Zinc Inhibition of cAMP Signaling*

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Zn$^{2+}$ is required as either a catalytic or structural component for a large number of enzymes and thus contributes to a variety of important biological processes. We report here that low micromolar concentrations of Zn$^{2+}$ inhibited hormone- or forskolin-stimulated cAMP production in N18TG2 neuroblastoma cells. Similarly, low concentrations inhibited hormone- and forskolin-stimulated adenylyl cyclase (AC) activity in membrane preparations and did so primarily by altering the $V_{max}$ of the enzyme. Zn$^{2+}$ also inhibited recombinant isoforms, indicating that this reflects a direct interaction with the enzyme. The $IC_{50}$ for Zn$^{2+}$ inhibition was $1-2 \mu M$ with a Hill coefficient of 1.33. The dose-response curve for Zn$^{2+}$ inhibition was identical for AC1, AC5, and AC6 as well as for the C411R mutant of AC5 whose defect appears to be in one of the catalytic metal binding sites. However, AC2 displayed a distinct dose-response curve. These data in combination with the findings that Zn$^{2+}$ inhibition was not competitive with Mg$^{2+}$ or Mg$^{2+}$/ATP suggest that the inhibitory Zn$^{2+}$ binding site is distinct from the metal binding sites involved in catalysis. The prestimulated enzyme was found to be less susceptible to Zn$^{2+}$ inhibition, suggesting that the ability of Zn$^{2+}$ to inhibit AC could be significantly influenced by the coincidence timing of the input signals to the enzyme.

In general, Zn$^{2+}$ is required as either a catalytic or structural component for a large number of enzymes and thus contributes to a wide variety of important biological processes including gene expression, replication, hormonal storage and release, neurotransmission, and memory. Zn$^{2+}$ is also critical for the structural integrity of cells, influencing membrane stability and cytoskeletal organization (reviewed in Refs. 1–3). In this light, it is not surprising that dietary Zn$^{2+}$ has been associated with a variety of abnormalities related to growth, sexual maturation, and wound healing (4). The concentration of Zn$^{2+}$ in the brain, Zn$^{2+}$ along with iron is the most concentrated metal (1–4). Significant levels of chelatable histochemically reactive Zn$^{2+}$ are present in a subset of glutamatergic neurons in which Zn$^{2+}$ appears to be localized to synaptic vesicles (5–7). These Zn$^{2+}$-containing neurons are primarily located in the hippocampus (mossy fibers), striatum, and neocortex. The concentrations of Zn$^{2+}$ within these vesicles have been estimated to be as high as millimolar levels (3). Neuronal firing results in the release of both glutamate and Zn$^{2+}$ into the synaptic cleft (4–7). Intense firing can result in Zn$^{2+}$ concentrations of several hundred micromolars (4). This estimate is based upon the accumulation of Zn$^{2+}$ in the perfusate of hippocampal slices. Thus, the actual localized concentrations of the metal may be significantly greater. The fate of neurally released Zn$^{2+}$ is not totally clear. It is likely that some of the metal is taken back up by the presynaptic neurons and reconstituted into vesicles (8). The movement of Zn$^{2+}$ from presynaptic to postsynaptic cell has been documented. In general, this translocation is associated with the neurotoxic effects of Zn$^{2+}$ (4). For example, excessive firing of mossy fibers of the hippocampus leads to selective Zn$^{2+}$ uptake and the destruction of the CA1 neurons that are innervated by these bundles. However, the fact that Zn$^{2+}$ translocation from presynaptic to postsynaptic cells occurs allows for the possibility that this metal is also used in the central nervous system as a transcellular messenger (1–3).

The immediate targets of intracellular Zn$^{2+}$ are unclear as are the physiological consequences of its actions on specific targets. In neuronal cells, targets such as glyceraldehyde 3'-phosphate dehydrogenase, NAD-catabolizing activities such as poly(ADP-ribose) synthetase, or the production of reactive oxygen species have been proposed as mediators of Zn$^{2+}$ neurotoxicity (for review, see Ref. 9 and references therein). However, Zn$^{2+}$ deprivation is also cytotoxic, and there are numerous examples where culturing cells in Zn$^{2+}$-deficient medium results in cell death, whereas higher external concentrations of Zn$^{2+}$ appear as antiapoptotic (for review, see Ref. 10 and references therein). In that case, Ca$^{2+}$/Mg$^{2+}$-dependent endonucleases, caspases, Bcl2/Bax ratios, and cytoskeletal components are some of the proposed targets that Zn$^{2+}$ modulates with a protective outcome.

