Stimulation of the Type III Olfactory Adenylyl Cyclase by Calcium and Calmodulin†

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ABSTRACT: Characterization of adenylyl cyclases has been facilitated by the isolation of cDNA clones for distinct adenylyl cyclases including the type I and type III enzymes. Expression of type I adenylyl cyclase activity in animal cells has established that this enzyme is stimulated by calmodulin and Ca^{2+}. Type III adenylyl cyclase is enriched in olfactory neurons and is regulated by stimulatory G proteins. The sensitivity of the type III adenylyl cyclase to Ca^{2+} and calmodulin has not been reported. In this study, type III adenylyl cyclase was expressed in human kidney 293 cells to determine if the enzyme is stimulated by Ca^{2+} and calmodulin. The type III enzyme was not stimulated by Ca^{2+} and calmodulin in the absence of other effectors. It was, however, stimulated by Ca^{2+} through calmodulin when the enzyme was concomitantly activated by either GppNHp or forskolin. The concentrations of free Ca^{2+} for half-maximal stimulation of type I and type III adenylyl cyclases were 0.05 and 5.0 μM Ca^{2+}, respectively. These data suggest that the type III adenylyl cyclase is stimulated by Ca^{2+} when the enzyme is activated by G-protein-coupled receptors and that increases in free Ca^{2+} accompanying receptor activation may amplify the primary cyclic AMP signal.

Adenylyl cyclases [ATP-pyrophosphate lyase (cyclizing), EC 4.6.1.1] catalyze the synthesis of cyclic AMP, an important intracellular second messenger in eukaryotic cells [reviewed by Levitzki (1987)]. Intracellular cyclic AMP regulates carbohydrate, lipid, protein, and nucleic acid metabolism as well as synaptic transmission, ion channel function, and transcription in neurons (Krebs & Beavo, 1979). Adenylyl cyclases are regulated by stimulatory and inhibitory receptors coupled to the catalytic subunit through the regulatory G proteins G_s and G_i [reviewed by Ross and Gilman (1980)]. In addition, modulation of adenylyl cyclase activity by Ca^{2+} has been demonstrated in several tissues and it has been proposed that cyclic AMP levels may be controlled by fluctuations in intracellular free Ca^{2+} [reviewed by Cheung and Storm (1982)]. Ca^{2+}-stimulated adenylyl cyclase activity has been reported in brain (Von Hungen & Roberts, 1973; Brostrom et al., 1975), pancreatic islets (Valverde et al., 1979), adrenal mdeulla (LeDonne & Coffee, 1979), olfactory bulb (Olianas & Onali, 1990), olfactory cilia (Anholt & Rivers, 1990), heart (Avdonin & Tkachuk, 1978; Potter et al., 1980), sperm (Gross et al., 1987), smooth muscle (Amiranoff et al., 1983; Piascik et al., 1983), kidney (Ausiello & Hall, 1981), retina (Gnegy et al., 1984), thyroid (Lakey et al., 1985), sea urchin sperm (Bookbinder et al., 1990), and an insect endocrine gland (Meller et al., 1990).

Although the mechanism for Ca^{2+} stimulation of adenylyl cyclase activity has not been fully defined for all tissues, calmodulin (CaM)[1] mediates Ca^{2+} stimulation of at least one form of adenylyl cyclase present in mammalian brain (Brostrom et al., 1975). The catalytic subunit of a CaM-sensitive adenylyl cyclase from brain has been purified to homogeneity (Yeager et al., 1985; Smigel, 1986) and shown to interact directly with CaM (Minocherhomjee et al., 1987). It has generally been assumed that Ca^{2+} stimulation of adenylyl cyclase activity in other tissues is mediated by CaM, although only the brain enzyme has been extensively characterized. The isolation of a cDNA clone for a CaM-sensitive adenylyl cyclase (type I) from a bovine brain cDNA library and its expression in several cultured animal cells including insect Sf9 cells (Tang et al., 1991) and human kidney 293 cells (Bakalyar & Reed, 1990) have greatly facilitated characterization of this enzyme.

Most mammalian tissues contain a mixture of adenylyl cyclases, and the Ca^{2+} sensitivity of other forms of adenylyl cyclase has not been examined. Therefore, it is not known whether or not Ca^{2+} stimulation of adenylyl cyclase activity in tissues other than brain is due to type I adenylyl cyclase or other forms of the enzyme that are less well characterized. Another adenylyl cyclase, type III, has been cloned and its expression is apparently localized to olfactory neurons (Bakalyar & Reed, 1990). Since olfactory cilia contain Ca^{2+}-stimulated adenylyl cyclase activity (Anholt & River, 1990), it was of interest to determine if type III adenylyl cyclase is stimulated by Ca^{2+}. In this study, the Ca^{2+} sensitivities of type I and type III adenylyl cyclases were compared by expression of each form of the enzyme in human 293 cells, a cell line that does not contain Ca^{2+}-stimulated adenylyl cyclase activity. We report that type III adenylyl cyclase is stimulated by Ca^{2+} but that its response to Ca^{2+} is significantly different from that of the type I enzyme.

