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CALCIUM-ACTIVATED, CALMODULIN-DEPENDENT PROTEIN KINASE ACTIVITY IN BOVINE THYROID CYTOSOL*

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Bovine thyroid 100,000 x g supernatant contained calcium-activated, calmodulin-dependent protein kinase (PK-CaM) activity. The PK-CaM was partially purified using ion-exchange chromatography and characterized. PK-CaM, using casein as exogenous substrate, was not stimulated by Ca^{2+}(0-500 μM) or calmodulin (1-10 μg) by themselves, but was stimulated by the combination of the two by 100%. The activation of the enzyme by Ca^{2+} and calmodulin was dose-dependent with maximal stimulation evident at 1 μM free-Ca^{2+} and 3 μg calmodulin. Both chlorpromazine and trifluoperazine inhibited the thyroid enzyme in a dose-related manner. The molecular weight (MW) of the PK-CaM, based on gel filtration, was approximately 500,000. PK-CaM could also be demonstrated using endogenous thyroid cytosol proteins as substrate. Separation of these ^{32}P-labelled proteins by SDS-PAGE and subsequent autoradiography revealed that one major protein of approximately 56,000 MW was phosphorylated by PK-CaM. In some experiments, a second, less-intense protein band of approximately 64,000 MW was also phosphorylated. Evidence is presented, suggesting that these two protein bands may result from the autophosphorylation of the PK-CaM holoenzyme. These results offer a molecular mechanism, in addition to protein kinase C, by which Ca^{2+} effects may be mediated in thyroid.

It is well-established that many of the stimulatory effects of TSH on thyroid metabolism are mediated by cyclic AMP (1), which in turn stimulates cyclic AMP-dependent protein kinase(s) (2). More recently, it has become increasingly evident that Ca^{2+} also functions as yet another intracellular regulatory signal and may act in conjunction with cyclic AMP as interrelated messengers for some cellular processes (3,4). The fact that Ca^{2+} has been implicated in the control of various thyroid functions (5,6) and the fact that TSH is involved in the compartmentalization and movement of Ca^{2+} in the thyroid (7) strongly implicates Ca^{2+} as an intracellular regulator in thyroid.

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There has recently been a growing interest in two novel protein kinase activities that require Ca^{2+} for activation as opposed to cyclic AMP. One being the diacylglycerol-activated, calcium/phospholipid-dependent protein kinase (i.e. protein kinase C) first described by Nishizuka (8) and the other being the calcium-activated, calmodulin-dependent protein kinase (PK-CaM) (9-11). These two kinases could, therefore, be the molecular mechanisms by which Ca^{2+} and/or diacylglycerol function as important second messengers in mediating hormonal responses (12-15).

We have previously reported on the existence of a protein kinase C activity in bovine thyroid cytosol (16) that is capable of phosphorylating several endogenous proteins. We, therefore, initiated these studies to determine if bovine thyroid also contains a PK-CaM activity, thus offering an additional means of modulating Ca^{2+} effects in this tissue. Additionally, we wished to determine if the PK-CaM could specifically phosphorylate endogenous proteins which could be indicative of its mode of action in vivo.

Materials and Methods

Materials: ATP, histone F_1 (lysine-rich subgroup), histone F_{2a} (slightly lysine-rich subgroup), histone F_{2b} (slightly lysine-rich subgroup), histone F_3 (arginine-rich subgroup), casein (partially dephosphorylated, C 4765), chlorpromazine, trifluoperazine, W-7, calmodulin (p 2277, 40,000 units/mg protein) and Sephacryl were purchased from Sigma Chemical Co. (St. Louis, Mo.) (γ-^{32}P) ATP (500 Ci/mmole) was purchased form ICN (Irvine, CA).

Methods: Tissue Preparation: Bovine thyroid glands were obtained fresh from a local slaughterhouse and immediately processed. The thyroid glands were trimmed of fat and fascia, finely minced, and homogenized in 4 volumes of 0.25M sucrose/20 mM Tris-HCl/2 mM EGTA; pH 7.4 in a Polytron homogenizer (Brinkman Inst.). After further homogenization in a dounce glass hand-homogenizer, the homogenate was filtered through a fine screen mesh and centrifuged at 800 x g for 10 min. The resulting supernatant was then centrifuged at 100,000 x g for 60 min. to yield the cytosol fraction. PK-CaM was partially purified using DE-52 ion-exchange chromatography (16).

