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Stimulatory Effects of Vanadate on Amylase Release from Isolated Rat Pancreatic Acini

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ABSTRACT. The effects of vanadate on exocrine pancreatic function were examined in isolated rat pancreatic acini. Vanadate caused a concentration-dependent stimulation of amylase release above a concentration of 1 mM. Co-incubation of vanadate with vasoactive intestinal polypeptide, 8-bromoadenosine 3′:5′-cyclic monophosphate, and the Ca^{2+} ionophore A23187 produced a synergistic pattern of amylase release, whereas co-incubation with cholecystokinin octapeptide (CCK-8), carbamylcholine, and 12-O-tetradecanoylphorbol 13-acetate produced an additive effect. Vanadate alone had no influence on acinar cyclic AMP content, Ca^{2+} efflux, or intracellular Ca^{2+} concentration. However, preincubation with vanadate prevented the plateau phase of CCK-8-induced Ca^{2+} transient increase from returning to baseline. Moreover, depletion of the intracellular Ca^{2+} pool by pretreatment of acini with CCK-8 in Ca^{2+}-free medium (plus ethyleneglycol bis[β-aminoethyl-ether]-*N*,*N*′-tetraacetic acid) had no effect on subsequent stimulation by vanadate, although it abolished the response to both CCK-8 and carbamylcholine stimulation. The protein kinase C (PKC) inhibitors staurosporine and calphostin C significantly inhibited vanadate-stimulated amylase release, whereas the protein tyrosine kinase inhibitor genistein had no inhibitory effect. Moreover, vanadate caused a significant translocation of PKC from cytosol to membrane fraction in pancreatic acinar cells. This translocation was inhibited significantly by staurosporine and calphostin C but not by genistein. These results suggest that vanadate acts directly on pancreatic acini and stimulates amylase release by activating PKC without an effect on Ca^{2+} mobilization, cyclic AMP, or protein tyrosine kinase. BIOCHEM PHARMACOL 55;5:677–685, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. vanadate; isolated pancreatic acini; amylase release; protein kinase C

Vanadium belongs to the group V transitional elements that exist in several valence states and is distributed widely in the animal and plant kingdom. Recent studies have demonstrated that vanadate has multiple biological effects including inhibition of (Na^+,K^+)-ATPase,§ Mg^{2+}-dependent, Ca^{2+}-activated ATPase (Ca^{2+}-ATPase), and PTPases in a variety of tissues [1–3]. Numerous effects of vanadate have been described in a number of tissues and attributed to changes in the activity of enzymes involved in phosphoryl-transfer reactions [4]. Vanadate has also been shown to activate certain signal transduction mechanisms [5]. Since pancreatic secretagogues, such as carbamylcholine and CCK, act via both DAG and Ca^{2+} to regulate pancreatic protein phosphorylation, presumably through activation of PKC and various calmodulin-dependent protein kinases and phosphatases [6], it is conceivable that pancreatic acini may represent a potentially important target for vanadate. However, the effects of vanadate on the exocrine pancreas have been evaluated in only one study using rat pancreatic tissue, which contains both exocrine and endocrine pancreas [7]. In that experimental system, vanadate was shown to stimulate amylase release through the release of calcium from an intracellular store.

In the present study, we characterized the direct effects of vanadate on pancreatic exocrine function, using rat pancreatic acini, and paid particular attention to signal transduction mechanisms.

MATERIALS AND METHODS

Chemicals

The following were purchased: carbamylcholine chloride, the calcium ionophore A23187, TPA, 8Br-cAMP, atropine, staurosporine, genistein, IBMX, HEPES, benzamidine, PMSF, β-mercaptoethanol, Nonidet P-40, EGTA, and soybean trypsin inhibitor (type 1-S) from the Sigma

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§ *Abbreviations:* ATPase, adenosine triphosphatase; PTPases, protein tyrosine phosphatases; CCK, cholecystokinin; DAG, diacylglycerol; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol 13-acetate; 8Br-cAMP, 8-bromoadenosine 3′:5′-monophosphate; IBMX, 3-isobutyl-1-methyl-xanthine; PMSF, phenylmethylsulfonyl fluoride; Fura-2/AM, acetoxymethyl ester of fura-2; CCK-8, CCK octapeptide; VIP, vasoactive intestinal polypeptide; KHB, Krebs-Henseleit bicarbonate buffer; HR, HEPES-buffered Ringer solution; LDH, lactic dehydrogenase; cAMP, cyclic AMP; and PKA, protein kinase A.

