TSH REGULATION OF cAMP-DEPENDENT PROTEIN KINASE ACTIVITY IN THE THYROID

Stephen W. Spaulding and Gerard N. Burrow

Yale University School of Medicine
and West Haven V.A. Hospital
New Haven, Connecticut 06510

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Summary - TSH-regulated activation of cAMP-dependent protein kinase has been demonstrated in calf thyroid slices. Despite the apparently high basal levels of cAMP in the thyroid, small changes in cAMP concentration in response to the phosphodiesterase inhibitor 1-methyl-3-isobutyl xanthine caused a significant activation of the kinase intracellularly. When TSH was added to the incubation medium there was a time-dependent and dose-dependent activation of the kinase which was closely correlated with the concentration of cAMP in the tissue. Homogenization of the tissue in NaCl to prevent re-association of the kinase subunits demonstrated that 16 mU/ml of TSH was sufficient for almost complete activation of kinase intracellularly.

Cyclic AMP-dependent protein kinase has been proposed as an important mediator of hormone action. To substantiate this hypothesis, it is necessary to demonstrate the activation of cAMP-dependent protein kinase by alteration of cAMP concentrations in intact tissue (1). Soderling, Corbin and Park recently demonstrated activation of fat-cell protein kinase in response to epinephrine, and Means et al. were able to show activation of testis kinase in response to FSH (2,3). Whether hormonal activation of kinases occurs in all such tissues has not been settled: the cAMP concentration in several organs, including the thyroid, has been reported to be sufficiently high that (if all the cAMP is free to act on the kinase) the kinase should be fully activated even in the absence of hormonal stimulation (1).

METHODS

Calf thyroids were placed on ice, sliced on a Stadie-Riggs microtome and incubated within an hour of slaughter. After 20 min. pre-incubation at 37°C, the slices were transferred to 15 ml fresh Krebs Ringer Bicarbonate buffer (KRB) with 200 mg % glucose containing different concentrations of TSH (Armour's Thytropar^{R}). Time-response was determined using 50 mU/ml TSH; dose-response was determined for a 10 minute incubation period. To end the incubation, the medium was rapidly decanted and the tissue immediately plunged into liquid nitrogen. The frozen tissue was then pulverized in liquid nitrogen and stored at -80°C until assayed. The activation ratio remained unaltered at this temperature for at least two weeks.

Protein kinase activity was measured as previously described, both with mixed calf thymus histone, 200 μg/ml (Schwartz-Mann #3601) and with no exogenous substrate and either with or without 10^{-6}M cAMP, (previously demonstrated to produce maximal kinase activation of purified thyroidal kinase) (4). Values for histone phosphorylation were obtained by subtracting the phosphorylation of endogenous protein from the activity measured with added histone. The degree of activation was expressed as the ratio of activity measured in the absence of added cAMP to the activity in the presence of added cAMP: complete activation would have an activity ratio "(-cAMP/+cAMP)" of 1.0. The frozen powdered tissue was prepared for the kinase assay by homogenizing it in 10 volumes of 2 mM Na_2 EDTA, 5 mM $KHPO_4$, 0.5 mM 1-methyl-3-isobutyl-xanthine (MIX), pH 7.0. Powdered tissue was also homogenized in this medium to which various concentrations of NaCl had been added. The homogenate was centrifuged for 5 minutes at 0°C, 20,000 xg and then samples (30-60 μg protein) immediately assayed for kinase activity in a final volume of 200 μl. Differences in the ratio of tissue-to-homogenizing medium from 1:5 to 1:40 did not significantly alter the percentage of the enzyme present in the active form if equal amounts of protein were used. Kinase activity did not increase linearly when protein aliquots above about 75 μg were used. cAMP determinations were performed on the frozen powdered tissue (5). cAMP was also measured in trichloroacetic acid (TCA) precipitates of the 20,000 xg supernatant used for the kinase. cAMP values per mg (Lowry) were only slightly lower than in the tissue powder directly extracted with TCA. Since the latter values more nearly reflect the levels in the slices immediately following the incubation and were less variable, they were used.

