Effects of vanadate on protein kinases in rat hepatocytes

Carlos VILLAR-PALASI,* Joan J. GUINOVART,† Anna M. GÓMEZ-FOIX, Joan E. RODRIGUEZ-GIL and Fàtima BOSCH

Department of Biochemistry, Autonomous University of Barcelona, School of Veterinary Medicine, 08193-Bellaterra (Barcelona), Spain

In rat hepatocytes, vanadate modifies neither the intracellular concentration of cyclic AMP nor the –cyclic AMP/+cyclic AMP activity ratio for cyclic AMP-dependent protein kinase. Vanadate can, however, counteract the increase in cyclic AMP and the increase in the –cyclic AMP/+cyclic AMP activity ratio of cyclic AMP-dependent protein kinase induced by glucagon. On the other hand, vanadate treatment of hepatocytes can produce a time- and concentration-dependent increase in cyclic AMP- and Ca^{2+} -independent casein kinase activity. Maximal activation at the optimal time with 5 mM-vanadate was about 70% over control. A clear relationship was observed between the activation of casein kinase and the inactivation of glycogen synthase after vanadate treatment. These results suggest that casein kinase activity may be involved in vanadate actions in rat hepatocytes.

INTRODUCTION

Vanadate exerts insulin-like effects in several cell types, and thus has been considered an important probe in studies of the mechanism of insulin action [1–6]. In rat hepatocytes [7], however, contrary to the reported insulin-like effects in rat adipocytes and skeletal muscle [4–6], vanadate activates glycogen phosphorylase and causes phosphorylation and inactivation of glycogen synthase, actions reminiscent of the behaviour of glycogenolytic hormones. In rat hepatocytes incubated in a medium depleted of Ca^{2+} , vanadate was still able to inactivate glycogen synthase, but its effect to activate phosphorylase was abolished, suggesting that vanadate action on these enzymes involves two different mechanisms: (1) a Ca^{2+} -mediated one on glycogen phosphorylase and (2) a Ca^{2+} -independent one on glycogen synthase.

In a previous paper [7] we demonstrated that, in vanadate-treated hepatocytes, glycogen synthase was phosphorylated at multiple sites, although it was not possible to determine which of the various protein kinases was involved in this action of vanadate. Glycogen synthase can be phosphorylated *in vitro* by Ca^{2+} -dependent protein kinases (phosphorylase kinase, calmodulin-dependent protein kinases and protein kinase C), by cyclic AMP-dependent protein kinase, and by several Ca^{2+} - and cyclic AMP-independent protein kinases (casein kinases I and II, and glycogen synthase kinase-3) (see [8] and [9] for reviews). Although the mechanisms of regulation *in vivo* of some kinases, such as cyclic AMP-dependent protein kinase or phosphorylase kinase, are well known [10], there is little understanding of the physiological regulation and actions of the casein kinases (reviewed in [11]).

In the present paper we report the effects of incubation of isolated rat hepatocytes with vanadate on cyclic AMP-dependent protein kinase and casein kinase activities. Our results demonstrate that, although the cyclic AMP-dependent protein kinase is not directly affected by vanadate, casein kinase activity is increased.

EXPERIMENTAL

Incubation of the cells

Suspensions of isolated parenchymal liver cells were prepared from 24 h-starved male Wistar rats (180–250 g) as in ref. [12]. Cells were finally resuspended in Krebs bicarbonate buffer (pH 7.4) free of glucose and pregassed with O_2/CO_2 (19:1). When stated, Ca^{2+} was omitted from this medium and 1 mM-EGTA was added. Samples $[(4-5) \times 10^6 \text{ cells/ml}]$ were incubated in stoppered vials at 37 °C with continuous shaking. To determine protein kinase activities, samples of the cell suspension (0.05 ml) were frozen in liquid N_2 and then rapidly thawed and homogenized in ice-cold buffer (10 mM- KH_2PO_4 , 0.5 mM-3-isobutyl-1-methylxanthine, 10 mM-EDTA, 150 mM-KCl, 0.5 mM-dithiothreitol and 0.5 mg of bovine serum albumin/ml). For the assay of Ca^{2+} - and cyclic AMP-independent protein kinase activity, 0.5 ml of buffer was used, but 2 ml was used to test the cyclic AMP-dependent protein kinase activity.

