The amounts of rat liver cyclic AMP-dependent protein kinase I and II are differentially regulated by diet

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1. The fluctuations in rat hepatocyte volume and protein content in response to dietary perturbations (starvation, protein restriction, refeeding) were accompanied by corresponding fluctuations in the amount of the regulatory (R) and catalytic (C) subunits of cyclic AMP-dependent protein kinase. Thus the intracellular concentration of this key enzyme was adjusted to be near constant. 2. The adjustment of cellular R was accomplished almost exclusively by regulating cytosolic RI (R subunit of type I kinase). The preferential down-regulation of cytosolic RI in response to starvation/protein restriction indicates that particulate RI and cytosolic as well as particulate RII are more resistant to breakdown during general catabolism in the hepatocyte. 3. The diet-induced fluctuations of kinase subunits were uniformly distributed in all populations of parenchymatous hepatocytes, regardless of their size and density. It is thus possible to isolate hepatocytes with uniformly altered RI/RII ratio from livers of rats with different feeding regimens. 4. The binding of endogenous cyclic AMP to RI and RII was similar in livers with high RI/RII ratio (fed rats) and low RI/RII ratio (fasted rats) as well as in hepatocytes isolated from fasted rats. Under the conditions of the experiment (short-term stimulation by glucagon), therefore, neither the dietary state nor the RI/RII ratio seemed to affect the apparent affinity of the isoreceptors for cyclic AMP. However, RI appeared to show a slightly higher co-operativity of intracellular cyclic AMP binding than did RII in all states.

INTRODUCTION

The cyclic AMP-dependent protein kinase is a tetramer composed of two catalytic (C) and two regulatory (R) cyclic AMP-binding subunits. In mammalian tissue the enzyme mediates most, if not all, effects of cyclic AMP (see Edelman *et al.*, 1987, for a review). It is known (Corbin *et al.*, 1975; Hofmann *et al.*, 1975) that the enzyme exists in two main types (I and II) with different regulatory subunits (RI, RII), which again occur in subforms (Titani *et al.*, 1984; Stein & Rubin, 1985; Weldon *et al.*, 1985; Jahnsen *et al.*, 1986; Uhler *et al.*, 1986; Scott *et al.*, 1987). The biological significance of the existence of isoreceptors is still enigmatic, there being no firm evidence (Sikorska *et al.*, 1988) for the attractive hypothesis that the R subunits may have effects of their own (Constantinou *et al.*, 1985).

Even if the sole function of R were to control C, the phosphorylation pattern resulting from a given intracellular concentration of cyclic AMP may depend on which isotype of R predominates. Thus the isoreceptors may 'target' the C subunit to specific subcellular sites, or the cyclic AMP-responsiveness of the isoenzymes may be different. It is also possible that the various types and forms of R give the cell greater flexibility in regulating the intracellular concentrations of total R under conditions when the cell volume and protein abruptly increase (anabolism) or decrease (catabolism).

The rat liver is very sensitive to food deprivation. The hepatocyte volume and protein content decrease by about 50% after a few days of starvation (Harrison,

1953; Uhal & Roehrig, 1982; the present work) and rapidly increase after refeeding (Uhal & Roehrig, 1982; the present work). We considered therefore that the nutritionally perturbed rat liver might give clues about how the concentration and subcellular distribution of RI and RII are affected under anabolic and catabolic conditions. We show that diet controlled the relative proportion of RI and RII as well as their partition between the soluble and particulate compartments, and that the effect was generally distributed throughout the hepatocyte population. This made it possible to study the titration of RI and RII by intracellular cyclic AMP in hepatocytes with different proportions of the two isoreceptors, both in the intact animal and in isolated hepatocytes.

It has been known for many years that rodent liver contains cyclic AMP-dependent protein kinase types I and II (Kumon *et al.*, 1972; Corbin *et al.*, 1975; Døskeland & Ueland, 1975), and that the latter is preferentially associated with the particulate fraction (Sommarin & Jergil, 1978). However, little is known about what factors may regulate the concentration, cyclic AMP-sensitivity and subcellular distribution of the isoenzymes: previous studies disagreed as to whether fasting decreases kinase isoenzyme I (Chan *et al.*, 1979) or not (McClung & Kletzien, 1981). Wittmaack *et al.* (1983) noted a steady increase in kinase type I during liver ontogeny, kinase type II showing a peak around birth. The same group (Weber & Hilz, 1986) found that RI turned over 4 times faster than RII in rat liver, and suggested that kinase type I was the isoenzyme preferentially activated *in vivo*. This agrees with an early

Abbreviations used: C, catalytic subunit, and R, regulatory subunit, of cyclic AMP-dependent protein kinase; RI, RII, regulatory subunit of kinase type I and type II; DTE, 1,4-dithioerythritol.

report by Schwach (1978), but not with observations on isolated hepatocytes (Ekanger *et al.*, 1985).

