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Insulin-like effects of vanadate on glucose uptake and on maturation in *Xenopus laevis* oocytes

Pierre Hainaut, Sophie Giorgetti, Aline Kowalski, and Emmanuel Van Obberghen
Institut National de la Santé
et de la Recherche Médicale, U145
Faculté de Médecine
F-06034 Nice Cedex, France

Vanadate, an inhibitor of phosphotyrosyl phosphatases that exerts insulin-like effects in intact cells, stimulated both maturation and glucose uptake in isolated *Xenopus laevis* oocytes. Vanadate enhanced the effects of insulin/IGF-I and progesterone on maturation in a dose-dependent manner, with an effective concentration of 750 μM and a maximum at 2 mM, whereas, in the absence of hormone, activation of maturation was seen at 10 mM vanadate. Further, vanadate at 2 mM increased glucose uptake, but this effect was not additive to that of the hormone. In cell-free systems, vanadate caused a 12-fold stimulation of autophosphorylation of the oocyte IGF-I receptor in the absence, but not in the presence, of IGF-I and inhibited largely, but not totally, receptor dephosphorylation induced by an extract of oocytes rich in phosphotyrosyl phosphatase activities. These effects were dose dependent, with effective concentrations of 50–100 μM and maxima at 2 mM. Moreover, using an acellular assay to study the effect of vanadate on the activation of maturation promoting factor (MPF), we found that vanadate at 2 mM stimulated the activation of the MPF H1 kinase. This suggests that vanadate did not prevent dephosphorylation of $p34^{cdc2}$ on tyrosine residues. Vanadate thus exerted insulin-like effects in oocytes, including stimulation of maturation. These effects might result from a direct or indirect action of vanadate on the IGF-I receptor kinase and on MPF activity.

Introduction

Vanadate (VO_4^{3-}) exerts insulin-like effects in many cell types. Added at millimolar concentrations in the culture medium, vanadate increases glucose transport and glycogen synthase activity in rat adipocytes (Tamura *et al.*, 1983, 1984) and decreases the expression of the gene for phosphoenolpyruvate carboxykinase in rat hepatoma cells (Bosch *et al.*, 1990). Moreover, it normalizes blood glucose levels when administered to streptozotocin-induced diabetic rats (Meyerovitch *et al.*, 1987). However, prolonged incubation of cultured cells with 1 mM vanadate resulted in cell rounding after 3–4 h and in cell death after 12 h, indicating that vanadate might transform the cells and even be toxic at high concentrations (Klarlund, 1985). Therefore, considerably lower concentrations have to be used to study the effects of vanadate over long periods of time (1–5 d). For example, vanadate at 5–100 μM regulates the expression of the glucose transporter Glut-1 in fibroblasts (Mountjoy and Flier, 1990), stimulates mitogenesis (Carpenter, 1981; Bingham Smith, 1983), and induces morphological transformation in several cell types (Klarlund, 1985). Vanadate in solution resembles inorganic phosphate and may interfere with many reactions involved in incorporation or removal of phosphate groups. However, vanadate is mostly known as a specific inhibitor of most, but not all, phosphotyrosyl phosphatases (PTPases) when used in the micromolar range in cell-free systems (Leis and Kaplan, 1982; Swarup *et al.*, 1982a,b; Brunati and Pinna, 1985; Mustelin *et al.*, 1989). In addition, vanadate stimulates the tyrosine kinase activity of insulin receptors from rat adipocytes (Tamura *et al.*, 1983, 1984).

The fate of intracellular vanadate is unclear. In adipocytes, vanadate uptake is linear over long periods and even exceeds the expected equilibrium concentration, suggesting that vanadate is either sequestered or modified, or both (Dubyak and Kleinzeller, 1980). Indeed, after entry into cells, most of the vanadate is reduced to the vanadyl ion (Cantley and Aisen, 1979), which binds to proteins and may exert actions that differ from those induced by the original orthovanadate. Although only little is known concerning the action of vanadyl on PTPases in cell-free systems, it is largely accepted that vanadate added to the external medium increases phosphotyrosine in cells by inhibiting intracellular PTPases (Klarlund, 1985; Lau *et al.*, 1989).

In this study, we have examined the effects of vanadate on glucose uptake and maturation in *Xenopus laevis* oocytes, a model system to investigate cellular signaling and growth control (Maller, 1990). In oocytes, glucose uptake and maturation are triggered by insulin and IGF-I through IGF-I receptors (Maller and Koontz, 1981; Hainaut *et al.*, 1991a). Glucose uptake occurs through transporters of the Glut-1 type and shows similarities with the facilitated diffusion glucose transport of higher vertebrates (Janicot and Lane, 1989; Vera and Rosen, 1989; Hainaut *et al.*, 1991b). Maturation is a multistep process during which oocytes, arrested at the G2/M border, resume cell division and progress to the M phase. The central event in this process is the activation of maturation promoting factor (MPF), which is a complex of a 34-kDa serine/threonine kinase, $p34^{cdc2}$, and a B-type cyclin of 45 kDa (reviewed by Maller, 1990). In G2, MPF exists in a latent form, in which $p34^{cdc2}$ is phosphorylated on both tyrosine and threonine residues and is associated with cyclin in an inactive complex. During the G2 to M transition, the cdc2 kinase is activated through dephosphorylation of $p34^{cdc2}$ by still unidentified phosphatases, and phosphorylatin of cyclin by $p34^{cdc2}$ and/or other kinases such as c-*mos* (Gautier *et al.*, 1989, 1990; Gould and Nurse, 1989; Morla *et al.*, 1989; Nurse, 1990; Roy *et al.*, 1990). Therefore, we were interested in determining whether vanadate would exert insulin-like effects and thus stimulate maturation or, alternatively, would block $p34^{cdc2}$ dephosphorylation and, by doing so, inhibit maturation.

