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Calmodulin Activates Adenylate Cyclase from Rat Anterior Pituitary

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SUMMARY

Bovine brain calmodulin activated adenlyate cyclase in calmodulin-deficient rat anterior pituitary membranes. This activation appeared to be specific by the following criteria: 1) calmodulin activation was Ca^{2+} dependent and responded biphasically to calcium, displaying activation at low and inhibition at higher concentrations; 2) calmidazolium, a potent calmodulin antagonist, inhibited calmodulin activation of adenylate cyclase; 3) activation of the enzyme occurred in a dose-dependent manner, at calmodulin concentrations normally found in most cells (1- to 20-μM range). However, this response was not saturated using calmodulin concentrations as high as 50 μM. The data suggest that endogenous calmodulin can be dissociated from normal anterior pituitary adenylate cyclase, that the enzyme can be subsequently stimulated by addition of micromolar concentrations of calmodulin, and that this enzyme appears to be at least 50-fold less sensitive to calmodulin than is the brain adenylate cyclase.

Calcium and cyclic adenosine 3′:5′ monophosphate (cAMP) play an important role in regulating hormone and transmitter secretion from numerous tissues. In some cells, the actions of these intracellular signals are interrelated such that Ca^{2+} can modulate cAMP levels (1) and cAMP can modulate the actions of Ca^{2+} (2). In the anterior pituitary, prolactin and growth hormone (GH) secretion are regulated by both cAMP and Ca^{2+}. In pituitary cell cultures, activation of adenylate cyclase elevates cAMP levels and promotes prolactin release (3, 4). Inhibition of adenylate cyclase by dopamine results in lowering of cAMP levels and is associated with inhibition of prolactin release (5, 6). Similarly, GH release is promoted by agents that normally stimulate adenylate cyclase, such as cholera toxin and forskolin (7). GH-releasing factor stimulates pituitary adenylate cyclase activity and this is associated with release of GH (8), whereas somatostatin inhibits GH-releasing-factor-induced cAMP accumulation and GH release (9). Extracellular Ca^{2+} supports prolactin and GH release, and lowering the Ca^{2+} diminishes this release (7, 10). In cultured pituitary cells, the Ca^{2+} ionophore, A23187, promotes both the release of prolactin and GH, and, concurrently, elevation of cAMP levels (8, 11). Calmodulin (CaM) inhibitors prevent both the increase in cAMP and in hormone release induced by A23187, suggesting that CaM may mediate these processes. However, the relationship between Ca^{2+} mobilization, increased cAMP levels, and increased hormone release has not been established. Although CaM may have several targets, one which would provide a direct link between elevated intracellular Ca^{2+} levels and increased cAMP is adenylate cyclase (EC 4.6.1.1).

CaM-sensitive adenylate cyclase was first identified in brain independently by Brostrom *et al.* (12) and Cheung *et al.* (13). The response of this enzyme to CaM has been extensively characterized, and apparent activation constants (K_A') for the particulate enzyme range from 100–200 nM CaM (14, 15). Experiments using partially purified brain adenylate cyclase indicate that CaM interacts directly with the catalytic subunit (16, 17), although additional interactions with other sites remain a possibility (18, 19). Recently, CaM-sensitive adenylate cyclases have also been reported in several peripheral tissues, including pancreatic islets (20), adrenal medulla (21), aortic smooth muscle (22), and GH_3 pituitary tumor cells (23). Using membranes from Ca^{2+}-depleted GH_3 cells, Brostrom *et al.* (23) observed a biphasic response of adenylate cyclase to added Ca^{2+}, which they proposed was mediated by calmodulin. Schettini *et al.* (24, 25) reported that addition of either Ca^{2+}, or Ca^{2+} plus CaM, to normal rat anterior pituitary membranes also caused adenylate cyclase activation. Apparently, efficient removal of endogenous CaM has hindered examination of this response, and to date a detailed characterization of the CaM-stimulated pituitary adenylate cyclase has not been performed.

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ABBREVIATIONS: cAMP, cyclic adenosine 3′:5′ monophosphate; GH, growth hormone; CaM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; ATP, adenosine triphosphate; GTP, guanosine-5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane.

