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Oxidizing Effects of Vanadate on Calcium Mobilization and Amylase Release in Rat Pancreatic Acinar Cells

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ABSTRACT. The effects of vanadate were examined by monitoring intracellular free calcium concentration ($[Ca^{2+}]_i$) and amylase secretion in collagenase-dispersed rat pancreatic acinar cells. Vanadate increased $[Ca^{2+}]_i$ by mobilizing calcium from agonist-releasable intracellular calcium stores, since this increase was observed in the absence of extracellular calcium and vanadate failed to increase $[Ca^{2+}]_i$ after treatment with thapsigargin in calcium-free medium. Moreover, pretreatment of acinar cells with vanadate prevented the cholecystokinin octapeptide (CCK-8)-induced signal of $[Ca^{2+}]_i$, whereas co-incubation with CCK-8 potentiated the plateau phase of calcium response to CCK-8 without modifying the transient calcium spike. The effects of vanadate on calcium mobilization were reversed by the presence of the sulfhydryl reducing agent dithiothreitol. Vanadate also activated the calcium influx, since an additional enhancement of calcium influx induced by thapsigargin-evoked intracellular store depletion was observed and vanadate reversed the inhibitory effect of lanthanum (an inhibitor of calcium entry) into acinar cells. In addition, vanadate evoked a concentration-dependent release of amylase from pancreatic acinar cells and moreover, reduced the secretory response to CCK-8. We conclude that, in pancreatic acinar cells, vanadate releases calcium from the agonist-releasable intracellular calcium pool and consequently induces amylase secretion. These effects are likely due to the oxidizing effects of this compound. BIOCHEM PHARMACOL **58**;1:77–84, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. vanadate; calcium; amylase secretion; pancreatic acinar cells

Vanadate is a trace element distributed widely in the animal kingdom. Several studies have shown that vanadate has multiple biological effects in a variety of mammalian tissues [1]. In isolated tissues, vanadate causes contractions in various smooth muscles [2–4], induces platelet aggregation [5], and exerts a positive inotropic effect in ventricular muscle [6]. It also inhibits the activities of several enzymes, including Na^+, K^+-ATPase [2], Ca^{2+}-ATPase [7], and protein tyrosine phosphatases [3]. Vanadate has also been shown to activate certain signal transduction mechanisms [8]. It has been reported that some actions of vanadate are associated with alterations on calcium homeostasis, and that these alterations are mediated by the ability of vanadate to inhibit Ca^{2+}-ATPase [7].

A rise in $[Ca^{2+}]_i$† is an important early signal by which physiological secretagogues elicit the release of digestive enzymes from the pancreatic acinar cells. However, the effects of vanadate on the exocrine pancreas have been evaluated in only a few studies. Early studies by Case and co-workers [9, 10] showed that vanadate causes an acceleration of $^{45}Ca^{2+}$ efflux and stimulates enzyme secretion from pancreatic tissue through the release of calcium from an intracellular store. More recently, the presence of a non-mitochondrial intracellular vanadate-sensitive calcium pool has been reported in pancreatic acinar cells [11], although very recently it has been suggested that vanadate acts directly on pancreatic acini and stimulates amylase release by activating PKC without an effect on calcium mobilization [12].

The purpose of this paper was to study the effects of vanadate on calcium mobilization in collagenase-dispersed rat pancreatic acinar cells and the mechanisms underlying these effects by using a digital microspectrofluorimetric system. Additionally, amylase release was also measured for comparison.

MATERIALS AND METHODS

Materials

All reagents were obtained from Sigma Chemical Co. except collagenase CLSPA, which was obtained from Worthington Biochemical Corporation. As sodium or-

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† *Abbreviations:* $[Ca^{2+}]_i$, intracellular free calcium concentration; $[Ca^{2+}]_o$, extracellular free calcium concentration; CCK-8, cholecystokinin octapeptide; DTT, dithiothreitol; and PKC, protein kinase C.

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thovanadate dissolved in water was alkaline, the pH of this solution was returned to 7.4 by the addition of HCl.

Preparation of Isolated Rat Pancreatic Acinar Cells

Acinar cells were prepared by previously described methods [13]. In brief, pancreata were excised from overnight-fasted male Wistar rats (150–200 g) killed by severance of the vertebral column and acinar cells prepared by enzymatic digestion with collagenase. Acinar cells were then suspended in a physiological salt solution containing 1.0 mL/L essential amino acid mixture, 0.1 mg/mL soybean trypsin inhibitor and (in mM): 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 10 HEPES, 10 glucose, 1 $CaCl_2$. The pH was adjusted to 7.4 and equilibrated with 100% oxygen. Cell viability, monitored by trypan blue exclusion, was always greater than 95% and was not significantly reduced by secretagogues.