It may be noted that most adult hepatocytes are not actively cycling, i.e. are in the G₀ phase of the cell cycle. This means that the interpretation of data on the expression of protein kinase subunits is not complicated by the cells being in different phases of the cell cycle.

EXPERIMENTAL

Animals, glucagon infusion and organ biopsies

Male Wistar rats (120–150 g) were kept at 20 °C and stabilized for at least 1 week on the complete diet. The animals had always free access to water. The selected diets c1000 (complete diet) or c1004 (low-protein diet) from Altromin, Lage, West Germany, or Vamin-N (amino acid solution) from Kabi Vitrum, Stockholm, Sweden, were always given *ad libitum*. For the intracellular titration of cyclic AMP binding sites, glucagon (0–50 μ M) dissolved in phosphate-buffered saline (0.14 M-NaCl/2.7 mM-KCl/1.5 mM-KH₂PO₄/7.6 mM-Na₂HPO₄), pH 7.4 at 37 °C, was infused for 45 s at a constant rate (0.2 ml/45 s) into the portal vein, with the rat under light ether anaesthesia. At 45 s after the infusion had been terminated, the liver was gently elevated and freeze-clamped with Wollenberger tongs precooled in liquid N₂. For determination of kinase subunits, the liver and other organs were freeze-clamped, and tissues finely ground in a mortar immersed in liquid N₂, and kept in liquid N₂ until homogenization.

Isolation and separation of hepatocytes

Hepatocytes were isolated by collagenase perfusion *in vitro* (Seglen, 1976; Christoffersen *et al.*, 1984). For cells that were separated by elutriation or analysed with a Coulter counter, the centrifugation steps were omitted, to avoid any preselection of hepatocytes.

The cells were separated into subpopulations by centrifugal elutriation (4 °C) in an IE-6 rotor (Beckman) equipped with a 4.5 ml separation chamber. The buffer was a modified iso-osmotic Krebs–Ringer solution (pH 7.3): Hepes/NaOH (20 mM) replaced NaHCO₃, and CaCl₂ (0.3 mM) and Na₂EDTA (0.3 mM) replaced the ordinary calcium supplements. Gelatin (0.5%) and gentamycin (40 μ g/ml) were added. The elutriation was performed at 850 rev./min with counter-flow rates ranging from 10 to 37.5 ml/min.

For isopycnic separation 5 ml of crude hepatocyte suspension was layered on top of a 45 ml linear density gradient of Percoll (22.5–90%) and spun for 30 min at 2000 rev./min (2 °C) in the H-1000 rotor of a Sorvall RT-6000 centrifuge.

Determination of hepatocyte volumes

This was done by three independent methods. The average volume was derived from the diameters of the cells as determined by analysis in a Coulter counter. The hepatocyte volume (i.e. the intracellular water space) was also determined by using ³H₂O and [U-¹⁴C]sucrose as markers for intra- and extra-cellular water respectively (Fisher & Pogson, 1984). Finally, packed cell volumes were determined.

Preparation of cytosol and particulate fractions of tissue and isolated cells

Tissue powder (100 mg) or cell pellet (10⁷ cells) was homogenized (2 × 10 s) with a Polytron PT 10/35

emulsifier at a setting of 4 in 2 ml of ice-cold 60 mM-Hepes/NaOH buffer, pH 7.2, containing 10 mM-EDTA, 20 mM-2-mercaptoethanol, 1.5 mM-DTE and the proteinase inhibitors benzamidine (10 mM), soya-bean trypsin inhibitor (0.3 mg/ml), leupeptin (0.05 mg/ml), aprotinin (0.05 mg/ml), chymostatin (0.07 mg/ml) and antipain (0.007 mg/ml). The homogenate was divided into three. One portion was kept for measurements of total DNA. The second portion was mixed with 0.01 vol. of Triton X-100 and incubated for 1 h at 0 °C (unfractionated homogenate). The third portion was centrifuged (120 000 g) for 10 min at 4 °C in a Beckman Airfuge with an A-95 rotor (*r*_{av} 13.3 mm) for preparation of cytosol (supernatant) and particulate (sediment). The sediment was resuspended and re-centrifuged three times in 2 ml of ice-cold homogenization buffer and finally incubated for 1 h at 0 °C in 1 ml of homogenization buffer containing 1 % of Triton X-100.