Experimental Procedures

Plasmid DNA Construction. The type I adenylyl cyclase cDNA clone pBBAC was isolated from a bovine brain cDNA

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[1] Abbreviations: CaM, calmodulin; CDM8(I-AC), the plasmid containing the mammalian expression vector CDM8 and the complete coding sequence of type I adenylyl cyclase; CDM8 (III-AC), the plasmid containing the mammalian expression vector CDM8 and the complete coding sequence of type III adenylyl cyclase; pBBAC, the plasmid pBluescript SK$^-$ containing the coding sequence of type I adenylyl cyclase and 102 bp of the 5′-untranslated region that has an additional ATG sequence; sATGpBBAC, pBBAC in which the the 5′-untranslated 102 bp was deleted to remove the additional ATG; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; 2′-dAMP, 2′-deoxyadenosine 3′-monophosphate; GppNHp, 5′-guanylyl imidodiphosphate.

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library in bacteriophage λ_{ZAP} as described by Xia et al. (1991). pBBAC contains the complete coding sequence of type I adenylyl cyclase and 102 bp of the 5′untranslated region that has an additional ATG sequence (Xia et al., 1991; Krupinski et al., 1989). In an attempt to improve the efficiency of protein expression, the 5′-untranslated 102 bp was deleted from pBBAC. The resulting plasmid was designated sATGpBBAC. For expression of type I adenylyl cyclase in human kidney 293 cells, the complete coding sequence of the enzyme was recovered from sATGpBBAC and ligated to the mammalian expression vector CDM8 (Seed & Aruffo, 1987; Liu et al., 1991). This plasmid was designated CDM8(I-AC). The type III adenylyl cyclase cDNA clone in pBluescript SK^- (Bakalyar & Reed, 1990) was obtained from R. R. Reed (The Johns Hopkins University, Baltimore, MD). The coding sequence of this enzyme was ligated to CDM8 for expression of type III adenylyl cyclase. This expression vector was designated CDM8(III-AC).

Cell Culture and DNA Transfection. Human embryonic kidney 293 cells (American Type Culture Collection) were grown at 36 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified incubator with 95% air/5% CO_2. For DNA transfections, confluent cultures of 293 cells were replated on 100-mm culture dishes at a density of 2×10^6 cells/plate. The newly seeded cells in culture were grown overnight and then transfected with expression plasmid vector (10 μg of DNA/plate) by the calcium phosphate method (Chen & Okayama, 1987).

Cell Membrane Preparation. Fifty hours after DNA transfection, cultured cells were washed three times with 150 mM NaCl, harvested into buffer A (20 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol, 5 μg/mL leupeptin, and 0.5 mM phenylmethanesulfonyl fluoride), and broken at 4 °C using a Dounce homogenizer. Cell homogenates were separated from unbroken cells and nuclei by centrifugation at 600*g* for 2 min, and the supernatants were subjected to centrifugation at 30000*g* for 20 min. The resulting membrane pellet was suspended in buffer A and assayed for adenyly cyclase activity. In some cases, membranes were depleted of endogenous CaM by washing the membrane pellet with buffer A containing 1 mM EGTA, followed by centrifugation at 30000*g* for 20 min. The washed membranes were resuspended in buffer A prior to adenylyl cyclase assays.

Adenylyl Cyclase Assay. The enzyme assay was performed at 30 °C for 25 min by adding membrane fractions (10–15 μg of protein) to the assay solution containing 1 mM [α-^{32}P]ATP (500 cpm/pmol), ^{3}H-labeled cyclic AMP (20,000 cpm/μmol), 5 mM $MgCl_2$, 0.2 mM EGTA, 1 mM EDTA, 2 mM cyclic AMP, 5 mM theophylline, 5% bovine serum albumin, 20 mM creatine phosphate, and 100 units/mL creatine phosphokinase in 20 mM Tris-HCl, pH 7.4, in a final assay volume of 250 μL. The reaction was stopped by adding 750 μL of 1.5% sodium dodecyl sulfate. The reaction mixture was heated at 100 °C for 2 min, and ^{32}P-labeled cyclic AMP generated was recovered using Dowex AG-50 WX-4 and neutral alumina columns, as described by Salomon et al. (1974). For every data point, except that shown in Figure 1, adenylyl cyclase activity due to the type I or type III enzyme was determined by subtracting enzyme specific activities in CDM8-transfected cells from those of cells transfected with either CDM8(I-AC) or CDM8(III-AC), respectively. The levels of Ca^{2+}-stimulated enzymatic activity due to endogenous adenylyl cyclase were less than 5% of that contributed by expressed type I or III adenylyl cyclases. When $CaCl_2$ was included in the assay, the concentrations of free Ca^{2+} in the presence of Ca^{2+} chelators were calculated by the method of Piascik et al. (1980). The values of adenylyl cyclase activities represent the mean of triplicate determinations. Protein concentrations in cell membranes were determined by the method of Hill and Straka (1988).

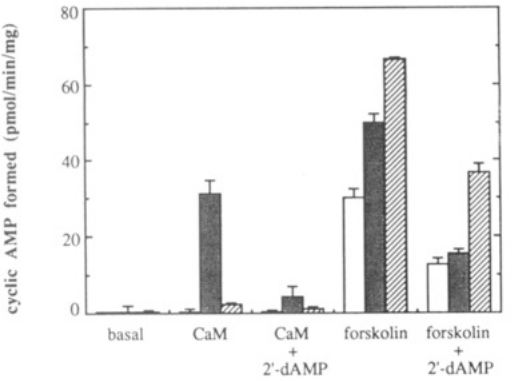

FIGURE 1: Transient expression of type I or type III adenylyl cyclase activity in human kidney 293 cells. Cultured 293 cells were transfected with either CDM8(I-AC) (shaded bars) or CDM8(III-AC) (hatched bars) for transient expression of either type I or type III adenylyl cyclase activity, respectively. The expression vector CDM8 without a cDNA insert (open bars) was used as a control for the DNA transfection. Cell homogenates were prepared from the transfected cells and assayed for adenylyl cyclase activity at 30 °C for 25 min. As indicated, the enzyme assay was performed in the presence of 2.4 μM CaM, 100 μM 2′-dAMP, 10 μM forskolin, or a combination of these effectors. The assay included 1.9 μM free Ca^{2+} when CaM was present.

