Selective regulation of the amount of catalytic subunit of cyclic AMP-dependent protein kinases during isoprenaline-induced growth of the rat parotid gland

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Stimulation of growth of the rat parotid gland by repeated injection of the β -agonist isoprenaline led to a significant decrease in the activity of cyclic AMP-dependent protein kinases. Immunochemical quantification of the catalytic (C) and regulatory (RI and RII) subunits of the cyclic AMP-dependent protein kinases type I and type II revealed a loss of 65 % of the immunochemically measurable amount of catalytic subunit C. The amount of the regulatory subunits, however, remained constant. The observed decrease in C-subunit was not due to a translocation of the molecule to cellular membranes or to an inhibiting effect of the heat-stable inhibitor of cyclic AMP-dependent protein kinases. A selective decrease in only the C-subunit was also observed after a brief exposure to isoprenaline leading to the stimulation of DNA synthesis. Under these conditions, the decrease was observed at the onset of DNA synthesis (17 h after injection), but not at the time of an earlier small cyclic AMP peak (13 h after injection) or at the time of maximal DNA synthesis (24 h after injection). The results indicate that the amount of the catalytic subunit of cyclic AMP-dependent protein kinases can be regulated independently from that of the regulatory subunits. The time-limited occurrence of the specific change in the amount of the C-subunit suggests that such a regulation is of physiological significance and that it may participate in cyclic AMP-mediated events involved in the control of cellular proliferation.

INTRODUCTION

An intracellular increase in cyclic AMP can induce a variety of different physiological effects in the same cell, and it is still far from being clear which mechanisms will determine if the one or the other response will be produced. One may be based on the existence of two classes of target enzymes for cyclic AMP in mammalian tissues, the cyclic AMP-dependent protein kinases type I and type II. The two types of kinases can be clearly distinguished by their different cyclic AMP-binding regulatory subunits RI and RII, but apparently share a common catalytic subunit C [1–3]. The physiological significance of the occurrence of two isoenzymes is indicated by the finding that they can be differentially activated by hormones [4–7]. An additional regulatory potential seems to come from variations in the concentration of the two types of kinase, since the ratio of the total enzyme activities of cyclic AMP-dependent protein kinases type I and type II varied under different experimental or physiological conditions (for review see [8]).

The observation of a relative increase in the total activity of the type I enzyme in proliferating cells led to the suggestion that the cyclic AMP-dependent protein kinase type I may be a positive regulator of cellular growth [9]. However, in other cases proliferation was found to be related rather to a relative decrease in the protein kinase type I [10–13] or, as in aging lung

fibroblasts [14], a loss of the proliferative potential of cells coincided with a significant increase in the activity of the type I kinase. This apparent contradiction could result at least in part from methodological problems. In most cases, the two types of cyclic AMP-dependent protein kinases were determined by their enzymic activity after separation of the isoenzymes on DEAE-cellulose. Previously, however, it has been shown that in some tissues the two kinases are not clearly separated by this method [15,16]. Furthermore, measurement of the enzyme activity alone will not be sufficient to establish the amount of the type I and type II protein kinase holoenzymes, since the measurement can be influenced by a number of cellular factors. In the parotid gland, for example, interference by ATPases and insufficient accessibility of membrane-bound protein kinase will not allow the conventional assay of cyclic AMP-dependent protein kinase activity to detect the relatively high amount of cyclic AMP-dependent protein kinase present in the particulate fraction of the cell [17]. The use of specific antibodies in an independent immunochemical determination of cyclic AMP-dependent protein kinases I and II may help to avoid such problems and to determine the amount of C-, RI- and RII-subunits by a similar method and in parallel to each other.