Determination of protein kinase subunits

The immunotitration of RI and RII was performed as previously described (Ekanger *et al.*, 1985; Ekanger & Døskeland, 1988), except that exchange between labelled cyclic AMP and endogenously bound cyclic AMP was achieved by incubation for 30 min at 37 °C.

For determination of C subunits, samples were diluted in 50 mM-potassium phosphate, pH 7.0, containing 1 mM-EGTA, 0.3 mM-EDTA, 2 mM-DTE, 0.15 % Triton X-100, 0.9 mg of bovine serum albumin/ml and 0.09 mg of soya-bean trypsin inhibitor/ml. The assay was started by adding 2 vol. of assay mixture to give the following final concentrations of assay components: 50 mM-potassium phosphate, 15 mM-Hepes, 10 mM-magnesium acetate, 70 μ M-Kemptide (a heptapeptide kinase substrate, from Sigma), 0.1 mM-[³²P]ATP (2 μ Ci/mmol), 0.5 mM-EGTA, 0.1 mM-EDTA, 1 mM-DTE, 0.2 mM-3-isobutyl-1-methylxanthine and 10 μ M-cyclic AMP (when present). The assay was done at 30 °C and pH 7.0. Samples (25 μ l) were spotted on phosphocellulose paper strips as described by Roskoski (1983).

Determination of the intracellular saturation of RI and RII with endogenous cyclic AMP

This was performed as previously described (Ekanger *et al.*, 1985; Ekanger & Døskeland, 1988), except that the homogenization solution contained 1.3 M-glycerol and cell concentration was 3 × 10⁶ cells/ml during incubation of the hepatocytes with glucagon.

Determination of cyclic AMP, protein and DNA

Cyclic AMP was measured by a competitive binding assay (Døskeland & Kvinnsland, 1980), protein by the Bio-Rad version of the Bradford (1976) assay, and DNA by a modification (Vintermyr & Døskeland, 1987) of the procedure of Patterson (1979).

RESULTS

Starvation decreases the RI/RII ratio in an organ specific and reversible manner

The ratio of type I to type II cyclic AMP-dependent protein kinase was determined in the cytosol of different tissues from rats either fed on a complete diet or starved for 3 days. During starvation, the initial liver RI/RII ratio of 2.0 decreased to 1.3 after 1 day, and was 0.8 after 3 days (Fig. 1a), i.e. 60 % decrease in 3 days. In

Fig 1. Effects of starvation, protein restriction and refeeding on the ratio of type I to type II cyclic AMP-dependent protein kinase in rat liver cytosol

(a) The RI/RII ratio was determined (for details, see the Experimental section) in rats fed on complete diet (Δ), or starved for 1, 2 or 3 days ($\bullet$) or starved for 3 days and then fed on either complete diet ($\blacktriangle$) or amino acid solution (Vamin-N) ($\blacksquare$). The points with error bars represent mean values $\pm$ S.E.M. ($n = 4$); points without error bars represent mean values ($n = 1-3$). Each liver biopsy was analysed in triplicate. (b) Rats stabilized on complete diet (Δ) were shifted to low-protein diet for 1-21 days ($\square$). After 16 days, some rats were shifted to amino acid solution (Vamin-N) ($\blacksquare$). Each point represents the RI/RII ratio of one liver analysed in triplicate.

skeletal muscle, cardiac muscle or adipose tissue the ratio decreased by less than 30%, and no decrease at all occurred in renal cortex. Refeeding with complete diet restored the liver RI/RII ratio completely within 7 days (Fig. 1a). It is therefore concluded that starvation rapidly and reversibly decreases the proportion between iso-receptor I and II in liver cytosol.