We have found that several washes of pituitary membranes with an ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) -containing buffer permits examination of CaM-stimulated adenylate cyclase activity. Our results suggest that CaM specifically activates anterior pituitary adenylate cyclase, but that this response differs from that of brain adenylate cyclase in that it requires much higher concentrations of CaM.

Materials and Methods

Materials. [α-^{32}P] Adenosine triphosphate (ATP) (specific activity 3000 Ci/mmol) was purchased from either ICN Pharmaceuticals (Irvine, CA) or New England Nuclear. Adenylate cyclase assay components and somatostatin were obtained from Sigma Chemical Co. (St. Louis, MO). Calmidazolium, forskolin, and guanosine-5′-triphosphate (GTP) were from Calbiochem (San Diego, CA). Fluphenazine was a gift from Schering-Plough Corp. (Bloomfield, NJ). Other materials were readily obtained from commercial sources.

Preparation of pituitary membranes. Male Sprague-Dawley rats (225–350 g) were killed by decapitation and each pituitary was placed in ice-cold buffer composed of 50 mM tris(hydroxymethyl)aminomethane (Tris)/HCl (pH 7.5)/1.0 mM EGTA/1.0 mM dithiothreitol (buffer A) containing 1.0 mM phenylmethylsulfonyl fluoride. All following procedures were performed at 4°C. Membranes were prepared from the dissected anterior pituitary lobes by homogenization using a glass homogenizer and motor-driven teflon pestle (3 ml buffer A/pituitary) and centrifugation at 10,000 × *g* for 20 min. The pellet was similarly washed twice in the same buffer in which phenylmethylsulfonyl fluoride was omitted, and EGTA was reduced to 250 μM (buffer B). The membranes were resuspended in buffer B at a protein concentration of 0.5–3.0 mg/ml for the adenylate cyclase assay. During the 10-min assay, the production of cAMP was linear within this range of protein concentration.

Adenylate cyclase assay. In a final volume of 250 μl, assays contained 0.1 mM [α-^{32}P]ATP (200 cpm/pmol), 2 mM [^{3}H]cAMP (20 cpm/nmol), 10 mM creatine phosphate, 50 U/ml creatine phosphokinase, 1 mM B-mercaptoethanol, 5 mM theophylline, 0.01% bovine serum albumin, 5 mM $MgCl_2$, 200 μM EGTA, 28 mM Tris-HCl (pH 7.5), and 50–200 μg pituitary membrane protein; $CaCl_2$, CaM, or calmidazolium were added as indicated. Ingredients were preincubated at 30°C for 15 min to facilitate equilibration between EGTA, CaM, Ca^{2+}, Mg^{2+}, and pH, and assays were initiated by the addition of membranes. Assays were incubated for 10 min at 30°C, and the reaction was stopped by the addition of 750 μl buffer containing final concentrations of 10 mM ATP, 5 mM EGTA, and 50 mM Tris-HCl (pH 7.5) and heating at 100°C for 2 min. Membranes were centrifuged for 10 min at 4,000 × *g*, and [^{32}P]cAMP was purified from the supernatant by the method of Salomon (26). Protein was measured by the Peterson method (27) using bovine serum albumin as a standard. Unless stated otherwise, results are expressed as the mean of triplicate determinations of adenylate cyclase activity ± SD from a single representative experiment. Each experiment was replicated at least once with similar results.

CaM preparation. CaM was partially purified from bovine cerebral cortex as described by LaPorte *et al.* (28). This CaM preparation was purified to homogeneity using fluphenazine-Sepharose according to Charbonneau (29). The pure CaM was dialyzed against 10 mM Tris-HCl (pH 7.5) containing 500 μM EGTA and stored at −20°C. The CaM concentration was calculated from the protein concentration of this stock using MW = 16,800 for CaM (30). CaM prepared by this method and a commercial CaM preparation (Calbiochem) elicited equivalent activities when assayed with *Bordetella pertussis* adenylate cyclase as in Ref. 31.

Calculation of free Ca^{2+} concentrations. All free Ca^{2+} concentrations were calculated using a computer program (32) at an ionic strength = 0.07, pH = 7.5, and assay temperature = 30°C. In the absence of added calcium, the total [Ca^{2+}] contaminating the assays, inclusive of membranes, was 27 μM as determined using an atomic absorption spectrophotometer with a graphite furnace. However, it was not clear that this contaminating Ca^{2+} was free and available to activate CaM, especially because the membranes had been washed three times with an EGTA-containing buffer. Because inclusion of the contaminating Ca^{2+} level could lead to overestimation of the effective, free Ca^{2+} concentration, it was not included in our calculations of free Ca^{2+} levels.