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Chemical Co.; calphostin C from the Kyowa Medics Co.; chromatographically purified collagenase (type CLSPA) from Cooper Biochemical; minimal Eagle's medium amino acid supplement from GIBCO Laboratories, Life Technologies Inc.; [^{45}Ca]Cl_2(1.5 GBq/mg Ca) and a PKC assay kit from Amersham International; Fura-2/AM from Dojinkagaku Laboratories; bovine plasma albumin (fraction V) from the Armour Pharmaceutical Co.; CCK-8 and VIP from the Peptide Institute, Protein Research Foundation; sodium orthovanadate (Na_3VO_4) from Janssen Chemica; a cAMP radioimmunoassay kit from the Yamasa Shoyu Co.; and a Phadebas amylase test (Amylase Test A) and an insulin radioimmunoassay kit from the Shionogi Pharmaceutical Co. Loxiglumide was supplied by the Kaken Pharmaceutical Co.

Animals

Male Wistar rats, weighing 250–280 g, were used throughout the experiments. The animals were kept at 23° on a 12-hr light–dark cycle with free access to water and a standard laboratory diet (CE-2 Kyudo).

Preparation of Isolated Pancreatic Acini

Isolated rat pancreatic acini were prepared by a method reported previously [8]. The medium used to prepare the isolated acini was modified KHB, containing 11.1 mM glucose, 0.1 mg/mL soybean trypsin inhibitor, and minimal Eagle's medium amino acid supplement, and was gassed with 95% O_2 and 5% CO_2. The preincubation and incubation medium was HR, and was similar to KHB but contained 10 mM HEPES (pH 7.4) as buffer and 5 mg/mL bovine plasma albumin. This HR was equilibrated with 100% O_2 and adjusted to pH 7.4.

Amylase Release

The acini were preincubated for 30 min at 37° in HR at a density of 1.0 to 1.5 mg acinar protein/mL while being shaken 60 times/min. After preincubation, the acini were centrifuged and resuspended in fresh HR at a density of 0.35 to 0.45 mg acinar protein/mL. Aliquots (2 mL) were distributed into 25-mL polycarbonate flasks. Amylase release during a 30-min incubation with various concentrations of vanadate was determined using a procedure reported previously [8]. As vanadate dissolved in water was alkaline, the pH of the acinar suspension was returned to 7.4 by the addition of HCl to the acinar suspension together with vanadate. The amylase-releasing effects of receptor-mediated secretagogues such as CCK-8, carbamylcholine, and VIP, and receptor bypassing agents such as TPA, A23187, and 8Br-cAMP were determined similarly in the presence or absence of 1 mM vanadate.

In another set of experiments, we examined the effects of the receptor antagonists atropine [9] and loxiglumide [10], the PKC inhibitors calphostin C [11] and staurosporine [12], and the protein tyrosine kinase inhibitor genistein [13] on vanadate-stimulated amylase release. Acini were preincubated with 1 μM staurosporine, 0.1 μM calphostin C, or 300 μM genistein for 30 min at 37°. After centrifugation, acini were resuspended in fresh incubation medium containing the same concentration of inhibitors and further incubated without or with 3 mM vanadate for 30 min at 37°. Since staurosporine has been shown to be a potent inhibitor of PKC activity, with complete inhibition of activity attained at a concentration of 1 μM [14], this concentration was chosen in the present study. Although calphostin C is a less potent but more specific PKC inhibitor than staurosporine, the concentration of 0.1 μM was chosen in the present study because we found in our preliminary study that calphostin C above 1 μM caused cell damage, demonstrated by increased release of amylase and LDH into the medium. Because a previous study has shown that 370 μM (100 μg/mL) genistein significantly inhibits amylase release from pancreatic acini [15] and because our preliminary study demonstrated that genistein above 500 μM caused cell damage, a 300 μM concentration was utilized in the present study.

To deplete the intracellular Ca^{2+} pool, the acini were incubated with 1 nM CCK-8 in Ca^{2+}-free HR containing 1 mM EGTA for 30 min at 37°. At the end of this pretreatment, acini were washed twice with fresh Ca^{2+}-free HR containing 1 mM EGTA and further incubated with 3 mM vanadate, 100 pM CCK-8, or 3 μM carbamylcholine in Ca^{2+}-free HR (plus 1 mM EGTA) for 30 min at 37°.

Amylase release in response to secretagogues was determined as previously described [8] and calculated as the percentage of the total content of enzyme in the acini at the beginning of the incubation. In all experiments, at least duplicate, but mostly triplicate, flasks were used to determine amylase release stimulated by each concentration of secretagogue.

Viability of Acini

The viability of acini after a 30-min incubation with vanadate was evaluated by determining LDH release into the incubation medium and by the trypan blue dye exclusion test. LDH activity was determined by the method of Wroblewski and LaDue [16].

Intracellular cAMP Content

cAMP content in the acini was measured according to a previously reported method [17]. Acini were incubated with 1 mM vanadate or 0.1 μM VIP in the presence of 1 mM IBMX for 30 min at 37°. At the end of the incubation period, 1 mL of sample was centrifuged at 3,000 × *g* for 10 sec. One milliliter of ice-cold 0.1 N HCl was added to each acinar pellet, the pellet was homogenized by ultrasonic disintegration (200 kHz, 10 sec), and cAMP and protein concentration were determined. The cAMP assay was carried out using a Yamasa cAMP radioimmunoassay kit.

