The modulation of neuronal adenyl cyclase by Ca\(^{2+}\), acting via calmodulin, is a long-established example of a positive interaction between the Ca\(^{2+}\)-mobilizing and cAMP-generating systems. In the present study, concentrations of Ca\(^{2+}\) that stimulate brain adenyl cyclase inhibit the adenyl cyclase of NCB-20 plasma membranes. These inhibitory effects of Ca\(^{2+}\) have been characterized and seem to be exerted at the catalytic unit of the enzyme; they are independent of calmodulin, G\(_i\), and phosphodiesterase. To determine whether this inhibition of adenyl cyclase by Ca\(^{2+}\) could occur in the intact cell, cAMP accumulation was measured in response to bradykinin. Bradykinin, which mobilizes Ca\(^{2+}\) in NCB-20 cells, as a consequence of stimulating inositol phosphate production, causes a transient inhibition of prostaglandin E\(_1\), stimulation of cAMP accumulation. The inhibitory action of bradykinin is attenuated significantly by treatment of cells with the cell-permeant Ca\(^{2+}\) chelator, 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid. It seems likely that the inhibition of adenyl cyclase by low concentrations of Ca\(^{2+}\) represents a novel means for a negative interaction between Ca\(^{2+}\)-mobilizing and cAMP-generating systems.

A growing volume of evidence demonstrates that significant interactions occur between individual signal transduction systems within cells (1-7). Although the number of signal-generating systems is limited, including adenyl cyclase, phospholipase C, phospholipase A\(_2\), and various ion channels, the patterns of interactions between these systems may vary between cell types, depending on the relative abundance and arrangement of system components and thereby leading to a considerable number of integrated cell signalling strategies. A long-established example of such interactions is the modulation of neuronal adenyl cyclase activity by Ca\(^{2+}\) ions acting via calmodulin (CaM); 8, 9). Brain adenyl cyclase activity is stimulated severalfold in the presence of CaM, by a range of Ca\(^{2+}\) ion concentrations which correspond to intracellular levels that are achieved upon neuronal depolarization or activation of the phospholipase C/Ca\(^{2+}\)-mobilizing system (6). It has been proposed that such stimulatory effects of Ca\(^{2+}\) on adenyl cyclase activity might promote cAMP-facilitated entry of Ca\(^{2+}\) via ion channels, thereby enhancing the rapid release of neurotransmitters from neurons and thus contributing to the synergism between the two systems in controlling neuronal activity (6, 10, 11).

Compelling evidence of synergistic interactions between cAMP- and Ca\(^{2+}\)-signalling pathways has been gathered from studies of intact brain preparations, although the underlying mechanisms have been difficult to identify or evaluate due to the heterogeneity of the preparations (2, 12). To evaluate the relative contributions of protein kinase C, calmodulin-dependent kinases, phosphodiesterases, or direct effects of Ca\(^{2+}\) on adenyl cyclase, it is necessary to study a homogenous cell line that is amenable to manipulation and dissection of the regulatory interplays. In the present studies, NCB-20 cells have been used as a model system both to examine the effects of Ca\(^{2+}\) ions on plasma membrane adenyl cyclase activity and to explore the effects of Ca\(^{2+}\)-mobilizing hormones on cAMP production in the intact cell. NCB-20 cells are hybrids derived from mouse neuroblastoma \(\times\) 18-day embryonic Chinese hamster brain explants (13, 14). Unexpectedly, we observed that concentrations of Ca\(^{2+}\) in the physiological range caused a substantial inhibition of adenyl cyclase activity in NCB-20 plasma membranes. The regulatory features of this Ca\(^{2+}\) inhibition were characterized in membrane preparations. The significance of this inhibition was confirmed in intact NCB-20 cells by the observation that bradykinin, which mobilizes intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)), via inositol phosphates, induced a significant reduction of cAMP accumulation in the intact cell. These observations provide a novel, inhibitory mode of interaction between Ca\(^{2+}\)-mobilizing and adenyl cyclase-signalling pathways.

**MATERIALS AND METHODS**

**Growth of NCB-20 Cells**—NCB-20 cells provided by Dr. Glyn Dawson (University of Chicago) and Dr. Fabian Guosovsky (National Institutes of Health) were grown in monolayer culture in the presence of Dulbecco’s minimum essential medium (DMEM) containing 5 mg/ml gentamicin, supplemented with 5% fetal bovine serum.

**Preparation of Plasma Membranes from NCB-20 Cells**—Cultured NCB-20 cells were detached from flask surfaces with phosphate-buffered saline (12 mM Na\(_2\)HPO\(_4\), 130 mM NaCl, pH 7.4) containing 0.02% EDTA. Membranes were prepared using a method described previously (15). Cell suspensions were centrifuged, washed with Philip’s buffer containing protease inhibitors (20 \(\mu\)g/ml soybean trypsin inhibitor, 4 \(\mu\)g/ml leupeptin, 12 units/ml kalikrein inactivator, 4 \(\mu\)g/ml antipain), and resuspended in Philip’s buffer diluted by 25% with water. The solutions were mixed gently by inversion and allowed to

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stand at room temperature for about 10 min. Following centrifugation and subsequent lysis of cells in hypotonic buffer containing protease inhibitors, the lysate was centrifuged at 270 × g for 10 min (at 4 °C). The supernatant was fractionated on a discontinuous gradient of 30 and 40% sucrose solutions in lysis buffer. Material collecting at the 30-40% interface was removed, washed, and resuspended in lysis buffer to a final protein concentration of 0.25–1.0 mg/ml (as determined by the method of Lowry et al. (16), using bovine serum albumin as standard), and stored in liquid nitrogen.

