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Hormonal control of fructose 2,6-bisphosphate concentration in the HT29 human colon adenocarcinoma cell line

α_2-Adrenergic agonists counteract effect of vasoactive intestinal peptide

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Vasoactive intestinal peptide (VIP) was found to cause a dose-dependent decrease in fructose 2,6-bisphosphatase concomitant with an increase in cyclic AMP in cultured HT29 cancer cells from human colon. The maximum effect was a 41% decrease obtained with 10 nM-VIP, and half-maximum effect was obtained with 0.75 nM-VIP. The effect of 2.5 nM-VIP was almost totally counteracted (i.e. fructose 2,6-bisphosphate concentration was restored) by either adrenaline (1 μM) or the α_2-adrenergic agonist UK-14304 (1 μM); the α_2-agonist clonidine (1 μM) was less efficient, since the VIP effect was decreased by 72% only. The adrenaline effect was totally antagonized by 1 μM-yohimbine. It is concluded that, in the HT29 cancer cells, the fructose 2,6-bisphosphate-producing system is sensitive to variations of cyclic AMP concentration and is under the dual control of VIP and α_2-adrenergic receptors.

INTRODUCTION

Fructose 2,6-bisphosphate (Fru-2,6-P_2) is known to be a powerful stimulator of phosphofructokinase-1 (PFK1) (Van Schaftingen *et al.*, 1980) as well as an inhibitor of fructose-1,6-bisphosphatase (FBPase1) (Pilkis *et al.*, 1981), thus playing a critical role in the control of glycolysis and gluconeogenesis in the liver (Hers & Hue, 1983).

Regulation of Fru-2,6-P_2 concentrations by hormonal signals has been extensively studied in some types of cells. In liver cells, glucagon decreases the Fru-2,6-P_2 concentration via cyclic AMP-dependent phosphorylation of the bifunctional enzyme (PFK2/FBPase2) which synthesizes and degrades Fru-2,6-P_2 (Bartrons *et al.*, 1983). By contrast, the Fru-2,6-P_2-producing system of islet β-cells was found to be insensitive to cyclic AMP variations (Sener *et al.*, 1984). In hepatocytes from fed rats, adrenaline and α-adrenergic agonists were reported to increase Fru-2,6-P_2, this effect being presumably due to an increase in fructose 6-phosphate concentration as a result of α_1-receptor stimulation and Ca^{2+}-mediated activation of glycogenolysis (Hue *et al.*, 1981). Some contradictory results were reported by Pilkis *et al.* (1983), showing a decrease in Fru-2,6-P_2 in liver cells after exposure to catecholamines, probably owing to an increase in cyclic AMP. In rat epididymal adipose tissue, adrenaline was found to decrease Fru-2,6-P_2 (Sobrino & Gualberto, 1985), but no significant change was observed after noradrenaline addition to isolated adipocytes from fed rats (Rider & Hue, 1985). The effect of insulin on the Fru-2,6-P_2-producing system is still puzzling: this hormone was found to counteract the effect of glucagon to decrease Fru-2,6-P_2 in hepatocytes from fed rats (Pilkis *et al.*, 1983), whereas in adipose tissue insulin was reported to elevate Fru-2,6-P_2 concentration (Sobrino & Gualberto, 1985) or to decrease it (Rider & Hue, 1985).

In fact, information about hormonal regulation of Fru-2,6-P_2 concentration in highly glycolytic cancer cells is scarce. Loiseau *et al.* (1985) showed that glucocorticoids were able to increase PFK2 activity and Fru-2,6-P_2 concentration in cultured rat hepatoma HTC cells, whereas dibutyryl cyclic AMP was without effect.