Measurement of Intracellular Ca^{2+}

Cell suspensions were incubated with 4 μM fura-2 AM (the acetoxymethyl ester form of fura-2) in presence of 0.025% pluronic acid at room temperature (20–22°) for 40 min and then washed and resuspended in fresh physiological salt solution. For quantification of fluorescence, aliquots of cell suspension were placed in a perifusion chamber placed in an inverted epifluorescence microscope and continuously superfused at a flow rate of 1.5 mL/min. To monitor changes in $[Ca^{2+}]_i$, samples were excited at 350/380 nm and the resultant emission at 505 nm was recorded by a cooled CCD camera using dedicated software (Hamamatsu). $[Ca^{2+}]_i$ values were calculated after calibration using a standard method [14].

Measurement of Amylase Release

To measure amylase secretion, aliquots (500 μL) of fresh acinar cells were incubated with appropriate secretagogue at 37° for 30 min followed by centrifugation at 1000 *g* for 30 sec. Amylase release was measured as described previously [15]. Amylase activities in the supernatant were determined using the Phadebas blue starch method [16] and expressed as percent of the total content of amylase at the beginning of the incubation.

Statistical Analysis

Values given are means ± standard errors of the mean (SEM). Statistical significance was calculated by one-way analysis of variance. $P < 0.05$ was considered as statistically significant.

RESULTS

In the presence of normal extracellular calcium, perifusion of pancreatic acinar cells with 1 mM vanadate elicited a gradual and slow increase in $[Ca^{2+}]_i$, with the peak value being reached after more than 25 min of exposure. Subsequent stimulation with 1 nM CCK-8 failed to induce the typical increase in $[Ca^{2+}]_i$ evoked by maximal concentrations of CCK-8 (31 of 40 studied cells from 4 experiments) (Fig. 1A). Similar results were obtained in the absence of extracellular calcium (calcium-free solution containing 0.5 mM EGTA) (Fig. 1B). It is worth noting that the cell was relatively impermeable to vanadate. This poor penetration of vanadate into the cell signified that either prolonged incubation or a very high concentration of vanadate was necessary to induce a biological effect [17, 18]. Interestingly, co-incubation of 1 mM vanadate with 1 nM CCK-8 did not significantly affect the transient calcium spike induced by 1 nM CCK-8, but the plateau phase of calcium response to CCK-8 remained elevated compared to the basal $[Ca^{2+}]_i$ (Fig. 2A), even in calcium-free solution (Fig. 2B).

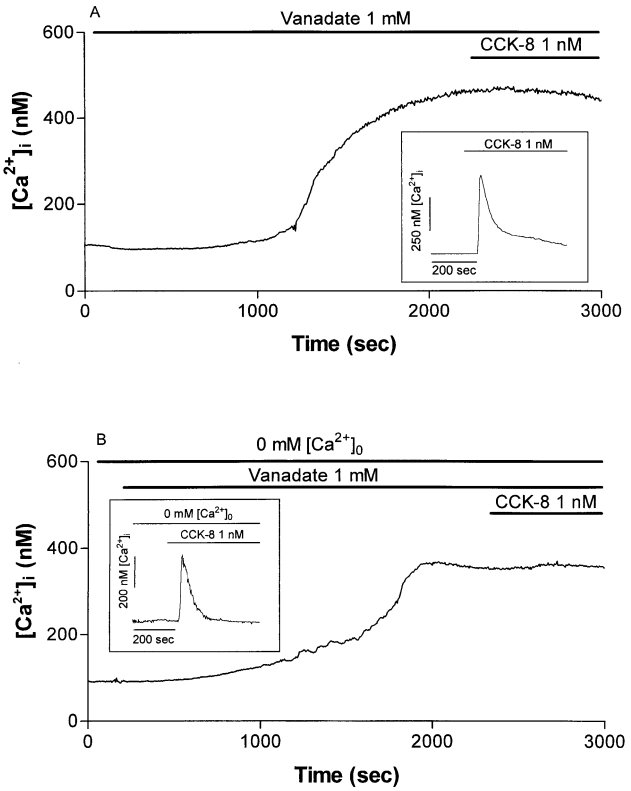

FIG. 1. Mobilization of calcium in response to CCK-8 in acinar cells pretreated with vanadate. Pancreatic acinar cells were initially perifused with 1 mM sodium orthovanadate followed by 1 nM CCK-8 in calcium-normal (A) or -free (B) solution. For comparison purposes, insets show the typical increase in $[Ca^{2+}]_i$ evoked by l nM CCK-8 in calcium-normal (inset of panel A) or -free (inset of panel B) solution. Traces are representative of 3–6 independent experiments employing 45–90 cells.