The aim of the present study was to determine the subunits of cyclic AMP-dependent protein kinases type I and type II with the specific purpose of assessing whether the effect of cyclic AMP on cellular proliferation was

Abbreviations used: C-subunit, catalytic subunit of cyclic AMP-dependent protein kinases; RI- and RII-subunit, regulatory subunits of cyclic AMP-dependent protein kinases type I or type II respectively; ELISA, enzyme-linked immunosorbent assay.

connected with a specific change in the concentration of cyclic AMP-dependent protein kinases and, if so, to clarify whether such a change affected the concentration of the subunits of only one specific type of the protein kinase isoenzymes. The parotid gland was chosen as a model, since proliferation in this tissue can be elicited *in vivo* by the injection of the specific β -receptor agonist isoprenaline (isoproterenol), in a relatively physiological way. Repeated injection of the agonist leads to a hyperplastic and hypertrophic enlargement of the gland [18,19]. However, even a single injection of isoprenaline induces DNA synthesis, with a maximum effect after about 24 h [19]. The initiation of DNA synthesis has been shown to depend on a small increase in intracellular cyclic AMP 12–14 h after the injection [20].

It is shown below that the stimulation by isoprenaline indeed leads to a change in the immunochemically measurable amount of cyclic AMP-dependent protein kinases. This, however, does not affect the amount of one specific type of protein kinase holoenzyme, but only that of the catalytic subunit. The time-limited occurrence of this effect after a single injection of isoprenaline suggests that a selective alteration in the concentration of the C-subunit can be an additional regulative tool in the transmission of the cyclic AMP signal to the one or the other biological response. Some of this work has been published in an abstract form [21].

EXPERIMENTAL

Materials

L-Isoprenaline, histones (type II-AS), *N*-*p*-tosyl-L-lysylchloromethane ('TLCK') and L-1-tosylphenylalanylchloromethane ('TPCK') were purchased from Sigma (Munich, Germany). Pentobarbital was from Ciba-Geigy (Basel, Switzerland). [γ - 32 P]ATP, cyclic [3 H]AMP and 125 I-Protein A were purchased from Amersham-Buchler (Baunschweig, Germany). ATP, cyclic AMP and phenylmethanesulphonyl fluoride were obtained from Boehringer (Mannheim, Germany). Bovine serum albumin was from Behring-Werke (Marburg, Germany) and calf thymus DNA from Calbiochem (Frankfurt/Main, Germany). Leupeptin, antipain and pepstatin were from the Peptide Institute Inc. (Osaka, Japan). Nitrocellulose filter paper was purchased from Sartorius (Göttingen, Germany), microtitre plates were from Nunc (Roskilde, Denmark), and alkaline phosphatase-conjugated pig antibody against rabbit IgG was from Orion (Helsinki, Finland). Other chemicals were of reagent grade and came from Merck (Darmstadt, Germany).

Preparations of proteins and antisera

The heat-stable inhibitor of cyclic AMP-dependent protein kinase was prepared from bovine skeletal muscle as described by Gilman [22]. The cyclic AMP-binding protein used in the determination of cyclic AMP was the regulatory subunit from a type I cyclic AMP-dependent protein kinase prepared from bovine skeletal muscle [23]. The catalytic subunit C of cyclic AMP-dependent protein kinase was purified from bovine heart as described by Peters *et al.* [24]. Rabbit antibodies against the catalytic subunit were produced and characterized in detail as described previously [25–27]. Rabbit antisera against the regulatory subunits RI and RII from rat heart [27] were

kindly given by Dr. S. M. Lohmann and Dr. U. Walter (Würzburg, Germany).

