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Adenylate cyclase stimulating agents and mitogens raise fructose 2,6-bisphosphate levels in human fibroblasts

Evidence for a dual control of the metabolite

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Fructose 2,6-bisphosphate, the most potent activator of 6-phosphofructo-1-kinase, has been demonstrated to mediate the increase of glycolytic flux induced by mitogens human fibroblasts. In the present work the molecular basis of transmembrane control of fructose 2,6-bisphosphate has been investigated. Prostacyclin and isoprenaline, known to activate adenylate cyclase, are able to increase fructose 2,6-bisphosphate levels, indicating that in human fibroblasts cyclic AMP plays a positive role in the control of the metabolite concentration, opposite to that exerted in hepatocytes. Substances known to activate protein kinase C such as phorbol 12-myristate 13-acetate, or to stimulate phosphoinositide turnover such as thrombin and bradykinin are also effective in raising fructose 2,6-bisphosphate. Therefore, we conclude that cyclic AMP and protein kinase C are likely involved in the control of fructose 2,6-bisphosphate levels in human fibroblasts.

Fructose 2,6-bisphosphate; Isoprenaline; Prostacyclin; Phorbol 12-myristate 13-acetate; Thrombin; Bradykinin; (Human fibroblast)

1. INTRODUCTION

We have previously observed that Fru-2,6-P_2, the most potent activator of PFK-1, is involved in the regulation of glycolysis in human fibroblasts. The stimulation of resting cells with mitogens is responsible for a marked increase in Fru-2,6-P_2 content that mediates the increase of the glycolytic flux elicited by these agents [1,2]. Studies on the molecular basis of transmembrane control of Fru-2,6-P_2 levels in different cell types have provided evidence for the involvement of well-known signal-transducing systems, such as that generating the second messenger cAMP or that leading to the activation of protein kinase C via phosphoinositide hydrolysis in the regulation of the metabolite concentration.

In this regard liver is the best characterized tissue. Fru-2,6-P_2 has been shown to play a fundamental role in the control of hepatic glycolysis and gluconeogenesis. It has been demonstrated that the hormonal effects on Fru-2,6-P_2 content are mediated by changes in cAMP levels and consequently in cAMP-dependent protein kinase activity. The decrease in Fru-2,6-P_2 levels by glucagon treatment has been explained by the property of cAMP-dependent protein kinase of phosphorylating the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase responsible for the synthesis/degradation of

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Abbreviations: Fru-2,6-P_2, fructose 2,6-bisphosphate; PFK-1, 6-phosphofructo-1-kinase (ATP:fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11); PMA, phorbol 12-myristate 13-acetate; PGI_2, prostacyclin; IBMX, 3-isobutyl-1-methylxanthine

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Fru-2,6-P_2, leading to the inactivation of the kinase and activation of the phosphatase [3].

On the other hand, in chicken embryo fibroblasts the control of Fru-2,6-P_2 levels appears to be disengaged from cAMP and a role for protein kinase C activation has been proposed, since the tumor promoter PMA and synthetic diacylglycerols, well known stimulators of protein kinase C, induce a rise of Fru-2,6-P_2 in these cells [4]. Recently we postulated a similar role in the rise of Fru-2,6-P_2 induced by thrombin in human platelets, showing that the positive effect of the agonist, known to activate phosphoinositide hydrolysis, can be mimicked by PMA and dioctanoylglycerol [5].

In view of these observations that indicate a complex and tissue-specific mechanism in the control of Fru-2,6-P_2 levels, we decided to investigate whether the above-mentioned membrane transducing systems were also involved in the regulation of Fru-2,6-P_2 in human fibroblasts. In this connection we have evaluated the effect on fibroblast Fru-2,6-P_2 levels of agents known either to increase fibroblast cAMP content, e.g. isoprenaline [6] and PGI_2 [7] or to activate directly protein kinase C such as PMA [8] or indirectly, through the stimulation of phosphoinositide turnover, for example thrombin [9] or bradykinin [10].

2. MATERIALS AND METHODS

Biochemicals and auxiliary enzymes for the assay of Fru-2,6-P_2 were purchased from Boehringer. Fru-2,6-P_2, (−)-isoprenaline, DL-propranolol, bradykinin, bovine thrombin, PMA were from Sigma (St. Louis, MO); prostacyclin was from Wellcome (London, England). Cyclic AMP assay kit was from Amersham International (Amersham, England).

