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PROTEIN-BOUND cAMP, TOTAL cAMP, AND PROTEIN KINASE ACTIVATION IN ISOLATED BOVINE THYROCYTES

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SUMMARY

Thyrocytes isolated from bovine thyroid tissue were subjected to thyrotropin treatment and analyzed for total cAMP, protein bound cAMP and protein kinase activation. The concentration of thyrotropin (~4 mU/ml) required for half-maximal elevation of total cAMP was 20 times greater than the concentration required for half-maximal elevation of protein kinase activity and bound cAMP levels (~0.2 mU thyrotropin/ml). In thyrocytes, bound cAMP and protein kinase activation showed a linear, one to one relationship indicating that bound cAMP obtained with the charcoal procedure is largely or completely identical with R·cAMP.

INTRODUCTION

In several steroid forming tissues large discrepancies between cAMP concentration and hormone response have been observed (cf. 1-3) leading some investigators to postulate a cAMP independent stimulation of steroidogenesis by low concentrations of the trophic hormones (1,2). However, such an interpretation has been questioned since only small elevations of cAMP were required to fully stimulate protein kinase and steroid formation in mouse Leydig cells (4). Also, half-maximal activation of protein kinase in the liver of glucagon-treated rats was reached when basal cAMP levels were only doubled (5). Activation of protein kinases by cAMP can be described by the equation (6)

$$(I) \qquad R_2 \cdot C_2 + 2\ cAMP \rightleftharpoons R_2 \cdot cAMP_2 + 2\ C$$

This equation suggests that 'bound cAMP' is a direct measure of the amount of active catalytic subunit. Dependence of protein kinase activation on total cAMP on the other hand should be non-linear since there is only a limited number of binding sites (R molecules) in a given tissue. Therefore, bound cAMP should be a better indicator of protein kinase activation than total cAMP. Attempts to measure bound cAMP by indirect (7) and direct (8) methods have been recently reported. A simple charcoal procedure has been used in this laboratory to evaluate protein bound cAMP (9, 5). This paper describes the application of the charcoal

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procedure to the analysis of bound cAMP in isolated thyrocytes and its correlation with total cAMP, and with protein kinase activation under conditions of TSH stimulation.

MATERIALS AND METHODS

Cell dispersion and incubation: The preparation of bovine thyrocytes was similar to the dispersion technique developed for the isolation of human thyrocytes (10) with the following modifications: The chopped tissue was washed only once with buffer (10 min, 4°); 1 mM $CaCl_2$ was added to the collagenase incubation mixture; the viocase incubation was performed for 20 - 30 min and remaining tissue fragments were reincubated twice; minimal essential medium with Earle's salts (11) was usually supplemented with 20 mM HEPES pH 7.3 (24°), 1.5% inactivated new born calf serum, streptomycin sulfate (50 mg/ml), and penicillin (10^5 u/l). The washed cells (10^6 cells/ml) were slowly stirred overnight (15 - 18 h, 37°) in medium + 10% inactivated serum. After incubation the cells were filtered through two layers of perlon gauze, sedimented (550 x g, 8 min), and resuspended in cold medium. The yield was about $2 \cdot 10^7$ cells from one gm of tissue. 2×10^6 cells were routinely incubated in a final volume of 400 µl medium containing 0.5 mM MIX ± TSH (organon) at 37° in a shaking water bath. The incubation was stopped by the addition of 1 ml cold medium and centrifugation for 50 sec (2300 x g, 4°). The cell pellet was either extracted with 400 µl 5% trichloroacetic acid for the determination of intracellular cAMP, or rapidly frozen (ethanol / dry ice) and stored at -80° until use for assay of protein kinase activity and protein bound cAMP.