Treatment of animals and preparation of parotid extracts

Male Wistar rats (180–210 g body wt.), obtained from Mus-Rattus G.m.b.H. (Brunnthal, Germany) and maintained on standard chow (Altromin, Lage/Lippe, Germany), were injected intraperitoneally with 2.5 mg of L-isoprenaline/100 g body wt., freshly dissolved in saline (0.9% NaCl), or with saline alone (controls). At the time points indicated, the animals were anaesthetized with pentobarbital (50 mg/kg body wt., intraperitoneally), killed by heart incision and exsanguinated. The parotid glands were removed, suspended in ice-cold oxygenated Ringer solution and micro-dissected under a stereo microscope [28]. The cleaned lobules were quick-frozen in liquid N₂ and stored at –80 °C. Tissue samples were powdered under liquid N₂ and homogenized at 0 °C in a glass/glass Potter homogenizer with 9 vol. of a buffer containing 10 mM-imidazole (pH 7.4), 4 mM-MgCl₂, 150 mM-NaCl, 0.22 M-sucrose, 1 mM-dithiothreitol and 0.5 mM-phenylmethanesulphonyl fluoride. For the immunological determination of cyclic AMP-dependent protein kinases, the buffer contained in addition 5 mM-benzamidine, 0.1 mM-tosyl-L-lysylchloromethane, 0.1 mM-tosyl-phenylalanylchloromethane and 4 mg each of leupeptin, antipain and pepstatin/ml. In pilot experiments these proteinase inhibitors, including tosyl-lysylchloromethane, which inactivates the free catalytic subunit [29], had been found not to interfere with the immunochemical determination of the protein kinase subunits (results not shown). The homogenate was centrifuged at 900 *g* (r_{av} 10.8 cm) for 10 min at 4 °C. Where mentioned, the 900 *g* supernatant was centrifuged again at 100 000 *g* (r_{av} 5.9 cm) for 1 h.

Determination of cyclic AMP

The cyclic AMP concentration was measured as described previously [4]. Frozen tissue samples were immediately homogenized in 7 vol. (v/w) of 5% trichloroacetic acid and centrifuged at 8000 *g* (r_{av} 7 cm) for 2 min. The supernatant was freed from trichloroacetic acid by diethyl ether extraction and from interfering nucleotides by BaCl₂/Na₂SO₄ precipitation. In the supernatant, cyclic AMP was determined by the method of Brown *et al.* [30], with bovine skeletal-muscle regulatory subunit type I as the cyclic AMP-binding protein.

Cyclic AMP-binding assays

The cyclic AMP-binding activity of parotid extracts was assayed in 0.1 ml of 50 mM-potassium phosphate buffer/10 mM-EDTA, pH 7.0, containing 2 mM-theophylline, 1 mg of bovine serum albumin/ml, 0–1 μ M-cyclic [3 H]AMP (about 10 000 c.p.m./pmol) and tissue samples corresponding to 0.2 mg of protein. The reaction mixtures were incubated on ice for 2 h, and the bound cyclic [3 H]AMP was determined by the (NH₄)₂SO₄-precipitation procedure of Døskeland & Ueland [31].

Cyclic AMP-dependent protein kinase activity

The protein kinase activity was determined as described previously [17,32]. Parallel determinations of the kinase activity in the presence of cyclic AMP and of saturating amounts of the heat-stable inhibitor served to dis-

criminate between the cyclic AMP-dependent protein kinase activity and other, cyclic AMP-independent, protein kinase activities. One unit of protein kinase activity is defined as the activity that catalysed the incorporation of 1 μ mol of [32 P]P_i into mixed histones in 1 min.

ELISA of cyclic AMP-dependent protein kinase subunits

Competitive ELISA assays of the protein kinase subunits were performed as described by Schwoch & Hamann [26]. Microtitre plates were coated with 3 ng of purified C-subunit/well or 4 ng of purified RI- or RII-subunit/well. Binding of antibodies from the mono-specific antisera diluted 1:4000 (anti-C, anti-RII sera) or 1:1000 (anti-RI serum) to the antigens adsorbed to the plastic surface of the microtitre plates in the absence or the presence of standard amounts of pure subunits or of tissue samples was detected by a second alkaline phosphatase-coupled anti-(rabbit IgG) antibody. The reaction of alkaline phosphatase with *p*-nitrophenyl phosphate was monitored in a SLT-210 microtitre-plate reader. Tissue samples and standard solutions were pretreated with 6 M-urea and by sonication as described previously [26].