Cellular cAMP was calculated relative to the protein concentration of the acinar pellet.

Intracellular Calcium Concentration

Intracellular calcium concentration was measured as described previously [18]. Acini were loaded with 10 μM Fura-2/AM in HR at room temperature for 1 hr. Then, acini were washed once with dye-free HR and kept at room temperature until used. A portion of the cell suspension was transferred to a perifusion chamber, the bottom of which consisted of a glass coverslip. This was placed on the stage of an inverted microscope, and perifusion with standard solution was begun. The volume of the perifusion solution in the chamber was kept constant at 130 μL by adding and withdrawing the solution at the same rate of 1 mL/min with a roller pump. Fluorescence intensity at an emission wavelength of 510 nm was measured in response to two excitation wavelengths at 340 and 380 nm. Digital imaging of Fura-2 fluorescence emitted during excitation at 340 and 380 nm was carried out with an inverted microscope and a digital image processor (Argus-50/CA; Hamamatsu Photonics) using a silicon-intensified target camera. Pairs of digital images were successively obtained at specified time intervals, and a ratio image from each pair was computed by the Argus 50.

Ca^{2+} Efflux

Efflux of $^{45}Ca^{2+}$ from the isolated pancreatic acini was measured as reported previously [8]. Acini were preincubated for 30 min at 37° in HR; then $^{45}CaCl_2$ (74 kBq/mL) was added and incubation continued for another 60 min. At the end of this loading period, the acini were centrifuged, washed once with ice-cold HR, resuspended in prewarmed HR, and further incubated in the presence or absence of 1 mM vanadate at 37°. Samples were taken at 0, 5, and 30 min and centrifuged at 10,000 × *g* for 20 sec in an Eppendorf microcentrifuge. Radioactivity in the medium was determined by liquid scintillation counting. At each time period, the $^{45}Ca^{2+}$ remaining in the acini was calculated as a percent of the $^{45}Ca^{2+}$ present at the beginning of the washout period.

PKC Enzyme Activity

Acini were incubated with the appropriate agents for 30 min at 37°. At the end of the incubation period, 1 mL of the sample was centrifuged at 3,000 × *g* for 20 sec. The acinar pellet was washed once with 1 mL of ice-cold solution A [50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 0.3% (w/v) β-mercaptoethanol, 10 mM benzamidine, 50 μg/mL PMSF]. The pellet was resuspended in 1 mL of ice-cold solution A, and then sonicated for 10 sec with a probe-type sonicator. The resulting suspension was centrifuged at 100,000 × *g* for 60 min at 4° and the supernatant was saved for assay of PKC (cytosol fraction),

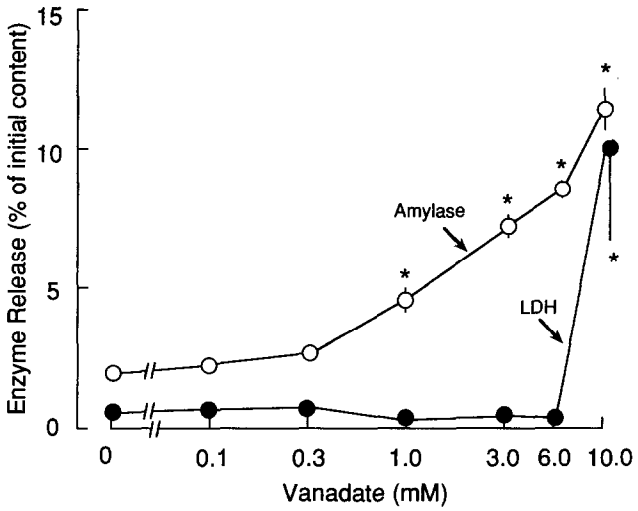

FIG. 1. Concentration dependence of amylase and LDH release stimulated by vanadate from isolated rat pancreatic acini. In the presence of various concentrations of vanadate, acini were incubated for 30 min at 37°. Amylase (○—○) and LDH (●—●) release into the medium over 30 min is expressed as a percentage of total enzyme activity initially present in acini. Values are the means ± SEM of three separate experiments. Key: (*) significant difference vs without vanadate ($P < 0.01$).

while the pellet was further homogenized with 1 mL of ice-cold solution A containing 1% Nonidet P-40 and left on ice for 1 hr. The homogenate was centrifuged at 100,000 × *g* for 60 min at 4°, and the supernatant was taken for assay of PKC (membrane fraction). PKC enzyme activity was measured using an enzyme assay kit from Amersham.

In another set of experiments, we examined the effects of the PKC inhibitors calphostin C and staurosporine and the protein tyrosine kinase inhibitor genistein on PKC translocation in response to 3 mM vanadate.

Statistics

Results are expressed as means ± SEM. The statistical significance of differences between means was assessed by Student's *t*-test or by ANOVA followed by Dunnett's test when more than two groups were compared. All *P* values <0.05 were considered significant.