Since vanadate has been shown to be an oxidizing agent [19], we tested whether the sulfhydryl reducing agent DTT could prevent the effect of vanadate on calcium mobilization. Both the vanadate-induced calcium increase and the inhibitory effect of vanadate on CCK-8-stimulated calcium increase described in Fig. 1 were abolished by addition of 2 mM DTT to the perifusion medium (Fig. 3).

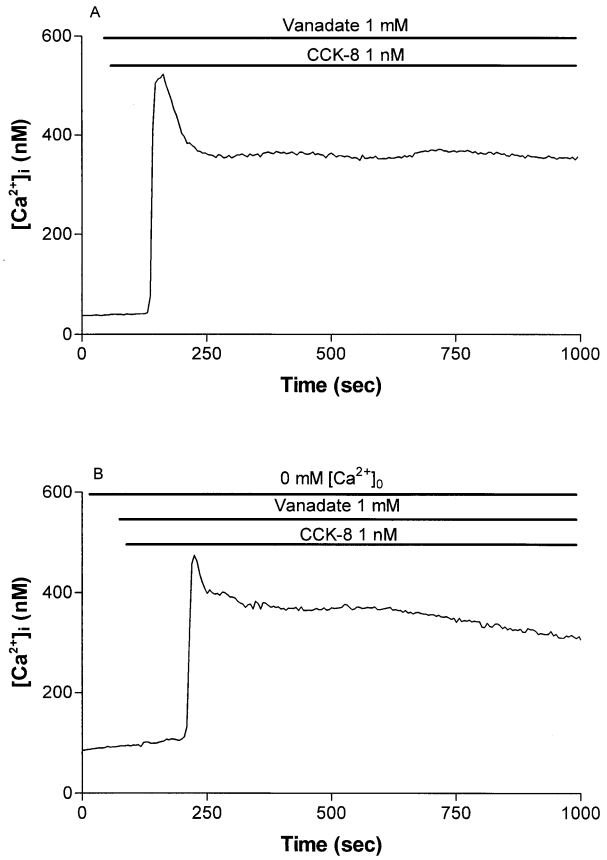

FIG. 2. Effect of vanadate on the plateau phase of calcium in response to CCK-8. Pancreatic acinar cells were co-incubated with 1 mM sodium orthovanadate and 1 nM CCK-8 in calcium-normal (A) or -free (B) solution. Traces are representative of 4–5 independent experiments employing 60–75 cells.

The application of 1 mM vanadate in acinar cells whose agonist-releasable calcium pools had previously been depleted by 0.5 μM thapsigargin in a calcium-free medium [20] is shown in Fig. 4. As expected, the application of

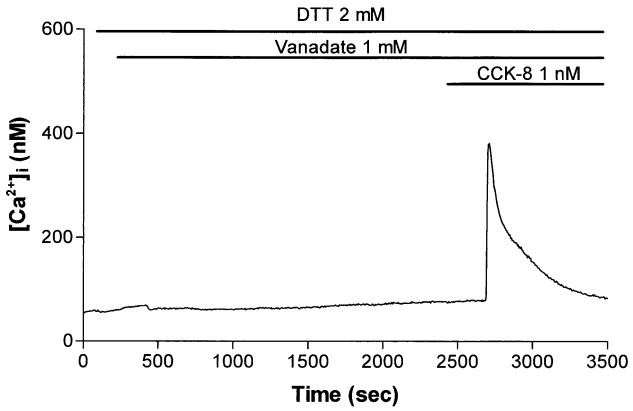

FIG. 3. Effect of DTT on vanadate-evoked calcium mobilization. Pancreatic acinar cells were perifused with 2 mM DTT followed by l mM sodium orthovanadate and 1 nM CCK-8. The trace is representative of 6 independent experiments employing 90 cells.