The purpose of the present work was to study the hormonal responsiveness of the Fru-2,6-P_2-producing system in the HT29 cancer cell line derived from human colon mucosa (Fogh *et al.*, 1977), paying special attention to variations of Fru-2,6-P_2 concentration induced by cyclic AMP-dependent mechanisms. HT29 cells were previously shown to possess an adenylate cyclase system which is very sensitive to vasoactive intestinal peptide (VIP) (Laburthe *et al.*, 1978). α_2-Adrenoceptors were also characterized in these cells (Carpéné *et al.*, 1983); when stimulated, these receptors were found to counteract the VIP-induced cyclic AMP accumulation in HT29 cells (Bouscarel *et al.*, 1985). We have previously reported that PFK1 could be a rate-limiting enzyme in the HT29 cells (Denis *et al.*, 1984) and that partially purified PFK1 from HT29 cells was very sensitive to allosteric activation by Fru-2,6-P_2: a concentration as low as 10 nM was found to activate the enzyme *in vitro*, and the maximum effect was reached at about 1 μM (Denis *et al.*, 1985*a*).

Therefore we decided to investigate, in HT29 cells, whether variations in Fru-2,6-P_2 concentration could be correlated with changes in cyclic AMP concentration as induced by VIP treatment and whether stimulation of α_2-adrenoceptors could modulate the response.

Abbreviations used: Fru-2,6-P_2, fructose 2,6-bisphosphate; VIP, vasoactive intestinal peptide; DMEM, Dulbecco's modified Eagle medium; PFK1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK2, 6-phosphofructo-2-kinase (EC 2.7.1.105); FBPase1, fructose-1,6-bisphosphatase (EC 3.1.3.11); FBPase2, fructose-2,6-bisphosphatase (EC 3.1.3.46).

MATERIALS AND METHODS

Drugs and chemicals

Yohimbine hydrochloride, (−)-adrenaline bitartrate, ascorbic acid and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (St. Louis, MO, U.S.A.). Clonidine hydrochloride was obtained from Boehringer (Ingelheim, Germany) and propranolol from ICI (U.K.). Bacitracin and Coomassie Blue were from Serva (Heidelberg, Germany). Bovine serum albumin (fraction V) and fetal-calf serum were also from Boehringer. Dulbecco's modified Eagle medium (DMEM) was from Eurobio (Paris, France). UK-14304 hydrochloride [5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline] was kindly provided by Pfizer (Sandwich, Kent, U.K.). VIP was generously given by Dr. M. Laburthe (Paris, France). Cyclic AMP ^{125}I-radioimmunoassay kit was from New England Nuclear (Boston, MA, U.S.A.). All other chemicals were purchased from Sigma or Serva, and were of the highest purity grade. All analytical enzymes were free of $(NH_4)_2SO_4$.

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 2×10^4 cells/cm^2 in plastic Petri dishes (60 mm diam.; Falcon; Becton Dickinson, Grenoble, France) and grown at 37 °C, under air/CO_2 (19:1) atmosphere, in DMEM containing 25 mM-glucose, supplemented with 10% fetal-calf serum.

During the exponential phase of growth, the culture medium was changed every 48 h. Under these conditions, cell confluency was reached after 10 days of culture. During stationary phase, medium was changed every 24 h, in order to avoid any nutrient exhaustion. We showed previously (Paris *et al.*, 1985) that the number of α_2-adrenoceptors present on HT29 cell membranes is a function of cell density within the dish. Thus, to avoid any interference with the α_2-adrenoceptor maturation process, all experiments were done at day 14 of the culture, cells being in stationary phase of growth.

Experimental protocol

All experiments were performed with attached post-confluent cells. At 6 h before zero time of the experiments, the standard culture medium was removed and replaced by 4 ml of serum-free DMEM. Experiments were started by adding 40 μl of hormone and/or drug at the appropriate concentration together with 0.5 mM-IBMX and 0.02% bacitracin. When adrenaline was tested, 0.75 mM-ascorbic acid was added to prevent oxidation and propranolol (1 μM) was added to avoid interference with β-adrenoceptors. For each experiment, six dishes were run in parallel for cyclic AMP and Fru-2,6-P_2 determinations. After 30 min at 37 °C, medium was removed and incubation was rapidly stopped by addition of either 4 ml of ice-cold methanol/formic acid (19:1, v/v) for cyclic AMP determination or 2.5 ml of 0.1 M-NaOH for Fru-2,6-P_2 and protein determinations (see below).