RESULTS

Figure 1 shows the results of amylase secretion and LDH release from isolated rat pancreatic acini as a function of the concentration of vanadate. A significant increase in amylase release was seen at a vanadate concentration of 1 mM. With increasing concentrations of vanadate, amylase secretion increased up to 10 mM, the highest concentration examined. Concentrations of vanadate above 6 mM caused cell damage demonstrated by increased release of LDH into the medium. Thus, the subsequent experiments were carried out using 1 or 3 mM vanadate. At this concentration, all the acini remained intact as shown by the insignificant

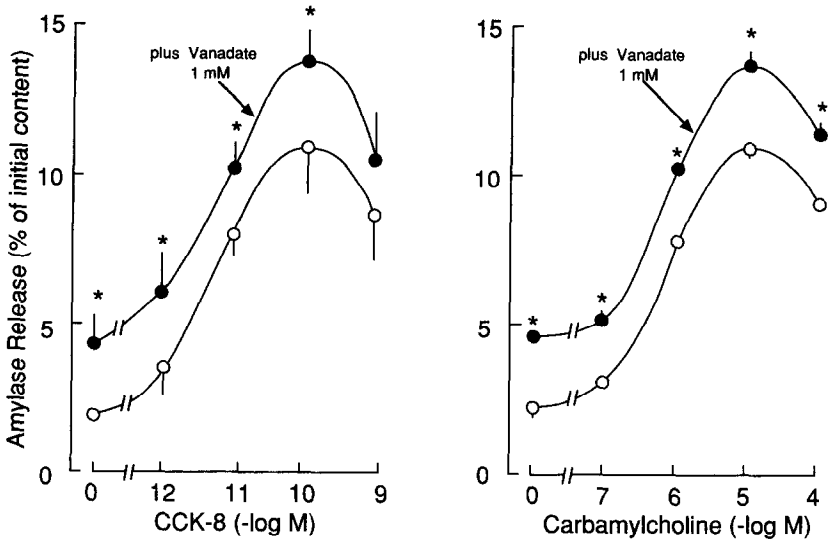

FIG. 2. Effect of vanadate on CCK-8 or carbamylcholine-stimulated amylase release from isolated rat pancreatic acini. Acini were incubated with various concentrations of CCK-8 (left) or carbamylcholine (right) in the absence (○—○) or presence (●—●) of 1 mM vanadate for 30 min at 37°. Amylase release is expressed as a percentage of total amylase activity initially present in acini. Values are the means ± SEM of five separate experiments. Key: (*) significant difference vs without vanadate ($P < 0.01$).

increase of LDH release (Fig. 1) and dye exclusion of trypan blue (data not shown).

The interaction between vanadate and both CCK-8 and carbamylcholine in stimulating amylase release was then studied (Fig. 2). Both CCK-8- and carbamylcholine-stimulated concentration–response curves were bell-shaped with high concentrations of secretagogue leading to secretion that was submaximal. For both secretagogues, the addition of vanadate produced an additive pattern of amylase release at every concentration of secretagogue without a shift in the concentration–response curve.

To confirm that vanadate was not activating either the CCK or muscarinic cholinergic receptors, we studied the effects of loxiglumide (10 μM) and atropine (10 μM), specific inhibitors of these two secretagogues, respectively [9, 10]. At concentrations completely blocking the effects of their respective agonists, neither loxiglumide nor atropine had an inhibitory effect on vanadate-stimulated amylase release (1 mM vanadate, 5.0 ± 0.7% vs plus 10 μM atropine, 4.6 ± 0.9%, NS; or vs plus 10 μM loxiglumide, 4.8 ± 0.5%, NS).

The interaction between vanadate and VIP in stimulating amylase release was then studied. The increase in amylase release caused by 1 mM vanadate plus 0.1 μM VIP was significantly greater than the sum of the increase caused by each agent alone, indicating a synergistic response (Table 1). Since the actions of VIP on pancreatic enzyme release are mediated by cAMP, we further studied the effects of 8Br-cAMP, a membrane-permeable synthetic analog of cAMP, on vanadate-stimulated amylase release. The addition of 8Br-cAMP to vanadate also caused an increase in amylase release of more than the sum of the response to each other (Table 1). These results suggest that vanadate acted via an intracellular transduction mechanism different from the cAMP pathway. To confirm that vanadate was not working through cAMP production, we then measured intracellular cAMP content after vanadate or VIP stimulation in the isolated rat pancreatic acini. VIP at a concentration of 0.1 μM increased intracellular cAMP concentration, reaching a level of 7-fold over the basal value (0.1 μM VIP, 17.1 ± 2.8 pmol/mg protein vs control, 2.5 ± 0.4 pmol/mg protein; $P < 0.001$). On the other hand, vanadate had no influence on intracellular cAMP concentration (1 mM vanadate, 2.5 ± 0.5 pmol/mg protein vs control).

