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Differential Activation of Type-I and Type-II Adenosine 3':5'-Cyclic Monophosphate-Dependent Protein Kinases in Liver of Glucagon-Treated Rats

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The protein-bound cyclic AMP and the activity of cytosolic protein kinases in the presence and absence of cyclic AMP were determined in rat liver up to 2h after injection of glucagon. On the basis of the different salt-sensitivities of the activated cyclic AMP-dependent protein kinases I and II, an activation of protein kinase II restricted to the high cyclic AMP concentrations present in the first 30min after hormone injection was found. Essentially the same result was obtained by chromatographic analysis on DEAE-cellulose of liver cytosol from untreated rats and from rats killed at 2 and 60min after glucagon injection. Protein kinase II activation was only detected at 2min after injection. In contrast, the cyclic AMP-dependent protein kinase I was found to be nearly totally activated at 2min and to be still almost as active at 60min after the hormone stimulus, whereas the amount of bound cyclic AMP and the activation of total cytosolic protein kinases had fallen to two-thirds of their maximal values during this time period. A third cyclic AMP-independent protein kinase, which co-chromatographed with protein kinase type II, could be clearly distinguished from the two cyclic AMP-dependent kinases by use of the heat-stable inhibitor from bovine muscle, which totally inhibited the cyclic AMP-dependent enzymes, but stimulated the cyclic AMP-independent protein kinase.

Most mammalian tissues contain two forms of cyclic AMP-dependent protein kinases, denoted types I and II, which can be separated by DEAE-cellulose chromatography (Corbin *et al.*, 1975). In the absence of cyclic AMP, both types of kinases show little if any catalytic activity. Under these conditions each enzyme appears to consist of four subunits, two regulatory and two catalytic. On addition of cyclic AMP the complex dissociates into the two catalytic subunits and a dimer of regulatory subunits to which two molecules of cyclic AMP are bound. The free catalytic subunits are the active form of the enzyme (see review by Rubin & Rosen, 1975).

The protein kinases I and II apparently have the same catalytic subunit, but different regulatory subunits (Kumon *et al.*, 1972; Hofmann *et al.*, 1977). Thus any differences between their properties may be attributed to the regulatory subunits. The two types of protein kinase differ with respect to their sensitivity to a number of factors that influence their state of association or dissociation. Such factors are the concentration of salt, substrate (Corbin *et al.*, 1975) or MgATP (Hofmann *et al.*, 1975) and the readiness with which autophosphorylation occurs (Rosen & Ehrlichman, 1975). In addition, other reports indicate that the two enzymes purified from liver need different cyclic AMP concentrations for half-maximal activation (Yamamura *et al.*, 1971; Kumon *et al.*, 1972; Granner, 1974). From these studies *in vitro* one may expect the activity *in vivo* of the two enzymes to be under multifactorial control, cyclic AMP apparently being the most important of these factors. So far, however, it is not known whether alterations of the cyclic AMP concentration *in vivo* influence the state of activity of the two protein kinases in a quantitatively equal or in a differential manner. To answer this question I attempted to analyse the state of activity of protein kinases I and II in liver cytosol as a function of the amount of cyclic AMP bound to high-affinity proteins, which in a previous paper (Schwoch & Hilz, 1977) has been shown to be a more meaningful parameter than the total cyclic AMP concentration.

The liver was the organ of choice for the present study, since in this tissue more than 90% of cyclic AMP-dependent protein kinase activity can be found in the cytosol and because protein kinases I and II are present in similar amounts (Chen & Walsh, 1971; Eil & Wool, 1971; Kumon *et al.*, 1972; Corbin *et al.*, 1975). I followed the enzyme activities and the concentration of bound cyclic AMP over a period of 2h after a single injection of glucagon. Evidence is

presented that the state of activation of protein kinases I and II *in vivo* is not uniform at different concentrations of bound cyclic AMP.

Materials and Methods

Materials

Glucagon was obtained from Eli Lilly G.m.b.H. (Giessen, Germany). [γ-^{32}P]ATP (sodium salt) and [8-^{3}H]adenosine 3′:5′-cyclic phosphate were from Amersham Buchler (Braunschweig, Germany). Calf thymus histone type II-A was from Sigma (St. Louis, MO, U.S.A.) and DEAE-cellulose (type DE-32) from Whatman (Maidstone, Kent, U.K.). Cyclic AMP was from Boehringer (Mannheim, Germany), bovine serum albumin from Behringwerke (Marburg, Germany) and charcoal from E. Merck (Darmstadt, Germany). Other chemicals were commercial preparations of reagent grade.