Preparation of Rat Cerebellar Plasma Membranes—Cerebellar plasma membranes were prepared from a continuous sucrose gradient and washed three times in a buffer containing 1 mM EGTA, as described previously (18).

Assay of Adenylyl Cyclase Activity—The adenylyl cyclase activity of purified NCB-20 or cerebellar plasma membranes (generally 6–10 μg of protein per assay) was measured in the presence of the following components (final concentrations): 4 mM phosphocreatine (disodium salt), 20 units/ml creatine phosphokinase, 0.1 mM CAMP, 1 unit/ml adenosine deaminase, 1 mM MgCl2, 0.08 mM ATP (disodium salt), 3–5 × 10^13 cpm/assay [α-32P]-ATP (tetra[triethylammonium] salt) (Amersham, 70 mM Tris-HCl, pH 7.4, and 0.01 mM GTP and 5 μM PGE2, unless indicated otherwise. The reaction mixture (final volume 100 μl) was incubated at 30 °C for 20 min. Reactions were stopped, and the [32P]-CAMP formed was quantified as described (17). Data points are presented as mean activities ± S.D. of triplicate determinations, unless indicated otherwise.

Free Ca2+ Concentrations—Free concentrations of Ca2+ were calculated as described previously (18). This involved an iterative computing program that solved the equations describing the complexes formed in a mixture composed of the assay ingredients which affect free divalent cation concentration, i.e. ATP, GTP, EGTA, Mg2+, Na+ and Ca2+ (19). Final assay mixture concentrations of CaCl2 (against a background of 200 μM EGTA) that gave rise to the free Ca2+ concentrations indicated in parentheses are as follows: 132 (0.080), 152 (0.10), 168 (0.22), 178 (0.33), 185 (0.49), 191 (0.81), 197 (1.7), 202 (4.0), 210 (10), 223 (23), 241 (40), and 260 (58) μM.

Treatment of Cells with Pertussis Toxin—Cell culture media were supplemented with 125 ng/ml pertussis toxin (List Biological Laboratories, Campbell, CA) for 24–30 h. Following exposure to the toxin, the cells were harvested and used for preparing plasma membranes for adenylyl cyclase assays.

Determination of Ca2+ Production in Intact Cells—Four-day cultures of NCB-20 cells were plated in 35-mm wells at a density of about 400,000 cells per well and used for obtaining base-line values. Prior to an experiment, the culture medium was aspirated and washed 3 times with 1 ml of DMEM without serum (37 °C). This medium was then aspirated, and serum-free DMEM incubation medium (1 ml) containing the following compounds was added to the wells for various periods of time: 10 μM PGE2, 500 μM IBMX, 100 μM Ro 20-1724, and 1 μM bradykinin, when indicated. Reactions were stopped by washing the cells with a buffer containing 100 μM MgCl2 and 1 μM bradykinin, and the suspensions centrifuged at 13,000 × g for 5–10 min in a Fisher 235B microcentrifuge at 4 °C. The supernatant was extracted five times with water-saturated ether and then frozen (~20 °C) until assay. The assay was performed by adding 1 N NaOH for heating for about 5 min, and assaying for protein (16).

Assay of Inositol Phosphate Production in Intact Cells—NCB-20 cells (about 1 × 10^6) were plated in 35-mm wells and allowed to attach for 24 h, at which time the culture medium was replaced with inositol-free DMEM, to which [3H]-inositol (22 Ci/mmol, Amersham) was added at a final concentration of 3–5 μCi/ml. Forty-eight hours later, the labeled medium was aspirated, and cells were washed for about 6 min with serum-free DMEM containing 10 mM LiCl (37 °C). This medium was aspirated, and serum-free DMEM (with 10 mM LiCl) was then added and increasing concentrations of bradykinin was added to cells wells for 10 min. Reactions were stopped with trichloroacetic acid (10% final) and all samples processed precisely as described above for CAMP assays, with the exception that the supernatant was adjusted to pH 7.0 with 2 M Tris base before storage at −80 °C. The sample supernatants were assayed for radio-labeled inositol phosphates by Dowex ion-exchange chromatography, involving sequential elutions with ammonium formate/formic acid, as described (20).

Intracellular Calcium Measurements—NCB-20 cells were detached with phosphate-buffered saline containing 1 mM EDTA. After recovery for 1 h in DMEM supplemented with 1.5% fetal bovine serum at room temperature, the cells were centrifuged at 1000 rpm in a table top centrifuge and resuspended in a concentration of approximately 10^6 cells/ml in DMEM with 1.5% serum. Fura-2 AM (5 μM) and pluronic (0.02%) (both from Molecular Probes, Eugene, OR) were added, and the cells were incubated for 20 min at room temperature. After loading, the cells were centrifuged, washed with medium, resuspended, and kept at room temperature until use.