To separate casein kinases (I and II) from other kinases, frozen pellets from 1 ml of cell suspension were homogenized with 150 μl of the above-mentioned buffer, centrifuged at 30 000 *g* for 15 min, and the extract was applied to a column (0.5 cm $\times$ 2.5 cm) of phosphocellulose (Whatman P11) equilibrated with 50 mM-Tris/HCl (pH 7.5) containing 1 mM-dithiothreitol, 0.1 mM-phenylmethanesulphonyl fluoride, 60 μg of leupeptin/l and 5% (v/v) glycerol. Columns were washed with 12 ml of this buffer containing 0.35 M-KCl. Casein kinase activity was eluted with the same buffer but containing 1.2 M-KCl, and fractions (0.25 ml) were assayed for casein kinase activity.

To determine glycogen synthase activity, 2 ml of cell suspension was centrifuged and cell pellets were homogenized with 160 μl of ice-cold 10 mM-Tris/HCl buffer (pH 7.0) containing 150 mM-KF, 15 mM-EDTA, 0.6 M-sucrose, 1 mM-phenylmethanesulphonyl fluoride, 1 mM-

* Permanent address: Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia, U.S.A.

† To whom all correspondence should be addressed.

benzamidine, 25 μg of leupeptin/ml and 50 mM- β -mercaptoethanol. To measure intracellular concentrations of cyclic AMP, neutralized HClO_4 extracts were prepared from 1 ml portions of cell suspension.

Enzyme and metabolite assays

Protein kinase activities were determined by the incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into the appropriate substrates. To 30 μl of test mixture, 20 μl of hepatocyte homogenate was added, and, after 10 min incubation at 30 $^\circ\text{C}$, 40 μl samples were spotted on 2 cm $\times$ 2 cm pieces of Whatman P-81 paper. The papers were washed in 75 mM- H_3PO_4 for 4 $\times$ 15 min, the acid being changed after each wash; then the papers were dried, and the radioactivity was counted. All assays were run in duplicate and the appropriate blanks subtracted.

For determination of cyclic AMP-dependent protein kinase activity, the test mixture was 20 mM-Tris/HCl, pH 8.1, 200 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (100–300 c.p.m./pmol), 24 mM- MgSO_4 and 0.1 mg of Kemptide/ml.

For assay of Ca^{2+} - and cyclic AMP-independent kinases, the test mixture contained 20 mM-3-(*N*-morpholino)-2-hydroxypropanesulphonic acid, pH 7.0, 50 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (100–600 c.p.m./pmol), 20 mM- MgSO_4 and 2 mg of dephosphorylated casein/ml. Where indicated, casein was replaced by β -casein A (0.1 mg/ml). This phosphorylation reaction mixture also contained 3 mM-EGTA and 0.1 mg of heat-stable Walsh inhibitor/ml [13], in order to inhibit Ca^{2+} - and cyclic AMP-dependent protein kinase activities. When stated, heparin (6 μg /ml) was included in the assay mixture. Under these conditions purified casein kinase II was completely inhibited, whereas purified casein kinase I activity was unaffected. The casein kinase activity inhibited by heparin was calculated as the difference between the ^{32}P incorporations into casein in the absence and presence of heparin.

Glycogen synthase activity ratio was measured by using the low- (0.25 mM) glucose 6-phosphate/high- (10 mM) glucose 6-phosphate method as described in ref. [14].

The concentrations of cyclic AMP were determined with an Amersham cyclic AMP assay kit.