In light of the importance of cAMP as a second messenger, we have examined whether Zn$^{2+}$ could elicit its effects by altering adenylyl cyclase (AC) activity. Biochemical studies of mutant ACs and recent crystallographic studies of a truncated

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¶ The abbreviations used are: AC, adenylyl cyclase; PGE$_{1}$, prostaglandin E$_{2}$; Gs$_{a}$, a subunit of G protein that stimulates adenylyl cyclase; PDE, phosphodiesterase.
soluble form of AC have indicated that the enzyme possesses two catalytic metal binding sites (11, 12). X-ray crystallography studies distinguished these two sites by their preferential occupancy by either Zn\(^{2+}\) or Mn\(^{2+}\). Although both sites are presumed to be occupied by Mg\(^{2+}\) in vivo (12), these observations allow for the possibility that Zn\(^{2+}\) could influence cAMP production particularly in the brain where Zn\(^{2+}\) concentrations are significant. In the studies reported here, we have examined the effects of Zn\(^{2+}\) on cAMP signaling in N18TG2 neuroblastoma cells. We have determined that low micromolar concentrations of Zn\(^{2+}\) inhibit hormone- and forskolin-stimulated cAMP accumulation directly by inhibiting AC. Furthermore, we have characterized the potent inhibitory effects of Zn\(^{2+}\) on AC in both isolated N18TG2 membranes and membranes isolated from S9 cells expressing recombinant isozymes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture Conditions**—N18TG2 cells were grown in Dulbecco’s modified Eagle’s/Ham’s F-12 (1:1) medium containing 10% heat-inactivated bovine calf serum and penicillin/streptomycin. The medium was changed 16–24 h prior to experimentation, and cells were confluent at the time of experimentation. Cells were dissociated from flasks by gentle trituration with phosphate-buffered saline containing 0.6 mM EDTA and resuspended to 2 x 10⁶ cells/ml in Gey’s balanced salt solution without calcium but containing 0.1 mg/ml fatty acid-free bovine serum albumin, 10 mM Na-Hepes, pH 7.4, and the phosphodiesterase (PDE) inhibitor Ro20-1724 at 0.1 μM. Cells were incubated in that buffer in a shaking 37 °C water bath for increasing time periods during which different concentrations of Zn\(^{2+}\) were added.

**cAMP Assay**—To determine cAMP levels in intact N18TG2 cells, we treated cells with or without stimulatory agents for a maximum of 4 min, a time within the linear range of stimulation, and then lysed them by boiling in sodium acetate, pH 4.5 (13). cAMP levels in the soluble fraction were measured using a modified Gilman assay (14). All assay points were taken in triplicate and assayed in triplicate. Triplicates generally agreed within 5% (13). Protein determinations were done according to Bradford (15).

**AC Assay**—Membrane-bound AC activity was assayed by incubating N18TG2 and H15 membranes for 20 min or S9 membranes for 10 min at 30 °C in a HEPES-buffered mixture containing Mg\(^{2+}\)-ATP, an ATP-regenerating system, cAMP, and PDE inhibitors (0.1 mM Ro20-1724 or isobutylmethylxanthine, respectively) and monitoring the conversion of [α-32P]ATP to [32P]cAMP (16–18). Unless indicated otherwise, ATP and MgCl₂ were added at 0.5 and 10 mM, respectively, and EDTA was not added to the reaction mixture. When PGE₁-stimulated AC activity in N18TG2 membranes was monitored, 10 μM Na-Hepes, pH 7.4, and the phosphodiesterase (PDE) inhibitor Ro20-1724 at 0.1 μM. Cells were incubated in that buffer in a shaking 37 °C water bath for increasing time periods during which different concentrations of Zn\(^{2+}\) were added.

**Zn\(^{2+}\) Inhibits cAMP Accumulation in N18TG2 Cells**—N18TG2 cells were incubated in the absence or presence of 300 μM ZnCl₂ for 2 h, and cAMP accumulation in response to forskolin and PGE₁ stimulation was monitored. As seen in Fig. 1, the incubation of cells with Zn\(^{2+}\) resulted in a dramatic attenuation of cAMP accumulation in response to both stimuli. It is unlikely that the low levels of cAMP reflect a stimulation of a PDE by Zn\(^{2+}\), because cells were preincubated throughout the experiment with PDE inhibitors. Moreover, Zn\(^{2+}\) had no effect on nonstimulated (basal) cAMP levels, also arguing against a Zn\(^{2+}\) effect on PDE activity. Because forskolin binds to and directly activates AC, the inhibition of cAMP accumulation would not likely reflect a primary effect on hormone receptors or Gs. When cells were preincubated with the heavy metal ionophore, pyrithione, a greater inhibition by Zn\(^{2+}\) was observed, suggesting that internalization of Zn\(^{2+}\) is necessary for the inhibition of cAMP production (Fig. 1).

