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Ca^{2+}/calmodulin sensitivity may be common to all forms of neural adenylate cyclase

(classical conditioning/cyclic AMP/associative learning)

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ABSTRACT **The Ca^{2+}/calmodulin (CaM)-activated adenylate cyclase has been implicated as playing an important associative role in classical conditioning in both *Aplysia* and *Drosophila*. Studies of the cyclase in mammalian cerebral cortex have suggested that Ca^{2+}/CaM sensitivity is confined to a subpopulation of total cyclase activity. We investigated the properties of cyclase from *Aplysia*, rat, and bovine central nervous system membranes by using CaM-Sepharose chromatography. Although only a minority of total cyclase activity bound to the CaM column, both bound and unbound fractions of cyclase from all three species showed comparable stimulation by Ca^{2+} in the presence of CaM. When solubilized bovine membranes were first depleted of most of their endogenous CaM by prior chromatography, binding to the CaM column was substantially increased and Ca^{2+} stimulation of the unbound fraction was somewhat reduced. However, this reduction in Ca^{2+} sensitivity resulted from the loss of Ca^{2+} sensitivity during prior chromatography, rather than from the more efficient separation of Ca^{2+}-sensitive and -insensitive forms. This finding, together with the fact that we never observed any enrichment for Ca^{2+} sensitivity in the bound fraction over the level in the starting preparation, suggests that the vast majority of the cyclase present in solubilized central nervous system membranes is Ca^{2+}/CaM-sensitive.**

Studies of the neural mechanisms underlying classical conditioning in both *Aplysia* and *Drosophila* have led to the proposal that the Ca^{2+}/calmodulin (CaM)-sensitive adenylate cyclase may serve as one site of associative interaction between inputs from the conditioned and unconditioned stimuli (1–6). Abrams *et al.* (7) recently presented evidence that both Ca^{2+}, the proposed molecular mediator of the conditioned stimulus, and modulatory transmitter, the mediator of the unconditioned stimulus, can activate the same population of CaM-binding adenylate cyclase molecules. In the present study, we ask the question: how much of the total cyclase population in the central nervous system (CNS) is capable of being activated by Ca^{2+}? Earlier work had indicated that Ca^{2+} sensitivity is confined to a fraction of the total adenylate cyclase activity in brain (8, 9). The strongest evidence for heterogeneity of cyclase came from the study of Westcott *et al.* (9), who found that only about 20% of the total adenylate cyclase activity in solubilized bovine brain membranes bound to a CaM-Sepharose column and that only this small bound fraction was capable of being activated by Ca^{2+}.

We have similarly observed that only a small fraction of the total cyclase activity in *Aplysia* CNS, as well as in rat and bovine CNS, binds to a CaM-Sepharose column in the presence of Ca^{2+}. But in contrast to the findings of Westcott *et al.* (9), we find for all three species that both the bound and the unbound fractions show comparable activation by Ca^{2+} in the presence of CaM. Moreover, despite this separation into bound and unbound CaM-Sepharose fractions, Ca^{2+} stimulation of the bound fraction was never enriched over the level of stimulation in the solubilized preparation; such enrichment would be expected if the original population contained a mixture of Ca^{2+}-sensitive and -insensitive cyclases. It is possible to isolate a largely Ca^{2+}-insensitive population of cyclase in the unbound fraction from a CaM-Sepharose column, but only when this column is preceded by other chromatographic methods. Our results suggest that this Ca^{2+}-insensitive population results from the degradation or loss of Ca^{2+} sensitivity during prior chromatography, rather than from the more efficient separation of Ca^{2+}-sensitive and Ca^{2+}-insensitive forms on CaM-Sepharose. Together, these results indicate that the vast majority of cyclase in the CNS can be activated by Ca^{2+}/CaM and furthermore suggest that much or all of the Ca^{2+}-insensitive cyclase that has been described in the literature may be a product of preparative degradation.