In an early report from Exton's group (Chan *et al.*, 1979), it was noted that starvation led to decreased ratio of type I to type II protein kinase in liver. However, another, more detailed, study failed to note any effect of fasting (McClung & Kletzien, 1981). The reason for these discrepancies is not known, but it should be noted that the method used in the present study greatly decreases the possibility of proteolysis and incomplete exchange between cyclic [3 H]AMP and endogenously bound cyclic AMP, and avoids pitfalls associated with DEAE-cellulose chromatography (Knight & Skala, 1977; Malkinson *et al.*, 1983; Butley & Malkinson, 1984).

Dietary protein affects the liver RI/RII ratio

Rats fed on complete diet were shifted to either a low-protein diet or amino acid solution. With the low-protein diet the RI/RII ratio in liver cytosol decreased steadily at about one-fifth the rate observed in starvation (Figs. 1a and 1b). Although the energy intake with this diet (230 kJ/day) was normal, the animals lost about 40% of body and liver weight. With the amino acid solution the RI/RII ratio decreased from 2.0 to 1.7 during 7 days (results not shown). During this period, the energy intake was 40% of normal, and the mean body and liver weight decreased by about 20%.

Additional information on the regulation of the liver RI/RII ratio was gained from refeeding experiments. In rats starved for 3 days and then fed on low-protein diet, no increase of the liver RI/RII ratio or body weight occurred (results not shown). In contrast, amino acid feeding of rats starved for 3 days (Fig. 1a) or pre-fed on low-protein diet for 16 days (Fig. 1b) doubled the RI/RII ratio within 18 h. The reason for the transient nature of this increase is not known. We have noted that the amino acid refeeding induced also a transient increase in the liver mass (40%), the hepatocyte volume (22%) and cytosolic protein (25%; Table 1). We have also noted that, after the first 2 days of refeeding, the intake of amino acids was decreased by 15%. It is possible that this decrease and a possible induction of amino acid-catabolic enzymes may have lowered the hepatocyte concentration of amino acids, and thereby diminished their effect [see Mortimore & Pösö (1986) for a review of the role of amino acids in the regulation of hepatic protein degradation].

Effects of diets on the cell content and subcellular distribution of protein kinase subunits

Several conclusions can be drawn from the determination of the amount of protein kinase subunits per mg of DNA in the unfractionated liver homogenate, the cytosol and the particulate fraction (Table 1). Firstly, it appeared that the decrease in the cytosolic RI/RII ratio upon starvation or protein restriction was due to a decrease in cytosolic RI rather than an increase in RII. Secondly, the decrease in cytosolic RI was not due to translocation to the particulate fraction, since the absolute content of particulate RI did not increase. Thirdly, the decrease in R (RI+RII) was accompanied by a near-corresponding decrease in C. It was also proportional to the decrease in liver protein (Table 1) and liver mass (results not shown). During amino acid feeding to pre-fasted or protein-deprived animals, the amount of cytosolic RI increased rapidly (Table 1). Since the cell contents of cytosolic RII and particulate RI and RII were relatively unaffected by the rather dramatic changes in cell volume and protein (Table 1), it can be concluded that the hepatocyte seemed to regulate selectively the amount of cytosolic RI to adjust total R (i.e. RI+RII) according to cell volume/protein content.

Dietary manipulations affect the ratio of type I to type II isoenzyme in all subpopulations of parenchymatous hepatocytes

The data just presented (Fig. 1, Table 1) are compatible with the RI/RII ratio either being positively correlated with hepatocyte (static) size or being correlated with an increase (dynamic) of the hepatocyte size. The finding

Table 1. Effect of diets on the cell content and the subcellular distribution of protein kinase subunits

The amounts of cyclic AMP-dependent protein kinase subunits were determined in the unfractionated Triton-treated liver homogenate, in the cytosol fraction and in the Triton-treated particulate fraction as described in the Experimental section. The amount of regulatory subunits (RI or RII) is expressed as the cyclic AMP-binding capacity (pmd) of each isoreceptor per mg of DNA. The amount of catalytic subunits (C) is expressed as units/mg of DNA (one unit is 1 nmol of phosphate incorporated into Kemptide/min under the present experimental conditions). Total protein (mg/mg of DNA) was determined in the cytosol fraction. For details of the dietary regimens, see the Experimental section. Results are means $\pm$ S.E.M. ($n = 6-10$). The significance of the diets was tested by Wilcoxon's two-sample rank sum test. The decrease in RI in homogenate and cytosol during starvation or protein restriction was highly significant ($P < 0.01$). The significance of the increase in RI after shifting to amino acids after either fasting or low-protein diet was $0.01 < P < 0.02$ and $P < 0.01$ in the homogenate and cytosol respectively.

