Quantitative changes in the catalytic and regulatory subunits of nuclear cAMP-dependent protein kinases type I and type II during isoproterenol-induced growth of the rat parotid gland

Gerhild Schwoch and Astrid Freimann

Abt. Klin. Biochemie, Universitätskliniken Göttingen, Humboldtallee 11, D-3400 Göttingen, FRG

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To quantify the cAMP-dependent protein kinases I and II in parotid gland nuclei independent of the enzyme activity, monospecific antisera against their subunits were applied in a sensitive enzyme immunoassay. About 3% of total catalytic subunit in the homogenate was found in the isolated nuclei. During β-agonist-induced proliferation of the parotid gland the nuclear concentration of catalytic and regulatory subunits changed. Related to the number of nuclei, the catalytic subunit and the regulatory subunit RI increased about 3-fold whereas the regulatory subunit RII remained unchanged.

Cell proliferation *cyclic AMP dependence* *Protein kinase* *(Parotid gland nucleus)*

1. INTRODUCTION

It is well established that cAMP is involved in the regulation of cellular growth (review [2]). Our understanding of the mechanism by which cAMP participates in cell proliferation, however, is still very limited. One possibility to convert an increase in the intracellular level of cAMP into a specific growth-related response could lie in the occurrence of two types of cAMP-dependent protein kinases. The two isozymes, composed of two different regulatory subunits, RI and RII, and of an apparently identical catalytic subunit C, have been reported to become differentially activated by cAMP [3,4] and to vary in their total cellular activities under different experimental and physiological conditions [5–9]. It has been suggested that the type I kinase may be a positive effector of growth whereas the type II kinase may be important in growth inhibition and cellular differentiation (cf. [5–7]). Other data, however, were not compatible with such an assumption [8–10]. For example, in the case of the in vivo induced proliferation of rat salivary gland by isoproterenol, no relative increase of type I protein kinase could be observed. In contrast, the activity of protein kinase type I became reduced to about 80% of the control value by isoproterenol treatment [8].

As in most other studies, this result was obtained with crude cellular supernatants from parotid homogenates. For specific regulation, however, one would expect a specific alteration of the cAMP-dependent protein kinases at specific intracellular sites. However, since in most eukaryotic tissues the majority of the kinases are soluble and measurements of their activities in the particulate fractions suffer from extraction problems as well as from the presence of cAMP-independent protein kinases or other interfering components, reports on site-specific changes are scarce. Alternative methods, e.g. immunological determinations, have rarely been applied as antibodies against the C-subunit of the cAMP-dependent pro-

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tein kinase have become available only recently [11,12].

Using antibodies we have shown [13] in experiments with rat parotid glands that measurement of the enzymatic activity alone will considerably underestimate the amount of C-subunit in the particulate fraction. Here, we have determined the catalytic and regulatory subunits of cAMP-dependent protein kinase in isolated nuclei of the parotid gland by an enzyme-linked immunosorbent assay (ELISA). In an attempt to follow the single steps leading to a cAMP-mediated control of proliferation, the concentrations of C, RI and RII were compared in nuclei isolated from parotid glands following stimulation of proliferation by repetitive injections of isoproterenol. The results show that, in contrast to the situation in the soluble compartment of the cells, the amount of C- and RI-subunits per nucleus increases during stimulation of growth whereas that of RII remains unchanged.