We show here that addition of millimolar concentrations of vanadate enhanced maturation induced by insulin, progesterone, or microinjected MPF. Vanadate also stimulated glucose uptake, but this effect was not additive to that of insulin/IGF-I. In cell-free systems, vanadate increased the autophosphorylation of the oocyte IGF-I receptor, inhibited its dephosphorylation, and enhanced the conversion of latent MPF from G2-arrested oocytes to the active form. Taken together, these results show that vanadate is not an inhibitor of oocyte maturation and suggest that vanadate exerts positive effects on the complex signaling cascade leading to MPF activation.

Results

Effect of vanadate on maturation

Maturation of *Xenopus* oocytes occurs 6–12 h after stimulation by IGF-I or progesterone and is evidenced by the appearance of a white spot at the animal pole of the oocyte, which follows germinal vesicle breakdown (GVBD). Each of these agents acts via specific receptors (Maller and Krebs, 1977; Maller and Koontz, 1981, Hainaut *et al.*, 1991a) and uses pathways that are, at least in part, distinct as far as induction of maturation is concerned (Le Goascogne *et al.*, 1984, Deshpande and Kung, 1987; Korn *et al.*, 1987). *Xenopus* oocytes have mainly IGF-I receptors and very few insulin receptors (Maller and Koontz, 1981; Hainaut *et al.*, 1991a). Insulin is able to bind to the oocyte IGF-I receptor and to induce maturation, but with an affinity 50–100 times lower than that of IGF-I. Generally, the effective concentration (EC_{50}) for the induction of maturation is 0.1 nM for IGF-I, 10 nM for insulin, and 0.1 μM for progesterone (unpublished observations). Although some variability in the extent of GVBD is observed among oocytes from different frogs, insulin/IGF-I is less potent than progesterone, because the extent of maturation at maximally stimulatory concentrations is 50–85% with progesterone, and 30–60% with insulin/IGF-I (see Figures 1 and 2). Note that to stimulate maturation we have used here a high concentration of insulin (10^{-6} M), which is very likely to act through the IGF-I receptor and is therefore referred to as an insulin/IGF-I effect.

Incubation of oocytes during 12 h in sodium orthovanadate stimulated oocyte maturation in a dose-dependent manner (Figure 1). Vanadate used at concentrations between 100 μM and 2 mM increased the effects of insulin or of pro-

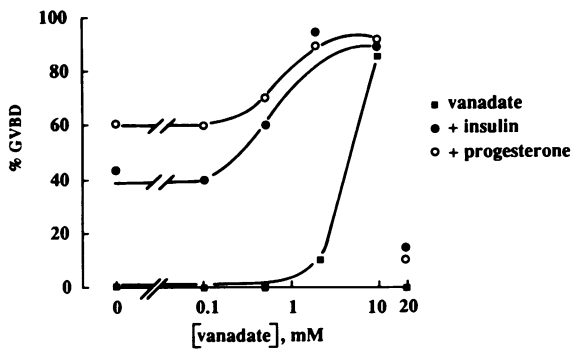

***Figure 1.* Effect of vanadate on oocyte maturation.** Oocytes (40–50 per point) were incubated during 12 h at 20°C in MBS containing 0.2% BSA and vanadate at concentrations ranging from 100 μM to 20 mM, in the absence or presence of insulin (1 μM) or progesterone (10 μM). After 12 h, oocytes were scored for the appearance of a "white spot" at the apical pole, which witnesses GVBD. Results are expressed as a percentage of oocytes that underwent GVBD, 100 being the number of intact oocytes at each point. A typical experiment is shown.

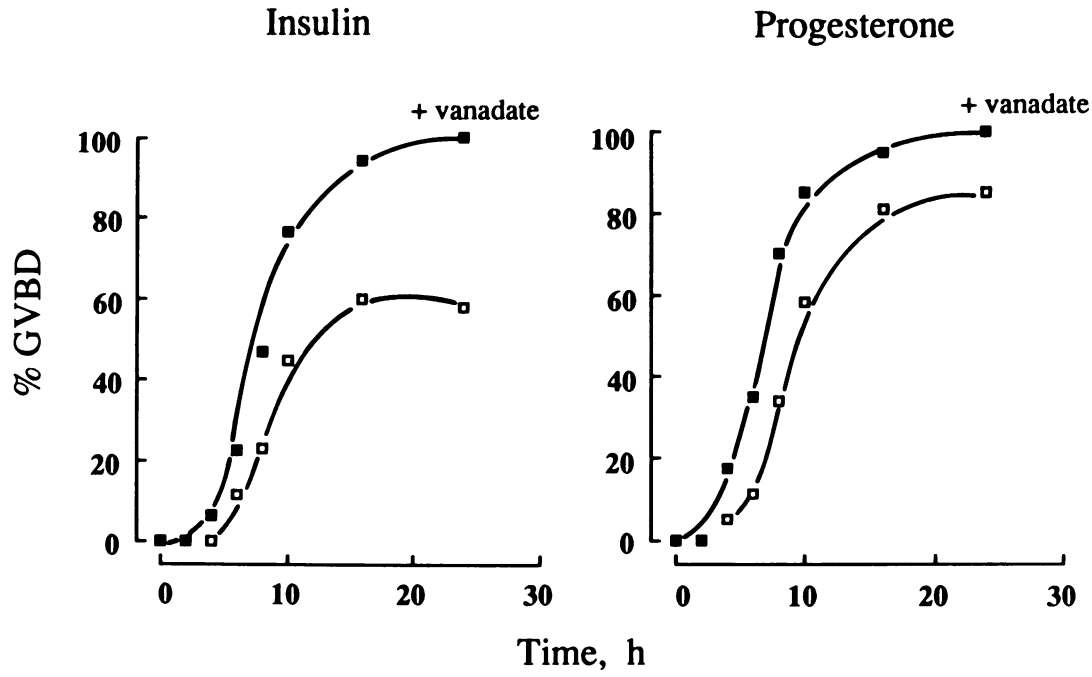

Figure 2. **Effect of vanadate on the time course of oocyte maturation induced by insulin or by progesterone.** Oocytes (40–50 per point) were incubated during 1 h at 20°C in MBS containing BSA 0.2% in the absence (□) or presence (■) of vanadate (2 mM). Then insulin (1 μM) or progesterone (10 μM) was added; after the indicated periods of time, oocytes were scored for maturation as described in legend to Figure 1.