Analytical procedures

Cyclic AMP was measured as previously described (Paris *et al.*, 1985). Briefly, the cell layer was scraped, the cell lysate was then sonicated and denatured particles were pelleted at 2500 *g* for 15 min. Samples of the methanol extract were evaporated and stored at −20 °C until analysis. At the time of analysis, the dried samples were re-diluted in the appropriate volume of sodium acetate buffer (0.2 M, pH 7.5) and cyclic AMP was measured by the radioimmunological method of Steiner *et al.* (1982).

For measuring Fru-2,6-P_2 concentration, frozen cell layers were scraped into ice-cold 0.1 M-NaOH (2.5 ml/dish) and sonicated for 10 s. A sample of the homogenate was used for protein determination as described by Bradford (1976). The rest of the homogenate was heated for 5 min at 80 °C to denaturate protein and to stabilize Fru-2,6-P_2; a 450 μl sample was mixed with 50 μl of 200 mM-Hepes, adjusted to pH 7 with 1 M-acetic acid and centrifuged down (9000 *g*, 1 min). Fru-2,6-P_2 was assayed in the supernatant by the method of Van Schaftingen *et al.* (1982) as modified by Van Schaftingen & Hers (1984).

Analysis of the data and statistics

All data were analysed with an Apple IIe computer by using the Biodata Handling programs written by Barlow (1983). All reported values are means ± S.E.M. for three separate experiments, and Student's *t* test was used for comparing the values, significance being admitted for $P < 0.05$.

RESULTS

Effects of VIP

The ability of this hormone to induce cyclic AMP accumulation in HT29 cells and subsequent changes in Fru-2,6-P_2 concentration was investigated in preliminary experiments. It was found that maximum cyclic AMP increase was obtained within 10 min, but that the most significant shift of Fru-2,6-P_2 concentration was observed after 30 min (results not shown).

As shown in Fig. 1, VIP produced a considerable accumulation of cyclic AMP. Under our experimental conditions, the basal concentration of cyclic AMP was 4.33 ± 0.07 ($n = 4$) pmol/mg of protein, and was increased 16-fold by VIP concentrations as low as 0.1 nM. The maximum effect (370 times basal) was observed with 2.5 nM-VIP, and half-maximum response was found at 0.53 ± 0.20 ($n = 3$) nM-VIP. Such values are in the same order of magnitude as those reported by Laburthe *et al.* (1978) for unattached HT29 cells in the exponential phase of growth and by Bouscarel *et al.* (1985) for attached HT29 cells.

Under basal conditions, the Fru-2,6-P_2 concentration in HT29 cells was 38.3 ± 2.7 ($n = 7$) pmol/mg of protein. Exposure to VIP produced a significant decrease in Fru-2,6-P_2, which appeared to be dependent on the hormone concentration: maximum effect was obtained with 10 nM-VIP, which decreased the basal value by 41%. Analysis of the dose–response curve indicates that the half-maximum effect occurs at 0.75 ± 0.41 ($n = 3$) nM-VIP, which is not significantly different from what was found for cyclic AMP.

Effects of α_2-adrenergic agonists

It has been previously shown that stimulation of α_2-adrenoceptors of HT29 cells by adrenaline or

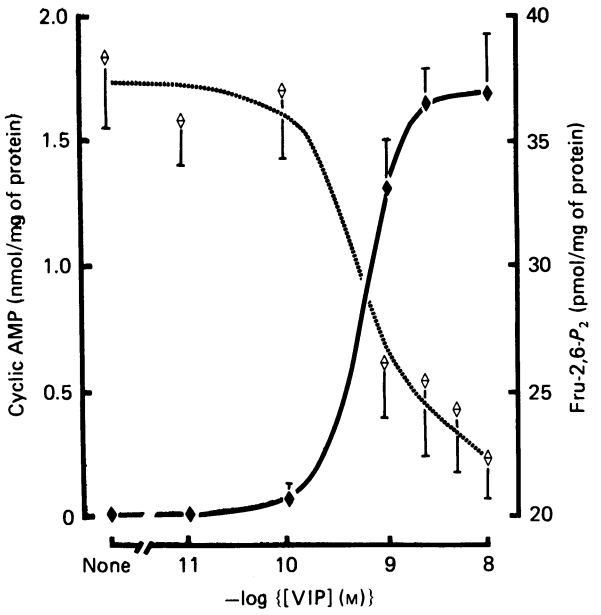

Fig. 1. Effect of VIP on cyclic AMP (◆) and Fru-2,6-P_2 (◈) concentrations in cultured HT29 cells