The extracellular levels of Zn\(^{2+}\) necessary to observe this inhibition were examined (Fig. 2). Little effect on forskolin-stimulated cAMP accumulation was seen when cells were preincubated with 1 or 10 μM ZnCl₂ for 2 h. Preincubation with 25 μM ZnCl₂ resulted in an approximate 25–30% attenuation of the forskolin response, whereas 150 μM resulted in an approximate 60–70% inhibition. When cells were incubated with Zn\(^{2+}\) and 5 μM pyrithione, concentrations of Zn\(^{2+}\) as low as 10 μM could significantly inhibit cellular cAMP accumulation in response to forskolin, and complete inhibition was observed at ~25 μM ZnCl₂. Thus, the relatively high extracellular concentrations of Zn\(^{2+}\) appear necessary to allow for the accumulation of sufficient intracellular levels. This is not unusual. For example, high concentrations of Zn\(^{2+}\) will suppress apoptosis in model cell culture systems, but concentrations approaching serum levels of Zn\(^{2+}\) (15–25 μM) will do so if pyrithione is present to facilitate uptake (19). In the experiment shown in this figure, pyrithione alone had a slight inhibitory action on forskolin-stimulated cAMP production. This effect was inconsistent and relatively minor such as not to alter the interpretation of the data.

In Fig. 3, indicates that when cells were preincubated with 100 μM ZnCl₂, a time period of more than 60 min was necessary to observe significant inhibition of forskolin-stimulated cAMP accumulation. The extent of inhibition increased with time and appeared maximum after 120 min. However, when pyrithione was present, the inhibition could be observed even after 30 min of incubation. In that case, maximum inhibition seemed to require between 60 and 90 min of incubation. The time dependence of the effects of Zn\(^{2+}\) is consistent with an uptake process that appears to be a limiting factor in eliciting the inhibition of cAMP accumulation.

It is generally accepted that the transfer of metals across the plasma membranes involves metal complexes that influence the efficiency with which the metal is delivered or transferred (8, 21–23). Thus, we evaluated the inhibition of cAMP accumulation by Zn\(^{2+}\) when the metal was presented as ZnCl₂ or as a
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FIG. 2. Concentration-dependent effects of Zn$^{2+}$. N18TG2 cells were preincubated with the indicated concentrations of ZnCl$_2$ for 2 h (solid bars) at which time the levels of forskolin-stimulated ($10^{-4}$ M) cAMP levels were determined. Parallel preincubations were performed in the presence of ZnCl$_2$ plus 0.5 $\mu$M pyrithione (hatched bars). The levels of cAMP are reported as the percentage found in forskolin-treated cells that had not been preincubated with Zn$^{2+}$ or pyrithione (open bar). Basal and forskolin-stimulated cAMP levels in control cells were 65 and 625 pmol/mg, respectively. The data are representative of three experiments.

FIG. 3. The effects of Zn$^{2+}$ are time-dependent. N18TG2 cells were preincubated without additions (open bars) with 300 $\mu$M ZnCl$_2$ (solid bars) or with 300 $\mu$M ZnCl$_2$ plus 0.5 $\mu$M pyrithione (hatched bars) for the indicated times. Forskolin-stimulated ($10^{-4}$ M) cAMP levels were then determined. 100% represents the levels achieved upon forskolin stimulation of cells preincubated in the absence of any additions. Basal levels were ~12% of those seen upon forskolin stimulation. The data are representative of two experiments.

FIG. 4. The effect of ascorbate on Zn$^{2+}$ inhibition. N18TG2 cells were preincubated for 2 h in the absence (open bar) or presence of the indicated concentrations of ZnCl$_2$ (solid bars) and 1 mM ascorbate (hatched bars). Samples were then assayed for forskolin-stimulated ($10^{-4}$ M) cAMP levels. 100% is the level attained in control cells. The data are representative of three experiments.

Complex of zinc ascorbate. As shown in Fig. 4, Zn$^{2+}$ became a more potent inhibitor of forskolin-stimulated cAMP accumulation when ascorbate was present. In that case, significant inhibition was observed with 25 $\mu$M Zn$^{2+}$ and appeared almost maximum with 75 $\mu$M. In contrast, zinc citrate and ZnSO$_4$ behaved in a fashion similar to ZnCl$_2$ (data not shown).

Zinc Inhibits AC Activity in N18TG2 Membranes—Plasma membranes purified by sucrose gradient centrifugation from N18TG2 cells were assayed for hormone-stimulated AC activity in the presence of varied concentrations of ZnCl$_2$. As shown in Fig. 5A, micromolar concentrations of Zn$^{2+}$ effectively inhibited AC activity such that at 60 $\mu$M ZnCl$_2$, little PGE$_1$-stimulated enzyme activity could be detected. The IC$_{50}$ for ZnCl$_2$ was 8–9 $\mu$M. Similar results were obtained using ZnSO$_4$. Because the assay for AC is performed in the presence of a PDE inhibitor and PDE activity is monitored by the tracer [3H]cAMP present in the assay, it is clear that the decreased levels of cAMP do not reflect the activation of PDE by Zn$^{2+}$. Low micromolar concentrations of Zn$^{2+}$ also effectively inhibited forskolin-stimulated AC activity (Fig. 5B). However, in this case, significant forskolin-stimulated activity could still be observed when 60 $\mu$M ZnCl$_2$ was present. The data, analyzed as a simple two-site binding model, suggested that two classes of binding sites could be present, one with an apparent IC$_{50}$ of 2–3 $\mu$M and another greater than 10-fold.