RESULTS AND DISCUSSION

Supernatants from slices incubated in KRB alone had basal specific activities of about 50 pmol P_i incorporated/mg protein/min and an activity ratio of 0.165 ± .006 (SEM). When slices were incubated with 0.5 mM MIX for 10 minutes, the activity ratio in the supernatant rose to 0.200 ± .006 (n=10, $p < .005$). The cAMP concentration in basal thyroid slices was 3.2 ± .3 pmol/mg protein and rose to 8.3 ± 1.0 with 0.5 mM MIX. Thus the kinase activity is sensitive to small changes in intracellular cAMP concentration.

In the time-response experiment, slices were incubated with 50 mU/ml TSH with 0.5 mM MIX for 2 to 10 minutes (Fig. 2). Activation of histone phosphorylation rose from 0.15 at zero time, to reach an activity ratio of 0.57 after a ten-minute incubation. The cAMP concentration in this experiment rose from 4.4 pmol/mg protein at zero time to 81 at ten minutes.

When thyroid slices were incubated with varying concentrations of TSH from 0.8 to 16.0 mU/ml in the presence of 0.5 mM MIX, there was a log-dose dependent activation of kinase activity, the (-cAMP/+cAMP) ratio reaching 0.60 at a TSH concentration of 16 mU/ml (Fig. 1). This TSH concentration

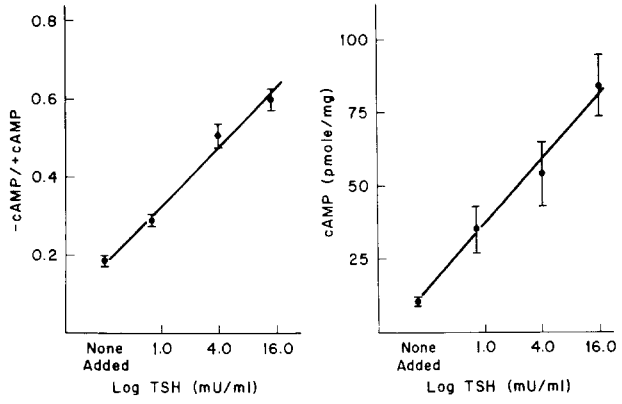

Figure 1. Dose-Response.

A) Activity Ratios: All values represent mean ± 1 S.E.M., 5 different sets of slices. The activity ratio was significantly different from endogenous with all doses of TSH. The response to 0.8 mU per ml TSH was also significantly different from all other concentrations, but the response to 16 mU per ml did not differ significantly from 4 mU per ml.

B) Cyclic AMP: all values represent the mean ± 1 S.E.M. (N=5). The cAMP response was significantly different from endogenous with all doses of TSH. The response to 0.8 mU per ml TSH was significantly different from 16 mU per ml but not from 4 mU per ml. The response to 16 mU per ml was significantly different from all other doses but the difference was borderline ($p < 0.1$) with respect to 4 mU per ml TSH.

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also produced a cAMP level of 85 pmol/mg protein. In slices incubated with various doses of TSH but without 0.5 mM MIX, the response of the kinase activity ratio was smaller and much more variable as were the cAMP concentrations. The linear regression between intracellular cAMP and activity ratio had correlation coefficients of 0.78 for the dose-response experiment and 0.85 in the time experiment, and the slope was .0045 for both.

In the above studies, the activity ratio did not reach 1.0 in the presence of maximal TSH stimulation. Soderling et al. reported that it is necessary to add 0.5 M NaCl during homogenization of the tissue to prevent re-association of the kinase subunits (2). We therefore examined the effect of adding various concentrations of NaCl to the homogenizing medium (Fig. 3). The activity ratio found in slices incubated with TSH was in fact raised to

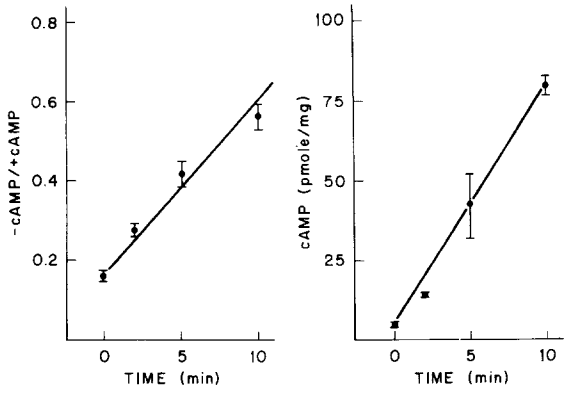

Figure 2. Time-Response.