METHODS

Tissue Preparation. For each preparation of *Aplysia* neural cyclase, abdominal and ring ganglia were removed from 20 *Aplysia californica* (100–200 g) and homogenized on ice in 10 ml of buffer A [20 mM Hepes, pH 7.6/5 mM $MgCl_2$/5 mM EGTA/1 mM dithiothreitol (DTT) containing phenylmethanesulfonyl fluoride (50 μg/ml) and protease inhibitors (benzamidine, 1 μM; leupeptin, 10 μg/ml; aprotinin, 10 μg/ml)]. The homogenate was filtered through gauze and then centrifuged at 30,000 × *g* for 30 min. All centrifugations, as well as all chromatography, were done at 4°C. The pellet was washed by resuspension in 10 ml of buffer A and recentrifugation. This membrane pellet (≈10 mg of protein) was solubilized on ice for 30 min in 6 ml of buffer B (25 mM Hepes, pH 7.6/1% Lubrol PX/1 mM EDTA/1 mM DTT containing phenylmethanesulfonyl fluoride and protease inhibitors). Unsolubilized material was pelleted by centrifugation at 100,000 × *g* for 1 hr. The supernatant (≈0.5 mg of protein per ml) was made 5 mM in $MgCl_2$ and then frozen in liquid N_2.

For each preparation of rat brain cyclase, the cerebral cortex of a single Sprague–Dawley rat was homogenized in 120 ml of buffer A and centrifuged as above. The first 30,000 × *g* pellet was resuspended and an aliquot (5 mg of protein) was washed three times in buffer A (15 ml per wash). Solubilization was as above.

For the preparation of bovine cerebral cyclase, the cortices of four cow brains were homogenized in a blender at 4°C with 2.5 liters of buffer A and centrifuged as above. One-tenth of this pellet was further washed three times in buffer A (400 ml

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Abbreviations: CaM, calmodulin; CNS, central nervous system; DTT, dithiothreitol; GTP[γS], guanosine 5′-[γ-thio]triphosphate.

per wash) and solubilized immediately in 400 ml of buffer B. The solubilization mixture was stirred for 30 min at 4°C and then centrifuged as above. The supernatant (2.2 mg of protein per ml) was frozen in liquid N_2. For experiments requiring extensive EGTA washing, a small piece (0.3–0.4 g) of the first membrane pellet, which had been frozen in liquid N_2, was thawed in buffer A and washed three to six times in buffer A (30 ml per wash). The final pellet (≈5 mg of protein) was solubilized in 5 ml of buffer B and centrifuged at 100,000 × *g* for 1 hr.

CaM-Affinity Chromatography. Solubilized neural tissue or concentrated column eluate (2–6 ml) was brought to 5 mM $MgCl_2$ and 2 mM $CaCl_2$ and then continuously recirculated for 3 hr over 1 ml of CaM-Sepharose (Pharmacia) that had been prewashed in buffer C (50 mM Hepes, pH 7.6/0.1% Lubrol PX/1 mM EDTA/5 mM $MgCl_2$/1 mM DTT containing protease inhibitors, 0.2 mg of human serum albumin per ml, and 2 mM $CaCl_2$). The resin was washed at 0.25 ml/min with 10–15 ml of buffer C, and then the bound proteins were eluted with buffer C containing 5 mM EGTA and lacking $CaCl_2$. Before assay of Ca^{2+} stimulation, the flow-through and eluate fractions were each brought to 5 mM Ca^{2+} and 5 mM EGTA; Ca^{2+} and EGTA were then reduced to <25 μM by repeated ultrafiltration on Centricon-30 (Amicon) membranes, using buffer D (50 mM Hepes, pH 7.6/0.1% Lubrol PX/1 mM DTT with protease inhibitors).

Affi-Gel Blue Chromatography. We followed the method of Westcott *et al.* (9) for Affi-Gel Blue chromatography. A 6- to 8-ml sample of solubilized bovine cerebral membranes was brought to 5 mM $MgCl_2$ and loaded on 2 ml of Affi-Gel Blue resin (Bio-Rad) that had been prewashed in buffer E (25 mM Hepes, pH 7.6/0.1% Lubrol PX/1 mM EDTA/5 mM $MgCl_2$/1 mM DTT with protease inhibitors). The column was washed at 0.25 ml/min with 10 ml of buffer E containing 3 mM EGTA, followed by 4 ml of buffer E, and bound material was eluted with 10 ml of buffer E containing 1 M KCl, 8 mM ATP, and 16 mM $MgCl_2$. The eluate, containing 20–50% of total applied activity, was pooled and desalted as above to <0.1% of its initial salt concentration, into buffer D.

