Insulinotropic Effects of Vanadate

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SUMMARY

Vanadium compounds are known to affect multiple membrane and cytosolic phosphoenzymes from various tissues; the most characterized effect is the inhibition of Na^+-K^+-ATPase. Since we previously reported that immunoreactive insulin (IRI) secretagogues tend to inhibit rat islet cation-dependent ATPases, we examined the effects of sodium vanadate on rat IRI secretion from incubated and perifused rat islets. In the presence of 2.4 mM Ca^{2+}, vanadate (10^{-3} M) induced biphasic IRI secretion with a background glucose of 100 mg/dl. In the absence of extracellular Ca^{2+}, IRI released from incubated islets by vanadate at 100 and 300 mg/dl glucose was doubled and tripled, respectively. Furthermore, this stimulatory effect was completely abolished by known inhibitors of IRI release such as somatostatin, epinephrine, and diphenylhydantoin. Although we found the expected dose-dependent inhibition by vanadate of islet membrane Na^+-K^+-ATPase activity, the mechanism of action of vanadate on IRI secretion remains unknown. Vanadate probably interacts in a complex fashion with different islet phosphoenzymes and may prove to be a useful probe to further unravel the mechanisms leading to insulin secretion. *Diabetes* 36:1448–52, 1987

Vanadium is a widely distributed element, the 21st most abundant in the Earth's crust (1). It has been found to be essential for normal growth and development in the chick and the rat (2). Although it is present in varying concentrations in most mammalian tissues (3), its biological role remains unclear. It appears that vanadium exists in body fluids in the 5+ oxidation state as vanadate (VO_3^-) and intracellularly in the 4+ state as vanadyl (VO^{2+}). Vanadate is structurally similar to phosphate (P_i) (4) and it has been suggested that the in vivo actions of VO_3^- may be due to its ability to mimic the structure of P_i in important phosphoenzymes involved in metabolism (5). Recently, VO_3^- has been shown to be a potent inhibitor of Na^+-K^+-ATPase (6,7), and it has been postulated that vanadium may be a physiological regulator of the Na^+-K^+ pump (8).

Work from our laboratory (9–11) and those of others (12–14) has suggested that modulation of cation-dependent ATPase activity may be involved in the process of insulin secretion. Immunoreactive insulin (IRI) secretagogues such as glucose, arginine, and ouabain inhibit islet Na^+-K^+-ATPase (9). Conversely, IRI secretory inhibitors such as diazoxide, diphenylhydantoin (DPH), and ammonium chloride enhance Na^+-K^+-ATPase activity (9).

Recently, there has been considerable interest in the insulin-like effects of vanadate. Vanadate stimulates glucose oxidation and transport in adipocytes, increases hepatic glycogenesis, and decreases liver gluconeogenesis and intestinal glucose transport (15). Furthermore, Heyliger et al. (16) have shown that vanadate administered for 4 wk was able to partially normalize the blood glucose of rats made diabetic with streptozocin with only marginal increases in their IRI levels. Although the authors believed vanadate to be acting as an IRI agonist, a concomitant stimulatory effect on IRI secretion was not excluded.

We thus examined the effects of sodium vanadate on IRI secretion by rat islets. We demonstrate that vanadate stimulates IRI release, particularly in the absence of extracellular Ca^{2+}. An inhibitory effect of vanadate on islet Na^+-K^+-ATPase is also shown.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Simonsen, Gilroy, CA) weighing 250–350 g were used. The animals were fed ad libitum and anesthetized with pentobarbital sodium (5 mg/100 g i.p.).