Immediately prior to the assay, approximately 4 × 10^6 cells were diluted with 3 ml of Krebs buffer (composition in mM: NaCl (120), KCl (4.75), CaCl2 (1.1), MgSO4 (1.44), KH2PO4 (1), Hepes (25), ethanol (1%), glucose (1%), and EGTA (0.1), pH 7.4), centrifuged, and resuspended in 3 ml of Krebs buffer. After a 1-min equilibration, base-line values were collected for 1 min, after which bradykinin was added.

Fluorescence measurements were performed at 30 °C on a H&L spectrofluorometer (Series 300), equipped with an IBM XT data acquisition system, at excitation wavelengths of 340 and 380 nm, and an emission wavelength of 500 nm. Fluorescence values were collected every 1.6 s and corrected for autofluorescence of the cells.

The data were transformed, using LOTUS 1-2-3, to calcium concentrations, using formula 1: [Ca2+]i = K0 × f × (R × Rmin)/(Rmex × Rmin), in which the K0 and R0 values of the fluorescence at 380 in the absence and presence of Ca2+, was generally 10 (21). Calibration was performed as follows: cells were lysed with Triton X-100 (0.1%); EGTA (2.5 mM) and Tris (30 mM) were added to obtain the ratio of absence of Ca2+ (Rmin): 3 mM CaCl2 was added to obtain Rmax. These values were multiplied by a correction factor for differences in viscosity between the intracellular and extracellular environment, calculated to be 0.75 according to the method of Poenie (22), to give the Rmin and Rmax values used in formula 1.

RESULTS

In plasma membranes prepared from rat cerebella that had been washed extensively with 1 mM EGTA, low concentrations of Ca2+ (from 0.1 to approximately 1 μM) stimulated adenylyl cyclase activity 3-fold in the presence of 1 μM CaM (Fig. 1a). This is the anticipated response of a brain adenylyl cyclase to physiologically significant concentrations of Ca2+. These same concentrations of Ca2+, when added to EGTA-washed NCB-20 plasma membranes, progressively inhibited adenylyl cyclase activity, reaching a maximum inhibition of about 50% at 1 μM (Fig. 1b). The addition of exogenous CaM (1 μM) did not enhance (or reverse) the inhibition (not shown). As Ca2+ concentrations were increased to 5 μM, the degree of inhibition remained constant; however, beyond 5 μM, the plateau in adenylyl cyclase activity was followed by a second inhibitory phase that continued up to 0.1 mM Ca2+ (Fig. 2). This second phase of Ca2+ inhibition is not considered to be of physiological significance because of the high concentrations involved; such inhibition has been observed widely with all adenylyl cyclases, including the enzyme from brain, liver, and other sources (23, 24), and probably reflects Ca2+ competition with Mg2+ at the catalytic site.

Hill plot analysis of inhibition of NCB-20 adenylyl cyclase activity by low concentrations of Ca2+ ions yielded a Hill coefficient of 1.67 ± 0.10 (Fig. 3), indicating that a cooperative mechanism was in effect. Agents that perturb the binding of Ca2+ to Ca2+-binding proteins (such as CaM), including trifluoperazine, W-7, and calmidazolium (18), at concentrations up to 10 μM, did not alter the Ca2+ inhibition of adenylyl cyclase activity (data not shown).

Because stimulated states of adenylyl cyclase are required for hormone/neurotransmitter-mediated inhibition of the enzyme (6, 11) in brain, the dependence of Ca2+ inhibition of NCB-20 cyclase on stimulated enzyme states was investigated. As shown in Fig. 4, as increasing concentrations of PGE2 stimulated adenylyl cyclase activity, the inhibition of the enzyme by Ca2+ was revealed progressively. This inhibi-
**Ca\textsuperscript{2+} Inhibition of NCB-20 Adenylylcyclase**

Fig. 1. Effects of Ca\textsuperscript{2+} and CaM on adenylylcyclase activity in EGTA-washed membranes prepared from rat cerebellum (a) and NCB-20 cells (b). Adenylylcyclase activity was measured in the absence of stimulating hormone (a) and in the presence of 5 \(\mu\)M PGE\textsubscript{1} and 10 \(\mu\)M GTP (b). Concentrations of free Ca\textsuperscript{2+} were determined by an iterative computer program, as described under “Materials and Methods.” Data are from a representative experiment that was replicated at least four times.

Fig. 2. Effects of an extended range of Ca\textsuperscript{2+} concentrations on adenylylcyclase activity in EGTA-washed membranes prepared from NCB-20 cells. Adenylylcyclase activity was measured in the presence of 5 \(\mu\)M PGE\textsubscript{1} and 10 \(\mu\)M GTP. Concentrations of free Ca\textsuperscript{2+} were determined as described under “Materials and Methods.” Data are from a representative experiment that was replicated at least four times.