Results

Transient Expression of Type I and Type III Adenylyl Cyclases in Human 293 Cells. The objectives of this study were to determine if the type III adenylyl cyclase is stimulated by Ca^{2+} and, if so, to compare the Ca^{2+} sensitivity of the type III enzyme to the type I adenylyl cyclase. This was accomplished by expression of each enzyme in 293 cells and analysis of the Ca^{2+} and CaM sensitivity of the expressed enzyme activities. Cultured human 293 cells were chosen as an expression system because it has been demonstrated that these cells are appropriate for expression of type III and type I adenylyl cyclases (Bakalyar & Reed, 1990), and the level of endogenous adenylyl cyclase activity in 293 cells is very low relative to the expressed activities. Most importantly, endogenous adenylyl cyclase activity in 293 cells was not significantly stimulated by Ca^{2+} and CaM, making it possible to analyze the Ca^{2+} sensitivity of the expressed enzyme activities.

Expression of type I and type III adenylyl cyclase in human 293 cells was accomplished using the expression vectors CDM8(I-AC) and CDM8(III-AC), respectively. The addition of CaM and Ca^{2+} did not have a statistically significant effect on adenylyl cyclase activity in cell homogenates from 293 cells transfected with either CDM8 or CDM8(III-AC) (Figure 1). In contrast, CaM and Ca^{2+} stimulated adenylyl cyclase activity in cell homogenates from CDM8(I-AC)-transfected cells. Forskolin stimulated enzyme activities in control cell homogenates and in cells transfected with either CDM8(I-AC) or CDM8(III-AC). We also examined the effects of 2′-dAMP on adenylyl cyclase activities expressed in 293 cells. This nucleotide is a specific adenosine P-site inhibitor of adenylyl cyclase(s) in brain and other tissues (Johnson et al., 1989). At a concentration of 100 μM, 2′-dAMP partially inhibited the forskolin-stimulated enzyme activity in CDM8(III-AC)-transfected cells. Moreover, both the CaM/Ca^{2+}-stim-

Table I: Effects of CaM and Ca^{2+} on Type III Adenylyl Cyclase Transiently Expressed in Human 293 Cells[a]

addition	type III adenylyl cyclase activity[b] [pmol min^{-1} (mg of protein)$^{-1}$]
basal	6 ± 20
Ca^{2+} + CaM	7 ± 4
forskolin	376 ± 11
forskolin + Ca^{2+}	394 ± 7
forskolin + Ca^{2+} + CaM	627 ± 20

[a] Cell membranes from human 293 cells transfected with CDM8 or CDM8(III-AC) were assayed for adenylyl cyclase activity. When indicated, the assay was carried out in the presence of 30 μM free Ca^{2+}, 2.4 μM CaM, 50 μM forskolin, or a combination of these chemicals. Type III adenylyl cyclase activity was calculated by subtracting adenylyl cyclase activities in control cells from those of cells transfected with CDM8(III-AC). [b] The data shown are the mean of triplicate determinations ± S.E.

ulated and the forskolin-stimulated adenylyl cylase activities in CDM8(I-AC)-transfected cells were significantly inhibited by 2′-dAMP. These observations are in agreement with the reported effects of P-site adenosine inhibitors on CaM-stimulated adenylyl cyclase from bovine brain (Yeager et al., 1986; Minocherhomjee et at., 1987; Johnson et al., 1989).

CaM Stimulation of Type I and Type III Adenylyl Cyclases. In order to examine the CaM stimulation of adenylyl cyclase activity expressed in 293 cells, it was necessary to remove endogenous CaM by washing the membranes with EGTA-containing buffers. CaM stimulated the type I enzyme activity in membranes from CDM8(I-AC)-transfected cells at a half-maximal concentration of approximately 20 nM (Figure 2A). Similar CaM sensitivities have been reported for the CaM-sensitive adenylyl cyclase purified from bovine brain (Minocherhomjee et al., 1987) and the type I adenylyl cyclase activity expressed in insect SF9 cells (Tang et al., 1991). Endogenous adenylyl cyclase in control cell membranes was not significantly stimulated by CaM (Figure 2A). In contrast to the type I enzyme, type III adenylyl cyclase was not stimulated by CaM and Ca^{2+} in the absence of other effectors (Figure 2B). However, CaM/Ca^{2+} did stimulate the type III enzyme activity in the presence of 10 μM forskolin. Half-maximal stimulation of the type III adenylyl cyclase occurred at approximately 1 μM CaM in the presence of forskolin. Moreover, forskolin-activated type III enzyme activity was further stimulated by Ca^{2+} only in the presence of CaM, suggesting that CaM mediates Ca^{2+} regulation of type III adenylyl cyclase (Table I).

