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Phosphofructokinase 2 and glycolysis in HT29 human colon adenocarcinoma cell line

Regulation by insulin and phorbol esters

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Kinetic properties of phosphofructokinase 2 (PFK2) and regulation of glycolysis by phorbol 12-myristate 13-acetate (PMA) and insulin were investigated in highly glycolytic HT29 colon cancer cells. PFK2 was found to be inhibited by citrate and, to a lesser extent, by phosphoenolpyruvate and ADP, but to be insensitive to inhibition by *sn*-glycerol phosphate. From these kinetic data, PFK2 from HT29 cells appears different from the liver form, but resembles somewhat the heart isoenzyme. Fructose 2,6-bisphosphate (Fru-2,6-P_2) levels, glucose consumption and lactate production are increased in a dose-dependent manner in HT29 cells treated with PMA or insulin. The increase in Fru-2,6-P_2 can be related to an increase in the $V_{max.}$ of PFK2, persisting after the enzyme has been precipitated with poly(ethylene glycol), without change in the K_m for fructose 6-phosphate. The most striking effects of PMA and insulin on Fru-2,6-P_2 production are observed after long-term treatment (24 h) and are abolished by actinomycin, cycloheximide and puromycin, suggesting that protein synthesis is involved. Furthermore, the effects of insulin and PMA on glucose consumption, lactate production, Fru-2,6-P_2 levels and PFK2 activity are additive, and the effect of insulin on Fru-2,6-P_2 production is not altered by pre-treatment of the cells with the phorbol ester. This suggests that these effects are exerted by separate mechanisms.

INTRODUCTION

The metabolism of many tumours and neoplastic or transformed cells in culture is characterized by very high rates of glycolysis even under aerobic conditions, but the biochemical mechanism of this stimulation is still poorly understood (Weinhouse, 1972; Eigenbrodt & Glossmann, 1980). In recent years, the discovery of fructose 2,6-bisphosphate (Fru-2,6-P_2), a potent activator of phosphofructokinase1 (PFK1) (Van Schaftingen *et al.*, 1980), has focused attention on this important control point of the glycolytic pathway. Indeed, Fru-2,6-P_2 is present in high concentrations in many neoplastic cells, and it is believed to play a key role in the maintenance of high glycolytic rates (Hue & Rider, 1987; de Miguel *et al.*, 1988). The HT29 cell line, derived from a human colon adenocarcinoma, also displays a very active glycolytic pathway (Rousset *et al.*, 1984; Viallard *et al.*, 1986). In these cells, PFK1 activity was shown to be controlled by Fru-2,6-P_2 (Denis *et al.*, 1985) and the rate of lactate production was found to be closely related to the level of Fru-2,6-P_2 (Denis *et al.*, 1986*a*).

Fru-2,6-P_2 concentration is controlled by the balance between the activities of phosphofructokinase 2 (PFK2), the enzyme which synthesizes Fru-2,6-P_2, and fructose 2,6-bisphosphatase (FBPase2), which hydrolyses this metabolite. These enzymes were extensively studied in liver, and it was established that both activities are supported by one and the same protein, regulated by cyclic AMP-dependent phosphorylation resulting in the inactivation of the kinase and activation of the phosphatase (for a review, see Pilkis & El-Maghrabi, 1988). Two other isoenzymes of PFK2 were identified in heart and muscle (Rider *et al.*, 1985; Taniyama *et al.*, 1988). These isoenzymes differ in their molecular mass, their relative ratio of PFK2/FBPase2 activities, their kinetic properties, and their ability to be phosphorylated by cyclic AMP-dependent protein kinase or protein kinase C (Rider & Hue, 1986; Van Schaftingen & Hers, 1986; Kitamura & Uyeda, 1988; Kitamura *et al.*, 1988).