Kinetic Analyses of the Effects of Zn$^{2+}$—We first determined the effects of Zn$^{2+}$ on forskolin-stimulated AC activity measured at increasing concentrations of substrate. The results obtained when AC activity in N18TG2 membranes was assayed in the presence or absence of 10 $\mu$M Zn$^{2+}$ are depicted in Fig. 6A. An analysis of the rate of substrate utilization indicated that the $K_m$ slightly increased from 0.23 ± 0.02 to 0.39 ± 0.09

B.

FIG. 5. Inhibition of AC activity in N18TG2 membranes by Zn$^{2+}$. Sucrose gradient-purified plasma membranes were assayed for PGE$_1$-stimulated (A) and forskolin-stimulated (B) AC activity in the presence of the indicated concentrations of ZnCl$_2$. The concentrations of activators were $10^{-5}$ and $10^{-4}$, respectively. Basal activity was 60 pmol/min/mg. The data are representative of three (A) and four (B) experiments. GraphPad Prism (GraphPad Software, Inc.) was used to fit the data to simple one-site (A) and two-site (B) binding models.
**FIG. 6. The effects of Zn^{2+} on the kinetic properties of the enzyme.** A, N18TG2 membranes were assayed for forskolin-stimulated (10^{-4} M) AC activity at increasing concentrations of ATP ranging from 0.005 to 2.0 mM. MgCl_{2} was added at 10 mM. When present, ZnCl_{2} was added at a final concentration of 10 μM. The data are representative of two experiments. B, N18TG2 membranes were assayed for forskolin-stimulated (10^{-4} M) AC activity at increasing concentrations of Mg^{2+} ranging from 0.75 to 10.0 mM. ATP was added at 0.5 mM. When present, ZnCl_{2} was added at a final concentration of 40 μM. The data are representative of two experiments.

mm in the presence of Zn^{2+}. The V_{\text{max}} of the activity in N18TG2 membranes showed a 2-fold decrease in the presence of Zn^{2+} going from 645 ± 19 pmol/min/mg to 318 ± 25 pmol/min/mg. Current data indicate that the active site of AC contains two metal ion binding sites referred to as A and B, which can be selectively occupied by Zn^{2+} and Mn^{2+}, respectively (12). To assess whether the inhibitory Zn^{2+} site is indeed the metal site A, we also assayed forskolin-stimulated activity using a constant ATP concentration and varying Mg^{2+} concentrations in the absence or presence of Zn^{2+}. As depicted in Fig. 6B, the presence of 40 μM ZnCl_{2} resulted in an approximate 4-fold decrease in V_{\text{max}}. The respective V_{\text{max}} values were 258 ± 11 pmol/min/mg and 73 ± 10 pmol/min/mg. K_{m} values were 0.6 ± 0.6 μM and 1.3 ± 0.12 μM when AC activity was measured in the absence and presence of 40 μM Zn^{2+}, respectively. With 10 μM ZnCl_{2}, we observed an approximate 2-fold decrease in both the apparent K_{m} and V_{\text{max}} for the Mg^{2+} dependence of AC activity (data not shown). The sum of the data suggests that the major effect of Zn^{2+} on AC activity is to reduce the rate of enzyme conversion of substrate to product.

Recombinant AC Is Inhibited by Zn^{2+}—N18TG2 cells appear to express AC6 as their predominant activity (18). To determine whether AC itself is the target of Zn^{2+} inhibition, we examined recombinant AC6. We also examined the other member of this isoform family, AC5, which is expressed primarily in the striatum, a site of relatively high neuronal levels of Zn^{2+} (reviewed in Refs. 1–3 and 24–26). Low micromolar concentrations of Zn^{2+} effectively inhibited the forskolin-stimulated activity of both isoforms (Fig. 7A). The data were identical to those obtained when ZnSO_{4} or ZnCl_{2}, plus ascorbate was evaluated (data not shown). The fact that Zn^{2+} inhibits the recombinant enzyme indicates that this reflects a direct interaction with the enzyme. The inhibition of forskolin-stimulated AC5 and AC6 activity was dramatic (100% at 10 μM) occurring over a narrow range of Zn^{2+} concentrations. An analysis of the data by Hill plot revealed a coefficient of 1.33, indicating that the inhibition was a cooperative process. The IC_{50} for ZnCl_{2} inhibition was ~1–2 μM, a value similar to that of the apparent higher affinity site seen when forskolin-stimulated AC activity in N18TG2 membranes was monitored. No evidence for the lower affinity site present in N18TG2 membranes was obtained in our experiments using recombinant enzyme. This would suggest that the apparent lower affinity site seen using N18TG2 membranes is not inherent to the AC enzyme but may reflect the participation of additional AC regulators present in those membranes. Because of the lower specific activity of the AC6 preparations, additional analyses were performed using AC5.