Methods for fibroblast cultures have been described elsewhere [2]. Approx. 5×10^4 cells were seeded in 35-mm multiwell dishes and grown at confluency. 24 h before the experiments cell cultures were shifted to a minimum essential medium in the absence of serum and the medium was further changed 1 h before the beginning of the experiments. The test substances were added to the dishes in small volumes and when the solvent was different from water, appropriate amounts were added to the controls. At various time intervals cells were processed for Fru-2,6-P_2 extraction, as described in [2]. Fru-2,6-P_2 was determined according to Van Schaftingen et al. [11]. For cAMP measurement the incubations were terminated by quickly removing the medium; dishes were added with 200 μl of cold 10% trichloroacetic acid in PBS and rapidly frozen. After thawing, medium was collected and treated with 1 vol. of 0.5 M tri-*n*-octylamine solution in freon [12]. The upper phase was used for radioimmunoassay of cAMP. Cells attached to dishes were solubilized with 1 N NaOH for determination of protein content [13].

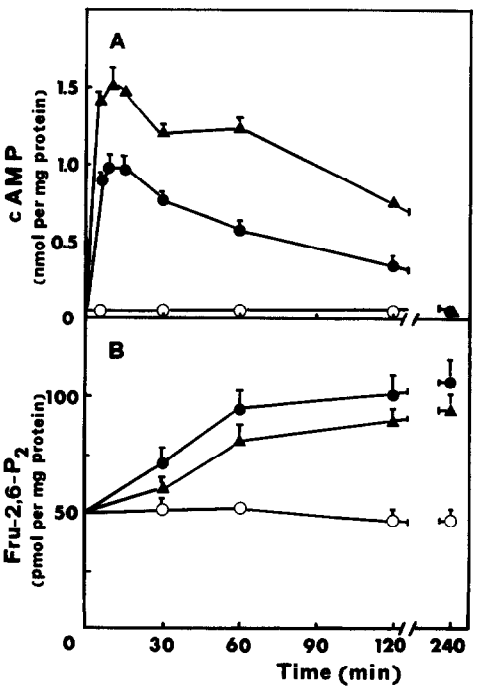

Fig.1. Time course of cAMP (A) and Fru-2,6-P_2 (B) levels in human fibroblasts stimulated with 5 μg/ml PGI_2 (▲) or 1 μM isoprenaline (●). Control values are reported (○). Experimental details are described in section 2.

Data reported are means ± SE of triplicate dishes from a single representative experiment which was repeated at least three times. In the figures the absence of an SE bar indicates that it is within the symbol.

3. RESULTS AND DISCUSSION

To evaluate whether the stimulation of the adenylate cyclase system could affect Fru-2,6-P_2 in

human fibroblasts, quiescent cells were stimulated with isoprenaline or PGI_2. Both compounds are responsible for a marked and sustained rise in cAMP which declines to control values within 2 h (fig.1A), followed by a progressive increase in Fru-2,6-P_2 levels (fig.1B). Analogous results were obtained on incubating the cells with 1 mM dibutyryl-cAMP in the presence of the phosphodiesterase inhibitor IBMX (0.1 mM) (not shown). The action of isoprenaline on the metabolite is entirely due to the interaction with the β-adrenergic receptor since 4 μM propranolol, a β-adrenergic antagonist, prevents the rise in both cAMP and Fru-2,6-P_2 (not shown). Therefore, it appears that cAMP plays a positive role in the control of Fru-2,6-P_2 in human fibroblasts, opposite to that occurring in hepatocytes where cAMP represents a negative signal for the metabolite [3]. Furthermore, human fibroblasts are regulated differently from chicken embryo fibroblasts where Fru-2,6-P_2 levels are not affected by dibutyryl-cAMP [4]. A positive role for cAMP in the control of Fru-2,6-P_2 levels has so far been demonstrated only in *Saccharomyces cerevisiae* [14] and this is the first evidence that a similar role is played in a higher eucaryotic cell system.

The possible involvement of protein kinase C in the control of fibroblast Fru-2,6-P_2 levels was investigated using PMA, a selective activator of the kinase in a variety of cells including fibroblasts [8]. The phorbol ester elicits a rise in Fru-2,6-P_2 content (fig.2B) in accordance with previous observations in chicken embryo fibroblasts [4] and does not affect cAMP levels (not shown). From the same figure it emerges that thrombin and bradykinin are also effective in raising Fru-2,6-P_2 levels. The two mitogens are able to stimulate phosphoinositide metabolism in cultured fibroblasts [9,10] and can also promote the release and metabolization of arachidonic acid to biologically active products such as prostaglandins [15,16]. These metabolites are responsible for the cAMP accumulation observed after stimulation of human fibroblasts with bradykinin [16]. The results in fig.2A confirm that bradykinin produces an increase in cAMP levels that peak at 5 min and then progressively decline to control values within 1 h. Noteworthily, thrombin is almost equipotent to bradykinin in increasing cAMP, in contrast to previous observations on hamster fibroblasts where it has been found to be unable to affect cAMP levels [17].