Loiseau *et al.* (1988) have purified the PFK2 from rat hepatoma cells (HTC cells), which does not have FBPase2 activity and differs by many criteria from the liver and muscle forms. This PFK2 more resembles the heart enzyme, but the authors suggested it might represent a fetal form re-expressed in the transformed state. However, the PFK2 from HT29 cell seems different, because we found that an increase in cyclic AMP induced by vasoactive intestinal peptide diminishes Fru-2,6-P_2 concentration, suggesting that this enzyme, like the liver one, can be phosphorylated and inhibited by cyclic AMP-dependent protein kinase (Denis *et al.*, 1986*b*). Furthermore, we have shown that insulin increases Fru-2,6-P_2 concentration in HT29 cells (Babia *et al.*, 1989), as is the case in heart (Rider & Hue, 1984), muscle (Hue *et al.*, 1982), human fibroblasts (Farnararo *et al.*, 1984) and chick-embryo fibroblasts (Bosca *et al.*, 1985), but not in hepatocytes from fed rats (Richards & Uyeda, 1982). The Fru-2,6-P_2-producing system in HT29 cells therefore appears original, and the aim of the present work was to gain more information on the properties of PFK2 from these cells, the way it is regulated by different agents and the impact of these treatments on the glycolytic rate. We investigated some kinetic properties of PFK2 and focused our attention on the metabolic responses after treatment of HT29 cells with insulin or phorbol esters (PMA). Phorbol esters, activating protein kinase C (for a review, see Blumberg, 1988), increase Fru-2,6-P_2 content in fibroblasts (Bosca *et al.*, 1985), lymphocytes (Bosca *et al.*, 1988) and rheumatoid synovial cells (Taylor *et al.*, 1988). The implication of protein kinase C in the proliferation as well as in the

Abbreviations used: DMEM, Dulbecco's modified Eagle medium; Fru-2,6-P_2, fructose 2,6-bisphosphate; PFK1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK2, 6-phosphofructo-2-kinase (EC 2.7.1.105); FBPase2, fructose-2,6-bisphosphatase (EC 3.1.3.46); PMA, phorbol 12-myristate 13-acetate.

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differentiation processes of many cell types is now well documented (for a review, see Berridge, 1987). It was therefore decided to see whether activation of this enzyme might influence carbohydrate metabolism in HT29 cells, especially at the step of Fru-2,6-P_2 production.

MATERIALS AND METHODS

Materials and cell culture

The HT29 cell line has been established in permanent culture from a human colon carcinoma by Dr. J. Fogh (Sloan Kettering Institute for Cancer Research, Rye, NY, U.S.A.) (Fogh *et al.*, 1977). The cells were routinely seeded at a density of 2.5 $\times 10^4$ cells/cm^2 in plastic Petri dishes (35 mm diam.) and grown at 37 °C under air/CO_2 (19:1) in DMEM (Eurobio), supplemented with 10% (v/v) fetal-calf serum (Boehringer). During the exponential phase of growth, the culture medium was changed every 48 h. Under these conditions, cell confluency was reached after 8 days of culture. During stationary phase, medium was changed every 24 h, to avoid any nutrient exhaustion.

All experiments were performed with attached post-confluent cells. At 24 h before starting the experiments, the standard culture medium was removed and replaced by serum-free medium. Bovine insulin (26 i.u./mg of protein; Boehringer) or PMA (Sigma) was added at the indicated concentrations. It was previously verified that the vehicle for PMA (dimethyl sulphoxide) at the concentrations used (0.1–0.001%, v/v) had no influence on the parameters studied. At given times, the medium was removed and kept for glucose and lactate determination, and the cell layer was rapidly frozen in liquid N_2.

Biochemical measurements

Glucose and lactate were measured in samples of the culture medium as previously described (Denis *et al.*, 1986*a*). For the determination of Fru-2,6-P_2, frozen cell layers were scraped into 0.1 M-NaOH and sonicated for 10 s. The homogenate was heated for 10 min at 80 °C and then used for Fru-2,6-P_2 assay as described by Van Schaftingen & Hers (1984).

PFK2 activity was determined as follows: the frozen cell layers were scraped into 1 ml of ice-cold 100 mM-Hepes buffer (pH 7.5) containing 50 mM-KCl, 2 mM-EDTA, 50 mM-NaF, 30 mM-2-mercaptoethanol and 0.1 mM-phenylmethanesulphonyl fluoride (medium A). The resulting suspension was homogenized by 10–12 passages through a 26-gauge needle and then centrifuged at 35000 *g* for 20 min. The supernatant was then tested for PFK2 activity. In some experiments, PFK2 activity was assayed in a poly(ethylene glycol) extract. For that purpose, poly(ethylene glycol) (M_r 6000) was added to the 35000 *g* supernatant to give a final concentration of 20%. The mixture was stirred for 2 h at 4 °C and centrifuged at 9000 *g* for 20 min. The pellet was then rinsed, resuspended in 0.5 ml of medium A and used for PFK2 assay.

