Mechanisms of inhibition of lipolysis by insulin, vanadate and peroxovanadate in rat adipocytes

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Vanadate and peroxovanadate (pV), potent inhibitors of tyrosine phosphatases, mimic several of the metabolic actions of insulin. Here we compare the mechanisms for the anti-lipolytic action of insulin, vanadate and pV in rat adipocytes. Vanadate (5 mM) and pV (0.01 mM) inhibited lipolysis induced by 0.01–1 μM isoprenaline, vanadate being more and pV less efficient than insulin (1 nM). A loss of anti-lipolytic effect of pV was observed by increasing the concentration of isoprenaline and/or pV. pV induced tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 to a greater extent than insulin, whereas vanadate affected these components little if at all. In addition, only a higher concentration (0.1 mM) of pV induced the tyrosine phosphorylation of p85, the 85 kDa regulatory subunit of phosphoinositide 3-kinase (PI-3K). Vanadate activated PI-3K-independent (in the presence of 10 nM isoprenaline) and PI-3K-dependent (in the presence of 100 nM isoprenaline) anti-lipolytic pathways, both of which were found to be independent of phosphodiesterase type 3B (PDE3B). pV (0.01 mM), like insulin, activated PI-3K- and PDE3B-dependent pathways. However, the anti-lipolytic pathway of 0.1 mM pV did not seem to require insulin receptor substrate-1-associated PI-3K and was found to be partly independent of PDE3B. Vanadate and pV (only at 0.01 mM), like insulin, decreased the isoprenaline-induced activation of cAMP-dependent protein kinase. Overall, these results underline the complexity and the diversity in the mechanisms that regulate lipolysis.

Key words: diabetes, insulin receptor substrate-1, phosphodiesterase type 3B, phosphoinositide 3-kinase, cAMP-dependent protein kinase.

INTRODUCTION

During the past 15 years, vanadium compounds have generated much interest for their ability to mimic insulin actions *in vitro*, including the stimulation of hexose uptake, the stimulation of lipogenesis and the inhibition of lipolysis (reviewed in [1,2]). *In vivo*, the oral administration of vanadate has been shown to normalize blood glucose concentrations in streptozotocin-induced diabetic rats [3]. In other rodent models of non-insulin-dependent diabetes mellitus, an ability of vanadate to decrease resistance to insulin has also been reported [4]. Studies performed in human subjects with non-insulin-dependent diabetes mellitus have shown that treatment with vanadate improved sensitivity to insulin [4,5]. Vanadium compounds are therefore considered to be potential therapeutic agents for diabetes, although side effects and toxicity need to be overcome [1,2]. Peroxovanadate (pV), generated by the combination of vanadate and H_2O_2, has been shown to be more powerful than vanadate in inducing insulin-like effects *in vitro* [6,7]. The main mechanism by which vanadate and pV elicit their insulin-like effects seems to be the inhibition of tyrosine phosphatases: vanadate is a competitive inhibitor of tyrosine phosphatases, whereas pV promotes irreversible inhibition [8]. A direct consequence of the inhibition of tyrosine phosphatases is an increase in tyrosine phosphorylation of proteins. pV and peroxovanadium compounds have been shown to promote tyrosine phosphorylation and activation of the insulin receptor (IR) tyrosine kinase [6]. However, it has been reported that vanadate stimulation does not increase the tyrosine phosphorylation of IR, indicating that pathways bypassing IR are involved in vanadate-mediated effects [9,10]. Nevertheless the mechanisms by which vanadium and peroxovanadium compounds exert their actions are not fully understood.

In the present study we compare the mechanism of inhibition of lipolysis by insulin, vanadate and pV in isolated rat adipocytes. By antagonizing catecholamine-induced lipolysis, insulin inhibits the release of fatty acids into the circulation. Increased concentrations of fatty acids have been suggested to be an important factor in the development of diabetes [11], underlining the importance of dissecting the anti-lipolytic action of insulin and insulin-mimicking agents. We have previously shown that insulin exerts its anti-lipolytic effect, to a large extent, by phosphorylating (on serine-302) and activating the phosphodiesterase type 3B (PDE3B), leading to a decrease in cAMP levels and thereby the inactivation of the cAMP-dependent protein kinase (PKA). Hormone-sensitive lipase, the rate-limiting lipolytic enzyme, is thereby dephosphorylated and inactivated [12]. Phosphoinositide 3-kinase (PI-3K), which associates with insulin receptor substrates (IRSs) and becomes activated during insulin stimulation (reviewed in [13]), has also been shown to be important in insulin's anti-lipolytic signalling pathway [14,15]. This study reveals the complexity and the diversity in the mechanisms of inhibition of lipolysis by vanadate and pV.

EXPERIMENTAL

Materials

Insulin was a gift from Novo Nordisk (Copenhagen, Denmark). Collagenase, BSA, Na_3VO_4, isoprenaline, wortmannin and protein kinase inhibitors were purchased from Sigma. Protease

Abbreviations used: IR, insulin receptor; IRS, insulin receptor substrate; KRH, Krebs–Ringer buffer supplemented with Hepes; p85, 85 kDa regulatory subunit of PI-3K; PDE3B, phosphodiesterase type 3B; PI-3K, phosphoinositide 3-kinase; PKA, cAMP-dependent protein kinase; pV, peroxovanadate; PY, anti-phosphotyrosine; TLE, thin-layer electrophoresis.

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inhibitors were from Peptide Institute. Protein A–Sepharose was from Pharmacia Biotech. Cellulose TLC plates were purchased from Merck; silica gel TLC plates precoated with 1% (w/v) potassium oxalate were from Analtech. Rabbit polyclonal antibodies against human IR (β subunit) and the 85 kDa subunit (p85) of the rat PI-3K were from Upstate Biotechnology. Rabbit polyclonal antibodies against a C-terminal peptide of IRS-1 were kindly provided by Dr. L. Mosthaf (Hagedorn Research Institute, Copenhagen, Denmark). Mouse polyclonal anti-phosphotyrosine antibodies (PY20) were purchased from Transduction Laboratories. Polyclonal peptide anti-PDE3B antibodies were raised in rabbits and used as described previously [14]. Glycerol kinase and glycerol-3-phosphate dehydrogenase were from Boehringer Mannheim. Enhanced chemiluminescence reagents (super signal substrate) were from Pierce. ^{32}P was from Amersham and [γ-^{32}P]ATP was synthesized as described previously [16].

Preparation and stimulation of rat adipocytes

Adipocytes were prepared from epididymal fat pads of 36–38-day-old male Sprague–Dawley rats (B&K Universal, Stockholm, Sweden) as described previously [17,18]. After digestion with collagenase, isolated fat cells were washed with Krebs–Ringer buffer, pH 7.4, containing 25 mM Hepes, 200 nM adenosine, 2 mM glucose and 1% (w/v) BSA; this buffer was called KRH. Adipocytes [2 ml of 10–12% (v/v) cell suspension] were incubated in KRH at 37 °C with shaking (120 cycles/min) with insulin, vanadate or pV at the concentrations and times indicated. pV was prepared 15 min before use by incubating 12 mM Na_3VO_4 with 12 mM H_2O_2 in 25 mM Hepes, pH 7.4.