The sensitivity of the type III adenylyl cyclase to CaM was also examined as a function of GppNHp, a nonhydrolyzable GTP analogue that persistently activates G proteins (Gilman, 1987). At an optimum GppNHp concentration of 100 μM, CaM stimulated the type III enzyme approximately 2-fold (Figure 3). The half-maximal concentration for GppNHp stimulation (5–10 μM) was not affected by the presence of CaM. These data indicate that the type III adenylyl cyclase is sensitive to CaM, but only when the enzyme is activated by other effectors such as GppNHp or forskolin.

Forskolin Sensitivity of Type I or Type III Adenylyl Cyclase. Type I and type III adenylyl cyclase activities expressed in 293 cells were both stimulated by forskolin; however, their sensitivities to forskolin were quite different (Figure 4). Half-maximal stimulations of type I and type III adenylyl cyclase occurred at about 2 and 20 μM forskolin, respectively. CaM stimulation of the type I adenylyl cyclase was independent of forskolin concentrations. In contrast, CaM stimulation of the type III enzyme was dependent upon the presence of forskolin.

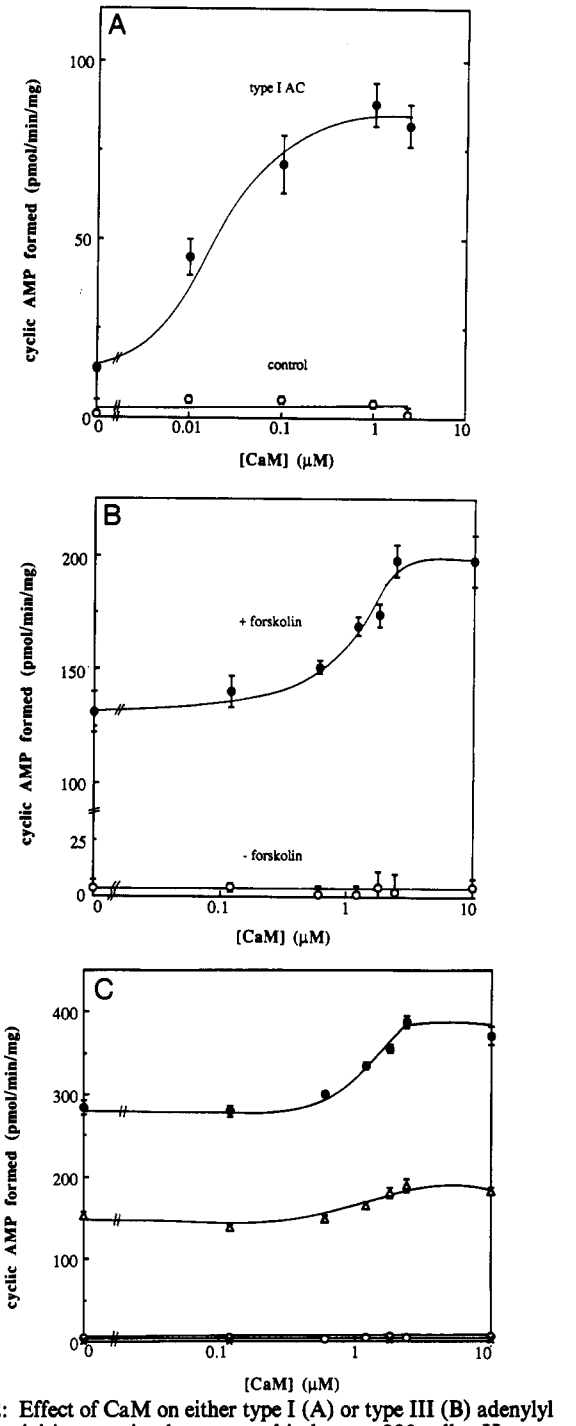

FIGURE 2: Effect of CaM on either type I (A) or type III (B) adenylyl cyclase activities transiently expressed in human 293 cells. Human 293 cells were transfected with control CDM8 vector, CDM8(I-AC), or CDM8(III-AC) as described in Experimental Procedures. Cell membranes prepared from transfected cells were washed with buffer A containing 1 mM EGTA to remove endogenous CaM and then assayed for adenylyl cyclase activity as a function of CaM concentration. (Panel A) Effect of CaM on control (○) or type I (●) adenylyl cyclase activity. The assay was performed in the presence of 1.9 μM free Ca^{2+} and varying concentrations of CaM. Control activity was the adenylyl cyclase activity in cell membranes from CDM8-transfected 293 cells. (Panel B) Effect of CaM on type III adenylyl cyclase activity in the absence (○) or in the presence (●) of 10 μM forskolin. The enzyme assay was performed in the presence of 30 μM free Ca^{2+} and varying concentrations of CaM. Adenylyl cyclase activities due to the type III enzyme were calculated from data shown in panel C by subtracting adenylyl cyclase activities in control cells from those of cells transfected with CDM8(III-AC). In panel C, cell membranes from control 293 cells (×, △) or cells transfected with CDM8(III-AC) (○, ●) were assayed for adenylyl cyclase activity with varying concentrations of CaM and 30 μM Ca^{2+} in the absence (×, ○) or presence (△, ●) of 10 μM forskolin.