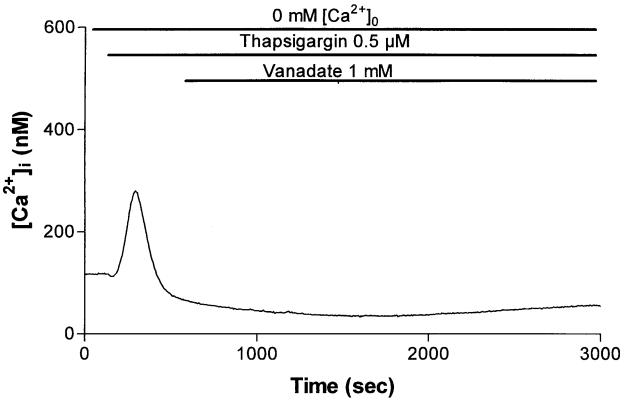

FIG. 4. Effect of thapsigargin-induced calcium depletion on vanadate-evoked calcium increase. Pancreatic acinar cells were perifused with 0.5 μM thapsigargin in calcium-free medium, followed by 1 mM sodium orthovanadate. The trace is representative of 5 independent experiments employing 75 cells.

thapsigargin induced a large transient increase in $[Ca^{2+}]_i$ followed by a rapid recovery of $[Ca^{2+}]_i$ to the prestimulation level as a result of the release and depletion of calcium from intracellular stores. Subsequent administration of vanadate was unable to induce a $[Ca^{2+}]_i$ increase. Similarly, vanadate was unable to release calcium following the depletion of intracellular calcium stores by a maximal concentration (1 nM) of CCK-8 [21] (data not shown).

To determine the need for extracellular calcium in vanadate-evoked calcium responses, the effects of the sequential applications of normal (1 mM $[Ca^{2+}]_o$) and free (0 mM $[Ca^{2+}]_o$) extracellular calcium in the presence of vanadate were evaluated. The results from this protocol are shown in Fig. 5. In the presence of normal extracellular calcium, vanadate induced an increase in $[Ca^{2+}]_i$; a subsequent imposition of zero calcium in the extracellular medium produced a small decrease in $[Ca^{2+}]_i$ (Fig. 5A). When calcium-free solution was applied before 1 mM $[Ca^{2+}]_o$ (Fig. 5B), it produced an additional increase in $[Ca^{2+}]_i$.

Since vanadate has been shown to increase the uptake of $^{45}Ca^{2+}$ into pancreatic lobules [9], we also evaluated the effect of vanadate on calcium influx induced by thapsigargin-evoked intracellular store depletion in dispersed pancreatic acinar cells. As shown in Fig. 6 (inset), the response to thapsigargin in a calcium-free medium was characterized by a transient increase in $[Ca^{2+}]_i$ followed by a rapid return to the basal value. On readmission of 10 mM calcium to the perifusion medium, a rapid increase in $[Ca^{2+}]_i$ was observed; this elevated level persisted, provided extracellular calcium was present, and was used as an indication of calcium influx [22, 23]. The effect of vanadate was assessed under these experimental conditions. As shown in Fig. 6, on readmission of calcium in the acinar cells pretreated with vanadate, an additional enhancement of calcium influx was observed. The first increase in $[Ca^{2+}]_i$ was due to the calcium gradient, while the second was probably due to the additional effect of vanadate on calcium influx. To