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TABLE 1
Effects of sodium vanadate (10^{-3} M) on insulin release (ng/ml) by rat islets incubated (10/tube) for 40 min in presence of different glucose concentrations with or without Ca^{2+}

	Glucose (mg/dl)			
	0	60	100	300
With Ca^{2+} (2.4 mM)(n)	5	5	16	6
Without sodium vanadate	2.52 ± 0.42	2.30 ± 0.73	6.20 ± 0.37	43.4 ± 7.3
With sodium vanadate (10^{-3} M)	2.36 ± 0.61*	3.27 ± 0.68*	7.80 ± 0.75†	49 ± 2.0*
Without Ca^{2+} (n)			6	7
Without sodium vanadate			2.50 ± 0.19	4.38 ± 0.64
With sodium vanadate (10^{-3} M)			5.15 ± 0.47‡	13.53 ± 1.91‡

*NS; †$P < .05$, ‡ $P < .001$, vs. insulin released in absence of sodium vanadate.

Islets were obtained by a modification of the method reported by Lacy and Kostianovsky (17). The pancreases of two rats were minced finely and suspended in 8 ml of Mg^{2+}-free Hanks' balanced salt solution (pH 7.3) at 37.5°C. Collagenase (Boehringer, Mannheim, FRG) was used in a three-step procedure. In the first period, the tissue was vortexed with 35 mg collagenase for 5 min. In the second period, 17.5 mg was used for 3 min. In the third, (when deemed necessary by visual inspection), 8.8 mg collagenase was employed for up to 3 min. Each period was followed by two washes with Hanks' solution. Finally, three washes were performed when the digestion was visually believed to be complete. Islets were then manually selected under a dissection microscope with a drawn-glass pipette.

Islet incubations. The islets were preincubated for 40 min in a Krebs-Ringer bicarbonate (KRB) buffer (Ca^{2+} 2.4 mM) with 0.5% bovine albumin and 60 mg/dl glucose (pH 7.35) in a Dubnov shaking water bath under an atmosphere of 95% O_2/5% CO_2. Next, 10 islets per test tube were incubated for 40 min with 1 ml KRB containing the additives indicated in RESULTS. At the end of this period, media was removed for insulin radioimmunoassay. Each data point was performed in triplicate or quadruplicate. In each experiment, control islets were incubated with high glucose (300 or 450 mg/dl) to assess viability and maximum responsiveness of the islet preparation. Data from experiments in which the IRI response of these control islets to high glucose stimulation was not at least fourfold the basal level were not included in final statistical assessment.

In the experiment with Ca^{2+}-free buffer, the islets were preincubated for 40 min in KRB plus Ca^{2+} as described above. Next islets were washed three times with Ca^{2+}-free KRB before being transferred to the Ca^{2+}-free incubation tubes. Less than 0.05 mM Ca^{2+} was present in these experiments as shown by measurements with a Perkin-Elmer atomic absorption spectrophotometer.

Islet perifusion. Eighty to 100 islets per millipore chamber were perifused with a flow rate of 1 ml/min via the method of Lacy et al. (18). We utilized a two-chamber system (Millipore filters, 5 μm pore diam) with one of the chambers receiving the vanadate-containing solution and the control chamber simultaneously perifused with the buffer without vanadate. Minute samples (1 ml/min flow rate) were collected from each chamber with a fraction collector and assayed for IRI. Background glucose concentrations and the timing of the vanadate infusion are described in RESULTS.

Immunoassay of insulin. Insulin was assayed by a solid-phase system, as described by Lundquist et al. (19).

Islet Na^+-K^+-ATPase activity. The method used has been previously described in detail (9). Briefly, after isolation, islets were collected in 1–2 ml of 0.25 M sucrose and 1 mM Tris-EDTA and frozen at −20°C. For each experiment, 1000 islets were used. Intact islets could be stored for up to 3 mo without loss of activity. After thawing, islets were homogenized manually in sucrose-Tris-EDTA at 4°C in a 2-ml Ten-Broeck tissue grinder with six or seven vigorous passes of the plunger. Homogenates were then centrifuged at 35,000 × g for 35 min in a refrigerated ultracentrifuge. Supernatants were discarded, and pellets were resuspended in 2.6 ml of cold 1 mM Tris-EDTA (no cation-dependent ATPase activities were found in these supernates nor were they measurable in 100,000 × g pellets of these supernatants). The volume used for subsequent assay, 0.1 ml of this solution, contained membrane tissue from 40 islets. This preparation was rapidly frozen in a test tube on dry ice in acetone and stored at −20°C. Enzyme assay was performed within 2–7 days of centrifugation to avoid loss of activity. At 4°C, 0.5 ml of incubation solution was added to 0.1 ml of the tissue homogenate suspension. This incubation solution consisted of 0.5 mM EDTA, 2 mM glycylglycine, 21 mM L-histidine, 3.6 mM $MgCl_2$, 100 mM NaCl, and 20 mM KCl. Ten microliters of test substance (various concentrations of sodium vanadate) or of 3 mM Tris buffer was then added. Duplicate samples were vortexed and preincubated at 37°C (pH 7.4) for 10 min.