A) Activity Ratios: All values represent the mean ± 1 S.E.M., 4 different sets of slices. The activity ratio response was significantly different from zero time at all times tested. The response at two minutes was also significantly different from all other times, but the difference between 5 and 10 minutes was not significantly different ($p < 0.2$).

B) Cyclic AMP: All values represent the mean ± 1 S.E.M. (N=4). The cAMP response at each time was significantly different from all other times tested.

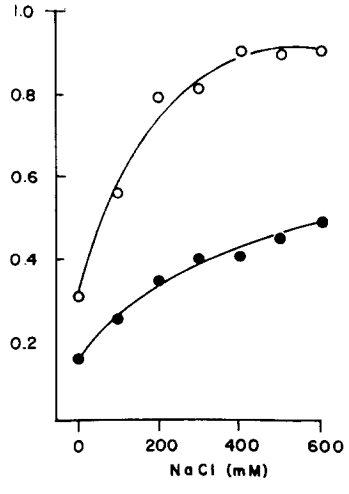

Figure 3. Effect of NaCl on Activity Ratio.
Slices incubated with 16 mU/ml TSH shown as open circles and slices incubated without TSH shown as closed circles. Frozen powdered sample were homogenized in usual medium to which various concentrations of NaCl had been added. Activity ratio shown on ordinate.

0.91. However, a lesser but significant activation of the unstimulated slices was also observed with increasing concentrations of NaCl. The largest difference in activity ratios between basal and maximally-stimulated slices was found with 0.2 M NaCl.

The levels of cAMP in our calf thyroid slices are similar to those reported by Gilman and Rall (5). The percent increase in cAMP in response to TSH stimulation was similar in both studies in the absence of phosphodiesterase inhibitor. Our observation that cAMP concentrations expressed in mg protein were essentially no different in whole tissue or 20,000 xg supernatant suggests that almost none of the nucleotide was associated with large subcellular particles, as also noted in skeletal muscle (7).

Purified thyroidal cAMP-dependent protein kinases have been reported to show half-maximal activation at cAMP concentrations between 4 and 9 x 10^{-8} M (4, 8). In our unstimulated bovine thyroid slices we have found cAMP concentrations are about 4 pmol/mg protein, so that the intracellular concentration approximates 3 x 10^{-7} M assuming uniform distribution in intracellular water. If all this cAMP were free to act on the kinase, there would be an activation of all protein kinase activity in the thyroid, even in the absence of TSH. However, it is clear that a significant fraction of total cellular cAMP is in the bound form. We have found that the 20,000 xg supernatant from the unstimulated thyroid homogenate is capable of binding about 1 pmol of cAMP per mg protein under the conditions which Gilman found to bind 12 pmol/mg protein in bovine muscle (9). Rat thyroid has been reported to bind about 2 pmol/mg protein (10). Obviously such determinations are only crude estimates of the real "free" cAMP concentration in the cell, since equilibration of endogenous bound cAMP with (^{3}H) cAMP is slow and the attachment of the binding-protein to the filter requires nonphysiological ionic conditions. Furthermore, cAMP binding is apparently affected by ATP and Mg^{++} concentrations (11) and possibly by the presence of a protein "inhibitor" as well (9).

There are several other problems in regard to both the estimation of the activity ratio and the measurement of the amount of cAMP binding. The concentration of cAMP is reduced by dilution with the homogenization medium. However, we did not find marked changes in the activity ratio of protein kinase in response to a wide range of tissue-to-medium ratios. Corbin et al. reported similar findings with skeletal muscle, but in the fat pad found lower activity ratios with greater dilution unless they prepared their tissue with 0.5 M NaCl (12). To demonstrate complete activation of the kinase with maximal doses of TSH we had to homogenize our tissue in 0.2 M NaCl, which also partially activated the kinase in the unstimulated slices. It is also possible

that cAMP and the kinase might be intracellularly segregated, and the apparent activation might actually occur during homogenization. The fact that the activity ratio was 0.17 in basal slices which contained enough cAMP to have completely activated the kinase makes this possibility less likely.

The demonstration that stimulation of adenyl cyclase by TSH results in activation of the kinase is of importance since it had seemed that the total cAMP concentration in the unstimulated thyroid was apparently high enough to fully activate cAMP-dependent protein kinase. While several problems have not yet been completely resolved, it is clear that TSH stimulation can be shown to alter the activity ratio of cyclic AMP-dependent kinase in calf thyroid slices.

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