Animals

Female Wistar rats (150–210g body wt.), maintained on standard chow (Altromin G.m.b.H., Lage/Lippe, Germany) in light/dark-regulated rooms, were injected intraperitoneally with 0.4mg of glucagon/100g body wt. At the indicated times the rats were killed by decapitation and their livers quickly frozen with aluminium clamps that had been cooled in liquid N_2. The livers were ground under liquid N_2 and stored at −80°C until use.

Determination of protein-bound cyclic AMP

The protein-bound cyclic AMP was determined as described by Schwoch & Hilz (1977). Approx. 300mg of frozen liver tissue was homogenized for 15–20s with 2.0ml of charcoal suspension [100mg of charcoal/ml of a buffer containing 50mM-Tris/HCl, 5mM-EDTA, in 5mM-theophylline and 2% (w/v) bovine serum albumin at pH7.4] in an Ultra-Turrax tissue homogenizer.

The suspension was centrifuged for 4min at 8000*g* and 0.5ml of the supernatant was transferred to 0.1ml of ice-cold 50% (w/w) trichloroacetic acid, mixed and centrifuged as described above. Then 300μl of the supernatant was extracted with 5×1ml of water-saturated diethyl ether. After incubation at 95°C for 4min, the tubes were transferred to an ice bath and 30μl each of 0.25M-$BaCl_2$ and of 0.25M-Na_2SO_4 were added to remove interfering nucleotides (Chan *et al.*, 1970). After mixing and centrifugation for 4min at 8000*g*, samples of the supernatant were used for the determination of cyclic AMP by the method of Brown *et al.* (1972). Calibration curves were obtained with standard cyclic AMP solutions homogenized with the buffer used for the charcoal suspension and carried through the entire procedure.

Determination of protein kinase activity

Frozen ground liver powder (150–160mg) was homogenized with 4ml of ice-cold buffer, containing 10mM-potassium phosphate, 4mM-EDTA and 2mM-theophylline, pH6.5, in a cooled glass homogenizer with two strokes of a motor-driven (1100rev./min) Teflon pestle. A portion of the homogenate was diluted with 4vol. of either the homogenization buffer or the buffer containing, in addition, KCl to give a final concentration of 150mM. Centrifugation was performed at 20000*g* for 5min at 4°C. The assay was carried out at 37°C as previously described (Schwoch & Hilz, 1977) in a total incubation volume of 0.11ml containing 20μl of enzyme, 7.5mM-magnesium acetate, 2mM-theophylline, 150mM-sodium acetate, 0.145mg of histone II-A and 0.22mM-[γ-^{32}P]ATP (about 150c.p.m./pmol), with or without 1.8μM-cyclic AMP at pH6.5. When liver extracts were used that were prepared in the presence of 150mM-KCl, the sodium acetate concentration was lowered to give comparable ionic strength in all assays. After addition of enzyme, incubations were carried out for 5 and 10min and then terminated by pipetting 50μl samples of the reaction mixtures on filter-paper discs and washing as described by Corbin & Reimann (1974). Corrections were made for radioactivity incorporated in the presence of heat-denatured (5min, 95°C) liver extracts. One unit of protein kinase activity is that amount of enzyme which catalyses incorporation of 1nmol of ^{32}P into histone in 1min. Protein kinase activity expressed as activity ratio means the ratio of the activity in the absence of cyclic AMP to the activity in the presence of 1.8μM-cyclic AMP.

DEAE-cellulose chromatography

Frozen powdered liver was homogenized (8ml/g) in cold 5mM-Tris/HCl, containing 4mM-EDTA, pH7.0, with 20 strokes of a Teflon pestle (1100rev./min) in a glass homogenizer. To a portion (3.6ml) of the homogenate, NaCl was added to a final concentration of 140mM. After centrifugation (20min, 20000*g*, 4°C) the supernatant was diluted to 15mM-NaCl, 4mM-EDTA and 5mM-Tris/HCl, pH7.0, and a portion containing the equivalent of 200mg of liver was applied to a DEAE-cellulose (DE-32) column (0.9cm×6.5cm) pre-equilibrated in the same buffer. After washing with 45ml of buffer, final elution was performed with 250mM-NaCl in 5mM-Tris/HCl/4mM-EDTA, pH7.0. Fractions (3.2ml) were collected at a flow rate of 17ml/h at 5°C. A 20μl sample of the fractions was assayed for protein kinase as described above.

Other procedures

The heat-stable inhibitor of protein kinase was prepared from bovine skeletal muscle as described by Gilman (1970).