Because CCK and carbamylcholine are known to elicit amylase release by phosphatidylinositol hydrolysis and DAG production resulting in intracellular Ca^{2+} mobilization and PKC activation [6], we then examined the interaction between vanadate and both the Ca^{2+} ionophore A23187 and the PKC activator TPA in stimulating amylase release. The increase in amylase release caused by 1 mM vanadate plus 1 μM A23187 was significantly greater than the sum of the increases caused by each secretagogue alone (synergistic effect), whereas that caused by 1 mM vanadate plus 1 μM TPA was the sum of the increases caused by each secretagogue (additive effect) (Table 1). An insignificant increase of LDH release and the exclusion of trypan blue indicated that synergistic release caused by

TABLE 1. Effect of vanadate on amylase release stimulated by various secretagogues

		Amylase release (% of initial content)	
		Alone	Plus vanadate (1 mM)
None		2.9 ± 0.3	4.6 ± 0.4*
VIP	(0.1 μM)	5.3 ± 0.1	10.0 ± 0.1*†
8Br-cAMP	(10 μM)	4.2 ± 0.4	8.6 ± 0.5*†
A23187	(1 μM)	3.6 ± 0.2	11.7 ± 0.6*†
TPA	(1 μM)	6.3 ± 0.2	8.4 ± 0.5*

Pancreatic acini were incubated with various secretagogues in the presence or absence of 1 mM vanadate for 30 min at 37°. Values are the means ± SEM from four separate experiments.

* Significant difference vs respective control ($P < 0.01$).

† Significant difference from additive response ($P < 0.05$).

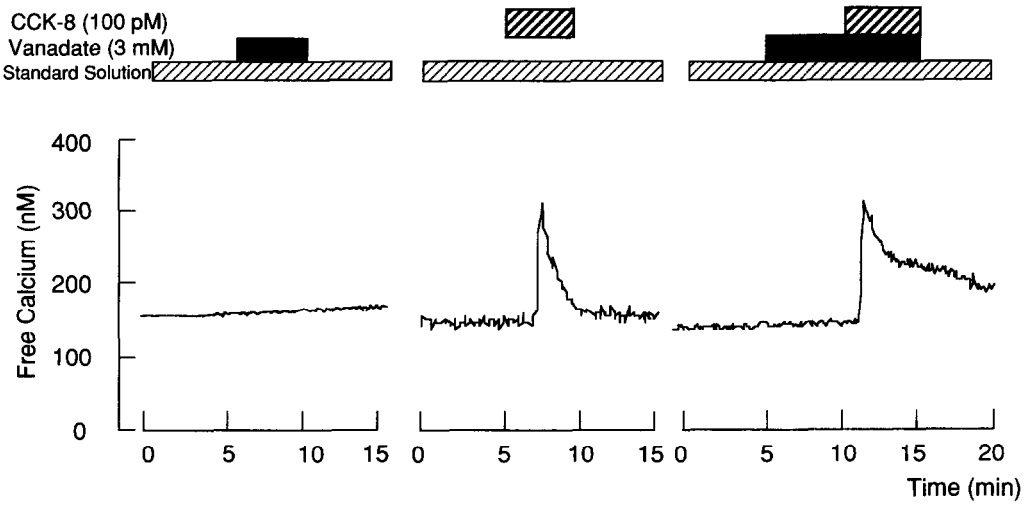

FIG. 3. Effects of vanadate or CCK-8 on cytosolic free calcium. Acinar cells were loaded with Fura-2/AM as described in "Materials and Methods" and were perfused with each agent in the time course indicated at the top of the figure. Intracellular calcium concentration was measured as described in "Materials and Methods." Results in this figure are representative of four other identical experiments.

vanadate plus A23187 was not due to cell damage (data not shown). These results suggest that vanadate acted via a mechanism similar to the DAG–PKC system but different from the Ca^{2+}-mediated pathway.

To further confirm that vanadate was not working via the Ca^{2+} pathway, we examined intracellular Ca^{2+} mobilization. Vanadate at a concentration of 3 mM did not alter intracellular Ca^{2+} concentration (Fig. 3, left panel) but 100 pM CCK-8 had its expected effect of increasing Ca^{2+} significantly (Fig. 3, middle panel). On the other hand, preincubation with 3 mM vanadate prevented the plateau phase of CCK-8-induced Ca^{2+} transient increase from returning to baseline (Fig. 3, right panel). However, a lower concentration of vanadate (1 mM) had no influence on CCK-8-induced intracellular Ca^{2+} mobilization (data not shown). Vanadate did not alter Ca^{2+} efflux at 5 min (percent remaining: 83.0 ± 2.5 vs control, 81.0 ± 2.8%; NS) or at 30 min (percent remaining: 54.8 ± 5.0 vs control, 67.8 ± 5.0%; NS). The possibility still remained that the action of vanadate was dependent on intracellular Ca^{2+}. We therefore depleted the intracellular Ca^{2+} pool by preincubation with CCK in the presence of EGTA [19]. Following this treatment, the effects of CCK or carbamylcholine were completely abolished completely, whereas the effect of vanadate was not reduced further (Fig. 4).

