Dual actions of lanthanides on ACTH-inhibited leak K⁺ channels

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Enyeart, John J., Lin Xu, and Judith A. Enyeart. Dual actions of lanthanides on ACTH-inhibited leak K⁺ channels. Am J Physiol Endocrinol Metab 282: E1255–E1266, 2002. First published February 26, 2002; 10.1152/ajpendo.00478.2001.—Bovine adrenal zona fasciculata cells express background K⁺ channels (Iₐcḥ) whose activity is potently inhibited by ACTH. In whole cell patch clamp recordings, it was discovered that the trivalent lanthanides (Ln³⁺) inhibit Iₐcḥ through Ca²⁺-sensing receptors on these cells. The mechanism involves the suppression of Ca²⁺-induced inhibition of Iₐcḥ by interaction with Ca²⁺-specific binding sites on the Iₐcḥ channel. These results identify Ln³⁺ as a relatively potent group of noncompetitive ACTH receptor antagonists. Allosteric actions of trivalent and divalent metal cations on hormone binding, mediated through Ca²⁺-specific sites, may be common to a variety of peptide hormone receptors.

K⁺ channels; nickel; calcium; adrenocorticotropic hormone receptor

The Lanthanides (Ln³⁺; elements 58–71 in the periodic table) are trivalent metal cations that interact with a range of biologically important proteins, including ion channels and G protein-coupled receptors. In many cases, the strong interaction of Ln³⁺ with these proteins occurs because these agents share biologically important properties with the divalent calcium (Ca²⁺) cation (12, 27, 38). Their similarity to Ca²⁺ with respect to ionic radii, coordination chemistry, and affinity for the oxygen donor groups underlies their strong interaction with Ca²⁺-binding sites on a wide range of proteins (7, 12, 27).

Ln³⁺ have been particularly valuable in the study of voltage-gated ion channels, including Ca²⁺ and K⁺ channels. Ln³⁺ inhibit low-voltage-activated T-type Ca²⁺ channels by pore occlusion with a potency that varies inversely with ionic radius (24). In contrast, Ln³⁺ inhibit L-type Ca²⁺ channels with a potency that varies directly with ionic radius (19). For both Ca²⁺ channel subtypes, Ln³⁺ effects are produced through interaction with Ca²⁺-binding sites.

Ln³⁺ also suppress ion flow through voltage-gated K⁺ channels with a potency that varies inversely with the ionic radius (11). However, the suppression of K⁺ currents by Ln³⁺ appears to occur by an entirely different mechanism. Rather than blocking K⁺ channels by pore occlusion, Ln³⁺ suppress K⁺ currents by altering voltage-dependent gating and kinetic parameters by interaction with binding sites that are not Ca²⁺ specific (11, 36). An additional effect on K⁺ permeation has not been ruled out. In this regard, several non-voltage-gated background K⁺ channels are inhibited by the Ln³⁺ gadolinium (Gd³⁺) with IC₅₀ values ≤ 100 μM (20, 29). However, an inward rectifier in rat corticotropes is insensitive to La³⁺ (18).

In addition to ion channels, Ca²⁺-specific and non-specific Ln³⁺-binding sites have been identified on several membrane receptors. Acetylcholine and insulin receptors possess two types of Ln³⁺-binding sites, only one of which accepts Ca²⁺ (30, 38). Ln³⁺ bind with high affinity to extracellular Ca²⁺-sensing receptors on bovine parathyroid cells (2).

Bovine adrenal cortical cells, including those of the fasciculata and glomerulosa, express ACTH receptors whose activation is coupled to the inhibition of a unique population of ATP-dependent background K⁺ channel (Iₐcḥ) (8, 22). These Iₐcḥ K⁺ channels set the resting potential of adrenal zona fasciculata (AZF) cells and couple ACTH receptors to depolarization-dependent Ca²⁺ entry and cortisol secretion (9, 22). A requirement for Ca²⁺ in the binding of ACTH to its receptor on these cells, as well as its continued receptor occupancy, has been well established (6). Optimum binding requires the presence of millimolar Ca²⁺. Accordingly, in the absence of extracellular Ca²⁺, ACTH fails to inhibit Iₐcḥ K⁺ channels (10). The mechanism

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that underlies the obligatory requirement for Ca\textsuperscript{2+} in ACTH binding and receptor occupation has not been determined.