gesterone in a dose-dependent manner, with an EC_{50} of ~750 μM, and a maximum at 2 mM, a concentration at which vanadate alone had no or only little effect on maturation. In the absence of hormones, a maximal response was observed with 10 mM vanadate. Concentrations > 10 mM were toxic, as judged by the rapid occurrence of deleterious morphological changes. Figure 2 shows the effect of vanadate at 2 mM on the time course of maturation induced by insulin and by progesterone at maximally stimulatory concentrations. In the absence of vanadate, the time courses of insulin and progesterone effects were similar (half-maximal effects after 7–8 h). Addition of vanadate reduced by 1 or 2 h the latency period preceding GVBD, whereas the rate of maturation observed with either hormone was increased to 100%.

Maturation can also be induced by microinjection in an oocyte of a small amount of active MPF (~1% of the MPF activity present in 1 unfertilized egg), which is sufficient to trigger the activation of dormant, endogenous pre-MPF (Masui and Markert, 1971; Wu and Gerhart, 1980; Cyert and Kirschner, 1988). In these conditions, the appearance of GVBD is more rapid than in hormone-induced maturation (2–3 h postinjection) and is independent of protein synthesis. Figure 3 shows that incubation in 2 mM vanadate increased maturation induced by microinjection of MPF. These data indicate that exposure of oocytes to vanadate stimulates

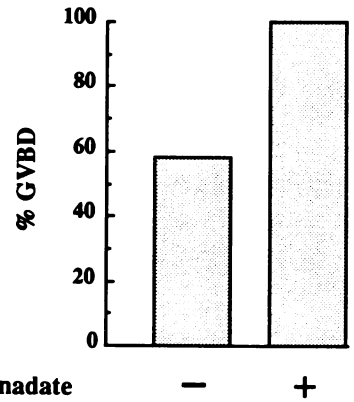

Figure 3. **Effect of vanadate on maturation induced by microinjected MPF.** Oocytes (50 per point) were incubated during 1 h at 20°C in MBS containing BSA 0.2% in the absence or presence of vanadate (2 mM). They were then microinjected with MPF (33% ammonium sulphate-precipitable fraction of the high-speed supernatant from unfertilized eggs, 6 mg/ml, 50 nl per oocyte at the dilution 1/30; this dilution gives a 50% response in 2–3 h at 20°C) and incubated for an additional 3 h in medium containing vanadate or not. Maturation was scored as in legend to Figure 1.

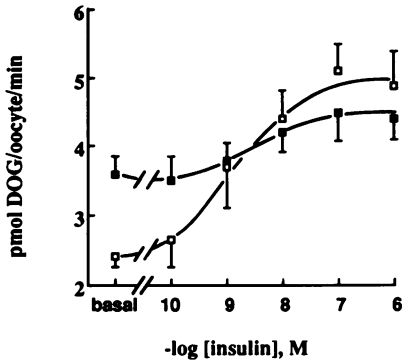

Figure 4. **Effect of vanadate on glucose uptake.** Ten oocytes per experimental point (in triplicate) were incubated in MBS containing BSA 0.2%, in the absence (□) or presence (■) of vanadate (2 mM). After 1 h, insulin at concentrations ranging from 0.1 nM to 1 μM was added for an additional hour (identical results could be obtained with IGF-I at concentration ranging from 1 pM to 10 nM). The 2-deoxy[^{3}H]glucose (DOG) was then added for 10 min. Uptake was stopped by three rapid washes in cold MBS, oocytes were lysed, and the amount of glucose incorporated was determined by scintillation counting. Results are expressed in pmol DOG per oocyte and per min, and are the means ± SE of triplicate points.

maturation induced by insulin/IGF-I, progesterone, or microinjected MPF.

Effect of vanadate on glucose uptake

In oocytes, insulin and IGF-I stimulated glucose uptake two- to threefold in a dose-dependent manner, with EC_{50} identical to those observed for the stimulation of maturation (Janicot and Lane, 1989; Vera and Rosen, 1989; Hainaut *et al.*, 1991b). However, in contrast to maturation, this effect was rapid (within a few minutes after stimulation, with a plateau being reached after 20 min and remaining constant for 2–3 h) and was not observed after progesterone stimulation (Hainaut *et al.*, 1991b). As seen in mammalian cells, vanadate enhanced glucose uptake in oocytes, with a maximum increase being observed at 2 mM. However, in contrast to maturation, vanadate exerted its effect in the absence of insulin (or IGF-I), increasing glucose uptake to a level that amounted to 50% of the maximal insulin/IGF-I response (Figure 4). This effect was not additive to that of insulin/IGF-I at maximally stimulatory concentrations.

Effect of vanadate on the phosphorylation of oocyte receptors

In rat adipocytes, the insulin-like effect of vanadate has been shown to result, at least in part, from its capacity to enhance the insulin receptor kinase activity and the tyrosine phosphorylation of the receptor β-subunit in the absence of insulin (Tamura *et al.*, 1983, 1984). In Figure 5 we used a cell-free phosphorylation assay to test the effect of vanadate on the autophosphorylation of oocyte receptors. *Xenopus* oocytes