Homogenates: 4×10^6 cells were sonicated for 5 sec without foaming in 1 ml ice cold 20 mM HEPES buffer pH 7.0 containing 20 mM $MgCl_2$, 0.5 mM MIX, 0.3 mM EGTA, and 0.1 M NaCl. Charcoal supernatant: Sonication was performed in 'HEPES buffer' (see above) containing 1% charcoal and 0.1% bovine serum albumin. The mixture was left on ice for 6 min, and centrifuged (14000 x g, 5 min). Aliquots of the resulting supernatant were immediately analyzed for protein kinase activity, (^{3}H)cAMP binding, and protein bound cAMP. Free labeled cAMP added after sonication was completely (> 99.9%) removed by the charcoal treatment.

Protein kinase activity was routinely measured for 5 min at 30° in 200 µl incubation mixture containing 20 mM HEPES pH 7.0, 10 mM $MgCl_2$, 0.5 mM MIX, 0.3 mM EGTA, 50 µM (^{32}P)ATP (1 µCi), 0.5 mg histone/ml and 1 µM cAMP. The reaction was started by the addition of 20 µl enzyme solution (10 - 20 µg protein) and stopped by spotting 120 µl of the mixture on paper discs (2 x 3 cm Whatman ET 31) soaked previously with 5 mM ATP and dried. This procedure led to a high reproducibility and low blanks. Washing in trichloroacetic acid solutions was performed according to (12). The dried paper discs were counted in 15 ml dioxane based scintillation cocktail.
Protein kinase activation is expressed as the activity ratio (activity in the absence to that in the presence of 1 µM cAMP). Kinase activity was linear for 10 min, and showed a linear dependence on the amount of extract up to 30 µg protein.

cAMP was determined acc. to Steiner et al. (13).

Protein bound cAMP: Aliquots of the charcoal supernatant (see above) were extracted with trichloroacetic acid and analyzed for cAMP. Control experiments revealed that this fraction could be degraded completely by phosphodiesterase. Although there was a time-dependent decrease in bound cAMP in the presence of

ABBREVIATIONS: TSH = thyroid stimulating hormone; MIX = 1-methyl-3-isobutyl xanthine; hCG = human chorionic gonadotropin

charcoal, changes between 2 and 6 min after homogenization were less than 6% of total. (^{3}H)cAMP binding: 100 µl of the charcoal supernatant were added to 50 µl HEPES buffer containing 14 pmol (^{3}H)cAMP (5×10^5 cpm), and incubated for 30 min at 0°. (^{3}H)cAMP bound to protein was collected on Millipore filters (0.45 µm) according to (14). Blanks were run with supernatants obtained from charcoal suspension sonicated without cells. No corrections were made for exchange between bound and free cAMP.

RESULTS

Determination of protein kinase activation and bound cAMP levels in TSH-treated thyrocytes: Bovine thyroid tissue contains cAMP dependent protein kinase type I and type II in comparable amounts (15, 16). The analysis of the protein kinase activation status, therefore, poses special problems since protein kinase II subunits tend to reassociate rapidly on diluting the tissue by homogenization. Although the reassociation of protein kinase II can largely be prevented by high salt, protein kinase I holoenzyme will be partially dissociated under the same conditions (17, 18). When thyrocytes stimulated with different TSH doses were homogenized with increasing NaCl and / or $MgCl_2$ concentrations, optimal preservation of the activation status was observed at 0.1 M NaCl - 0.02 M $MgCl_2$. Under these conditions highest kinase activity ratios obtained were 0.90 - 0.95 while basal values rose from 0.15 to 0.25 (from 0.25 to 0.35 in the presence of 0.5 mM MIX).

As indicated by equation (I), bound cAMP is closely correlated with the dissociation and reassociation of protein kinase subunits. It could be assumed, therefore, that the optimal homogenization conditions stated above were also optimal for the determination of bound cAMP. In order to allow determination of protein kinase activation and R·cAMP formation in the same extract, thyrocytes were sonicated with buffered salt solution, and in the presence of charcoal. From the supernatant obtained by centrifugation, bound cAMP levels as well as protein kinase activation were determined. Control experiments showed that 85 - 90% of bound cAMP and of protein kinase activity were in the soluble fraction (cf. 16).