In addition to trivalent cations, divalent cations including Ni\textsuperscript{2+} also interact with Ca\textsuperscript{2+}-binding sites on membrane channel proteins. Ni\textsuperscript{2+} preferentially blocks low-voltage-activated T-type Ca\textsuperscript{2+} channels (15, 24). Ni\textsuperscript{2+} also modulates ion flux through voltage-gated K+ channels and cyclic nucleotide-gated channels (13, 25).

Using whole cell patch clamp recording, we have studied the interaction of Ln\textsuperscript{3+} of large (La\textsuperscript{3+}) and small (Yb\textsuperscript{3+}) ionic radii and Ni\textsuperscript{2+} on K+ current through I\textsubscript{AC} channels in the absence and in the presence of ACTH.

MATERIALS AND METHODS

Tissue culture media, antibiotics, fibronectin, and fetal calf serum (FCS) were obtained from Gibco-BRL (Grand Island, NY). Culture dishes were purchased from Corning (Corning, NY). Coverslips were from Belco (Vineland, NY). Lanthanide chlorides (≥99.9% purity) were obtained from Aldrich Chemical (Milwaukee, WI). All other chemicals were purchased from Sigma (St. Louis, MO).

Isolation and culture of AZF cells. Bovine adrenal glands were obtained from steers (age range 1–3 yr) within 30 min of slaughter at a local slaughterhouse. Fatty tissue was removed immediately, and the glands were transported to the laboratory in ice-cold PBS containing 0.2% dextrose. Removed immediately, and the glands were transported to the laboratory in ice-cold PBS containing 0.2% dextrose. Isolated AZF cells were prepared as previously described (10). After isolation, cells were either resuspended in DMEM-F12 (1:1) with 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and the antioxidants 1 μM tocopherol, 20 nM selenite, and 100 μM ascorbic acid (DMEM-F12+) and plated for immediate use or resuspended in FCS-5% DMSO, divided into 1-ml aliquots each containing ~2 × 10\textsuperscript{6} cells, and stored in liquid nitrogen for future use. Cells were plated in 35-mm dishes containing 9-mm\textsuperscript{2} glass coverslips that had been treated with fibronectin (10 μg/ml) at 37°C for 30 min and then rinsed with warm, sterile PBS immediately before cells were added. Dishes were maintained in DMEM-F12+ at 37°C in a humidified atmosphere of 95% air-5% CO\textsubscript{2}.

Solutions and bath perfusion. For recording whole cell K+ currents, the standard pipette solution was 120 mM KCl, 2 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, 10 mM HEPES, 11 mM 1,2-bis-(2-aminophenoxy)ethane-N,N,N,N',N'-tetracetic acid, 200 μM GTP, and 5 mM MgATP, with pH buffered to 7.2 using KOH. With this composition, the free Ca\textsuperscript{2+} concentration was determined to be 2.3 × 10\textsuperscript{−8} M by use of the “Bound and Determined” program (1). Pipette solutions were filtered through 0.22-μm cellulose acetate filters. The external solution consisted of (in mM) 140 NaCl, 5 KCl, 2 CaCl\textsubscript{2}, 2 MgCl\textsubscript{2}, 10 HEPES, and 5 glucose, pH 7.55, using NaOH.

Handling of Ln\textsuperscript{3+} is restricted by their chemical properties (12). To avoid formation and precipitation of insoluble Ln(OH)\textsubscript{3} and Ln(CO\textsubscript{3})\textsubscript{3}, as well as formation of radiocolloids and loss of Ln\textsuperscript{3+} ions to the container surface, millimolar aqueous stock solutions of LnCl\textsubscript{3} were prepared daily in polyethylene vials. Stock solutions were diluted to final concentration directly in the bath perfusion vessel immediately before use. The perfusion system consisted of polyethylene and polypropylene containers and tubing, because the Ln\textsuperscript{3+} strongly bind to negatively charged groups on glass surfaces. The recording chamber (volume ~1 ml) was continuously perfused by gravity at a rate of 5–6 ml/min. Bath solution exchange was done by a manually controlled six-way rotary valve.