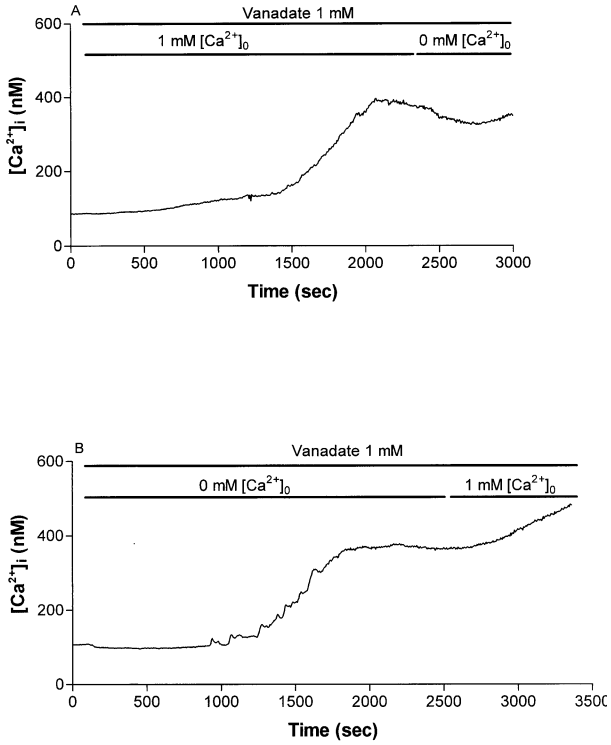

FIG. 5. Effect of extracellular calcium on vanadate-evoked calcium mobilization. (A) Pancreatic acinar cells were perifused with 1 mM sodium orthovanadate in calcium-normal solution (1 mM $[Ca^{2+}]_o$) followed by calcium-free solution (0 mM $[Ca^{2+}]_o$). (B) Pancreatic acinar cells were perifused with 1 mM sodium orthovanadate in calcium-free solution (0 mM $[Ca^{2+}]_o$) followed by calcium-normal solution (1 mM $[Ca^{2+}]_o$). Traces are representative of 3–5 independent experiments employing 45–75 cells.

further support the idea that vanadate modulates calcium entry, we investigated the inhibition calcium influx induced by the inhibitor of calcium influx lanthanum [24] in the presence or absence of vanadate. As expected, calcium depletion-stimulated calcium influx was markedly inhibited by 100 μM $LaCl_3$ (Fig. 7, inset). However, when vanadate was included in the perifusion medium prior to addition of lanthanum, a stimulation of calcium influx could be observed (Fig. 7), though this stimulatory effect was significantly ($P < 0.0001$) smaller than that obtained in the absence of lanthanum (see Fig. 6) (330 ± 13 nM vs 644 ± 36 nM).

Since the main function of pancreatic acinar cells is the secretion of digestive enzymes, we also wanted to examine the effect of vanadate (0.1–10 mM) on CCK-8-stimulated amylase secretion (Table 1). Similarly to calcium experiments, vanadate alone caused a significant increase in amylase secretion in a concentration-dependent manner. Additionally, vanadate inhibited the stimulation of amylase secretion induced by 320 pM CCK-8 (a dose that corresponds to maximal stimulatory response for this secretagogue on rat pancreatic amylase secretion; see Ref. 25). This inhibition proved to be statistically significant ($P < 0.05$) at higher concentrations of vanadate. On the contrary, the treatment with DTT, which was effective in blocking either the vanadate-induced calcium increase or the inhibitory effect of vanadate on CCK-8-stimulated calcium increase, did not abolish the effects of vanadate on either basal or CCK-8-induced amylase secretion. Since it has been suggested that vanadate stimulates amylase release in rat pancreatic acinar cells by activating the PKC [12], we tested this hypothesis by studying the effect of GF 109203 X, a potent and selective inhibitor of PKC [26], on amylase secretion stimulated by vanadate. As shown in Table 1, 5 μM GF 109203 X, an agent that itself does not modify basal amylase secretion, slightly decreased vanadate-stimulated amylase release, though not in a statistically significant manner; however, it was able to significantly inhibit the stimulation of amylase secretion induced by the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA), a stim-

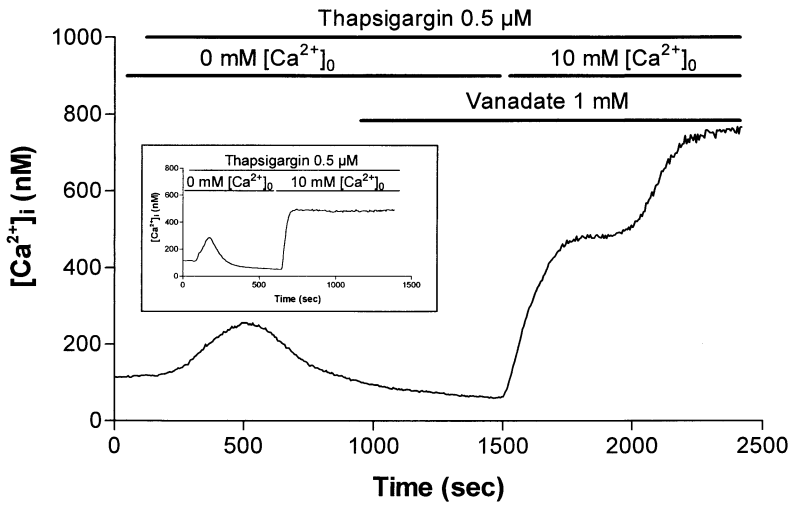

FIG. 6. Effect of vanadate on calcium influx induced by thapsigargin in calcium-free medium. Pancreatic acinar cells were perifused with 0.5 μM thapsigargin in calcium-free medium, followed by 1 mM sodium orthovanadate and 10 mM Ca^{2+}. The inset shows the typical calcium influx induced by thapsigargin-evoked intracellular store depletion. Traces are representative of 3–4 independent experiments employing 45–60 cells.

