Calcineurin Feedback Inhibition of Agonist-evoked cAMP Formation*

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The effects of immunosuppressant blockers of calcineurin (protein phosphatase 2B) on cAMP formation and hormone release were investigated in mouse pituitary tumor (AT20) cells. Immunosuppressants enhanced corticotropin-releasing factor- and isoproterenol-evoked cAMP production in proportion with their potency to block calcineurin. Further analysis of cAMP production revealed that intracellular Ca\(^{2+}\) derived through voltage-regulated calcium channels reduces cAMP formation induced by corticotropin releasing-factor or \(\beta\)-adrenergic stimulation and that this effect of Ca\(^{2+}\) is inhibited by blockers of calcineurin. AT20 cells were found to express at least three species of adenyl cyclase mRNA-encoding types 1 and 6 as well as a novel isotype, which appeared to be the predominant species. In two cell lines expressing very low or undetectable levels of the novel cyclase mRNA (NCB20 and HEK293 cells respectively), corticotropin-releasing factor-induced cAMP formation was not altered upon blockage of calcineurin activity. These data identify calcineurin as a Ca\(^{2+}\)/calmodulin sensor that mediates the negative feedback effect of intracellular Ca\(^{2+}\) on receptor-stimulated cAMP production. Furthermore, the effect of calcineurin on cAMP synthesis appears to be associated with the expression of a novel adenyl cyclase isotype, which is highly abundant in AT20 cells.

Calcineurin (protein phosphatase 2B) is a Ca\(^{2+}\)/calmodulin-regulated protein phosphatase first discovered in brain, where it is highly abundant (0.5–1% of total protein) (1). Elucidation of the physiological role of this protein phosphatase has been relatively slow due to the lack of specific inhibitors of its enzymatic activity. It is now well established that the major immunosuppressant compounds cyclosporin A and FK506 are potent and, with appropriate controls, specific blockers of calcineurin (2) and, with appropriate controls, specific blockers of calcineurin are used widely in the treatment of organ transplant patients (3). This observation has led to the discovery that calcineurin is an essential element of the signal transduction pathway activated by the T-cell receptor (5, 6).

In excitable cells, the functions of calcineurin are less well understood. Calcineurin has been implicated in the control of voltage regulated ion channel activity (7), particularly with respect to \(\alpha\)-type calcium channels (8). More recent studies applying immunosuppressant blockers of calcineurin have shown that the synaptic vesicular protein dynamin, which is thought to participate in synaptic vesicle recycling in nerve endings, is a prominent substrate for calcineurin (9) and that blockage of calcineurin enhances glutamate release by synaptosomes prepared from rat brain (10). In pituitary corticotrope tumor (AT20) cells (4, 11), immunosuppressants block calcineurin activity and stimulate Ca\(^{2+}\)-dependent hormone release in correlation with their calcineurin blocking potency. In hippocampal brain slices, calcineurin is involved in the induction of long-term synaptic depression (12). Finally, ligand-operated ion channels such as the NMDA receptor (13) or the \(5\)HT\(_3\) receptor (14) are desensitized by calcineurin.

Taken together, these data indicate multiple roles for calcineurin in diverse signal transduction cascades of excitable cells. A common feature of all of these proposed functions is the Ca\(^{2+}\)-dependent inhibition of cellular activation. This is the opposite of what has been observed in nonexcitable cells, such as lymphocytes (5, 6) and adrenocortical glomerulosa cells (15), where calcineurin is an intracellular mediator of the action of stimulatory agents.

The effects of immunosuppressants on the cAMP signaling system in excitable cells have not been previously examined. cAMP is a cardinal signaling molecule in pituitary corticotropes (16), where its synthesis is activated by 41-amino acid residue CRF (17). Increased levels of cAMP augment intracellular free Ca\(^{2+}\) concentration ([(Ca\(^{2+}\)]\(_i\)) (4); in turn, Ca\(^{2+}\) synergizes with cAMP to trigger the release of adrenocorticotropic hormone (ACTH) (18). As [Ca\(^{2+}\)]\(_i\), is also known to inhibit cAMP formation in several systems (19) and because immunosuppressants enhanced CRF-induced ACTH release in AT20 cells (20), we have examined the effects of immunosuppressants on CRF-induced cAMP production. The results indicate that in AT20 cells, calcineurin inhibits CRF-induced cAMP formation and that this is associated with the expression of a novel isotype of adenyl cyclase.