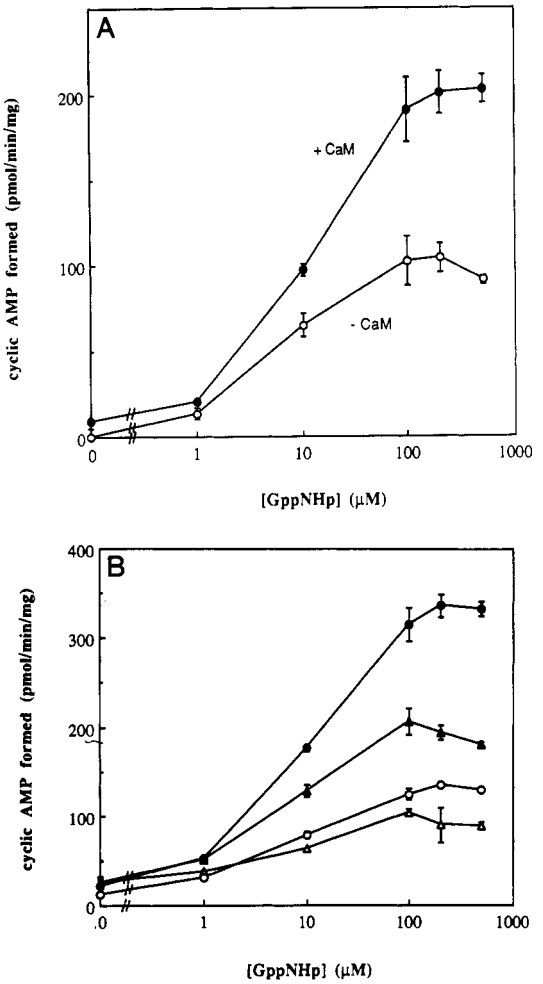

FIGURE 3: Effect of GppNHp on type III adenylyl cyclase activity. Cell membranes from 293 cells transfected with control CDM8 vector or CDM8(III-AC) were assayed for adenylyl cyclase activity. (Panel A) Effect of GppNHp on type III adenylyl cyclase activity either in the absence (○) or in the presence (●) of 30 μM free Ca^{2+} and 2.4 μM CaM. Type III adenylyl cyclase activity was calculated from data shown in panel B by subtracting adenylyl cyclase activities in control cells from those of cells transfected with CDM8(III-AC). In panel B, cell membranes from control cells (△, ○) or cells transfected with CDM8(III-AC) (▲, ●) were assayed for adenylyl cyclase activity with varying concentrations of GppNHp in the absence (△, ▲) or presence (○, ●) of 30 μM free Ca^{2+} and 2.4 μM CaM.

Ca^{2+} Sensitivity of Type III Adenylyl Cyclase. The differences in CaM sensitivity exhibited by type I and type III adenylyl cyclases suggested that the two enzymes may have significantly different Ca^{2+} sensitivities because there is generally energy coupling between the binding of Ca^{2+} and CaM-binding proteins to CaM (Olwin & Storm, 1985). Therefore, the activity of each enzyme was examined as a function of free Ca^{2+} in the presence of saturating CaM. Neither enzyme was stimulated by CaM in the absence of Ca^{2+}, suggesting that Ca^{2+} directly mediates CaM regulation of both enzymes (Figure 5). Half-maximal stimulation of type I adenylyl cyclase occurred at approximately 0.05 μM free Ca^{2+} (Figure 5A), which is consistent with the Ca^{2+} sensitivity of the CaM-stimulated adenylyl cyclase isolated from bovine brain calculated from the data of Westcott et al. (1979) and reported by Piascik et al. (1980). Endogenous adenylyl cyclase in control 293 cell membranes showed little stimulation by free Ca^{2+}. Type III adenylyl cyclase activity was stimulated by Ca^{2+} in the presence of 2.4 μM CaM and 100 μM GppNHp (Figure 5B). Half-maximal Ca^{2+} stimulation of type III adenylyl cyclase was at about 5 μM. Similar results were