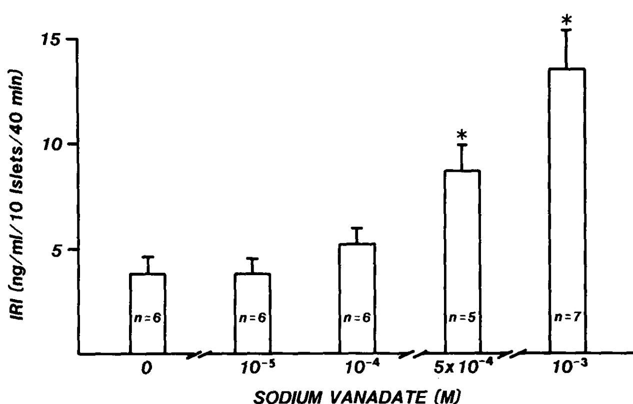

FIG. 1. Effects of different concentrations of sodium vanadate on immunoreactive insulin (IRI) secretion by islets incubated in presence of 300 mg/dl glucose and <0.05 mM Ca^{2+}. n = number of paired incubations. *$P < .01$ vs. no vanadate control.

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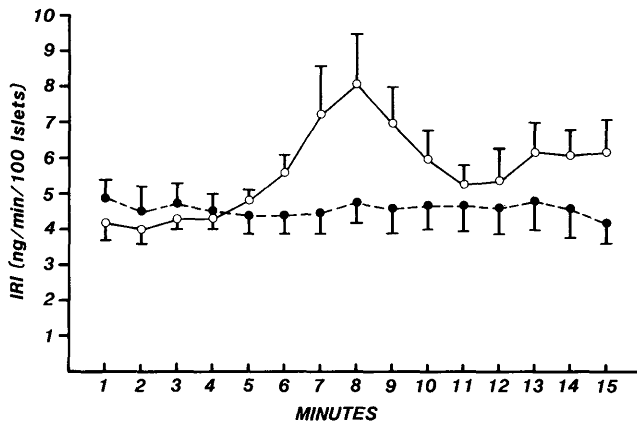

FIG. 2. Effects of sodium vanadate (10^{-3} M) on immunoreactive insulin (IRI) secretion in perifused rat islets in presence of 100 mg/dl glucose and 2.4 mM Ca^{2+}. Islets were perifused with 100 mg/dl glucose for 60 min before introduction of vanadate. n = 7 paired-chamber (control-vanadate) experiments. Mean summated secretion from 5–15 min was significantly different in vanadate (*solid line*) versus control chambers (*dashed line*) ($P < .025$).

Next, 50 μl Tris-ATP (final concn 3 mM, Sigma, St. Louis, MO) was added to all tubes. Samples were incubated in the shaker bath for 10 min at 37°C. All incubation tubes were then placed in an ice bath, and the reaction was stopped with 50 μl ice-cold 50% trichloroacetic acid. Correction for spontaneous hydrolysis (nonenzymatic) of ATP was made with tubes that did not contain tissue. Samples were then centrifuged at 1100 × g for 10 min, and supernatants were assayed for P_i with the method of Fiske and Subbarow (20). Na^+-K^+-ATPase activity was defined as the difference between the P_i liberated in the presence and absence of Na^+ and K^+. ATPase activity is expressed as micromoles of P_i released per milligram of protein per hour. Mean islet protein per incubation tube was 13.5 ± 0.65 μg.