Protein was measured after precipitation with trichloroacetic acid by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Results

Protein-bound cyclic AMP and protein kinase activity in liver cytosol prepared in the absence or presence of 150mM-KCl at various times after glucagon injection

A single intraperitoneal injection of glucagon (0.4mg/100g body wt.) leads to a rapid rise of protein-bound cyclic AMP in rat liver to about 2.5 times the control values. After a small decline, the value stabilized at about 2-fold above controls for the time of investigation (Fig. 1, inset). As shown previously (Schwoch & Hilz, 1977), the fraction of bound cyclic AMP represents mainly, if not exclusively, cyclic AMP bound to the regulatory subunit of cyclic AMP-dependent protein kinases and therefore reflects the activation status of these enzymes. The biphasic decay of bound cyclic AMP could suggest a differential inactivation of the two types of liver cytosolic protein kinases involved in the response to glucagon. To test this suggestion, I correlated the changes in non-dissociated inactive protein kinase (specific activity of protein kinase in presence of 1.8μM-cyclic AMP minus specific activity in absence of cyclic AMP), as determined in cytosolic extracts prepared in either the presence or the absence of 150mM-KCl, with the concentrations of bound cyclic AMP measured in the same livers.

As shown in Fig. 1, the proportion of non-dissociated protein kinase decreases similarly at both salt conditions with rising concentrations of bound cyclic AMP up to a value of 0.67pmol/mg of tissue, i.e. about 2pmol/mg of tissue total cyclic AMP. Beyond this value a clear change in slope can be recognized in both curves. The change is more pronounced when the protein kinase activities were measured in cytosol samples not supplemented with KCl. This means that a further increase of bound cyclic AMP up to the saturation value (0.95pmol/mg of tissue; see Schwoch & Hilz, 1977) is not accompanied by a correspondingly high decrease in the inactive holoenzyme.

That the curves do not intersect the abscissa at the value for maximally bound cyclic AMP, as would result from extrapolation of the initial slope of the curves, may be because of the enzyme-determination procedure. Although the protein-bound cyclic AMP is rapidly separated from the free cyclic AMP by direct homogenization of frozen liver with charcoal, the measurement of protein kinase requires a more long-lasting preparation of the tissue, thus facilitating changes in their original dissociation status. It is known (Corbin *et al.*, 1975) that protein kinases type

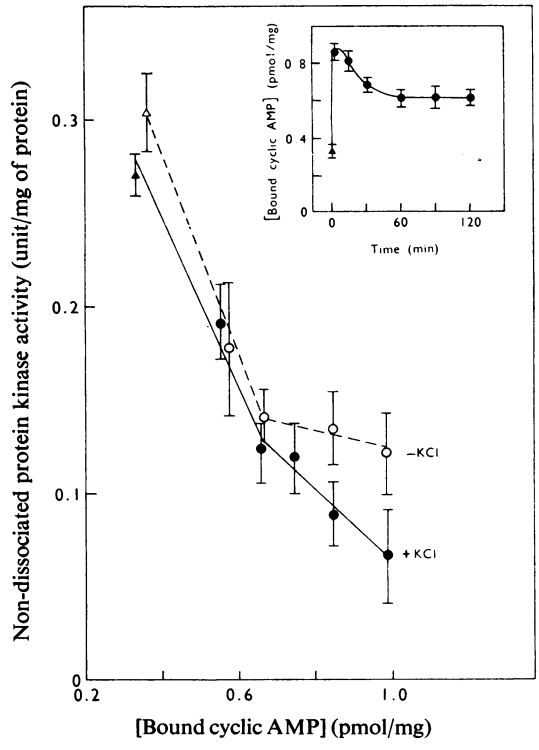

Fig. 1. *Correlation between non-dissociated inactive protein kinase and protein-bound cyclic AMP*

The specific activity of protein kinase (units/mg of protein) and the protein-bound cyclic AMP were determined in the same rat livers obtained at various times after injection of glucagon as described in the Materials and Methods section. The values for protein-bound cyclic AMP were arranged in the order of their magnitude and divided into subsets, each subset covering a range of 0.1 pmol/mg of tissue. The mean of the values within each subset is plotted against the mean of the corresponding values for non-dissociated protein kinase (specific activity measured after addition of 1.8μM-cyclic AMP minus specific activity measured without cyclic AMP). ●, Cytosol samples prepared in the presence of 150mM-KCl from rats injected with glucagon; ▲, control cytosols prepared in the presence of 150mM-KCl from rats injected with 0.9% NaCl; ○, cytosols prepared without KCl from rats injected with glucagon; △, cytosols prepared without KCl from rats injected with 0.9% NaCl. Each point represents the mean± S.E.M. of results for 13 (●, ▲) or nine (○, △) rats, except the highest point, which is the mean of results for three rats. The inset shows the time-course of protein-bound cyclic AMP in rat liver after injection of glucagon. Data points represent means±S.E.M. of results for 10–14 rats.