2. MATERIALS AND METHODS

Male Wistar rats (180–200 g) were used. For stimulation, the rats were injected twice daily for 3.5 days with either isoproterenol (2.5 mg/100 g body wt, dissolved in saline, Sigma, St. Louis, MO) or saline (controls). About 17 h after the last injection, animals were anesthetized with nembutal (90 mg/kg), killed by heart incision and exsanguinated. The parotid glands were excised and cleaned from lymph nodes, connective and adipose tissue under a stereomicroscope. They were homogenized with 6 vols of 0.25 M sucrose in buffer A (10 mM imidazole, 4 mM $MgCl_2$, 150 mM NaCl, 1 mM dithioerythritol, 0.5 mM PMSF, pH 7.4) in a glass-teflon homogenizer. The homogenate was filtered through 4 layers of cheese-cloth, spun at $70 \times g_{max} \cdot$ min and the pellet discarded. For the preparation of nuclei, the supernatant was centrifuged for 10 min at $180 \times g_{max}$ and the pellet collected. This step was repeated twice. After resuspension in the homogenization buffer the combined pellets were underlayered with 0.8 vol. buffer A containing 2.15 M sucrose and centrifuged for 1 h at $105\,000 \times g_{max}$. The pellet obtained was resuspended in buffer A containing 0.32 M sucrose, spun for 10 min at $1000 \times g_{max}$ and the resulting pellet designated 'nuclei'. The number of nuclei was determined in a Neubauer hemocytometer (Hecht, Sonderheim, FRG) by counting at least 1000–2000 nuclei.

Protein was determined by a modified Lowry procedure [14] and DNA according to [15]. The activity of α-amylase was measured according to [16].

Monospecific antisera against bovine heart catalytic subunit [11,17] and rat heart regulatory subunits [18] were used in an ELISA method as described [17].

3. RESULTS AND DISCUSSION

3.1. *Isolation of nuclei*

In a previous investigation [13] we found about 14% of the catalytic subunit of rat parotid cAMP-dependent protein kinase in the $900 \times g$ nuclear

Table 1

Purification of rat parotid nuclei

	Homogenate	Particulate fraction	Nuclei, purified on sucrose		
			1.8 M	1.95 M	2.15 M
Protein (mg)	87.08 (100)	9.31 (10.7)	2.63 (3.0)	1.47 (1.7)	1.06 (1.2)
DNA (mg)	1.98 (100)	0.91 (46.0)	0.72 (36.6)	0.68 (34.4)	0.53 (26.7)
Amylase (U)	15 840 (100)	1680 (10.6)	200 (1.3)	43 (0.27)	23 (0.15)
Protein/DNA	44	10.23	3.63	2.16	2.00

A crude nuclear particulate fraction was prepared as described in section 2, resuspended and layered on top of a sucrose cushion containing either 1.8, 1.95 or 2.15 M sucrose. The nuclei were sedimented by centrifugation for 1 h at $105\,000 \times g$, washed, resuspended and analyzed for protein, DNA and α-amylase as described in section 2. The data give the amount obtained from 1 g wet wt of parotid gland as the starting material. Numbers in parentheses represent the percentage of the values measured in the filtered homogenate

pellet. The major part of the enzyme was present in other particulate structures of the cell. As secretory granules tend to sediment similarly to the nuclei, very pure nuclei had to be prepared for an analysis of nuclear cAMP-dependent protein kinases.

Table 1 gives an example for the properties of parotid nuclei isolated by centrifugation of an initial crude nuclear pellet through sucrose of different density. For all subsequent experiments the centrifugation through 2.15 M sucrose was chosen. Under these conditions the yield of nuclei as indicated by the DNA concentration was not essentially less than with the lower sucrose concentrations but, as indicated by the activity of amylase, contamination by secretory granules and cytosol – which contains the bulk of the total amylase activity in the homogenate – becomes negligible. Also indicative of a 'good' nuclear preparation is the protein:DNA ratio of 2 in this preparation [19].

3.2. *cAMP-dependent protein kinase in the parotid nuclei*

When the protein kinase activity was measured in nuclei separated through increasing sucrose concentrations, the fraction of the activity which could be inhibited by the heat-stable inhibitor of cAMP-dependent protein kinase became increasingly smaller and was hardly detectable in the most pure nuclei (Hoffman and Schwoch, unpublished). This indicates, that measurement of any nuclear cAMP-dependent protein kinase activity will require the application of special extraction procedures.

For the immunochemical determination by ELISA, extraction of the nuclear protein kinase subunits was ensured by pretreatment of the samples with sonication in the presence of 6 M urea. These conditions had previously been found to increase the sensitivity of determination of C-subunit in ELISA [17] and to remove any interfering cAMP bound to the R-subunits [18].