Unless otherwise indicated, PFK2 activity present in 50 μl was determined at 30 °C in a final volume of 0.5 ml containing 50 mM-Hepes (pH 7.1), 100 mM-KCl, 5 mM-KCl, 5 mM-KH_2PO_4, 7 mM-$MgCl_2$, 15 mM-2-mercaptoethanol and 5 mM-fructose 6-phosphate. Glucose 6-phosphate was always present in the proportion of 3 glucose 6-phosphate to 1 fructose 6-phosphate. After 5 min preincubation, the reaction was started by addition of 5 mM-ATP. At times 0, 5, 10 and 15 min, 100 μl samples were taken and mixed with 100 μl of 0.25 M-NaOH to stop the reaction. After neutralization with 0.1 M-acetic acid in the presence of 20 mM-Hepes, Fru-2,6-P_2 concentration was determined. One unit of PFK2 is defined as the amount of enzyme that catalyses the formation of 1 μmol of Fru-2,6-P_2/min.

Protein concentration was determined as described by Bradford (1976), with bovine serum albumin (Sigma) as standard.

Statistical significance of differences was assessed by Student's unpaired *t* test. Values reported are means ± S.E.M. For the kinetic and dose/response studies, curves were fitted and analysed by computer by using the Biodata Handling Program written by Barlow (1983).

RESULTS

Kinetic properties of PFK2 from HT29 cells

PFK2 activity measured in the supernatant of HT29 cells is 27.80 ± 7.63 μ-units/mg of protein ($n = 14$), whereas precipitation of the enzyme by 20% poly(ethylene glycol) increases the specific activity to 40.92 ± 2.53 μ-units/mg ($n = 3$). All the kinetic studies presented below were made with poly(ethylene glycol) extracts. The saturation curves for fructose 6-phosphate and ATPMg (Fig. 1) are hyperbolic, and the apparent K_m values obtained by linearization of the curves are 25 ± 7 μM ($n = 3$) for fructose 6-phosphate and 950 ± 210 μM ($n = 3$) for ATPMg.

The effects of some metabolites on PFK2 activity are presented in Fig. 2. Citrate, a well-known negative effector of PFK2, also inhibits PFK2 from HT29 cells. However, a 80% inhibition only can be obtained, and the calculated K_i is 210 ± 80 μM ($n = 4$). By contrast, *sn*-glycerol phosphate fails to inhibit the enzyme, and ADP appears to be only a weak inhibitor. Phosphoenolpyruvate decreases the enzyme activity to 20% of the control value, and the estimated K_i is 1.8 ± 0.3 mM ($n = 7$). Although it was not possible to work with a purified enzyme, the kinetic behaviour of the PFK2 from HT29 cells seems to be different from that described for liver, but somewhat resembles that described for heart (Rider *et al.*, 1985) or HTC hepatoma cells (Loiseau *et al.*, 1988). To obtain more information on the properties of PFK2 from HT29 cells, we investigated the regulation of the enzyme and of the glycolytic rates by insulin and PMA.

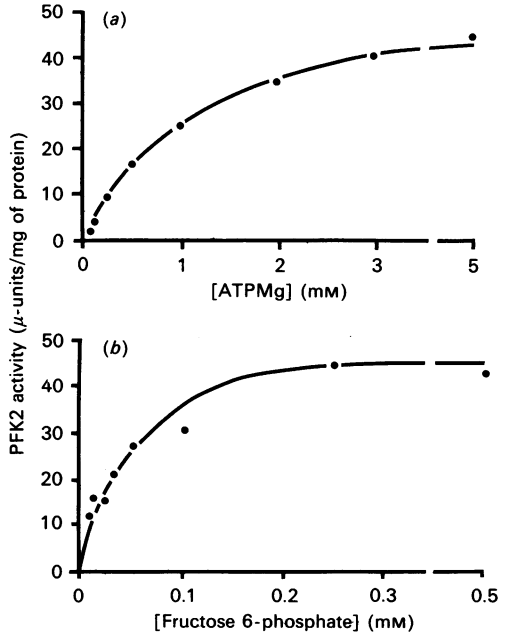

Fig. 1. PFK2 activity in 20%-poly(ethylene glycol) extracts of HT29 cells

The saturation curves were established for ATPMg (*a*) and fructose 6-phosphate (*b*). In (*a*) the concentration of fructose 6-phosphate was 1 mM, and in (*b*) the concentration of ATPMg was 1 mM. The curves are from one typical experiment out of four.