II, in contrast with protein kinases type I, exhibit a rapid reassociation of their subunits at salt concentrations below 0.5M-KCl or -NaCl. A sufficiently high ionic strength to maintain the activation status of protein kinase II, however, was not included in my experiments, since salt concentrations over 150mM-KCl would introduce another sort of error, namely dissociation of protein kinase I (Cherrington *et al.*, 1976).

Thus, even in the presence of 150mM-KCl, a partial reassociation of protein kinase II has to be expected during preparation of the enzyme, the reassociation, i.e. inactivation of the enzyme, becoming more pronounced when no KCl was added to the homogenate. The change of slope of the curves presented in Fig. 1 therefore strongly suggests a preferential activation of the type-II protein kinase at the high concentrations of cyclic AMP obtained after the hormone treatment.

Chromatographic analysis of liver cytosol protein kinases at various times after glucagon injection

The above conclusion was corroborated by chromatographic data. Liver cytosol samples prepared in the presence of 150mM-KCl were fractionated on DEAE-cellulose columns. Three states of activation were analysed; livers from animals killed 2min after glucagon, known to exhibit nearly complete activation of protein kinases (Schwoch & Hilz, 1977), livers from rats killed 60min after hormone injection, which show persistence of bound cyclic AMP values at about 2-fold basal values (cf. Fig. 1, inset), and livers from unstimulated controls. All protein kinase assays were performed at the same time intervals after preparation of the tissues to avoid differences due to instability of the separated enzymes. Preliminary experiments, moreover, showed stability for 48h of protein kinases fractionated from control livers. The results are shown in Fig. 2.

By stepwise elution of columns loaded with cytosol from control livers, two peaks of protein kinase activity are obtained that can both be considerably stimulated by the addition of 1.8μM-cyclic AMP to the assay. The first peak, eluted by the equilibration buffer, may be identified as a type-I protein kinase as described by Corbin & Keeley (1977). The second peak, eluted by 0.25M-NaCl, may contain protein kinase type II, since in a pilot experi-

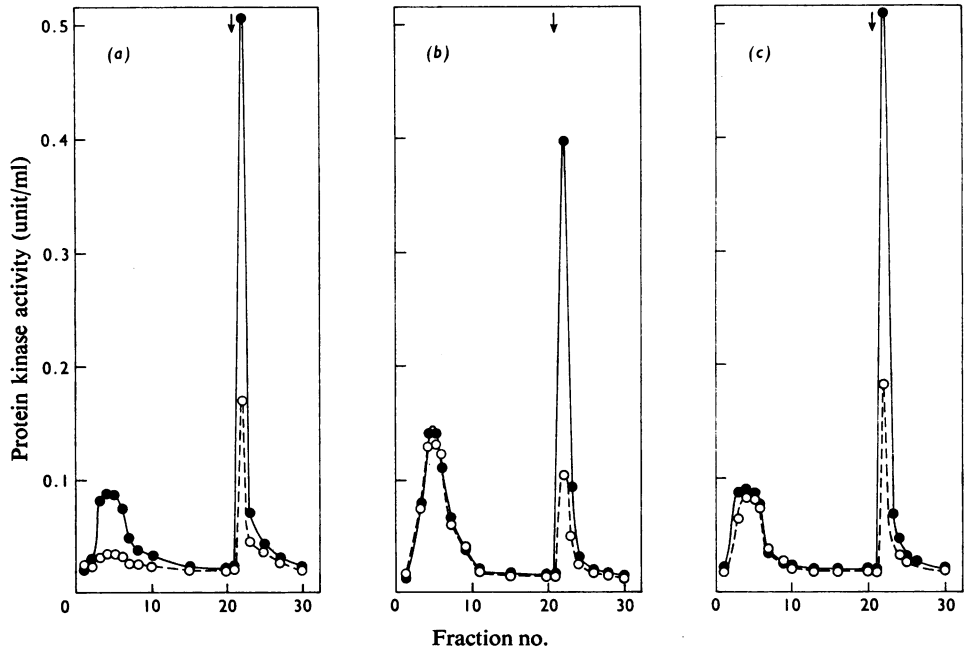

Fig. 2. *DEAE-cellulose chromatography of liver cytosol protein kinase of rats killed without treatment (control, a) and at 2min (b) and 60min (c) after injection of glucagon*