Immunoprecipitation and immunoblotting

Homogenization buffer (2 vol.) [10 mM Tris/HCl (pH 7.4)/250 mM sucrose/1 mM EDTA/100 μM Na_3VO_4/0.1 mM diisopropyl fluorophosphate/1 μg/ml pepstatin A/10 μg/ml antipain and leupeptin/10 mM NaF/20 mM sodium pyrophosphate] was added to the adipocyte incubations to stop the reactions. Homogenates free from fat were supplemented with 1% (v/v) Triton X-100 (final concentration) and solubilized for 1 h at 4 °C. Insoluble material was removed by centrifugation (12500 ***g***) for 10 min at 4 °C. Lysates were incubated overnight with the different antibodies at 4 °C and with 30 μl of Protein A–Sepharose 4B for 30–60 min at 4 °C. The immunocomplexes were washed three times with PBS containing 0.1% (v/v) *N*-laurylsarcosine, boiled in Laemmli sample buffer and subjected to SDS/PAGE [6% or 7% (w/v) gel]. Proteins were transferred to PVDF membranes (Immobilon-P; Millipore). After blocking with 0.5% gelatin [or 3% (v/v) non-fat milk when anti-IR antibody was used] in TBST buffer [10 mM Tris/HCl (pH 7.6)/150 mM NaCl/0.1% (v/v) Tween 20], the membranes were incubated with the appropriate antibodies at 1:1000 dilution. Immunoblot analysis was performed by using the enhanced chemiluminescence system and horseradish peroxidase conjugated to anti-rabbit or anti-mouse IgG (Amersham). To reprobe the membrane with a second antibody, the membrane was stripped by incubation in 2% (w/v) SDS/62.5 mM Tris/HCl (pH 6.7)/100 mM 2-mercaptoethanol for 30 min at 50 °C.

Labelling of adipocytes with ^{32}P, tryptic phosphopeptide mapping of ^{32}P-IRS-1 and two-dimensional phosphoamino acid analysis

Adipocytes were incubated in KRH containing 300 μM KH_2PO_4 and 1 mCi/ml ^{32}P for 75 min before stimulation with the different agents, to study the phosphorylation state of PDE3B and IRS-1. PDE3B was immunoisolated from solubilized membranes and subjected to SDS/PAGE as described previously [14]. IRS-1 immunoprecipitates were separated by SDS/PAGE and electrotransferred to PVDF membranes as described above. ^{32}P-phosphorylated PDE3B and IRS-1 were localized by digital imaging of ^{32}P (Fujix Bas 2000; Fuji). The piece of membrane containing phosphorylated IRS-1 was excised and the phosphoprotein was either subjected to phosphoamino acid analysis or digested with trypsin. Tryptic phosphopeptides were subjected to two-dimensional phosphopeptide mapping with the Hunter thin-layer electrophoresis (TLE) apparatus (HTLE-7000; C.B.S Scientific Co.) in the first dimension and TLC in the second dimension as described previously [19]. Phosphoamino acid analysis was performed as described by Boyle et al. [20]. The phosphorylated proteins (IRS-1 or PI-3K) were hydrolysed in 6 M HCl for 1 h at 110 °C. The hydrolysates were vacuum-centrifuged, washed in 50 μl of water, vacuum-centrifuged again, dissolved in 10 μl of buffer, pH 1.9, containing 1 mg/ml of phosphoserine, phosphothreonine or phosphotyrosine respectively (as standards) and spotted on cellulose TLC plates. The phosphoamino acids were separated through TLE in buffer, pH 1.9, for 25 min at 2000 V in the first dimension and through TLE in buffer, pH 3.5, for 20 min at 1800 V in the second dimension. The labelled phosphoamino acids were detected by digital imaging of ^{32}P and identified by comparison with the standards stained with 0.25% ninhydrin in acetone.

PI-3K assay

PI-3K activity was measured in PY20 or IRS-1 immunoprecipitates as described previously [21]. In brief, immunoprecipitates prepared as mentioned above were washed twice with 25 mM Hepes buffer (pH 7.4)/1% (v/v) Nonidet P40, twice with 100 mM Tris/HCl (pH 7.4)/500 mM LiCl/100 μM Na_3VO_4 and twice with 10 mM Tris/HCl (pH 7.4)/100 mM NaCl/1 mM EDTA/100 μM Na_3VO_4. Finally, immunoprecipitates were resuspended in a final volume of 50 μl of assay buffer containing 40 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, a mixture of phosphatidylinositol and phosphatidylserine at final concentrations of 0.2 mg/ml and 0.1 mg/ml respectively and 50 μM [γ-^{32}P]ATP (1 μCi). Wortmannin (100 nM) was added to some samples as indicated. After 15 min at 30 °C, the reactions were stopped by the addition of 40 μl of 4 M HCl and 160 μl of ethanol/chloroform (1:1, v/v). The phospholipids in the organic phase were recovered and spotted on a silica gel TLC plate precoated with 1% (w/v) KOH/oxalate. Migration was performed in ethanol/chloroform/water/25% (v/v) NH_4OH, (45:35:7:3, by vol.). Phosphatidylinositol 3-phosphate was used as a standard. ^{32}P incorporated into phosphatidylinositol was detected and quantified with the Fujix Bas 2000 system.

PDE assay

Adipocytes [1.8 ml of 10% (v/v) cell suspension in KRH] were stimulated for 10 min with 1 nM insulin and for 30 min with 5 mM vanadate or 100 μM pV at 37 °C. Crude membranes were prepared and assayed for PDE3B activity as described previously [14].

PKA assay

Adipocytes [800 μl of 8% (v/v) cell suspension in KRH] were stimulated for 5 min with 100 nM isoprenaline alone or in the presence of insulin, vanadate or pV at 37 °C. The cell suspensions were poured into homogenizers containing 200 μl of ice-cold

buffer [50 mM Tris/HCl (pH 7.4)/50 mM EDTA/50 μM 3-isobutyl-1-methylxanthine/10 μM OPC 3911/1 μg/ml pepstatin A/10 μg/ml antipain/10 μg/ml leupeptin], homogenized and centrifuged (12500 ***g***) for 10 min at 4 °C. The supernatants (10 μl after having removed the floating fat cake) were incubated for 20 min at 30 °C with 5 μl of a phosphorylation mixture containing 20 mM Tes, pH 7.4, 50 mM $MgSO_4$, 5 mM dithioerythritol, 250 mM sucrose, 0.2 mM ATP, 10 mg/ml Kemptide and [γ-^{32}P]ATP (23 μCi) in the presence or absence of protein kinase inhibitor (25 μM) or 16 μM cAMP. The reactions were stopped by the addition of 10 μl of 1% (w/v) BSA/1 mM ATP (pH 3.0) and 10 μl of 12.5% (w/v) trichloroacetic acid. Samples were put on ice for at least 10 min and were then centrifuged. An aliquot (15 μl) of the supernatants was applied to phosphocellulose paper (P81-membrane). After three washes with 75 mM H_3PO_4 and one wash with acetone, the amount of ^{32}P incorporated was determined by scintillation counting. Corrections were made for non-PKA activity as reported previously [18].