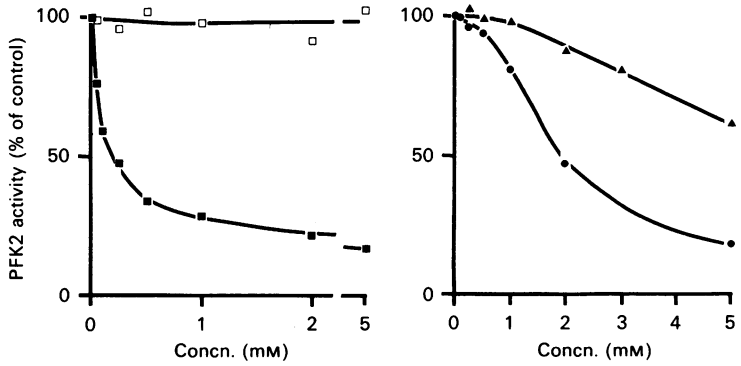

Fig. 2. Effect of citrate (■), *sn*-glycerol phosphate (□), phosphoenolpyruvate (●) and ADP (▲) on PFK2 activity measured with sub-optimal concentrations of fructose 6-phosphate (0.1 mM) and ATPMg (1 mM)

Each point represents the mean of four separate experiments. For clarity S.E.M. is not presented, but was less than 7 % in all cases. The 100 % value was 24.15±4.85 μ-units/mg of protein.

Effects of insulin and PMA on Fru-2,6-P_2 concentration and glycolytic rates

Fru-2,6-P_2 concentration was studied as a function of time after changing the culture medium. As shown in Fig. 3, in control cells (incubated in serum-free DMEM), replacement of the medium induces an increase in Fru-2,6-P_2 concentration to reach more than twice the basal value after 2 h. This peak can be attributed to the removal of inhibitory metabolites, especially lactate, as discussed in a previous paper (Denis *et al.*, 1986*a*). After 10 h, Fru-2,6-P_2 concentration has returned to basal value and no further change is observed later.

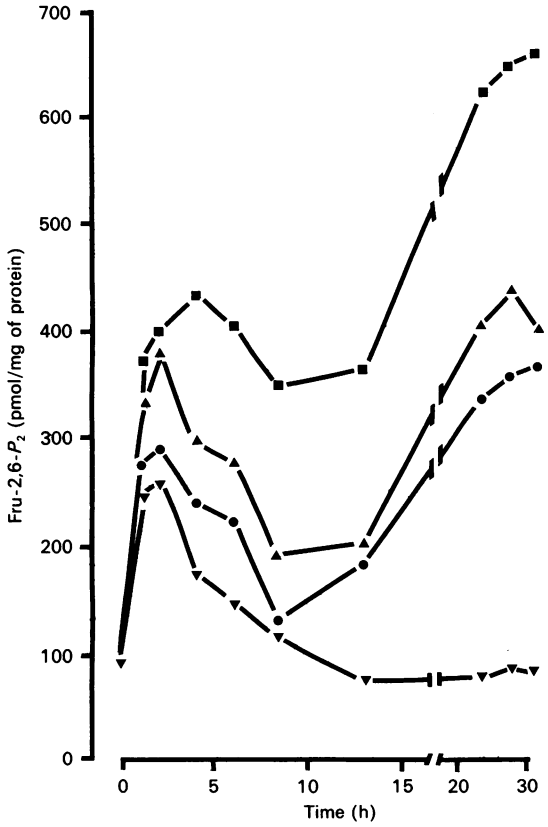

Fig. 3. Time course of Fru-2,6-P_2 concentration in HT29 cells incubated in serum-free DMEM without addition (▼), with 850 nM-insulin (▲), with 1620 nM-PMA (●) or with 850 nM-insulin and 1620 nM-PMA (■)

The curve is from one representative experiment out of three, and each value is calculated from triplicate assays.

When insulin (850 nM) or PMA (1620 nM) is present, a different pattern is observed. The peak observed at 2–4 h is greatly enhanced. After 4 h, Fru-2,6-P_2 levels tend to decrease, as observed with control cells. But, after 12 h, a further and long-lasting increase is observed, leading to very high Fru-2,6-P_2 concentrations. At 24 h, Fru-2,6-P_2 level is increased 4- and 5-fold with PMA and insulin respectively, compared with the control value. It is noteworthy that this second peak occurs although glucose is nearly exhausted in the culture medium and lactate is present at very high concentration [40.87±3.29 mM (n = 12) with insulin and 36.48±2.69 mM (n = 11) with PMA]. It is likely that the effect of PMA is mediated by the activation of protein kinase C, since other phorbol derivatives, which are ineffective in stimulating protein kinase C (4 α-phorbol 12,13-didecanoate and 4 α-phorbol), do not modify Fru-2,6-P_2 levels (results not shown). Furthermore, PMA was shown by Franklin *et al.* (1989) to increase the membrane-associated protein kinase C activity in HT29 cells.