**FIG. 7. Zn^{2+} inhibition of recombinant ACs at different Mg^{2+} concentrations.** A, S9 membranes expressing AC5 (■) and Hi5 membranes expressing AC6 (▲) were assayed for forskolin-stimulated (10^{-4} M) AC activity in the presence of the indicated concentrations of ZnCl_{2}. MgCl_{2} was added at 10 mM. The activity seen with 0.1 μM ZnCl_{2} was equivalent to that seen when no Zn^{2+} was added. The results are representative of three experiments. The data are presented as the percent of maximum activity as the specific activity of the AC6 preparation is ~15-fold lower than that of AC5. B, the dose-response curve for Zn^{2+} inhibition of AC5 was examined when activity was measured in the presence of 40 mM MgCl_{2}. The data are representative of two experiments.
Zn\(^{2+}\) (data not shown). Such findings are consistent with the premise that Zn\(^{2+}\) does not bind to a catalytic metal binding site unless Zn\(^{2+}\) binds to such a site in a manner that appears kinetically irreversible.

Several experiments were performed to address the reversibility of Zn\(^{2+}\) inhibition. In the experiment shown in Fig. 8A, we monitored the time course of AC5 inhibition by 15 \(\mu\)M Zn\(^{2+}\). As shown, the rate of forskolin-stimulated enzyme activity in the presence of Zn\(^{2+}\) was reduced but linear over the time frame of the experiment, suggestive of a reversible reaction (27, 28). The reversibility of the inhibition was further evaluated by adding EDTA to the assay after the first 10 min of incubation with Zn\(^{2+}\). In that case, the rate of forskolin-stimulated cAMP synthesis was substantially recovered. The addition of EDTA to control samples did not alter the rate of cAMP formation. Similar observations were made when AC activity in N18TG2 membranes was monitored (data not shown). The data indicate that the inhibition of AC by Zn\(^{2+}\) is reversible.

To address whether the site of Zn\(^{2+}\) inhibition is located at the other metal site, the preferential binding site of Mn\(^{2+}\) (site B), we assessed the effects of Mn\(^{2+}\) on the efficacy of Zn\(^{2+}\) inhibition of AC5. The data depicted in Fig. 8B shows that 0.1, 1, and 10 mM Mn\(^{2+}\) had no effect on the IC\(_{50}\) for Zn\(^{2+}\). Note that Mn\(^{2+}\) is a potent activator of AC activity, and thus higher maximal activities accompany increasing Mn\(^{2+}\) concentrations. The data support the premise that the site of Zn\(^{2+}\) inhibition is distinct from the catalytic metal binding sites.

Additional support for this premise was obtained in our studies of the C441R mutant of AC5 (11). This mutant AC5 displays a dramatic decrease in catalysis with only a slight change (2-fold) in the ability to bind substrate. The mutation is located at the residue adjacent to Asp-440, one of the critical aspartates that participate in coordinating metal binding (12). The close proximity of Cys-441 led the authors to suggest that a mutation in this residue results in a conformational change that disrupts the orientation of Asp-440 and thus the metal binding pocket (11). As shown in Fig. 9A, the C441R mutant behaves in a fashion identical to wild-type AC5 in response to ZnCl\(_2\) inhibition of forskolin-stimulated activity (IC\(_{50}\) of 1.8 \(\mu\)M). Thus, this mutation did not supplant the Zn\(^{2+}\) inhibition of AC5.

The Effects of Zn\(^{2+}\) Are Isoform-specific—To examine the effects of Zn\(^{2+}\) on other AC isoforms, we focused our attention on recombinant AC1 as representative of the AC1, AC3, and AC8 isoform family, and AC2 as representative of the AC2, AC4, and AC7 family (24–26). AC1 and AC2 are of particular interest, because like AC5 they are expressed in regions of the brain with relatively high vesicular levels of Zn\(^{2+}\) (1–3, 24–26). As illustrated in Fig. 9B, recombinant AC1 was effectively inhibited over a narrow range of Zn\(^{2+}\) concentrations, displaying an IC\(_{50}\) of 1.4 \(\mu\)M, similar to that of AC5. The dose-response curve of AC2, however, was distinct from the other isoforms (Fig. 9C). AC2 was not inhibited by the low micromolar con-
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Fig. 10. The effects of Zn\(^{2+}\) on activated AC. PGE\(_2\)-stimulated (10\(^{-5}\) M) (A) and forskolin-stimulated (10\(^{-4}\) M) (B) AC activity was measured in N18TG2 membranes in the absence (solid bars) or presence (last two bars in 20-min group) of 10 or 40 \(\mu\)M ZnCl\(_2\). Alternatively, Zn\(^{2+}\) was added to the assay of hormone- or forskolin-stimulated activity 5 min after the assay had been initiated (open and hatched bars). Cyclic AMP synthesis was determined after an additional 5 and 15 min, resulting in a total incubation time of 10 and 20 min, respectively. The data are representative of three experiments.