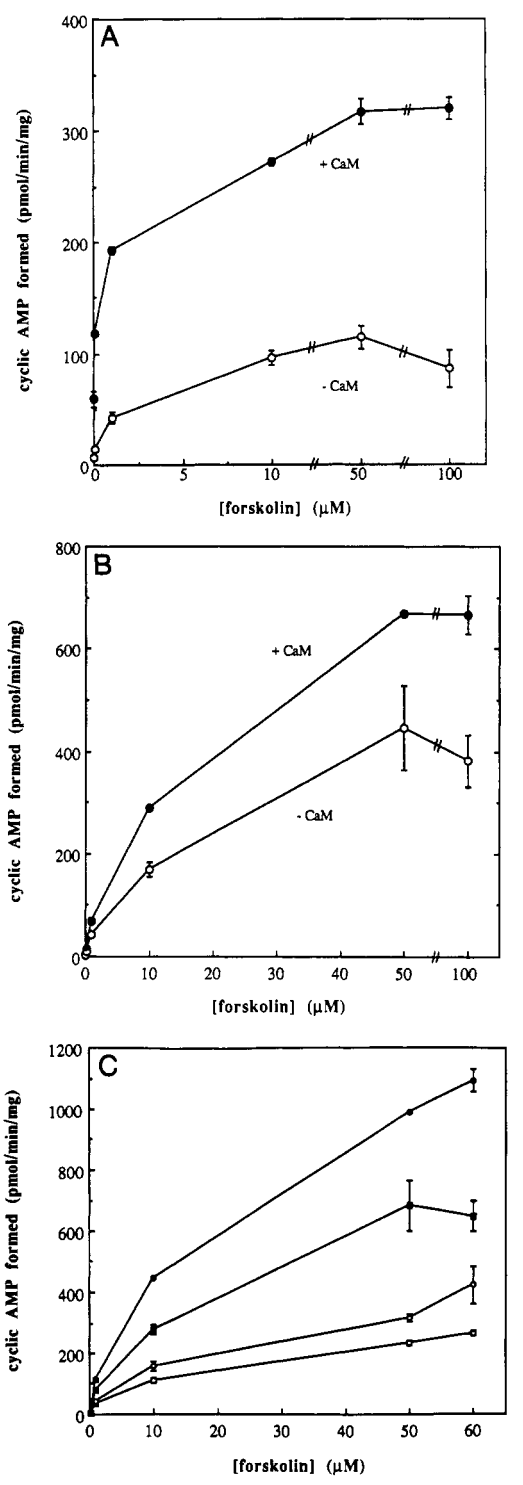

FIGURE 4: Effect of forskolin on type I (A) or type III (B) adenylyl cyclase activities. Human 293 cells were transfected with CDM8, CDM8(I-AC), or CDM8(III-AC). Cell membranes from the transfected cells were assayed for adenylyl cyclase activity in the presence of varying concentrations of forskolin. (Panel A) Effect of forskolin on type I adenylyl cyclase activity examined in the absence (○) or in the presence (●) of 1.9 μM free Ca^{2+} and 2.4 μM CaM. Type I adenylyl cyclase activity was calculated as described in Experimental Procedures. (Panel B) Effect of forskolin on type III adenylyl cyclase activity in the absence (○) or in the presence (●) of 30 μM free Ca^{2+} and 2.4 μM CaM. Type III adenylyl cyclase activity was calculated from data shown in panel in panel C by subtracting adenylyl cyclase activities in control cells from those of cells transfected with CDM8(III-AC). In panel C, cell membranes from control cells (□, ○) or cells transfected with CDM8(III-AC) (■), ●) were assayed for adenylyl cyclase activity with varying concentrations of forskolin in the absence (□, ■) or presence (○, ●) of 30 μM free Ca^{2+} and 2.4 μM CaM.

