Time course of vanadate-sensitive Na^+ uptake by rat liver plasma membrane vesicles. Effect of vasopressin. Vesiculation and preincubation were performed in the presence of 100 nM hormone (○) or in its absence (●).

10 nM vasopressin had practically the same effect as 100 nM but with 1 nM no stimulation was observed (not shown), indicating that the concentration range of

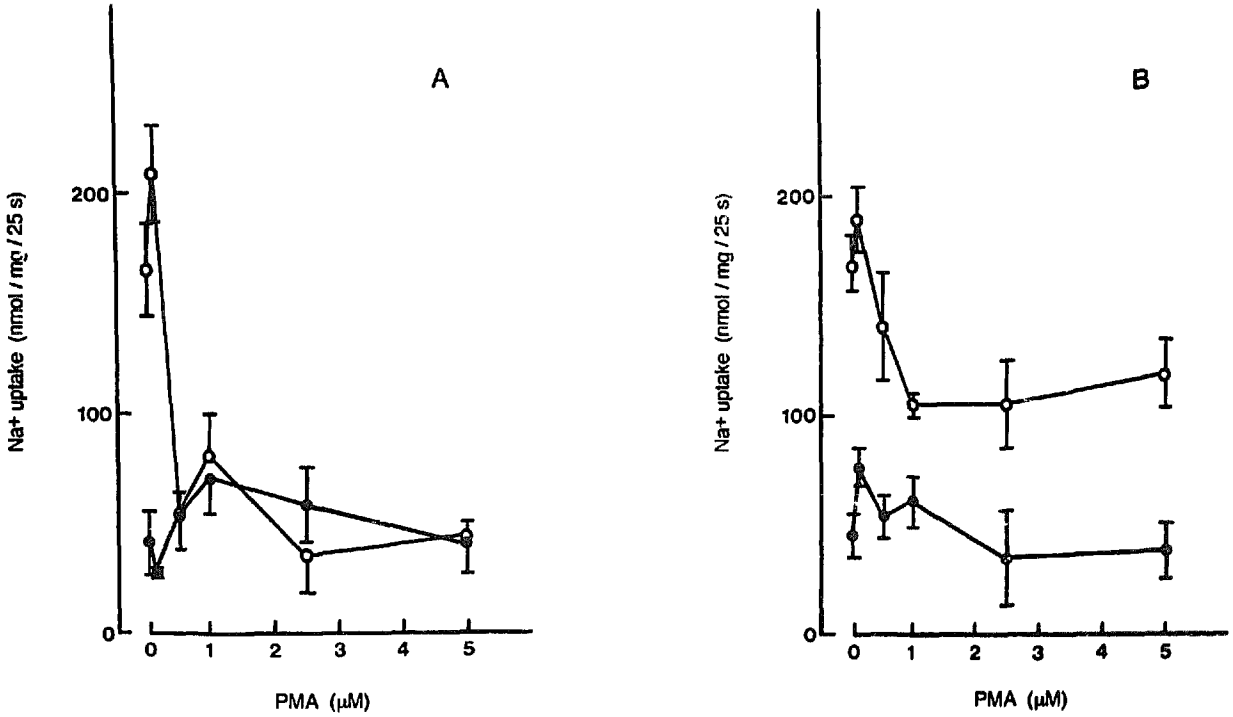

Fig. 2. Effect of phorbol ester on Na^+ uptake in the absence (●) or presence (○) of 100 nM vasopressin. Vesiculation and preincubation were performed with different concentrations of PMA in the absence (A) or presence (B) of 1 mM EGTA. Solvent alone was added to controls.

vasopressin acting on Na^+ transport was similar to that required for other responses in liver [4].