Suppliers

Purified rat liver casein kinase I [15] and casein kinase II [16] were gifts from Dr. E. Itarte. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was obtained from Amersham Corp. Sodium orthovanadate and glucagon were from Sigma. All other reagents were analytical grade.

RESULTS

Effects of vanadate on intracellular cyclic AMP concentrations and cyclic AMP-dependent protein kinase activity

Vanadate inactivation of glycogen synthase in hepatocytes is independent of the presence of Ca^{2+} in the medium, indicating that this action is not mediated by Ca^{2+} -dependent kinases, and implicating other kinases such as the cyclic AMP-dependent protein kinase and/or the cyclic AMP- and Ca^{2+} -independent kinases. Therefore our first experiments examined the effects of vanadate on cyclic AMP concentrations and cyclic AMP-dependent protein kinase activity.

Incubation of hepatocytes from livers of 24 h-fasted

Fig. 1. Time-dependent effects of vanadate on (a) basal and glucagon-stimulated cyclic AMP concentrations and (b) basal and glucagon-increased cyclic AMP-dependent protein kinase – cyclic AMP/ +cyclic AMP activity ratio

Hepatocytes were incubated for different times, with 2 mM-vanadate (○), 1 nM-glucagon (▲) or 2 mM-vanadate plus 1 nM-glucagon (●). Results represent mean values $\pm$ S.E.M. from three (a) or five (b) separate cell preparations each incubated in duplicate.

rats with 2 mM-vanadate, in the absence of glucose and for different times, did not modify the intracellular concentration of cyclic AMP (Fig. 1a). On the other hand, when cells were incubated with 1 nM-glucagon, a marked rise in cyclic AMP concentration was observed, with a maximal effect after 2 min of incubation with the hormone. However, when hepatocytes were incubated with both vanadate and glucagon together, it was found that vanadate decreased the increase in cyclic AMP caused by glucagon (see Fig. 1a). Identical effects of vanadate to decrease glucagon-stimulated concentrations of cyclic AMP were also found in hepatocytes incubated without Ca^{2+} in the medium, indicating that this action of vanadate is not mediated through a mechanism involving Ca^{2+} (results not shown).

We next studied whether this effect of vanadate on cyclic AMP concentrations was reflected in cyclic AMP-

dependent protein kinase activity. As before, cells were incubated with 2 mM-vanadate, 1 nM-glucagon or both. As shown in Fig. 1(b), vanadate had no significant effect on the $-$ cyclic AMP/ $+$ cyclic AMP activity ratio for cyclic AMP-dependent protein kinase, whereas incubation with glucagon resulted in a large increase in the state of activation of the enzyme. Again, vanadate, in agreement with the results of the determination of the concentrations of cyclic AMP, counteracted the increased activation of the cyclic AMP-dependent kinase induced by glucagon.

Fig. 2. Time-course of the effects of vanadate on the Ca²⁺- and cyclic AMP-independent casein kinase activity

Isolated hepatocytes were incubated for the indicated times with 2 mM-vanadate. Then cells were homogenized and the Ca²⁺- and cyclic AMP-independent casein kinase activity was measured as described in the Experimental section. Cyclic AMP- and Ca²⁺-independent casein kinase activity is expressed as a percentage of the control. Values are means $\pm$ S.E.M. for three independent experiments.

These results support the idea that vanadate does not exert its effects through a cyclic AMP-mediated mechanism, but that it is able to counteract the effects of glucagon, which does act through this mechanism.

Effects of vanadate on the cyclic AMP- and Ca²⁺-independent protein kinase activity

The possibility that vanadate could activate a cyclic AMP- and Ca²⁺-independent protein kinase was next studied. Fig. 2 shows that the incubation of rat hepatocytes with 2 mM-vanadate for different times resulted in a time-dependent increase (about 70 %) in the activity of the total cyclic AMP- and Ca²⁺-independent casein kinase activity. The specific activation of casein kinase activity by incubation of the cells with 2 mM-vanadate was determined with β -casein A as a substrate (results not shown). β -Casein A, especially at low concentrations, is very poorly phosphorylated by glycogen synthase kinase 3 [17], but is the preferred substrate for casein kinases I and II.