Bound cAMP as a direct measure of R·cAMP and of protein kinase activation: When isolated thyrocytes were incubated with a saturating dose of TSH in the presence of 0.5 mM MIX, they responded with a continuous increase in total cAMP (fig. 1). Rather different kinetics were observed for the activation of protein kinase which reached maximal levels within 2 - 5 min after hormone addition, the activity ratio remaining at a value of 0.9 throughout the rest of the incubation period. When cAMP bound to high affinity receptors was analyzed by the charcoal method, a close correlation with protein kinase activation was seen. Evidently,

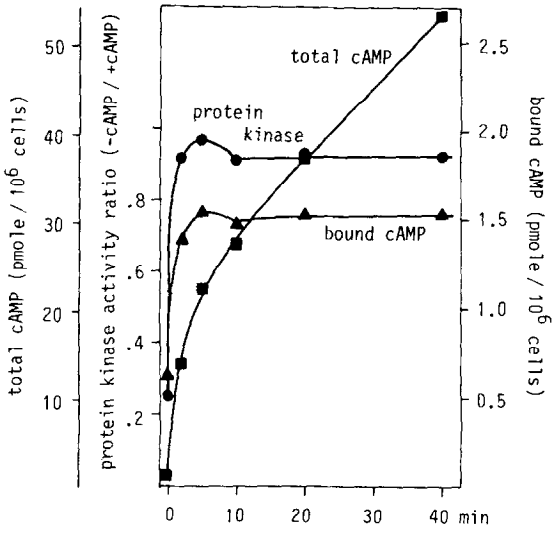

Figure 1. Kinetic analysis of TSH induced increase in cAMP, bound cAMP, and protein kinase activation. - 2 x 10^6 bovine thyrocytes were incubated with 50 mU TSH/ml in the presence of 0.5 mM MIX under conditions described in methods. Total cAMP was obtained by trichloroacetic acid extraction of cells and medium. Each point represents the mean value of triplicate determinations. Control incubations without TSH (+ 0.5 mM MIX) showed a slight increase of all three parameters. Control values at 40 min were: total cAMP = 1.8 pmol/10^6 cells; protein kinase activity ratio = 0.35; bound cAMP = 0.68 pmol/10^6 cells.

bound cAMP reflects protein kinase activation better than total cAMP. Bound cAMP levels and protein kinase activation showed also a parallel dependence on TSH concentration (fig. 2). In this experiment, bound cAMP, protein kinase activation and (^{3}H)cAMP binding were determined from the same charcoal supernatant, and compared with total intracellular cAMP. Intracellular cAMP reached half-maximal values at 3 - 5 mU TSH / ml. However, a much lower dose of TSH (0.1 - 0.2 mU/ml) was required to effect half-maximal activation of protein kinases. The same low hormone dose was also sufficient to bring about half-maximal levels of bound cAMP indicating a close correlation between bound cAMP as obtained with the charcoal procedure, and R·cAMP. This interpretation is further supported by the analysis of (^{3}H)cAMP binding (fig. 2). A half-maximal value for residual binding sites was found at similar TSH concentrations as required for half-maximal levels of bound cAMP (= half-maximal occupancy of binding sites). Fig. 2 also shows the limitations of the (^{3}H)cAMP titration procedure: Although titration was performed in the charcoal supernatant which eliminated interference of free cAMP by isotope dilution, zero levels of apparent residual binding sites (R·C) were not approached in spite of nearly complete activation

