Calcium Dependence of Hormone-stimulated cAMP Accumulation in Intact Glial Tumor Cells*

Margaret A. Brostrom, Charles O. Brostrom, and Donald J. Wolff

From the Department of Pharmacology, Rutgers Medical School, College of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854

The Ca²⁺ content of glial tumor (C6) cells was reduced approximately 5-fold by repeated treatment with media containing ethylene glycol bis(β-aminoethyl ether) N,N'-tetraacetic acid (EGTA) without loss of cellular viability. The ability of the cells to accumulate cAMP in response to β-adrenergic agonists was reduced 60 to 70% following Ca²⁺ depletion. Ca²⁺ did not affect the apparent Kₐ₅ for norepinephrine, nor did it change the concentration of norepinephrine required to produce 50% inhibition of the maximal norepinephrine response. Phentolamine did not alter the Ca²⁺ dependence of the response. The binding of dihydroalprenolol by intact C6 cells was not influenced by Ca²⁺. Furthermore, pre-treatment with norepinephrine did not affect the Ca²⁺ dependence of cAMP accumulation. The effects of Ca²⁺ therefore, appeared to be exerted on components of the adenylate cyclase system other than the catecholamine receptor.

Micromolar free Ca²⁺ concentrations in the extracellular medium were sufficient to restore a maximal norepinephrine response to Ca²⁺-depleted cells. The effect of Ca²⁺ on cAMP accumulation in response to hormone was immediate and was rapidly reversible upon the addition of EGTA in excess of the cation. Cells in media containing Ca²⁺ exhibited a characteristic biphasic time course of cAMP accumulation; with Ca²⁺-depleted cells cAMP was accumulated more slowly and the subsequent decline in cAMP content was also reduced. Verapamil, an inhibitor of plasmalemmal Ca²⁺ influx, decreased the Ca²⁺-dependent component of the cAMP accumulation when added prior to the cation. The effect of Ca²⁺ on cAMP accumulation was reduced more extensively by pretreatment of cells at 45°C under Ca²⁺-depleted (80% loss) than under Ca²⁺-restored (30% loss) conditions. Trifluoperazine at micromolar concentrations decreased the Ca²⁺-dependent increment in accumulation of cAMP in Ca²⁺-restored cells. This inhibition was not overcome by increasing concentrations of norepinephrine or of extracellular Ca²⁺.

Calcium-dependent regulation of brain adenylate cyclase is now recognized to be mediated through a specific Ca²⁺-binding protein. The Ca²⁺-dependent regulator protein (CDR)¹ has been shown to activate the enzyme from detergent-dispersed (1, 2) as well as from particulate (3, 4) preparations of cerebral cortex. The adenylate cyclase activity of washed, particulate preparations from brain is comprised of two contributing components, one of which requires CDR; the CDR-dependent activity, like other adenylate cyclase activities, is enhanced by NaF (3) and by GTP (5). Furthermore, cholera activation of the detergent-dispersed brain enzyme requires CDR (6). However, the role of CDR in hormone receptor-adenylate cyclase coupling in neural tissue has not been defined.

Ca²⁺ has been implicated in the control of responses of intact cells to hormones. The rate of accumulation of cAMP in response to norepinephrine in rat cerebral cortex slices or in response to histamine in guinea pig cerebral cortex slices was reduced when extracellular Ca²⁺ concentrations were lowered with EGTA (7). In rat brain slices, accumulation of cAMP elicited by α-adrenergic agonists depended completely on the presence of extracellular Ca²⁺ whereas accumulation of cAMP with β-adrenergic agents was influenced minimally by extracellular Ca²⁺ (8). Formation of cAMP in response to isoproterenol in rat parotid slices (9, 10), to epinephrine in adipocytes (11), and to adrenocorticotropic in perfused adrenal glands (12) was reduced when these tissues were incubated in Ca²⁺-depleted media. In contrast, hepatocytes depleted of Ca²⁺ exhibited an enhanced response to α-adrenergic agonists (13), a result compatible with the hypothesis that Ca²⁺ exerts an inhibitory action on α-adrenergic receptor-stimulated adenylate cyclase activity in this cell.

The C6 glial tumor cell responds to β-adrenergic agonists with 100-fold increases in cAMP (14) and has been used as a model for the study of the β-adrenergic receptor-adenylate cyclase system. β-Adrenergic receptor binding sites have been characterized in this cell line (15-17). C6 cells have been shown to contain CDR (18), and the catecholamine-stimulated adenylate cyclase activity of particulate preparations washed with Ca²⁺ chelators was observed to be enhanced approximately 40% by Ca²⁺ and CDR (19). CDR was found to lower the Ca²⁺ concentration required for maximal stimulation of the enzyme, but an absolute requirement of the hormone-dependent activity of cell-free preparations for Ca²⁺ and CDR was not demonstrated. Furthermore, evidence that Ca²⁺ or CDR regulates adenylate cyclase activity in vivo is lacking. The present study was undertaken to assess the role of Ca²⁺ in the regulation of cAMP synthesis in response to norepinephrine in intact C6 cells. The results obtained are consistent with the hypothesis that intracellular Ca²⁺ is required for β-adrenergic receptor-stimulated cAMP formation in these cells. CDR is proposed to be the intracellular mediator of this Ca²⁺-dependent process.