Detection of the catalytic subunit by immunoblotting

Immunoblotting of the catalytic subunit was performed by a modification of the procedure of Towbin *et al.* [33] as described by Lohmann *et al.* [27]. Parotid-gland extracts or pure bovine heart catalytic subunit were separated by SDS/polyacrylamide-gel electrophoresis and transferred on to nitrocellulose paper by electroblotting. The catalytic subunit was detected by incubation with the antiserum against bovine heart catalytic subunit (diluted 1:400), followed by incubation with 125 I-Protein A (1 μ Ci/10 ml) and autoradiography as described previously [27].

Determination of protein and DNA

Protein was determined by a modified Lowry procedure [34], with bovine serum albumin as standard. DNA was determined as described by Burton [35], with calf thymus DNA as standard.

RESULTS

Effect of repeated injection of isoprenaline on parotid-gland mass, protein and DNA concentration and cyclic AMP-dependent protein kinase activity

For a comparison of cyclic AMP-dependent protein kinase activities and/or concentrations in isoprenaline-stimulated and unstimulated parotid glands, it was important that the parameters to which they were related did not change independently. For example, in rat hepatomas the protein/DNA ratio was found to be less than half of that in the normal liver cell [36]. The same could happen in the parotid, especially since the stimulation by isoprenaline leads also to an enhanced secretion which decreases, at least transiently, the intracellular protein pool in the stimulated tissue.

As shown in Table 1, injections of isoprenaline twice daily for 3.5 days increased the parotid-gland mass about 3-fold as compared with the control value. The DNA and protein concentrations were elevated to about the same extent. A quantification of cyclic AMP-dependent protein kinases in control and isoprenaline-treated animals will therefore lead to similar values, whether they are expressed per g wet wt., per g of protein or per μ g of DNA. The activities of the soluble cyclic AMP-dependent protein kinases were decreased in the stimulated parotid glands to about one-third of the control value. A small part of the protein kinase activity measured in the presence of cyclic AMP (about 25% of the total activity) that could not be inhibited by the heat-stable inhibitor of cyclic AMP-dependent protein kinase was unchanged by the hormone treatment (results not shown).

Immunochemical determination of cyclic AMP-dependent protein kinase subunits in the growth-stimulated parotid gland

To determine possible changes in the amount of the cyclic AMP-dependent protein kinase isoenzymes under the different physiological conditions, the catalytic (C) and regulatory (RI and RII) subunits of the kinases were determined by the use of specific antibodies. Mono-specific antisera which had been characterized in detail previously [17,25–27] were used in an ELISA [26] as an

Table 1. Effect of repeated treatment with isoprenaline on the mass, protein and DNA concentration and the cyclic AMP-dependent protein kinase activity of the rat parotid gland

Rats were injected intraperitoneally twice a day for 3.5 days with either isoprenaline (2.5 mg/100 g body wt.) or saline (control). The parotid glands were removed, trimmed, weighed, and their protein and DNA concentrations were measured. The cyclic AMP-dependent protein kinase activity was determined in a 100000 g supernatant fraction (60 min, 4 °C) as the phosphotransferase activity measured in the presence of cyclic AMP that could be inhibited by the heat-stable inhibitor. For details see the Experimental section. The data represent means $\pm$ S.D. for four (protein kinase), seven (protein, DNA) or 17 (mass) values.

	Control	Isoprenaline	Isoprenaline Control
Parotid gland			
Mass (g/kg body wt.)	2.08 $\pm$ 0.40	6.14 $\pm$ 0.80	2.95
Protein (g/kg body wt.)	0.23 $\pm$ 0.03	0.57 $\pm$ 0.07	2.48
DNA (mg/kg body wt.)	4.45 $\pm$ 0.51	13.54 $\pm$ 1.90	3.04
Cyclic AMP-dependent protein kinase (μ units/mg of protein)	337.7 $\pm$ 8.2	136.6 $\pm$ 28.2	0.36

Fig. 1. ELISA determinations of cyclic AMP-dependent protein kinase subunits in growth-stimulated parotid glands