Liver extracts were prepared as described in the Materials and Methods section and applied to columns (0.9cm × 6.5cm) of DEAE-cellulose equilibrated with 15mM-NaCl/5mM-Tris/HCl/4mM-EDTA, pH7.0. The columns were eluted with the same buffer, followed by a step (arrow) of 250mM-NaCl/5mM-Tris/HCl/4mM-EDTA, pH7.0. Fractions (3.2ml) were collected at a flow rate of 17ml/h at 5°C. Portions (20μl) were assayed for protein kinase activity in the absence (○) or presence (●) of 1.8μM-cyclic AMP, with histone II-A as substrate.

ment (results not shown) with a continuous NaCl gradient the protein kinase was eluted with a peak at 175mM-NaCl, which agrees with the elution behaviour of other type-II protein kinases from mammalian tissues (Corbin *et al.*, 1975). No additional protein kinase activity was eluted up to 0.35M-NaCl.

In contrast with the controls, protein kinase in peak 1 is strongly activated at 2min and is still highly active at 60min after glucagon injection. However, although at 60min the total activity in the first peak is as high as in control livers, at 2min the peak-1 activity with or without cyclic AMP had nearly doubled at the expense of peak 2. This indicates activation of protein kinase type II at this stage, since its free catalytic subunit, which has a higher isoelectric point than the holoenzyme and which is probably identical with the active subunit of protein kinase I (Chen & Walsh, 1971; Kinzel & Kübler, 1976), is to be expected in the low-salt fractions.

Table 1 contains the data of Fig. 2 and in addition, to demonstrate the reproducibility, a second set of data (Expt. 2) obtained under identical experimental conditions. The activity in both chromatographic peaks is calculated by summation of the activity of the individual fractions. The same results were obtained when pooled fractions were assayed. The quantitative evaluation of the column chromatography shows a small increase (about 10%) in the recovery of total enzyme activity at 2min after hormone injection, which may be related to some stabilizing effect of the higher cyclic AMP concentration in these livers (Brostrom & Kon, 1974). This small increase in total activity, however, cannot impair the significance of displacement of protein kinase activity from peak 2 to peak 1.

As Fig. 2 shows, approx. 30% of the protein kinase activity eluted in the position of the type-II enzyme is active without addition of cyclic AMP to the assay. This activity is independent of cyclic AMP and is obtained in all experiments to nearly the same amount with cytosol from either control or stimulated livers. In accordance with similar findings in mouse liver (Ueland & Døskeland, 1976), we suggest that this activity represents a cyclic AMP-independent protein kinase that co-chromatographs with cyclic AMP-dependent protein kinase II. Experiments with the heat-stable protein kinase inhibitor (Fig. 3) indeed show that only the activity obtained in the first peak can be completely suppressed by the inhibitor. This applies to control livers as well as to livers removed 2min after glucagon injection, and thus confirms that at both stages only activity of cyclic AMP-dependent protein kinases has been eluted in the low-salt fractions. The activity of peak 2, however, shows inhibition only to some extent, the inhibition being partly reversed at higher concentrations of the inhibitor. This latter effect probably is not due to a non-specific desorption of protein kinase from the incubation-tube walls by the higher amounts of inhibitor protein, as proposed elsewhere (Rousseau & DeVischer, 1976), since the same curves are obtained when bovine serum albumin is included in the assay.

Table 1. *Activity of protein kinases separated by DEAE-cellulose chromatography of cytosol from untreated controls and glucagon-treated rats*

The activity of protein kinases eluted by the equilibration buffer (peak 1) and by 0.25M-NaCl (peak 2) from DEAE-cellulose columns was calculated by summarizing the activity assayed in the individual fractions with and without 1.8μM-cyclic AMP (for details see the Materials and Methods section). These data were corrected for background activity (fractions 15–20 in Fig. 2) and differences in the amount of protein applied to the column. Each experiment represents a chromatographic separation of cytosol prepared from three pooled livers. The protein-bound cyclic AMP in the same livers was measured as described in the Materials and Methods section. The difference between Expts. 1 and 2 is explained in the text.