Additional experiments were performed after chromatography on phosphocellulose to separate casein kinases from interfering enzymes and inhibitors. The increase in casein kinase activity caused by vanadate was also observed in the fractions where casein kinases I and II are eluted from the phosphocellulose columns. Casein kinase activity in the eluates from cells treated with 2 mM-vanadate for 15 min was $39 \pm 5\%$ greater than that observed in eluates from control cells. These results indicate that a casein kinase is involved in vanadate action.

In order to obtain further information about which type of casein kinase was activated by incubation of hepatocytes with vanadate, the specific inhibition of casein kinase II by heparin was used as a criterion to differentiate between the two casein kinase activities. As shown in Fig. 3(a), 2 mM-vanadate caused a time-dependent increase in casein kinase activity which was not inhibited by heparin, without modifying the casein kinase activity inhibited by heparin. This effect on casein kinase

Fig. 3. Effects of vanadate on casein kinase activities inhibited and not inhibited by heparin

(a) Cells were incubated with 2 mM-vanadate for different times. (b) Cells were incubated for 8 min with increasing concentrations of vanadate. At the end of the incubations, casein kinase activity inhibited (●) or not inhibited (○) by heparin was determined. The activities are expressed as percentages of the control. Results are means $\pm$ S.E.M. for five independent experiments.

Fig. 4. Correlation between glycogen synthase activity ratio and casein kinase activity not inhibited by heparin

Hepatocytes were incubated for 8 min with increasing concentrations of vanadate: 0 (○), 0.1 mM (●), 0.5 mM (▲), 1 mM (■), 2 mM (▼) and 5 mM (◆). At the end of the incubations, low glucose 6-phosphate/high glucose 6-phosphate glycogen synthase activity ratio and casein kinase activity in the presence of heparin were determined. Casein kinase activity is expressed as a percentage of the control. Results are means $\pm$ S.E.M. for five independent experiments.

activity in the presence of heparin was maximal (the activity was increased more than 1.5-fold) after 8 min of incubation and was maintained for periods up to 30 min.

This effect of vanadate was also concentration-dependent (Fig. 3b). At 5 mM-vanadate the activity of casein kinase not inhibited by heparin was increased approx. 1.9-fold. Concentrations of vanadate higher than 5 mM were not used, since they produce a decrease in the intracellular concentration of ATP [7].

Correlation between the activation of casein kinase activity and glycogen synthase activity ratio

When the activation state of glycogen synthase was plotted against the degree of activation of casein kinase activity which was not inhibited by heparin, an inverse correlation between the variables was apparent (Fig. 4), suggesting that this casein kinase activity may be involved in the inactivation of glycogen synthase.

DISCUSSION

The aim of this work was to understand further the mechanism of action of vanadate in rat hepatocytes. Since vanadate inactivates glycogen synthase even in Ca^{2+} -depleted hepatocytes, it could logically be concluded that this effect was mediated through a Ca^{2+} -independent mechanism. This ruled out the possibility of vanadate acting through Ca^{2+} -dependent kinases, leaving only as alternative possibilities the cyclic AMP-dependent protein kinase and/or the cyclic AMP- and Ca^{2+} -independent protein kinases. Therefore, we first examined whether vanadate affected the intracellular concentrations of cyclic AMP in isolated rat hepatocytes, since contradictory results concerning the action of vanadate

on adenylate cyclase in several tissues have been reported [18–22].