To ascertain whether the observed increase in cAMP content could mediate at least in part the effect on Fru-2,6-P_2 elicited by thrombin and bradykinin, analogous experiments were perform-

Table 1

Effect of indomethacin on cAMP and Fru-2,6-P_2 levels in human fibroblasts stimulated with thrombin or bradykinin

	1 μM indomethacin	cAMP (pmol/mg protein)	Fru-2,6-P_2 (pmol/mg protein)	
		Time of incubation		
		5 min	1 h	4 h
Control	–	50 ± 2	51.7 ± 4.7	50.3 ± 4.1
	+	40 ± 3	48.5 ± 5.2	52.4 ± 3.9
Thrombin, 4 U/ml	–	1709 ± 40	85.1 ± 7.5[a]	112.4 ± 10.6
	+	70 ± 5	59.2 ± 5.8[b]	84.3 ± 6.4
Bradykinin, 1 μM	–	2751 ± 52	110.3 ± 9.7[a]	190.4 ± 11.6
	+	59 ± 3	61.3 ± 8.5[b]	168.6 ± 15.0

[a] Significantly different from control $p < 0.05$

[b] The effect of indomethacin was significant ($p < 0.05$) vs agents alone

Statistical analysis was by Student's t-test for paired data

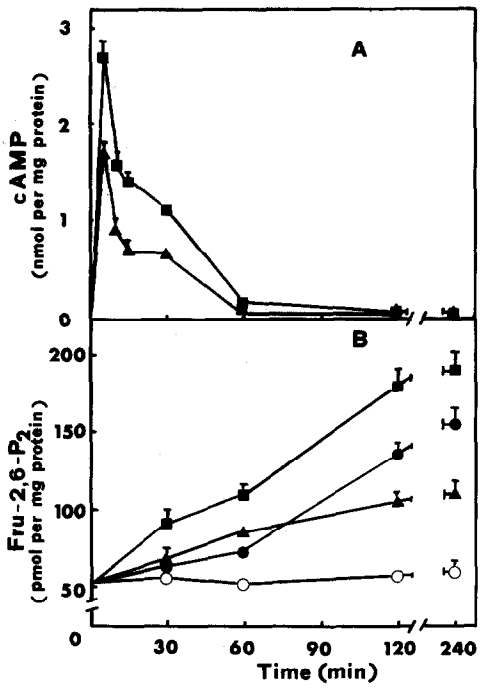

Fig.2. Time course of cAMP (A) and Fru-2,6-P_2 (B) levels in human fibroblasts stimulated with 100 ng/ml PMA (●), 4 U/ml thrombin (▲) or 1 μM bradykinin (■). Control values for Fru-2,6-P_2 are reported; the basal cAMP content was 50 pmol/mg protein. Experimental details are described in section 2.

ed in the presence of indomethacin in order to prevent prostaglandin synthesis (table 1). The cyclooxygenase inhibitor, as reported in [16], completely reduces bradykinin-stimulated cAMP formation and is similarly efficacious in preventing the rise in the cyclic nucleotide induced by thrombin, demonstrating that prostaglandins also mediate the action of thrombin. Indomethacin significantly reduces the rise in Fru-2,6-P_2 elicited by thrombin and bradykinin at 1 h of incubation indicating that, at early times of incubation, the effect on the metabolite is largely mediated by cAMP. On the other hand, the effect of the mitogens on Fru-2,6-P_2 levels at longer times of incubation is likely due to molecular events linked to the activation of phosphoinositide hydrolysis induced by both the agents. This hypothesis is supported by the observation that indomethacin does not impair the effectiveness of thrombin and bradykinin at 4 h of incubation and by the similar ability to increase Fru-2,6-P_2 displayed by PMA, a known activator of protein kinase C.

In summary, the levels of Fru-2,6-P_2, previously reported to regulate glycolysis in human fibroblasts [1,2], appear to be affected by changes in the concentration of cAMP: the rise of the cyclic nucleotide induced by stimulatory agonists of adenylate cyclase such as isoprenaline and PGI_2, or by the analogous dibutyryl-cAMP in the presence of the phosphodiesterase inhibitor IBMX, or secondary to the activation of the arachidonic acid cascade caused by thrombin or bradykinin, is always responsible for an increase in Fru-2,6-P_2 levels. In addition, protein kinase C activation may also affect Fru-2,6-P_2 levels in human fibroblasts, as indicated by the finding that PMA, a direct activator of protein kinase C, increases Fru-2,6-P_2 levels; moreover, the effectiveness of thrombin and bradykinin at raising Fru-2,6-P_2 levels at longer times of incubation, under experimental conditions preventing the rise in cAMP, may be ascribed to their ability to activate phosphoinositide hydrolysis, further supporting this hypothesis.

This is the first evidence for the involvement of cAMP- and protein kinase C-dependent pathways in the control of a specific metabolic effect in human fibroblasts, indicating that the two signal-transducing systems act in the same direction in this particular cell type, both eliciting the rise in Fru-2,6-P_2.

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