To further evaluate the possibility that vanadate acted via an activation of PKC, we examined the effect of the PKC inhibitor calphostin C and a more potent but less specific PKC inhibitor, staurosporine, on vanadate-stimulated amylase release. Calphostin C at a concentration of

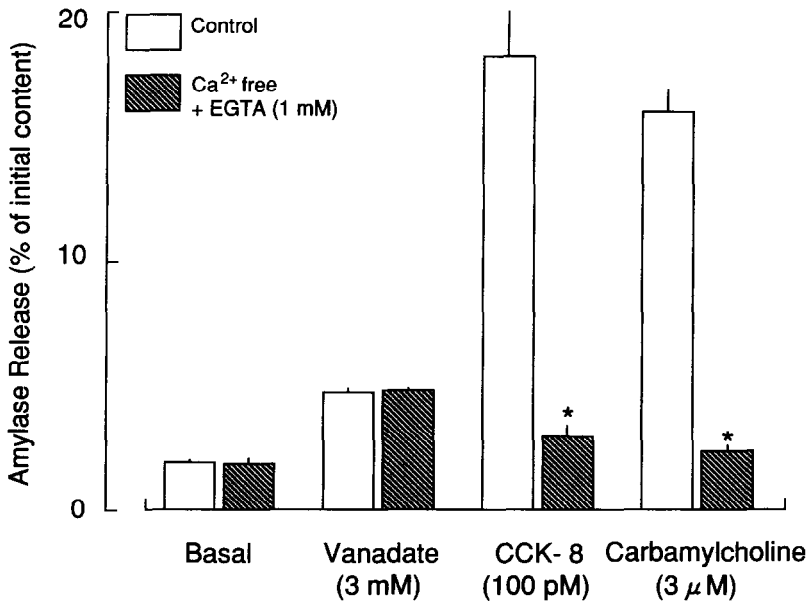

FIG. 4. Effect of Ca^{2+} removal on basal, vanadate-, CCK-8-, and carbamylcholine-stimulated amylase release from isolated rat pancreatic acini. Acini were incubated with 1 nM CCK-8 in Ca^{2+}-free HR containing 1 mM EGTA for 30 min at 37°. At the end of this pretreatment, acini were washed twice with fresh HR containing no added Ca^{2+} and 1 mM EGTA, and further incubated with 3 mM vanadate, 100 pM CCK-8, or 3 μM carbamylcholine in Ca^{2+}-free HR (plus 1 mM EGTA) for 30 min at 37°. Values are the means ± SEM of three separate experiments. Key: (*) significant difference vs respective control ($P < 0.05$).

TABLE 2. Effect of calphostin C and staurosporine on vanadate-stimulated amylase release

	Amylase release (% of initial content)			
	Alone	Plus calphostin C (0.1 μM)	Plus staurosporine (1 μM)	Plus genistein (300 μM)
None	1.9 ± 0.3	1.9 ± 0.1	1.8 ± 0.1	1.8 ± 0.3
Vanadate (3 mM)	7.3 ± 1.1	4.9 ± 0.4*	4.2 ± 0.4*	6.4 ± 0.9

Pancreatic acini were incubated with 0.1 μM calphostin C, 1 μM staurosporine, or 300 μM genistein for 30 min at 37°. After centrifugation, acini were resuspended in fresh incubation medium containing the same concentration of the inhibitor and further incubated without or with 3 mM vanadate. Values are the means ± SEM from four separate experiments.

* Significant difference vs vanadate alone ($P < 0.05$).

0.1 μM partially but significantly inhibited 3 mM vanadate-induced amylase release (Table 2). Staurosporine at a concentration of 1 μM significantly inhibited amylase release stimulated by vanadate. On the other hand, genistein, a specific inhibitor of tyrosine-specific kinases, at a concentration of 300 μM had no influence on amylase release caused by vanadate (Table 2).

We then investigated the effects of vanadate on the subcellular distribution of PKC. Vanadate at a concentration of 3 mM caused a significant redistribution of PKC enzyme activity from cytosol to membrane fraction (Fig. 5, Table 3). Moreover, the PKC inhibitors calphostin C and staurosporine, but not the protein tyrosine kinase inhibitor genistein, inhibited PKC translocation in response to 3 mM vanadate (Table 3).

DISCUSSION

Most tissues of higher animals contain vanadium in cells at concentrations varying between 0.1 and 1 μM [20–22]. It has been shown that vanadate has multiple biologic effects that are very diverse, depending on the target cells and the concentration of vanadate [23–27]. In the present study, we found that vanadate directly stimulates amylase release from isolated rat pancreatic acini in part by directly activating PKC but with yet other unknown intracellular mechanisms not involving cAMP production and Ca^{2+} mobilization.