Fig. 3. Hill plot analysis of the inhibition by [Ca\textsuperscript{2+}] in the submicromolar range of NCB-20 adenylylcyclase activity. Data are from four independent experiments similar to the one shown in Fig. 1b, each performed in triplicate.

Fig. 4. Dependence of Ca\textsuperscript{2+} inhibition of NCB-20 adenylylcyclase activity on PGE\textsubscript{1}. Activity was measured in the presence of the indicated PGE\textsubscript{1} concentrations, in the presence of 10 \(\mu\)M GTP. Data are representative of two experiments performed in triplicate.

Because inhibitory effects of Ca\textsuperscript{2+} on adenylylcyclase appeared to depend upon hormone-stimulated states of the enzyme, the possibility that this inhibition was mediated via a G-protein such as G\textsubscript{i} was tested. Following overnight exposure of NCB-20 cells to pertussis toxin (PT), plasma membranes were prepared and adenylylcyclase activity assayed. PGE\textsubscript{1}-stimulated levels of adenylylcyclase activity were similar in membranes prepared from untreated and PT-treated membranes (Fig. 5a). Ca\textsuperscript{2+} inhibition was unaffected by the toxin treatment, although G\textsubscript{i}-mediated, d-Ala-d-Met-enkephalinamide (Enk) inhibition of adenylylcyclase activity was abolished. Consistent with this finding, inhibitory effects of a combination of maximally effective concentrations of Ca\textsuperscript{2+} and the opioid were additive (65%), compared to either Ca\textsuperscript{2+} (40%) or Enk alone (24%; Fig. 5b). These combined data suggest that Ca\textsuperscript{2+} and Enk inhibit NCB-20 adenylylcyclase...
The presence or absence of high concentrations of phosphodiesterase inhibitors, along with Ca\(^{2+}\) ions (1.7 M) and hormone (Enk) (1 M) in NCB-20 plasma membranes. Adenylylcyclase activities were measured in the presence of 5 M PGE\(_{1}\) and 10 M GTP, with the other additions indicated. Data are the mean activities ± S.D. from three experiments.

Activity by different mechanisms.

As a further comparison between Ca\(^{2+}\)- and receptor-mediated inhibition of adenylylcyclase, the effects of NaCl on the inhibition of adenylylcyclase activity by Ca\(^{2+}\) ions were examined (Fig. 6). In many tissues, NaCl enhances GTP-dependent, receptor-mediated inhibition of adenylylcyclase (25). In Fig. 6, it appears that the inclusion of NaCl in assay mixtures enhances the magnitude of the inhibition by Ca\(^{2+}\) slightly, although this effect may reflect the stimulatory effects of NaCl on adenylylcyclase activity.

Harden et al. (26) demonstrated previously, that in intact astrocytoma cells, muscarinic cholinergic agonists, as a consequence of mobilizing intracellular Ca\(^{2+}\), activate cAMP phosphodiesterase activity, leading to reduced levels of cAMP. The possibility was explored that a Ca\(^{2+}\)-regulated phosphodiesterase played a role in the present apparent inhibition of the adenylylcyclase activity. Experiments were performed in the presence or absence of high concentrations of phosphodiesterase inhibitors (in addition to the 100 M cAMP "trap" routinely employed to protect enzymatically formed \(^{32}\)P-cAMP). The presence of the phosphodiesterase inhibitors did not affect the inhibition of adenylylcyclase activity by 1.7 M Ca\(^{2+}\) (Table I). These data make it unlikely that the Ca\(^{2+}\) inhibition of adenylylcyclase is due to an enhancement of the degradation of \(^{32}\)PCAMP.

The possibility was evaluated that Ca\(^{2+}\) inhibition of adenylylcyclase activity was due to Ca\(^{2+}\)-dependent activation of a membrane-associated protease. Adnot et al. (27) reported such an activity in platelets which could inactivate adenylylcyclase. Thus, plasma membranes were prepared from NCB-20 cells in the presence and absence of a mixture of various protease inhibitors (i.e. leupeptin, antipain, aprotinin, pepstatin A, and soybean trypsin inhibitor). These preparations were then assayed in the presence and absence of the same protease inhibitors. Under each assay condition, Ca\(^{2+}\) inhibited adenylylcyclase activity to similar extents, with similar potency (not shown). Along similar lines, the proteolytic susceptibility of the elements mediating Ca\(^{2+}\)-inhibitory effects on adenylylcyclase was assessed using trypsin. Receptor-mediated, G\(_i\)-dependent inhibition of adenylylcyclase is selectively sensitive to mild trypsinization (28, 29). Increasing periods of preincubation of membranes with trypsin (from 15 s to 2 min) resulted in a progressive loss of hormone (Enk)-mediated inhibition of adenylylcyclase in subsequent assays, but did not affect Ca\(^{2+}\) inhibition of the enzyme (Fig. 7). Mild conditions of trypsinization were chosen, which did not alter adenylylcyclase catalytic activity. In this context, it should also be pointed out that the inhibition by Ca\(^{2+}\) was reversible.