Post-confluent cells were incubated for 30 min at 37 °C in the presence of different concentrations of the hormone added to the culture medium. Experimental protocol and analytical procedures are described in the Materials and methods section. Each point is the mean ± S.E.M. of six values obtained from three separate experiments.

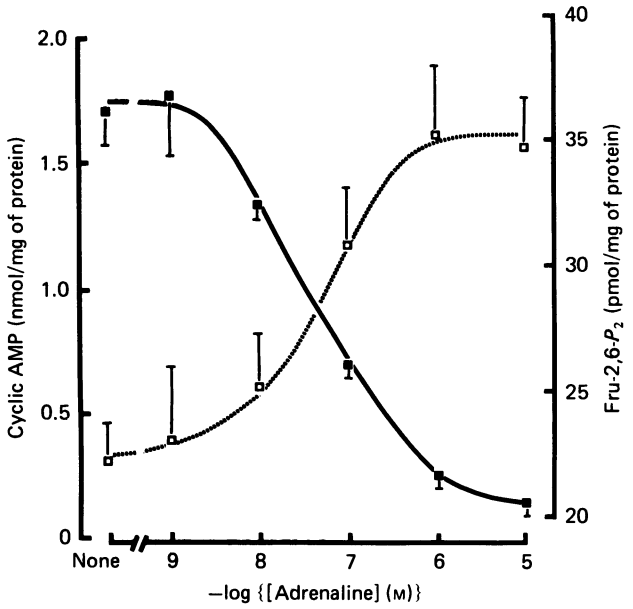

Fig. 2. Modulation by adrenaline of the effect of 2.5 nM-VIP on cyclic AMP (■) and Fru-2,6-P_2 (□) concentrations in cultured HT29 cells

Adrenaline, together with 1 μM-propranolol, was added at different concentrations to the medium, before VIP. HT29 cells were then incubated for 30 min at 37 °C. Experimental protocol and analytical procedures are described in the Materials and methods section. Each point is the mean ± S.E.M. of six values obtained from three separate experiments.

clonidine failed to modify the basal cyclic AMP concentration, but was very efficient in lowering high concentrations of cyclic AMP such as those induced by VIP (Bouscarel *et al.*, 1985; Paris *et al.*, 1985). For this reason, the effects of adrenaline and α_2-adrenergic agonists were normally tested on VIP (2.5 nM)-stimulated cells.

When adrenaline plus propranolol was added to the incubation medium before VIP, a significant decrease in cyclic AMP accumulation was observed (Fig. 2). The maximum effect was obtained with 1 μM-adrenaline and represented a 91% inhibition of the VIP-induced cyclic AMP accumulation, which is in agreement with what was reported by Paris *et al.* (1985) in post-confluent HT29 cells. Such a decrease in cyclic AMP restores Fru-2,6-P_2 concentration in the cell up to almost the basal value, the VIP effect being thus inhibited by more than 90%.

From computer-assisted study of the dose–response curves, it was found that the half-maximum effect of adrenaline on Fru-2,6-P_2 concentration occurs at 37.6 ± 7.7 ($n = 3$) nM-adrenaline, which is not significantly different from the adrenaline concentration (29.7 ± 14.3 nM, $n = 3$) required to obtain the half-maximum effect on cyclic AMP accumulation.