EXPERIMENTAL PROCEDURES

Materials—(−)-Norepinephrine bitartrate, dopamine hydrochloro-
ride, histamine dihydrochloride, 5-hydroxytryptamine hydrochloride, 1-methyl-3-isobutylxanthine, N-[Tris(hydroxymethyl)methyl-2-amino]ethane sulfonic acid (Tes), cAMP, and ATP were purchased from the Sigma Chemical Co. Histoquinone, glucose-6-phosphate dehydrogenase, and NADP* were obtained from Boehringer/Mannheim. Hams F-10 medium was purchased from the Grand Island Biological Co. and fetal bovine serum from Flow Laboratories, Inc. The following materials were gifts: I(-)-isoproterenol hydrochloride and I(-)-phenylephrine from the Sterling-Winthrop Research Institute, dl(-)-propranolol hydrochloride from Ayerst Laboratories, I(-)-alpranolol from Hassie, l(-)-sotalol from the Regis Chemical Co., and I(-)-phenylephrine from the Sterling-Winthrop Research Institute, dl(-)-propranolol hydrochloride from Ayerst Laboratories, I(-)-alpranolol from Hassie, l(-)-sotalol from the Regis Chemical Co., and I(-)-phenylephrine from the Sterin-Winthrop Research Institute.

Solutions of agonists and antagonists in the testate, dl(-)-propranolol hydrochloride from Ayerst Laboratories, I(-)-alpranolol from Hassie, l(-)-sotalol from the Regis Chemical Co., and I(-)-phenylephrine from the Sterin-Winthrop Research Institute, dl(-)-propranolol hydrochloride from Ayerst Laboratories, I(-)-alpranolol from Hassie, l(-)-sotalol from the Regis Chemical Co., and I(-)-phenylephrine from the Sterling-Winthrop Research Institute.

The viability of the cell preparation was determined by two procedures. The capacity of the Ca2+-depleted cells to exclude the dye trypan blue or eosin Y was monitored; by this technique the cells routinely were 90 to 95% viable. Alternatively, when Ca2+-depleted cells were prepared under fully aseptic conditions and replated in complete culture medium at the same density as that of a preparation of stock culture cells, cell number and protein increased with a doubling time equivalent to that of the stock culture cells (21 h).

Determination of CAMP Content—One-milliliter aliquots of cell suspensions or of buffered saline alone were treated with 0.2 ml of 30% trichloroacetic acid. Samples were held on ice for 30 min and were centrifuged at 2000 x g for 10 min. Pellets were dissolved in 1 N NaOH and analyzed for protein content. Each supernatant fraction was reacted with 50 ml of 1 N HCl, and trichloroacetic acid was removed by five extractions with 2 ml of water-saturated ether. The trichloroacetic acid-free samples were lyophilized, reconstituted with distilled water, and analyzed for cAMP content as previously described (21) with the following exception. Binding of [3H]cAMP to the regulatory subunit of cAMP-dependent protein kinase, adenosine 3',5'-monophosphate (cAMP), and adenosine 3',5'-cyclic monophosphate (cGMP) was determined by a model 2200 HGA graphite furnace. At least five determinations were made for each sample. Protein was determined according to the method of Lowry et al. (25).

Cell number was measured with a Levy Corpocule Counting Chamber.

RESULTS

Effect of Ca2+ Depletion on CAMP, cAMP, and ATP Content—To verify that cells prepared with solutions containing EGTA were Ca2+-depleted, the CAMP content of cells incubated in medium containing EGTA was compared with that of cells incubated in medium containing Ca2+ in excess of EGTA. Cells were separated from their extracellular fluid by centrifugation and were analyzed for Ca2+ content by atomic absorption spectrophotometry (Table IA). The contribution of extracellular Ca2+ was estimated from the inulin space of the pellet and subtracted. When two independent preparations of Ca2+-depleted and Ca2+-restored cells were examined, it was observed that the Ca2+ content of the Ca2+-depleted cells was 21% of that present in Ca2+-restored cells. It was presumed that extracellular bound Ca2+ did not contribute significantly to these measurements since pretreatment with LaCl3 (26) did not alter the values obtained.

Ca2+-depleted and Ca2+-restored cells were examined initially for cAMP and ATP content in the absence and presence of 10 µM norepinephrine (Table IB). The ATP content of the
were analyzed for CAMP and ATP as described under "Experimental Procedures." After allowing the tubes to drain, the insides of the tube were wiped dry with tissue paper and the pellet of cells was suspended in Ca"+-free water. Cells were analyzed for Ca"+ content by atomic absorption spectrophotometry.

To determine the contribution of extracellular bound Ca"+ to total Ca"+ content, 5 mM LaCl3 (26) was added to portions of each preparation of cells and the suspensions incubated an additional 15 min. The Ca"+ content of the cells was not found to be altered by the LaCl3 treatment. The Ca"+ content of the extracellular media was also determined by atomic absorption spectrophotometry and was found to be 3 mM for Ca"+-restored cells and 30 pM for Ca"+-depleted cells. The contribution of extracellular Ca"+ was estimated from the inulin paper method (26). Cells were separated from their extracellular media by centrifugation at 600 x g for 3 min. After allowing the tubes to drain, the insides of the tube were wiped dry with tissue paper and the pellet of cells was suspended in Ca"+-free water. Cells were analyzed for Ca"+ content by atomic absorption spectrophotometry.

The Ca"+ content of the extracelullar media was also determined by atomic absorption spectrophotometry and was found to be 3 mM for Ca"+-restored cells and 30 pM for Ca"+-depleted cells. The contribution of extracellular Ca"+ was estimated from the inulin paper method (26). Cells were separated from their extracellular media by centrifugation at 600 x g for 3 min. After allowing the tubes to drain, the insides of the tube were wiped dry with tissue paper and the pellet of cells was suspended in Ca"+-free water. Cells were analyzed for Ca"+ content by atomic absorption spectrophotometry.