**FIG. 5.** Comparison between opiate and Ca\(^{2+}\) inhibition of NCB-20 adenylylcyclase. a, effects of pertussis toxin on Ca\(^{2+}\) and hormone (Enk)-mediated inhibition of NCB-20 adenylylcyclase. Cultures of NCB-20 cells were exposed to 125 ng/ml pertussis toxin in their growth medium overnight, and plasma membranes were prepared as described under "Materials and Methods." b, additivity of the inhibition of adenylylcyclase produced by maximally effective concentrations of Ca\(^{2+}\) ions (1.7 M) and hormone (Enk) (1 M) in NCB-20 plasma membranes. Adenylylcyclase activities were measured in the presence of 5 M PGE\(_{1}\) and 10 M GTP, with the other additions indicated. Data are the mean activities ± S.D. from three experiments.

**FIG. 6.** Effect of NaCl (80 mM) on the Ca\(^{2+}\) inhibition of NCB-20 adenylylcyclase activity, measured in the presence of 5 M PGE\(_{1}\) and 10 M GTP. Data are representative of two experiments.

**TABLE I**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Adenylylcyclase activity (pmol/mg-min)</th>
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<tbody>
<tr>
<td>Control</td>
<td>94.7 ± 2.2</td>
</tr>
<tr>
<td>+500 M IBMX + 100 M Ro 20-1724</td>
<td>91.2 ± 1.1</td>
</tr>
<tr>
<td>+1.7 M Ca(^{2+})</td>
<td>61.0 ± 2.6</td>
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As assays were performed as described under "Materials and Methods," including 10 M PGE\(_{1}\) and 0.1 M cAMP in each assay, with the addition of phosphodiesterase inhibitors as indicated. Data values are from a single experiment performed three times with similar results and are expressed as the mean activity ± S.D. of at least triplicate determinations.
were examined for their influence on the inhibition. Consist-
tants at each time point were within 10% of each other. Data are
mediated inhibition, the inhibitory effects of Ca2+ did not
inhibition by 1.7 μM Ca2+ (Table II). Stimulation by MgCl2, however, did curtail inhibition by Ca2+. This an-
tagonism probably reflects competition by the 1000-fold ex-
cess of Mn2+ for the Ca2+-binding site. Inhibition of adenylyl-
cyclase by "P-site" adenosine analogs shares some of the
properties of the Ca2+ inhibition observed presently; for in-
stance, it is enhanced by hormone stimulation (30). Thus
the possibility was explored that the Ca2+ inhibition might be
mediated at the P-site of adenylylcyclase; it would then be
expected to be nonadditive with the effects of P-site analogs.
However, while dideoxyadenosine inhibited cyclase activity
by approximately 45%, this inhibition did not preclude a
further 44% inhibition by 1.7 μM Ca2+ (Table II). Protein
kinase C did not appear to play any direct role in these Ca2+-
inhibitory effects, because the addition of sphingosine (up to
10 μM) to plasma membranes for a 30-min preincubation prior
to assay did not preclude the inhibition of adenylylcyclase
activity by 1.7 μM Ca2+ (not shown).

In view of the apparently physiological concentrations of
Ca2+ which inhibited adenylylcyclase activity in plasma mem-
branes, the possibility was explored that hormones that could
mobilize Ca2+ in intact cells might reduce cAMP levels by this
mechanism. Cultures of NCB-20 cells were tested for their
responsiveness to bradykinin. As shown in Fig. 8, bradykinin
increased inositol phosphate production in a concentration-
dependent manner, consistent with previous reports (31).
Bradykinin also mobilizes Ca2+ in these cells, from experi-
ments that were performed on suspensions of NCB-20 cells,
using the intracellular Ca2+ indicator, fura-2 (Fig. 9). There-
fore, we examined the effects of bradykinin on cAMP produc-
tion by intact NCB-20 cells under analogous experimental
conditions. As seen in Fig. 10, bradykinin elicited a marked
inhibition of PGE2-stimulated cAMP accumulation over the
concentration range which enhanced inositol phosphate pro-
duction and [Ca2+] mobilization.

This inhibition of cAMP accumulation by bradykinin in
whole cells was not due to any direct effects of the hormone

![Graph](image)

**Fig. 7. Effects of trypsin treatment on inhibition by Ca2+**

(open circles) compared to that elicited by Enk (closed circles).
NCB-20 plasma membranes were treated with 2 μg/ml trypsin or
bovine serum albumin (as control) for increasing periods of time (at
37°C), until soybean trypsin inhibitor was added at a final concen-
tration of 1 μg/ml. Five minutes following the addition of soybean
trypsin inhibitor, membranes were added to adenylylcyclase assay
mixtures plus or minus either 1.7 mM Ca2+ or 1 μM Enk and this
incubation proceeded for 20 min (at 37°C). The adenylylcyclase
activities of bovine serum albumin control and trypsinized mem-
branes at each time point were within 10% of each other. Data are
expressed as the percent inhibition of adenylylcyclase activity deter-
mined at each time point in the absence of added Ca2+ or Enk, and
are from a single experiment performed in triplicate, repeated
twice with similar results.