Lipolysis measurements

Adipocytes [1 ml of 5% (v/v) cell suspension in KRH] were incubated at 37 °C with shaking (150 cycles/min) with different concentrations of isoprenaline (a β-adrenergic agonist) without or with insulin, vanadate or pV at the indicated concentrations. After 30 min, the incubation tubes were placed on ice and 200 μl of cell medium was removed for the enzymic determination of glycerol as described previously [22]. Total lipids were evaluated gravimetrically after extraction by the method of Dole and Meinertz [23]. Results were expressed as μmol of glycerol released by 100 mg of lipids in 30 min.

Statistical analysis

The statistical significance of differences was analysed with Student's *t* test (paired data) for simple comparison and with analysis of variance for multiple comparisons. Results are given as means ± S.E.M.

RESULTS

Anti-lipolytic effect of insulin, vanadate and pV

In the presence of 1 nM insulin, lipolysis stimulated by 10 nM, 100 nM and 1 μM isoprenaline was inhibited by 95.4 ± 2.4%, 76.0 ± 4.4% and 60.2 ± 4.4% respectively ($n = 7$–9). Vanadate at a concentration of 0.1 mM inhibited lipolysis weakly, whereas 5 mM vanadate inhibited lipolysis fully at all isoprenaline concentrations used (Figure 1). pV (0.01 mM) was almost as potent as 1 nM insulin or 5 mM vanadate in inhibiting lipolysis induced by 10 nM isoprenaline. However, in the presence of 100 nM isoprenaline the anti-lipolytic effect of 0.01 mM pV was less pronounced and was no more significant at 1 μM isoprenaline. H_2O_2 alone tested at the concentration used in the preparation of 0.01 and 0.1 mM pV (corresponding to a final concentration of 0.012 and 0.12 mM H_2O_2 respectively) did not significantly inhibit lipolysis (results not shown) and was therefore not responsible for the anti-lipolytic effect of pV. Increasing the concentration of pV did not overcome the weak anti-lipolytic effect of pV observed at higher isoprenaline concentrations; rather, 0.1 mM pV was less efficient than 0.01 mM pV in inhibiting the lipolysis induced by 100 nM and 1 μM isoprenaline. The combination of 1 μM isoprenaline and 0.1 mM pV even resulted in the stimulation of lipolysis [0.1 mM pV by itself did not induce lipolysis (results not shown)]. Several of the signalling components thought to be involved in the anti-lipolytic pathway of insulin, such as IR, IRS-1, PI-3K, PDE3B and PKA, were investigated with regard to their role in the anti-lipolytic pathways activated by vanadate and pV.

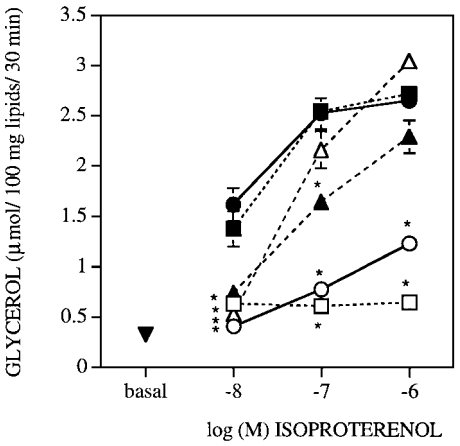

Figure 1 Effect of insulin, vanadate and pV on isoprenaline-stimulated lipolysis

Isolated adipocytes [1 ml of 5% (v/v) cell suspension] were incubated for 30 min at 37 °C in KRH buffer, pH 7.4, without (▼) or with 0.01, 0.1 or 1 μM isoprenaline (isoproterenol) alone (●) or together with 1 nM insulin (○), 0.1 mM vanadate (■), 5 mM vanadate (□), 0.01 mM pV (▲) or 0.1 mM pV (△). The glycerol released into the incubation medium was measured. Results are means ± S.E.M. for six to nine experiments. *$P < 0.001$ compared with stimulation by isoprenaline, with analysis of variance.

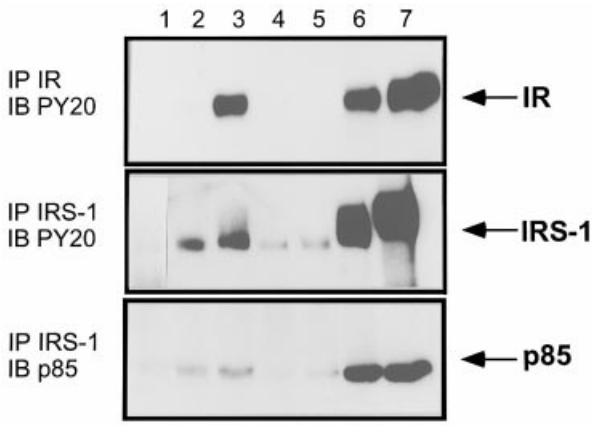

Figure 2 Effect of insulin, vanadate and pV on tyrosine phosphorylation of IR and IRS-1 and on PI-3K recruitment

Isolated adipocytes [2 ml of 10–12% (v/v) cell suspension in KRH] were treated for 30 min at 37 °C without (lane 1) or with 1 nM insulin (lane 2), 100 nM insulin (lane 3), 0.1 mM vanadate (lane 4), 5 mM vanadate (lane 5), 0.01 mM pV (lane 6) or 0.1 mM pV (lane 7). After homogenization, proteins from whole cell lysates were immunoprecipitated (IP) with anti-IR antibody (top panel) or anti-(IRS-1) antibody (middle and bottom panels) as described in the Experimental section, separated by SDS/PAGE [7% (w/v) gel], transferred to PVDF membrane and probed (IB) with the indicated antibodies. This result is representative of five separate experiments.

pV, but not vanadate, markedly stimulates the tyrosine phosphorylation of IR and IRS-1

IR and IRS-1 were immunoprecipitated from whole-cell lysates by using anti-IR and anti-(IRS-1) antibodies respectively; the phosphotyrosine contents of these proteins were analysed by Western blotting with an anti-PY20 antibody. Vanadate (5 mM)