Parotid-gland supernatants (900 *g*, 10 min) prepared from rats that had been treated repeatedly either with isoprenaline (○) or with saline (●) were incubated with monospecific antisera against the catalytic subunit (C) or the regulatory subunits type I and type II (RI, RII). The mixtures were applied to microtitre plates coated with pure C-, RI- or RII-subunits, and that part of the antibody which still bound to the antigen in the solid phase was detected by a second, alkaline-phosphatase-conjugated, antibody. For details see the Experimental section. Each point represents the mean from two determinations performed with cellular extracts prepared from three rats. A/A_0 is the absorbance measured in the presence of competing antigen relative to that measured in the absence of competing antigen.

immunochemical method which was suitable also for quantitative studies.

Fig. 1 shows the curves resulting from the competition of antibody binding to pure antigen coated to the microtitre plate surface by C-, RI- and RII-subunits present in the 900 *g* supernatant from control and growth-stimulated parotid glands. For the regulatory subunits RI or RII, the curves were not different in stimulated and unstimulated glands. For the C-subunit, however, a considerably higher amount of protein from stimulated glands was necessary to achieve the same degree of competition as in the controls. A similar result was obtained in a cytosolic fraction (100 000 *g* supernatant) of parotid-gland tissue (not shown). This indicates that the isoprenaline treatment had led to a selective decrease in the concentration of the catalytic subunit of the parotid-gland cyclic AMP-dependent protein kinases.

For quantification, a series of ELISA determinations was performed, and the concentrations of subunits were calculated by comparison with the parallel competition curves obtained with pure C-, RI- and RII-subunits as standards (Table 2). In the unstimulated gland the C- and R-subunits were present in equal amounts, and most of the R-subunits were of the type II form (see also [17]). In the stimulated tissue, the specific concentration ($\mu\text{mol/g}$ of protein) of the catalytic subunit C was decreased to about one-third of that in the control glands. This value was similar to that measured for the decrease in the cyclic AMP-dependent protein kinase activity (Table 1). No change could be observed in the amount of the regulatory subunit RII in seven independent experiments. The amount of the regulatory subunit RI showed a slight but statistically insignificant decrease in the stimulated tissue. Moreover, in five out of ten independent experiments no difference between

Table 2. Quantification of cyclic AMP-dependent protein kinase subunits in growth-stimulated and in control parotid glands

Rats were injected twice daily for 3.5 days with isoprenaline or saline (control). The animals were killed 16–17 h after the last injections, and the C- and R-subunits were measured by ELISA as described in the Experimental section. Absolute concentrations were calculated by comparison of the competition curves obtained with pure standard solutions of C- or R-subunits. Values are means $\pm$ S.D. for *n* independent experiments. In each experiment, duplicate determinations were made of the competition of six or seven dilutions of parotid extracts (900 *g* supernatant) prepared from three stimulated or unstimulated rats for antibody binding to pure subunits adsorbed to the microtitre-plate surface.

Subunit	<i>n</i>	Protein kinase subunit concn.		
		$(\mu\text{mol/kg of protein})$		Isoprenaline
		Control	Isoprenaline	Control
C	9	2.43 ± 0.32	0.85 ± 0.09	0.35
RI	10	0.86 ± 0.11	0.65 ± 0.08	0.76
RII	7	1.88 ± 0.33	1.88 ± 0.33	1.00

control and stimulated tissue could be measured. We assume, therefore, that, in those five experiments where a decrease of RI-subunit occurred, this had resulted from a degradation during the experimental procedure and does not represent a physiological regulation. The presence of various proteinase inhibitors (see the Experimental section) may not fully exclude a proteolytic breakdown of RI-subunit, especially in view of the fact

that the stimulated samples contained a decreased amount of C-subunit. A protection of R-subunits against proteolytic breakdown by holoenzyme formation has indeed been described [3]. Furthermore, S49 lymphoma