**Table II**

Effects of various regulators of adenylylcyclase on the Ca2+-
inhibition of NCB-20 adenylylcyclase activity

Assays were performed as described under "Materials and Meth-
ods," with 1 mM MgCl2 present in each assay. Data are expressed as
the mean activity ± S.D. of at least triplicate samples from three
separate experiments.

| Additions       | Adenylylcyclase activity
<table>
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<tr>
<td></td>
<td>−Ca2+ +1.7 μM Ca2+ Inhibition</td>
</tr>
<tr>
<td></td>
<td>pmol/mg-min %</td>
</tr>
<tr>
<td>−GTP, −PGE</td>
<td></td>
</tr>
<tr>
<td>No addition</td>
<td>3.08 ± 0.4 2.1 ± 0.37 32</td>
</tr>
<tr>
<td>+1 μM forskolin</td>
<td>29.1 ± 0.7 20.5 ± 1.3 27</td>
</tr>
<tr>
<td>+10 μM GppNHp</td>
<td>29.7 ± 2.9 19.0 ± 1.2 36</td>
</tr>
<tr>
<td>+10 μM GTP, −PGE</td>
<td>5.8 ± 0.52 3.74 ± 0.4 33</td>
</tr>
<tr>
<td>+2 mM MnCl2</td>
<td>40.9 ± 1.9 35.3 ± 1.5 13</td>
</tr>
<tr>
<td>+10 μM GTP, +10 μM PGE</td>
<td>107 ± 3.1 57.4 ± 3.5 47</td>
</tr>
<tr>
<td>No addition</td>
<td>+100 μM ddA* 60.9 ± 2.1 34.6 ± 2.0 44</td>
</tr>
</tbody>
</table>

* ddA, 2', 5'-dideoxyadenosine.

i.e. if membranes were exposed to 1 μM Ca2+, then diluted,
centrifuged, and reassayed, the inhibition by Ca2+ was still
observed (not shown).

To characterize further the inhibition by Ca2+ of adenylyl-
cyclase, a variety of compounds that interact with the enzyme
were examined for their influence on the inhibition. Consist-
ent with data presented above in which Ca2+ inhibition was
insensitive to pertussis toxin, and additive with hormone-
mediated inhibition, the inhibitory effects of Ca2+ did not
absolutely require GTP (in the absence and presence of GTP,
inhibition by 1.7 μM Ca2+ was 32 and 33%, respectively; Table
II). As characterized in detail earlier (Fig. 4), the addition of
PGE2 enhances the inhibitory effect to 47% (Table II).
GppNHp stimulation, which precludes hormonal inhibition

![Graph](image)

**Fig. 8. Effect of bradykinin on the production of [3H]inos-
itol phosphates ([3H]IP1,2,3) from NCB-20 cells.** Cells were incubated with the indicated concentrations of bradykinin for 15 s
and assayed for [3H]IP1,2,3 formation as described under "Materials and Methods." LiCl (10 mM) was present throughout the experiment.
Data are from a single experiment performed in triplicate and are expressed as the mean counts per min of [3H]IP1,2,3 per mg of protein.
**Ca²⁺ Inhibition of NCB-20 Adenylyl Cyclase**

**Fig. 9. Effect of bradykinin on intracellular calcium concentrations in NCB-20 cells.** Cells were loaded with fura-2 AM (5 μM) in DMEM with 1.5% serum and 0.02% pluronic for 20 min at room temperature. Fluorescence measurements were performed at 30 °C. The excitation wavelengths used were 340 and 380 nm, the emission was measured at 500 nm. The indicated concentrations of bradykinin (BK) were added after 1 min. The data were transformed to [Ca²⁺] concentrations as described under “Materials and Methods.” Values are from a representative experiment, performed in duplicate, that was repeated twice with virtually identical results.

on adenylyl cyclase. As is evident in Fig. 11, bradykinin did not affect [³²P]cAMP formation in NCB-20 plasma membrane preparations, in which potential subcellular interactions were precluded. In the same plasma membrane preparation, direct inhibition of adenylyl cyclase activity by Enk could be demonstrated (Fig. 11), indicating that the failure of bradykinin to inhibit adenylyl cyclase activity was not due to the lack of a viable receptor-Gi-coupled adenylyl cyclase system under these experimental conditions.

The time course of bradykinin inhibition of cAMP production in intact NCB-20 cells was investigated. PGE₁-stimulated cAMP accumulation was linear over the time period examined, in the absence of bradykinin (Fig. 12, inset). However, at the shortest incubation time measured (3 s), a significant reduction in cellular cAMP accumulation was observed upon the addition of 1 μM bradykinin (Fig. 12). This bradykinin inhibition of cAMP formation was maximal at about 1 min, thereafter diminishing until it was no longer apparent by 5 min. The transient nature of the response suggested that the inhibition was likely due to the transient mobilization by bradykinin of intracellular Ca²⁺. In addition to PGE₁, high...
Inhibition of NCB-20 Adenylyl cyclase