Animal treatment	Protein-bound cyclic AMP (pmol/mg of tissue)	Total protein kinase activity (units) Peak 1	Peak 2	Protein kinase activity ratio (−cyclic AMP/+cyclic AMP) (peak 1)
Controls				
Expt. 1	0.31	0.89	1.86	0.13
Expt. 2	0.34	0.94	2.02	0.22
Glucagon-treated				
2 min				
Expt. 1	0.96	1.65	1.58	0.82
Expt. 2	0.99	1.49	1.67	0.83
60 min				
Expt. 1	0.60	0.94	2.10	0.83
Expt. 2	0.72	0.59	1.98	0.75

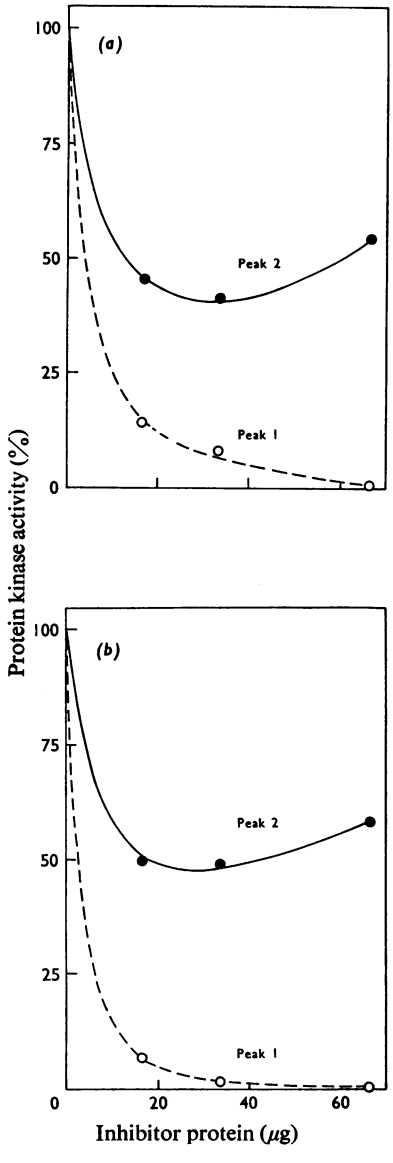

Fig. 3. *Effect of the heat-stable inhibitor on protein kinase activity of peak* 1 *and peak* 2

Liver extracts from untreated controls (*a*) or rats killed 2min after injection of glucagon (*b*) were separated on columns of DEAE-cellulose. The protein kinase eluted by the starting buffer (peak 1, ○) or with 0.25M-NaCl (peak 2, ●) was assayed in the presence of 1.8μM-cyclic AMP and with histone II-A as substrate. The activity assays were performed for 10min at 37°C in the presence of various amounts of bovine muscle inhibitor protein. Means of duplicate determinations of the eluates of two chromatographic runs are expressed as percentages of the protein kinase activity measured in the absence of inhibitor (about 82units/ml).

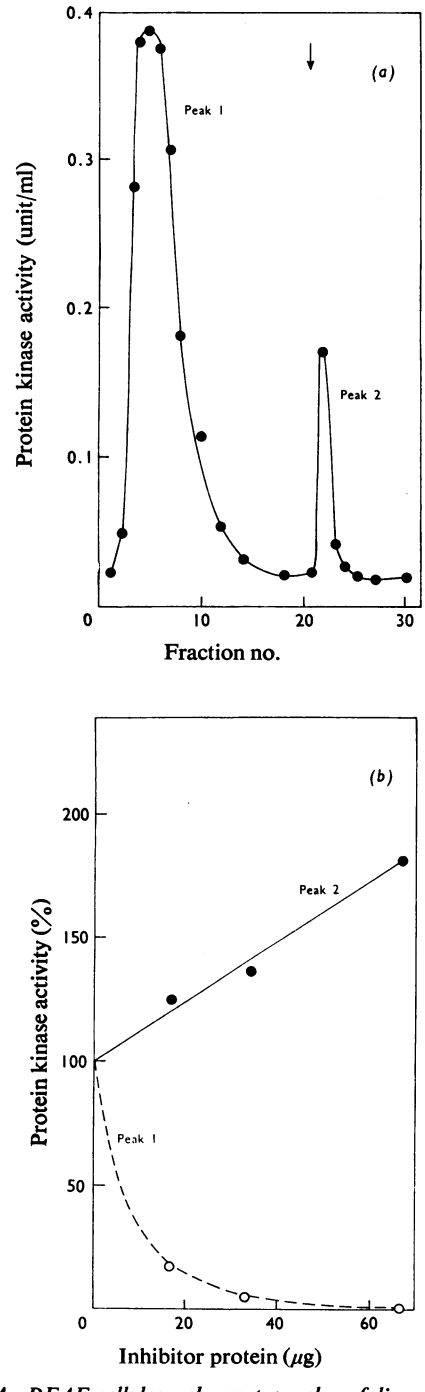

Fig. 4. *DEAE-cellulose chromatography of liver cytosol protein kinase in the presence of* 10μM-*cyclic AMP* (*a*) *and effect of the heat-stable inhibitor* (*b*)