Statistical analysis. All values are expressed as means ± SE. The effects of the different drugs or additives on insulin secretion were compared with the appropriate control incubations or perifusions with Student's paired or unpaired t test as appropriate.

RESULTS

Table 1 shows the effects of 10^{-3} M sodium vanadate on IRI release by rat islets incubated for 40 min at different glucose concentrations (0, 60, 100, and 300 mg/dl). In the presence of 2.4 mM Ca^{2+}, vanadate stimulated IRI release ($P < .05$) with a background of 100 mg/dl glucose but not significantly at other glucose concentrations. When Ca^{2+} was omitted, the stimulatory effects of vanadate were doubled and tripled, respectively, at both glucose levels tested (100 and 300 mg/dl; $P < .001$).

Figure 1 shows the insulin response to different vanadate concentrations by islets exposed to 300 mg/dl glucose in the absence of added Ca^{2+}. There was a stepwise increase in IRI released by the increasing vanadate concentrations, which became significant at 5 × 10^{-4} M ($P < .01$).

To determine whether vanadate was exerting toxic effects on the islets, they were incubated for 40 min in the presence of 100 mg/dl glucose with or without 10^{-3} M sodium vanadate. After a washout period (60 mg/dl glucose) of 40 min, they were exposed to 300 mg/dl glucose for a further 40 min. Islets that had been preexposed to vanadate secreted 24.6 ± 3.7 ng/ml (n = 12) in response to the glucose challenge versus 28.4 ± 3.3 ng/ml (n = 11) for control islets (not significant).

The effects of vanadate on the dynamics of IRI release in perifused islets can be seen in Fig. 2. Islets were perifused with 100 mg/dl glucose for 60 min before introduction of the test substance. At this time, islets were secreting IRI in a steady-state fashion. Summated IRI released by islets receiving the vanadate perifusate (5–15 min) was 44% greater than that of controls, 69.2 ± 8.1 vs. 48.2 ± 7.1 ng · ml^{-1} · 100 $islets^{-1}$, respectively ($P < .025$). Both first-phase IRI secretion (6–10 min) and second-phase release (11–15 min) were significantly stimulated by vanadate ($P < .05$).

Because vanadate-induced IRI secretion was more striking in the absence of extracellular Ca^{2+} and with 300 mg/dl glucose, the effects of various inhibitors of IRI release were examined under these conditions (Fig. 3). Somatostatin (5 μg/ml), epinephrine (1.8 μg/ml), and DPH (phenytoin, 50 μg/ml) were all similarly effective in blocking vanadate-induced IRI secretion. Although the DPH diluent (40% propylene glycol, 10% ethanol) also inhibited IRI release, the effect of DPH was significantly greater than that of its diluent alone.

Figure 4 shows a dose-dependent inhibition of Na^+-K^+-ATPase activity in islet membranes by sodium vanadate. We found no interference with the phosphate standard curve at 10^{-7}, 10^{-6}, or 10^{-5} M vanadate. However, 10^{-4} M vanadate interfered with color development by 20%.

DISCUSSION

We have shown that sodium vanadate elicits IRI secretion in a concentration-dependent manner from rat islets. This effect is evident with physiologic Ca^{2+} concentrations, and, with substimulatory background glucose, vanadate evokes a biphasic secretory pattern in a perifusion system. In the ab-