Fig. 2. Cyclic AMP binding in growth-stimulated parotid glands

Supernatants (900 g, 10 min) were prepared from homogenates of the parotid glands of either three isoprenaline-treated (○) or three saline-treated (●) rats, and a sample (0.2 mg of protein) was incubated in 50 mM-potassium phosphate buffer/10 mM-EDTA (pH 7.0)/2 mM-theophylline/0.1% bovine serum albumin and cyclic [3 H]AMP at the concentrations indicated. Bound cyclic [3 H]AMP was determined by the $(\text{NH}_4)_2\text{SO}_4$ -precipitation method. Values are means from three experiments.

mutants lacking functional C-subunit have a RI-subunit that is about 10 times more labile than in wild-type cells [37]. Our explanation is further supported by the fact that the direct determination of the total cyclic [3 H]AMP-binding capacity in freshly prepared parotid extracts revealed no difference in the binding activities of control and stimulated parotid glands (Fig. 2).

Therefore the decrease in cyclic AMP-dependent protein kinase activity observed after injection of isoprenaline apparently was due to a selective decrease in the concentration of catalytic subunit of cyclic AMP-dependent protein kinases in the growth-stimulated gland, to about one-third of the control value.

Concentration of cyclic AMP-dependent protein kinase subunits at different times after a single injection of isoprenaline

We have also examined the concentration of the protein kinase subunits during the period of stimulation of DNA synthesis caused by a single injection of isoprenaline. The subunits were determined by enzyme immunoassay at 13 h (time of a cyclic AMP peak related to DNA synthesis [20]) and at 17 h after injection as an intermediate time point.

At no time point did any significant change occur in the amount of the regulatory subunits RI or RII (Fig. 3). On the other hand, the amount of the catalytic subunit again was decreased. After the single injection it was decreased to about 50% of the control value. However, under these conditions the decrease was observed only at 17 h, but not at 13 h, where the second increase in cyclic AMP is maximal ([20] and Fig. 3, inset) or at the time of

Fig. 3. ELISA determination of cyclic AMP-dependent protein kinase subunits in rat parotid glands at different times after a single injection of isoprenaline

Parotid-gland supernatants (900 g, 10 min) were prepared from rats killed either 17 h after intraperitoneal injection of saline (●) or 13 h (△), 17 h (○) and 24 h (▲) after intraperitoneal injection of isoprenaline (2.5 mg/100 g body wt.). ELISA determinations were performed as described in the legend of Fig. 1 and in the Experimental section. The values represent means from two experiments. In each experiment duplicate determinations were made with five or six concentrations of the parotid extracts pooled from three animals. Inset: concn. of cyclic AMP as percentage of the control value (6.58 pmol/mg of protein). Determinations were made in duplicate in the homogenates from the pooled parotid glands.

Fig. 4. ELISA determination of the C-subunit in the 900 g particulate fraction from growth-stimulated and from control parotid glands

Experiments were performed under the same conditions as described in the legend of Fig. 1, but with the particulate fraction from the centrifuged (900 g, 10 min) homogenates of control glands (●) and isoprenaline-stimulated glands (○). Mean values from two experiments are shown.

maximal DNA synthesis ([20]; results not shown) at 24 h. As in the long-term-stimulated parotid glands, a similar result to that in the 900 g supernatant was obtained with the 100 000 g supernatant (results not shown).

Possible mechanisms for the decrease in catalytic subunit in the growth-stimulated parotid gland

A repeatedly described phenomenon in cyclic AMP-mediated cell regulation is a possible translocation of cyclic AMP-dependent protein kinase from the soluble to the nuclear fraction in stimulated cells [38]. To prove whether a translocation could have caused the observed decrease in C-subunit in the 900 g supernatant, the 900 g sediments derived from the same homogenates were also analysed for the amount of catalytic subunit. The amount of C-subunit measured in the particulate fraction was not different from the control either 17 h after a single injection of isoprenaline (results not shown) or after repeated stimulation with the catecholamine (Fig. 4). Therefore the decrease in C-subunit obtained in the extranuclear fraction of growth-stimulated parotid glands apparently was not due to any physiological or unphysiological [39] binding of C-subunit to the 900 g particulate fraction.