* A preliminary account of parts of this work has been published (11). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\(^\text{TM}\)EMBL Data Bank with accession number(s) MNU30602, Z50190, and Z46958.

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1 The abbreviations used are: CRF, corticotropin releasing factor; ACTH, adrenocorticotropic hormone; IBMX, isobutylmethylxanthine; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N',N"-tetraacetic acid tetra(acetoxymethyl)ester; PCR, polymerase chain reaction; bp, base pair(s); ANOVA, analysis of variance.

2 MATERIALS AND METHODS

Cdl Culture—AT20 D16:16 mouse anterior pituitary tumor cells were maintained in culture as described previously (21). NCB20 mouse neuroblastoma × hamster brain hybridoma cells (courtesy of Dr. Beth Hoffman, National Institute of Mental Health, Bethesda, MD) were cultured in 10% newborn calf serum and Dulbecco’s minimal essential
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medium with hypoxanthine/aminopterin/thymidine supplement (Life Technologies, Paisley, Scotland, United Kingdom). Human embryonic kidney (HEK293) cells (courtesy of Dr. Lorraine Anderson, Medical Research Council Reproductive Biology Unit, Edinburgh, Scotland, UK) were maintained as AtT20 cells.

For measurements of ACTH release, cAMP production, or calcineurin activity, the cells were plated on 24-well tissue culture plates (5 × 10^4 cells/well) and used 4–6 days afterwards. ACTH (21) and cAMP (22) were measured by specific radioimmunoassays. Calcineurin protein phosphatase activity was determined by the ^32P-labeled casein assay (23) or the RII phosphopeptide assay (24) adapted to measure calcineurin phosphatase activity in AtT20 cell extracts as described previously (25).

Measurement of cAMP Responses to Agonists—This protocol was established using AtT20 cells and was also applied in experiments with the other two cell lines. Experiments were all carried out in Hank’s balanced salt solution containing 2 mM CaCl_2 and 1 mM MgSO_4 buffered with 25 mM HEPES, pH 7.4, and supplemented with 0.25% (w/v) bovine serum albumin. The cells were preincubated in serum-free medium for 1 h, after which fresh medium containing blockers of phosphodiesterase, 0.5–1 mM IBMX, and/or 0.1 mM rolipram along with various other agents as specified below and in the figure legends were applied for 30 min at 37°C. Subsequently, the cells were cooled to 24°C (5–10 min in a water bath), and agonists were added for 10 min. The reaction was stopped by the addition of 0.2 M HCl to achieve a final concentration of 0.5 M (25).

In the absence of phosphodiesterase blockers, agonist-induced changes of total cAMP content were undetectable at 24°C. In the presence of IBMX, total cAMP content (cells + medium) increased with time up to 20 min after the addition of CRF and remained constant for up to 30 min. In contrast, intracellular cAMP peaked between 2 and 5 min and subsequently declined to basal levels even in the presence of the phosphodiesterase blockers. Hence, after establishing that immunosuppressant drugs had the same effect on peak cellular and total cAMP content under these conditions, all experiments shown here report total cAMP content.

Depletion and Replacment of Intracellular Calcium Pools—Calcium depletion during preincubation in the presence of phosphodiesterase blockers, which were applied as described in the preceding section. Cells were preincubated for 30 min in medium containing 2 mM EGTA and no added Ca_2^+ supplemented with 5 μM A23187 and 2.5 μM nifedipine in order to deplete rapidly mobilized cellular stores of Ca_2^+ and to ensure that L-type Ca_2^+ channels, the principal avenue of voltage-regulated Ca_2^+ influx in AtT20 cells (26, 27, 28) were fully blocked. Thus, Ca_2^+ added extracellularly at the extracellular fluid at the time agonist stimulation was initiated, would enter largely through the pores made by the ionophore A23187. The rationale for this protocol was that calcineurin reportedly influences L channel activity at 80°C and prehybridization at 42°C for 2 h in 50% deionized formamide, 5 × saline/nucleotide phosphate buffer (EDTA, 0.5 × Denhardt’s solution, 0.1% (w/v) SDS, 0.2 mg/ml denatured salmon sperm carrier DNA, and 10% Dextran sulfate. Random-primer [^32P]cDNA probe (50 ng; Sequenase 2.0 kit, U. S. Biochemical Corp., Clone) PI 34, containing 180 bp of cDNA sequence was isolated and used for generating cDNA and cRNA probes for RNA detection.