To complete this study, further experiments using two specific α_2-adrenergic agents, clonidine and UK-14304, were performed. Clonidine, described as a partial agonist (Bouscarel *et al.*, 1985), was found to counteract the VIP-induced cyclic AMP accumulation in our model, but to a lesser extent than adrenaline did (Fig. 3). The decrease in cyclic AMP obtained with 1 μM-clonidine

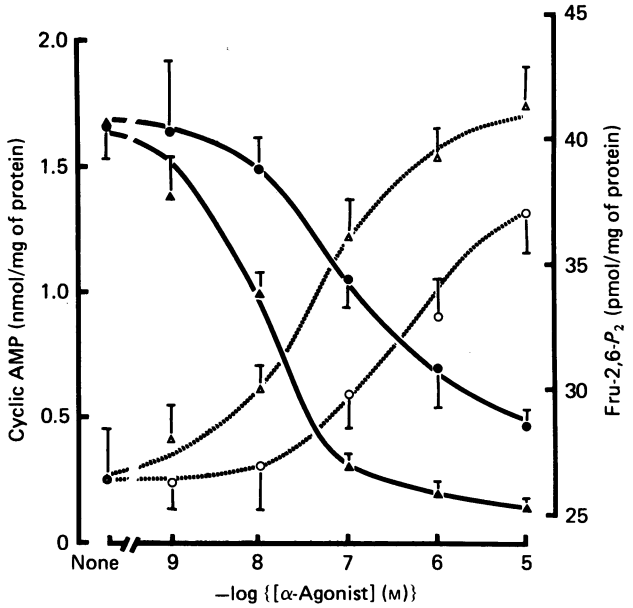

Fig. 3. Modulation by either clonidine (●, ○) or UK-14304 (▲, △) of the effect of 2.5 nM-VIP on cyclic AMP (●, ▲) and Fru-2,6-P_2 (○, △) concentrations in cultured HT29 cells

The α_2-adrenergic agent was added at different concentrations to the medium, before VIP. HT29 cells were then incubated for 30 min at 37 °C. Experimental protocol and analytical procedures are described in the Materials and methods section. Each point is the mean ± S.E.M. of six values obtained from three separate experiments.

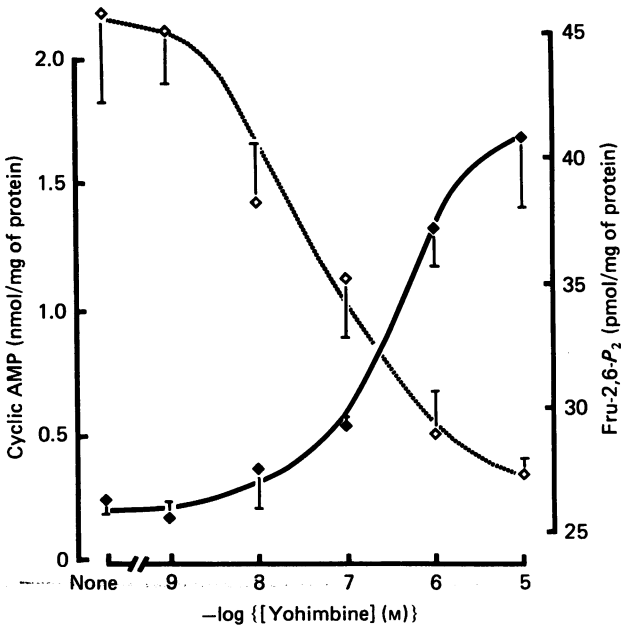

Fig. 4. Abolition by yohimbine of the inhibiting effect of 10 μM-adrenaline on the VIP (2.5 nM)-induced changes in cyclic AMP (◆) and Fru-2,6-P_2 (◇) concentrations in cultured HT29 cells

The α_2-antagonist was added at different concentrations to the culture medium before adrenaline and VIP. HT29 cells were then incubated for 30 min at 37 °C. Experimental protocol and analytical procedures are described in the Materials and methods section. Each point is the mean ± S.E.M. of six values obtained from three separate experiments.