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Table I

| Additions to Ca"+-depleted cells | Ca"+ content | ATP content |
|----------------------------------|-------------|-------------|
| None                             | 19 ± 3      | 19.4 ± 1.7  |
| Ca"+                             | 24 ± 2      | 17.8 ± 1.2  |
| Norepinephrine                   | 1220 ± 200  | 204 ± 1.7   |
| Ca"+ + norepinephrine            | 2940 ± 220  | 21.0 ± 0.8  |

The effect of increasing concentrations of norepinephrine on CAMP accumulation in Ca"+-depleted and Ca"+-restored cells was shown in Fig. 1. With both cell preparations a KAC2 for norepinephrine of 80 nM was obtained. However, at saturating concentrations of norepinephrine the CAMP accumulation in Ca"+-restored cells was 3-fold greater than that in Ca"+-depleted cells.

The time dependences of hormone-stimulated CAMP accumulation in Ca"+-depleted and Ca"+-restored cells (Fig. 2A) and of the CAMP content of their extracellular media (Fig. 2B) were examined. A rapid increase in the CAMP content of Ca"+-restored cells was apparent during the first 20 min of incubation with hormone; this increase was followed by a decline in CAMP content which continued throughout the 3-h period following hormone addition. In Ca"+-depleted cells, the CAMP content of the extracellular medium was slow, but linear with time for at least 2 h following norepinephrine treatment. Thus, at earlier time points, the CAMP content of cell suspensions is due primarily to the cyclic nucleotide inside the cells whereas at later time points the CAMP content of cell suspensions comes primarily from the extracellular medium. The CAMP content of medium from Ca"+-restored cells was greater than that of medium from Ca"+-depleted cells throughout the 3-h period following hormone addition.

a- and β-adrenergic blocking agents have been reported to have opposing effects on CAMP formed in response to norepinephrine in adipocytes (28) and in cultured cells of dissociated perinatal mouse brain (29); β-adrenergic blockers inhibit while α-adrenergic blockers potentiate the hormonal response. The α-receptor is postulated to be linked to a Ca"+-dependent process inhibitory to CAMP synthesis (13). Therefore adrenergic blocking agents were tested for their ability to influence CAMP accumulation in response to norepinephrine in Ca"+-depleted and Ca"+-restored cells (Table III). Agents possessing β-adrenergic receptor-blocking ability, i.e. propranolol, alprenolol, and sotalol, inhibited CAMP accumulation both in Ca"+-depleted and Ca"+-restored cells. Propranolol and alprenolol were more effective antagonists than sotalol, but each agent inhibited CAMP accumulation in Ca"+-restored cells to approximately the same extent that it did in Ca"+-depleted cells. Butoxamine, a selective β-adrenergic antagonist, was without effect in either cell preparation. The α-
were incubated with or without Ca\(^{2+}\) for 30 min. Varying concentrations of norepinephrine accumulation in Ca\(^{2+}\)-depleted or Ca\(^{2+}\)-restored C6 cells. Cells determined after 20 min of incubation. Ca\(^{2+}\)-depleted cells (○); Ca\(^{2+}\)-restored cells (●).

Acid or with 5% trichloroacetic acid alone as controls. The protein made 5% with respect to trichloroacetic acid. Not more than 5 min elapsed between removal of samples and addition of trichloroacetic acid. Samples were analyzed for CAMP as described under "Experimental Procedures" with buffered saline containing 5% trichloroacetic acid. The extracellular medium was made 5% with respect to trichloroacetic acid. Not more than 5 min elapsed between removal of samples and addition of trichloroacetic acid. Samples were analyzed for CAMP as described under "Experimental Procedures" with buffered saline containing 5% trichloroacetic acid or with 5% trichloroacetic acid alone as controls. The protein concentration of the original cell suspension was 0.33 mg/ml. Panel A, CAMP contents of cells; Panel B, CAMP contents of extracellular media. Ca\(^{2+}\)-depleted cells (○); Ca\(^{2+}\)-restored cells (●).

Adrenergic blocker phentolamine only produced a small (20%) inhibition of CAMP accumulation in both preparations of cells. None of the blocking agents altered significantly the CAMP contents of unstimulated cells in the absence or presence of Ca\(^{2+}\). Increasing concentrations of propranolol were observed to inhibit CAMP accumulation following norepinephrine to the same degree in Ca\(^{2+}\)-depleted as in Ca\(^{2+}\)-restored cells. 0.2 μM propranolol was sufficient to produce 50% inhibition of CAMP accumulation in response to 10 μM norepinephrine (data not shown).

Divalent cations such as Mg\(^{2+}\) and Mn\(^{2+}\) are reported to influence markedly the affinity of α-adrenergic receptors for α-adrenergic agonists (30). Yet the results obtained here with β-adrenergic agonists and antagonists are in agreement with the hypothesis that Ca\(^{2+}\) does not alter the affinity of the β-receptor for these agents. The decreased maximal rates of CAMP accumulation seen in Ca\(^{2+}\)-depleted cells could be explained by an action of Ca\(^{2+}\) at a site other than the hormone receptor. An alternative explanation is a decrease in the number of receptor sites following Ca\(^{2+}\) depletion. To investigate the latter possibility, the binding of [\(^3\)H]dihydroalprenolol, a potent β-adrenergic antagonist demonstrated to bind

**FIG. 1.** Norepinephrine concentration dependence of CAMP accumulation in Ca\(^{2+}\)-depleted or Ca\(^{2+}\)-restored C6 cells. Cells were incubated with or without Ca\(^{2+}\) for 30 min. Varying concentrations of norepinephrine were then added, and CAMP content was determined after 20 min of incubation. Ca\(^{2+}\)-depleted cells (○); Ca\(^{2+}\)-restored cells (●).