Another reason considered for the decrease in the activity (Table 1) and amount (Figs. 1 and 3) of catalytic subunit was an enhanced blockage of active or immunoreactive sites by the heat-stable inhibitor in the isoprenaline-stimulated glands. Catalytic subunit was therefore incubated with and without an amount of the heat-stable inhibitor which in a pilot experiment had been found to contain more than twice the activity necessary to inhibit the kinase activity totally. The samples were then treated as usual and an ELISA was performed. As

Fig. 5. Effect of the heat-stable inhibitor on the ELISA determination of the C-subunit

Pure C-subunit from bovine heart was incubated (15 min, 30 °C) in homogenization buffer with (○) or without (●) a concentration of the heat-stable inhibitor 2.5 times that necessary to suppress the enzymic activity of C-subunit completely. Then the samples were processed and determined in an ELISA procedure exactly as described for the tissue samples (see the Experimental section).

shown in Fig. 5, the competition curves were identical in the presence or in the absence of heat-stable inhibitor of cyclic AMP-dependent protein kinase. Therefore the decrease in the immunochemically determined amount of C-subunit in the growth-stimulated parotid glands was not due to interference by the heat-stable inhibitor.

A determination of C-subunit by an immunoblot procedure expanded and confirmed these results: the amount of detectable C-subunit was clearly decreased in the cytosol as well as in total homogenates from growth-stimulated parotid glands (Fig. 6). A comparison of the radioactivity incorporated into the C-subunit band revealed a decrease to about 40 % of the control value, which was in good agreement with the value calculated by ELISA (Table 2). Since the immunoblot procedure included a separation of cellular proteins by SDS/polyacrylamide-gel electrophoresis, a heat-stable inhibitor or any other competing protein should have been removed before the detection of the C-subunit. In addition, the initial immediate treatment of the samples with SDS at 95 °C should have further limited any unphysiological breakdown of the C-subunit during the experiment and should have freed the C-subunit from

Fig. 6. Immunoblot of the C-subunit in growth-stimulated and in control parotid glands

Parotid-gland homogenates and cytosols (100000 *g* supernatant, 60 min at 4 °C) were prepared from control rats and from rats which had been stimulated by repeated injection of isoprenaline (IPR). Samples of the material pooled from three rats (homogenate, 380 μ g of protein; cytosol, 720 μ g of protein) as well as 50 ng of pure bovine heart catalytic subunit (C) were boiled (95 °C, 2 min) in a buffer containing 0.2 mM-Tris/HCl, pH 6.7, 15% (v/v) glycerol, 6% (v/v) SDS and 10% (v/v) 2-mercaptoethanol, separated by electrophoresis on SDS-containing gels which were 8% in acrylamide, electroblotted on nitrocellulose paper, and the C-subunit was detected by incubation with anti-(catalytic subunit) serum and 125 I-Protein A, followed by autoradiography.

any intracellular membranes which possibly occluded it.

DISCUSSION

The specific immunochemical determination of C- and R-subunits of cyclic AMP-dependent protein kinases type I and II revealed that a decrease measured in the activity of cyclic AMP-dependent protein kinase in growth-stimulated parotid glands was due to a decrease in the amount of the catalytic subunit C. This decrease was not accompanied by a corresponding decrease in the amount of the regulatory subunit of either type RI or RII.

As one consequence, not all R-subunits can re-associate with C-subunits. The residual C-subunit will

not necessarily recombine to only one specific type of cyclic AMP-dependent protein kinase holoenzyme, since re-association of C-subunit with either the RI- or the RII-subunit can be regulated by additional factors [1–3]. This could possibly explain why the relative total activity of the type I cyclic AMP-dependent protein kinase holoenzyme has been found to be increased [8,9] as well as decreased [10–14] during cellular proliferation. Support for the assumption that an alteration of the C-subunit may be more critical in growth control than one of the type I or II holoenzyme comes also from investigations on several mutants of Chinese-hamster ovary cells [12,13]. All of these mutants were resistant to the inhibitory effect on growth that cyclic AMP normally has in these cells, and all had defects in the cyclic AMP-dependent protein kinase activity. Their type I and type II protein kinase holoenzymes, however, were differentially affected: either the type I enzyme [13] or the type II enzyme [12] was missing, or the activities of both types of enzyme appeared to be decreased [12].