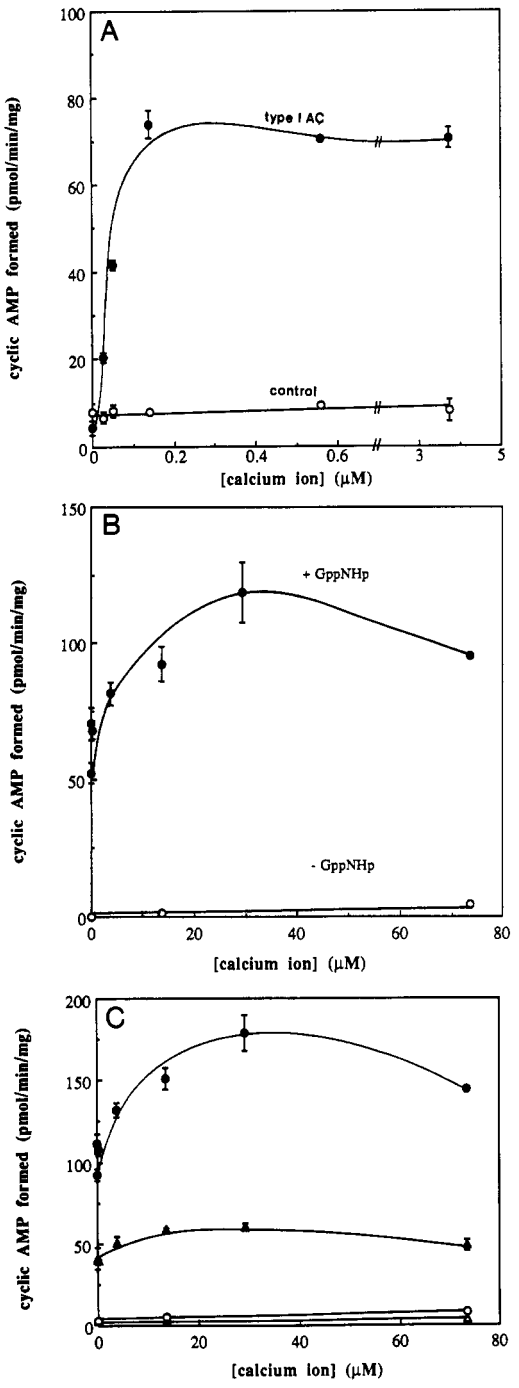

FIGURE 5: Effect of Ca^{2+} on type I (A) or type III (B) adenylyl cyclase activities expressed in 293 cells. Cell membranes from human 293 cells transfected with CDM8, CDM8(I-AC), or CDM8(III-AC) were examined for adenylyl cyclase activity as a function of free Ca^{2+} concentration by varying concentrations of $CaCl_2$ in the presence of 0.2 mM EGTA in the assay. The concentration of free Ca^{2+} for each addition of $CaCl_2$ was calculated by the general method of Piascik et al. (1980). (Panel A) Effect of Ca^{2+} on control (○) or type I (●) adenylyl cyclase activity. The enzyme assay was performed in the presence of 2.4 μM CaM and various cencentrations (0–3.7 μM) of free Ca^{2+}. Type I adenylyl cyclase activity was calculated as described in Experimental Procedures. (Panel B) Effect of Ca^{2+} on type III adenylyl cyclase activity in the absence (○) or in the presence (●) of 100 μM GppNHp. The enzyme assay was performed in the presence of 2.4 μM CaM and various concentrations (0–74 μM) of free Ca^{2+}. Type III adenylyl cyclase activity was calculated from data shown in panel C by subtracting adenylyl cyclase activities in control cells from those of cells transfected with CDM8(III-AC). In panel C, cell membranes from control cells (△, ▲) or cells transfected with CDM8(III-AC) (○, ●) were assayed for adenylyl cyclase activity with varying concentrations of free Ca^{2+} and 2.4 μM CaM in the absence (△, ○) or presence (▲, ●) of 100 μM GppNHp.

observed when we examined the Ca^{2+} sensitivity of type III enzyme in the presence of CaM and forskolin.[2] Although the type III adenylyl cyclase exhibited CaM-dependent stimulation by Ca^{2+}, its sensitivity to Ca^{2+} was approximately 100 times less than that of the type I enzyme.

Discussion

There is now convincing evidence for a family of adenylyl cyclases with different regulatory properties. This was first directly demonstrated by the separation of CaM-sensitive and CaM-insensitivie adenylyl cyclases using CaM-Sepharose affinity chromatography (Westcott et al., 1979) and by the isolation of antibodies that distinguished between these two forms of the enzyme (Rosenberg & Storm, 1987; Mollner & Pfeuffer, 1988). The isolation of cDNA clones encoding type I CaM-sensitive (Krupinski et al., 1989) and type II CaM-insensitive adenylyl cyclase (Tang et al., 1991) has confirmed the existence of CaM-sensitive and CaM-insensitive adenylyl cyclases. cDNA clones have now been isolated for a minimum of four distinct mammalian adenylyl cyclases (Tang et al., 1991) and the number of members in this family of enzymes is likely to grow. The diversity of this enzyme system undoubtedly reflects the need for different mechanisms for regulation of cyclic AMP levels in animal cells and the variety of physiological processes that are regulated by intracellular cyclic AMP.

The existence of Ca^{2+}-stimulated adenylyl cyclases in animal cells allows coordination of the Ca^{2+} and cyclic AMP signal transduction systems. This coupling mechanism is thought to be an important regulatory mechanism in neurons, and there is evidence that Ca^{2+}-stimulated adenylyl cyclases may play an important role in learning and memory (Kandel & Schwartz, 1982; Walters & Byrne, 1983). For examople, rutabaga, a memory mutant of *Drosophila melanogaster*, is deficient in CaM-sensitive adenylyl cyclase activity (Livingstone et al., 1984), and the gene for the CaM-sensitive enzyme maps within a region on the X chromosome that includes the *rut* locus (Krupinski et al., 1989). Furthermore, mRNA for the type I adenylyl cyclase is expressed in areas of rat brain associated with learning and memory (Xia et al., 1991). Since type III adenylyl cyclase is highly enriched in olfactory neurons (Bakalyar & Reed, 1990) and the olfactory system exhibits Ca^{2+}-sensitive adenylyl cyclase activity (Anholt & Rivers, 1990), it was the objective of this study to determine if type III adenylyl cyclase is also regulated by Ca^{2+}.

Purification of specific forms of mammalian adenylyl cyclases has been very difficult and only the type I enzyme has been purified to homogeneity (Yeager et al., 1985; Smigel, 1986). The availability of cDNA clones for distinct adenylyl cyclases and expression of their activities in animal cells have made it possible to examine the properties of each enzyme (Krupinski et al., 1989; Tang et al., 1991; Bakalyar & Reed, 1990). Prior to this study, the only documented Ca^{2+}-sensitive mammalian adenylyl cyclase was the type I enzyme (Tang et al., 1991). The data reported in this study establish that type III adenylyl cyclase is stimulated by Ca^{2+} with half-maximal stimulation at 5 μM free Ca^{2+}. Ca^{2+} stimulation of the enzyme is apparently mediated by CaM since the Ca^{2+} stimulation required the presence of CaM. Quite interestingly, Ca^{2+} activation of the type III adenylyl cyclase was only seen in the presence of forskolin or an activator of G proteins, GppNHp. One possible interpretation of these data is that the enzyme is stimulated by Ca^{2+} only when it is activated by other ef-

[2] E. J. Choi and D. R. Storm, unpublished observations.

fectors (e.g., activated G_s or G_{olf}). Presumably, conformational changes in the catalytic subunit induced by these effectors influence its interactions with CaM/Ca^{2+}. Alternatively, Ca^{2+}/CaM may stimulate type III enzyme in the absence of other effectors but the stimulated enzyme activity might be below a detectable level under our assay conditions.