PK-CaM Assay: PK-CaM activity was assayed by measuring the incorporation of ^{32}P from (γ-^{32}P) ATP into casein. The reaction mixture (0.2 ml) contained 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 2 mM dithiothreitol, 20 µM ATP, 0.25 µCi (γ-^{32}P) ATP, 0.25 mM EGTA, and casein (500 µg). Where indicated, various concentrations of Ca^{2+} and/or calmodulin were also added. After 10 min. at 37°C, the reaction mixture was terminated and TCA precipitated material counted, as previously described (17).

Phosphorylation of Endogenous Proteins: The assay for endogenous phosphorylation was essentially as described above except that casein was deleted and the ATP concentration was reduced to 0.5 µM. Samples were analyzed by SDS-PAGE and autoradiography as recently described (18). Protein was determined by the method of Lowry, et al. (19).

Results

PK-CaM activity could be demonstrated in a crude bovine thyroid 100,000 x g supernatant fraction using casein as exogenous substrate; the addition of Ca^{2+} and calmodulin increased kinase activity by 60% (data not shown). In order to more accurately characterize the PK-CaM activity, the enzyme was partially purified using DE-52 ion-exchange chromatography (16). PK-CaM was predominantly found in Fraction #1 and was used for the following characterization studies since it contained no protein kinase C activity (16) which is also affected by Ca^{2+}. When the DE-52 fraction containing the PK-CaM was put on a Sephacryl S-400 column a single peak of activity was obtained at a MW of 500,000 indicating that the thyroid enzyme is of the calcium/calmodulin-dependent protein kinase II variety (20) (data not shown).

The partially purified PK-CaM was unaffected by the addition of Ca^{2+} (0-500 μM) or calmodulin (1-10 μg) by themselves, whereas the combination of Ca^{2+} plus calmodulin increased the protein kinase activity by approximately 100% (Table I).

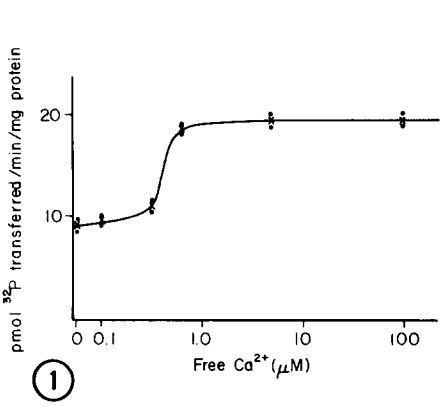
The activation of PK-CaM was dose-dependent for both calmodulin as well as Ca^{2+}. Thus, in the presence of Ca^{2+}, maximal protein kinase stimulation was observed at 3 μg of calmodulin added with 50% stimulation at 0.5 μg (data not shown). Using EGTA-Ca^{2+} buffers (21), it was shown that the activation of the enzyme by Ca^{2+} occurs at a very narrow range of free-Ca^{2+} concentration of approximately 0.1 to 1 μM (Figure 1), which is in the range of resting levels of free intracellular Ca^{2+} in cells (4,22). Both chlorpromazine and trifluoperazine, previously shown to in-

TABLE I

DEPENDENCY OF BOVINE THYROID PK-CaM ACTIVITY ON CALCIUM AND CALMODULIN

Addition	pmol ^{32}P transferred/min/mg protein±SD
1) Basal (0.25 mM EGTA)	9.4±0.9
2) Ca^{2+} (500 μM)	9.4±0.7
3) Calmodulin (5 μg)	10.5±0.6
4) Ca^{2+}+Calmodulin	19.4±2.2

PK-CaM was assayed as described in the text using casein as substrate, the results are expressed as the mean±SD and are from a representative experiment (5 experiments).



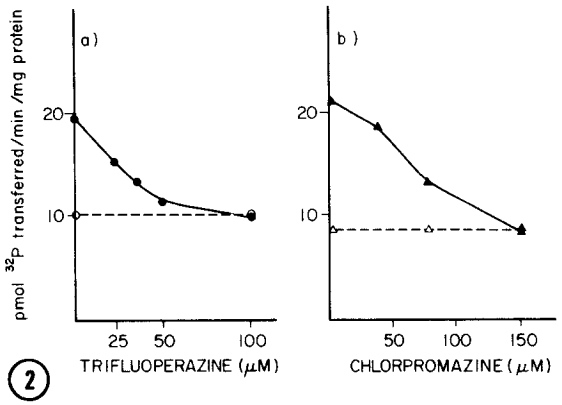


Figure 1. Effect of calcium on bovine thyroid PK-CaM activity: PK-CaM activity was assayed as described in the test. Free Ca^{2+} concentration was varied by the use of Ca^{2+}/EGTA buffers containing 0.25 mM EGTA. Free-Ca^{2+} concentration was estimated as previously described (21). Each point represents the mean±SD from a representative experiment.