Gel Permeation HPLC. An 8-ml sample of solubilized bovine cerebral membranes was concentrated to 1 ml by ultrafiltration on Centricon-30. The concentrate (10–15 mg of protein) was injected onto a series of two 7.5 mm × 60 cm TSK-gel columns (LKB), a 63000SW followed by a 64000SW column. The mobile phase contained 10 mM Hepes, pH 7.2/100 mM NaCl/10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)/1 mM $MgCl_2$/1 mM EDTA/2 mM EGTA/0.02% NaN_3; the flow rate was 0.33 ml/min. Adenylate cyclase was eluted in a single peak, just after the void fraction, that was well separated from the CaM peak. Recovery of total applied activity was ≈50%.

Adenylate Cyclase Assays. Cyclase was assayed by the method of Salomon (10), as detailed in ref. 9, except that the reaction mixture included 50 μM cAMP. Unlabeled ATP was 5 or 20 μM for assays of *Aplysia* enzyme and 100 μM for assays of rat or bovine enzyme. All assays were carried out for 10 min in triplicate, at 30°C. For assays of Ca^{2+} stimulation, we used titrated buffers (11) containing 10 mM free Mg^{2+}, and free Ca^{2+} ranging from 0.3 nM to 1 mM. Following the method of Westcott *et al.* (9), elution profiles were routinely assayed in Mn^{2+}. This was particularly important for *Aplysia* cyclase, where the total amount of CNS tissue is limiting, because Mn^{2+} stimulates its activity about 20-fold as compared to Mg^{2+}.

Other Assays. CaM was measured by RIA (New England Nuclear). Protein was determined by the method of Bradford (12) with human serum albumin as the standard.

RESULTS

Fractionation of *Aplysia* Adenylate Cyclase on CaM-Sepharose: Both Bound and Unbound Fractions Are Comparably Stimulated by Ca^{2+}. When solubilized proteins from *Aplysia* nervous-tissue membranes were applied to a CaM-Sepharose column in the presence of Ca^{2+}, 8–35% (mean ± SEM, 19.4 ± 1.9%; n = 17) of the total adenylate cyclase activity bound to the column and could be eluted with EGTA (Fig. 1*A*). Total recovered activity was 94.5 ± 2.9% of the applied activity. Elution of the bound enzyme was not due to the increase in ionic strength with addition of 5 mM EGTA, because a larger increase in ionic strength (by addition of 25 mM KCl) did not elute detectable cyclase activity. Binding was blocked by the addition of bovine CaM (1.8 mg/ml) to the solubilized preparation prior to incubation with CaM-Sepharose, whereas human serum albumin (1.8 mg/ml) did not alter binding. These results indicate that the binding of cyclase to immobilized CaM is due to a specific and Ca^{2+}-dependent interaction.

Despite the observation that only a limited percentage of total cyclase activity bound to CaM-Sepharose, we found that both bound (fraction I) and unbound (fraction II) fractions showed similar stimulation by Ca^{2+} in the presence of 3 μM exogenous CaM (3.1 ± 0.6-fold, fraction I; 2.8 ± 0.2-fold, fraction II, n = 3; Fig. 1*B*). Omitting exogenous CaM from the assay in one experiment did not alter the stimulation of fraction I (3.2-fold), whereas it reduced the stimulation of fraction II to just 1.5-fold.

These results suggest that all of the cyclase in *Aplysia* is Ca^{2+}-sensitive and that despite extensive washing of the membranes in EGTA-containing buffers, fraction I apparently contains enough endogenous CaM to allow near-maximal