**FIG. 2.** Accumulation of CAMP with time in Ca\(^{2+}\)-depleted and Ca\(^{2+}\)-restored C6 cells and the respective CAMP content of their extracellular fluids following addition of norepinephrine. Cells were incubated for 30 min with or without Ca\(^{2+}\) and then challenged with 10 μM norepinephrine. At the indicated times, 1-ml aliquots of cell suspension were removed and centrifuged immediately at 1000 × g for 2 min at 4°C. The pellet of cells was suspended in 1 ml of ice-cold 5% trichloroacetic acid. The extracellular medium was made 5% with respect to trichloroacetic acid. Not more than 5 min elapsed between removal of samples and addition of trichloroacetic acid. Samples were analyzed for CAMP as described under "Experimental Procedures" with buffered saline containing 5% trichloroacetic acid or with 5% trichloroacetic acid alone as controls. The protein concentration of the original cell suspension was 0.33 mg/ml. Panel A, CAMP contents of cells; Panel B, CAMP contents of extracellular media. Ca\(^{2+}\)-depleted cells (○); Ca\(^{2+}\)-restored cells (●).

**FIG. 3.** Binding of [\(^3\)H]dihydroalprenolol to Ca\(^{2+}\)-depleted and Ca\(^{2+}\)-restored C6 cells as a function of ligand concentration. Ca\(^{2+}\)-depleted and Ca\(^{2+}\)-restored cells were incubated for 30 min and then treated with 10 μM blocking agents for another 10 min. Norepinephrine (10 μM) was added where indicated, and the CAMP content was determined after 20 min of incubation.

**TABLE III**

Effects of adrenergic blocking agents on CAMP content of Ca\(^{2+}\)-depleted and Ca\(^{2+}\)-restored cells

| Additions | Ca\(^{2+}\)-depleted cells | Ca\(^{2+}\)-restored cells |
|-----------|---------------------------|---------------------------|
| None      | 23 ± 2                    | 24 ± 2                    |
| Propranolol| 27 ± 2                    | 27 ± 2                    |
| Alprenolol| 29 ± 3                    | 30 ± 1                    |
| Sotalol   | 26 ± 5                    | 25 ± 5                    |
| Butoxamine| 27 ± 5                    | 28 ± 2                    |
| Phentolamine| 25 ± 1                  | 28 ± 2                    |

Results are expressed as the mean of determinations performed in triplicate. The number of cells in the preparation was determined to be 1.1 × 10⁰/mg of protein. Ca\(^{2+}\)-depleted cells (○, A); Ca\(^{2+}\)-restored cells (●, A). Aliquots of the original cell preparations were saved for analyses of CAMP accumulation in response to treatment with 10 μM norepinephrine for 20 min. Values obtained were: Ca\(^{2+}\)-depleted cells, 1.28 ± 0.08 nmol of CAMP/mg of protein; Ca\(^{2+}\)-restored cells, 3.82 ± 0.10 nmol of CAMP/mg of protein.
to the β-adrenergic receptor of C6 cells (16), was measured in intact Ca²⁺-depleted and Ca²⁺-restored cells. As shown in Fig. 3, specific dihydroalprenolol binding was a saturable process as a function of ligand concentration while nonspecific binding increased linearly. At limiting dihydroalprenolol concentrations, specific binding was the predominant form whereas nonspecific binding predominated at saturating ligand concentrations. A dissociation constant for specific binding of 4 nM was obtained using either Ca²⁺-depleted or Ca²⁺-restored cells. Furthermore, specific binding at saturating dihydroalprenolol was not changed by Ca²⁺ depletion, and 1.8 × 10⁴ receptors/cell were calculated to be present in both preparations. This value is in close agreement with that obtained by Lucas and Bockaert (16) for washed membrane preparations of C6 cells (1.0 × 10⁴ receptors/cell). Nonspecific binding was similarly unaffected by Ca²⁺ depletion. The results therefore do not support the assertion that cells depleted of Ca²⁺ have fewer β-receptors.

C6 cells are known to exhibit a decreased responsiveness to catecholamines when the cells are pretreated for several hours with these hormones (31, 32). The development of tachyphylaxis results, at least in part, from a decrease in catecholamine-stimulated adenylate cyclase activity (33). It was of interest to determine whether cells which had become subsensitive to norepinephrine retained a Ca²⁺ requirement for cAMP accumulation in response to hormone. One monolayer culture of cells was pretreated with norepinephrine for 3 h in Hams F-10 medium while a second monolayer culture was pretreated in medium alone. Following the pretreatment, suspensions of Ca²⁺-depleted and Ca²⁺-restored cells were prepared from each monolayer culture and amounts of cAMP accumulated in response to fresh norepinephrine were determined (Fig. 4). The pretreated culture (Panel B) exhibited a 70% reduction in cAMP accumulation as compared to the control culture (Panel A). Both cultures, however, showed a 3-fold dependence on Ca²⁺ for cAMP accumulation suggesting that modification of hormonal effectiveness in tachyphylaxis is not attributable to modification of the Ca²⁺ requirement for cAMP synthesis. These observations are suggestive of a Ca²⁺ effect at a site beyond that of the catecholamine receptor.