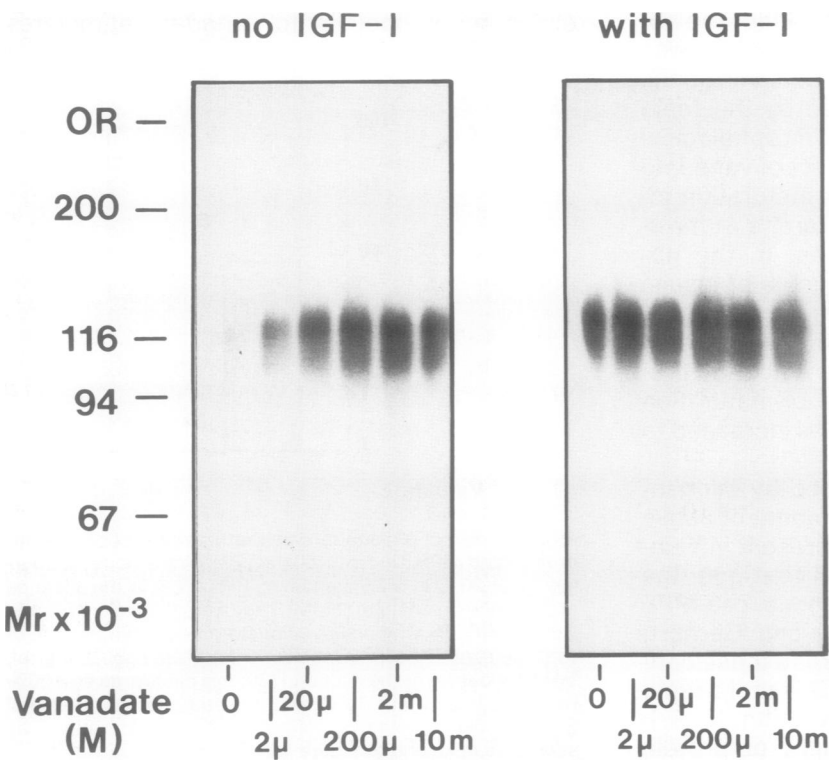

Figure 5. **Phosphorylation of partially purified oocyte IGF-I receptor β-subunits in the absence or presence of vanadate.** Partially purified receptors (20 μl/point) were incubated during 90 min at 20°C without or with IGF-I (10 nM) in the absence or presence of vanadate (from 2 μM to 10 mM) and were then phosphorylated with [γ-^{32}P]ATP for 30 min, followed by precipitation with anti-phosphotyrosine antibodies, and analysis by 7.5% SDS-PAGE under reducing conditions. An autoradiogram of the gel is shown.

carry predominantly IGF-I receptors compared with insulin receptors (Maller and Koontz, 1981; Hainaut *et al.,* 1991a). These receptors show a typical $\alpha_2\beta_2$ heterotetrameric structure, but with two distinct β-subunits of 95 and 105 kDa that may correspond to two different receptor subtypes (Hainaut *et al.,* 1991a). Partially purified receptors, stimulated or not with IGF-I at 10 nM, were incubated in the absence or in the presence of vanadate at concentrations ranging from 2 μM to 10 mM and were then exposed to [γ-^{32}P]-ATP. After immunoprecipitation with antibodies to phosphotyrosine, proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 5). In reducing conditions, only receptor β-subunits (95–105 kDa) are visible, because of their ability to undergo autophosphorylation on tyrosine residues. Stimulation with IGF-I resulted in a 13-fold increase in receptor β-subunit phosphorylation. Vanadate was found to stimulate in a dose-dependent manner the receptor β-subunit phosphorylation in the absence of IGF-I (left panel) and to be without significant effect in the presence of the ligand (right panel). Although the stimulating action of vanadate was already detected at 2 μM, maximal stimulation, almost identical to that induced by IGF-I, was achieved with 0.5–2 mM (EC_{50}: 50–100 μM). As found by Tamura *et al.* (1984) with rat adipocytes insulin receptors, this result was not brought about by the inhibition of PTPases, because the receptor preparations used were essentially devoid of such activities. Indeed, the spontaneous dephosphorylation of receptors amounted to <10% over a 4-h period (not shown). Taken together, our data show that vanadate stimulated the autophosphorylation of *Xenopus* oocyte IGF-I receptors by mechanisms other than PTPase inhibition.

Xenopus oocytes have been found to be rich in PTPase activities (Cicirelli *et al.,* 1990). We thus analyzed the effect of vanadate on oocyte receptor dephosphorylation in the presence of an oocyte extract containing PTPase activities (Figure 6). Partially purified receptors were stimulated by IGF-I and phosphorylated with [γ-^{32}P]-ATP in the absence of vanadate. They were then exposed to the oocyte extract for 10–30 min, without or with vanadate at concentrations ranging from 2 μM to 10 mM. Finally, the phosphorylation level of receptors was analyzed by SDS-PAGE. In the absence of vanadate, 80% of the radioactive phosphate incorporated in the β-subunit was removed after 10 min of exposure to the oocyte extract (90% after 30 min). This phosphatase activity was largely, but not totally, inhibited by vanadate, because 25% of the incorporated phosphate was still removed by the extract in the presence of vanadate at 10 mM. The inhibition of phosphatase activity by vanadate was dose dependent, being already visible at 2 μM vanadate and reaching a maximum at 2 mM (EC_{50}:50–100 μM).

As a whole, these results show that vanadate could exert at least two distinct effects on oocyte IGF-I receptor phosphorylation. First, it caused a net increase in the phosphorylation of receptor β-subunits that was not additive to the IGF-I effect. Second, vanadate stabilized the phosphorylation level of activated receptors by inhibiting receptor dephosphorylation. Similar results were found with the use of partially purified human insulin receptors (not shown).