When insulin and PMA are added in combination, an additive effect is observed. After incubation for 32 h with both drugs, the concentration of Fru-2,6-P_2 reaches 698±28 pmol/mg of protein (n = 5), which represents more than 8 times that in control cells. Assuming that the cell water volume is 4.8 μl/mg of protein in HT29 cells (Rousset *et al.*, 1984), this corresponds to an intracellular concentration as high as 145 μM, which is far above that (1 μM) necessary to obtain maximal activation of PFK1 *in vitro* (Denis *et al.*, 1985).

As the most striking effects of either insulin or PMA occur with long-term incubation, the effects of these compounds were studied in the following experiments after 24 h incubation with the drugs. Table 1 shows the effects of insulin and PMA, separately or in combination, on glycolytic rate. Both compounds clearly increase the rates of glucose consumption and lactate production. An additive effect is also observed on these parameters. Lactate production is increased by 30 % with insulin, by 21 % with PMA and by 53 % when both are added in combination. By contrast with the results of Franklin *et al.* (1988), who observed that insulin activates glycolysis in HT29 cells but leads to the accumulation of phosphorylated intermediates, we found that glucose consumption and lactate production are increased in the proportion of 2 lactate molecules produced per glucose molecule consumed, indicating that stimulation by insulin and PMA concerns the whole glycolytic pathway.

Table 1. Effects of insulin (850 nM) and PMA (1620 nM) on the glycolytic rate of HT29 cells

Glucose and lactate concentrations were determined in the culture medium at 2 h intervals during 12 h. Numbers of determinations are given in parentheses. Significant differences from control are shown by ** $P < 0.01$ and *** $P < 0.001$.

	Glucose consumption (nmol/h per mg of protein)	Lactate production (nmol/h per mg of protein)
Control	441 ± 30 (8)	867 ± 37 (10)
Insulin	567 ± 25 (8)**	1128 ± 34 (10)***
PMA	562 ± 21 (8)**	1048 ± 21 (10)***
Insulin + PMA	669 ± 66 (8)**	1327 ± 52 (10)***

The additivity of the effects of PMA and insulin on the glycolytic rate and the Fru-2,6-P_2 concentration suggests that they act by different pathways, i.e. that PMA effects are mediated by the activation of protein kinase C, whereas the transducing pathway of insulin does not involve this enzyme. As shown in Table 2, the additivity is also observed with lower concentrations of insulin or PMA. Furthermore, a 24 h preincubation, of the cells with PMA, which is known to down-regulate protein kinase C, does not modify the effects of insulin.

Dose/response curves for Fru-2,6-P_2 concentration and lactate production are presented in Fig. 4. Throughout the experiments (repeated three times at least in triplicate) insulin is more effective than PMA in stimulating Fru-2,6-P_2 production. The values presented in Fig. 4 are somewhat lower than the corresponding values reported in Fig. 3. In fact, we observed a variability in the intensity of the response, the effect being more pronounced when cells were left for longer periods in stationary phase. However, the curves remained identical in shape. Like Fru-2,6-P_2, lactate production is also increased in a dose-dependent manner by insulin and PMA, and the maximum effect is almost equivalent with each compound. For insulin, the half-maximum effect on Fru-2,6-P_2 synthesis is obtained with 129 ± 8 nM ($n = 3$), whereas the half-maximum effect on lactate production occurs at one-tenth the concentration, i.e. 13.1 ± 3.7 nM ($n = 3$). This means that lactate production is more sensitive to the stimulation by insulin than is Fru-2,6-P_2 synthesis. Maximum production of lactate can be obtained even if maximum Fru-2,6-P_2 levels are not reached; in other words, Fru-2,6-P_2 production can be over-stimulated when maximum glycolytic rate is already obtained. With PMA the same difference is observed, although less pronounced: the half-maximum effects on Fru-2,6-P_2 synthesis and lactate production are obtained with 334 ± 26 nM ($n = 4$) and 135 ± 6 nM ($n = 3$) respectively.

