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Calmodulin Plays a Dominant Role in Determining Neurotransmitter Regulation of Neuronal Adenylate Cyclase

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Ca^{2+}, through the mediation of calmodulin, stimulates the activity of brain adenylate cyclase. The growing awareness that fluctuating Ca^{2+} concentrations play a major role in intracellular signalling prompted the present study, which aimed to investigate the implications for neurotransmitter (receptor) regulation of enzymatic activity of this calmodulin regulation. The role of Ca^{2+}/calmodulin in regulating neurotransmitter-mediated inhibition and stimulation was assessed in a number of rat brain areas. Ca^{2+}/calmodulin stimulated adenylate cyclase activity in EGTA-washed plasma preparations from each region studied—from 1.3-fold (in striatum) to 3.4-fold (in cerebral cortex). The fold-stimulation produced by Ca^{2+}/calmodulin was decreased in the presence of GTP, forskolin, or Mn^{2+}. In EGTA-washed membranes, receptor-mediated inhibition of adenylate cyclase was strictly dependent upon Ca^{2+}/calmodulin stimulation in all regions, except striatum. A requirement for Mg^{2+} in combination with Ca^{2+}/calmodulin to observe neurotransmitter-mediated inhibition was also observed. In contrast, receptor-mediated stimulation of activity was much greater in the absence of Ca^{2+}/calmodulin. The findings demonstrate that ambient Ca^{2+} concentrations, in concert with endogenous calmodulin, may play a central role in dictating whether inhibition or stimulation of adenylate cyclase by neurotransmitters may proceed.

Key words: rat brain, hormones, calcium, cAMP

Ca^{2+} acts as a second messenger in many situations through the mediation of the Ca^{2+}-binding protein calmodulin [1]. Calmodulin is a key sensing device which can translate the signal of alterations in intracellular Ca^{2+} concentrations into modification of the activity of intracellular enzymes. These enzymes are often critical

Abbreviations used: GPPNHP, 5′-guanylylimidodiphosphate; GABA, γ-aminobutyric acid; EGTA, ethyleneglycolbis β-aminoethylether)-N,N′-tetraacetate; PIA, N^6-phenylisopropyladenosine; N_s, N_i, GTP-regulatory proteins that mediate stimulation and inhibition, respectively, of the catalytic unit of adenylate cyclase (C).

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regulators of cellular activity. They include calmodulin-dependent protein kinase [2,3], calmodulin-regulated cyclic AMP-phosphodiesterase [4], and adenylate cyclase [5–7]. Calmodulin is present in high concentrations (ca. 10 μM) in mammalian brain [8,9]; consequently, it is to be expected that the coexistence of neurotransmitters that can elevate intracellular Ca^{2+} and enzymes whose activity are regulated by calmodulin would lead to significant regulatory interactions. In the case of neuronal adenylate cyclase, the implications of its being subject to fluctuating Ca^{2+} concentrations on its activity have never received detailed experimental attention. Although the enzyme was first described to be calmodulin-regulated in 1975 [5,6] and the calmodulin-stimulated form has now been purified to apparent homogeneity [10], the implications of this regulation have not yet been fully evaluated with respect to neurotransmitter regulation of its activity. In nonneuronal tissues adenylate cyclase is regulated primarily by the actions of stimulatory and inhibitory receptors that are coupled via distinct GTP regulatory complexes to the enzyme [11,12]. However, in neuronal systems, the stimulation of activity that is elicited by neurotransmitters is modest by comparison with what is observed in peripheral systems (50–100% compared to 5–20-fold). Previous studies by Gnegy's group [13,14] indicate that some synergism occurs between the effects of dopamine, acting through D1-receptors, and calmodulin in stimulating the activity of neuronal adenylate cyclase. On the other hand, earlier studies from this laboratory indicated that, at least in the case of the regulation of activity that was mediated by nonhydrolyzable guanine nucleotide analogs, such as GPPNHP[2], calmodulin stimulation reduced the stimulation that could be elicited by guanine nucleotides, whereas the opposite was true for the inhibitory effects of these nucleotides; ie, calmodulin enhanced the inhibitory effects of GPPNHP [15,16]. The present studies were undertaken with the goal of attempting to predict—within a physiological context—the relevance of calmodulin regulation of neuronal adenylate cyclase to the ability of neurotransmitters either to stimulate or inhibit the enzyme.