Factors which Influence the Ca²⁺ Requirement for Norepinephrine-stimulated cAMP Accumulation—The extracellular CaCl₂ concentration dependence of the cAMP content of unstimulated cells and of cells exposed to norepinephrine for 20 min is shown in Fig. 5. cAMP in unstimulated cells did not change as Ca²⁺ concentrations in the extracellular media were increased (Panel A). cAMP accumulated in response to hormone (Panel B) was dependent on external Ca²⁺ concentration with maximal rates being obtained at 1 mM added CaCl₂. This actually represented micromolar concentrations of free Ca²⁺ since the EGTA concentration in the external medium was 1 mM.

The time required for Ca²⁺ to enhance accumulation of cAMP when added to Ca²⁺-depleted cells was investigated (Fig. 6A). A suspension of Ca²⁺-depleted cells was divided in half and each half was treated with hormone. One-half was treated with Ca²⁺ at 4½ min after addition of hormone. In the absence of external Ca²⁺, cAMP was accumulated in a nonlinear fashion during the 16 min following hormone addition. Ca²⁺ produced an immediate increase in cAMP accumulation which was linear with time for approximately 10 min after Ca²⁺ addition. The ability of EGTA to reduce the Ca²⁺-dependent component of cAMP accumulation was also examined (Fig. 6B). A suspension of Ca²⁺-depleted cells was divided into equal portions and each portion treated with norepinephrine. One portion of cells was treated with EGTA in excess of Ca²⁺ at 4½ min after norepinephrine, and cAMP accumulation in the two suspensions of cells was compared. Accumulation of the nucleotide in the presence of Ca²⁺ was linear with respect to time for at least 12 min following hormone addition. When EGTA was added in excess of Ca²⁺, the extent of cAMP accumulation was immediately reduced to that of the Ca²⁺-depleted cells.

It was desired to ascertain whether the Ca²⁺ necessary for cAMP accumulation in response to hormone was extracellular or intracellular. Preliminary evidence was consistent with an extracellular Ca²⁺ requirement for the hormonal response. First, repeated washing of the cells in monolayer culture with EGTA-containing medium was necessary to obtain a cell preparation exhibiting a decreased rate of cAMP accumulation in response to norepinephrine. Second, micromolar free
Ca\textsuperscript{2+} concentrations, which are believed to exist in intracellular fluids (34), were sufficient for maximal accumulation of cAMP (Fig. 5). On the other hand EGTA rapidly abolished the Ca\textsuperscript{2+}-dependent component of cAMP accumulation when added to suspensions of Ca\textsuperscript{2+}-restored cells (Fig. 6B), an observation consistent either with an extracellular Ca\textsuperscript{2+} requirement or a requirement for an intracellular Ca\textsuperscript{2+} pool in the Ca\textsuperscript{2+}-dependent CAMP accumulation of C6 cells (34). Suspensions of Ca\textsuperscript{2+}-depleted cells in buffered saline containing 1 mM Ca\textsuperscript{2+} but lacking EGTA. The suspension was incubated for 30 min and divided into equal portions. Each portion was challenged with 10 \mu M norepinephrine. One-milliliter aliquots of cells were removed at 1-min intervals and analyzed for cAMP content. At 4\% min, half of the suspension was treated with 2 mM CaCl\textsubscript{2} (●) and the other half with an equivalent volume of buffered saline (○). Sampling was continued to 16 min. Panel B, time dependence of EGTA effect. Ca\textsuperscript{2+}-restored cells were prepared by suspending Ca\textsuperscript{2+}-depleted cells in buffered saline containing 1 mM CaCl\textsubscript{2}, and the other with an equivalent volume of buffered saline (●). Sampling was continued to 16 min. Each point shown in Panels A and B represents a cAMP determination on a single sample of cells.

The adenylate cyclase activity of cerebral cortex homogenates exists in two forms, one of which is stabilized by Ca\textsuperscript{2+} to thermal denaturation (36). When intact C6 cells were pretreated at 45°C for varying lengths of time in the absence or in the presence of Ca\textsuperscript{2+}, two components of hormone-stimulated cAMP accumulation could be distinguished (Fig. 8). The capacity of the cells to accumulate cAMP in response to norepinephrine decreased. This effect of verapamil was competitive with respect to Ca\textsuperscript{2+} concentration; at 10 mM CaCl\textsubscript{2}, the inhibition was almost fully overcome. Verapamil had no effect on cAMP accumulation in the absence of Ca\textsuperscript{2+}.

The capacity of the cells to accumulate cAMP in response to norepinephrine decreased as a function of time of pretreatment. Accumulation of cAMP determined in the absence of Ca\textsuperscript{2+} (Panel A) was reduced 80\% regardless of whether Ca\textsuperscript{2+} was present during the 45\degree C pretreatment period. However, cAMP accumulation determined in the presence of Ca\textsuperscript{2+} (Panel B) was not identical for cells pretreated with and without Ca\textsuperscript{2+}; cells pretreated with Ca\textsuperscript{2+} accumulated more of the nucleotide. The Ca\textsuperscript{2+}-dependent component of cAMP accumulation, defined as the increment in cAMP accumulation due to the presence of Ca\textsuperscript{2+}, accounted for two-thirds of the total cAMP accumulated in nonpretreated cells whereas in cells pretreated with Ca\textsuperscript{2+} at 45\degree C the Ca\textsuperscript{2+}-dependent
component accounted for 80% of the total. Thus, exposing the cells to high temperature with Ca\(^{2+}\) increased the Ca\(^{2+}\) dependency of the hormonal response by selectively inactivating the component of the process which did not depend on Ca\(^{2+}\). In contrast, thermal pretreatment in the absence of Ca\(^{2+}\) inactivated both components of the process equally.