Effect of vanadate on MPF activity in a cell-free system

The observation that vanadate did not prevent oocyte maturation suggested to us that vanadate did not inhibit the putative PTPase activities responsible for $p34^{cdc2}$ dephosphorylation and MPF kinase activation. To verify this hypothesis, we have used a cell-free system to reconstitute MPF activation. Extracts of G2-arrested oocytes containing inactive MPF (pre-MPF) were incubated for 1 h in the presence of a small amount of active MPF (1%) and of an ATP-regenerating system containing ATP, creatine phosphate, and creatine kinase (Cyert and Kirschner, 1988). In this system, tyrosine dephosphorylation of $p34^{cdc2}$ occurs and is strictly correlated with an increase in MPF kinase activity, which can be monitored by the use of histone H1 as a substrate (Dunphy and Newport, 1989). The MPF kinase is by far the major, and perhaps the sole, Ca^{2+}- and cyclic nucleotide-independent H1 kinase activity that can be extracted from *Xenopus* oocytes (Cicirelli *et al.,* 1988; Labbé *et al.,* 1988; Norburry and Nurse, 1989). In Figure 7, we have incubated pre-MPF with a small amount of active MPF and an ATP regenerating system, without or with vanadate at 2 mM. Vanadate was found to stimulate by ~1.5-fold the H1 kinase activity in both pre-MPF (lanes 1 and 2) and MPF (lanes 7 and 8). Moreover, when pre-MPF was incubated for 1 h at 20°C with an ATP regenerating system (lanes 3 and 4), or with an ATP regenerating system plus a small amount of active MPF (lanes 5 and 6), vanadate stimulated H1 kinase activity by factors of 5 and 3, respectively. In particular, the extent of H1 kinase activation in lane 4 (pre-MPF + ATP regenerating system + vanadate)

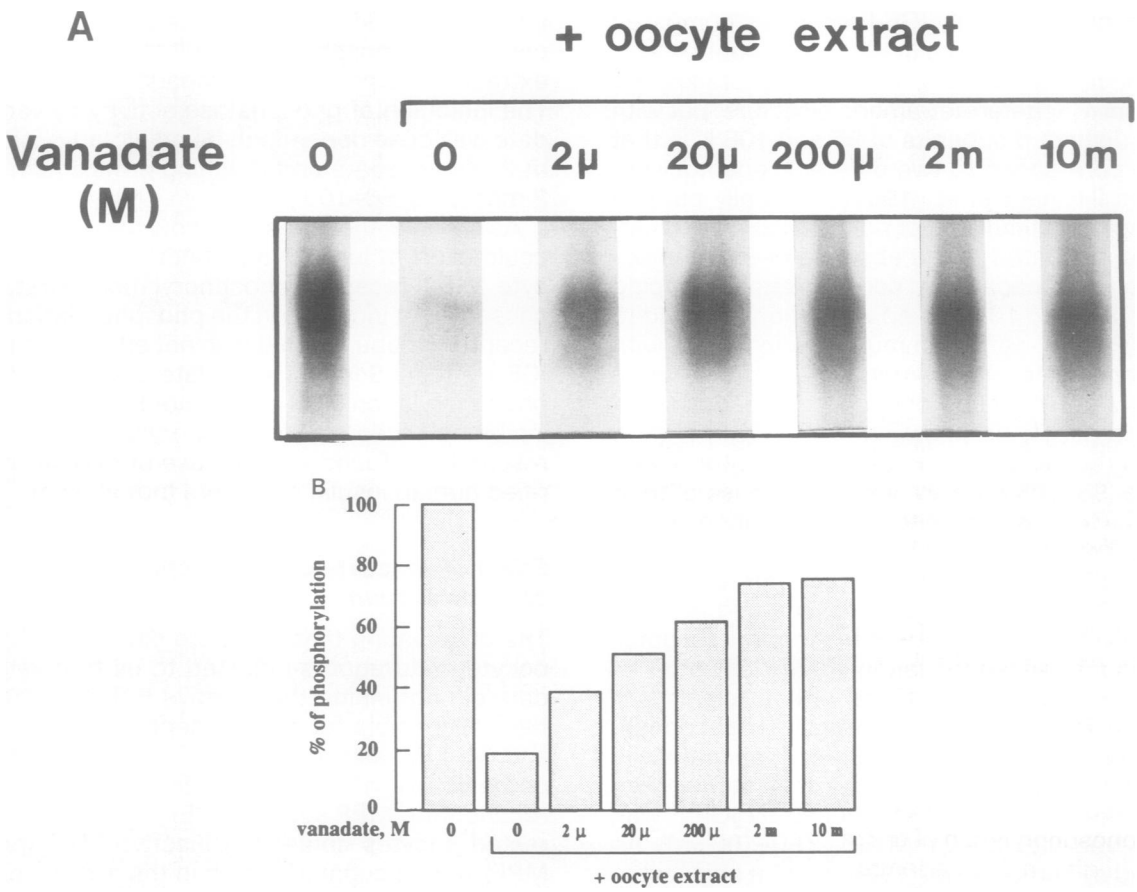

Figure 6. **Effect of vanadate on the dephosphorylation of oocyte IGF-I receptor β-subunits in the presence of an oocyte extract.** Partially purified receptors (20 μl/point) were stimulated with IGF-I (10 nM) and phosphorylated with [γ-^{32}P]ATP (15 μM) in the absence of vanadate. The incorporation of radiolabeled phosphate was then stopped by addition of 10 mM unlabeled ATP, and samples were exposed for 10 min to an extract of oocytes that contained phosphatase activities (40 μl/assay). The oocyte extract (protein content: 13 mg/ml) was prepared as described under Methods. Samples were analyzed by 5% SDS-PAGE under reducing conditions. (A) Autoradiogram of the gel. (B) Densitometric analysis of the autoradiogram presented in A. Results are expressed as a percentage, 100% being the autoradiographic density of the phosphorylated receptor after 10 min of incubation without vanadate and in the absence of oocyte extract.

suggested that, in the presence of ATP, vanadate was able to enhance the conversion of pre-MPF into active MPF. These results show that vanadate at 2 mM did not prevent the activation of MPF in a cell-free system and suggest that the stimulatory effect of vanadate on maturation could be due, at least in part, to its ability to increase the MPF kinase activity itself.