MATERIALS AND METHODS

Preparation of Plasma Membranes

The protocol for preparation of rat plasma membranes has been previously described [15–18]. Freshly dissected cerebella, striata, hippocampi, and cortices from male Sprague-Dawley rats (170–250 gm) were placed in a buffer containing 50 mM Tris (pH 7.4), 1 mM dithiothreitol, 0.1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 6 U/ml aprotinin, and 1 μg/ml trypsin inhibitor, to which 10% sucrose (w/w) was added. The tissue was homogenized by hand and the suspension centrifuged at 121g for 10 min; the pellet was discarded. Subsequently, the supernatant was centrifuged at 12,000g for 10 min. The supernatant was discarded, and the pellet was resuspended in preparation buffer (without sucrose), plus 0.5 M NaCl. This suspension was placed on ice for 10 min before centrifugation at 12,000g for 10 min. The pellet was resuspended in preparation buffer without sucrose and centrifuged at 12,000g for 10 min. This pellet was resuspended in a small volume of preparation buffer containing sucrose (10%); plasma membranes were collected from sucrose gradients and diluted approximately fourfold with preparation buffer without sucrose and centrifuged at 12,000g for 10 min. The pellet was resuspended in the same buffer at 0.5–1 mg protein per ml. Aliquots were frozen and stored in liquid nitrogen.

Adenylate Cyclase Assay

In order to remove endogenous Ca^{2+} and calmodulin, extensive washes of the membranes were employed prior to the adenylate cyclase assay. Frozen membranes were thawed and diluted threefold with buffer consisting of 50 mM Tris (pH 7.4), 1 mM EGTA, and 1 mg/ml BSA. This suspension was centrifuged at 12,000g for 10 min. The supernatant was discarded, and the pellet was resuspended in 3 ml of the above buffer. This process was repeated twice (for a total of three washes). Following the third centrifugation, the pellet was suspended in 40 mM Tris (pH 7.4), 0.8 mM EGTA, and 0.8 mg/ml BSA to give a final EGTA concentration of 200 μM in the adenylate cyclase assay. In the hippocampus, the actions of calmodulin were inferred by performing the experiments in the presence or absence of 50 μM EGTA [15].

Adenylate cyclase activity was measured by the method of Salomon et al [19]. The assay mixture contained α-[^{32}P]ATP (1 μCi), 0.1 mM cyclic AMP, 80 mM Tris (pH 7.4), 20 mM NaCl, 10 μM GTP, 1 U/ml adenosine deaminase, 4 mM creatine phosphate, 25 U/ml creatine phosphokinase, and 0.2 mg/ml BSA. ATP concentrations varied with the brain region, ie, 200 μM in cerebellum and striatum, 100 μM in hippocampus, and 25 μM in cortex. In most cases, 10 μg of membrane protein, as determined by the method of Lowry et al [20], was assayed. Generally, reactions were conducted for 10 min at 24°C and were stopped by the addition of 0.1 ml of 2% sodium lauryl sulfate containing 1.3 mM cyclic AMP and 49 mM ATP. Recovery was monitored with 15,000 cpm of ^{3}H-cyclic AMP.

Determination of Free Divalent Cation Concentration

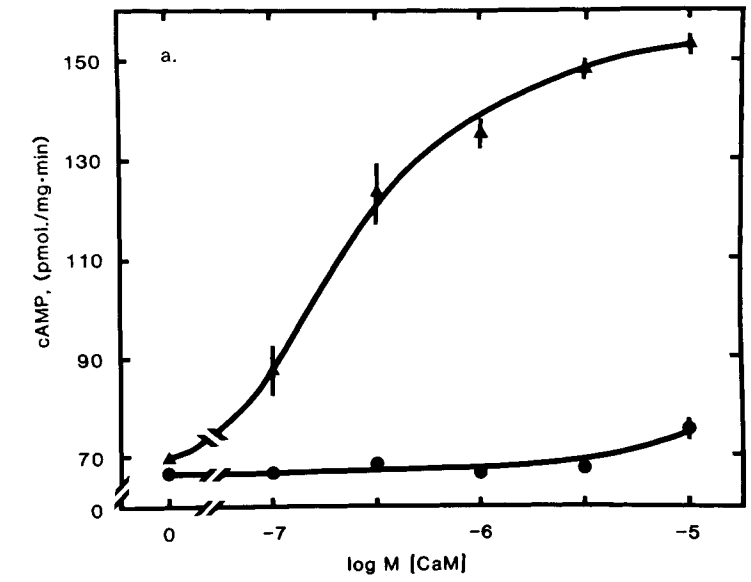
Free concentrations of Ca^{2+} were calculated as previously described [15,18] by using an iterative computing procedure for the solution of the equations describing the complexes formed in a mixture comprising the ingredients involved in the assay of adenylate cyclase that can affect free divalent cation concentration.