CaM content of pituitary membranes. Fourteen anterior pituitaries were homogenized in 900 μl of buffer A containing 1 mM phenylmethylsulfonyl fluoride. Three 330-μl aliquots were taken for determination of CaM content in the whole homogenate, in the particulate and supernatant fractions, and in washed membranes. One aliquot was centrifuged at 12,000 × *g* for 20 min in a microfuge to separate supernatant from particulate fractions, and the pellet was resuspended in 250 μl buffer A. A second aliquot was diluted with 14.7 ml buffer B, processed as described above for preparation of anterior pituitary membranes, and resuspended in 250 μl buffer A. CaM was extracted from each of the four samples by heating at 95°C for 4.5 min and centrifuging at 12,000 × *g* for 20 min (33). The supernatants were saved and combined with a 100-μl wash of each respective pellet. After dialysis against 10 mM Tris-HCl (pH 7.5) containing 500 μM EGTA, serial 10-fold dilutions were assayed in duplicate in the presence of 200 μM EGTA/200 μM $MnCl_2$/0.05% Triton X-100 for their ability to activate *B. pertussis* adenylate cyclase (31). EC_{50} values (the dilution of extract causing 50% of maximal activation) were determined from log-probit plots, and the CaM content of each sample was calculated by comparison of the EC_{50} with the average EC_{50} obtained from standard log-probit plots using pure bovine brain CaM.

Results

Using 7 μM CaM, adenylate cyclase activity responded biphasically to $CaCl_2$ addition (Fig. 1). Concentrations of free Ca^{2+} from 0.2–25 μM stimulated the enzyme, whereas higher concentrations inhibited the activity. At Ca^{2+} concentrations below 1 μM, the adenylate cyclase activity measured in the presence of CaM did not exceed the basal activity measured in the absence of Ca^{2+}. Hence, most of the CaM stimulation in this region may be caused by prevention of Ca^{2+} inhibition of basal adenylate cyclase activity mediated by the Ca^{2+} chelating action of CaM. However, it seems more likely that this stimulation may be caused by direct CaM activation of the enzyme because the CaM antagonist, calmidazolium, completely inhibited stimulation of adenylate cyclase by 2 μM CaM down to basal activity measured in the presence of 25 μM free Ca^{2+} (see below and Fig. 3). At free Ca^{2+} concentrations between 1–25 μM, CaM stimulated the adenylate cyclase activity above the basal activity measured in the absence of Ca^{2+}. Because the Ca^{2+} levels in the resting and stimulated cell are estimated to be 0.1 and 10 μM, respectively, it appears that CaM activation of pituitary adenylate cyclase can occur in a physiological range of Ca^{2+}. In the absence of CaM, Ca^{2+} caused only inhibition of basal adenylate cyclase activity. Therefore, it appears that the remaining endogenous CaM that was not eliminated by our wash procedure was not sufficient for activating the enzyme. Hence, CaM activation of pituitary adenylate cyclase is dependent on both added CaM and Ca^{2+}.

Measurement of endogenous CaM levels in the membranes before and after washing indicated that CaM had been lowered from 1.22 μg/mg protein to 0.33 μg/mg protein (Table 1). Using this latter value, the concentration of endogenous CaM contributed to the adenylate cyclase assay by the washed membranes is calculated to be ≤ 16 nM, approximately 10-fold below