Discussion

This study shows that vanadate, added at millimolar concentrations in the incubation medium, stimulates both glucose uptake and maturation in *Xenopus* oocytes. Although vanadate increased glucose uptake in the absence, but not in the presence, of insulin, it enhanced hormone-induced maturation but had no effect *per se* unless used at higher concentrations (10 mM). The concentrations of vanadate that maximally stimulated glucose uptake and hormone-induced maturation were similar to those required for the induction of rapid insulin-like effects in isolated rat adipocytes (0.5–3 mM: Dubyak and Kleinzeller, 1980; Tamura *et al.*, 1983, 1984; Bernier *et al.*, 1988). In the latter system, the insulinomimetic effects of vanadate have been clearly dissociated from its ability to exert other effects, such as inhibition of (Na^+-K^+)ATPase (Dubyak and Kleinzeller, 1980; Tamura *et al.*, 1984), and appeared to arise from an increase in insulin receptor tyrosine phosphorylation. This increased phosphorylation resulted from direct stimulation of the insulin receptor kinase and/or from inhibition of PTPases (Tamura *et al.*, 1983, 1984; Mountjoy and Flier,

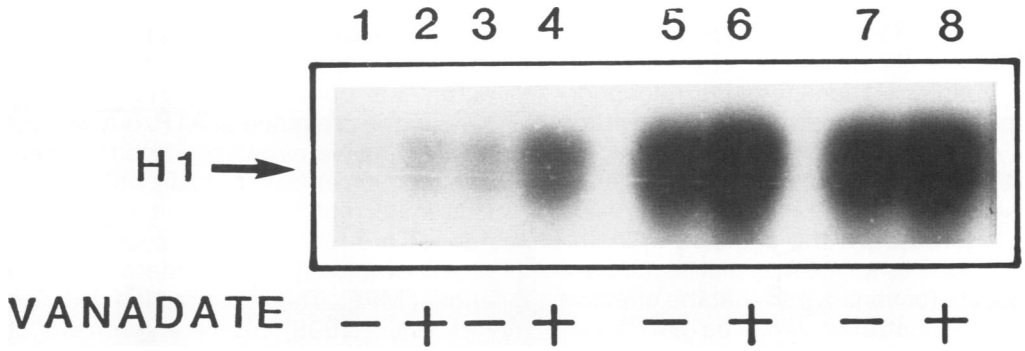

Figure 7. **Effect of vanadate on the activity of the MPF kinase.** The H1 kinase activity of a G2-arrested oocytes extract was tested after various incubation conditions. Samples were incubated during 1 h at 20°C in a final volume of 20 μl, in the absence or presence of vanadate (2 mM). (1–2) pre-MPF (100 μg); (3–4) pre-MPF (100 μg), plus ATP (ATP regenerating system consisting of 1 mM ATP, 10 mM creatine phosphate and 50 μg/ml creatine kinase); (5–6) pre-MPF (100 μg), plus ATP, plus MPF (1 μg); (7–8) MPF (40 μg), plus ATP. After incubation, 2 μl of each sample were assayed for the cdc2 H1 activity during 10 min at 20°C in a final volume of 20 μl, using H1 as a substrate. Each sample was analyzed by 10% SDS-PAGE. The conditions in the cdc2 kinase assay were adjusted so that the final concentrations of ATP, creatine phosphate, and creatine kinase were, respectively, 0.1 mM, 1 mM, and 5 μg/ml.

1990). According to Tamura *et al.* (1984), vanadate decreases the K_m of the insulin receptor kinase for ATP, thus enhancing the kinase reaction and causing a net increase in tyrosine, but not serine or threonine, phosphorylation of insulin receptor β-subunit. Similar to these observations, we found that vanadate stimulated the phosphorylation of the oocyte IGF-I receptor β-subunits in the absence of IGF-I. Moreover, in a cell-free system, vanadate also inhibited, at least partially, oocyte IGF-I receptor dephosphorylation by an oocyte extract containing PTPase activities. The EC_{50} for these two effects, i.e., activation of the oocyte receptor kinase and inhibition of oocyte PTPases, was 50–100 μM, although significant effects could be detected with 2 μM vanadate. These values are in agreement with those observed by Tamura *et al.* (1983, 1984) for the activation of the murine insulin receptor kinase, but are ~5–10 times higher than those reported by Swarup *et al.* (1982a,b) for the specific inhibition of PTPases by vanadate. Indeed, all PTPases are not inhibited by vanadate (Brunati and Pinna, 1985), and several examples of weak inhibition by vanadate have been reported (Boivin and Galand, 1986). In contrast with the results of Swarup *et al.* (1982a,b), but similar to ours, Mustelin *et al.* (1989) found that vanadate inhibited the PTPase activity of CD45 in preparations of T cell membranes with an EC_{50} of 300 μM. Most likely, oocyte extracts contain several distinct PTPase activities with different sensitivities to vanadate. At any rate, the insulinomimetic effects of vanadate could, at least in part, result from a vanadate-induced direct or indirect activation of the oocyte IGF-I receptor kinase. However, the effects of vanadate in oocytes are probably not limited to the IGF-I receptor kinase, because we also observed an effect on maturation induced by progesterone and by microinjected MPF. Indeed, vanadate might also modulate the activity of other phosphotyrosyl proteins such as pp15, possibly involved in stimulation of glucose uptake (Bernier *et al.*, 1988), or $pp60^{src}$ (Brown and Gordon, 1984; Ryder and Gordon, 1987), which may be a downstream effector of MPF action during maturation (Morgan *et al.*, 1989; Shenoy *et al.*, 1989; Maller, 1990). Finally, we cannot totally exclude that vanadate could also affect other ATP-dependent reactions. For example, an inhibition of $(Na^+\text{-}K^+)$ATPase by vanadate might enhance maturation, as suggested by the observation that ouabain facilitates oocyte maturation (Vitto and Wallace, 1976). In contrast, vanadate is known to stimulate adenylyl cyclase (Lichstein *et al.*, 1982), an effect that would be expected to inhibit maturation (Maller and Krebs, 1977).