Materials

Calmodulin (from bovine brain, activity reported to be greater than 40,000 U/mg protein), GTP, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, BSA (Cohn fraction V), adenosine deaminase (1,500 U/ml, from calf intestinal mucosa, in glycerol), leupeptin, and trypsin inhibitor were from Sigma. Aprotinin was from Calbiochem, and pepstatin, GPPNHP, and PIA were from Boehringer-Mannheim.

RESULTS AND DISCUSSION

The first requirement in evaluating the role of calmodulin in the regulation of adenylate cyclase by neurotransmitters is a preparation in which a clear calmodulin and Ca^{2+} dependency can be demonstrated. Calmodulin antagonists, such as calmidazolium and trifluoperazine, can be useful in this regard, but their potency is variable depending on the endogenous calmodulin content of a particular preparation. We have recently demonstrated [18] that by depleting the endogenous calmodulin content of brain membranes and restoring a finite quantity of the protein, potent antagonism by such agents is demonstrable. In the present case, we have investigated the effect of adding calmodulin, or Ca^{2+}, to calmodulin-depleted cerebellar plasma membranes. Increasing calmodulin concentrations progressively stimulate adenylate cyclase activity (in the presence of 1.3 μM Ca^{2+}) by twofold (Fig. 1a). At a fixed calmodulin



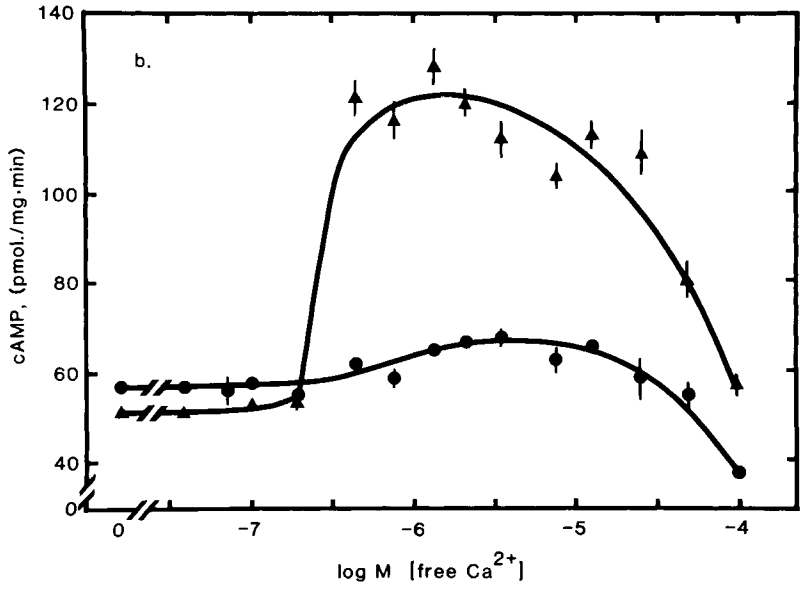


Fig. 1. Effects of Ca^{2+}/calmodulin on adenylate cyclase activity from EGTA-washed rat cerebellar plasma membranes. **a:** Calmodulin dose-response curves in the presence (▲) or absence (●) of 1.3 μM Ca^{2+}. **b:** Ca^{2+} dose-response curves in the presence of (▲) or absence (●) of 1 μM calmodulin. Cerebellar plasma membranes were prepared as described in Materials and Methods.