The action of vanadate to induce amylase secretion appears to involve multiple mechanisms of which the major one bypasses the initial steps in the physiological stimulus–secretion coupling pathway. Normal amylase secretion from rat pancreatic acini is initiated by secretagogues binding to their specific receptors. The ligand–receptor complex then causes stimulation of phosphoinositide turnover with subsequent mobilization of intracellular Ca^{2+} and activation of PKC by DAG [6] and/or stimulation of cAMP formation with subsequent activation of PKA. It is currently believed that calcium and DAG act as intracellular messengers, activating, respectively, a calcium, calmodulin-dependent kinase and PKC [28–30].

Our current results suggest that vanadate bypasses the initial steps in stimulus–secretion coupling and directly activates the secretory mechanism that is normally con-

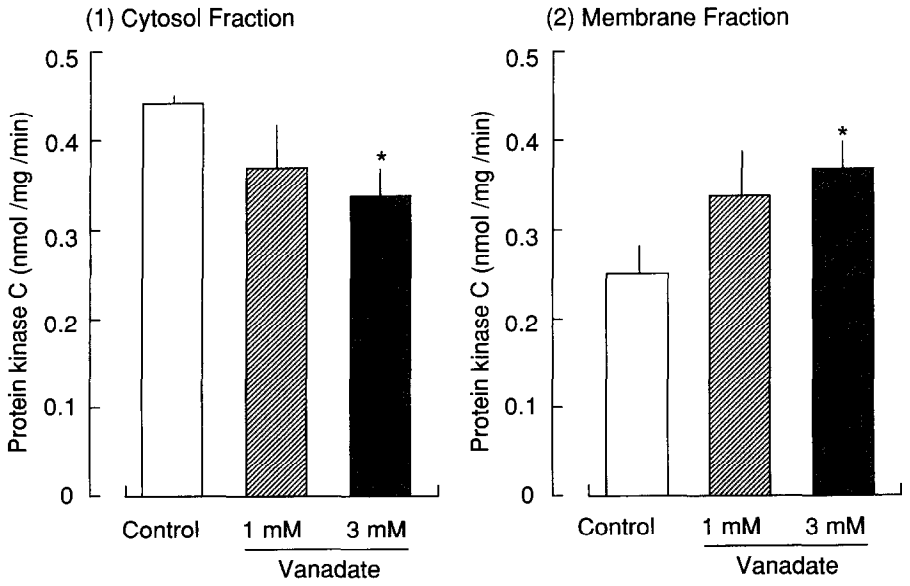

FIG. 5. Effect of vanadate on PKC enzyme activity in cytosol and membrane fractions from pancreatic acini. Acini were incubated with 1 or 3 mM vanadate for 30 min at 37°, and then PKC enzyme activity was determined in membrane and cytosol fractions. Values are the means ± SEM of four separate experiments. Key: (*) significant difference vs control ($P < 0.05$).

TABLE 3. Effects of calphostin C, staurosporine, and genistein on vanadate-induced PKC translocation

	PKC enzyme activity (nmol/mg/min)			
	None	Plus calphostin C (0.1 μM)	Plus staurosporine (1 μM)	Plus genistein (300 μM)
Cytosol fraction				
Control (none)	0.444 ± 0.01	0.431 ± 0.01	0.447 ± 0.02	0.442 ± 0.01
Vanadate (3 mM)	0.338 ± 0.03*	0.426 ± 0.01†	0.463 ± 0.04†	0.273 ± 0.04*
Membrane fraction				
Control (none)	0.251 ± 0.03	0.243 ± 0.01	0.251 ± 0.01	0.234 ± 0.01
Vanadate (3 mM)	0.368 ± 0.03*	0.246 ± 0.01†	0.275 ± 0.04†	0.377 ± 0.03*

Pancreatic acini were incubated with 0.1 μM calphostin C, 1 μM staurosporine, or 300 μM genistein for 30 min at 37°. After centrifugation, acini were resuspended in fresh HR containing the same concentration of inhibitor and further incubated without or with 3 mM vanadate. Values are the means ± SEM from four separate experiments.

* Significant difference vs respective control ($P < 0.01$).

† Significant difference vs 3 mM vanadate alone ($P < 0.01$).

trolled by PKC. Although vanadate could release amylase by an action on either the secretagogue receptors or the Ca^{2+} mobilizing mechanism, these possibilities do not appear to account for the major action of vanadate. First, the amylase releasing action of vanadate was blocked by neither loxiglumide nor atropine, well-known competitive antagonists of CCK and muscarinic receptors, respectively. Second, vanadate had no effect on $^{45}Ca^{2+}$ efflux and intracellular Ca^{2+} concentration. Third, the action of vanadate on amylase release was synergistic with the Ca^{2+} ionophore A23187.