Figure 2. Dose-response for the inhibition of bovine thyroid calcium-activated, calmodulin-dependent protein kinase (PK-CaM) by a) trifluoperazine and b) chlorpromazine: a) Increasing doses of trifluoperazine were added to basal (O--O or to PK-CaM) (i.e. Ca^{2+} plus calmodulin) (●—●). b) Increasing doses of chlorpromazine were added to basal (△--△) or to PK-CaM (i.e. Ca^{2+} plus calmodulin) (▲—▲). Each point represents the mean±SD of triplicate determinations from a representative experiment.

hibit PK-CaM in other systems (4,23), effectively inhibited the thyroid enzyme in dose-related fashions while having no effect on basal protein kinase activity (Figure 2).

Substrate specificity studies showed that the enzyme was most active when casein was used as an exogenous substrate. However, histones F_{2a}, F_{2b}, and F_3 could also function as exogenous substrates, albeit to a lesser extent. Histone F_1, the preferred substrate for protein kianse C (16) was totally ineffective as a substrate for PK-CaM (data not shown).

In order to implicate PK-CaM as a possible effector in thyroid physiology and function, experiments were performed to determine if PK-CaM could specifically phosphorylate endogenous thyroid cytosol proteins. The results showed that there was one major protein band of 56,000±3000 MW that was specifically phosphorylated by PK-CaM (Figure 3). Quantitation of this band showed that the addition of Ca^{2+} plus calmodulin increased its phosphorylation by 160%. In some experiments, a second less intense band of 64,000±3000 MW was also stimulated by PK-CaM. The phosphoryla-

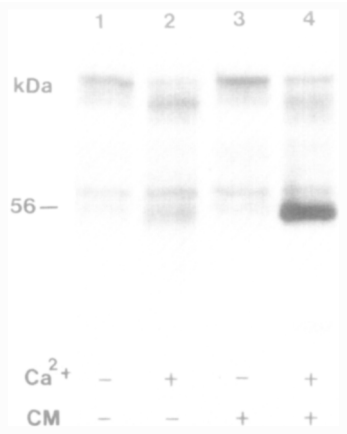


Figure 3. Autoradiograph of PK-CaM phosphorylation in bovine thyroid cytosol: Phosphorylation reactions were carried out as described in the "Methods" section. EGTA (0.25 mM) was present in all the fractions, Ca^{2+} was 100 µM above the EGTA concentration, and calmodulin (CM) was 5 µg. A protein of 56,000±3000 MW was specifically phosphorylated by PK-CaM. The quantitative cpm's of the 56,000 MW band (average of 8 experiments) were as follows: Basal = 55±10 and Ca^{2+} plus calmodulin = 145±18.

tion of both of these protein bands was totally inhibited by trifluoperazine and chlorpromazine. (data not shown).

Although not detailed here, calcium/calmodulin-dependent phosphorylation could also be demonstrated using myosin light chain (MLC) as exogenous substrate. However, the MLC was phosphorylated to a lesser extent than the 56,000 MW band. Furthermore, the addition of exogenous MLC to the assay mixture did not reduce the phosphorylation of the 56,000 MW protein.

Experiments were performed to determine if the 56,000 and 64,000 MW proteins could be derived form an autophosphorylation of the PK-CaM holoenzyme (24-26). Thus, when Sephacryl purified PK-CaM holoenzyme was reacted under phosphorylating conditions in the presence of Ca^{2+} and calmodulin, the same two protein bands were apparently phosphorylated, with the major phosphorylation occurring in the 51,000± 3000 MW protein band (Figure #4); trifluoperazine, chlorpromazine, and W-7 (27) totally inhibited this phosphorylation.