concentration, increasing Ca^{2+} concentrations stimulate activity by 2.4-fold between 0.4 and .8 μM Ca^{2+}. Following this stimulation, activity declines (Fig. 1b). The lack of any stimulation by Ca^{2+} in the absence of added calmodulin testifies to the effectiveness of the depletion of endogenous calmodulin by the EGTA washing procedure. The Ca^{2+} concentrations that are effective in stimulating adenylate cyclase activity (in the presence of calmodulin) are very much in the appropriate range for that which is achieved physiologically upon either neuron depolarization or the hydrolysis of phosphatidylinositolbisphosphate. The sharp rise in activity that is observed over a very narrow Ca^{2+} concentration range presumably occurs as a consequence of the cooperative binding of 4 Ca^{2+} ions [1] to calmodulin, which results in its activation of adenylate cyclase. Such cooperative interactions would ensure a rapid response to small changes in ambient Ca^{2+} concentrations. The inhibition that is evoked by high Ca^{2+} concentrations is a general effect of Ca^{2+} on adenylate cyclase from all tissues and is not dependent on calmodulin, but presumably it reflects competitive interaction for Mg^{2+} regulatory sites.

The implications of calmodulin stimulation for regulation by an endogenous receptor-mediated stimulation were examined in the experiment described in Table I; β-adrenergic receptors are well represented in the cerebellum; consequently the stimulation that could be evoked by calmodulin and isoproterenol (a β-adrenergic agonist) was compared. (It should be noted that greater receptor-mediated stimulation of activity can be elicited in the presence of GPPNHP, rather than GTP. However, receptor-mediated inhibition will not proceed in the presence of GPPNHP [24]. Consequently, for the purposes of attempting to examine both receptor-mediated stimulation and inhibition, as well as for the purpose of preserving a quasi-physiological context in which to compare effects of calmodulin with those mediated by receptors, only GTP was employed in the majority of these studies.) Isoproterenol evoked only a 25% stimulation of activity under assay conditions in which calmodulin evoked a 2.2-fold stimulation in activity (Table I). The stimulation produced by isoproterenol, though modest, was highly significant and not atypical of the stimulation that is commonly encountered in the central nervous system by receptor-mediated regulators of adenylate cyclase. In fact, one of the differences between neuronal and peripheral adenylate cyclase is in the degree of stimulation that can be evoked by receptors in both cases. In neuronal systems, stimulation tends to be maximally 50–100% (depending on the assay conditions that are used) whereas in peripheral cyclase

TABLE I. Effects of Ca^{2+}/Calmodulin on Isoproterenol-Mediated Stimulation of Rat Cerebellar Adenylate Cyclase*

	Adenylate cyclase activity (pmol cAMP/mg protein·min)	
	0.1 μM Ca^{2+}[a]	0.75 μM Ca^{2+}[a]
Control (GTP 10 μM)	116.0 ± 2.5[b]	252.6 ± 6.8
Isoproterenol (100 μM) + GTP (10 μM)	144.2 ± 10.3	261.1 ± 5.6
Control	56.6 ± 1.9	139.2 ± 4.0
Gpp(NH)p (0.3 μM)	73.6 ± 1.9	111.0 ± 3.5

*Rat cerebellar plasma membranes were washed with EGTA as described in Materials and Methods.
[a]Calmodulin concentration was 1 μM.
[b]Values represent mean ± SEM of at least six determinations.

systems, stimulation as high as 20-fold is not uncommon. This discrepancy, coupled with the greater effectiveness of calmodulin compared to the β-adrenergic receptor-mediated effect, suggests that calmodulin regulation in the CNS may supercede receptor-mediated effects. It might be argued that in heterogeneous neuronal tissues, no single stimulatory receptor would be capable of recruiting all of the N_s complexes that could stimulate adenylate cyclase, with the result that comparisons between calmodulin stimulation and that evoked by β-adrenergic stimulation are inappropriate. Consequently, the nonhydrolyzable guanine nucleotide analog, GPPNHP, was investigated to determine whether it would mimic the effects of the neurotransmitter and also whether by recruiting the total pool of stimulatory GTP regulatory proteins that were present in the preparation similar findings would be encountered. The findings with regard to the effects of the stimulatory neurotransmitter were repeated with GPPNHP; ie, in the absence of calmodulin, GPPNHP elicited approximately a 40% stimulation, whereas in the presence of calmodulin, this stimulation was eliminated; in fact, the effect of the guanine nucleotide was converted to an inhibitory effect (Table I). This inhibition appeared to reflect activation of the inhibitory pathway of adenylate cyclase regulation, as explored in the next series of experiments.