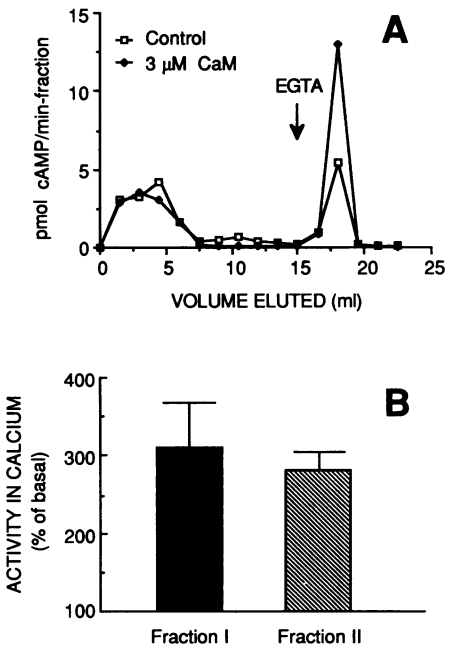

FIG. 1. CaM-affinity chromatography of *Aplysia* adenylate cyclase and Ca^{2+} stimulation of the resulting fractions. (*A*) CaM-Sepharose elution profile. Solubilized *Aplysia* CNS membranes were applied to the column in 2 mM $CaCl_2$ and then eluted with buffer containing 5 mM EGTA (at arrow). Fractions were assayed in the presence of 5 mM $MnCl_2$, 2.5 mM EGTA, and 1 mM $CaCl_2$ with (●) and without (□) 3 μM added CaM. (*B*) Ca^{2+} stimulation (activity in the presence of Ca^{2+} as a percentage of basal activity) of fractions I (flow-through) and II (EGTA eluate); n = 3. CaM was added (3 μM) to desalted fractions and each fraction was assayed in 0.3 nM (basal) or ≈10 μM free Ca^{2+}. Basal activities were 0.91 ± 0.38 and 0.29 ± 0.16 pmol/(min·ml), respectively, for fractions I and II.

stimulation by elevated Ca^{2+}. In support of this interpretation, residual endogenous CaM was measured by RIA to be ≈0.5 μM in fraction I, at least an order of magnitude greater than the CaM in fraction II. This excess CaM in fraction I may be competing with the immobilized CaM to prevent more complete binding to the affinity column.

This substantial amount of endogenous CaM in fraction I also leads to an underestimation of the percentage of cyclase activity bound to the column, because we routinely used Mn^{2+} to assay the elution profiles of CaM affinity columns (9). Since Mn^{2+} can also substitute for Ca^{2+} in activating CaM (13), the cyclase in fraction I, which contains CaM, would be preferentially activated. To compensate for this problem, in one experiment we included 3 μM exogenous CaM in the elution-profile assay and found that fraction II increased from an apparent 32% to 54% of the total eluted activity (Fig. 1*A*).

Ca^{2+}/CaM Sensitivity of Mammalian Brain Adenylate Cyclase. The apparent lack of a Ca^{2+}-insensitive species of adenylate cyclase in *Aplysia* suggested that the cyclase in *Aplysia* differs from the cyclase in mammalian brain in that the latter has generally been believed to comprise separate Ca^{2+}-sensitive and Ca^{2+}-insensitive species (8, 9). We therefore reexamined the heterogeneity of mammalian brain cyclase. Bovine cerebral cortex membranes were extensively washed with EGTA, solubilized, and then chromatographed on CaM-Sepharose. Eleven ± 2.5% of the total eluted activity bound to the column, as assayed with Mn^{2+} and 6 μM CaM, or 4.3 ± 0.9%, as assayed with Mn^{2+} alone (n = 3). In each experiment, recovery from the CaM-Sepharose column exceeded 95% of total applied activity. As was the case for the *Aplysia* enzyme, both fractions of bovine adenylate cyclase showed comparable stimulation by Ca^{2+} in the presence of CaM (maximal stimulation of 7.0 ± 0.1-fold for fraction I and 6.1 ± 0.3-fold for fraction II, n = 3; Figs. 2 and 3*C*). Similar results were obtained for two preparations of rat brain cyclase, where fractions I and II showed, respectively, 11.0 ± 0.8-fold and 9.0 ± 0.6-fold stimulation by Ca^{2+}. When CaM was omitted from the assay of rat brain cyclase, the unbound fraction showed nearly the same maximal stimulation (9.6 ± 1.6-fold, n = 2), indicating that fraction I contains sufficient endogenous CaM for Ca^{2+} to activate the cyclase; in contrast, the second fraction showed only 2.2 ± 0.1-fold Ca^{2+} stimulation in the absence of added CaM. These results suggest that in mammalian brain, as in *Aplysia*, the vast majority of adenylate cyclase can be activated by Ca^{2+}.