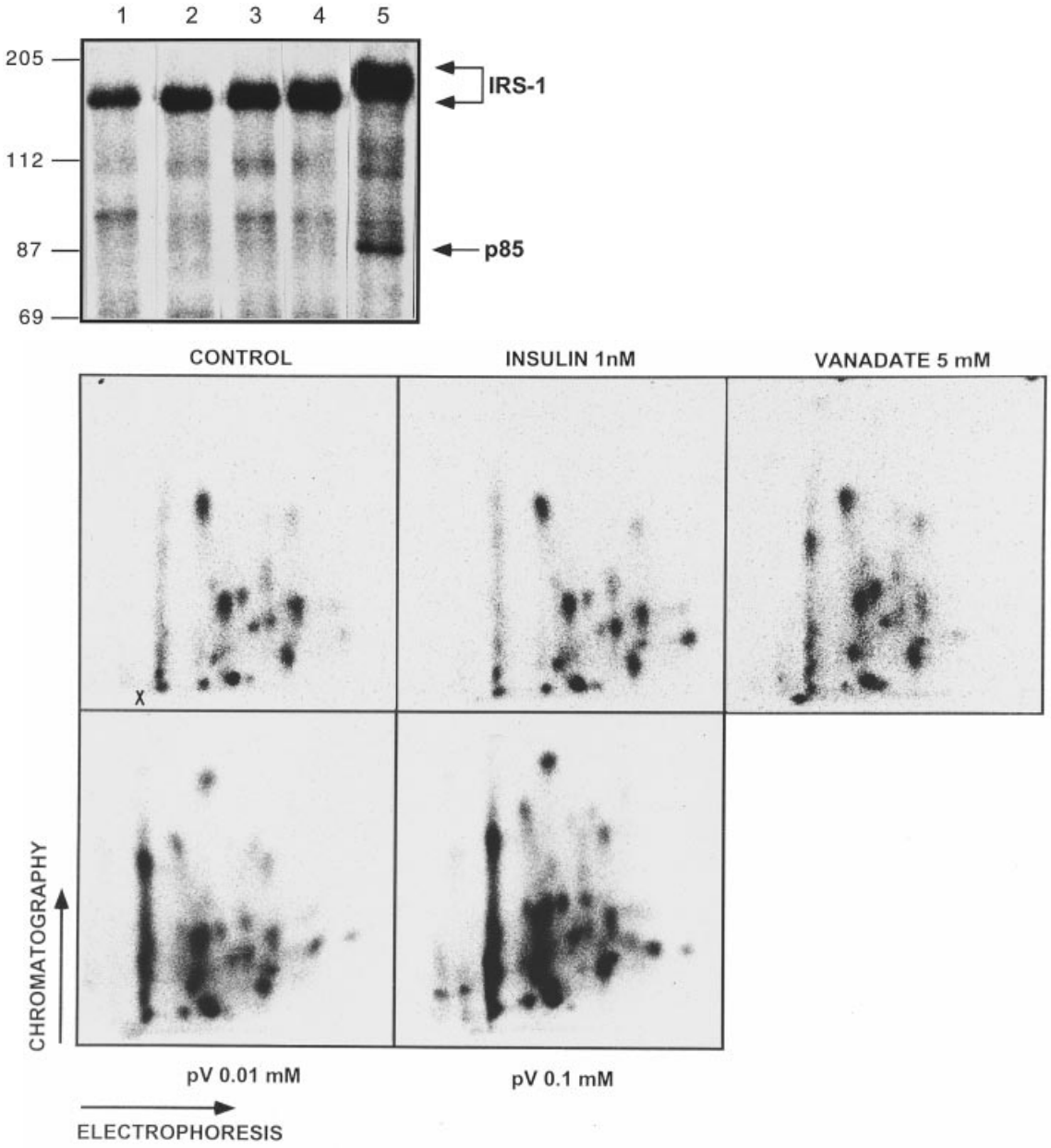

Figure 3 Two-dimensional tryptic phosphopeptide maps of IRS-1 from intact ^{32}P-labelled adipocytes stimulated with insulin, vanadate or pV

Upper panel: SDS/PAGE and Western blot of ^{32}P-IRS-1. ^{32}P-labelled adipocytes [2 ml of 10–12% (v/v) cell suspension] were stimulated for 30 min without (lane 1) or with 1 nM insulin (lane 2), 5 mM vanadate (lane 3), 0.01 mM pV (lane 4) or 0.1 mM pV (lane 5). IRS-1 from cell lysates was immunoprecipitated with anti-(IRS-1) antibody, subjected to SDS/PAGE [7% (w/v) gel] and transferred to PVDF membrane. Phosphoproteins were detected by digital imaging of ^{32}P. The positions of molecular mass markers are indicated (in kDa) at the left. Lower panels: tryptic phosphopeptide maps of IRS-1. The piece of membrane corresponding to IRS-1 was excised and the phosphoprotein was digested with trypsin. ^{32}P-phosphopeptides were separated by two-dimensional TLE/TLC and detected by digital imaging of ^{32}P. X marks the origin. The same results were obtained in five separate experiments.

induced no detectable tyrosine phosphorylation of the β-subunit of IR and less tyrosine phosphorylation of IRS-1 than did 1 nM insulin. However, vanadate was able to potentiate the insulin-induced tyrosine phosphorylation of both IR and IRS-1 (results not shown). pV (0.01 mM) induced tyrosine phosphorylation of the β-subunit of IR to the same extent as 100 nM insulin and induced higher tyrosine phosphorylation of IRS-1 than 1 or 100 nM insulin. In response to 0.1 mM pV, tyrosine phosphorylations of IR and IRS-1 were even more pronounced (Figure 2). H_2O_2 alone induced only a very weak tyrosine phosphorylation of IRS-1 (results not shown). The phosphorylations of IRS-1 and IR were also studied in the presence of isoprenaline, because (1) it has been reported that agents that increase cAMP levels block the insulin-mediated tyrosine phosphorylation of IR [24] and (2) the anti-lipolytic effect of pV was decreased at higher concentrations of isoprenaline (Figure 1). Isoprenaline did not significantly alter the tyrosine phosphorylation of IRS-1 and IR induced by pV (results not shown). To study phosphorylated IRS-1 in more detail, ^{32}P-labelled adipocytes were stimulated with 0.01 or 0.1 mM pV, 1 nM

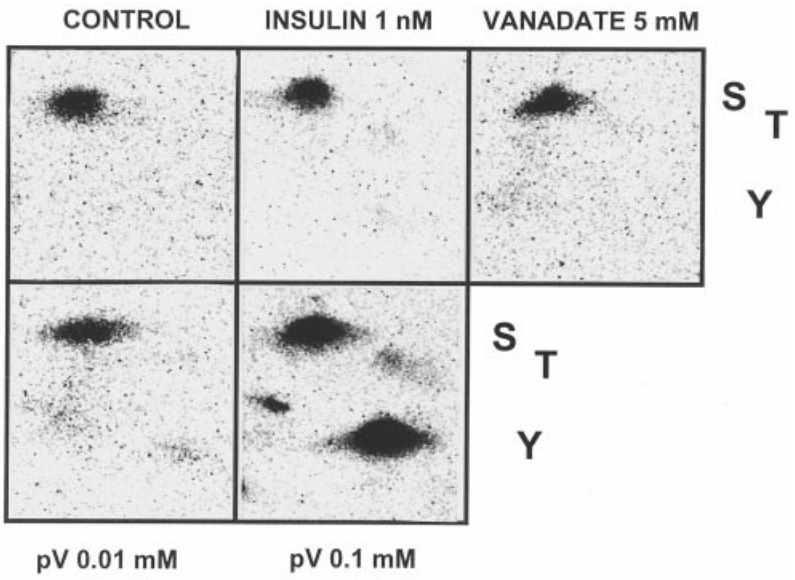

Figure 4 Phosphoamino acid analysis of PI-3K

The piece of membrane corresponding to p85 from the blot shown in Figure 3 was excised and the phosphoprotein was subjected to acid hydrolysis (6 M HCl for 60 min at 110 °C). Phosphoserine, phosphothreonine and phosphotyrosine were separated by two-dimensional TLE as described in the Experimental section and ^{32}P-phosphoamino acids were detected by digital imaging of ^{32}P. S, T and Y indicate the relative positions of phosphorylated serine, threonine and tyrosine respectively. The same results were obtained in four separate experiments.