By comparing the competition curves produced by pretreated nuclear extracts in ELISA with the parallel competition curves produced by equally pretreated standard solutions of pure catalytic subunit, the absolute concentration of the protein kinase catalytic subunit could be determined (table 2).

The specific concentration of C-subunit in the isolated nuclei was found to be the same as that in the total homogenate and about 150% of that in the soluble cell fraction. With the protein concentration measured and a yield of nuclei of 27% (table 1), it was calculated that about 3% of the total catalytic subunit in the homogenate was present in the nuclei.

Table 2

Concentration of the catalytic subunit of cAMP-dependent protein kinase in purified rat parotid nuclei and in the crude homogenate and cytosol

	Protein (mg/g wet wt)	Catalytic subunit (pmol/mg protein)
Homogenate	100.03 ± 17.61	2.45 ± 0.35
Cytosol	61.72 ± 15.18	1.66 ± 0.05
Nuclei	3.13 ± 0.26	2.47 ± 0.37

Values represent means ± SD from 3 experiments. In each experiment the concentration of the catalytic subunit C was determined by ELISA [17] from the parallel competition curves produced with 6–8 dilutions of parotid extracts or of pure bovine heart catalytic subunit as a standard. Each point of the curves was determined in duplicate. Cytosol is defined as the supernatant after centrifugation of the homogenate for 1 h at 100000 × *g*. The nuclear protein was calculated based on a 27% yield of nuclei in the preparation used (see table 1)

To our knowledge, this is the first quantitation of catalytic subunit in isolated nuclei by a method which does not rely on the determination of its catalytic activity. The data, therefore, cannot be directly compared with those obtained from other tissues. They agree insofar with data from activity measurements in different tissues [20] as only a small part of the total cellular concentration was found in the parotid nuclei. The relatively high specific concentration found in the nucleus by the immunoassay, however, may suggest a significant role of the catalytic subunit in the regulation of nuclear events even under unstimulated, basal physiological conditions.

3.3. *cAMP-dependent protein kinase subunits in the nuclei of the growth-stimulated parotid gland*

Using specific antisera against the regulatory subunits RI and RII [18] in ELISA performed similarly to the determination of the C-subunit,

both RI- and RII-subunits could be detected in the parotid nuclei. This is in accordance with a recent report [21] that both R-subunits could be detected in a parotid nuclear fraction by an affinity labelling procedure. The competition curves obtained in a typical ELISA experiment for the determination of the catalytic as well as of the regulatory subunits of cAMP-dependent protein kinase in nuclei of control glands and of glands which had been stimulated to growth by isoproterenol are shown in fig.1.

In the case of the C- and RI-subunits, significantly less nuclei from isoproterenol-treated glands than from control glands were necessary to achieve the same inhibition of antibody binding to the antigen fixed on the plastic surface of the microtiter plate. In the case of the RII-subunit no difference between the stimulated and unstimulated nuclei could be detected. The results indicate a selective increase of the cAMP-dependent protein kinase type I in the nuclei of growth-stimulated parotid glands.

This phenomenon was quantified in a series of 4 independent experiments. The results are summarized in table 3.

The nuclei isolated from the isoproterenol-treated rats contain about 3-times as much C and RI as nuclei isolated from control rats. The nuclear concentration of RII is not different in the control and stimulated tissue. Stimulation of growth of the salivary gland by isoproterenol leads to the formation of enlarged, polyploid nuclei [22]. Therefore, another situation occurs when the competition produced by the subunits in ELISA is related not to the number of nuclei introduced into the immunoassay, but to the amount of nuclear protein or DNA. Then, the concentration of C and RI is unchanged by isoproterenol treatment, but RII is decreased to about 40% of the original value (table 3).