Ca\(^{2+}\) Inhibition of NCB-20 Adenylyl cyclase

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**FIG. 12.** Time course of the effect of bradykinin (1 \(\mu\)M) on cAMP production in cultured NCB-20 cells. Cells were incubated in the presence and absence of bradykinin (BK) for varying periods of time, and assayed for cAMP accumulation as described under "Materials and Methods." PGE\(_1\) (10 \(\mu\)M) and the phosphodiesterase inhibitors IBMX (500 \(\mu\)M) and Ro 20-1724 (100 \(\mu\)M) were present throughout the incubation period. Data are expressed as the percent cAMP accumulation measured in the absence of bradykinin at each time point, and represent the mean percent control value ± S.E. from two to four experiments. Inset, the absolute amount of cAMP accumulation in the absence of bradykinin, expressed as picomoles per mg of protein, is shown as a function of incubation time.

concentrations of the phosphodiesterase inhibitors, Ro 20-1724 (100 \(\mu\)M) and IBMX (500 \(\mu\)M), were present in the incubation media. Thus, it seems unlikely that enhanced (Ca\(^{2+}\)-dependent) phosphodiesterase activity could account for the inhibitory effects of bradykinin on whole cell cAMP accumulation. In most of the studies performed on cAMP accumulation in intact cells, Ro 20-1724 was present at 100 \(\mu\)M and IBMX at 500 \(\mu\)M. However, to explore further the possibility that Ca\(^{2+}\) activation of a phosphodiesterase might play a role in the observed inhibition, analogous to the situation described earlier for astrocytoma cells in the studies by Harden et al. (26), concentrations of phosphodiesterase inhibitors were varied. If Ro 20-1724 was excluded, there was little change in cAMP accumulation or in bradykinin inhibition; however, if both inhibitors were omitted, cAMP accumulation fell from 371 ± 9.1 to 148 ± 9.8 pmol/mg, in a 1-min assay. Nevertheless, the percentage inhibition by bradykinin remains constant. Activity measured in the presence of bradykinin in the above cases was 280 ± 23.8 and 110 ± 4.6, respectively. In both cases this represents approximately a 25% inhibition. If there were a phosphodiesterase playing a role in the observed inhibition by bradykinin, an increased inhibition by bradykinin might have been anticipated in the absence of phosphodiesterase inhibitors. In addition, when greater than usual concentrations of phosphodiesterase inhibitors were employed (viz. 200 \(\mu\)M Ro 20-1724 and 1 mM IBMX), no alteration in either the time course of cAMP accumulation or of the magnitude of the bradykinin inhibition was detected (not shown). It seems unlikely therefore that [Ca\(^{2+}\)], stimulation of phosphodiesterase activity underlies the inhibition by bradykinin of cAMP-AM accumulation in these cells. Indeed these data are consistent with a direct effect of elevated [Ca\(^{2+}\)], on adenylyl cyclase activity.

We pursued further the possibility that inhibition by bradykinin of cAMP accumulation in intact cells was due to the mobilization of [Ca\(^{2+}\)], which then directly inhibited the adenylyl cyclase. Intact NCB-20 cells were preincubated with the cell-permeant Ca\(^{2+}\) chelator BAPTA-AM (32) with the intention of precluding a rise in free intracellular Ca\(^{2+}\) in response to bradykinin. As shown in Fig. 13, bradykinin-induced inhibition of cAMP formation was diminished significantly in cells preincubated with BAPTA (15%) as compared with control cells preincubated with vehicle alone (35%). These data support the contention that a significant component of bradykinin's inhibitory effects on cAMP generation in the intact cell is due to direct effects of elevated [Ca\(^{2+}\)], on adenylyl cyclase.

**DISCUSSION**

In the present study, concentrations of Ca\(^{2+}\) within the range achieved intracellularly exert a potent inhibition on NCB-20 adenylyl cyclase. The evidence accruing from characterization of the inhibition suggests that it is mediated directly at the catalytic unit of adenylyl cyclase. This evidence can be summarized as follows: it is reversible, additive with hormonal inhibition, and not sensitive to pertussis toxin; although it is enhanced by stimulatory conditions, it does not depend on stimulation of activity; the inhibition is also additive with the effects of P-site adenosine analogs. The inhibition is not mediated by a readily dissociable protein, nor is it selectively sensitive to proteolysis; nor can the inhibition be modulated by phosphodiesterase inhibitors, calmodulin or calmodulin antagonists, or protein kinase C inhibitors.

The concentrations of Ca\(^{2+}\) exerting the present inhibition and the cooperative effects of the ion indicate that the effect is of physiological significance. In addition, inhibition of adenylyl cyclase by these concentrations of Ca\(^{2+}\) is not encountered universally; in brain, the same Ca\(^{2+}\) concentrations...
stimulate adenylyl cyclase, whereas in liver, such concentrations are without effect (not shown). However, a number of similar reports of potent inhibitory effects of Ca^2+ on adenylyl cyclase activity, from rat pituitary, GH3 cells, and Leydig MA-10 cells have appeared recently (23, 34).