The effects of calmodulin regulation of adenylate cyclase on regulation by a neurotransmitter that is linked to the inhibition of adenylate cyclase were examined. The compound baclofen, which is a $GABA_B$ selective agonist, was investigated. In the presence of Ca^{2+}/calmodulin, increasing concentrations of baclofen caused a steady decline in activity (Fig. 2). This result confirms an earlier finding [21]; it is a GTP-dependent (not shown), presumably N_i-mediated regulation of activity. However, in the absence of calmodulin stimulation, baclofen was unable to inhibit activity significantly (Fig. 2).

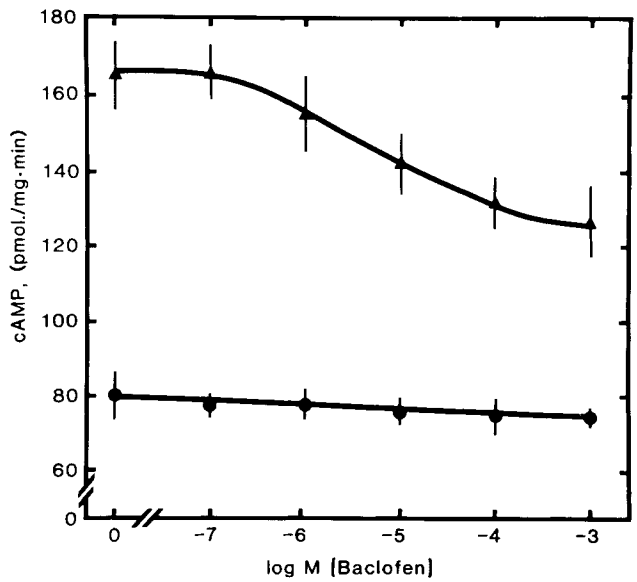


Fig. 2. Effects of Ca^{2+}/calmodulin on the inhibition of adenylate cyclase activity produced by (−)-baclofen in EGTA-washed rat cerebellar plasma membranes. (−)-Baclofen dose-response curves were performed in the absence (●) or presence (▲) of Ca^{2+}/calmodulin (1.3 and 1 μM, respectively).

The effect of baclofen was stereospecific as anticipated for a $GABA_B$-receptor-mediated response. In the presence of Ca^{2+}/calmodulin, the maximum inhibition of activity produced by (−)-baclofen was 23%, while (+)-baclofen (1 mM) inhibited by only 10% (not shown). The native neurotransmitter, GABA (1mM), produced 18% inhibition in the presence of Ca^{2+}/calmodulin (not shown).

Previous studies from this [22] and other [23] laboratories suggest that Mg^{2+} plays an important role in the coupling of receptors to the inhibition of adenylate cyclase. Thus, a potential interaction between Mg^{2+} and Ca^{2+}/calmodulin in regulating the inhibition of adenylate cyclase was examined. A Mg^{2+} dose-response curve was generated in the absence of Ca^{2+}/calmodulin and in the absence or presence of 1 μM PIA (Fig. 3a). Although Mg^{2+} stimulated adenylate cyclase activity in a dose-dependent manner at concentrations from 0.1 to 100 mM (concentrations greater than 100 mM lead to decreases in activity), PIA was unable to inhibit adenylate cyclase activity at any Mg^{2+} concentration. When the Mg^{2+} dose-response curve was repeated in the presence of Ca^{2+}/calmodulin, Ca^{2+}/calmodulin stimulated activity above that which could be elicited by Mg^{2+} alone, at concentrations of Mg^{2+} from 3 to 30 mM (Fig. 3b). Coincident with the Ca^{2+}/calmodulin-dependent stimulation, PIA was able to inhibit adenylate cyclase activity at these Mg^{2+} concentrations. Thus, in order to observe receptor-mediated inhibition of activity, Ca^{2+}/calmodulin must be present in combination with concentrations of Mg^{2+} from 3 to 30 mM.