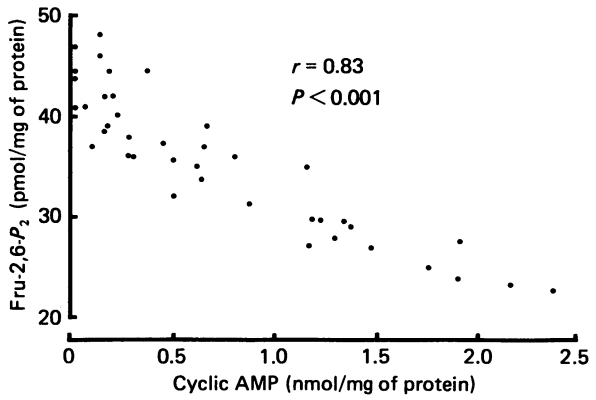

Fig. 5. Fru-2,6-P_2 concentration plotted as a function of cyclic AMP concentration in cultured HT29 cells

Paired values of both parameters from all experiments were used. The value of the linear regression coefficient was obtained from classical calculation.

was, however, sufficient to give an increase in Fru-2,6-P_2, which returned to 81% of the basal value. In this case, the shape of the dose–response curve did not allow us to calculate accurately the K_D value, but the half-maximum effect could be estimated to require at least 300 nM-clonidine.

On the contrary, and as would be expected from results by Turner *et al.* (1985), UK-14304 was found to be a powerful agonist for α_2-adrenoceptors in our model, exhibiting nearly the same potency as adrenaline (Fig. 3): cyclic AMP accumulation was inhibited by over 92% with 1 μM-UK-14304, half-maximal effect being observed with a drug concentration of 17.1 ± 16.2 ($n = 3$) nM. The same increase in Fru-2,6-P_2 content as with adrenaline was also observed, 86.9% of basal value being reached with 1 μM-UK-14304 and half-maximum effect being obtained with 41.4 ± 20.9 ($n = 3$) nM.

Effect of yohimbine

To confirm the α_2 nature of the adrenoceptor that mediates the decrease in cyclic AMP and the increase in Fru-2,6-P_2 in HT29 cells, the effect of yohimbine, a specific α_2-adrenergic antagonist, was studied.

As seen in Fig. 4, 1 μM-yohimbine abolished the inhibiting effect of 10 μM-adrenaline plus 1 μM-propranolol on the VIP-induced cyclic AMP accumulation, and Fru-2,6-P_2 was again decreased by 40%, compared with data in Fig. 3.

This result clearly shows the implication of α_2-adrenoceptors when adrenaline counteracts the biological effects of VIP.

Fru-2,6-P_2 concentration as a function of cyclic AMP concentration

The relationship between the concentrations of cyclic AMP and Fru-2,6-P_2 in the cells was investigated by pooling the paired values of both parameters from all experiments. In Fig. 5, Fru-2,6-P_2 values are plotted as a function of cyclic AMP values: the correlation appears to be almost linear and inverse, which underscores the functional correlation between the increase in cyclic AMP and the decrease in Fru-2,6-P_2, or vice versa, in our model.

DISCUSSION

Our results indicate that Fru-2,6-P_2 concentration is subject to hormonal regulation in HT29 cells. VIP causes a rise in cyclic AMP concentration and a 41% decrease in Fru-2,6-P_2 content of the cell; furthermore, changes in cyclic AMP and Fru-2,6-P_2 concentrations are inversely and well correlated. The hormone effect could be due to phosphorylation of PFK2/FBPase2 by the cyclic AMP-dependent protein kinase which was demonstrated to be present and functional in HT29 cells (Marvaldi *et al.*, 1979; Rousset *et al.*, 1981).

The intensity of the response obtained in HT29 cells is, however, lower than that obtained in glucagon-treated hepatocytes. Bartrons *et al.* (1983) and Pilkis *et al.* (1983) showed that 1 nM-glucagon caused a total fall in Fru-2,6-P_2 content in rat hepatocytes, whereas we never observed more than a 41% decrease in VIP-stimulated HT29 cells. When 3 μM-forskolin was used synergistically with 5 nM-VIP, which induced a considerable increase in intracellular cyclic AMP (3000-fold above basal), the Fru-2,6-P_2 content was diminished by only 65% (results not shown).