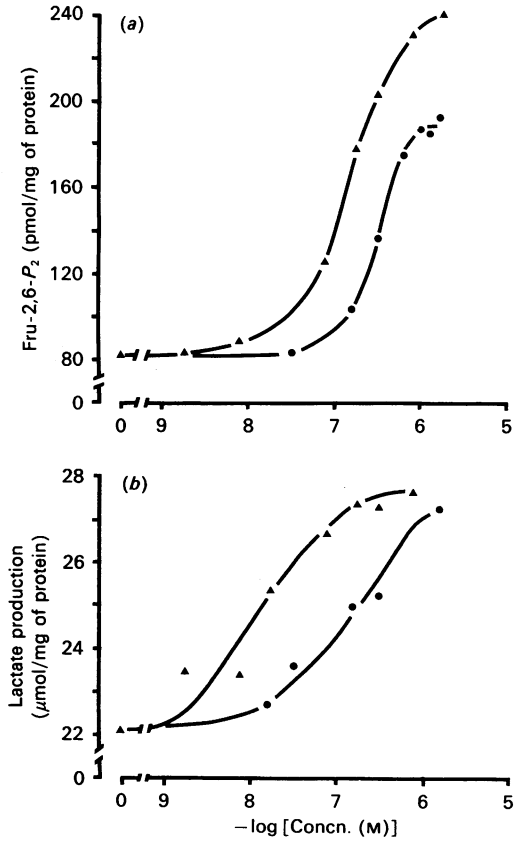

Fig. 4. Dose/response curves for the effects of insulin (▲) and PMA (●) on Fru-2,6-P_2 concentration (*a*) and lactate production (*b*) after 24 h incubation

The curves are from one and the same representative experiment out of three, and each point is the mean of four samples.

It is noteworthy that when the dose/response curves are established for a shorter incubation time (4 h) (results not shown), the half-maximum effect on Fru-2,6-P_2 level is obtained at a lower concentration [28 ± 7 nM ($n = 3$) for insulin and 238 ± 35 nM ($n = 3$) for PMA]. At least for insulin, this result suggests that the short-term effect on Fru-2,6-P_2 occurs via a general activation of the glycolytic pathway (probably by increasing substrate availability for PFK2), whereas the long-term effect is somewhat independent and directed more specifically towards PFK2.

Table 2. Additivity of the effects of PMA and insulin on lactate production and Fru-2,6-P_2 concentration

Treated cells were incubated for 24 h in the presence of either PMA or insulin or both. Pre-treated cells were incubated for 24 h with 1620 nM-PMA, then the medium was changed and the cells were further incubated for 24 h with no addition or with 850 nM-insulin. Results are means ± S.E.M. for three experiments.

Cells	Lactate production (μmol/24 h per mg of protein)	Fru-2,6-P_2 concn. (pmol/mg of protein)
Control	24.5 ± 1.7	68.8 ± 1.2
Treated		
PMA (162 nM)	26.3 ± 0.9	72.4 ± 3.2
Insulin (170 nM)	29.6 ± 1.4	200.2 ± 23.4
PMA + insulin	35.8 ± 0.9	305.8 ± 18.3
Pre-treated 24 h with PMA (1620 nM)		
No further addition	25.2 ± 1.0	120.5 ± 22.4
+ insulin (850 nM)	34.8 ± 0.7	319.7 ± 11.9

PFK2 activity in HT29 cells treated with insulin or PMA

The increase in Fru-2,6-P_2 concentration after treatment with insulin or PMA suggests that PFK2 is stimulated in HT29 cells. Therefore the enzyme activity was investigated, and the results are presented in Table 3. When the enzyme activity is measured in the supernatant of HT29 cells, a significant increase is noticed, the basal value being increased 1.9-fold with 850 nM-insulin or 1620 nM-PMA and 3.2-fold when both are added in combination. The additive effect observed on Fru-2,6-P_2 is also evidenced with respect to PFK2 activity. The effects of insulin or PMA persist when the enzyme is precipitated by poly(ethylene glycol), which is believed to separate small-molecular-mass regulators from the enzyme. In such a case, PFK2 activity is increased by 1.7-, 1.6- and 2.8-fold with insulin, PMA or the combination of both, respectively. In poly(ethylene glycol) extracts again, the additivity of the effects of insulin and PMA are remarkable. The stimulation of the enzyme is almost the same as in supernatants, suggesting that the presence of small-mass effectors is not critical for the activation of PFK2, which would rather rely on a covalent modification (such as phosphorylation) or an increased number of enzyme molecules. However, the delay necessary to see the increase in activity seems to favour an enhancement of PFK2 synthesis (or a decreased rate of the enzyme turnover) on treatment with insulin or PMA.