Since it was proposed that stimulation of the hepatic Na^+ pump by Ca^{2+} mobilizing hormones is the consequence of an enhanced diacylglycerol formation and activation of protein kinase C in the absence of changes in cellular Ca^{2+} levels [4], we used PMA to study the role played by membrane-bound protein kinase C in the regulation of Na^+ transport. In the absence of vasopressin the tumor promotor (0.2–5 μM) had no effect, whereas the hormonal stimulation of Na^+ transport was suppressed by 0.5 μM and higher concentrations (Fig. 2A). The fact that the phorbol ester was able to antagonize the stimulatory effect of vasopressin, suggested that an activation of protein kinase C was responsible for the suppression of the effect of the hormone in this instances. Because ATP and Mg^{2+} were routinely added to the incubation medium for measurements of Na^+ transport, protein kinase C may have been active during transport assays and contaminating traces of Ca^{2+} present in the membrane preparation may have potentiated its activation by PMA. Additional experiments performed in the presence of 1 mM EGTA showed that the effect of the tumor promotor was diminished but an inhibition of Na^+ transport by approx. 30% was still observed in the presence of vasopressin (Fig. 2B). Presumably, PMA was able to activate protein kinase C to some extent in the absence of Ca^{2+}. To test the possibility of an unspecific effect of phorbol esters on Na^+ transport, the response to an analogue, known to be unable to activate protein kinase C [20,21], was studied. The finding that it had no measurable effect in the concentration range of 0.1–5 μM (not shown), further supported the hypothesis that protein kinase C was responsible for the loss of sensitivity of the Na^+ transport system to stimulation by vasopressin. Consequently, the enhancement of Na^+ transport by the hormone could not be due to an increased production of diacylglycerol by phospholipase C but was rather mediated by another agent. The possibility that an alternative product of phospholipids degraded by phospholipase C was responsible for the stimulation was studied in additional experiments.

Since exogenous phospholipase C from bacterial origin was reported to stimulate the Na^+ pump in isolated hepatocytes [4], the effects of phospholipase C from *Bacillus thuringiensis* were investigated in a first step. Subsequently, Ins(1,4,5)P_3 or unphosphorylated inositol were added to membrane vesicles to study their effects. In accordance with the results obtained by others from rat hepatocytes [22] it was found that phosphatidylinositol glycan-anchored alkaline phosphatase and 5'-nucleotidase were released from liver membranes during incubation with PI-PLC. In contrast, glucose-6-phosphatase anchored by a hydrophobic domain of the peptide was not solubilized (Fig. 3).

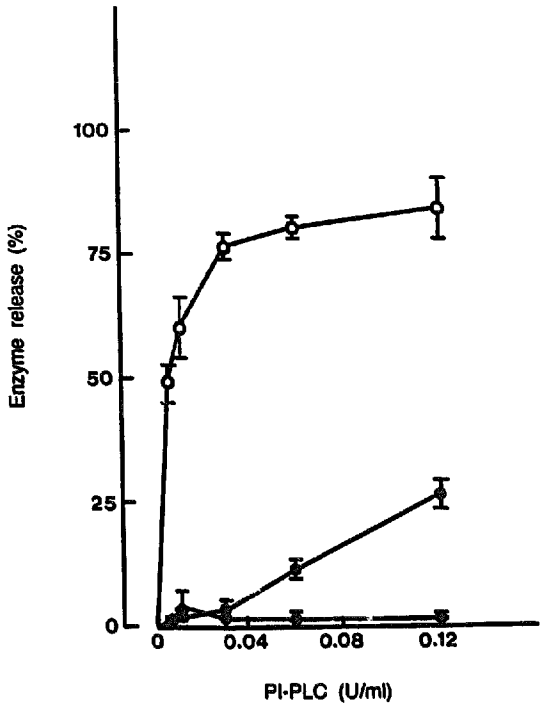

Fig. 3. Release of membrane-bound enzymes by PI-PLC. Vesiculation and preincubation were performed as described in Materials and Methods, with different concentrations of PI-PLC, at 37°C for 1 h. Afterwards the vesicles were sedimented at 100000 × *g* (Sorvall, rotor TST 60.4) for 1 h at 4°C, and the supernatants were assayed for enzyme activities: alkaline phosphatase (○); 5'-nucleotidase (●) and glucose 6-phosphatase (◆) The 100% values measured in the absence of added PI-PLC were 1063 ± 264, 2320 ± 286 and 129 ± 21 nmol/min per mg for 5'-nucleotidase, alkaline phosphatase and glucose 6-phosphatase, respectively, in the presence of 0.12 U/ml PI-PLC they were 1066 ± 264, 2335 ± 282 and 136 ± 21 nmol/min per mg, respectively.