Detection of mRNA Expression—Northern analysis was performed using standard procedures. Briefly, 10 μg of total RNA was separated by formaldehyde gel electrophoresis and transferred by blotting onto positively charged nylon membrane (Amersham) and then fixed by baking at 80°C and prehybridized at 42°C for 2 h in 50% deionized formamide, 5 × saline/nucleotide phosphate EDTA, 0.05 × Denhardt’s solution, 0.1% (w/v) SDS, 0.2 mg/ml denatured salmon sperm carrier DNA, and 10% Dextran sulfate. Random-primer[^32P]cRNA probe (50 ng: 10^-10 cpm/μg) was then added, and hybridization was then carried out overnight at 42°C in a humidified chamber. The blot was then washed in 2 × SSC, 0.1% SDS, followed by 20 min in 1 × SSC, 0.1% SDS at 50°C and finally in 20 min in 0.5 × SSC, 0.1% SDS at 50°C before wrapping in cling-film and exposing to autoradiographic film at −70°C or to Molecular Dynamics Phosphorimager cassettes and quantified with the ImageQuant software using the 28 S RNA band as a standard for RNA loading. Division of the integrated volume of pixels of the selected radiolabeled band with the integrated volume of the internal standard band yields the relative hybridization intensity, which was used to compare the intensity of labeled RNA bands within blots.

Ribonuclease protection assays were performed using an RPA II kit (Ambion, AM5 BioTechnology, Wixton, Oxford, UK) according to the manufacturer’s instructions. Briefly, 10 μg of total RNA was hybridized overnight at 45°C to 10^6 cpm of radiolabeled P134 antisense ribo-probe. Following hybridization, reactions were digested with single strand-specific RNase and protected fragments were resolved on a 6% polyacrylamide denaturing gel, and various fragments were quantified with the ImageQuant software using the 28 S RNA band as a standard for RNA loading. Division of the integrated volume of pixels of the selected radiolabeled band with the integrated volume of the internal standard band yields the relative hybridization intensity, which was used to compare the intensity of labeled RNA bands within blots.

Enhancement of CRF-stimulated cAMP Production by Immunosuppressants—Blockers of calcineurin activity enhanced cAMP production in AtT20 cells. Measurement of cAMP responses to CRF was for 30 min at 24°C, to allow comparisons with the conditions used in cAMP accumulation experiments. Immunosuppressants were applied as in cAMP experiments; blockers of phosphodiesterase were not used.
The effect of FK506 on CRF-induced cAMP production could be antagonized by the nonimmunosuppressant analogue L685,818 (30), which also blocked the inhibitory effect of FK506 on calcineurin-mediated dephosphorylation of phosphocasein (Fig. 3, A and B).

Receptor-evoked Synthesis of cAMP Is under Inhibitory Control by Intracellular Ca\(^{2+}\) and Calcineurin—Lowering of [Ca\(^{2+}\)], by a variety of methods all markedly increased the cAMP response to 10 nM CRF (control, 100 ± 9; Ca\(^{2+}\) depletion protocol, 199 ± 18; nifedipine (0.1 \(\mu\)M) in preincubation, 205 ± 22; BAPTA-AM (20 \(\mu\)M) in preincubation, 275 ± 25). Data are means ± S.E. of the increment over unstimulated cAMP levels and are expressed as percentage of the control CRF group run in each experiment, \(n = 6\)group. \(p < 0.05\) compared with respective control group (one-way ANOVA followed by orthogonal contrasts), \(n = 6\)group. Data are means ± S.E., \(n = 6\)group. * \(p < 0.05\) compared with respective vehicle treated group (one-way ANOVA followed by orthogonal contrasts), \(n = 6\)group.
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Through Calcineurin—Isoproterenol stimulates cAMP production (4), to stimulate ACTH release, and to augment cAMP accumulation by extracellular Ca2+ (not shown).