centrations of ZnCl\(_2\) that were effective against AC1 and AC5. It appeared that such concentrations were actually somewhat stimulatory to the enzyme. Only at concentrations of ZnCl\(_2\) of >10 \(\mu\)M did we observe an inhibitory effect. This would suggest that the high affinity inhibitory site seen in AC1 and AC5 is different in AC2. Because the residues surrounding the metal binding sites are strictly conserved across all isoforms, these data would be consistent with the premise that Zn\(^{2+}\) binds to a site distinct from the catalytic metal binding sites.

The Effects of Zn\(^{2+}\) Are Attenuated When It Is Added to an Active Enzyme—In the experiment shown in Fig. 10A, hormone-stimulated AC activity of N18TG2 membranes was inhibited ~60–70% when assayed in the presence of 10 or 40 \(\mu\)M ZnCl\(_2\) (Fig. 10A, last two bars). However, if Zn\(^{2+}\) was added 5 min after hormone activation of AC was initiated, the inhibition was attenuated (being almost negligible with 10 \(\mu\)M ZnCl\(_2\) and only ~20% with 40 \(\mu\)M ZnCl\(_2\)). Similarly, when Zn\(^{2+}\) was added after the assay of forskolin-stimulated AC had been initiated, the inhibition was also attenuated (Fig. 10B). The calculation of the possible chelation of Zn\(^{2+}\) by ATP in the presence of Mg\(^{2+}\) using WinmaxC indicated that minimal changes in the free concentration of Zn\(^{2+}\) would be expected. This finding rules out the possibility that ATP is simply chelating Zn\(^{2+}\). The data suggest that Zn\(^{2+}\) is a less potent inhibitor of AC activity when the enzyme has achieved an activated state.

DISCUSSION

We have demonstrated that the incubation of neuroblastoma cells with Zn\(^{2+}\) attenuates their ability to synthesize cAMP in response to hormone or forskolin stimulation. This appears to reflect a reversible inhibition of AC and thus would be compatible with a homeostatic role for this regulation. This would also be consistent with the fact that little or no loss in cell viability (trypan blue exclusion) upon incubation with Zn\(^{2+}\) was observed during the time course of our experiments.\(^2\) The concentrations of Zn\(^{2+}\) that are effective in attenuating the cell response to hormone or forskolin between 25 and 150 \(\mu\)M may be encountered in vivo (1–5, 19). However, the extracellular concentrations needed to inhibit cAMP accumulation in N18TG2 cells could be significantly reduced under conditions that facilitated cellular uptake of the metal. One condition was the use of the heavy metal ionophore, pyrithione, whereas another condition more apt to reflect a physiological relevant condition was the addition of ascorbate. It is not uncommon that the form of metal chelation influences the efficiency of metal transport (8, 21–23). Ascorbate is of particular interest, because it accumulates in the brain (being maintained at relatively high (1–2 \(\mu\)M) concentrations (29, 30)). It is further concentrated in synaptic vesicles of glutamatergic neurons and released into the extracellular space as a result of neuronal activity (29, 30). Therefore, ascorbate has the potential to ligate Zn\(^{2+}\), rendering the metal a more potent neuromodulator. It is also noted that we are probably manifesting the effects of a passive influx of Zn\(^{2+}\). Passive influx through the neuronal membrane has been demonstrated to occur when extracellular levels of Zn\(^{2+}\) are elevated, e.g., when Zn\(^{2+}\) is added to the cell culture medium or when Zn\(^{2+}\) is released from presynaptic vesicles (31). However, experiments using cultured neuronal cells have also demonstrated that Zn\(^{2+}\) uptake can be stimulated upon activation of voltage-gated Ca\(^{2+}\) or NMDA channels, for example (1–3, 32). Thus, physiological conditions that stimulate Zn\(^{2+}\) uptake will potentially facilitate a more effective inhibition of AC.