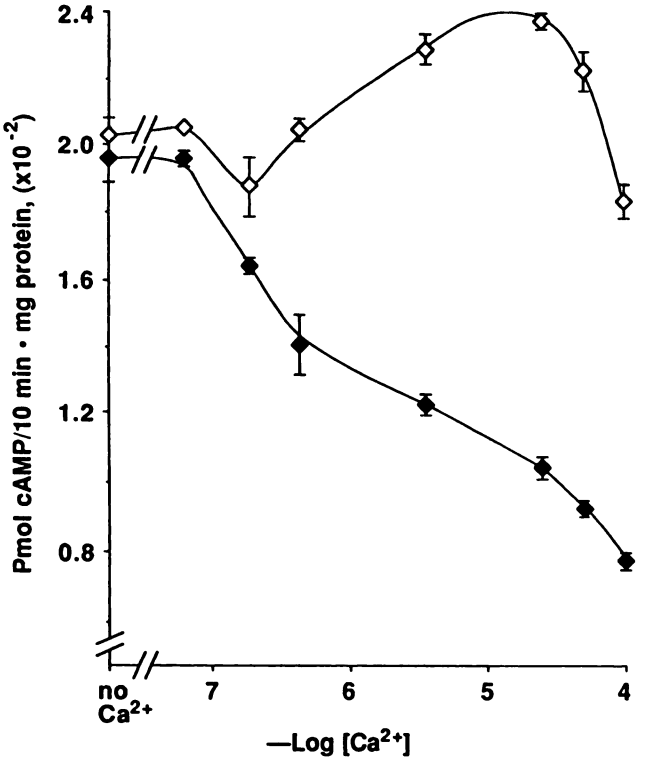

Fig. 1. CaM activation of pituitary adenylate cyclase is Ca^{2+} dependent. Pituitary membranes were assayed for adenylate cyclase activity in the presence of the indicated free Ca^{2+} concentrations and in the absence (♦) or presence (◇) of 7 μM CaM. Where error bars are not shown, the SD lie within the symbol.

TABLE 1

CaM content of anterior pituitary lobe fractions and washed membranes

Fraction	Total CaM content	Total mg of protein in extract[a]	(μg CaM)/(mg protein)
	μg		
Whole homogenate	6.13 ± 0.19[b]	5.04 ± 0.32	1.22 ± 0.07
Supernatant	4.32 ± 0.48	2.29 ± 0.07	1.89 ± 0.19
Particulate	1.13 ± 0.19	1.96 ± 0.20	0.57 ± 0.07
Washed membranes	0.284 ± 0.098	0.86 ± 0.06	0.33 ± 0.10

[a] Protein content was measured in each fraction directly before heating.
[b] Results are the mean values ± SD from three separate experiments.

the K_A' established for CaM activation of brain adenylate cyclase. Therefore, it appeared that our wash procedure sufficiently lowered endogenous CaM levels such that the full response of adenylate cyclase to CaM activation could be observed, assuming that the pituitary enzyme displayed a similar K_A' for CaM activation as that of brain adenylate cyclase. However, pituitary adenylate cyclase exhibited only minimal CaM stimulation in the 100-nM range (Fig. 2). Using the optimal Ca^{2+} conditions determined in Fig. 1, increasing CaM concentrations caused a dose-dependent activation of the enzyme up to 25 μM CaM, whereas higher concentrations caused apparent inhibition. The apparent inhibition by high CaM concentrations was eliminated when higher Ca^{2+} concentrations were used (Fig. 2, *dashed line*), suggesting that the "inhibition" was caused by depletion of Ca^{2+} caused by high CaM levels. Hence, the results of Fig. 2 indicate that micromolar CaM levels are required to activate pituitary adenylate cyclase.