Preactivation with Guanosine 5′-[γ-thio]Triphosphate (GTP-[γS]) Does Not Alter the Predominance of Ca^{2+} Sensitivity. It remained possible that a substantial portion of the cyclase was GTP-sensitive, but Ca^{2+}-insensitive, and that this population

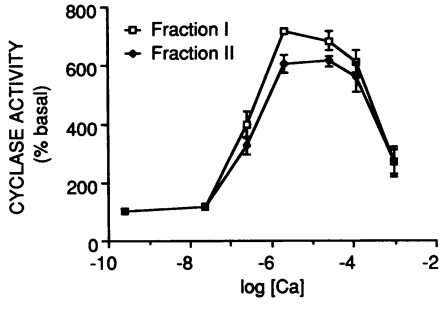

FIG. 2. Ca^{2+} stimulation of bovine brain adenylate cyclase in fractions I and II from CaM-affinity chromatography. Values are averages of three experiments in which bovine brain membranes were extensively washed with EGTA, solubilized, and chromatographed on CaM-Sepharose, and each desalted fraction was assayed in the presence of 6 μM CaM for stimulation by Ca^{2+}. Basal activities were 72.4 ± 27.1 and 5.3 ± 1.0 pmol/(min·ml) for fractions I and II, respectively.

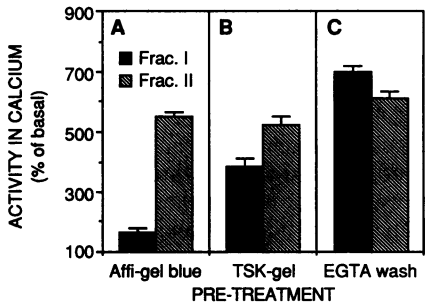

FIG. 3. Ca^{2+} stimulation of bovine brain adenylate cyclase in fractions I and II from CaM-affinity chromatography following various pretreatments. (*A*) Average of two experiments in which solubilized membranes were chromatographed on Affi-Gel Blue prior to CaM-affinity chromatography. Basal activities were 260 and 134 pmol/(min·ml) for fractions I and II, respectively. (*B*) Results from seven experiments in which TSK-gel HPLC preceded CaM-Sepharose chromatography. Basal activities were 149 ± 57 pmol/(min·ml) for fraction I and 64 ± 27 pmol/(min·ml) for fraction II. (*C*) Results from three experiments on cyclase from EGTA-washed, solubilized membranes (from Fig. 2). All assay mixtures contained 6 μM CaM. Ca^{2+} stimulation is an average of activity measured at 2 and 26 μM free Ca^{2+} (the two concentrations that gave maximal stimulation; see Fig. 2), expressed as a percent of the basal activity measured at 0.3 nM Ca^{2+}. Fraction II constituted 26.5% of the total recovered activity from the CaM-Sepharose column (as assayed in Mn^{2+} without CaM) in *A*, 20.9% in *B*, and 4.3% in *C*.

was not detected because its activity was very low in the absence of GTP. To test this possibility, the stimulatory GTP-binding protein (G_s) was preactivated (14) by incubating bovine brain membranes with 100 μM GTP[γS] and 100 mM $MgCl_2$ prior to EGTA washing. Preactivation neither altered the amount of binding to the CaM-affinity column (9.3% vs. 10.8% for control membranes) nor reduced the Ca^{2+} stimulation of fraction I relative to fraction II, although it elevated the basal activity of both by 5- to 8-fold. For the GTP[γS]-preactivated preparation, fraction I gave 3.9-fold and fraction II, 2.6-fold stimulation by Ca^{2+}, while in the control, fraction I gave 6.7-fold and fraction II, 4.7-fold stimulation. These findings argue against the existence of a large GTP-sensitive, but Ca^{2+}-insensitive, cyclase population in fraction I.

Loss of Ca^{2+} Sensitivity During Chromatography Prior to CaM-Sepharose. The relatively poor binding of mammalian cyclase to the CaM-affinity column probably reflects competition by residual endogenous CaM (measured as about 0.4 μM in bovine fraction I). We thus attempted to deplete endogenous CaM prior to affinity chromatography by using two different methods. First, we tried Affi-Gel Blue chromatography, following Westcott *et al.* (9), and then we used gel permeation HPLC. Both methods substantially increased binding to CaM-Sepharose. Twenty-seven ± 10% of the Affi-Gel Blue eluate (n = 2) and 21 ± 3% of the TSK-gel eluate (n = 7) bound to the affinity column, as compared with 4.3% binding after EGTA washing alone (measured in Mn^{2+} in the absence of added CaM). Neither pretreatment substantially altered the Ca^{2+} stimulation of the bound fraction; fraction II showed an average maximal stimulation of 5.5 ± 0.1-fold following Affi-Gel Blue pretreatment and 5.3 ± 0.3-fold following TSK-gel pretreatment, as compared with 6.1 ± 0.3-fold following EGTA washing alone (Fig. 3). However, both methods reduced the Ca^{2+} stimulation of fraction I: to 3.8 ± 0.3-fold following TSK-gel pretreatment, and only 1.6 ± 0.2-fold following Affi-Gel Blue pretreatment, compared to 7.0 ± 0.1-fold following EGTA washing.