That Zn\(^{2+}\) uptake is required to observe the inhibition of AC would indicate that inhibition does not result from the nonspecific binding of Zn\(^{2+}\) either to the plasma membrane or to the exofacial residues of the AC. That Zn\(^{2+}\) inhibition of AC1, AC5, and AC6 appears identical could argue that amino acid residues of the transmembrane domains, which show limited homology, are not involved but that Zn\(^{2+}\) binds to the cytosolic CI and/or CII regions, which exhibit considerable homology. This assumption is substantiated by the fact that a soluble AC construct composed of the CI region of AC5 and the CII region of AC2 is inhibited by low micromolar concentrations of Zn\(^{2+}\) (12) and that this reflects a decrease in \(V_{\text{max}}\) of the enzyme.\(^3\) It is of interest to note that the IC\(_{50}\) of the soluble construct is ~15 \(\mu\)M, an intermediate value among that of the contributing isoforms. Although this value may reflect the loss of regions outside of the domains in question, it may also reflect the contribution of both subunits in defining the Zn\(^{2+}\) binding site.

In our attempts to determine the mechanism by which Zn\(^{2+}\) inhibits AC, we have made a number of observations to support the premise that it does not function at the catalytic metal binding sites. We wish to emphasize that our findings do not preclude Zn\(^{2+}\) binding to site A, a possibility indicated by cryostallographic studies. Rather, the biochemical effects of Zn\(^{2+}\) binding to this “novel” inhibitory site may be of an affinity such that we are not in a position to observe enzymatically the effects of Zn\(^{2+}\) binding to alternative sites. We also note that Zn\(^{2+}\) does not generally inhibit two-metal ion-requiring enzymes as are ACs (34). Perhaps upon the disruption of the Zn\(^{2+}\) site that inhibits AC activity, we will observe that Zn\(^{2+}\) functions more typically, supporting or enhancing AC activity when it occupies a catalytic metal binding site. In that light, it is tempting to speculate that for AC2, which has a lower affinity Zn\(^{2+}\) inhibitory site (IC\(_{50}\) = 25 \(\mu\)M), the stimulation of activity observed at the lower Zn\(^{2+}\) concentrations reflects the binding of Zn\(^{2+}\) to site A. Such questions are currently being explored.

This still leaves the question of how Zn\(^{2+}\) binding to this novel site leads to an attenuation of AC activity. The dose-response curve indicates that this is a cooperative process, reflecting either the binding of two (more than one) Zn\(^{2+}\) molecules or a Zn\(^{2+}\)-induced protein-protein interaction. Although we can only speculate at this point, several observations could

\(^2\) T. Y. Hudson, unpublished observations.

\(^3\) R. K. Sunahara, unpublished observations.
favor the latter interpretation. Based on current crystallographic data, the physical relationship of Cl to CII is altered upon occupancy of the substrate binding site. Subsequent association of AC with Go or forskolin is believed to allosterically influence the interaction to enhance the catalytic capacity of the enzyme (20, 33). Thus, there is precedence for the regulation of AC via changes in protein-protein interactions. We have also determined that a preassociation of AC with forskolin or Go6 protects the enzyme from the inhibitory effects of Zn2+. We interpret such findings to suggest that both activators induce a conformational change(s) in the enzyme that minimizes the effects of Zn2+. This interpretation as opposed to one evoking steric hindrance of the Zn2+ binding site is favored, because forskolin and Go6 bind to adjacent but distinct regions of AC and thus would obscure different residues of the enzyme (12, 33).

That the Go6-AC complex would display an altered sensitivity to Zn2+ could explain the biphasic dose-response curve for forskolin-stimulated AC activity in N18TG2 membranes (Fig. 5B) compared with that of the recombinant enzyme (Fig. 7A). It would also be expected that a population of enzyme in N18TG2 membranes is associated in equilibrium with Go and as such exhibits a decreased sensitivity to Zn2+. A greater population of enzyme would be associated with Go6 when PGE1-stimulated AC activity in N18TG2 membranes is monitored, thus explaining the higher IC50 value for Zn2+ inhibition (Fig. 5A). Having shown that AC1, AC5, and AC6 display an IC50 of ~1–2 μM while AC2 inhibition occurs with an IC50 of ~20–25 μM, we could also account for the data obtained using N18TG2 membranes if a population of AC isoforms were present. Our Northern analysis of AC expression in N18TG2 cells did not detect the mRNA for AC2 (18), but we did not probe for the presence of the other members of this family. Although other isoforms may be present, they are not typically predominant in neuronal cells, and biochemical evidence for their presence in N18TG2 cells has not been reported. The aforementioned observations also suggest that the coincidence timing requirements may affect an inhibition of AC by Zn2+. Cyclic AMP synthesis would be relatively refractory to an influx of Zn2+ should that occur subsequent to hormonal stimulation of the enzyme. It would also appear that the consequences of Zn2+ relative to cAMP synthesis will not depend upon the timing of the input signals but will reflect the nature of the AC isoform predominant in a particular cell or tissue. In regions of the brain in which AC2 is expressed, the stimulation of cAMP synthesis could still occur in the presence of Zn2+ and may actually be enhanced under conditions that effectively limit cAMP synthesis supported by either AC1 or AC5.