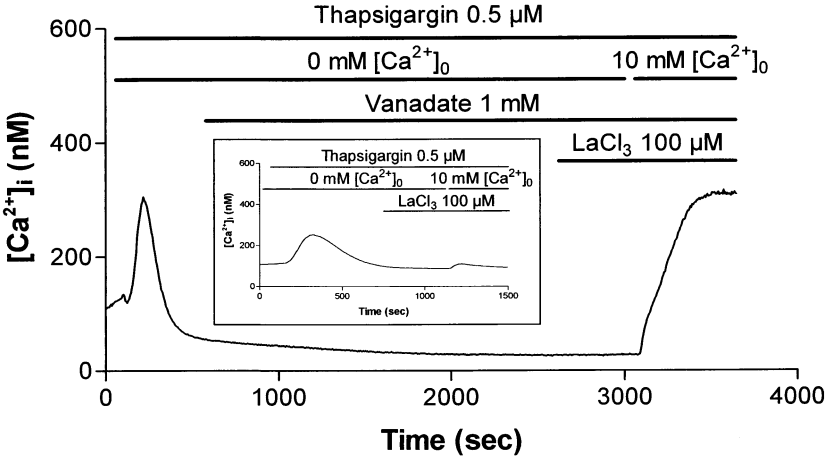

FIG. 7. Effect of vanadate on the inhibition of calcium influx induced by lanthanum. Pancreatic acinar cells were initially perifused with 0.5 μM thapsigargin in calcium-free solution, followed by 1 mM sodium orthovanadate, 100 μM $LaCl_3$, and 10 mM Ca^{2+}. For comparison purposes, the inset shows the typical inhibition of thapsigargin-induced calcium influx evoked by 100 μM $LaCl_3$. Traces are representative of 3–6 independent experiments employing 45–90 cells.

ulator of PKC [27] (10.3 ± 0.4 vs 16.1 ± 0.6% of total content, $P < 0.001$).

DISCUSSION

The present study shows that vanadate induced both a $[Ca^{2+}]_i$ increase and amylase release in rat pancreatic acinar cells. Our results also indicate that the $[Ca^{2+}]_i$ increase is mainly due to release from intracellular calcium stores, since it was observed in calcium-free solution. Our findings are consistent with the results obtained by Proffitt and Case [9, 10], who found that vanadate stimulated pancreatic secretion by an effect on an intracellular calcium store. Furthermore, it has been reported that pervanadate, which is generated by vanadate peroxidation in the presence of hydrogen peroxide, increases amylase secretion in differentiated AR4-2J pancreatic acinar cells [28]; however, our results are only partially similar to those of Hirohata *et al.* [12], who showed that vanadate stimulates amylase release without an effect on calcium mobilization. On the other hand, vanadate blocked CCK-8-evoked calcium release, which is correlated to the inhibition of CCK-8-induced amylase release. In addition, we present evidence that vanadate acts by a mechanism that involves sulfhydryl group oxidation.

In pancreatic acinar cells, the stimulatory effect of vanadate on $[Ca^{2+}]_i$ and its inhibitory effect on CCK-8-induced calcium mobilization could be due to a direct effect on the calcium release process and not a consequence of the opposing action in the calcium pathway. Vanadate has been widely used as a Ca^{2+}-ATPase inhibitor [1, 7]. Furthermore, the presence of a non-mitochondrial intracellular vanadate-sensitive [11] but thapsigargin-insensitive [29] calcium pool has been reported in pancreatic acinar cells. Our results, demonstrating that vanadate releases calcium from intracellular stores, suggest that the failure of CCK-8 to induce calcium mobilization after lengthy vanadate application is related to complete depletion of the stores by this agent. Our data also suggest that vanadate inhibits calcium uptake by an intracellular store, thereby elevating $[Ca^{2+}]_i$ and hence evoking amylase release. The vanadate-sensitive calcium pool is similar to that released by agonists, as indicated by the finding that vanadate failed to increase $[Ca^{2+}]_i$ after treatment with thapsigargin in calcium-free medium. On the other hand, co-incubation of vanadate with CCK-8 potentiates the plateau phase of