The loss of Ca^{2+} sensitivity in CaM-Sepharose fraction I after Affi-Gel Blue chromatography and, to a lesser extent, after TSK-gel HPLC may be accounted for in one of two ways. One possibility is that there is greater binding of

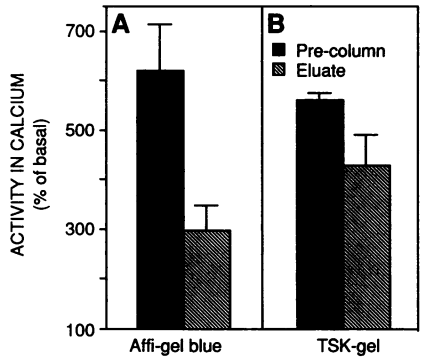

FIG. 4. Effect of Affi-Gel Blue chromatography and gel permeation HPLC on the Ca^{2+} sensitivity of adenylate cyclase. For each chromatographic procedure, an aliquot of "pre-column," solubilized bovine brain membranes was desalted in parallel with an aliquot of the column eluate, into buffer D. Each fraction was then assayed for stimulation by 26 μM Ca^{2+} in the presence of 12 μM exogenous CaM. (*A*) Ca^{2+} stimulation of bovine adenylate cyclase before and after Affi-Gel Blue chromatography; $n = 3$. (*B*) Ca^{2+} stimulation before and after TSK-gel permeation HPLC; $n = 3$.

Ca^{2+}-sensitive cyclase to the CaM-affinity column after these two pretreatments and that this results in the unmasking of a large Ca^{2+}-insensitive subpopulation of the cyclase in fraction I. Another possibility is that these pretreatments eliminate the Ca^{2+} sensitivity from some of the cyclase population. Thus, if during prior chromatography, some of the Ca^{2+}-sensitive enzyme were to lose its ability to bind and be stimulated by CaM, this population would remain exclusively in fraction I of the CaM-affinity column. The result would be an overall reduction in the Ca^{2+} sensitivity of fraction I, but no change in the Ca^{2+} sensitivity of fraction II.

In support of the second possibility, we found that both chromatographic pretreatments reduced Ca^{2+} stimulation from its level in solubilized membranes. Ca^{2+} stimulation decreased 52% during Affi-Gel Blue chromatography (from 6.2 ± 0.9-fold in the solubilized preparation to 3.0 ± 0.5-fold in the eluate, $n = 3$; Fig. 4*A*) and 23% during TSK-gel HPLC (from 5.6 ± 0.1-fold in the solubilized preparation to 4.3 ± 0.6-fold in the eluted peak, $n = 3$; Fig. 4*B*). For the Affi-Gel Blue column, this reduction in Ca^{2+} sensitivity cannot be accounted for by segregation of the Ca^{2+}-sensitive cyclase into the Affi-Gel Blue flow-through fraction, because when we examined Ca^{2+} stimulation of the flow-through's cyclase in one experiment, we found that it was comparable (2.8-fold) to that of the eluate (2.2-fold); both were less than the Ca^{2+} stimulation of the pre-column solubilized preparation (4.7-fold). A similar concern does not apply to the gel permeation column, because cyclase was eluted from this column in only one peak. Thus Affi-Gel Blue chromatography and, to a lesser extent, TSK-gel HPLC reduced the Ca^{2+} sensitivity of adenylate cyclase.

This reduction is apparently due to the loss of Ca^{2+} sensitivity in a fraction of the cyclase population, as opposed to a reduction of the Ca^{2+} sensitivity in the entire population, because regardless of pretreatment, the CaM-binding species (fraction II) retains its 5- to 6-fold stimulation by Ca^{2+} (Fig. 5). If one assumes that fraction I is a mixture of Ca^{2+}-insensitive and Ca^{2+}-sensitive species, with the latter showing the same amount of stimulation as fraction II, then the Ca^{2+}-insensitive form represents 0% of the total activity following EGTA washing, 27% following gel permeation HPLC, and 63% following Affi-Gel Blue chromatography.§

§Calculated by assuming that the level of stimulated activity in fraction I is a weighted average of the basal activity of the Ca^{2+}-insensitive fraction and the stimulated activity of the Ca^{2+}-sensitive fraction that it contains.