However, the possibility still remains that vanadate has some effect on Ca^{2+} mobilization, because the action of vanadate was additive with CCK, carbamylcholine, and TPA. In support of this view, previous studies have demonstrated that vanadate evokes a transient increase in Ca^{2+} efflux and a rise in amylase release [7], and it inhibits Ca^{2+}-ATPase in both microsomes (intracellular Ca^{2+} pool) and plasma membrane isolated from pancreatic acini [31]. In the present study, however, vanadate was unable to alter intracellular free Ca^{2+} concentration or to stimulate $^{45}Ca^{2+}$ efflux. There are two possible explanations for these discrepant results. One is the difference in pH of the incubation medium. Ca^{2+} influx is enhanced in alkaline and inhibited in acidic conditions [32, 33]. In the present study, the pH of the acinar suspension was adjusted to 7.4. Indeed, we observed that vanadate evoked a transient increase in the intracellular Ca^{2+} concentration at pH 8.4 (unpublished observation). The other explanation is the difference of experimental materials. In the previous study [31], isolated microsomal vesicles from rat pancreatic acini were used, whereas in the present study we used intact acinar cells. The results of the current study indicate that the amylase releasing effect of vanadate is independent of intracellular Ca^{2+} (Fig. 4). It is conceivable, therefore, that vanadate acts in a manner different from thapsigargin, a well-known inhibitor of Ca^{2+}-ATPase in microsomes [34]. On the other hand, preincubation with vanadate prevented the plateau phase of CCK-8-induced Ca^{2+} transient from returning to baseline (Fig. 3, right panel). This indicates that vanadate inhibits Ca^{2+}-ATPase in plasma membrane and the Ca^{2+} efflux evoked by CCK-8. However, the possibility still remains that vanadate is involved in the Ca^{2+} channel in the plasma membrane and that Ca^{2+} influx from the extracellular Ca^{2+} pool is induced and/or maintained by vanadate.

Although vanadate is found to activate adenylate cyclase and to increase cAMP levels in certain tissues [35, 36], it has no direct effects on the cAMP-dependent protein kinase in hepatocytes [37] and on intracellular cAMP formation in pancreatic acinar cells (present study). Moreover, vanadate when combined with VIP or 8Br-cAMP caused a synergistic effect on amylase release (Table 1). It is unlikely that the changes in amylase release produced by vanadate involve the cAMP pathway. Taken together, it is likely that vanadate stimulates amylase release through a mechanism other than intracellular cAMP activation and the Ca^{2+} mobilization pathway.

Along with calcium, DAG plays a central role in regulating pancreatic acinar secretory activity as an intracellular messenger by activating PKC [6, 30]. Phorbol ester TPA can selectively and directly activate PKC and thus stimulates amylase release via a Ca^{2+}-independent cascade [38, 39]. Co-incubation of TPA with the Ca^{2+} ionophore A23187 in concert exerts a synergistic effect on amylase release in pancreatic acini [40]. In the present study, potentiation of amylase release was seen when vanadate was combined with the Ca^{2+} ionophore A23187, whereas only an additive effect was obtained when vanadate was combined with TPA. Our current results support the view that vanadate mimics the action of TPA on amylase release from isolated pancreatic acini.

A different approach is to directly inhibit the kinase using potent and selective inhibitors of PKC. Calphostin C is a specific PKC inhibitor [11], whereas staurosporine inhibits not only PKC but also cAMP- and cGMP-dependent kinase and tyrosine kinase activities, although with much more potency [14]. These agents significantly inhibited vanadate-induced amylase release. Since, however, the inhibitory effect of PKC inhibitors on vanadate-stimulated amylase release was partial, there is a possibility that other effectors or messengers in stimulus–secretion coupling are

involved in amylase release evoked by vanadate. In addition, there is a possibility that staurosporine reduced vanadate-stimulated amylase release by inhibiting tyrosine kinase as well as PKC, because a recent study has suggested the potential role of protein tyrosine phosphorylation in regulating pancreatic acinar cell secretion [15]. In contrast, a specific tyrosine kinase inhibitor, genistein, was unable to inhibit vanadate-stimulated amylase release.

Finally, we measured PKC enzyme activity in cytosol and membrane fractions from pancreatic acini. Vanadate caused a significant redistribution of PKC enzyme activity from cytosol to membrane fraction. Moreover, this translocation was inhibited significantly by the PKC inhibitors calphostin C and staurosporine but not by the protein tyrosine kinase inhibitor genistein. These results suggest that activation of PKC participates in the stimulation of amylase release brought about by vanadate. Recent biochemical, immunologic, and molecular cloning studies have demonstrated the existence of two classes of isoforms of PKC, i.e. Ca^{2+}-dependent and -independent isoforms. In the present study, we could not confirm which class of isoforms of PKC was activated by vanadate. Since, however, the amylase releasing effect of vanadate was independent of intracellular Ca^{2+} (Fig. 4), it is conceivable that vanadate releases amylase by Ca^{2+}-independent PKC isoforms.

In conclusion, we showed that vanadate stimulates amylase release in a concentration-dependent fashion by directly activating PKC. In addition, unknown intracellular mechanisms other than cAMP production and Ca^{2+} mobilization are likely to be involved in the action of vanadate.

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