TABLE 1. Effect of vanadate on both basal and CCK-8-evoked amylase secretion

		plus vanadate (mM)		
Additions	**Alone**	**0.1**	**1**	**10**
None	4.6 ± 0.3	4.8 ± 0.3	5.8 ± 0.5*	7.0 ± 0.5*
CCK-8 (320 pM)	28.2 ± 1.1	26.4 ± 1.5	23.7 ± 1.4†	18.5 ± 1.5†
DTT (2 mM)	4.3 ± 0.2	5.4 ± 0.5	5.1 ± 0.5	7.8 ± 1.2
CCK-8 (320 pM) + DTT (2 mM)	27.5 ± 1.2	23.0 ± 1.3†	23.8 ± 1.4†	19.9 ± 2.0†
GF 109203 X (5 μM)	4.9 ± 0.4	4.1 ± 0.5	4.9 ± 0.6	6.2 ± 0.6

Amylase secretion was expressed as percent of the total content of amylase at the beginning of the incubation.
Results are means ± SEM from six separate experiments.
*Significantly different than the corresponding value alone.
†Significantly different than the value obtained with CCK-8 alone.

calcium response to CCK-8 without modifying the transient calcium spike. This observation is in agreement with a previous study in a rat thyroid cell line [23], where vanadate positively affected the plateau phase of noradrenaline-induced $[Ca^{2+}]_i$ increase without modifying the pattern of response, due to the activation of adrenergic receptor. Similar results have been observed in pancreatic acinar cells by Hirohata *et al.* [12], who found that vanadate prevented the plateau phase of CCK-8-induced calcium transient increase from returning to baseline. This stimulatory effect of vanadate on the plateau phase of the calcium response to CCK-8 could be due to the blockade of extrusion mechanisms of calcium, as indicated by the finding that vanadate was still able to positively affect the plateau phase of CCK-8 stimulation in the absence of extracellular calcium. In fact, vanadate has been shown to be an inhibitor of plasma membrane Ca^{2+}-ATPase pump in red cell [7] and smooth muscle [30] membrane. Taken together, our observations clearly suggest that vanadate inhibits both the Ca^{2+}-ATPase involved in the intracellular sequestration of calcium within the acinar cell (the mechanism probably involved in the effects of Figs. 1, 5, and 6) and the plasma membrane Ca^{2+}-ATPase pump (the mechanism probably involved in Fig. 2), thereby increasing $[Ca^{2+}]_i$.

The effects of vanadate on calcium mobilization were strongly blocked by the presence of the sulfhydryl reducing agent DTT. It has previously been shown that sulfhydryl-oxidizing reagents mobilize calcium in different cell types [31, 32]. In pancreatic acinar cells, it has been shown that the sulfhydryl-oxidizing agent thimerosal-induced calcium spikes were reversibly blocked by DTT [33]. Furthermore, it is known that sulfhydryl groups play a critical role in Ca^{2+}-ATPase function and that sulfhydryl-oxidizing reagents markedly inhibit ATP-dependent calcium uptake in liver plasma membrane fractions [34]. Our results, taken together with the known effects of vanadate as an oxidizing agent [19], suggest that the effects of vanadate on calcium mobilization are mediated by a mechanism that involves sulfhydryl group oxidation. Thus, the reduced form of DTT, which protects sulfhydryl groups from oxidation, blocked the effects of vanadate, presumably by reducing sulfhydryl groups. However, DTT was unable to block the effects of vanadate on either basal or CCK-8-induced amylase secretion. It has previously been reported that vanadate stimulates amylase release by activating PKC [12]. We tested this hypothesis using a selective inhibitor of PKC, GF 109203 X [26], on vanadate-evoked amylase secretion. However, our results indicate that GF 109203 X was also unable to abolish the stimulatory effect of vanadate on basal amylase secretion. Vanadate may well have multiple sites of action, and we cannot from our experiments exclude the possibility that vanadate may also act in a step of the terminal part of the exocytosis mechanism. In agreement with this hypothesis, it has been reported that vanadate, which has also been shown to be a powerful protein tyrosine phosphatase inhibitor [3], stimulates the secretion of amylase by increasing tyrosine phosphorylation of different proteins in a step distal to PKC [28]. Moreover, Lutz *et al.* [35] reported that the tyrosine phosphorylation of paxillin (protein is thought to be essential for regulated fusion of zymogen granules with the plasma membrane) induced by CCK is involved in the secretory response to the hormone.