To document this point, the action of several inhibitors of protein synthesis was tested, and results are shown in Table 4. The inhibitors did not modify the basal PFK2 activity at the concentrations used, and similar results were obtained when the enzyme was assayed in supernatants or in poly(ethylene glycol) extracts. Actinomycin, puromycin and cycloheximide significantly decrease the activation of PFK2 induced by insulin, PMA or both: in the presence of the inhibitors, PFK2 activity is no longer different from that measured in control cells. Taken together, these results strongly suggest that the increase in PFK2 activity observed in the presence of insulin or PMA relies on a mechanism involving protein synthesis.

DISCUSSION

The experiments presented in this paper show that PFK2 from HT29 cells is different from liver PFK2 but shares common characteristics with the heart form (Rider *et al.*, 1985) or the PFK2 from HTC hepatoma cells (Loiseau *et al.*, 1988). Fru-2,6-P_2 levels are regulated in HT29 colon-cancer cells by different hormones. Vasoactive intestinal peptide induces a decrease in Fru-2,6-P_2 concentration which can be counteracted by α_2-adrenergic agonists (Denis *et al.*, 1986*b*), whereas insulin increases Fru-2,6-P_2 (Babia *et al.*, 1989). The present study demonstrates that phorbol esters are also able to enhance PFK2 activity and Fru-2,6-P_2 production.

The regulation of glucose metabolism by insulin was investigated in HT29 cells by Franklin *et al.* (1988). In agreement with the present study, they found that insulin activates glucose consumption and lactate production, although it does not stimulate glucose transport. However, they raised the possibility that insulin may act via activation of receptors for insulin-like growth factor I (IGF-I). Under our experimental conditions, maximum stimulation of glycolysis is obtained at an insulin concentration (100 nM) which does not inhibit IGF-I binding (Franklin *et al.*, 1988). However, the maximum effect on Fru-2,6-P_2 production requires about 1 μM-insulin, a concentration at which 30% of IGF-I binding is displaced. Therefore, we cannot exclude that a part of the effects of insulin is mediated through activation of IGF-I receptors. The fact that insulin modulates Fru-2,6-P_2 production was already reported in heart (Rider & Hue, 1984) and chick-embryo fibroblasts (Bosca *et al.*, 1985). Since the increase in $V_{max.}$ persisted after the enzyme had been precipitated by poly(ethylene glycol), it was postulated by these authors that a stable change in PFK2, such as a covalent

Table 3. PFK2 activity in HT29 cells incubated for 24 h in the presence of 850 nM-insulin, 1620 nM-PMA or both in combination

PFK2 activity was measured as described in the Materials and methods section, either in the 35000 *g* supernatant or in the 20%-poly(ethylene glycol) extract. Numbers of determinations are given in parentheses: significant differences from control are shown by ** $P < 0.02$ and *** $P < 0.001$. Abbreviation: Fru-6-*P*, fructose 6-phosphate.

	PFK2 activity in supernatant	PFK2 activity in poly(ethylene glycol) extracts	
	$V_{max.}$ (μ-units/mg of protein)	$V_{max.}$ (μ-units/mg of protein)	K_m for Fru-6-*P* (μM)
Control	27.80 ± 2.03 (14)	40.92 ± 2.53 (3)	25 ± 7 (3)
Insulin	53.96 ± 5.15 (11)***	68.04 ± 6.61 (3)**	34 ± 10 (3)
PMA	51.94 ± 6.14 (9)***	65.92 ± 6.65 (3)**	18 ± 9 (3)
Insulin + PMA	88.19 ± 7.22 (9)***	113.21 ± 17.90 (3)**	21 ± 7 (3)

Table 4. Effect of protein synthesis inhibitors on PFK2 activity in HT29 cells incubated in serum-free DMEM with no addition (control), with 850 nM-insulin (insulin), with 1620 nM-PMA (PMA) or with 850 nM-insulin and 1620 nM-PMA (insulin + PMA)

Actinomycin (0.2 μg/ml), puromycin (100 μg/ml) or cycloheximide (2 μg/ml) were introduced 45 min before insulin or PMA and left during the whole incubation period. After 24 h, the cell layers were frozen and analysed for PFK2 activity in the supernatant. Means ± S.E.M. of four separate experiments are presented: †† $P < 0.02$ and ††† $P < 0.005$ compared with control values; * $P < 0.05$, ** $P < 0.02$, *** $P < 0.005$ compared with the activity measured in the absence of inhibitor.