FK506 had no significant effect on cAMP accumulation evoked by 10 or 30 μM forskolin, a drug that at these concentrations activates adenylyl cyclase independent of Gs. Loading of the cells with BAPTA-AM caused a small (15%), statistically significant (p < 0.05) enhancement of forskolin-evoked cAMP accumulation (not shown).

Finally, in contrast to the effects of FK506 and cyclosporin A, pretreatment with other blockers of protein phosphatases such as calyculin A (1–30 nM) and okadaic acid (0.2–5 μM), caused a concentration-dependent inhibition (up to 80%) of CRF-induced cAMP accumulation (not shown and Ref. 36).

Enhancement of CRF-stimulated ACTH Release by FK506—Blockage of calcineurin activity by FK506 enhanced the release of ACTH evoked by CRF (Fig. 5A), and this action was prevented by L-685,818 (Fig. 5B). Note that the apparent EC50 for FK506 to inhibit calcineurin activity in AtT20 cells (4), to stimulate ACTH release, and to augment cAMP accumulation induced by CRF are all approximately 10 nM. β-Adrenergic Stimulation is Under Similar Regulation by Calcineurin—Isoproterenol stimulates cAMP production through β2-adrenergic receptors in AtT20 cells (37), and this was enhanced by both BAPTA-AM and FK506 (Table I).

Effect of Immunosuppressants on cAMP Accumulation in AtT20 Cells Correlates with the Expression of a Novel Adenylyl Cyclase mRNA—In order to determine the profile of adenylyl cyclase isoforms present in AtT20 cells, two sets of degenerate oligonucleotide primers were used to analyze AtT20 cell total RNA for adenylyl cyclase-related sequences by means of reverse transcriptase PCR. Using primer set B, a PCR product of approximately 180 bp was obtained. DNA sequence analysis revealed that approximately 8% of the subcloned 180-bp cDNA fragments amplified proved to be identical to type 6 adenylyl cyclase. The majority (>90%), however, gave a novel sequence that was highly homologous to the primary amino acid sequences of known mammalian adenylyl cyclases found in current data bases but was not identical to any of these (Fig. 6). Type 1 adenylyl cyclase was detected in AtT20 cells using primer set A.

Northern blot analysis of total RNA using the novel adenylyl cyclase 180-bp cDNA fragment as a probe indicated hybridization to an approximately 9-kilobase mRNA expressed in AtT20 cells (Fig. 7), and a single hybridizing species of RNA of similar size was detected in NCB20 and HEK293 cells at much lower intensity (Relative hybridization intensity of 9-kb band (arbitrary units): AtT20, 0.38; NCB20, 0.03; HEK293, 0.07).

Table I

| Isoproterenol | Controla | FK506a | BAPTA-AMa |
|---------------|----------|--------|-----------|
| 0.4 | 0.43 ± 0.4 | ND | ND |
| 0.5 | 0.7 ± 0.04 | 0.94 ± 0.09 | 0.96 ± 0.06 |
| 1.0 | 2.0 ± 0.08 | 3.3 ± 0.7 | 3.6 ± 0.6 |
| 10 | 9.5 ± 0.33 | 15.3 ± 2.5 | 11.4 ± 0.9 |
| 100 | 19.5 ± 0.5 | 31.4 ± 1.9 | 35.5 ± 3.4 |
| 1000 | 32.1 ± 0.5 | 45.3 ± 9 | 46.9 ± 9 |

*a Pretreatment.

As a potentially more sensitive alternative, mRNA expression was also assayed by ribonuclease protection using a radiolabeled antisense riboprobe transcribed from the novel adenylyl cyclase cDNA. An approximately 160-bp ribonuclease-resistant RNA species (Fig. 7) indicates that the novel adenylyl cyclase mRNA is highly abundant in AtT20 cells, whereas much lower levels are present in NCB20 cells, and in HEK293 cells the mRNA was undetectable.

Calcineurin protein phosphatase activity (substrate RI1 sub-
unit peptide (24)) in cell extracts prepared from AtT20, HEK293, and NCB20 cells fell to 25, 19, and 42% of the respective control activities after pretreatment with 1 \( \mu M \) FK506. Similar to AtT20 cells, CRF-stimulated cAMP formation in HEK293 cells (Fig. 8) as well as NCB20 cells (not shown), and this was enhanced by the depletion of intracellular calcium stores as described for AtT20 cells. However, while in AtT20 cells FK506 consistently enhanced CRF-induced cAMP forma-

tion, in NCB20 cells only one out of four experiments gave a statistically significant enhancing effect of 1 \( \mu M \) FK506 on CRF-induced cAMP accumulation, and in none out four experiments in the case of HEK293 cells (Fig. 8). No effects of cyclosporin A were found in either system (not shown).