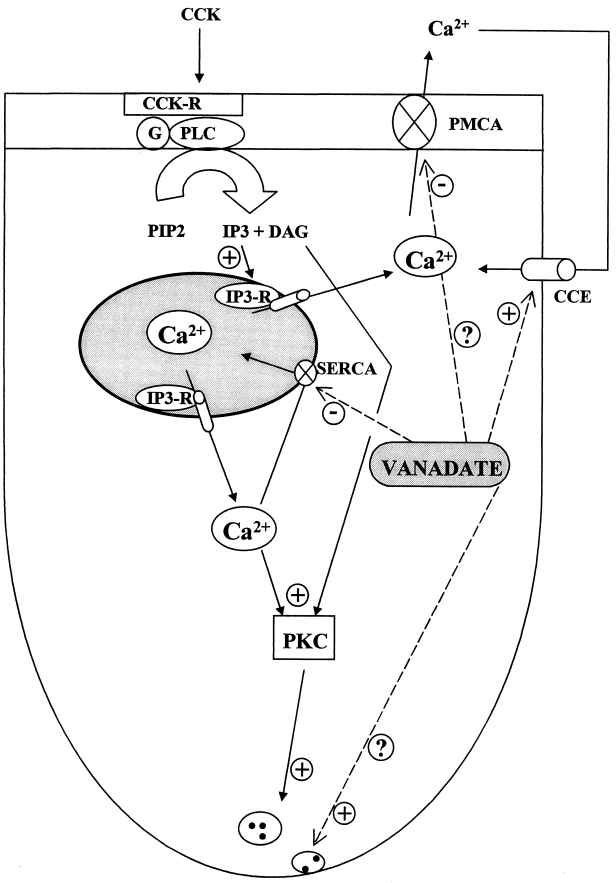

FIG. 8. A schematic model of a rat pancreatic acinar cell showing the different effects of vanadate on the stimulus–secretion coupling process. CCE, capacitative calcium entry; CCK-R, CCK receptor; DAG, diacylglycerol; G, G-protein; IP_3, inositol trisphosphate; IP_3-R, IP_3 receptor; PIP_2, phosphatidylinositol bisphosphate; PLC, phospholipase C; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase.

Finally, our studies of the effects of vanadate on calcium influx induced by thapsigargin-evoked intracellular store depletion indicate that vanadate caused an increase in calcium influx. Under our experimental conditions, vanadate induced an additional increase in calcium influx upon readmission of extracellular calcium in pancreatic acinar cells whose agonist-releasable calcium pool had previously been depleted by thapsigargin in a calcium-free solution. Moreover, vanadate reverses the inhibitory effect of lanthanum (an inhibitor of calcium entry) [24] on calcium influx. In this context, it is important to note that it has been widely reported that vanadate induces contractions in various smooth muscle [2–4], and that these contractions depend on the presence of extracellular calcium [36].

However, our data suggest that calcium mobilization induced by vanadate is independent of the presence of extracellular calcium in pancreatic acinar cells. The effects of vanadate were observed in calcium-free solutions, as mentioned above, although the sequential imposition of normal calcium (1 mM $[Ca^{2+}]_o$) in the extracellular medium produced a small increase in $[Ca^{2+}]_i$.

In summary, the results of this study (see Fig. 8 for main conclusions), taken together, provide further evidence that vanadate can be used as a pharmacological tool in calcium mobilization. Our findings show that the pretreatment of acinar cells with vanadate resulted in the release of calcium from agonist-sensitive intracellular stores and an inhibition of CCK-8-stimulated calcium increase, whereas co-incubation of vanadate with CCK-8 potentiated the plateau phase of calcium response to CCK-8. These effects of vanadate could be blocked by the use of DTT, indicating that its action is likely mediated by oxidation of sulfhydryl groups of Ca^{2+}-ATPases. The present results also suggest that vanadate has a stimulatory effect on calcium influx evoked by thapsigargin-induced store depletion. Additionally, these effects of vanadate on calcium mobilization are correlated, in large part, to vanadate-induced amylase secretion.

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