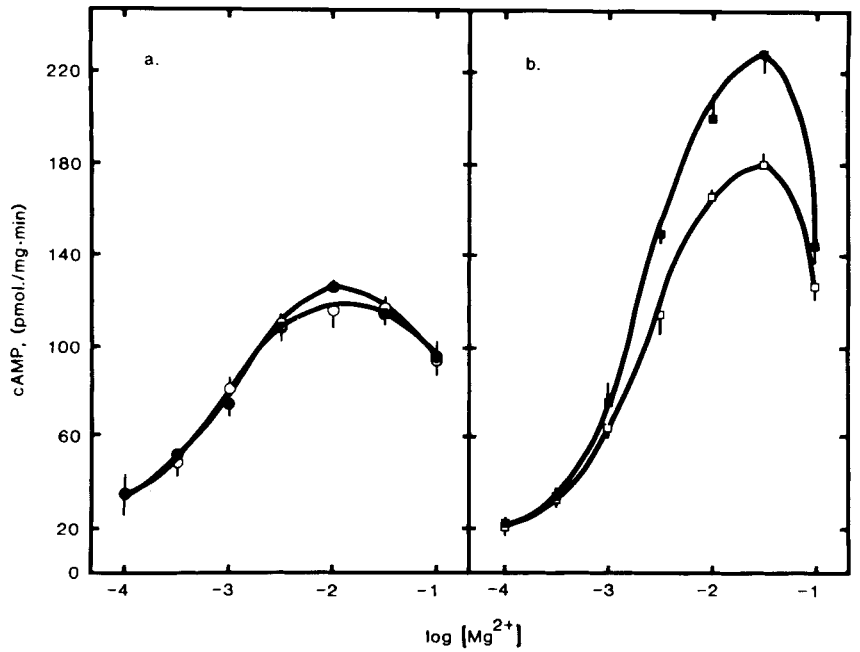


Fig. 3. Effect of Mg^{2+} and Ca^{2+}/calmodulin on the inhibition of adenylate cyclase activity produced by PIA in EGTA-washed rat cerebellar plasma membranes. **a:** Mg^{2+} dose-response curves in the absence of Ca^{2+}/calmodulin and in the absence (●) or presence (○) of 1 μM PIA. **b:** Mg^{2+} dose-response curves in the presence of Ca^{2+}/calmodulin (1.3 and 1 μM, respectively) and in the absence (■) or presence (□) of 1 μM PIA.

Since Ca^{2+}/calmodulin-dependent stimulation of activity could only be observed at Mg^{2+} concentrations greater than or equal to 3 mM (Fig. 3b), it was possible that other agents which promote N_S-C interactions might regulate the stimulation of enzyme activity produced by Ca^{2+}/calmodulin. Therefore, the stimulation produced by Ca^{2+}/calmodulin was determined in the presence or absence of GTP, GTP plus forskolin, and GTP plus Mn^{2+} (Table II). In the absence of any effectors, Ca^{2+}/calmodulin produced a 2.4-fold increase in adenylate cyclase activity. In the presence of GTP, the fold-stimulation produced by Ca^{2+}/calmodulin decreased to 1.9-fold. When forskolin was added with GTP, Ca^{2+}/calmodulin produced stimulation of enzyme activity that approximated that produced in the presence of GTP alone (2.1-fold). When Mn^{2+} was combined with GTP, the stimulation produced by Ca^{2+}/calmodulin decreased further—to 1.6-fold. These data indicate that the relative stimulation produced by Ca^{2+}/calmodulin is decreased in the presence of effectors which are able to promote N_S-C interactions. It could be argued that although the fold-stimulation produced by Ca^{2+}/calmodulin decreases in the presence of these agents, the absolute amount of cAMP produced is increased. For example, in the absence of GTP, a net increase of 79.4 pmol/mg protein·min is produced by Ca^{2+}/calmodulin, while in the presence of GTP this value is increased to 99.4; and in the case of GTP combined with forskolin, the value is increased to 229. However, in the presence of these effectors, the basal activity increases significantly (up to sixfold in the case of GTP plus Mn^{2+}). Thus, the relative stimulation produced by Ca^{2+}/calmodulin in the presence of stimulatory effectors may have less significance in the whole cell.