The present inhibition of adenylyl cyclase by concentrations of Ca^2+ that are achieved inside cells is reminiscent of a number of recent similar inhibitory effects by such Ca^2+-concentrations on other signal transduction pathways. For instance, Koch and Stryer (35) describe potent inhibitory regulation of guanylate cyclase activity by Ca^2+ ions in retinal rod outer segments. Numerous reports describe Ca^2+ activation of outward K+ channels (reviewed in Ref. 36) which reduces elevated [Ca^2+]i, in a negative feedback manner, as well as Ca^2+ inhibition of voltage-gated Ca^2+ channels (37). These findings suggest a widespread regulatory role that may be played by elevated [Ca^2+]i, which could be mediated by a conserved structural motif. Given also that adenylyl cyclase from brain, liver, and NCB-20 cells, for instance, are regulated differently by Ca^2+ (see above), it seems likely that these activities represent different molecular forms of the enzyme. The recent cloning of four distinct species of adenylyl cyclase (38) raises the possibility that one of these, or another unique molecular species, will represent the form that is susceptible to inhibition by low concentrations of Ca^2+.

In keeping with the present results suggesting that intracellular Ca^2+ concentrations in the physiological range can inhibit plasma membrane adenylyl cyclase, evidence from studies in intact cells also suggests that hormones/neurotransmitters that elevate intracellular Ca^2+ can decrease the production of cAMP (2). In some cell types, this effect was due to the stimulation of a Ca^2+-dependent phosphodiesterase activity. Harden et al. (26) demonstrated in 1321N1 astrocytoma cells that the phosphodiesterase inhibitor IBMX (500 µM) reversed the inhibition of agonist-stimulated cAMP accumulation elicited by muscarinic cholinergic M1 receptors. However, in NCB-20 cells, in the present study, the inclusion of higher concentrations of phosphodiesterase inhibitors did not perturb Ca^2+ inhibition of adenylyl cyclase. In addition, the absence of phosphodiesterase inhibitors did not increase Ca^2+ inhibition of adenylyl cyclase. These data suggest that Ca^2+ acts directly on a component of the adenylyl cyclase system.

The present inhibition of adenylyl cyclase activity by Ca^2+ ions is saturable and of sufficient potency and magnitude to suggest that Ca^2+-signalling systems may play a significant role in the modulation of the activity of the cAMP-generating system in intact cells. This possibility is supported by the effects of bradykinin, a Ca^2+-mobilizing hormone in NCB-20 cells, on the production of cAMP. Bradykinin caused a significant reduction in the amount of cAMP produced by intact cells. The fact that the bradykinin-induced inhibition of cAMP accumulation was diminished by treatment of the cells with the intracellular Ca^2+ chelator, BAPTA, also suggests that a major component of the bradykinin-induced inhibition was due to mobilization of Ca^2+]. The fact that BAPTA pretreatment did not eliminate fully the bradykinin-induced inhibition of cAMP accumulation raises two possibilities: (a) that these conditions did not fully buffer the rise in [Ca^2+], stimulated by bradykinin or (b) that part of the effect of bradykinin was due to the promotion of some unidentified Ca^2+-independent process. Further experiments that measure cAMP and [Ca^2+]i in parallel are required to address these possibilities.

The present findings of a negative interaction between the two major signalling systems may form the basis for the physiological antagonism that is seen between these systems in certain regulatory processes (1, 2). For instance, platelet aggregation is promoted by Ca^2+ signals and antagonized by the elevation of cAMP. In platelet plasma membranes, submicromolar Ca^2+ concentrations also elicit a profound inhibition of adenylyl cyclase analogous to those presently reported. Thus, factors such as thrombin, which elevate [Ca^2+]i, could overcome the actions of hormones that elevate cAMP. In general terms, a potent and cooperative inhibition of adenylyl cyclase elicited by Ca^2+ ions upon exposure of intact cells to Ca^2+-mobilizing hormones could reinforce cellular [Ca^2+]-sensing mechanisms. By such means, cAMP-facilitated Ca^2+ influx, via protein kinase A phosphorylation of Ca^2+ channels (39), would be under negative feedback control. However, it is clear that although the inhibition of cAMP production reported in the present study provides a potentially major negative effect of the Ca^2+-mobilizing system on cAMP accumulation, the overall outcome of the interaction will be determined by the sum of positive and negative influences mediated by Ca^2+, along with protein kinase C. In the case of NCB-20 cells, protein kinase C also appears to inhibit cAMP accumulation. Hollingsworth and Daly (40) demonstrated significant reductions in POE-stimulated (but not forskolin-stimulated) cAMP production in NCB-20 cells upon administration of the phorbol ester 12-myristate 13-acetate. Future studies may quantify the influences of the various interactions that are possible between Ca^2+ and cAMP-signalling pathways, how they change with time, and ultimately uncover the regulatory sophistication in physiological terms afforded by such interactions. For the present, however, these observations provide the first demonstration of a negative influence of Ca^2+-signalling pathways on cAMP accumulation that are likely due to direct inhibition by Ca^2+ of the catalytic activity of adenylyl cyclase.

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REFERENCES

Ca\textsuperscript{2+} Inhibition of NCB-20 Adenylylcyclase