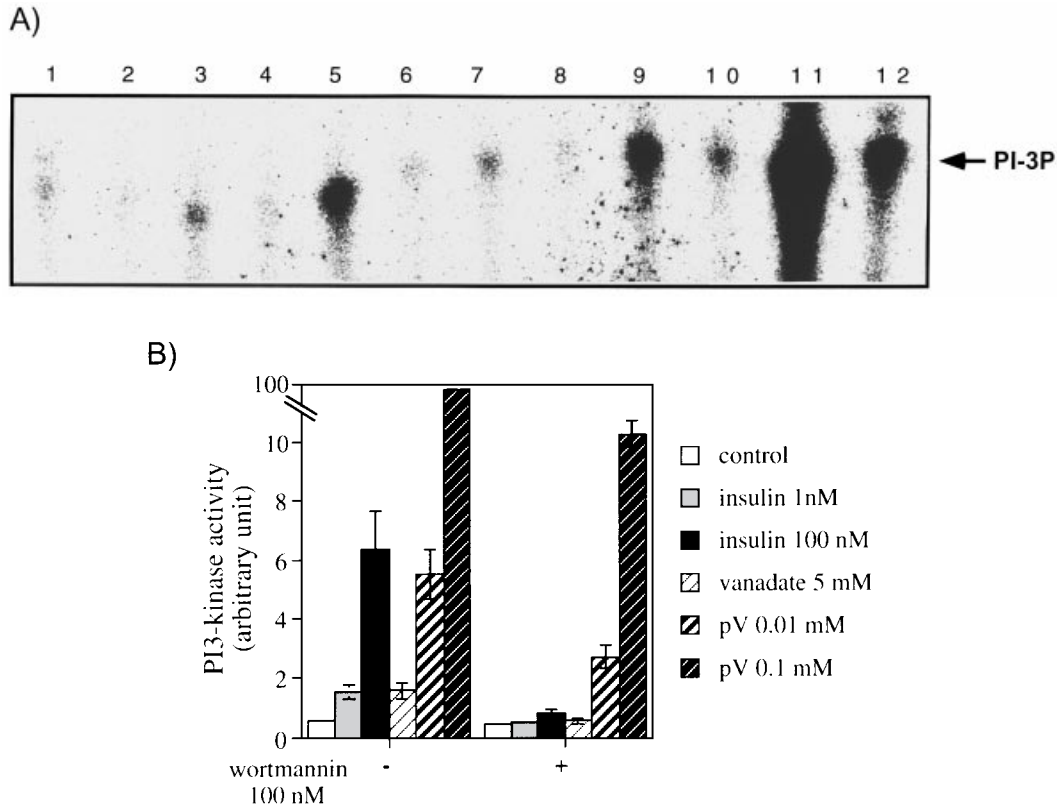

Figure 5 Effect of wortmannin on insulin-, vanadate- and pV-induced activation of PI-3K

(**A**) Isolated adipocytes [2 ml of 10–12% (v/v) cell suspension] were treated for 10 min at 37 °C without (lanes 1 and 2) or with 1 nM insulin (lanes 3 and 4), 100 nM insulin (lanes 5 and 6), 5 mM vanadate (lanes 7 and 8), 0.01 mM pV (lanes 9 and 10) or 0.1 mM pV (lanes 11 and 12). After homogenization, PI-3K from whole-cell lysates was immunoprecipitated with anti-PY20 antibody. A PI-3K assay was then performed in the absence (lanes 1, 3, 5, 7, 9 and 11) or the presence of 100 nM wortmannin (lanes 2, 4, 6, 8, 10 and 12). Phospholipids were separated by TLC as described in the Experimental section. Incorporation of ^{32}P into PI-3P was detected by digital imaging of ^{32}P. (**B**) Quantification of labelled PI-3P was performed with a Fujix Bas 2000 system. Results are means ± S.E.M. for five independent experiments.

Table 1 Effect of wortmannin on the anti-lipolytic action of insulin, vanadate and pV

Isolated adipocytes [1 ml of 5% (v/v) cell suspension] were preincubated without or with wortmannin (100 nM) for 10 min at 37 °C in KRH buffer, pH 7.4, before the addition of 10 nM isoprenaline (columns 1 and 2) or 100 nM isoprenaline (columns 3 and 4) and insulin, vanadate or pV for 30 min at the indicated concentrations. Basal lipolysis is also reported. Results (means ± S.E.M.) are from four to nine experiments. $^{*}P < 0.05$, $^{***}P < 0.0005$ compared with the anti-lipolytic effect of the different agents in the absence of wortmaninn; ††$P < 0.005$ for 0.1 mM pV compared with wortmannin/0.1 mM pV.

Conditions	Release of glycerol into medium (μmol/30 min per 100 mg of lipids)			
	− Wortmannin	+ Wortmannin	− Wortmannin	+ Wortmannin
Basal lipolysis	0.33 ± 0.04	0.34 ± 0.04	0.32 ± 0.04	0.33 ± 0.04
Isoprenaline	1.64 ± 0.13	2.13 ± 0.19	2.42 ± 0.12	2.75 ± 0.11
Isoprenaline + 1 nM insulin	0.40 ± 0.05	1.92 ± 0.22***	0.80 ± 0.07	2.71 ± 0.11***
Isoprenaline + 5 mM vanadate	0.65 ± 0.04	0.80 ± 0.07	0.61 ± 0.03	2.87 ± 0.13***
Isoprenaline + 0.01 mM pV	0.74 ± 0.11	1.71 ± 0.30*	1.62 ± 2.44	2.60 ± 0.15*
Isoprenaline + 0.1 mM pV	0.47 ± 0.05	0.77 ± 0.05	2.20 ± 0.17	1.54 ± 0.12††