Increasing concentrations of PI-PLC did not influence Na^+ uptake by membrane vesicles in the absence of vasopressin (Fig. 4). In the presence of the hormone,

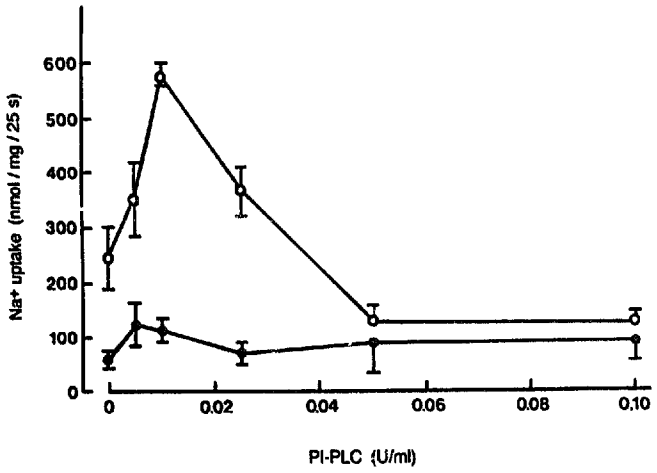

Fig. 4. Effect of phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis* (PI-PLC) on Na^+ uptake, in the absence (●) or presence (○) of 100 nM vasopressin. Vesiculation and preincubation were performed with the concentrations of phospholipase C shown.

low concentrations of phospholipase C (5–10 mU/ml) stimulated Na^+ transport by 40% and maximally 130%. At higher concentrations (25 mU/ml) the stimulation became less pronounced and a further increase of the concentrations of PI-PLC (50–100 mU/ml) even resulted in suppression of the stimulatory effect of vasopressin. The results of the same experiments performed with PC-PLC (not shown) indicated that either phosphocholin and the type of diacylglycerol produced by this enzyme as well as their degradation products had no regulatory influence on Na^+ transport or that PC-PLC was unable to split the cholin phospholipids of membranes under the experimental conditions used.

The results showing that PMA contributed to the loss of stimulation of Na^+ transport by activating protein kinase C, suggested that PI-PLC at high concentrations acted by a mechanism similar to that of PMA. Staurosporine, a nonspecific inhibitor of protein kinase C, was used to test this hypothesis (Fig. 5). In the absence of vasopressin, staurosporine (5–100 nM) and PI-PLC (60 mU/ml) had no effect. Low concentrations (up to 25 nM) of the inhibitor enhanced the stimulatory effect of the hormone approximately 5- and 6-fold in the absence and presence of PI-PLC, respectively, thus confirming the view that protein kinase C was involved in the inhibitory effect of high concentrations of phospholipase C. In the absence of added bacterial enzymes, 50 and 100 nM staurosporine were less effective than at 25 nM, whereas Na^+ transport was further enhanced (up to approx. 8-fold) by high concentrations and in the presence of PI-PLC. Presumably, staurosporine was able to interfere with the response to diacylglycerols formed by endogenous phospholipase C and PI-PLC. Its antagonistic effect on Na^+ transport at high concentrations could tentatively be explained

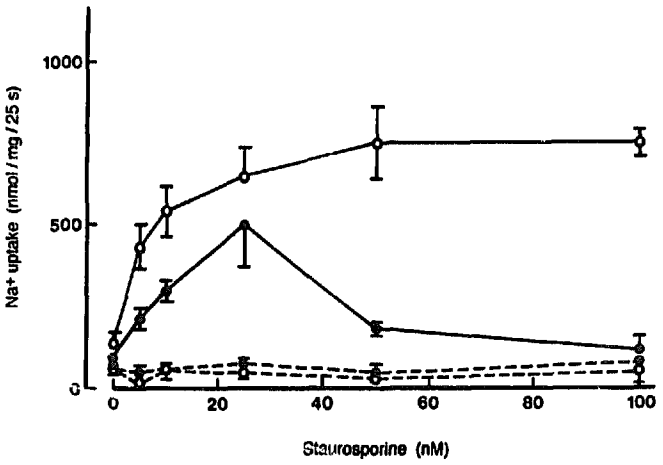

Fig. 5. Effect of staurosporine on Na^+ uptake in the absence (●) or presence (○) of PI-PLC; 0.05 U/ml, and in the absence (— — —) or presence (———) of vasopressin (100 nM). Vesiculation and preincubation were performed in the presence of the concentrations of staurosporine shown, with or without PI-PLC and in the absence or presence of the hormone.