According to Pilkis *et al.* (1983), the half-maximum effect on Fru-2,6-P_2 concentration is obtained in hepatocytes with 12 μM-cyclic AMP. In HT29 cells, assuming that cell water volume is 4.8 μl/mg of protein

(Rousset *et al.*, 1984), it can be calculated that half-maximum effect occurs at 166 μM-cyclic AMP. This could indicate a weaker sensitivity of the Fru-2,6-P_2-producing system to cyclic AMP shifts.

Whether such a decrease in Fru-2,6-P_2 brought about by VIP stimulation has a physiological significance remains an open question. However, we have previously shown that PFK1 from HT29 cells is highly sensitive to Fru-2,6-P_2 activation: when determined with 1 mM-fructose 6-phosphate and 1.5 mM-ATP, half-maximum activation of PFK1 is obtained with 0.06 μM- and maximum effect with 1.12 μM-Fru-2,6-P_2 (Denis *et al.*, 1985*a*). In HT29 cells, the basal concentration of Fru-2,6-P_2 is about 40 pmol/mg of protein, which represents an intracellular concentration of 8.5 μM. It was proposed by MacGrane *et al.* (1983) and Hue *et al.* (1984) that 90% of Fru-2,6-P_2 could be bound to intracellular proteins. FBPase1, which accounts for about 80% of this binding, is present in HT29 cells (Denis *et al.*, 1984). Thus the free Fru-2,6-P_2 concentration should be about 0.85 μM, which is in the range of concentrations that might actually control the PFK1 activity (Denis *et al.*, 1985*a*). Therefore a 41% decrease in Fru-2,6-P_2, as induced by VIP stimulation, could cause a decrease in PFK1 activity and decrease the glycolytic flux.

The present study clearly shows that α_2-adrenergic stimulation can modulate VIP response and modify Fru-2,6-P_2 concentrations in HT29 cells: we found that adrenaline and UK-14304 can restore Fru-2,6-P_2 concentrations up to more than 90% of basal value.

α_2-Adrenoceptors are also found in normal enterocytes (Nakaki *et al.*, 1982) and in human colon mucosa (Boige *et al.*, 1984). They were demonstrated to decrease the sensitivity of the cyclic-AMP-generating system to various secretagogues such as VIP and prostaglandin E1 (Laburthe *et al.*, 1982).

However, the possible involvement of these receptors in regulation of carbohydrate metabolism was up to now poorly documented. McMahon & Schimmel (1981) showed that stimulation of α_2-adrenoceptors inhibits glycogen phosphorylase activation in hamster adipocytes, but no clear implication in the regulation of glycolysis or gluconeogenesis was then demonstrated.

We found that, in a highly glycolytic cancer cell, catecholamines can modulate cyclic AMP concentration and the concentration of one key metabolite controlling the carbohydrate metabolism.

As the α_2-adrenoceptor present in HT29 cells appears identical with that found in human intestinal cells (Boige *et al.*, 1984; Bouscarel *et al.*, 1985), it is questionable whether they can exert such a regulatory effect in normal colonocytes.

Like other rapidly dividing cells, normal colonocytes exhibit a high rate of glycolysis, but were found to possess enzymes for gluconeogenesis, the activity of which was increased during starvation (Ardawi & Newsholme, 1985).

Although glycolysis is the main degradative pathway of glucose in HT29 cells, FBPase1 activity was also detected (Denis *et al.*, 1984), the cells being able to develop a gluconeogenic pathway when cultured without glucose (Denis *et al.*, 1985*b*). Fru-2,6-P_2 is supposed to play a critical role in these cells, as in the normal colonocyte, controlling the balance between glycolysis and gluconeogenesis at the PFK1/FBPase1 step.

HT29 cells which, like the normal corresponding intestinal cells, possess functional VIP and α_2-adrenergic receptors, could constitute an interesting model for further studies *in vitro* on the hormonal regulation of carbohydrate metabolism.

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