**DISCUSSION**

These data show that receptor-stimulated cAMP formation may be inhibited by calcineurin and that this regulation is associated with the expression of a novel adenylyl cyclase mRNA.

All studies of cAMP formation reported here were carried out in the presence of blockers of phosphodiesterase, and hence the effects observed relate to changes in the rate of synthesis of cAMP rather than to its degradation.

Evidence for the involvement of calcineurin in the control of cAMP accumulation is provided by the use of immunosuppressant compounds previously (4) shown to block calcineurin activity in AtT20 cells with the same order of potency that they influenced cAMP accumulation (present study). The EC\(_{50}\) for FK506 to block calcineurin activity is considerably higher in AtT20 cells (\( 10^{-8} \) M) than in T lymphocytes (\( 0.8 \) nM), which is probably attributable to differences in the respective cellular levels of calcineurin and FKBP12 in these systems. Importantly, L685,818, an analogue of FK506 (30) that binds to the prolyl isomerase FKBP-12 in a manner similar to FK506 but does not give rise to a drug-protein complex that inhibits the activity of calcineurin, reversed the effects of FK506 on cAMP formation or ACTH secretion, further suggesting that the changes observed upon treatment with FK506 are due to the inhibition of calcineurin and not due to the blockage of the prolyl isomerase activity of FKBP12. Finally, neither cyclosporin A was effective in cells deprived of Ca\(^{2+}\) (20).

Taken together, these characteristics justify the conclusion that the effects of immunosuppressants described here are attributable to the inhibition of calcineurin.

**The production of cAMP in AtT20 cells is under inhibitory control by Ca\(^{2+}\)**

Stimulation with cAMP activates protein kinase A (PKA), which phosphorylates calcineurin activity and cAMP formation as well as ACTH release. When given alone, L685,818 had no discernible effect on cAMP formation or ACTH secretion, further suggesting that the changes observed upon treatment with FK506 are due to the inhibition of calcineurin and not due to the blockage of the prolyl isomerase activity of FKBP12. Finally, neither FK506 nor cyclosporin A was effective in cells deprived of Ca\(^{2+}\) (20).

Taken together, these characteristics justify the conclusion that the effects of immunosuppressants described here are attributable to the inhibition of calcineurin.

The production of cAMP in AtT20 cells is under inhibitory control by Ca\(^{2+}\). Stimulation with cAMP is known to elicit a rise of Ca\(^{2+}\), which is largely derived from the extracellular pool by influx through dihydropyridine-sensitive Ca\(^{2+}\)-channels (26, 27, 28). Thus the Ca\(^{2+}\) signal is a measure of the activity of the electrical activity of the cells and, in addition to triggering hormone release, provides feedback inhibition to the chemical messenger system that generates it. In the case of...
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CRF-induced cAMP formation, this feedback appears to be mediated by calcineurin.

Several possibilities have to be considered with respect to the site of action of Ca$^{2+}$/calcineurin in the signal transduction cascade.

An action of calcineurin at the receptor level is conceivable; however, the prevailing concept of G-protein-coupled receptors (38) dictates that receptor down-regulation or uncoupling is largely due to the action of protein kinases while protein phosphatases reverse this process. In contrast, the present data implicate calcineurin as an inhibitor of receptor-stimulated cAMP production.

D Dephosphorylation of the coupling protein Gs is also a possible site of regulation by calcineurin (39). Once more, current evidence in the literature associates protein phosphorylation with down-regulation of G-protein function and implicates protein phosphatases in the restoration of the cellular response (40, 41).