insulin or 5 mM vanadate, IRS-1 was immunoprecipitated from whole-cell lysates, subjected to SDS/PAGE and transferred to PVDF membrane. As shown in Figure 3 (upper panel), IRS-1 was phosphorylated in unstimulated adipocytes. The total phosphorylation was increased in response to insulin, vanadate and pV. The electrophoretic mobility of IRS-1 was decreased only in the presence of 0.1 mM pV, suggesting that high concentrations of pV induce phosphorylation(s) of IRS-1 on sites not phosphorylated by insulin or vanadate. Membrane pieces containing IRS-1 were excised and the phosphoprotein was either subjected to acid hydrolysis for phosphoamino acid analysis, or digested with trypsin to generate phosphopeptide maps of IRS-1 by two-dimensional TLC/TLE. Phosphoamino acid analysis demonstrated that IRS-1 is phosphorylated mainly on serine residues in unstimulated adipocytes. After stimulation of the cells by insulin or pV (both concentrations), the phosphotyrosine content of IRS-1 was increased. This increase was most pronounced after stimulation with 0.1 mM pV (results not shown), in agreement with the results in Figure 2. Taken together, these results therefore indicate that the phosphotyrosine content of IRS-1 after stimulation by insulin constitutes a very small fraction of the total phosphate content; under these conditions, serine phosphorylation dominates. Tryptic phosphopeptide maps of IRS-1 from unstimulated adipocytes or from adipocytes stimulated by insulin, vanadate or pV are shown in Figure 3 (lower panels). The same patterns of detectable IRS-1 phosphopeptides were generated in unstimulated cells and in response to insulin and vanadate, except for one phosphopeptide that was generated as a result of stimulation by insulin. This phosphopeptide and additional phosphopeptides were obtained in maps from adipocytes stimulated by 0.01 and 0.1 mM pV. Importantly, the presence of isoprenaline did not alter the patterns of IRS-1 phosphopeptides (results not shown).

Insulin, vanadate and pV induce the activation of PI-3K but only pV induces the tyrosine phosphorylation of p85

Next we studied the translocation of p85 to IRS-1. In IRS-1 immunoprecipitates from vanadate-stimulated adipocytes, p85 was hardly detectable on a Western blot with anti-p85 antibody, whereas in response to pV much more PI-3K was translocated to IRS-1 than in response to 1 or 100 nM insulin (Figure 2). As seen in Figure 3 (upper panel), a phosphoprotein corresponding to p85, as judged by Western blotting (results not shown), was mainly detected in IRS-1 immunoprecipitates from ^{32}P-labelled adipocytes stimulated by 0.1 mM pV. Phosphoamino acid analysis indicated that although insulin, vanadate and pV induced the phosphorylation of serine residues of p85, only 0.1 mM pV induced a strong phosphorylation of tyrosine residues (Figure 4) and an increase in threonine phosphorylation. PI-3K activity was then measured in PY20 immunoprecipitates from adipocytes stimulated by insulin, vanadate and pV (Figure 5). Like 1 nM insulin, 5 mM vanadate induced the activation of PI-3K; 100 nM wortmannin, a potent inhibitor of the PI-3K, blocked this effect (Figure 5A). pV (0.01 mM) induced the activation of PI-3K to a similar extent as 100 nM insulin did, whereas a higher concentration of pV (0.1 mM) resulted in 15-fold more activation of PI-3K than that caused by 100 nM insulin. Although wortmannin inhibited 90% of the PI-3K activity induced by 0.1 mM pV, the residual activity was still higher than that observed in the presence of 100 nM insulin (Figure 5B). Increasing the concentration of wortmannin to 1 μM did not completely block the effect of 0.1 mM pV (results not shown). The activation of PI-3K induced by 0.01 mM pV was also partly inhibited by 100 nM wortmannin. In contrast, the pV-induced activation of PI-3K in IRS-1 immunoprecipitates was completely blocked by wortmannin (results not shown). The presence of 100 nM isoprenaline did not change the activation of PI-3K by the different agents (results not shown).

Next we evaluated the role of PI-3K activation in anti-lipolysis mediated by vanadate and pV. In the presence of 10 nM isoprenaline, the anti-lipolytic effects of 1 nM insulin and 0.01 mM pV were blocked by wortmannin, but not those of 5 mM vanadate and 0.1 mM pV (Table 1). Surprisingly, at a higher concentration of isoprenaline (100 nM), wortmannin efficiently blocked the anti-lipolytic effect of vanadate. In addition, 0.1 mM pV, which did not efficiently inhibit the lipolysis induced by 100 nM isoprenaline, could inhibit lipolysis in the presence of wortmannin (Table 1). Thus, although vanadate apparently inhibited lipolysis via a PI-3K-independent pathway, at higher isoprenaline concentrations its effect on lipolysis was clearly dependent on PI-3K. pV (0.01 mM), like insulin, inhibits lipolysis via a PI-3K-dependent pathway, whereas IRS-1-associated PI-3K does not seem to be required in the anti-lipolytic pathway of higher concentrations of pV.

Insulin, vanadate and pV induce activation of PDE3B but PDE3B is apparently not essential in vanadate-induced anti-lipolysis

As a next step we explored the role of PDE3B in the anti-lipolytic pathway mediated by vanadate and pV. PDE3B has previously been identified as an important component in the anti-lipolytic pathway of insulin [12]. Phosphorylation of PDE3B (serine-302)

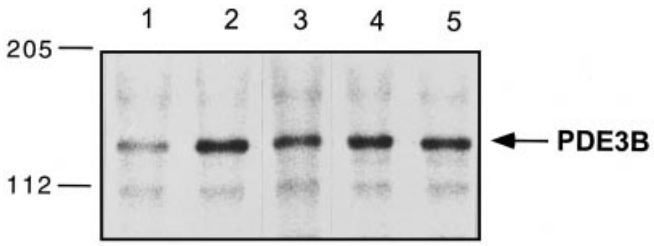

Figure 6 Effect of insulin, vanadate and pV on PDE3B phosphorylation

^{32}P-labelled adipocytes [1.5 ml of 10–12% (v/v) cell suspension] were stimulated for 30 min without (lane 1) or with 1 nM insulin (lane 2), 5 mM vanadate (lane 3), 0.01 mM pV (lane 4) or 0.1 mM pV (lane 5). PDE3B from solubilized membrane fractions was immunoprecipitated with anti-PDE3B antibody as described in the Experimental section and subjected to SDS/PAGE [7% (w/v) gel]; this was followed by digital imaging of ^{32}P. Results are representative of four separate experiments. The positions of molecular mass markers are indicated (in kDa) at the left.

Table 3 Effect of insulin, vanadate and pV on isoprenaline-induced activation of PKA

Isolated adipocytes [800 μl of 8% (v/v) cell suspension] were incubated for 5 min at 37 °C in KRH buffer with 100 nM isoprenaline alone or in the presence of insulin (1 nM), vanadate (5 mM) or pV (0.01 or 0.1 mM). The PKA assay was performed as described in the Experimental section. Results are expressed as corrected PKA activity ratios and are means ± S.E.M. for five separate experiments. ***$P < 0.0005$ compared with control value; †$P < 0.05$, ††$P < 0.005$ compared with isoprenaline value.