The key finding of our report is that vanadate did not prevent MPF activation and oocyte maturation, although there is compelling evidence that MPF activation requires dephosphorylation of $p34^{cdc2}$ on tyrosine and threonine residues (Gautier *et al.*, 1989; Morla *et al.*, 1989). In G2-arrested cells, inactive $p34^{cdc2}$ is phosphorylated on a tyrosine residue lying within the ATP binding site. This phosphorylation event is thought to prevent kinase activation (Gould and Nurse, 1989; Nurse, 1990). Therefore, a still unidentified PTPase activity might be necessary for MPF activation. At present, the reason that vanadate

does not inhibit MPF activation in our experiments is unclear. Recent evidences show that before dephosphorylation, tyrosine phosphorylation of $p34^{cdc2}$ is required for MPF activation (Solomon *et al.,* 1990). Therefore, vanadate might have a positive effect on MPF activation by increasing the amount of phosphotyrosine in $p34^{cdc2}$. Alternatively, the absence of inhibition might be due to an intracellular sequestration of vanadate (or vanadyl) so that the effective cytoplasmic concentration would be much lower than expected. This hypothesis is supported by the observation that the EC_{50} for vanadate effects in intact oocytes is 5–10 times higher than in cell-free systems, as also found by Tamura *et al.* (1983, 1984) in adipocytes. However, using a cell-free assay with extracts of G2-arrested oocytes, we have found that vanadate was unable to prevent MPF activation also in vitro. This assay was based on the one developed by Dunphy and Newport (1989) to demonstrate that activation of MPF kinase was accompanied by tyrosine dephosphorylation of $p34^{cdc2}$ and that both events were prevented in the presence of the *Xenopus* protein p13 (homologous to the product of the *Schizosaccharomyces pombe* gene *suc1*). In contrast with our results, Morla *et al.* (1989) have found that vanadate induced a reversible mitotic arrest of NIH 3T3 cells at the G2/M border. This inhibition was accompanied by an increase in tyrosine phosphorylation of many proteins, including $p34^{cdc2}$. Indeed, the use of different experimental models might explain in part such a discrepancy between these results and ours. However, the report by Morla *et al.* (1989), that vanadate could act as an inhibitor of mitosis, is at variance with a whole series of studies showing that vanadate 1) stimulates DNA synthesis in human and mouse fibroblasts (Carpenter, 1981; Bingham Smith, 1983); 2) induces cell proliferation and appearance of a transformed phenotype in at least three different cell lines, including NIH 3T3 cells (Klarlund *et al.,* 1985; Mountjoy and Flier, 1990); 3) stimulates the activity of oncogenic proteins such as $pp60^{v\text{-}src}$ (Brown and Gordon, 1984; Ryder and Gordon, 1987); and 4) increases the expression of the Glut-1 glucose transporter, a gene with expression linked to cellular growth and proliferation (Mountjoy and Flier, 1990).

Interestingly, we found that vanadate was able to stimulate MPF activation in a cell-free system. Activation of pre-MPF in extracts of G2-arrested oocytes is an ATP-dependent reaction, which requires the presence of a small amount of active MPF (Cyert and Kirschner, 1988; Dunphy and Newport, 1989). Our results suggest that vanadate is able to activate at least to some extent the conversion of pre-MPF into active MPF in the presence of ATP, but without added MPF. Thus, vanadate renders the MPF activation reaction at least partially MPF-independent. It is important to note that partially or highly purified pre-MPF activates spontaneously in the presence of ATP, but in the absence of exogenous MPF. This observation led Cyert and Kirschner (1988) to the hypothesis that the crude extract of G2-arrested oocytes contained an inhibitory component, INH, that prevents spontaneous activation of pre-MPF and that is turned off by the addition of active MPF. This suggests that some regulatory mechanisms of MPF activity are still functioning in these extracts. In agreement with this notion, the biological properties of the highly purified MPF kinase are strikingly different from that of crude MPF. For example, sodium β-glycerophosphate and sodium fluoride stabilize the activity of MPF in oocyte extracts or in 33% ammonium sulfate fractions but inhibit the purified MPF kinase, suggesting that they exert their positive effect by acting on regulators of MPF activity rather than on the $p34^{cdc2}$ kinase itself (Erikson and Maller, 1989). Likewise, vanadate could increase MPF activity by modulating the activity of other component(s) in the cell-free system that would, in turn, stimulate the MPF kinase. Although the regulation of MPF activation in vertebrates is still poorly understood, a large body of evidence suggests that several kinases and phosphatases may be involved (Huchon *et al.,* 1981; Russell and Nurse, 1987; Cyert and Kirschner, 1988; Goris *et al.,* 1989; Brautigan *et al.,* 1990; Felix *et al.,* 1990; Rime and Ozon, 1990). Further experiments are needed to analyze the effect of vanadate in these processes.

In conclusion, our results show that in oocytes, vanadate exerts insulin-like effects on glucose uptake and maturation. In cell-free systems, vanadate increases the phosphorylation of the oocyte IGF-I receptor, inhibits oocyte PTPases, and enhances MPF activation. Although we cannot exclude that vanadate affects several different phosphorylation reactions in intact cells, we favor the hypothesis that inhibition of PTPases is a major mechanism of vanadate action. Indeed, microinjection in oocytes of a purified protein PTPase (PTPase 1B) inhibits an early insulin effect (Cicirelli *et al.,* 1990) and markedly delays oocyte maturation (Tonks *et al.,* 1990), two effects that are the opposite of those observed in oocytes treated with vanadate. Moreover, zinc ions (Zn^{2+}), which are also potent

PTPase inhibitors (Brautigan *et al.,* 1981), stimulate oocyte maturation and increase the effects of either insulin or progesterone (Wallace and Misulovin, 1980). Finally, our results suggest a new explanation for the growth-promoting properties of vanadate, because it appears to enhance the activation of MPF, a key regulator of the cell cycle.