Feeding regimen	Unfractionated homogenate			Particulate fraction		Cytosol fraction		Total protein
	RI	RII	C	RI	RII	RI	RII	
Complete diet	197 $\pm$ 12	150 $\pm$ 5.0	178 $\pm$ 6.6	45 $\pm$ 2.0	57 $\pm$ 1.0	161 $\pm$ 14	94 $\pm$ 13	115 $\pm$ 8.5
Starvation (3 days)	109 $\pm$ 6.3	129 $\pm$ 7.9	123 $\pm$ 2.9	38 $\pm$ 3.1	58 $\pm$ 6.4	66 $\pm$ 4.6	87 $\pm$ 11	71 $\pm$ 4.6
Starvation + amino acid feeding (18 h)	188 $\pm$ 21	131 $\pm$ 8.7	149 $\pm$ 17	42 $\pm$ 9.0	49 $\pm$ 5.8	141 $\pm$ 16	82 $\pm$ 6.5	98 $\pm$ 9.7
Low-protein diet (16 days)	111 $\pm$ 6.5	118 $\pm$ 8.3	108 $\pm$ 7.8	34 $\pm$ 2.8	52 $\pm$ 5.8	66 $\pm$ 3.2	70 $\pm$ 3.7	75 $\pm$ 7.8
Low-protein diet + amino acid feeding (18 h)	177 $\pm$ 4.6	118 $\pm$ 2.3	130 $\pm$ 5.5	37 $\pm$ 1.2	43 $\pm$ 2.2	139 $\pm$ 3.0	71 $\pm$ 3.2	83 $\pm$ 4.2

that the RI/RII ratio was independent of hepatocyte (static) size (Table 2) argues that the effects of diet on the RI/RII ratio are due to metabolic conditions related to the alteration of cell size. It was remarkable that all subpopulations of hepatocytes, whether separated on the basis of size (Table 2) or buoyant density (results not shown), had similar RI/RII ratio. The effects of diet on whole-liver RI/RII ratio were reflected in the findings for the isolated hepatocytes, and thus could not be ascribed to the non-parenchymatous cells.

Intracellular titration of the endogenous binding of cyclic AMP in the liver of fed and starved rats

The results presented in the preceding paragraphs show that the ratio of type I to type II protein kinase in liver is easily altered by dietary manipulation. This allowed us to test whether an altered isoenzyme ratio could affect the intracellular titration of cyclic AMP binding to RI or RII. For this, the amount of hepatic cyclic AMP was manipulated in livers of fed and starved (3 days) rats by the intraportal infusion of glucagon. The

titration curves were essentially similar in cells whether the RI/RII ratio was 0.8:1 (Fig. 2, starved rats) or 2:1 (Fig. 2, fed rats). The saturation of RII appeared to be slightly higher than that of RI at low cyclic AMP concentrations, whereas RI was slightly more saturated than RII at high cyclic AMP concentrations. Similar results were obtained also in hepatocytes isolated from rats fasted for 1 or 3 days (results not shown). This indicates that the slightly higher co-operativity of the intracellular cyclic AMP binding of RI, as compared with RII, is significant.

DISCUSSION

The present study shows that dietary protein affects the ratio of type I to type II cyclic AMP-dependent protein kinase in liver. The results indicate that the expression of liver RI is closely related to the metabolic conditions. As a response to either fasting or protein deprivation, both leading to catabolic hepatocyte shrinkage (Kosterlitz, 1944; Hutson & Mortimore, 1982;

Table 2. Effects of dietary conditions on the RI/RII ratio in subpopulations of parenchymatous hepatocytes separated by centrifugal elutriation

Crude hepatocyte suspensions were prepared from rats on various feeding regimens. The cytosolic RI/RII ratio was determined in the crude cell suspension or in subpopulations of cells separated by centrifugal elutriation at counter flow rates from 10 to 37.5 ml/min (for details, see the Experimental section). Results are means of three separate experiments.