With respect to the effector enzyme adenyl cyclase, these proteins have lately emerged as dynamic sites of signal integration (42). At least two types of cyclase, types 5 and 6 (43), are inhibited by Ca$^{2+}$, but the mechanism of this effect has not been elucidated (33). The inhibition of type 5 and 6 cyclase by Ca$^{2+}$ is most marked after stimulation by agonists such as isoproterenol in chick heart cells (44), or VIP in GH$_3$ pituitary tumor cells (45), but much less prominent after activation with forskolin in GH$_3$ cells (46). Overall this is analogous to the observations made here, which in the first instance suggest a prominent action of calcineurin at or before the level of G-protein effector coupling. However, as multiple types of adenyl cyclase coexist in all cell types analyzed to date (33, 46, 47), and forskolin appears to activate these by different efficiencies and mechanisms, an effect of Ca$^{2+}$ on catalytic activity as opposed to the interaction with the Gs α-subunit-coupling site cannot be excluded (43). Reverse transcriptase PCR analysis and sequencing of amplified cDNAs clearly show that at least three types of adenyl cyclase mRNA (type 1 and 6 as well as a novel isotype) are co-expressed in AtT20 cells, and thus the above considerations also apply to this system.

It is unlikely, that type 1 cyclase is involved in the effects reported here as it is invariably stimulated by Ca$^{2+}$, whereas Ca$^{2+}$ was strongly inhibitory to both CRF and β-adrenergic stimulation of cAMP. Type 6 adenyl cyclase could be implicated as it is inhibited by Ca$^{2+}$. However this isotype is abundant in NCB20 (33) as well as HEK293 cells (47) where the stimulation of cAMP accumulation through endogenously expressed receptors for CRF was not altered by immunosuppressants, despite a marked inhibition of calcineurin activity measured using the R1I substrate phosphopeptide. Importantly, the novel adenyl cyclase homologue mRNA was found in very low amounts in NCB20 cells and HEK293 cells, whereas it appears to be the predominant adenyl cyclase isotype mRNA in AtT20 cells. A partial mammalian sequence that is identical except for a single amino acid to the one reported here has been previously designated as adenyl cyclase type 10 (48). Results from this laboratory (49) show that the 9-kb mRNA detected in AtT20 cells contains a full-length adenyl cyclase coding sequence giving rise to an adenyl cyclase inhibited by calcineurin.

A previous study (15) has reported that calcineurin is stimulatory to cAMP formation; immunosuppressants blocked the enhancement of ACTH-evoked cAMP production by angiotensin II and activators of protein kinase C in bovine adrenal cortical cells as well as transfected COS-7 cells. As adenyl cyclase type 10 mRNA is undetectable in COS-7 cells and protein kinase C activation is inhibitory to cAMP production in AtT20 cells (36), it is unlikely that adenyl cyclase type 10 is involved in the enhancement of cAMP production by calcineurin as reported by Baukal and co-workers (15). Another study, using partially purified solubilized bovine brain adenyl cyclase (50) reported inhibition of cyclase activity by calcineurin; however, this was attributed to the sequestration of endogenous calmodulin in the enzyme preparation by calcineurin, and the consequent inhibition of a calmodulin-stimulated cyclase activity. Thus, whether calcineurin regulates cyclase type 10 directly or through an intermediary phosphoprotein specific to AtT20 cells remains to be determined by future studies.

Taken together, the present data indicate that in AtT20 cells calcineurin is a Ca$^{2+}$-operated feedback inhibitor of CRF or β-adrenergic receptor-evoked cAMP responses. As [Ca$^{2+}$], is largely derived through voltage-regulated Ca$^{2+}$ channels in AtT20 cells, these cells exemplify a case where calcineurin functions as a link between the cAMP-generating and electrical signaling systems of the cell. The potential functional significance of this mechanism is illustrated by changes of hormone secretion that parallel the enhancement of the cAMP signal. Immunosuppressants augmented CRF-induced ACTH secretion and attenuated the inhibitory effect of adrenal corticosteroids (20). Our findings conform with previous reports (7, 10, 13, 14) in showing that calcineurin is a fundamental negative feedback regulator of cellular responses in excitable cells and extend this function to the cAMP signal transduction cascade. In this latter respect the data also support the earlier notion (51), that calcineurin is a generic antagonist of cAMP-induced stimulatory mechanisms.

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Addendum—The cDNA sequence of the novel adenyl cyclase cloned from AtT20 cells (now called type 9) has been deposited in GenBank by two groups under accession numbers M96002 and Z50190. A further highly homologous sequence from Xenopus laevis is found under accession number Z46958.

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