Methods

Materials

Triton X-100, bovine serum albumin (BSA, ref 7030 Sigma), aprotinin, leupeptin, progesterone, histone IIIS, pregnant mare's serum gonadotropin (PMSG), human chorionic gonadotropin (hCG), cyclic AMP-dependent protein kinase inhibitor peptide (PKI), and sodium orthovanadate were from Sigma (St. Louis, MO). Bacitracin and phenylmethylsulphonyl fluoride (PMSF) were from Serva (Heidelberg, FRG). γS-ATP was from Boerhinger Mannheim (Mannheim, FRG). Pig insulin was from Novo (Copenhagen, Denmark). Human recombinant IGF-I was from Euromedex (Strasbourg, France). 2-deoxy [^{3}H]glucose was from CEA (Saclay, France). [γ-^{32}P]ATP was from Amersham (Bucks, UK). Frogs were from the CRBM (Centre de Recherche de Biochimie Macromoléculaire, Montpellier, France).

Oocytes isolation and microinjection

Stage V–VI oocytes were removed from frogs 3–6 d after injection of PMSG (100 IU). Follicles were isolated by manual dissection, and maintained in modified Barth solution (MBS) as described (Colman, 1984). During dissection, most of the outer layer surrounding oocytes (theca) was removed, but follicular cells remained essentially unaltered. This method was preferred to enzymic stripping, because we found that the latter procedure altered insulin/IGF-I receptors at the oocyte surface (Hainaut *et al.,* 1991a,b). However, in some instances, oocytes were freed of follicular cells by treatment with collagenase II (2 mg/ml) and soybean trypsin inhibitor (0.4 mg/ml) and used as controls, but no significant difference was observed as far as the effect of vanadate is concerned. In all experiments, follicles (i.e., oocytes and follicular cells) were used and were incubated in MBS containing 0.2% (vol/vol) BSA, without or with vanadate. Vanadate was prepared as a 200 mM water solution, adjusted to pH 7.6 with concentrated HCl, and stored at −20°C for several weeks. Microinjections (50 nl/oocyte) were performed with the use of an automatic microinjector (Inject-Matic, Geneva, Switzerland).

Receptor phosphorylation and dephosphorylation assays

Receptors were partially purified from oocytes, stimulated with IGF-I (10 nM), phosphorylated with [γ-^{32}P]-ATP, immunoprecipitated with antibodies to phosphotyrosine, and analyzed by SDS-PAGE as described elsewhere (Hainaut *et al.,* 1991a). For dephosphorylation experiments, receptors were exposed to IGF-I and phosphorylated as above; incorporation of radioactive phosphate was stopped after 30 min by addition of unlabeled ATP in excess (10 mM). The phosphorylated receptors were incubated for 10–30 min in the absence or presence of vanadate with a freshly prepared oocyte extract (40 μl per experimental point) before being analyzed by SDS-PAGE. Autoradiograms were scanned using a densitometer (Hoefer Scientific Instrument, San Francisco, USA). The oocyte extract was prepared by homogenization of oocytes in 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.6, containing 30 mM NaCl, 0.1% Triton X-100 (vol/vol), 0.5 mM PMSF, 100 IU/ml Trasylol, 10 μg/ml leupeptin, and 1 mM bacitracin. After centrifugation for 15 min at 13 000 × *g,* the supernatant (protein concentration: 13 mg/ml) was kept on ice. Before use, the phosphatase activity of the extract was tested by a colorimetric assay using para-nitrophenyl-phosphate as a substrate (Swarup *et al.,* 1982b).

Biological responses

Glucose uptake was measured essentially as described by Janicot and Lane (1989), except that 2-deoxy [^{3}H]glucose (0.1 mM, 28 mCi/mmol) was used and that [^{14}C]sucrose (0.2 μCi/ml) was added in each assay as a marker of nonspecifically trapped radioactivity (Hainaut *et al.,* 1991b). Maturation was scored by the appearance of a "white spot" at the animal pole, which is a consequence of GVBD. When the presence of a white spot was questionable, oocytes were fixed in trichloroacetic acid 10% and dissected to confirm that GVBD had occurred.

Preparation of oocyte extracts, MPF and pre-MPF

Extracts of G2-arrested oocytes were prepared essentially as described (Cyert and Kirschner, 1988). Oocytes were homogeneized in extraction buffer (EB: 80 mM sodium β-glycerophosphate [pH 7.3], 20 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid [EGTA], 15 mM $MgCl_2$) containing 1 mM dithiothreitol, 0.5 mM PMSF, 100 IU/ml aprotinin, and 10 μg/ml leupeptin. After centifugation at 160 000 × *g* (r_{av}: 11.2 cm) for 90 min at 4°C, the supernatant was recovered (pre-MPF). MPF was prepared from unfertilized eggs, obtained by injection of a PMSG-treated frog with 1000 IU hCG. After dejellying in 2% cysteine (pH 7.6), eggs were extracted in EB as above, containing γS-ATP (0.5 mM). The high-speed supernatant was fractionated in 33% ammonium sulphate. The precipitate was dialyzed against EB containing protease inhibitors and 0.1 mM γS-ATP, and kept at −80°C. When injected into oocytes (50 nl/oocyte), this MPF preparation (protein concentration 6 mg/ml) induced 50% GVBD in 2–3 h at the dilution 1/30.

Cdc2 H1 kinase assays

Histone H1 kinase assays were performed exactly as described by Dunphy and Newport (1989), using histone IIIS as a source of H1. The composition of the kinase assay buffer was 20 mM HEPES, pH 7.3, 5 mM EGTA, 10 mM $MgCl_2$, containing 0.3 mg/ml histones, 0.1 mM ATP (specific activity 500 mCi/mmol), and 5 μM PKI. To study the effect of vanadate on pre-MPF activation, we incubated 10 μl of pre-MPF (100 μg protein) during 1 h at 20°C in a final volume of 20 μl, in the absence or in the presence of the following: an ATP-regenerating system containing 1 mM ATP, 10 mM creatine phosphate, and 50 μg/ml creatine kinase; active MPF (1 μg); and vanadate (2 mM). MPF (7 μl, 40 μg protein, in a final volume of 20 μl) was used as control. Two microliters of each sample, diluted to 10 μl with water, were used to test H1 kinase activity.

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