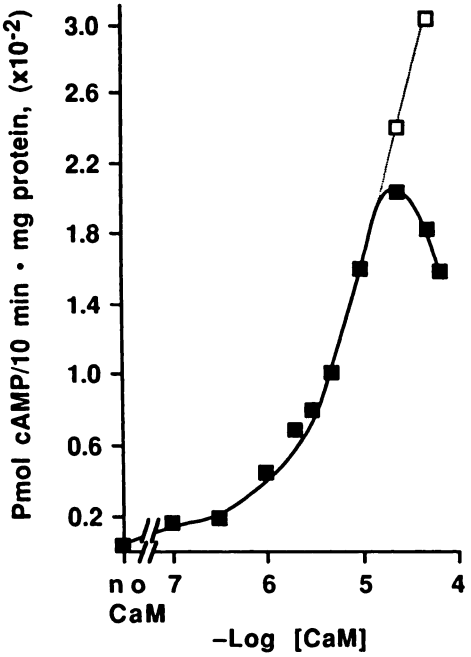

Fig. 2. CaM dose-dependently activates pituitary adenylate cyclase. Membranes were assayed for adenylate cyclase activity using optimal Ca^{2+} conditions determined in Fig. 1 (25 μM free Ca^{2+}) in the presence of increasing concentrations of CaM (■). Basal activity (126 pmol cAMP/10 min-mg protein) was substracted from each point. To demonstrate that the apparent inhibition of adenylate cyclase activity observed at high CaM concentrations was caused by insufficient Ca^{2+}, $CaCl_2$ concentrations yielding optimal CaM-stimulated adenylate cyclase activity were determined as in Fig. 1 for 25 and 50 μM CaM. CaM-stimulated adenylate cyclase activity was then determined at the respective optimal $CaCl_2$ concentrations by taking the difference between the total activity and basal activity, measured in the presence and absence of CaM, respectively (□). SD of total adenylate cyclase activities were within 8% of each mean value.

From these results it is also clear that the CaM dose-response was not complete up to 50 μM CaM. Therefore, in the absence of other endogenous factors that might increase the affinity for CaM, the pituitary adenylate cyclase appears to be at least 50-fold less sensitive to CaM than is brain adenylate cyclase.

To determine whether the wash procedure altered the responsiveness of adenylate cyclase to stimulatory and inhibitory agonists, the ability of both CaM and forskolin to activate adenylate cyclase, and the ability of somatostatin to inhibit the forskolin-stimulated enzyme, were examined at each stage of the washing procedure (Table 2). Although some protein is lost during the wash procedure (Table 1), no significant differences in the adenylate cyclase activities measured in the presence of GTP, CaM, forskolin, or forskolin plus somatostatin were observed throughout all three wash steps. Because the adenylate cyclase activities measured in the presence of somatostatin were not significantly different from those activities containing only forskolin and GTP, we cannot evaluate the effect of washing on the ability of somatostatin to inhibit adenylate cyclase. However, the slight decrement in activity in the presence of somatostatin (approximately 13%) was consistent between washes. Although activities varied between experiments, within each experiment the percentage of stimulation of adenylate cyclase by forskolin or by CaM was very reproducible (Table 2). These results indicate that washing the membranes had no detrimental effect on the ability of these agents to stimulate adenylate cyclase.

TABLE 2

Effect of washing on the responsiveness of particulate adenylate cyclase to stimulatory and inhibitory agents[a]

Addition to assay	Adenylate cyclase activity			Percentage of stimulation or inhibition (−)		
	P1	P2	P3	P1	P2	P3
	pmol cAMP/10 min-mg protein					
GTP	554 ± 76	563 ± 16	522 ± 24	450 ± 22	442 ± 25	423 ± 25
GTP + forskolin	2467 ± 300	2475 ± 163	2210 ± 245			
GTP + forskolin + somatostatin	2180 ± 197	2148 ± 69	1943 ± (132)	−15 ± 4	−13 ± 4	−14 ± (4)
$CaCl_2$	185 ± 53	170 ± (6)	136 ± 58			
$CaCl_2$ + CaM	405 ± 112	338 ± 69	326 ± 126	219 ± 2	215 ± (23)	245 ± 30

[a] Pituitary membranes were prepared as described in Materials and Methods and assayed at each stage of washing for stimulation of adenylate cyclase activity by GTP (10 μM), forskolin (30 μM), and CaM (10 μM), and for inhibition of adenylate cyclase activity by somatostatin (1 μM). Where present, assays contained 25 μM free Ca^{2+}. P1 represents the pellet obtained by centrifugation of the first homogenate (crude membranes), P2 represents the pellet obtained from the first wash, and P3 represents the pellet obtained from the second wash. Values are averages from 2–4 experiments ± SD. Where an average was obtained from only two experiments, the range of the averages is given in parenthesis. None of the adenylate cyclase activities resulting from a particular drug addition were significantly different between P1–P3 when analyzed by Student's *t* test using 90% confidence limits. Within each wash, adenylate cyclase activities measured in the presence of forskolin and GTP and in the absence or presence of somatostatin were not significantly different when analyzed as above using the Student's *t* test.