Effects of Trifluoperazine on Norepinephrine-stimulated cAMP Accumulation in C6 Cells—Phenothiazines competitively inhibit the stimulation by CDR of brain adenylate cyclase (5). Calcium-dependent binding sites for these drugs on CDR have been described and evidence provided that the phenothiazine Ca\(^{2+}\)-CDR complex is not an activating species (37). It was therefore of interest to determine whether phenothiazines inhibit Ca\(^{2+}\)-dependent cAMP accumulation in intact cells. The effect of trifluoperazine concentration on the cAMP content of nonstimulated cells and of norepinephrine-treated cells is presented in Fig. 9. At concentrations up to 30 \(\mu M\), trifluoperazine had no effect on the cAMP content of Ca\(^{2+}\)-depleted or Ca\(^{2+}\)-restored cells in the absence of hormone (Panel A) or on the cAMP content of Ca\(^{2+}\)-depleted cells exposed to hormone (Panel B). However, hormone-stimulated cAMP accumulation in Ca\(^{2+}\)-restored cells was progressively inhibited by increasing drug concentrations; at 30 \(\mu M\) trifluoperazine, cAMP accumulation in these cells was reduced to a value similar to that of Ca\(^{2+}\)-depleted cells. Ca\(^{2+}\)-dependent cAMP accumulation was reduced 50% by 15 \(\mu M\) drug.

Other workers have observed that phenothiazines inhibit dopamine-sensitive (38) or norepinephrine-sensitive (39) adenylate cyclase of brain in a manner competitive with catecholamine. To ascertain that the trifluoperazine effect described above was not due to occupation of the \(\beta\)-adrenergic receptor by the drug, the norepinephrine concentration dependence of cAMP accumulation was determined in the absence or presence of 15 \(\mu M\) trifluoperazine (Fig. 10). The \(K_{\text{ACT}}\) for norepinephrine in the absence of drug was 80 nM for Ca\(^{2+}\)-depleted and Ca\(^{2+}\)-restored cells (Panel A); in the presence of the drug the \(K_{\text{ACT}}\) for hormone was shifted to 300 nM in both preparations of cells (Panel B). Inhibition by the drug of cAMP accumulation in Ca\(^{2+}\)-depleted cells, however, was fully overcome at saturating norepinephrine. In contrast, the trifluoperazine inhibition in Ca\(^{2+}\)-restored cells was not overcome at saturating hormone. These observations are consistent with two distinct effects of trifluoperazine: competition with hormone for its receptor and Ca\(^{2+}\)-dependent inhibition of cAMP accumulation at limiting or at saturating hormone concentrations.

The two inhibitory actions of trifluoperazine in C6 cells are summarized and contrasted in Table IV as a function of drug concentration. The \(K_{\text{ACT}}\) for norepinephrine was seen to increase gradually over a large range of trifluoperazine concentrations; a doubling of \(K_{\text{ACT}}\) occurred at 8 \(\mu M\). On the other hand, Ca\(^{2+}\)-dependent accumulation of cAMP was decreased over a narrow and high range of drug concentrations. At 30 \(\mu M\) trifluoperazine and 30 \(\mu M\) norepinephrine (a concentration 90 times the \(K_{\text{ACT}}\)), the Ca\(^{2+}\)-dependent component of cAMP

![Fig. 9. Effect of trifluoperazine concentration on cAMP content of Ca\(^{2+}\)-depleted and Ca\(^{2+}\)-restored C6 cells. Cells were incubated for 30 min with or without Ca\(^{2+}\) and treated with the indicated concentrations of trifluoperazine. After 10 min of incubation with the drug, norepinephrine (10 \(\mu M\)) or saline was added and cAMP content determined after 20 min of incubation. Ca\(^{2+}\)-depleted cells (C); Ca\(^{2+}\)-restored cells (O). Panel A, without norepinephrine; Panel B, with norepinephrine.](http://www.jbc.org/)

![Fig. 10. Effect of trifluoperazine on the norepinephrine concentration dependence of cAMP accumulation in Ca\(^{2+}\)-depleted and Ca\(^{2+}\)-restored C6 cells. Cells were pretreated for 30 min with or without Ca\(^{2+}\). Trifluoperazine (15 \(\mu M\)) was then added to half of the cells and an equivalent volume of saline to the other half. After 10 min of incubation cells were challenged with varying concentrations of norepinephrine, incubated 20 min and analyzed for cAMP content. Ca\(^{2+}\)-depleted cells (C); Ca\(^{2+}\)-restored cells (O). Panel A, without trifluoperazine; Panel B, with 15 \(\mu M\) trifluoperazine.](http://www.jbc.org/)

![Table IV](http://www.jbc.org/)