In this study, we have described a novel regulation of AC by Zn2+, whereby the presence of this metal reversibly inhibits its activity. Such a regulation raises the possibility that in the regions of the brain like the hippocampus where Zn2+ is believed to function as either a neuromodulator or neurotoxic agent, it could do so in part by altering cAMP levels. In addition to defining a previously unappreciated mechanism of Zn2+ action, these experiments suggest a novel target for mediating the physiological effects of Zn2+ in the central nervous system as well as in other tissues in which Zn2+ concentrations may be significant.

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REFERENCES
1. Cuajungco, M. P., and Lees, G. J. (1997) Neurobiol. Dis. 4, 137–169
2. Valley, B. L., and Falchuk, K. H. (1993) Physiol. Rev. 73, 79–118
3. Fredericksen, C. J., Suh, S. W., Silva, D., Fredericksen, C. J., and Thompson, R. B. (2000) J. Nutr. 130, 13471–13483
4. Choi, D. W. (1996) Cold Spring Harb. Symp. Quant. Biol. 61, 385–387
5. Assaf, S. Y., and Chung, S. H. (1984) Nature 308, 734–736
6. Fredericksen, C. J. (1989) Int. Rev. Neurobiol. 31, 145–238
7. Xie, X. M., and Smart, T. G. (1991) Nature 349, 521–524
8. Wensink, J., Molenaar, A., Wanrowiecka, U., and Van den Hamer, C. (1988) J. Neurochem. 50, 782–788
9. Sheline, C. T., Behrens, M. M., and Choi, D. W. (2000) J. Neurosci. 20, 3139–3146
10. Troung-Tran, A. Q., Ho, L. H., Chai, F., and Zalewski, P. D. (2000) J. Nutr. 130, 3145–3146
11. Zimmermann, G., Zhou, D., and Taussig, R. (1998) J. Biol. Chem. 273, 19650–19653
12. Tesmer, J. J. G., Sunahara, R. K., Johnson, R. A., Gosselin, G., Gilman, A. G., and Sprang, S. R. (1999) Science 285, 756–760
13. Tao, Y.-P., Najafi, L., Shipley, S., Howlett, A., and Klein, C. (1998) J. Pharmacol. Exp. Ther. 286, 266–273
14. Brostrom, C., and Kon, C. (1974) Anal. Biochem. 58, 459–468
15. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
16. Howlett, A. C. (1982) Mol. Pharmacol. 21, 664–670
17. Roth, R. L., Beinfeld, M. C., and Howlett, A. C. (1984) J. Neurochem. 42, 1145–1152
18. McVey, M., Hill, J., Howlett, A., and Klein, C. (1999) J. Biol. Chem. 274, 18887–18892
19. Zalewski, P. D., Forbes, I. J., and Betts, W. H. (1993) Biochem. J. 296, 403–408
20. Sunahara, R. K., Dessauer, C. W., Whisnant, R. E., Kleuss, C., and Gilman, A. G. (1997) J. Biol. Chem. 272, 22265–22271
21. Kaplan, J., Jordan, L., and Sturrock, A. (1991) J. Biol. Chem. 266, 2997–3004
22. Powell, S. R. (2000) J. Nutr. 130, S1447–S1454
23. Trombley, P. Q., Horning, M. S., and Blakemore, L. J. (2000) Biochemistry (Moscow) 65, 807–816
24. Iyengar, R. (1993) FASEB J. 7, 768–775
25. Miao, N., and Cooper, D. M. F. (1995) Trends Neurosci. 18, 536–542
26. Sunahara, R. K., Dessauer, C. W., and Gilman, A. G. (1996) Annu. Rev. Pharmacol. Toxicol. 36, 461–480
27. Zhang, R.-Q., Chen, Q.-X., Xiao, R., Xie, L. P., Zeng, X.-G., and Xhou, H.-M. (2001) Biochim. Biophys. Acta 1545, 6–12
28. Tou, C. L. (1988) Adv. Enzymol. Relat. Areas Mol. Biol. 61, 381–438
29. Oke, A. F., May, L., and Adams, R. N. (1987) Ann. N. Y. Acad. Sci. 498, 1–12
30. Gurwmid, R. A. (1993) Brain Res. Rev. 18, 123–133
31. Colvin, R. A., Davis, N., Nipper, R. W., and Carter, P. A. (2000) J. Nutr. 130, S1484–S1487
32. Weiss, J. H., Hartley, D. M., Koh, J. Y., and Choi, D. W. (1993) Neuron 10, 43–49
33. Tesmer, J. J. G., and Sprang, S. R. (1998) Curr. Opin. Struct. Biol. 8, 713–719
34. Derbyshire, V., Pinonneault, J. K., and Joyce, C. M. (1995) Methods Enzymol. 262, 363–385