Under our assay conditions, no effect of vanadate on cyclic AMP concentrations or –cyclic AMP/+cyclic AMP activity ratio for cyclic AMP-dependent protein kinase were observed. Therefore, cyclic AMP-dependent protein kinase is unlikely to be responsible for the effects of vanadate in rat hepatocytes. In contrast, vanadate counteracted the increased cyclic AMP concentrations and the resulting activation of cyclic AMP-dependent protein kinase induced by glucagon. This effect of vanadate resembles the action of insulin [12,23,24] and of epidermal growth factor [24], which are both able to counteract the increased concentrations of cyclic AMP caused by glucagon in rat hepatocytes. There is evidence that insulin can increase the activity of cyclic AMP phosphodiesterase [25,26]. Therefore it seems possible that vanadate may act in hepatocytes by increasing the activity of this enzyme. In fact, it has been reported that vanadate activates phosphodiesterase activity in adipocytes [27].

Several Ca^{2+} - and cyclic AMP-independent protein kinases which can phosphorylate and inactivate glycogen synthase *in vitro* have been described [8]. However, there are very few reports about the regulation of these kinases *in vivo* [16,28–30]. In the present report we show that vanadate increases casein kinase activity. Because of the presence of EGTA in the assay mixture, we can reasonably assume that this activity is independent of Ca^{2+} , ruling out a possible casein kinase activity by Ca^{2+} -dependent protein kinases. Because of the presence of heat-stable Walsh inhibitor [13], it can be assumed that a cyclic AMP-dependent protein kinase does not contribute to this activity. This vanadate-stimulated casein kinase activity does not appear to be glycogen synthase kinase 3, because the effect of vanadate is still observed when low concentrations of β -casein A are used for the assay. Moreover, the effect of vanadate is still observed after phosphocellulose chromatography of extracts, which separates casein kinases I and II from other kinases and potential inhibitors. Therefore our results strongly indicate that vanadate increases a Ca^{2+} - and cyclic AMP-independent casein kinase activity.

Employing the differential inhibition by heparin of the two casein kinases as a discriminating measure, we have been able to demonstrate that only casein kinase activity that was not inhibited by heparin is increased in extracts of hepatocytes pretreated with vanadate. These results suggest that vanadate stimulates a casein kinase activity that behaves, according to the heparin-inhibition criterion, as casein kinase I. The activity inhibited by heparin, which corresponds to casein kinase II, remains completely unaffected. Although casein kinase I phosphorylates and inactivates muscle glycogen synthase *in vitro*, its effects *in vivo* in rabbit skeletal muscle have been questioned [31]. However, liver and muscle isoenzymes of glycogen synthase show different susceptibility to phosphorylation by various glycogen synthase kinases [8]. In rat hepatocytes, the inverse relationship between the activity of the casein kinase not inhibited by heparin and the glycogen synthase activity ratio would suggest that casein kinase I may be involved in the inactivation of glycogen synthase by vanadate.

We thank Dr. E. Itarte for providing us with purified rat liver casein kinases I and II and for his helpful discussions in the

preparation of the manuscript, and Ms. Anna Vilalta and Ms. Teresa Babiá for their skilled technical assistance. The work was supported by a grant from the 'Fundación Banco Exterior'. C. V.-P. was the recipient of a fellowship from the CAICYT for 'Estancias temporales de científicos en situación de sabático'.