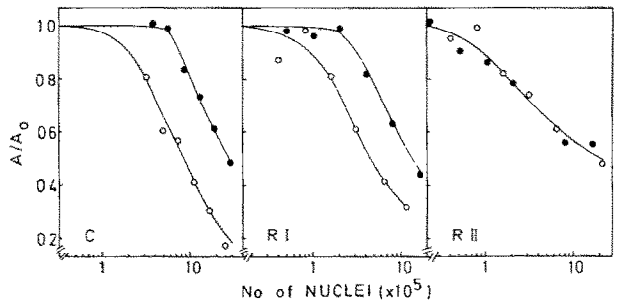

Fig.1. ELISA of the catalytic (C) and regulatory (RI and RII) subunits of cAMP-dependent protein kinase in nuclei isolated from parotid glands whose growth had been stimulated by isoproterenol (○) or from control glands (●). The nuclei were incubated for 30 min at 30°C, sonicated (3× for 5 s) and introduced in an ELISA as described before [13,17].

Our results show that repetitive stimulation with isoproterenol leads in rat parotid gland to different changes in cAMP-dependent protein kinases in different cellular compartments. While measurement of the catalytic activity of protein kinases type I and II separated from the soluble cell fraction on DEAE-cellulose revealed 80% reduction in type I activity ([8] and unpublished), the amounts of the C- and RI-subunits in the isolated nuclei increase without an accompanying change in the amount of RII-subunit. Whether the alterations observed are caused by intracellular translocation of subunits [20] or differences in the turnover of C and RI relative to RII cannot be decided at present. The same holds for the question; is it the increase in C and RI related to the number of nuclei or the decrease in RII related to nuclear protein which is of significance for the isoproterenol-induced proliferation processes? A recent report [23] that the regulatory subunit RII possesses topoisomerase activity may stress the latter. Independently of these problems, parallel quantitation of the intracellular concentration of all protein kinase subunits may also serve in understanding a step in the cAMP-mediated regulation of different processes in one subcellular compartment. By an immunocytochemical method, the regenerative response of rat liver after partial hepatectomy was found to be accompanied by an increase in the nuclear staining of C and RI but not of RII [24]. This would be in accordance with the finding reported here for proliferating parotid gland tissue. In glucagon-stimulated liver, however, an increase in staining of nuclear subunits was observed only for the C-subunit of cAMP-dependent protein kinase [25]. Therefore, it seems possible that cAMP regulation of enzyme induction might be connected with a change of only the catalytic subunit concentration in the nucleus, while cAMP-mediated regulation of cell proliferation may require also changes in the local amount of the RI-subunit.

Table 3

Catalytic (C) and regulatory (RI, RII) subunits of cAMP-dependent protein kinase in the nuclei of parotid glands stimulated by isoproterenol

	cAMP-dependent protein kinase subunit concentration		
	C	RI	RII
(pmol/10^8 nuclei)			
Control	2.62 ± 0.54	2.02 ± 0.16	-*
Isoproterenol	7.62 ± 1.67	5.92 ± 0.49	-*
Isoproterenol/control	2.91 ± 0.24	2.93 ± 0.32	1.03 ± 0.10
(pmol/mg DNA)			
Control	4.74 ± 0.71	3.70 ± 0.52	-*
Isoproterenol	5.76 ± 0.56	3.08 ± 0.46	-*
Isoproterenol/control	1.21 ± 0.33	0.83 ± 0.23	0.39 ± 0.06
(pmol/mg protein)			
Control	2.47 ± 0.37	1.93 ± 0.27	-*
Isoproterenol	2.99 ± 0.29	1.60 ± 0.24	-*
Isoproterenol/control	1.21 ± 0.33	0.83 ± 0.23	0.39 ± 0.06

* For the RII-subunit no absolute values are given, as the available standard of purified rat heart RII tended to unstable reactions in the ELISA

Values are calculated as means ± SD from 4 experiments. In each experiment 5–8 rats were injected with either saline (controls) or isoproterenol. The parotid nuclei were isolated, counted and analyzed for DNA, protein and kinase subunits by ELISA as described in section 2. Absolute values were calculated by comparison of the competition curves obtained with the nuclear material in the ELISA with the parallel curves obtained with standard solutions of pure C or RI. The relative values (isoproterenol/control) were calculated by comparison of the curves obtained with isoproterenol-stimulated nuclei with those obtained with nuclei from the saline-injected control animals

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