The data presented thus far were derived exclusively from rat cerebellum. In order to determine whether Ca^{2+}/calmodulin-dependent modulation of neurotransmitter-mediated regulation of adenylate cyclase activity is a more general phenomenon, inhibitory and stimulatory neurotransmitters were tested in the presence and absence of Ca^{2+}/calmodulin in rat hippocampus, striatum, and cortex as well as cerebellum (Table III). Ca^{2+}/calmodulin stimulated adenylate cyclase activity in every region tested, with the degree of stimulation ranging from 1.3-fold in striatum to 3.4-fold in cortex. Inhibition of activity by PIA was observed only in the presence of Ca^{2+}/calmodulin in cortex and cerebellum ($GABA_B$-receptor-mediated inhibition is also Ca^{2+}/calmodulin dependent in cerebellum, Fig. 2). In the hippocampus, the inhibition produced by cyclohexyladenosine and D-ala-met-enkephalin was also strictly dependent on the presence of Ca^{2+}/calmodulin. The striatum was unique in this

TABLE II. Effects of GTP, Forskolin, and Mn^{2+} on Ca^{2+}/CaM-Dependent Stimulation of Rat Cerebellar Adenylate Cyclase*

	cAMP (pmol/mg protein·min)		
	$-Ca^{2+}$/CaM	$+Ca^{2+}$/CaM[a]	Fold-stimulation
Control	56.2 ± 5.5[b]	135.6 ± 9.0	2.41
GTP 10 μM	112.5 ± 4.2	212.1 ± 14.7	1.89
GTP 10 μM + Forskolin 3 μM	199.3 ± 13.4	428.3 ± 7.4	2.15
GTP 10 μM + Mn^{2+}	303.0 ± 16.7	488.4 ± 26.1	1.61

*Rat cerebellar plasma membranes were washed with EGTA as described in Materials and Methods.
[a]Ca^{2+} and CaM concentrations were 1.3 and 1.0 μM, respectively.
[b]Values represent the mean ± standard deviation of at least three determinations.

TABLE III. Calmodulin Regulation of Neurotransmitter-Mediated Inhibition and Stimulation of the Adenylate Cyclase Activities of Various Rat Brain Regions*

	Brain region							
	Hippocampus		Striatum		Cerebellum		Cortex	
	$-Ca^{2+}$/CaM	$+Ca^{2+}$/CaM[a]	$-Ca^{2+}$/CaM	$+Ca^{2+}$/CaM	$-Ca^{2+}$/CaM	$+Ca^{2+}$/CaM	$-Ca^{2+}$/CaM	$+Ca^{2+}$/CaM
Control	184.1[b]	426.2	97.1	127.4	103.1	199.5	45	152
Inhibitory neurotransmitter[c]	166.8	313.7	79.9	108.2	110.6	150.1	80	110
Control[d]	499.5	1,965	60.2	92.7	116.9	252.6	—	—
Stimulatory neurotransmitter[e]	832.5	2,331	110.9	109.2	144.2	261.1	—	—

*Some of the results compiled here are derived from previously published work, ie, hippocampus [15]. The adenylate cyclase assays in different regions were performed under different conditions. For details, see the appropriate references (above) and/or Materials and Methods.

[a]Ca^{2+} and calmodulin concentrations were 1.3 and 1 μM, respectively.

[b]Values represent the mean of at least three determinations (pmol cAMP/mg protein·min). SD were 5% or less of the mean.

[c]Inhibitory neurotransmitters were: hippocampus, 1 μM cyclohexyladenosine—similar results were obtained with 0.1 μM D-ala-met-enkephalin; striatum, 10 μM morphine—similar results were obtained with 1 μM norpropylapomorphine (a dopaminergic agonist) and 100 μM carbachol; cerebellum, 1 μM phenylisoproplyadenosine—similar results were obtained with 1 mM GABA; cortex, 1 μM phenylisopropyl adenosine.

[d]In all regions except cerebellum, different adenylate cyclase assay conditions (eg, higher temperature) were used for testing the effect of stimulatory vs inhibitory neurotransmitters. For hippocampus, see reference 15. In striatum, the assay was performed for 10 min at 30°C with the stimulatory neurotransmitter (dopamine) vs 10 min at 24°C with the inhibitory neurotransmitter (morphine).