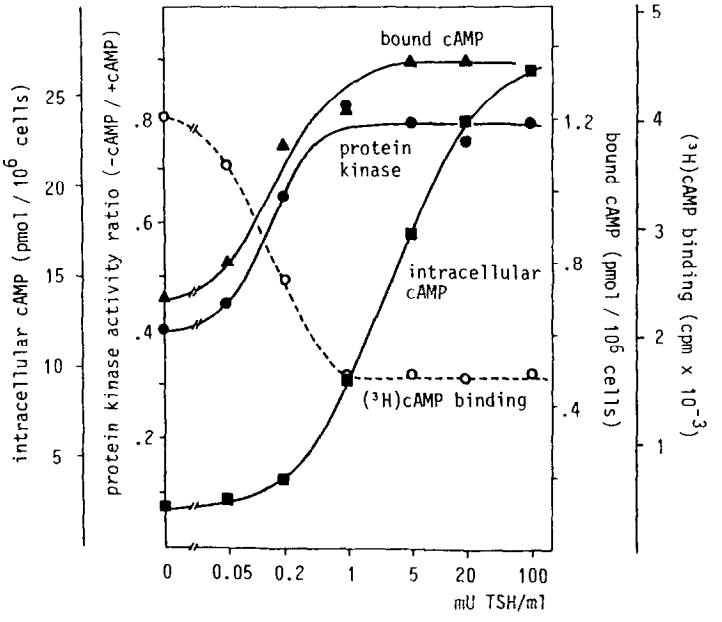

Figure 2. Formation of intracellular cAMP and of bound cAMP, protein kinase activation and (^{3}H)cAMP binding in bovine thyroid cells in response to different TSH concentrations. - Incubations were performed for 20 min at 37° in the presence of 0.5 mM MIX. All parameters were analyzed from the same charcoal supernatant except intracellular cAMP (see methods). Mean values from triplicate determinations. Total soluble protein kinase activity (determined in the presence of 1 µM cAMP) remained constant at all TSH concentrations.

of protein kinases. The high residual binding at hormone saturation presumably is the consequence of an exchange between free (^{3}H)cAMP and R·cAMP, which has been shown to occur in isolated R proteins without significant loss in total bound cAMP (Weber, unpublished experiments).

The data obtained from fig. 2 were used to show the dependence of protein kinase activation as well as of bound cAMP formation on intracellular cAMP (fig. 3A). These saturation type curves apparently reflect 'titration' of R·C molecules by a TSH induced rise in intracellular cAMP with the concomitant formation of R·cAMP and active catalytic subunits C. Half-maximal values of both parameters were obtained at about 2.2 pmol cAMP / 10^6 cells. A rather small increase in intracellular cAMP from basal values (1.15 $\pm$ 0.18 pmol / 10^6 cells) was therefore sufficient to bring about half-maximal activation of protein kinase. The similarity of the 'titration' curves for bound cAMP and for protein kinase activation pointed to a close correlation of bound cAMP with R·cAMP. Indeed, when protein kinase activation was plotted against bound cAMP, a linear one to one relation-