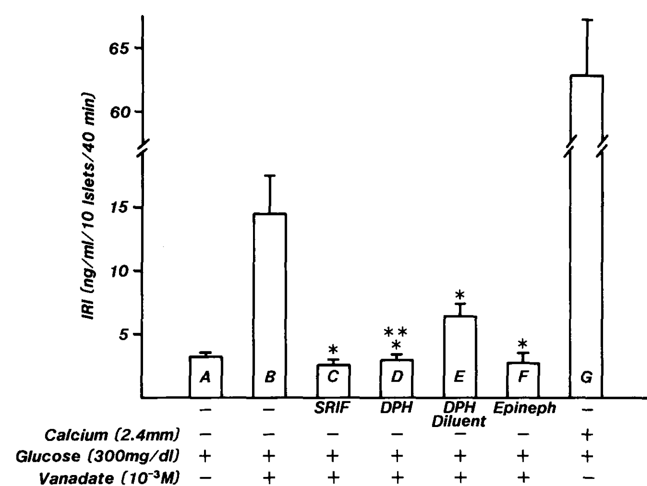

FIG. 3. Effects of somatostatin (SRIF), diphenylhydantoin (DPH), DPH diluent, and epinephrine on 10^{-3} M vanadate-stimulated insulin release in presence of 300 mg/dl glucose and in absence of added Ca^{2+} (<0.05 mM) in incubated islets. Results indicate means ± SE of 4 experiments. *$P < .05$ vs. *B*. **$P < .05$ vs. *E*.

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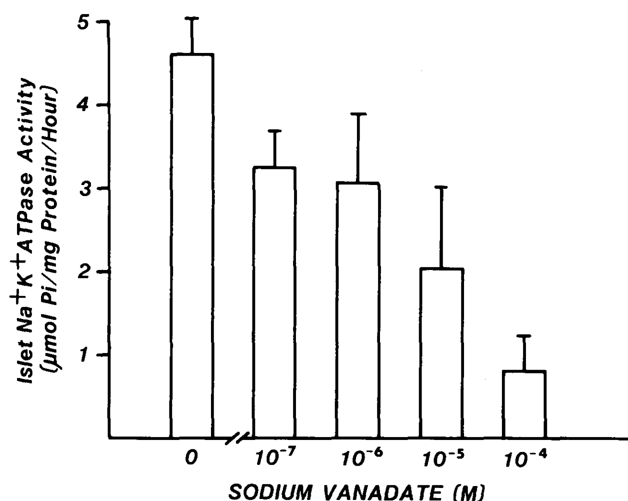

FIG. 4. Islet Na^+-K^+-ATPase activity in presence of different concentrations of vanadate. Each *point* represents mean ± SE of 3 separate experiments.

sence of extracellular Ca^{2+}, the stimulation of IRI release is greater than threefold at high glucose concentrations, and this effect can be abolished by known inhibitors of IRI secretion such as somatostatin, epinephrine, and DPH. Finally, as has been described in other tissues (1,6–8), vanadate exhibits a dose-dependent inhibition of islet Na^+-K^+-ATPase activity.

Recently, there has been increasing interest in the biochemical effects of vanadium compounds. At physiologic pH, vanadium exists predominantly as vanadate in extracellular fluids. Vanadate may be reduced intracellularly to vanadyl, putatively by cellular glutathione and/or catechols (5). Both oxidation states of the metal influence numerous phosphoenzymes. The most characterized effects of vanadate are those on the membrane Na^+-K^+-ATPase (6–8). This enzyme is more sensitive to vanadate than to vanadyl, which has prompted the speculation that vanadium may be a regulator of the Na^+-K^+ pump via an intracellular redox mechanism (8). However, when speculating on the mode of action of vanadium, note that this metal has been found to influence many other phosphoprotein systems such as ribonucleases (4) and adenylate cyclase (21) from various tissues. More recently, Tamura et al. (22) have demonstrated that the known insulin-like effect of vanadate on rat adipocytes is at least partly secondary to phosphorylation of the IRI receptor. Thus, when examining the complex mechanisms of action of vanadium on a particular target tissue, the fact that different oxidation compounds of the element may be acting simultaneously on different enzymatic reactions must be considered.