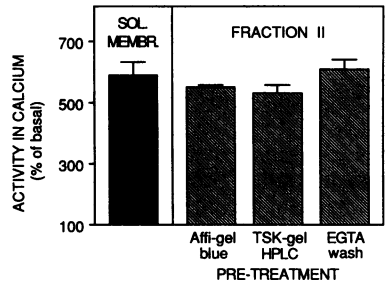

FIG. 5. Summary of results presented in Figs. 3 and 4. The isolation of CaM-binding cyclase in fraction II does not enrich Ca^{2+} stimulation over its starting level in solubilized membranes (Sol. Membr.).

These numbers correlate well with the amount of Ca^{2+} sensitivity lost during gel permeation HPLC and Affi-Gel Blue chromatography (23% and 52%, respectively).

This loss of Ca^{2+} sensitivity during Affi-Gel Blue chromatography and gel permeation HPLC could reflect the conversion of some of the Ca^{2+}-sensitive cyclase to a Ca^{2+}-insensitive form through degradation of the CaM binding site during chromatography. Such degradation seems plausible, since many CaM-dependent enzymes can be rendered CaM-independent by limited proteolysis (15). Indeed, Malnoe and Cox (16) found that Ca^{2+}/CaM stimulation of cyclase was selectively eliminated by low concentrations of chymotrypsin.

A Larger CaM-Sepharose Column Produces More Cyclase Binding. The presence of Ca^{2+}-sensitive cyclase in fraction I indicated that the volume of CaM-Sepharose resin we were using, although generous, was nonetheless insufficient for separating Ca^{2+}-sensitive and -insensitive cyclases. To test this possibility, we loaded the same amount of TSK-gel eluate on two CaM-Sepharose columns of different volumes. We found that a 5-fold increase in the amount of resin more than doubled binding (from 19% to 46%). Concomitant with this shift of Ca^{2+}-sensitive cyclase from the unbound to the bound fraction with chromatography on a larger column, the Ca^{2+} stimulation of fraction I decreased slightly (from 4.0-fold to 3.3-fold), while the Ca^{2+} stimulation of fraction II remained nearly the same (6.2-fold for the 1-ml column and 5.7-fold for the 5-ml column). This result demonstrates that a substantial proportion of total bovine brain cyclase activity is capable of binding to the affinity column. It also provides evidence that there was not a large, Ca^{2+}-insensitive population that had been masked in fraction I when we used the smaller column.

DISCUSSION

The Principal Form of Neural Adenylate Cyclase Is Ca^{2+}-Sensitive. The results presented here indicate that the predominant form of adenylate cyclase in the CNS can be stimulated by Ca^{2+}. When CaM-Sepharose chromatography was preceded only by extensive EGTA washing of CNS membranes, both bound and unbound fractions showed virtually identical Ca^{2+} stimulation in the presence of CaM. This was the case for the cyclase from all three preparations studied, *Aplysia* CNS, rat brain, and bovine brain.

Further evidence that Ca^{2+} sensitivity may be a universal property of neural adenylate cyclase comes from the fact that isolation of CaM-binding cyclase produced no enrichment for Ca^{2+} stimulation compared to the level of stimulation in the solubilized preparation. If native cyclase were truly a mixture of Ca^{2+}-sensitive and -insensitive forms, then the total Ca^{2+} stimulation should increase with the isolation of Ca^{2+}-sensitive cyclase by CaM-affinity chromatography. We found, however, that regardless of pretreatment, Ca^{2+} stimulation of the bound fraction (fraction II) never exceeded the

level present in the solubilized preparation. Fig. 5 summarizes these results. Cyclase in the original preparation averaged 5.9-fold Ca^{2+} stimulation, while the cyclase in fraction II ranged from 5.3- to 6.1-fold Ca^{2+} stimulation, depending on pretreatment.