REFERENCES

1. Ramasarma, T. & Crane, F. L. (1981) *Curr. Top. Cell. Regul.* **20**, 247–301
2. Dubyak, G. & Kleinzeller, A. (1980) *J. Biol. Chem.* **255**, 5306–5312
3. Schechter, Y. & Karlsh, S. J. D. (1980) *Nature (London)* **284**, 556–558
4. Tamura, S., Brown, T. A., Dubler, R. E. & Larner, J. (1983) *Biochem. Biophys. Res. Commun.* **113**, 80–86
5. Tamura, S., Brown, T. A., Whipple, J. H., Fujita-Yamaguchi, Y., Dubler, R. E., Cheng, K. & Larner, J. (1984) *J. Biol. Chem.* **259**, 6650–6658
6. Clark, A. S., Fagan, J. M. & Mitch, W. E. (1985) *Biochem. J.* **232**, 273–276
7. Bosch, F., Ariño, J., Gómez-Foix, A. M. & Guinovart, J. J. (1987) *J. Biol. Chem.* **262**, 218–222
8. Roach, P. J. (1986) *Enzymes* 3rd Ed. **27**, 499–539
9. Edelman, A. M. & Krebs, E. G. (1987) *Annu. Rev. Biochem.* **56**, 567–613
10. Exton, J. H. (1987) *Diabetes/Metab. Rev.* **3**, 163–183
11. Hathaway, G. M. & Traugh, J. A. (1982) *Curr. Top. Cell. Regul.* **21**, 101–127
12. Massagué, J. & Guinovart, J. J. (1977) *FEBS Lett.* **82**, 317–320
13. Ashby, C. D. & Walsh, D. A. (1974) *Methods Enzymol.* **38**, 350–358
14. Guinovart, J. J., Salavert, A., Massagué, J., Ciudad, C. J., Salsas, E. & Itarte, E. (1979) *FEBS Lett.* **106**, 284–288
15. Itarte, E., Mor, A. M., Salavert, A., Pena, J. M., Bertomeu, J. F. & Guinovart, J. J. (1981) *Biochim. Biophys. Acta* **658**, 334–347
16. Martos, C., Plana, M., Guasch, M. D. & Itarte, E. (1985) *Biochem. J.* **225**, 321–326
17. Donella-Deana, A., Pinna, L. A., Hemmings, B. & Cohen, P. (1983) *Biochim. Biophys. Acta* **745**, 149–153
18. Grupp, G., Grupp, I., Johnson, C. L., Wallick, E. T. & Schwartz, A. (1979) *Biochem. Biophys. Res. Commun.* **88**, 440–447
19. Schwabe, V., Puchstein, C. H., Hannemann, H. & Söchtig, E. (1979) *Nature (London)* **277**, 143–145
20. Krawietz, W., Downs, R. W., Spiegel, A. M. & Aurbach, G. D. (1982) *Biochem. Pharmacol.* **31**, 843–848
21. Catalan, R. E., Martínez, A. M. & Aragonés, M. D. (1980) *Biochem. Biophys. Res. Commun.* **96**, 672–677
22. Hayashi, Y. & Kimura, T. (1985) *Biochem. Biophys. Res. Commun.* **130**, 945–951
23. Blackmore, P. F., Assimacopoulos-Jeannet, F., Chan, T. M. & Exton, J. H. (1979) *J. Biol. Chem.* **254**, 2828–2834
24. Bosch, F., Bouscarel, B., Slaton, J., Blackmore, P. F., & Exton, J. H. (1986) *Biochem. J.* **239**, 523–530
25. Loten, E. G., Assimacopoulos-Jeannet, F. D., Exton, J. H. & Park, C. R. (1978) *J. Biol. Chem.* **253**, 746–757
26. Heyworth, C. M., Wallace, A. V., Wilson, S. R. & Houslay, M. D. (1984) *Biochem. J.* **222**, 183–187
27. Souness, J. E., Thompson, W. J. & Strada, S. J. (1985) *J. Cyclic Nucleotide Res.* **10**, 383–396
28. Cobb, M. H. & Rosen, O. M. (1983) *J. Biol. Chem.* **258**, 12472–12481
29. Akatsuka, A., Singh, T. J., Nakabayashi, H., Lin, M. C. & Huang, K.-P. (1985) *J. Biol. Chem.* **260**, 3239–3242
30. Sommercom, J., Mulligan, J. A., Lozeman, F. J. & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8834–8838
31. Poulter, L., Ang, S.-G., Gibson, B. W., Williams, D. H., Holmes, C. F. B., Caudwell, F. B., Pitcher, J. & Cohen, P. (1988) *Eur. J. Biochem.* **175**, 497–510

Received 19 September 1988/4 April 1989; accepted 24 April 1989