by an unspecific inhibition of endogenous phospholipase C. The experiments with PI-PLC suggested that the stimulation of Na^+ transport by vasopressin was linked to the degradation of membrane inositolphospholipids even if the addition of exogenous phospholipase C in the absence of hormone was ineffective. In an attempt to further characterize the stimulatory mechanisms, the effects of Ins(1,4,5)P_3 and *myo*-inositol on Na^+ uptake were studied. As shown in Fig. 6A low concentrations of Ins(1,4,5)P_3 (up to 0.1 μM) resulted in an approximately 4-fold enhancement of Na^+ transport if no vasopressin was added, whereas at high concentrations the stimulation disappeared again. In the presence of the hormone, already low concentrations of Ins(1,4,5)P_3 suppressed the stimulatory effect of the hormone. As in the case of phospholipase C, stimulatory and antagonistic effects were closely associated. However, Ins(1,4,5)P_3 alone was able to mimic the stimulation by the hormone, suggesting that this agent itself or a degradation product generated by membrane associated enzymes was responsible. Among several degradation products and analogues, *myo*-inositol was tested and found to be stimulatory although at higher concentrations than Ins(1,4,5)P_3 (Fig. 6B). In preliminary experiments glucose 6-phosphate (0.1 mM) stimulated Na^+ transport approximately 2-fold (not shown). Because of this unspecificity a further characterization of the stimulatory mechanism was not attempted. A similarly unspecific effect of Ins(1,4,5)P_3 on the activation of the pyruvate dehydrogenase complex in rat adipose tissue was observed previously, however, at approximately 100-fold higher concentrations [23].

Because the use of EGTA and staurosporine appeared successful in our earlier attempts to link protein kinase C activity with antagonistic effects, the same approach was used to analyse the response to Ins(1,4,5)P_3 at high concentrations (0.25–1.0 μM). In the presence of 1 mM EGTA a stimulation of Na^+ transport was observed with all these concentrations (Fig. 6C) indicating that in the experiment shown in Fig. 6A traces of Ca^{2+} present in the membrane preparation were mobilized by Ins(1,4,5)P_3 and were able to antagonize the stimulation by activating protein kinase C. The additional production of diacylglycerol in response to vasopressin seemed to potentiate the inhibitory effect of Ca^{2+} even at low concentrations of Ins(1,4,5)P_3 (Fig. 6A). Because a continuous, hyperbolic increase of Na^+ transport was not observed in Fig. 6C, EGTA was apparently not able to eliminate the inhibitory contribution of inositol trisphosphate completely. In an attempt to further reduce it, staurosporine and EGTA were used together to inhibit protein kinase C as much as possible (Fig. 6D). Eventually, the stimulation of Na^+ uptake by low concentrations of Ins(1,4,5)P_3 was enhanced to some extent but