Although type I and type III adenylyl cyclases show some sequence homology and similar hydropathy plots, there are significant sequence differences between the two enzymes, particularly in the membrane-spanning regions (Bakalyar & Reed, 1990). Type I and type III adenylyl cyclases were both stimulated by Ca^{2+} and CaM; however, there are some very significant differences between the Ca^{2+} sensitivities of the two enzymes. Whereas type I adenylyl cyclase was half-maximally stimulated at approximately 0.05 μM free Ca^{2+}, the calcium sensitivity of type III was about 100-fold less. In addition, the two enzymes differed considerably in their sensitivity to CaM, with type I being approximately 10-fold more sensitive to CaM than type III. In contrast to type III adenylyl cyclase, type I adenylyl cyclase was stimulated by Ca^{2+} and CaM in the absence of other effectors. This suggests that the type I enzyme may directly couple increases in intracellular Ca^{2+} to cyclic AMP elevations, regardless of the presence of other activators of the enzyme.

Is Ca^{2+} regulation of the type III adenylyl cyclase at concentrations of 1–10 μM Ca^{2+} physiologically significant, and if so, what role could it play in signal transduction pathways in olfactory sensory neurons? Free Ca^{2+} in many animal cells generally varies from less than 0.1 to 10 μM. Local Ca^{2+} concentrations at membrane surface in neurons may rise up to 100 μM or even higher during action potentials (Smith & Augustine, 1988). Since the type III adenylyl cyclase is only activated at the higher end of the free Ca^{2+} range and when the enzyme is activated by other effectors, the enzyme may allow Ca^{2+} amplification of cyclic AMP signals. For example, the existence of cyclic AMP gated ion channels in olfactory sensory neurons (Nakamura & Gold, 1987) suggests that initial cyclic AMP signals, generated through olfactory receptors coupled to adenylyl cyclase, may be further amplified by increases in intracellular Ca^{2+}. Regardless of the physiological function of this enzyme, the data in this study demonstrate the existence of at least two distinct Ca^{2+}-stimulated adenylyl cyclases with different sensitivities to Ca^{2+}.

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Registry No. AC, 9012-42-4; Ca^{2+}, 7440-70-2; forskolin, 66575-29-9.

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Ability of Different Chemically Modified Heparins To Potentiate the Biological Activity of Heparin-Binding Growth Factor 1: Lack of Correlation with Growth Factor Binding

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ABSTRACT: A range of chemically modified heparins was examined for their ability to bind heparin-binding growth factor 1 (HBGF-1; acidic fibroblast growth factor) and potentiate the in vitro mitogenic and neurotrophic activity of HBGF-1. It was found that carboxyl-reduced heparin bound HBGF-1 as effectively as the native heparin molecule. Totally desulfated heparin and N-desulfated heparin lack HBGF-1-binding capacity, and substitution of the exposed amino group with acetyl or acetoacetyl groups only partially restored binding capacity, indicating that *N*-sulfates only play a limited role in growth factor binding. However, the failure of totally desulfated, N-resulfated heparin to interact with HBGF-1 demonstrated that *N*-sulfates alone are insufficient and ester sulfates are absolutely essential for HBGF-1 binding. In contrast, the ability of the modified heparins to potentiate the mitogenic activity of HBGF-1 correlated only to a limited extent with their affinity for HBGF-1. Thus, the carboxyl-reduced molecule which displayed similar affinity for HBGF-1 as native heparin was consistently less potent in augmenting mitogenesis. Similarly, the N-acetylated and the N-acetoacetylated species, which had much lower affinity for HBGF-1 than the carboxyl-reduced molecule, conferred similar biological activity to HBGF-1 whereas N-desulfated heparin, which was unable to bind growth factor, potentiated the mitogenic activity of HBGF-1 for both 3T3 and HUVE cells. In contrast, the neurotrophic activity of HBGF-1 was potentiated by modified heparin species which failed to bind HBGF-1 and were without activity in the mitogenic assays. In fact, native heparin was much less effective at potentiating the neurotrophic activity of HBGF-1 than several of the modified heparins. Thus, heparin exerts its effects not only by binding of HBGF-1 but also by mechanisms independent of its binding activity probably via cell-surface heparin receptors.

Heparin and heparan sulfate proteoglycans (HSPG)[1] are involved in a range of biological functions, including cell–cell (Cole et al., 1986) and cell–substratum (Culp et al., 1980) adhesion, cellular proliferation and differentiation (Fritze et al., 1985), neurite outgrowth (Hantaz-Ambroise et al., 1987), synaptic function (Anderson & Fambrough, 1983), myelination (Carey et al., 1987), matrix assembly (Laurie et al., 1986), in vivo coagulation (Marcum & Rosenberg, 1989), and capillary permeability (Farquhar, 1981).

Heparin will bind to—and in many cases alter the biological activity of—a number of protein and glycoprotein ligands. Heparin is now known to potentiate the mitotic (Schreiber et al., 1985), chemotactic (Terranova et al., 1985), neurotrophic (Unsicker et al., 1987), and angiogenic (Lobb et al., 1985) properties of the pure acidic mitogen heparin-binding growth factor 1 (HBGF-1; also known as acidic fibroblast growth factor or aFGF), and heparin affinity chromatography forms the basis of the purification of HBGF-1 (Maciag et al., 1984).

The binding of heparin to HBGF-1 acts to potentiate its biological activity (Burgess & Maciag, 1989). Indirect evidence for such a mechanism has been derived from experiments that show heparin protects HBGF-1 against proteolytic attack (Lobb, 1988), as well as acid and heat denaturation

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[1] Abbreviations: DMEM, Dulbecco's modified Eagle's medium; HBGF-1, heparin-binding growth factor 1; HSPG, heparin and heparan sulfate proteoglycan(s); HUVE, human umbilical vein endothelial cell(s).

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