	PFK2 activity (μ-units/mg of protein)			
Addition	Control	Insulin	PMA	Insulin + PMA
None	22.40 ± 1.35	40.29 ± 3.78†††	33.81 ± 3.88††	56.15 ± 5.02†††
Actinomycin	24.69 ± 2.66	26.45 ± 2.54*	25.64 ± 3.75	22.36 ± 2.78***
Puromycin	27.03 ± 5.54	24.41 ± 1.16**	21.68 ± 2.13*	26.69 ± 5.14***
Cycloheximide	25.54 ± 3.07	28.57 ± 2.16*	27.80 ± 3.46**	32.05 ± 15.33**

modification, is involved. This could also be the case in HT29 cells. By contrast, in muscle, Fru-2,6-P_2 concentration is increased by insulin (Hue *et al.*, 1982), whereas PFK2 activity is not modified (Carreras *et al.*, 1988).

Phorbol esters also stimulate glycolytic flux, Fru-2,6-P_2 production and PFK2 activity in HT29 cells. Franklin *et al.* (1989) observed that the treatment of HT29 cells with PMA induces the translocation and rapid activation of protein kinase C followed, after 2 h, by the progressive disappearance of its activity. Therefore, the long-term effects observed in our study might be due to the down-regulation of protein kinase C rather than to its activation. However, PMA is also known to induce gene expression (Angel *et al.*, 1987), and the abolition of PFK2 activation on treatment with actinomycin, cycloheximide or puromycin rather suggests that RNA and protein synthesis (*de novo* or continuous) is involved in the activation of PFK2. In mouse epidermal cells treated with phorbol esters, Tejwani *et al.* (1985) observed an enhancement of glucose-6-phosphate dehydrogenase, hexokinase, PFK1 and pyruvate kinase activities, owing to an increase in the number of enzymes molecules. Insulin is also known to regulate the expression of several genes, especially those coding for the glycolytic enzymes such as pyruvate kinase or glyceraldehyde-phosphate dehydrogenase (Munnich *et al.*, 1984; Dani *et al.*, 1986). In liver, this hormone is necessary for the maintenance of Fru-2,6-P_2 level, because the latter is decreased in starved or diabetic states (Pilkis *et al.*, 1983). This decrease is due to a lower content of PFK2, although no change in mRNA level is observed (Colosia *et al.*, 1988; Crepin *et al.*, 1988). In HT29 cells, the long-term effects of insulin on PFK2 are abolished by protein-synthesis inhibitors, suggesting that the synthesis of at least one protein is necessary to obtain activation of the enzyme.

Interestingly, in HT29 cells, PMA and insulin present additive effects on glucose consumption, lactate production, Fru-2,6-P_2 levels and PFK2 activation, suggesting they act by separate mechanisms. This was also shown for the stimulation of glucose uptake in L6 muscle cells (Klip & Ramlal, 1987), for the accumulation of p33 mRNA in rat hepatoma cells (Sato *et al.*, 1988) and for the induction of ornithine decarboxylase in H 35 hepatoma cells (Goodman *et al.*, 1988).

Rochette-Egly *et al.*, (1988) investigated the effects of PMA on the differentiation of HT29 cells and found that the phorbol ester is able to induce, after 4 weeks treatment, a limited expression of differentiation, preceded by a decrease in the growth rate. However, the increase in the glycolytic flux and the activation of Fru-2,6-P_2 synthesis that we observed after 24 h treatment are more often associated with the proliferative process rather than with differentiation. Proliferating cells are characterized by a high content of Fru-2,6-P_2 (Hue & Rider, 1987). In HT29 cells treated with insulin and PMA, the level of Fru-2,6-P_2 is amost 150 times that needed for PFK1 activation. It is remarkable that Fru-2,6-P_2 concentration is further increased even when the maximum rate of lactate production is obtained. The significance of these elevated Fru-2,6-P_2 levels in response to proliferative signals is not yet understood, but they seem necessary for the maintenance of the 'glycolytic phenotype' in neoplastic or transformed cells.

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