Several facts suggest that the vanadate effects on IRI secretion were not due to toxicity. First, islets preexposed to 10^{-3} M vanadate were capable of secreting equivalent amounts of IRI after a washout period in response to high glucose compared with control islets not preincubated with vanadate. Furthermore, the existence of a dose-dependent stimulation of IRI secretion in response to increasing concentrations of the metal and the fact that known inhibitors of IRI release were able to block the vanadate effect argue against the presence of "leaky" or irreversibly damaged islets. Note that the concentrations of sodium vanadate used to promote IRI release were on the order of 0.5–1 mM, higher than those believed to occur physiologically in extracellular fluids (23). As has been reported, free intracellular vanadate would be expected to be completely reduced to vanadyl with existing cellular concentrations of glutathione (5). However, at high but nontoxic concentrations of vanadate (1 mM), effects compatible with those of free intracellular vanadate on the Na^+-K^+-ATPase can be obtained in cultured heart cells (24). This suggests that under some conditions, cells can maintain the vanadate ion intracellularly. Because this oxidation state of vanadium may exist normally in the process of cellular redox events (5,8), our experiments exemplify one circumstance in which high concentrations of a substance in vitro might help to explore its potential physiological importance. As shown in Figs. 1 and 4, vanadate has a more powerful effect on islet Na^+-K^+-ATPase than on IRI secretion. In other systems, VO_2^+ has been found to be a weaker inhibitor of Na^+-K^+-ATPase than VO_3^- (25). If this is the case in islet membranes, the dose-response curves of vanadate on IRI release and vanadyl on Na^+-K^+-ATPase activity may more closely approximate each other, providing further grounds to suggest that modulation of Na^+-K^+-ATPase activity may play a role in vanadate-induced IRI secretion.

Vanadate was relatively ineffective in stimulating IRI release at low glucose concentrations. The glucose dependency of the vanadate-induced IRI secretion suggests that vanadate may be magnifying some effect of glucose, probably involving intracellular mobilization of Ca^{2+} or interference with Na^+-Ca^{2+} exchange. Redistribution of Ca^{2+} within cellular compartments is fundamental to the secretory process, and it is reasonable to speculate that vanadate may be affecting Ca^{2+} translocation. Furthermore, acting as an inhibitor of Ca^{2+}-ATPase (1) either in the plasma membrane or in the endoplasmic reticulum, vanadate could influence Ca^{2+} efflux or intracellular Ca^{2+} mobilization. Indeed, islets possess Ca^{2+}-ATPase (10,14), the inhibition of which may be a mechanism coupled to IRI secretion (9).

At concentrations $>10^{-5}$ M, vanadate has been found to enhance adenylate cyclase activity from various sources (21,26). Because cAMP generation can promote IRI secretion in the absence of extracellular Ca^{2+} (27), vanadate may be stimulating IRI secretion through this mechanism. Furthermore, somatostatin and epinephrine, which antagonized the vanadate stimulation of IRI release, have been reported to inhibit islet cAMP generation (28–30).

Note that islets exposed to either ouabain or K^+ depletion, conditions known to inhibit Na^+-K^+-ATPase activity, show a similar pattern of IRI stimulatory activity as we report herein (31). Ouabain evokes modest biphasic IRI release in the presence of 1 mM Ca^{2+} and 300 mg/dl glucose. In the absence of added Ca^{2+}, however, the stimulation is three- to fourfold. A similar stimulation of IRI release occurs when both Ca^{2+} and K^+ are omitted from the media (31). Because Siegel et al. (31) also encountered an inhibition of Ca^{2+} efflux in response to the above conditions, they speculated that interference with Na^+-Ca^{2+} exchange by ouabain and K^+ depletion may induce IRI secretion.

Somatostatin, DPH, and epinephrine were equally capable of blocking the vanadate effect on IRI release in the absence

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of Ca^{2+}. This indicates that the sites of action of these inhibitors were distal to that of vanadate. Because the concentration of epinephrine used was relatively high, we cannot exclude that it acted as a reducing agent converting VO_3^- to VO_2^+ or by binding VO_3^- as has been reported in other systems (32). In contrast with sodium vanadate, however, somatostatin and DPH enhance islet Na^+-K^+-ATPase activity (9,11). Catecholamines have been found to reverse the vanadate inhibition of Na^+-K^+-ATPase in human red cell membranes (33). Thus, the coordinate effects of these agents on IRI release and on the Na^+-K^+ pump suggest that modulation of Na^+-K^+-ATPase may be a central event coupled to the β-cell secretory process.

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