Discussion

The results presented herein demonstrating PK-CaM activity in bovine thyroid offers a potential molecular mechanism for the mediation of Ca^{2+} effects in this tissue (5-7). Our findings differ from those reported by Kasai and Field

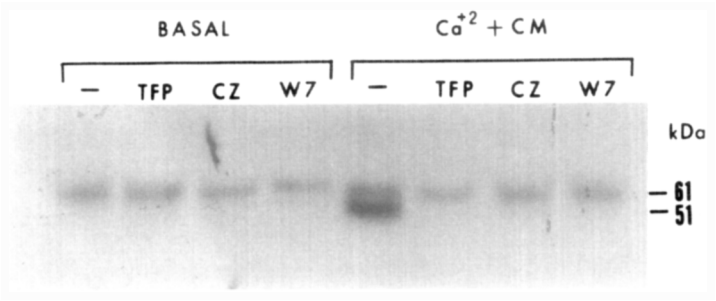


Figure 4. Autoradiograph of the inhibition of bovine thyroid PK-CaM activity by trifluoperazine (TFP), chlorpromazine (CZ), and W-7, using a Sephacryl purified enzyme preparation: Phosphorylation reactions were carried out as described in the "Methods" section using a Sephacryl purified enzyme preparation. EGTA (0.25 μM) was present in all the fractions, Ca^{2+} was 100 μM above the EGTA concentration, and calmodulin (CM) was 5 μg. The quantitative cpm's of the 51,000±3000 MW band was as follows: Basal = 32; Ca^{2+}+CM = 100; Ca^{2+}+CM+TFP (40 μM) = 30; Ca^{2+}+CM+CZ (80 μM) = 31; Ca^{2+}+CM+W-7 (80 μM) = 36.

(28) in which they failed to demonstrate a PK-CaM activity in thyroid. However, this discrepancy may be due to the fact that they tested for PK-CaM in a plasma membrane preparation and used histone as substrate.

Although very little data is available regarding PK-CaM activity in thyroid, Tawata, et al. (29) have reported on the existence of a myosin light chain kinase (MLCK) in bovine thyroid that shares many of the characteristics observed in our system. However, while it has been shown that MLCK is activated by Ca^{2+} and calmodulin (29,30), the general consensus is that the two activities are distinct entities (20,31). Our data in which the addition of exogenous MLC did not reduce the phosphorylation of the 56,000 MW protein would support this hypothesis.

While there are few reports on the presence and possible role of calmodulin in thyorid cell metabolism, recent reports lend support for a role of calmodulin in this tissue. These include the findings of Ollis, et al. (27) and Lakey, et al. (32) showing the presence of calmodulin in human thyroid and that calmodulin-dependent events are involved in the activation of the adenylate cyclase-cyclic AMP system by TSH in thyroid. Additionally, it has been shown that bovine thyroid plasma membrane has calmodulin-binding proteins (33) and that calmodulin levels may be increased in Graves' disease (34).

The present results showing the effects of various concentrations of Ca^{2+} on PK-CaM activation are in agreement to the findings of Nose' and Schulman (22) in bovine brain cytosol in which PK-CaM was stimulated at a free-Ca^{2+} concentration of 0.5 to 5 μM. This sensitivity to Ca^{2+} makes the thyroid PK-CaM responsive to fluctuations in Ca^{2+} levels thought to occur in vivo (4,22). This sensitivity for Ca^{2+} by PK-CaM would, therefore, be consistent with the observation that the initial rise in cytosolic Ca^{2+} activates a calmodulin-dependent pathway (12-15).

The finding that PK-CaM phosphorylated endogenous cytosol proteins suggests that it may function in a physiologic role in thyroid. It is noteworthy that in most other systems tested that PK-CaM phosphorylates a protein with approximate MW of 54,000 (11,35-38). Furthermore it has been suggested that this phosphorylation results form an autophosphorylation of the PK-CaM holoenzyme (20;24-26;35-37). This apparently may also be true for the thyroid PK-CaM, in that the fraction containing the holoenzyme was autophosphorylated with the major phosphorylation occurring in the 51,000 MW protein and to a lesser extent in the 61,000 MW protein, which are thought to be the sub-units of the PK-CaM holoenzyme (20;24-26).

In summary, these studies as well as our previous report on protein kinase C activity in thyroid (16) show that thyroid has the necessary molecular machinery to modulate both Ca^{2+} and diacylglycerol effects via their respective protein kinases. Studies are currently in progress to further delineate the physiologic roles of the various proteins that are phosphorylated by the respective protein kinases and how they may function in mediating basal as well as TSH-stimulated events in thyroid.

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