The mechanism of the apparently independent regulation of C-subunit in the stimulated parotid needs to be clarified by further studies. The detection of a proteinase that specifically cleaves the free C-subunit [40] could suggest that, in the stimulated tissue, C-subunit is degraded by specific and regulated proteolysis. The physiological significance, however, of this proteinase, which until now has been found only in intestinal brush-border and in kidney membranes [40], is still unknown. Furthermore, extracts from growth-stimulated parotid glands did not change the activity or amount either of pure catalytic subunit or of C-subunit present in unstimulated parotid glands (U. Jung & G. Schwach, unpublished work). Another explanation for the observed decrease in C-subunit could be an impaired synthesis of C-subunit in the stimulated tissue. Normally, C- and R-subunits are present in equimolar concentrations in unstimulated cells [41] and may have a similar turnover rate in each type of cyclic AMP-dependent protein kinase [42]. In the isoprenaline-stimulated parotid gland, such a balance may have been upset by additional and so far unknown regulatory factors. Also, it cannot be excluded that the decrease in the activity and amount of the C-subunit is due to an as-yet unknown modification of the molecule in the stimulated cell that affects its active as well as its immunoreactive site(s).

An independent change in the amount of a single subunit of cyclic AMP-dependent protein kinases had been observed occasionally also previously. For example, a specific increase in the amount of RI-subunit in dibutyryl-cyclic-AMP-stimulated neuroblastoma-glioma cells [27], a specific increase of RII in hormone-stimulated granulosa cells [43] and, more recently, a specific decrease of the C-subunit in hormone-stimulated epithelial cells [44], have been reported. In all these cases, however, the effect occurred only under conditions of questionable biological significance, that exposed the cells to very high cyclic AMP concentrations for long periods. Correspondingly, a decrease in the total activity of the free C-subunit observed in human chorionadotropin-stimulated Leydig cells was seen only at hormone concentrations that led to an excessive accumulation of cyclic AMP [45]. Therefore, it seems of particular importance that we observed the decrease in C-subunit not only after repeated injection of isoprenaline, but also

at a specific time after a single injection of isoprenaline. Under the latter condition, parotid-gland cells isolated up to 36 h after the injection were found to be morphologically and functionally intact [46]. Moreover, in contrast with the findings cited above, the very late decrease in C-subunit at 17 h after the injection was accompanied by only a moderate rise in the concentration of intracellular cyclic AMP. Very high concentrations of cyclic AMP are measured only during the first minutes after the injection of isoprenaline [20]. During this early phase, however, total cyclic AMP-dependent protein kinase activity was either unchanged (J. Hofmann & G. Schwoch, unpublished work) or tended to increase [47].

The marked increase in cyclic AMP measured directly after injection stimulates exocytosis, but is not needed for the stimulation of DNA synthesis [20]. On the other hand, the small second increase in cyclic AMP which occurs about 13 h after stimulation seems to be essential for proliferation in the parotid, as in hepatocytes and lymphocytes (for review see [48]). Therefore, it seems reasonable to assume that the specific decrease in the amount of the catalytic subunit of cyclic AMP-dependent protein kinase obtained in the time between the late small increase in cyclic AMP and maximal DNA synthesis may be involved in the events which finally lead to cell proliferation.

I thank Dr. S. M. Lohmann and Dr. U. Walter for a generous gift of RI and RII antisera and for help in the immunoblot experiment, Mrs. U. Jung and Ms. C. Bode for skilful technical assistance, Professor H. D. Söling for helpful discussions, and the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie for financial support.

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