## Table IV

Comparison of the effect of trifluoperazine concentration on the \(K_{\text{ACT}}\) for norepinephrine with the effect of trifluoperazine concentration on cAMP accumulation in C6 cells

| Trifluoperazine | \(K_{\text{ACT}}\) for norepinephrine | Total cAMP accumulation in Ca\(^{2+}\)-restored C6 cells following 30 \(\mu M\) norepinephrine | Ca\(^{2+}\)-dependent cAMP accumulation following 30 \(\mu M\) norepinephrine |
|-----------------|--------------------------------|---------------------------------|---------------------------------|
| \(\mu M\)       | \(\mu M\)        | nmol/mg protein                  | nmol/mg protein                  |
| 0               | 0.074           | 3.50 ± 0.14                      | 2.45 ± 0.24                      |
| 0.1             | 0.081           | 3.45 ± 0.22                      | 2.55 ± 0.27                      |
| 0.3             | 0.091           | 3.45 ± 0.23                      | 2.40 ± 0.28                      |
| 1               | 0.11            | 3.37 ± 0.15                      | 2.32 ± 0.40                      |
| 3               | 0.12            | 2.86 ± 0.27                      | 1.83 ± 0.47                      |
| 10              | 0.16            | 2.65 ± 0.13                      | 1.53 ± 0.21                      |
| 30              | 0.34            | 0.93 ± 0.05                      | 0.28 ± 0.17                      |
accumulation was virtually abolished but the capacity of norepinephrine to saturate the receptor was unaffected. Increasing concentrations of Ca\(^{2+}\) were ineffective in reversing the inhibition of Ca\(^{2+}\)-dependent cAMP accumulation by 15 or by 30 \text{pM} trifluoperazine (Fig. 11). Both concentrations of drug decreased hormone-sensitive CAMP accumulation at all Ca\(^{2+}\) concentrations tested with the exception of no added Ca\(^{2+}\).

**DISCUSSION**

The results presented in this report demonstrate that Ca\(^{2+}\) depletion alters the capacity of C6 cells to accumulate cAMP in response to \(\beta\)-adrenergic agonists. The data are consistent with the concept that intracellular, as opposed to extracellular, Ca\(^{2+}\) is a requirement for maximal CAMP formation in these cells in response to hormone. For example, simple replacement of the extracellular growth medium with medium containing EGTA had no effect on the ability of cells in monolayer culture to accumulate cAMP. Rather, repeated washing of the cells in EGTA-containing medium was necessary to observe a decrease in the accumulation of cAMP. Paradoxically, when Ca\(^{2+}\)-depleted cells were subsequently restored with Ca\(^{2+}\), EGTA effectively removed the Ca\(^{2+}\)-dependent component of cAMP accumulation suggesting an extracellular Ca\(^{2+}\) requirement (Fig. 6). It is possible, however, that cells which have been re-exposed to the cation are not fully Ca\(^{2+}\)-restored, but have only certain Ca\(^{2+}\) pools repleted. If the pool of intracellular Ca\(^{2+}\) which is needed for cAMP accumulation were in rapid equilibrium with the pool of extracellular Ca\(^{2+}\), then EGTA addition would be expected to deplete both of these Ca\(^{2+}\) pools. The finding that cells in monolayer culture with normal stores of Ca\(^{2+}\) require more extensive EGTA treatment to inhibit cAMP accumulation may reflect a sequential mobilization of a strongly sequestered intracellular pool(s) of Ca\(^{2+}\) to a free Ca\(^{2+}\) pool which can then be depleted by EGTA. Without a knowledge of the Ca\(^{2+}\) contents of these intracellular pools for either cell preparation, it is difficult to substantiate or disprove such an argument. A second observation implying the involvement of intracellular Ca\(^{2+}\) was the restoration of full hormonal responsiveness at equimolar concentration of Ca\(^{2+}\) and EGTA (Fig. 5). If the \(K_d\) for Ca\(^{2+}\)-EGTA at pH 7.5 is assumed to be 0.1 \text{mM} (40), then free Ca\(^{2+}\) under these conditions would be present in the micromolar range. Micromolar free Ca\(^{2+}\) concentrations are believed to exist in intracellular fluids (34). If extracellular Ca\(^{2+}\) were required, then millimolar free concentrations such as are present in plasma should have been necessary to restore Ca\(^{2+}\)-dependent cAMP accumulation. Lastly, Ca\(^{2+}\)-dependent cAMP accumulation could be inhibited by verapamil, an agent which reduces Ca\(^{2+}\) influx, when the drug was added prior to Ca\(^{2+}\) restoration (Fig. 7).