Compounds such as calmidazolium, the sulfonamide derivative W-7, and phenothiazines are thought to bind CaM and thereby antagonize CaM activation of enzymes. However, these compounds are not entirely specific for CaM because they also inhibit protein kinase-C and perturb membranes (1). Calmidazolium appears to be the most selective because it is the most potent antagonist of CaM activation of phosphodiesterase (34, 35) and Ca^{2+} ATP'ase (35). Calmidazolium caused a dose-dependent inhibition of CaM activation of pituitary adenylate cyclase (Fig. 3). Using 2 μM CaM, the IC_{50} (concentration of drug needed to inhibit 50% of the CaM-stimulated activity) was 3.3 ± 1.8 μM (N = 4). The IC_{50} values were shifted to higher concentrations as the CaM concentration was increased, suggesting that the antagonist was acting competitively with CaM. Complete inhibition of the CaM-stimulated adenylate cyclase activity was not obtainable when higher CaM concentrations were used because calmidazolium concentrations above 20 μM inhibited the basal adenylate cyclase activity.

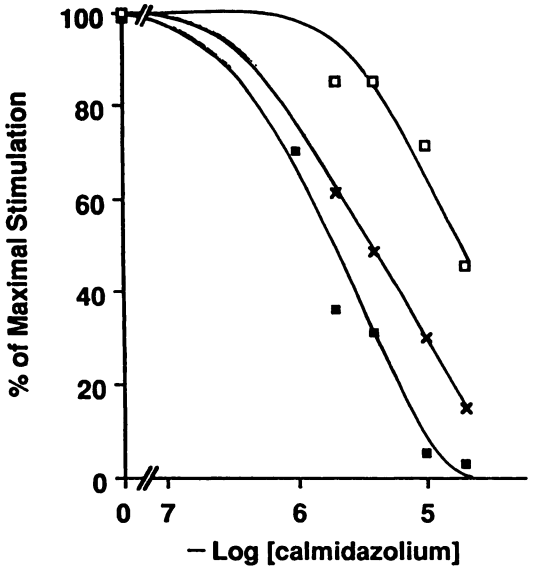

Fig. 3. Calmidazolium inhibits CaM activation of pituitary adenylate cyclase. Membranes were assayed for adenylate cyclase activity using optimal Ca^{2+} conditions determined in Fig. 1 (25 μM free Ca^{2+}) in the presence of increasing concentrations of calmidazolium, and in the absence (basal) or presence of 2 μM (■), 5 μM (×), and 10 μM (□) CaM. The percentage of maximal stimulation was derived by dividing the CaM-stimulated adenylate cyclase activity measured in the presence of calmidazolium by the CaM-stimulated adenylate cyclase activity, measured in the absence of drug, and multiplying by 100. Each CaM-stimulated adenylate cyclase activity was obtained by subtracting the basal adenylate cyclase activity, measured at a respective calmidazolium concentration, from the total adenylate cyclase activity measured in the presence of CaM. The basal activities, in pmol cAMP/10 min-mg protein, were 151 ± 6, 156 ± 0, 152 ± 6, 142 ± 4, 149 ± 4, and 128 ± 4 at calmidazolium concentrations of 0, 1, 2, 4, 10, and 20 μM, respectively. SD of total adenylate cyclase activities were within 5% of each mean value.

Throughout these experiments, basal adenylate cyclase activity has varied usually between 150–250 pmol cAMP/10 min-mg protein. Because the anterior pituitary is highly vascularized, it is possible that contaminating protease activity was responsible for this variation. We are currently investigating this possibility. We have also observed that freezing decreased CaM-stimulated adenylate cyclase activity in washed membranes, whereas basal activity, measured in the presence of Ca^{2+}, was minimally affected. In pmoles cAMP/10 min-mg protein, CaM-sensitive adenylate cyclase activities were 301 ± 57 for fresh versus 70 ± 43 for thawed membranes measured in the presence of 25 μM CaM, and basal activities were 64 ± 4 for fresh versus 49 ± 29 for thawed membranes (N = 3) measured in the absence of CaM. This suggests that the factor mediating CaM stimulation of pituitary adenylate cyclase is sensitive to freezing.