[e]Stimulatory neurotransmitters were: hippocampus, 1 μM vasointestinal peptide; striatum, 100 μM dopamine; cerebellum, 100 μM isoproterenol.

respect, in that the inhibition produced by morphine (as well as carbachol, acting via M2 muscarinic-cholinergic receptors, and norpropylapomorphine, acting via dopaminergic D2 receptors) was completely independent of Ca^{2+}/calmodulin. The relatively low degree of stimulation (30% above basal) produced by Ca^{2+}/calmodulin may reflect a diminished role for Ca^{2+}/calmodulin with respect to the regulation of adenylate cyclase in the striatum. Furthermore, since at least two neurotransmitter systems were tested in every region except cortex, it is likely that the regulation of receptor-mediated inhibition by Ca^{2+}/calmodulin is conserved throughout these regions despite the variety of cell types and neurotransmitters that are involved.

In hippocampus, striatum, and cerebellum, the stimulation produced by vasointestinal peptide, dopamine, and isoproterenol, respectively, was much greater in the absence than in the presence of Ca^{2+}/calmodulin. In fact, in cerebral cortex a similar effect is seen, in that PIA, acting through A1-adenosine receptors, inhibits activity in the presence of Ca^{2+}/calmodulin but stimulates activity (acting via A2-adenosine receptors—which are also present in cerebral cortex) in the absence of Ca^{2+}/calmodulin. Thus, it appears that the modulation of neurotransmitter-mediated inhibition and stimulation of adenylate cyclase by calmodulin is a general phenomenon, which occurs in most tissues that possess calmodulin-sensitive adenylate cyclase activity.

CONCLUSIONS

One of the major findings to emerge from these studies is that Ca^{2+} concentrations in the range that are achieved physiologically—ie, near micromolar—are capable of causing a profound stimulation of the activity of adenylate cyclase in all brain regions studied, through the mediation of the endogenous regulator, calmodulin. Generally, this stimulation exceeds that which can be evoked by neurotransmitters that act via receptors that exert their effects through stimulatory GTP regulatory proteins. In addition, upon stimulation of the adenylate cyclase activity by Ca^{2+}/calmodulin, no further stimulation can be evoked by neurotransmitters that act through receptors that are directly linked to the enzyme, although pharmacological agents (such as forskolin) can exert additional stimulation of activity. The opposite situation is encountered when neurotransmitters are considered that act to inhibit the enzyme activity through the mediation of inhibitory GTP regulatory proteins. In this case, inhibition of activity can only be detected when the activity is stimulated by Ca^{2+}/calmodulin, even though the unstimulated levels of activity are quite high when compared to peripheral adenylate cyclase activity, which can be inhibited in the basal state [24]. It thus appears reasonable to propose that ambient Ca^{2+} levels (through the mediation of calmodulin) may have a dominant effect on the ability of neuronal adenylate cyclase to be regulated by neurotransmitter receptors that are directly linked to the enzyme. It is possible that the regulation of receptor-mediated stimulation and inhibition of adenylate cyclase by Ca^{2+}/calmodulin may be restricted to certain cell types or neurotransmitter systems within specific brain regions. This possibility cannot be discounted with respect to calmodulin regulation of receptor-mediated stimulation, since only one neurotransmitter system was tested in the various regions. However, in the case of receptor-mediated inhibition, at least two receptor systems were probed in striatum, hippocampus, and cerebellum with similar results, suggesting that the interaction between calmodulin and inhibitory receptors is conserved throughout discrete brain regions, despite the apparent heterogeneity of cell types and

neurotransmitter systems within each region. The proposal that Ca^{2+}-mobilizing hormones might play a central role in the ability of cAMP levels to be modulated by receptors that directly regulate adenylate cyclase must be tempered with the fact that many neuronal cells also contain a Ca^{2+}/calmodulin-dependent cAMP-phosphodiesterase activity [4], which degrades cAMP and which therefore would also effect cAMP levels. In addition, protein kinase C, which is present in high concentrations in brain, is regulated by Ca^{2+}; in some cases, this enzyme appears capable of removing tonic inhibitory influences of inhibitory GTP regulatory proteins on adenylate cyclase activity [25]. It seems likely, therefore, that the consequences of Ca^{2+}-mobilization for cAMP levels and for their modulation by hormones will vary depending on the balance of calmodulin-regulated adenylate cyclase, phosphodiesterase, and protein kinase C in individual cell types. The important implication of the present studies is that Ca^{2+}/calmodulin—and not neurotransmitters that are directly linked to adenylate cyclase—may play a dominant role in determining cAMP production in neurons.

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