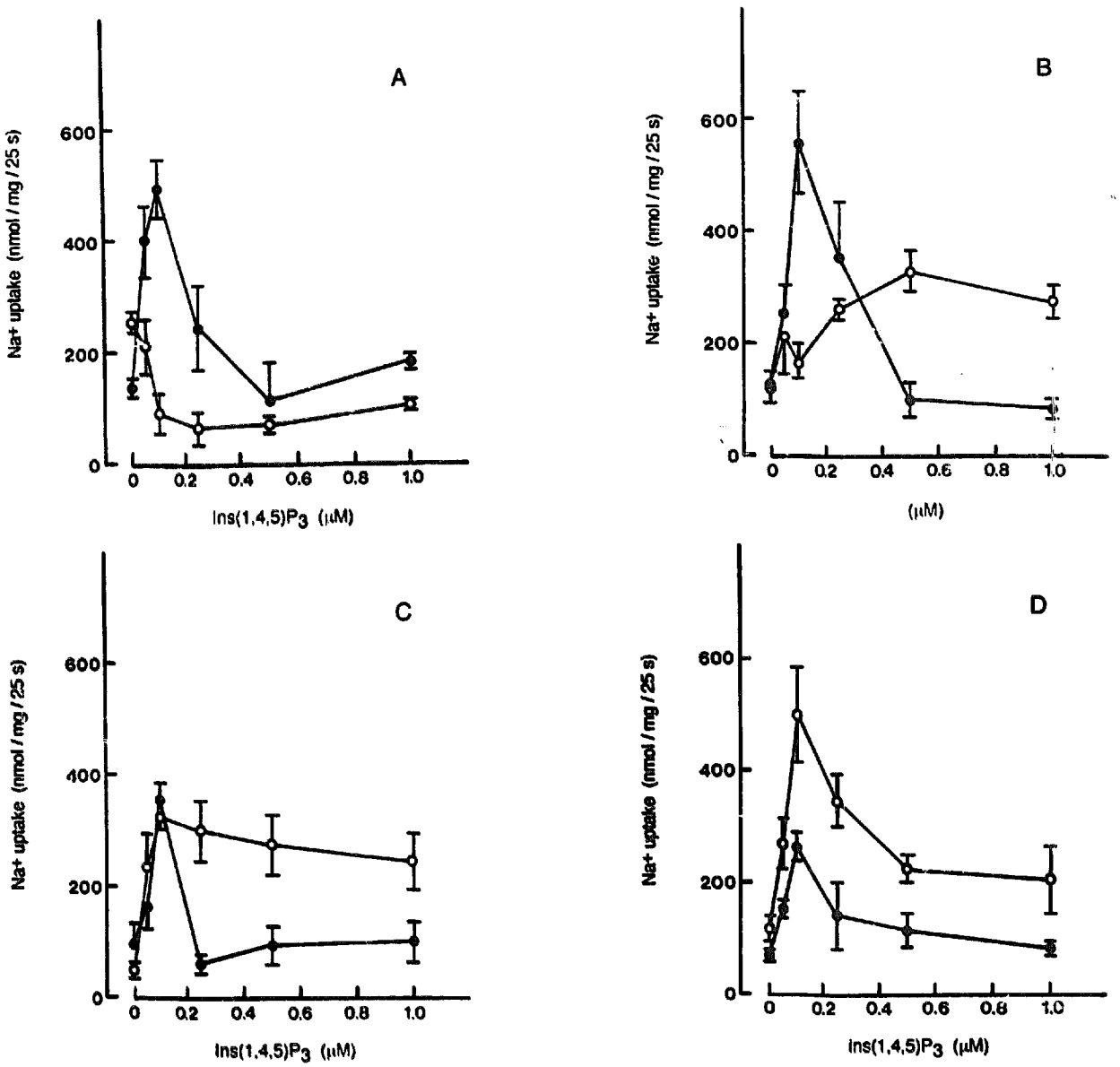

Fig. 6. Effect of Ins(1,4,5)P_3 and *myo*-inositol on Na^+ uptake. Vesiculation and preincubation were performed at different concentrations of Ins(1,4,5)P_3 and in the absence (●) or presence (○) of 100 nM vasopressin (A). Effect of different concentrations of *myo*-inositol (○) and Ins(1,4,5)P_3 (●) (B). Influence of EGTA on Na^+ uptake in the presence of Ins(1,4,5)P_3. Intra and extravesicular media were prepared without EGTA (●) or 1 mM EGTA (○) (C). Influence of staurosporine (50 nM) and EGTA (1 mM) on Na^+ uptake in the presence of Ins(1,4,5)P_3. Vesiculation and preincubation were performed in the absence (●) and presence (○) of staurosporine plus EGTA (D).

antagonistic effects of higher concentrations were still observed, raising the question whether protein kinase C is the only factor responsible for antagonistic effects.

Discussion

The hepatic Na^+ pump is known to be stimulated in response to several hormones [2,4,5,7] but except for insulin which appears to result in a secondary activation of Na^+/K^+-ATPase following a stimulation of the Na^+/H^+ exchanger [2], the mechanisms involved have not been characterized. Earlier experiments have shown that the activation of Na^+/K^+-ATPase observed after incubation of hepatocytes with vasopressin is retained during the preparation of membrane vesicles from hormone-treated cells [7,8]. It is possible that a phosphorylation step involved in the transduction of the hormonal signal is responsible for the stability of this effect on Na^+ transport. Since vasopressin mobilizes cellular Ca^{2+} by activating phospholipase C and Ins(1,4,5)P_3 formation a Ca^{2+}/calmodulin-dependent phosphorylation is conceivable but others have concluded that a stimulation of the hepatic Na^+ pump may occur in the absence of changes in cytosolic and total cellular Ca^{2+} [4]. An involvement of a phosphorylation step catalyzed by protein kinase C in the

stimulation of Na^+ transport is also conceivable but appears difficult to reconcile with our present observations, because Ca^{2+}-mobilizing hormones have been shown to stimulate the formation of diacylglycerol in hepatocytes [24,25]: Our results indicate that an enhanced protein kinase C activity antagonizes the stimulatory effect of vasopressin on Na^+ transport. In addition they show that it is extremely difficult or even impossible to fully differentiate between stimulatory and antagonistic effects occurring simultaneously. In spite of the difficulties in separating stimulatory and antagonistic effects experimentally, they will be discussed separately in the following sections for the sake of clarity in presentation.