The only recognized intracellular Ca\(^{2+}\) receptor which has a stimulatory effect on the synthesis of cAMP is CDR. Although the experiments presented here do not demonstrate a CDR involvement in Ca\(^{2+}\)-dependent cAMP accumulation in intact C6 cells, several observations favor a role for CDR in this process. First, Ca\(^{2+}\) did not affect the concentration of the substrate for adenylate cyclase in these cells. Although an obligatory relationship is known to exist between Ca\(^{2+}\) accumulation and energy utilization at the level of the mitochondrial respiratory chain (41), the ATP contents of Ca\(^{2+}\)-depleted and Ca\(^{2+}\)-restored cells were similar (Table I). Second, no effects of Ca\(^{2+}\) were noted on the interaction of agonist or antagonist with the adrenergic receptor; Ca\(^{2+}\) altered neither the \(K_{act}\) for norepinephrine (Fig. 1) nor the extent of inhibition by propranolol of the response to norepinephrine. Dihydraldrenolol binding was unaffected by the absence or presence of Ca\(^{2+}\) (Fig. 3). Furthermore the development of \(\beta\)-receptor subsensitivity did not change the Ca\(^{2+}\) requirement for cAMP accumulation (Fig. 4). Third, cAMP accumulation in the presence of Ca\(^{2+}\) was inhibited by the drug trifluoperazine. This observation directly implicates CDR since phenothiazines have been demonstrated (a) to inhibit competitively the CDR-dependent activation of brain adenylyl cyclase (5) and cyclic nucleotide phosphodiesterase (42, 43) and (b) to bind in Ca\(^{2+}\)-dependent manner to specific sites on CDR (37) creating an apparently ineffective species. The specific effect of the drug on Ca\(^{2+}\)-dependent cAMP accumulation was not attributed to competition with norepinephrine for its receptor because inhibitions by the drug of the Ca\(^{2+}\)-dependent component of the activity were obtained at saturating concentrations of hormone (Fig. 10, Table IV). Concentrations of trifluoperazine which inhibited Ca\(^{2+}\)-dependent cAMP accumulation were in the micromolar range (Fig. 9), in agreement with those reported to be required for binding to CDR in vitro (37). Increasing concentrations of Ca\(^{2+}\) did not overcome the trifluoperazine inhibition of Ca\(^{2+}\)-dependent cAMP accumulation (Fig. 11). Similarly, inhibition of CDR-dependent phosphodiesterase (43) or adenylyl cyclase by phenothiazines in vitro is not overcome by Ca\(^{2+}\); the inhibition is reversed solely by CDR. A fourth observation consistent with a CDR involvement was the relative stability of the Ca\(^{2+}\)-dependent process of cAMP accumulation when cells were exposed to elevated temperature in the presence as compared to the absence of Ca\(^{2+}\) (Fig. 8). A similar observation has been made in brain homogenates; Ca\(^{2+}\) selectively stabilized to thermal denaturation the component of adenylyl cyclase which depended on Ca\(^{2+}\) for activity (5). The stabilizing factor was identified to be CDR. The Ca\(^{2+}\)-dependent form of brain adenylyl cyclase in washed, particulate preparations from which CDR had been removed was protected by Ca\(^{2+}\)-CDR, but not by Ca\(^{2+}\) alone (3). A final piece of evidence supportive of a CDR involvement is that adenylyl cyclase of cell-free preparations of C6 cells is stimulated to some extent by Ca\(^{2+}\) and CDR (19). Although CDR was not fully removed from

3 M. A. Brostrom, unpublished observations.
and was not demonstrated to be a requirement of the enzyme, the addition of CDR served to increase the sensitivity of the enzyme to Ca⁺⁺. As was seen for cAMP accumulation in intact C6 cells, the adenylate cyclase activity of particulate fractions of these cells was influenced by micromolar free Ca⁺⁺ concentrations. The K_{AC} for norepinephrine was unaltered by Ca⁺⁺ in either system or by CDR itself in the cell-free system.

Investigations of the adenylate cyclase activity of homogenates of rat cerebral cortex revealed two contributing components, only one of which required CDR for activity (3). Although the CDR-requiring component from cerebral cortex is not known to be activated by hormones, it has several properties in common with Ca⁺⁺-dependent cAMP accumulation in response to hormone in intact C6 cells. Both processes require micromolar free Ca⁺⁺ concentrations for optimal activity. are more stable to elevated temperatures in the presence of Ca⁺⁺, are inhibited by phenothiazines, and are present 65 to 80% of the total activity of the system. The basal adenylate cyclase activity of brain homogenates which did not depend on Ca⁺⁺ shared several features with cAMP accumulation in Ca⁺⁺-depleted C6 cells. Each of these activities represented 20 to 35% of the total activity, was inactivated equally well at elevated temperatures in the absence or presence of Ca⁺⁺, and was unaffected by phenothiazines at concentrations which inhibit the Ca⁺⁺-dependent processes. While it is clear that two components of the process of cAMP synthesis can be recognized in vivo as well as in vitro, the relationships between the Ca⁺⁺-dependent and Ca⁺⁺-independent processes are at this time unclear.

The cAMP content of Ca⁺⁺-restored cells was observed to increase rapidly during the first 20 min after norepinephrine addition and then to decline at a constant rate for approximately 90 min. The decline phase, which was noted for the cAMP content of cells but not for the cAMP content of the extracellular medium, was almost completely eliminated by Ca⁺⁺ depletion (Fig. 2). Since C6 cells are known to contain a Ca⁺⁺-dependent phosphodiesterase activity (18), it is attractive to speculate that this enzyme may be responsible for the decline in cAMP content of cells seen during longer incubations with hormone and that depletion of cellular Ca⁺⁺ interferes with this activity. Such an interpretation should be viewed cautiously, however, in light of the complexity of the known adaptive changes in cAMP metabolism in C6 cells following prolonged hormonal exposure (31, 33).

Participation of Ca⁺⁺ in the hormonal responses of other intact cell systems has been reported but important differences exist between the requirements of these systems and those of the C6 cell system. For example, formation of cAMP in brain slices in response to norepinephrine or histamine is dependent on Ca⁺⁺ (7). However, the source of the Ca⁺⁺ required for these responses is believed to be extracellular since EGTA added immediately before hormone to minimize disturbances of the intracellular Ca⁺⁺ pools was found to reduce the rate of cAMP accumulation. Furthermore, millimolar concentrations of Ca⁺⁺ as are ordinarily found in extracellular fluids were required to achieve maximal rates of cAMP synthesis. Norepinephrine-stimulated, Ca⁺⁺-dependent cAMP accumulation in brain slices was also shown to require the presence of adenosine. Yet neither adenosine nor high concentrations of adenosine deaminase had any effect on Ca⁺⁺-dependent cAMP accumulation in C6 cells (3). Another major difference between the two intact cell systems is that the Ca⁺⁺ requirement for norepinephrine-stimulated cAMP formation in brain slices was mediated primarily by an α-adrenergic receptor; Ca⁺⁺ effects on β-receptor-stimulated cAMP accumulation were marginal (8). Since the C6 cell is a glial tumor cell, it will be of interest to determine whether the

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