The loss of Ca^{2+} sensitivity with both Affi-Gel Blue and TSK-gel chromatographic pretreatments may reflect some degradation of the CaM binding site during chromatography. However, another possibility is that various chromatographic procedures may create a Ca^{2+}-insensitive population by removing some factor that may be responsible for conferring Ca^{2+} sensitivity on the catalytic unit of the cyclase. Indeed, Toscano *et al.* (17) reported that it is possible to restore Ca^{2+} sensitivity to the unbound fraction from CaM-Sepharose (following Affi-Gel Blue pretreatment) by reconstituting it with a dilute preparation of solubilized membranes. Their results are consistent with our finding that most of the cyclase in bovine brain, including that which fails to bind to CaM-Sepharose, can be activated by Ca^{2+}/CaM.

Inefficient Binding of Adenylate Cyclase to CaM-Sepharose. In all cases studied, Ca^{2+} stimulation of adenylate cyclase has been found to be mediated by CaM (18). Since we found that most of the adenylate cyclase present in neural tissue is capable of stimulation by Ca^{2+}, one would expect the majority of neural cyclase activity to bind to a CaM-Sepharose column. The generally inefficient binding we observed probably reflects the combined hindrances of competition for binding by endogenous CaM and the rather low affinity of the enzyme for the immobilized ligand. Even when endogenous CaM was reduced 20-fold by TSK-gel HPLC, to ≈50 nM, which is 3 orders of magnitude lower than the concentration of CaM on the affinity column, binding was still incomplete, as is evident from the fact that the unbound fraction retains considerable Ca^{2+}-sensitive cyclase.

Does a Ca^{2+}-Insensitive Cyclase Exist in the CNS? The primary conclusion of this paper is that the vast majority of cyclase activity in CNS membranes is Ca^{2+}/CaM-sensitive. This finding raises the issue of whether a Ca^{2+}-insensitive form of cyclase exists at all in the brain. It is virtually impossible, with the methods we have employed, to rule out the possibility that a small Ca^{2+}-insensitive population of cyclase is present in CNS membranes. At the very least, however, we can estimate that any Ca^{2+}-insensitive cyclase population that does exist could not represent >20% of total cyclase activity in the CNS, because any larger population would cause the Ca^{2+} stimulation of fraction I to be detectably lower than that of fraction II when EGTA washing is the only pretreatment.

Our conclusions differ from those of Rosenberg and Storm (19). Those authors prepared an antiserum against a preparation of guanosine 5′-[β-γ-imido]triphosphate-preactivated bovine brain adenylate cyclase that had been purified through a number of steps, including CaM-Sepharose. This antiserum effectively precipitated adenylate cyclase activity only from the bound, and not from the flow-through, fraction of a CaM-Sepharose column. It also precipitated only 60% of the total cyclase activity in bovine brain membranes. The authors concluded that bovine brain contains two distinct isozymes of adenylate cyclase, with the Ca^{2+}-sensitive form presumably representing 60% of the total activity. However, Rosenberg and Storm did not demonstrate that the precipitated cyclase activity showed Ca^{2+} stimulation while the activity in the supernatant did not. Thus, it is not clear that this antiserum truly segregates Ca^{2+}-sensitive and -insensitive forms of cyclase.

Given the lability of the Ca^{2+}/CaM sensitivity of cyclase, the convincing identification of a Ca^{2+}-insensitive form probably requires the cloning, expression, and characterization of all adenylate cyclase species in the CNS. Recently, one species of adenylate cyclase that binds to CaM has been cloned from bovine brain (20). The enzyme expressed from this clone was stimulated by Ca^{2+}/CaM. A second, highly homologous cDNA was subsequently isolated, but it is not yet known whether the cyclase encoded by this clone is Ca^{2+}-sensitive (R. Reed, personal communication).

Dual Regulation of Adenylate Cyclase and Associative Learning. Abrams *et al.* (7) recently demonstrated that the CaM-binding adenylate cyclase can be dually activated by Ca^{2+} and the stimulatory GTP-binding protein, G_s. The finding that the vast majority of cyclase in *Aplysia* and mammalian CNS is Ca^{2+}-sensitive similarly suggests that the same catalytic units that are stimulated by receptor via G_s will also be stimulated by Ca^{2+}/CaM. Indeed, these results indicate that Ca^{2+} regulation may be a general feature of adenylate cyclase activation in the CNS. Thus, the enzyme could function generally in nervous systems to temporally associate transmitter input with neuronal activity or any of a variety of other stimuli that increase intracellular Ca^{2+}.

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