A direct stimulation of Na^+ transport has been observed in response to vasopressin, low concentrations of Ins(1,4,5)P_3 and unphosphorylated inositol (Figs. 1 and 6), whereas the stimulatory action of PI-PLC (Fig. 5) is indirect because it depends on the presence of the hormone. The direct effect of vasopressin may occur via G protein-dependent stimulation of endogenous phospholipase C and the formation of a mediator acting on the Na^+ transport system. Whether Ins(1,4,5)P_3 is the mediator remains questionable because of the unspecificity of its effect which is mimicked by inositol and other phosphorylated or possibly also unphosphorylated sugars. It appears reasonable to assume that the putative mediator is formed by membrane-associated enzymes from Ins(1,4,5)P_3 as well as from inositol or other sugars which may eventually become phosphorylated in the presence of ATP and Mg^{2+}. Several phosphatases and kinases involved in the metabolism of inositol phosphates may be present in our membrane preparation.

Indirect effects of vasopressin could explain the enhancement of Na^+ transport by exogenous phospholipase C in the presence of the hormone: Either PI-PLC is unable to split membrane phospholipids in the absence of vasopressin in spite of its ability to mobilize phosphatidylinositol-anchored enzymes or cyclic inositol 1,2-phosphate generated by the bacterial enzyme is converted to the active mediator only in the presence of the hormone. Alternatively, the fact that the stimulation of Na^+ transport by vasopressin is potentiated by low concentrations of bacterial phospholipase C may be explained by a sensitization of the transport system to the putative mediator.

If membrane vesicles are incubated with phorbol myristate acetate, the stimulation of Na^+ transport by vasopressin is decreased or totally suppressed (Fig. 2A). Since the phorbol ester has no effect on basal Na^+ transport if added alone, the term heterologous desensitization appears appropriate to describe its action. Comparably, high concentrations (50–100 mU/ml) of PI-PLC and Ins(1,4,5)P_3 also appear to desensitize the Na^+ transport to stimulation by vasopressin (Figs. 4A and 6A). Because EGTA partially prevents desensitization to Ins(1,4,5)P_3 (Fig. 6C) and since staurosporine (up to 25 nM) appears to counteract desensitization to vasopressin by high concentrations of phospholipase C (Fig. 4A and 5), we propose that membrane-associated protein kinase C mediates desensitization. The observation that PMA causes desensitization and that this effect is not shared by an analogue of the tumor promotor which is known to be unable to activate protein kinase C further supports this proposal, even if diacylglycerol and protein kinase C activity were not measured. However, activation of protein kinase C may not be the only mechanism of desensitization because EGTA and staurosporine together were only partially effective in enhancing the stimulatory effect of high concentrations of Ins(1,4,5)P_3 (Fig. 6D).

As pointed out before, we have never been able to clearly isolate the stimulatory effect experimentally by completely blocking desensitization and a close association between stimulation and inhibition may be physiologically important. A characteristic mixture of the two effects is observed in response to vasopressin. Depending the experimental conditions used the observed actions of the hormone appear to be the expression of agonistic and antagonistic effects, proceeding simultaneously and balancing each other to various extents, finally resulting in a more or less pronounced stimulation of Na^+ transport. If the balance observed in response to vasopressin alone or in combination with exogenous phospholipase C is disturbed by inhibiting the desensitization process by low concentrations of staurosporine the apparent stimulation is enhanced (Fig. 5). Conversely, the balanced stimulation by the hormone is shifted to virtually zero by adding PMA and favouring desensitization (Fig. 2A). These considerations exemplify the value of our experimental model which offers the opportunity to study a hormone sensitive subcellular system comprising all the signal transfer components between receptor and response element and including protein kinase C mediated 'crosstalk' as defined by Houslay [26]. With subcellular preparations containing only segments of the 'crosstalk' system the study of complex, multistep processes is more difficult because it has to be reconstituted in vitro with different components and in intact and permeabilized cells such studies may be impeded by toxic effects of inhibitors or agonists and by cell damage.

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