Feeding regimen	Cells . . .	RI/RII ratio				
		Crude suspension	N ₁₀ fraction	N ₂₀ fraction	N ₃₀ fraction	N _{37.5} fraction
Complete diet		1.8	1.7	1.8	2.0	1.6
Starvation (3 days)		0.6	0.7	0.7	0.6	0.8
Low-protein diet (16 days)		0.8	1.0	0.9	1.0	1.0
Low-protein diet (16 days) + amino acid feeding (18 h)		1.7	1.8	1.9	1.9	1.4

Fig. 2. Intracellular saturation of hepatic RI and RII with endogenous cyclic AMP as a response to intraportal infusion of glucagon in starved and fed rats

Glucagon was injected in the portal vein in rats starved for 3 days (a) or fed on complete diet (b) by a standardized procedure, and total liver cyclic AMP ($\square$; right ordinates, log scale), and the intracellular saturation of RI ($\circ$) or RII ($\bullet$) (left ordinates) were determined as described in the Experimental section. The results are means ($n = 1-3$). Each liver biopsy was analysed in triplicate

Uhal & Roehrig, 1982; the present work), a significant decrease in liver RI/RII ratio occurred very uniformly in all populations of parenchymatous liver cells. This change was a result of decreased cytosolic RI content per cell. In contrast, the cellular amounts of cytosolic RII and particulate RI and RII were only slightly changed. This shows that the amount of cytosolic RI is regulated differently from that of RII and particulate-associated RI. The preferential decrease of cytosolic RI is compatible with RI being more susceptible to intracellular proteolysis than RII, as reported by Weber & Hilz (1986). However, our data do not support their hypothesis that the higher rate of degradation of RI was a result of preferential activation of type I protein kinase. In contrast with Schwach (1978), we found slightly higher endogenous cyclic AMP saturation of RII than of RI in the unstimulated liver (Fig. 2). However, as a response to glucagon stimulation, RI bound cyclic AMP slightly more co-operatively than did RII.

The down-regulation of RI was reversed by anabolic stimulation of the liver, and both the RI/RII ratio (Fig. 1a) and liver mass were permanently restored by refeeding with complete diet. The amount of RI per cell increased more dramatically, albeit transiently, as a response to amino acid feeding of starved or protein-deprived rats (Table 1). The rapidity of the increase in RI suggests that it cannot be explained by diminished breakdown only, but that increased synthesis must be involved. In

accordance with this, we have recently observed (G. Houge, R. Ekanger & S. O. Døskeland, unpublished work) a 5-fold increase in the mRNA for RI as a response to amino acid refeeding. On the basis of the present data, we conclude that RI, which repeatedly has been implicated as a positive effector of proliferative cellular growth (Døskeland *et al.*, 1975; Fuller *et al.*, 1978; Russell, 1978; Miyazaki *et al.*, 1980) also correlates positively with anabolic conditions in a non-proliferating cell type.

The main, if not only, function of the regulatory subunits of cyclic AMP-dependent protein kinase is to regulate the activity of the catalytic subunits. Since the intracellular cyclic AMP-binding properties of both isoenzymes were unchanged in the catabolically perturbed liver (Fig. 2), and since also the ratio between the total amounts of regulatory and catalytic subunits was unchanged (Table 1), the decreased RI/RII ratio would not by itself alter the regulation of C. However, kinase localization in membranes and other structures has been shown to depend on the compartmentalization of the R-proteins (Rubin *et al.*, 1972; Corbin *et al.*, 1977; Walter *et al.*, 1978), and this localization may partially determine which substrates are the most accessible targets for phosphorylation. Thus the different expression of cytosolic and particulate-associated RI in the metabolically perturbed liver indicates that the targeting function of the isoenzymes is altered. Of interest in this respect is the notion that particulate-associated RI behaves quite differently in regenerating liver, always fluctuating in close correlation with cytosolic RI (R. Ekanger & S. O. Døskeland, unpublished work).

Our study shows that dietary manipulation allows the study of cyclic AMP-dependent protein kinase isoenzyme expression in liver. Studies on cultured hepatocytes may help to characterize the effect of separate amino acids on the transcriptional regulation of cyclic AMP-dependent protein kinase subunits.

The technical assistance of Erna Finsås, Nina Lied Larsen and Dao Kim Khanh is highly appreciated. This work was supported by grants from the Nordic Insulin Foundation and the Norwegian Society for Fighting Cancer (N.F.T.K.B.).

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Received 18 April 1988/14 July 1988; accepted 21 July 1988