Conditions	Corrected PKA activity ratio
Control	0.21 ± 0.04
Isoprenaline	0.39 ± 0.05***
Isoprenaline + 1 nM insulin	0.26 ± 0.05††
Isoprenaline + 5 mM vanadate	0.27 ± 0.04†
Isoprenaline + 0.01 mM pV	0.22 ± 0.04††
Isoprenaline + 0.1 mM pV	0.42 ± 0.04

has been shown to be associated with PDE3B activation [19]. Incubation of ^{32}P-labelled adipocytes with 1 nM insulin, 5 mM vanadate, 0.01 mM pV or 0.1 mM pV resulted in phosphorylation of PDE3B (Figure 6). Vanadate and pV were as efficient as insulin in inducing phosphorylation of PDE3B. Moreover, vanadate and pV (0.1 mM) induced activation of PDE3B to a similar extent as insulin (187.21 ± 18.45% activity of control for insulin, 228.50 ± 27.94% for vanadate and 201.80 ± 26.79% for 0.1 mM pV; $n = 4$–6). To evaluate the role of PDE3B phosphorylation/activation in the anti-lipolytic pathways activated by vanadate and pV, we used OPC 3911, a cell-permeable specific PDE3 inhibitor. OPC 3911 completely blocked the anti-lipolytic effect of 1 nM insulin and 0.01 mM pV in the presence of 10 or 100 nM isoprenaline (Table 2). In contrast, the ability of vanadate to antagonize lipolysis was not changed in the presence of OPC 3911. In the presence of 10 nM isoprenaline, OPC 3911 blocked 57 ± 14% ($n = 6$) of the anti-lipolytic effect of 0.1 mM pV and completely reversed the weak anti-lipolytic effect of 0.1 mM pV observed in the presence of 100 nM isoprenaline.

Insulin, vanadate and low concentration of pV block isoprenaline-induced PKA activation

As a final step in the dissection of the anti-lipolytic pathways activated by vanadate and pV, we studied isoprenaline-induced PKA activation. It has been suggested that the anti-lipolytic effect of insulin can be explained to a large extent by the ability of insulin to lower cAMP levels and thereby PKA activity [12]. As shown in Table 3, 1 nM insulin, 5 mM vanadate and 0.01 mM pV decreased the PKA activity induced by 100 nM isoprenaline. pV (0.1 mM), which did not efficiently inhibit lipolysis in the presence of 100 nM isoprenaline, did not inhibit isoprenaline-induced PKA activation; this is in agreement with the anti-lipolytic responses observed in Figure 1.

DISCUSSION

The present study reveals an intriguing level of complexity and important differences in the mechanism of inhibition of lipolysis by vanadate and pV. The exploration of the exact molecular mechanisms involved in the signal transduction of vanadate and pV is important because they are candidate drugs for the treatment of diabetes. pV (0.01 mM) most probably promotes the appropriate cascade initiated by insulin, involving PI-3K and PDE3B, whereas at higher concentration pV exerts its anti-lipolytic effect independently of IRS-1-associated PI-3K and partly independently of PDE3B. Vanadate acts by mechanisms distinct from that of insulin: it does not require PDE3B and the involvement of PI-3K is determined by the concentration of isoprenaline used to stimulate lipolysis.

Tyrosine phosphorylation of IR and IRS proteins has an important role in mediating the effects of insulin [13]. Vanadate-induced anti-lipolysis was associated with no detectable tyrosine

Table 2 Effect of OPC 3911 on the anti-lipolytic action of insulin, vanadate and pV

Isolated adipocytes [1 ml of 5% (v/v) cell suspension] were incubated without or with 10 μM OPC 3911 for 30 min at 37 °C in KRH buffer, pH 7.4, with 10 nM isoprenaline (columns 1 and 2) or 100 nM isoprenaline (columns 3 and 4) and insulin, vanadate and pV at the concentrations indicated. Basal lipolysis is also reported. Results (means ± S.E.M.) are from three to seven experiments. *$P < 0.01$, **$P < 0.05$, ***$P < 0.0005$ compared with the anti-lipolytic effect of the different agents in the absence of OPC.

	Release of glycerol into medium (μmol/30 min per 100 mg of lipids)			
Conditions	− OPC 3911	+ OPC 3911	− OPC 3911	+ OPC 3911
Basal lipolysis	0.31 ± 0.05	0.45 ± 0.08	0.27 ± 0.03	0.48 ± 0.07
Isoprenaline	1.39 ± 0.15	2.17 ± 0.16	2.35 ± 0.12	2.65 ± 0.17
Isoprenaline + 1 nM insulin	0.34 ± 0.03	2.49 ± 0.22**	0.81 ± 0.10	2.83 ± 0.14***
Isoprenaline + 5 mM vanadate	0.59 ± 0.03	0.78 ± 0.04	0.61 ± 0.03	0.81 ± 0.05
Isoprenaline + 0.01 mM pV	0.74 ± 0.11	2.55 ± 0.40*	1.62 ± 0.24	3.00 ± 0.31*
Isoprenaline + 0.1 mM pV	0.41 ± 0.04	1.36 ± 0.31*	2.05 ± 0.17	2.83 ± 0.33**

phosphorylation of IR and very low tyrosine phosphorylation of IRS-1, suggesting that vanadate mediates its effect primarily downstream of the receptor. A signalling pathway bypassing IR has previously been proposed for vanadium compounds [1,10] (discussed below). In contrast with vanadate, pV induced a marked stimulation of tyrosine phosphorylation of IR and IRS-1. However, the extent of tyrosine phosphorylation of IRS-1 was not correlated with the efficiency of pV in blocking isoprenaline-induced lipolysis, especially at high concentrations of both pV and isoprenaline. Furthermore, vanadate, which marginally induced the tyrosine phosphorylation of IRS-1, was found to be a more potent anti-lipolytic agent than pV. To study the phosphorylation of IRS-1 in more detail, we also performed phosphoamino acid analysis and two-dimensional phosphopeptide mapping of IRS-1 from ^{32}P-labelled adipocytes. Whereas serine residues in IRS-1 were phosphorylated in unstimulated and stimulated adipocytes, phosphorylated tyrosine residues were detected mainly in IRS-1 from cells stimulated by pV. The phosphopeptide maps of IRS-1 isolated from pV-stimulated adipocytes showed the same pattern of phosphopeptides as generated from insulin- and vanadate-stimulated adipocytes as well as several other phosphopeptides. We have no indication that these additional phosphorylations of IRS-1 negatively influence the association of p85 with IRS-1 and the activation of PI-3K. Rather, pV stimulation resulted in the efficient translocation of PI-3K to IRS-1 and activation of PI-3K. Thus the poor anti-lipolytic effect of especially a high concentration of pV in the presence of a high concentration of isoprenaline cannot be explained by an inability of pV to activate PI-3K. It is possible that other pathways, activated at the level of IRS-1 due to the additional phosphorylations, can negatively influence the anti-lipolytic pathway of pV.