(*a*) Cytosol from livers of three untreated rats was activated by 10μM-cyclic AMP, applied to a column (0.9cm×6.5cm) of DEAE-cellulose pre-equilibrated

The validity of the above conclusions is further documented by an experiment in which control cytosol was completely activated by 10 μM-cyclic AMP and chromatographed in the presence of cyclic AMP, thus avoiding reassociation of protein kinase II. As shown in Fig. 4(*a*), most of the protein kinase activity is eluted at the low salt concentration, where the free catalytic subunits of both cyclic AMP-dependent protein kinases should be eluted from the column. A small part of kinase activity remains in the peak-2 position. The enzymes thus separated can be clearly distinguished by use of the heat-stable inhibitor (Fig. 4*b*). The enzymes eluted at low ionic strength are completely inactivated by the inhibitor. The residual activity eluted by 0.25 M-NaCl, however, cannot be inhibited, and is even stimulated by the inhibitor preparation in a dose-dependent manner. Thus the dual effect of the inhibitor preparation on liver cytosol protein kinase activity eluted from the column by 0.25 mM-NaCl seen in Fig. 3 results from inhibition of the cyclic AMP-dependent protein kinase II on the one hand and stimulation of a cyclic AMP-independent protein kinase on the other. Furthermore, the complete inhibition of the 'low-salt' fractions obtained from livers withdrawn at 2 min after glucagon injection, together with the findings presented in Fig. 4, clearly shows that the increase of protein kinase activity with or without cyclic AMP in the lower-salt fractions observed at this stage is not due to a rearrangement of cyclic AMP-independent protein kinase, but represents activation of the cyclic AMP-dependent protein kinase type II.

Discussion

Chromatographic analysis of rat liver cytosol prepared in the presence of 150 mM-KCl revealed the presence of two cyclic AMP-dependent protein kinases, types I and II, and a third, cyclic AMP-independent protein kinase.

Injection of glucagon led to a rapid increase in the activity ratio of liver cytosol protein kinase to about 0.9, thus indicating activation of protein kinases I and II, which both are present in comparable amounts. During the decay of intracellular cyclic AMP in the 2 h after hormone treatment, however, the two enzymes became inactive to different extents. By use of the specific property of protein kinase II to reassociate rapidly in media of low ionic strength (Corbin *et al.*, 1975) and the difference in charge of inactive holoenzyme and the free catalytic subunit, it could be shown that protein kinase II remained activated at the high concentration of cyclic AMP up to 30 min after glucagon injection, whereas protein kinase I remained nearly fully activated for at least 1 h.

In accordance with other reports (Keeley *et al.*, 1975; Cherrington *et al.*, 1976), low salt concentrations in the homogenate led to a considerable decrease in total cytosolic protein kinase activity in the highly stimulated liver. This loss of activity may be the result of non-specific adsorption of free catalytic subunit on the particulate fraction (Keeley *et al.*, 1975). The decreased activation rate of protein kinase that is pronounced in the low-salt extracts at high intracellular cyclic AMP concentrations (Fig. 1), however, apparently is not due to such an artifact, but must be exclusively attributed to a reassociation of protein kinase II during the experimental procedure. A loss of catalytic subunit would decrease the protein kinase activity by the same value whether determined in the absence or in the presence of cyclic AMP and thus would not alter the difference of the two determinations, which is taken here as a measure of residual inactive protein kinase, i.e. as the parameter of protein kinase activation. A decrease in the amount of holoenzyme by low-salt conditions, which cannot be fully excluded, on the other hand, would change the rate of protein kinase activation, calculated in this way, in a manner opposite to that observed. Finally, a marked activation of protein kinase II restricted to the time immediately after hormone treatment could also be concluded from the determinations performed with liver extracts supplemented with 150 mM-KCl. Under these conditions, as was also reported for hepatocytes (Cherrington *et al.*, 1976), the same values for total protein kinase activity were maintained at 2 min after glucagon injection as in the controls.