Discussion

We report here that micromolar concentrations of CaM can activate anterior pituitary adenylate cyclase and that this activation appears to be specific by several criteria. Calcium is required for CaM activation (Fig. 1), and the observed biphasic response to Ca^{2+} also occurs with the CaM-sensitive adenylate cyclase from bovine brain (15) and from *B. pertussis* (31). These results are consistent with observations of Schettini *et al.* (24, 25), who found that Ca^{2+} was required for maximal activation of adenylate cyclase activity using micromolar CaM levels and normal rat anterior pituitary membranes. Because Ca^{2+}-dependent CaM stimulation occurs in the same range of Ca^{2+} found in the resting and stimulated cell (0.1–10 μM, respectively), it seems possible that CaM may cause activation of adenylate cyclase *in vivo*.

Using GH_3 pituitary tumor cell membranes, Brostrom *et al.* (23) reported that basal adenylate cyclase activity responded biphasically to Ca^{2+}, even after extensive washing of the membranes with EGTA. It was suggested that endogenous CaM was responsible for the Ca^{2+} activation and that CaM may be an

integral and nondissociable component of the enzyme. Using normal rat anterior pituitary membranes, Schettini *et al.* (24, 25) similarly observed that adenylate cyclase activity responded biphasically to $CaCl_2$ addition. However, these membranes had not been extensively washed to remove endogenous CaM. In contrast, addition of Ca^{2+} to washed, normal anterior pituitary membranes caused only inhibition of basal adenylate cyclase activity (Fig. 1). This lack of responsiveness to added Ca^{2+} cannot be attributed to damaged adenylate cyclase caused by the wash procedure because activation of the enzyme by added GTP, forskolin, and CaM remained unchanged throughout this treatment (Table 2). It seems more likely that endogenous CaM mediated the Ca^{2+} activation of adenylate cyclase reported by others, and that our washing procedure lowers endogenous CaM levels such that stimulation of the enzyme by added Ca^{2+} alone no longer occurs. Based on these results, CaM does not appear to be a nondissociable component of normal pituitary adenylate cyclase. Furthermore, comparison of the CaM content between washed and unwashed pituitary membranes revealed that endogenous CaM levels could be reduced approximately 50%, such that they contributed ≤ 16 nM CaM to each assay. Using similar extraction procedures, endogenous CaM was evenly distributed between the particulate and cytosol in GH_3 cells (23), whereas the distribution in normal anterior pituitary tissue was approximately 75–80% cytosolic and 20–25% particulate (Table 1). This suggests that GH_3 pituitary tumor cell membranes may have a greater affinity for CaM than do normal anterior pituitary membranes. These observations may represent important differences between normal anterior pituitary and GH_3 pituitary tumor cells, especially if CaM partially modulates hormone release (8, 11).

Calmidazolium inhibited stimulation of anterior pituitary adenylate cyclase by 2 μM CaM with an apparent IC_{50} = 3.3 μM. This antagonist inhibited CaM activation of partially purified phosphodiesterase with an IC_{50} ranging from 0.005–0.150 μM (35, 36); higher concentrations of calmidazolium were required at higher CaM concentrations (range 2–30 nM). This compound was less potent at inhibiting a crude particulate preparation of CaM-sensitive Ca^{2+} ATP′ase (IC_{50} = 0.15–0.35 μM), possibly because of nonspecific membrane interactions (35). Because both a crude membrane preparation and high CaM concentrations (≥2 μM) were used in our experiments, it is reasonable to expect a higher apparent IC_{50} value than was observed with these other enzymes.

Together, the above data are consistent with the idea that activation of pituitary adenylate cyclase is specifically mediated by CaM. However, it is clear from Fig. 2 that the CaM dose-response could not be completed even at 50 μM CaM. In contrast with the particulate brain adenylate cyclase, where the K_A' ranges from 100–200 nM CaM (14, 15), the K_A' for pituitary adenylate cyclase appears to be greater than 5 μM CaM. Although we have not measured intracellular CaM levels, it is interesting to note that estimates of CaM concentrations in different cell types range from 1–20 μM (37). It seems possible that the CaM concentration in the microenvironment of the membrane-bound adenylate cyclase may be even higher than these estimates, because CaM has been shown to selectively distribute between the membrane and cytosol in response to extracellular hormones and transmitters (38, 39). Therefore, it appears plausible that activation of adenylate cyclase by the micromolar CaM levels shown here may also occur *in vivo*, although saturation of this effect probably would not occur because of the high CaM levels required.

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