In addition to IRS-1, IRS-2 and IRS-3 proteins are also substrates that are tyrosine-phosphorylated by IR in rat adipocytes [25]. Two recent studies in rat and 3T3-L1 adipocytes have shown that IRS proteins as well as the associated PI-3K activity exhibit different cellular localizations [25,26]. Tyrosine phosphorylation of IRS-2 and association with PI-3K has been reported to be more transient than that of IRS-1 in response to insulin [27]. The main IRS-docking protein for PI-3K could be different between insulin, vanadate and pV owing to different distributions and/or functions. Experiments to address this point are continuing in our laboratory.

Platelet-derived growth factor has been shown to induce tyrosine phosphorylation of p85 in 3T3-L1 adipocytes [28], as well as in other cells [29]. Insulin-mediated tyrosine phosphorylation of p85 in rat adipocytes has, to our knowledge, been shown only in one study [30]. This is the first report showing that, in rat adipocytes, pV stimulation induced mainly the tyrosine phosphorylation of p85. Although pV treatment in intact rat liver increased the translocation of PI-3K to IRS-1, tyrosine phosphorylation of p85 was not observed [31]. In our study, tyrosine phosphorylation of p85 in IRS-1 immunoprecipitates was hardly detectable on a Western blot with anti-PY20 antibody. However, phosphoamino acid analysis of p85 from ^{32}P-labelled pV-stimulated adipocytes clearly showed that p85 was tyrosine phosphorylated. What, then, is the significance of this tyrosine phosphorylation? Stimulation by pV resulted in a more powerful activation of PI-3K than by insulin or vanadate; this was associated either with an efficient inhibition of lipolysis (using 10 nM isoprenaline to stimulate lipolysis) or no inhibition of lipolysis (using higher concentrations of isoprenaline). Thus the importance of the tyrosine phosphorylation of p85 is unclear.

The involvement of PI-3K in the anti-lipolytic pathways activated by vanadate and pV was also addressed. Although vanadate induced the activation of PI-3K, wortmannin did not block vanadate-mediated anti-lipolysis when lipolysis was induced by 10 nM isoprenaline, suggesting a PI-3K-independent pathway, as has also been reported by Li et al. [32]. A non-receptor protein tyrosine kinase (staurosporine-sensitive), recently identified by Elberg et al. [33], has been suggested to be involved in vanadate's anti-lipolytic pathway. This kinase was also found to interact with PI-3K in response to vanadate. The role of this interaction is unclear because, under their conditions, wortmannin was reported to not inhibit the anti-lipolytic effect of vanadate [32]. Our finding that vanadate also induced a PI-3K-dependent anti-lipolytic pathway suggests that the interaction of PI-3K with this protein tyrosine kinase could have an important role. Although we have not yet identified the mechanism responsible for directing vanadate signalling through PI-3K-dependent and PI-3K-independent pathways, it seems clear that isoprenaline-sensitive mechanisms are involved, perhaps mediated by PKA, the $\beta\gamma$ subunit of G-proteins or by other components.

When lipolysis was induced by 10 or 100 nM isoprenaline, wortmannin blocked the anti-lipolytic effect of pV (0.01 mM), suggesting that pV, like insulin, utilized PI-3K in the anti-lipolytic pathway. In contrast, the anti-lipolytic effect of a higher concentration of pV (0.1 mM) in the presence of 10 nM isoprenaline was affected only slightly by wortmannin. If we consider the PI-3K activity measured in IRS-1 immunoprecipitates (which was blocked by wortmannin), we can assume that the PI-3K associated with IRS-1 is at least not involved in the anti-lipolytic action of 0.1 mM pV. Similarly, Ida et al. [34] found that pV induced hexose uptake by a wortmannin-sensitive mechanism at low concentrations of pV but by a wortmannin-resistant mechanism at a high concentration of pV. In contrast, we cannot exclude the possibility that a wortmannin-insensitive PI-3K (detected in PY20 immunoprecipitates) is responsible for the anti-lipolytic effect observed, thus indicating a PI-3K-dependent pathway.

Ueki et al. [35] showed that vanadate activates PDE3 in rat fat pads. We found that vanadate and pV induced PDE3B phosphorylation and PDE3B activation to the same extent as 1 nM insulin. Although vanadate induced the phosphorylation and activation of PDE3B, OPC 3911, a specific inihibitor of PDE3, did not block the anti-lipolytic effect of vanadate (lipolysis stimulated by 10 or 100 nM isoprenaline), suggesting that PDE3B is not a major component in vanadate's anti-lipolytic pathway as with insulin's pathway. Brownsey and Dong [36] have also reported that the anti-lipolytic effect of vanadate persisted in the presence of cilostamide, another specific PDE3 inhibitor. In contrast with vanadate, the anti-lipolytic effect of pV (0.01 mM) was blocked by OPC 3911, suggesting an insulin-like anti-lipolytic pathway. In response to a higher concentration of pV, OPC 3911 blocked 57 % of the anti-lipolytic effect of pV (in the presence of 10 nM isoprenaline), indicating that the anti-lipolytic pathway was PDE3B-dependent but also PDE3B-independent.

Finally, we analysed the effect of pV and vanadate on PKA activity. To our knowledge, the effect of pV on PKA activity has not been reported in rat adipocytes. However, studies performed on a purified preparation of the catalytic subunit of PKA revealed dose-dependent inhibition by vanadium [36]. The decrease of isoprenaline-induced PKA activation observed in response to vanadate and a low concentration of pV would presumably lead to a lowering of PKA-mediated phosphorylation and activation of hormone-sensitive lipase [37] that could explain the anti-lipolytic effect of these agents. However, a direct effect of these agents on hormone-sensitive lipase also cannot be excluded. The inability of a high concentration of pV to decrease

PKA activity could also be explained by the fact that the combination of high concentrations of pV and isoprenaline (1 μM) even resulted in a stimulation of lipolysis. Bahouth et al. [38] have also shown that pV did not efficiently inhibit lipolysis induced by 1 μM isoprenaline but inhibited lipolysis induced by 100 nM isoprenaline. pV was suggested to inhibit preferentially β1-agonist-mediated lipolysis by increasing the desensitization of β1-adrenergic receptors. Although we cannot exclude such a mechanism, other mechanisms are involved because by using different concentrations of pV and isoprenaline we found that pV could utilize the same components as those involved in insulin's anti-lipolytic pathway.

In conclusion, we have shown that by increasing the concentration of isoprenaline to stimulate lipolysis, (1) the anti-lipolytic pathway of vanadate switched from a PI-3K-independent to a PI-3K-dependent pathway, neither of which was PDE3B-dependent, and (2) the efficiency of the anti-lipolytic effect of pV decreased; this was even more pronounced when the concentration of pV was increased. Increasing the concentration of pV also resulted in the tyrosine phosphorylation of p85 and induced a pathway that did not seem to involve IRS-1-associated PI-3K and only partly involved PDE3B. In contrast, the anti-lipolytic pathway of insulin involves PI-3K and PDE3B whatever the concentration of isoprenaline used to stimulate lipolysis. Taken together, these results reveal several important complexities in the regulation of lipolysis, provide further insight into the molecular mechanisms of vanadate and pV and could therefore be helpful to define better the use of these agents in diabetes mellitus.

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