In the chromatographic experiments, partial reassociation of protein kinase II also cannot be fully avoided, since separation of the enzymes requires an initial low osmolarity. The absolute value for protein kinase II activation *in vivo* therefore may be underestimated. Yet my main point, a differential activation of protein kinases I and II, is still clearly seen from the chromatographic data, and the high activation status of protein kinase I found at 60 min after hormone stimulation shows that

with 15 mM-NaCl / 5 mM-Tris / HCl / 4 mM-EDTA, pH 7.0, containing 10 μM-cyclic AMP and eluted in the presence of cyclic AMP as described in Fig. 2. Samples (0.02 ml) of the fractions were assayed for protein kinase activity with histone II-A as substrate. The same values were obtained whether or not additional cyclic AMP (1.8 μM) was included in the assay. For further details see the Materials and Methods section. (*b*) Protein kinase obtained in peaks 1 and 2 after separation in the presence of 10 μM-cyclic AMP was measured in the presence of different amounts of inhibitor protein with histone II-A as substrate. The reaction mixture, containing additional 1.8 μM-cyclic AMP, was incubated for 10 min at 37°C. The data, represented as the percentage of the activity measured without inhibitor (about 90 units/ml), are means of two determinations.

the decrease in total activation during this time (from 0.9 to 0.6 when assayed in the crude liver cytosol) essentially takes place at the expense of protein kinase II. This is further corroborated by the biphasic decrease in the protein-bound cyclic AMP *in vivo*, a parameter that was determined independently and by a different method in the same livers.

The high activity of protein kinase I at relatively low intracellular cyclic AMP concentrations suggests that physiological conditions that only slightly increase the cyclic AMP concentration, for instance starvation (Pauk & Reddy, 1971), primarily activate the protein kinase type I. This would be in accordance with determinations of the dissociation constants of isolated liver protein kinases, indicating a lower affinity for cyclic AMP for protein kinase II and a higher one for protein kinase I (Yamamura *et al.*, 1971; Kumon *et al.*, 1972; Granner, 1974). However, it should be noted that the results presented here are obtained under conditions of decreasing cyclic AMP concentrations subsequent to a near-maximal stimulation, and not under conditions of a gentle increase in the intracellular cyclic AMP concentration. Furthermore, it has been shown that the rate of protein kinase dissociation and reassociation *in vitro* can be considerably influenced by MgATP (Hofmann *et al.*, 1975) or autophosphorylation of protein kinase II (Rosen & Ehrlichman, 1975; Rangel-Aldao & Rosen, 1976). This implies that under other stimulation conditions the activation status of the two cyclic AMP-dependent protein kinases could be changed in a manner different from that observed here.

The different duration of the responses of protein kinases I and II to glucagon may indicate different functions of the two enzymes in the cell. This can be also suggested from reports of a special synthesis of protein kinase II during the postnatal development of the rat testis (Lee *et al.*, 1976) and during the boundary between G_1- and S-phases in the cell cycle of the hamster ovary (Costa *et al.*, 1976), or an increase in protein kinase I during isoproterenol-induced cardiac hypertrophy (Byus *et al.*, 1976*a*). Then the extent and duration of a metabolic effect regulated preferentially by one of the two kinases would be considerably misinterpreted on the basis of changed values for intracellular cyclic AMP or of total protein kinase activation. In liver, for example, activation of phosphorylase may be maximal at a time when the intracellular cyclic AMP concentration had already fallen substantially from its peak value (Cherrington & Exton, 1976). In addition, in hepatocytes, maximal activation of phosphorylase has been observed after doses of glucagon that only partially activated the protein kinases (Byus *et al.*, 1976*b*). If under these conditions phosphorylase is not activated by a possible cyclic AMP-independent mechanism (Birnbaum & Fain, 1977), my findings would explain these observations by a specific regulation of liver phosphorylase by the cyclic AMP-dependent protein kinase I. On the other hand, the catalytic subunits of the two protein kinases are nearly identical, and *in vitro* no difference in their substrate specificity has yet been demonstrated (Chen & Walsh, 1971; Yamamura *et al.*, 1973). If this holds true also for their activity towards the cellular substrates, the biological relevance of having two protein kinases could lie in the extension of the stimulation range by activation of enzymes with different affinities for cyclic AMP. Alternatively the differences in activation time as shown here may be connected with different biological responses.

The cyclic AMP-independent protein kinase that was eluted from the DEAE-cellulose column in the same position as protein kinase II comprised about 20% of the sum of all protein kinase activities, and therefore seems to be partly responsible for the relatively high (0.36) protein kinase activity ratio obtained in the non-stimulated liver. A crude preparation of protein kinase inhibitor from bovine muscle stimulated the enzyme. Stimulatory actions of the inhibitor have been described for cyclic GMP-dependent protein kinases and for the cyclic AMP-dependent phosphorylation of substrates other than histone (Donelly *et al.*, 1973; Kuo, 1975). That the inhibitor, which according to the authors just cited should better be called a 'modulator', affects the cyclic AMP-dependent and cyclic AMP-independent protein kinases under identical experimental conditions in an opposite way, inhibiting the former and stimulating the latter, may be of physiological significance in that it expands the variability of the regulation of cellular activities by protein phosphorylation.

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