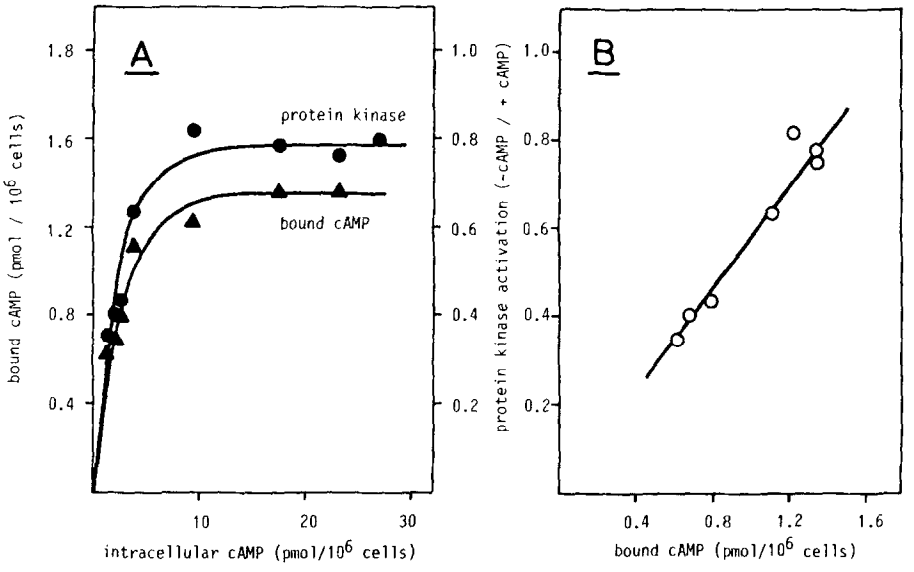

Figure 3. A) Dependency of bound cAMP and protein kinase activation from intracellular cAMP. - B) Correlation of protein kinase activation with bound cAMP. - The values were derived from the experiment presented in figure 2, and from control samples incubated without MIX.

ship was seen (fig. 3B) indicating identity or near identity of R·cAMP and bound cAMP as determined by the charcoal procedure. Extrapolation to the activity ratio 1.0 indicated a maximal level of 1.7 pmol bound cAMP ($\approx$R·cAMP) / 10^6 thyrocytes which, at the same time, is a measure for the concentration of total R molecules.

DISCUSSION

This study describes the correlation of protein-bound cAMP levels and protein kinase activation in hormone stimulated thyrocytes. The presence of protein kinase I and II in bovine thyrocytes required homogenization of the stimulated cells at intermediate salt concentrations in order to preserve the activation status (17, 18). These salt concentrations represent a compromise largely preventing reassociation of protein kinase II subunits and avoiding to a greater extent dissociation of protein kinase I.

A second problem arose from the poorly understood phenomenon that *in vitro* much smaller concentrations of cAMP are effective in activating protein kinase than the basal levels found *in vivo* (18, 19). The capacity of fully stimulated cells to raise cAMP levels appears to be far in excess of what is needed for the complete activation of protein kinases. Since the use of phosphodiesterase inhibi-

tors leads to additional increases in cAMP, artefactual activation of protein kinases by cAMP during homogenization is to be expected especially in mixed cell populations, in which only one species of cells responds to the added hormone. Artificial activation of this kind can be avoided by homogenizing the tissue in the presence of charcoal. When the charcoal procedure was performed in the presence of salt, both protein kinase activation and the level of bound cAMP could be determined from the same charcoal supernatant. Under these conditions, a linear correlation between the two parameters was obtained indicating identity (or near identity) of bound cAMP with R·cAMP.

The data allowed also an estimation of R molecules in thyrocytes. Assuming binding of 1 mol cAMP per mol R (6), a value of 1.7 pmol R / 10^6 cells can be deduced. This is somewhat higher than basal cAMP levels and higher than indirect estimates of cAMP binding sites using (^{3}H)cAMP binding without corrections for isotope dilution and exchange reaction (18, 20).

Bound cAMP ($\approx$ R·cAMP) as the biologically active form of the cyclic nucleotide comprised less than one half of total cAMP in non-stimulated cells. By contrast to total cAMP, it was a direct measure of protein kinase activation, and small elevations of total cAMP (from basal 1.15 pmol to 2.2 pmol / 10^6 cells) were sufficient to bring it to half-maximal levels with a concomitant half-maximal activation of protein kinase. TSH concentrations leading to half-maximal activation of protein kinase were 20 times lower than those required for half-maximal accumulation of total cAMP, which is similar to the situation in Leydig cells (1, 4), Barely visible changes in total cAMP, therefore, can effect significant activation of protein kinases and significant stimulation of the ultimate hormonal response (cf. 4). Apparent discrepancies in hormone-stimulated steroidogenesis (1 - 3) may thus be explained.

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Addendum: During the preparation of the manuscript, a paper by the group of Catt appeared (21), in which occupancy of cAMP receptors was determined in hCG stimulated Leydig cells. In these cells, too, cAMP receptor occupancy correlated much better with protein kinase activation and steroidogenesis than total cAMP (cf. also (4)).

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