Recording conditions and electronics. AZF cells were used for patch clamp experiments 2–12 h after plating. Coverslips with cells were transferred from 35-mm culture dishes to the recording chamber. Cells with diameters of 10–15 μm and capacitances of 8–15 pF were used for recording. Patch electrodes with resistances of 1–2 MΩ were fabricated from 01010 glass (Corning) using a Brown-Flaming model P-87 microelectrode puller (Sutter Instruments, Novato, CA). Access resistance during recording, estimated from the transient cancellation controls of the patch clamp amplifier, was 2–5 MΩ. The combination of access resistance and cell capacitance yielded voltage clamp time constants of <100 μs.

Whole cell currents were recorded at room temperature (22–24°C) following the procedure of Hamill et al. (14), using a List EPC-7 (List-Medical, Darmstadt, Germany) patch clamp amplifier. Pulse generation and data acquisition were done using a personal computer and PCLAMP software with TL-1 interface (Axon Instruments, Burlington, CA). Currents were digitized at 2–10 KHz after being filtered with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA). Linear leak and capacity currents were subtracted from current records by use of summed scaled hyperpolarizing steps of one-half to one-quarter pulse amplitude.

Data were analyzed using PCLAMP 6.04 (CLAMPAN and CLAMPFIT) and SigmaPlot (version 5.0) software. Inhibition curves are least square regression fits, where current in control saline is normalized to 1 and complete block of current with sufficient concentration of antagonists is assumed.

RESULTS

Bovine AZF cells express two types of K+ current. These include a voltage-gated, rapidly inactivating Kv1.4 current and a noninactivating background K+ current that is activated by intracellular ATP (8, 22, 25). In whole cell recordings, I\textsubscript{AC} grows dramatically over a period of minutes, provided that ATP or other nucleotides are present at millimolar concentrations in the recording pipette (8, 22).

The absence of time- and voltage-dependent inactivation of I\textsubscript{AC} K+ channels allows the corresponding membrane current to be easily isolated in whole cell recording with the use of either of two voltage clamp protocols. When voltage steps of 300 ms duration are applied from a holding potential of ~80 mV, I\textsubscript{AC} can be measured near the end of a voltage step when the transient Kv1.4 current has inactivated (Fig. 1A, left traces). Alternatively, I\textsubscript{AC} can be selectively activated by an identical voltage step after a 10-s prepulse to ~20 mV has fully inactivated the Kv1.4 current (Fig. 1A, right traces). Measurement of I\textsubscript{AC} by either method yielded nearly identical results.

Inhibition of I\textsubscript{AC} by Yb\textsuperscript{3+} and La\textsuperscript{3+}. To determine whether Ln\textsuperscript{3+} inhibited K+ flow through the nonvoltage-gated background I\textsubscript{AC} K+ channels, we examined the effects of the largest Ln\textsuperscript{3+}, La\textsuperscript{3+} (ionic radius 1.160 Å), and one of the smallest Ln\textsuperscript{3+}, Yb\textsuperscript{3+} (ionic radius 0.985 Å), on whole cell I\textsubscript{AC} currents in AZF cells. Both of these Ln\textsuperscript{3+} produced concentration-dependent inhibition of I\textsubscript{AC} K+ current (Fig. 1). In contrast to their effect on the voltage-gated Kv1.4 current (11), these two Ln\textsuperscript{3+} inhibited I\textsubscript{AC} at nearly identical concentra-
tions, with IC50s for La3+ and Yb3+ of 52.5 and 50.1 µM, respectively (Fig. 1C).

Ln3+-induced increase in IAC. Although La3+ and Yb3+ inhibited IAC with similar potency, it was observed that the initial inhibition of IAC produced by these two agents was often followed by a delayed increase in IAC amplitude (Fig. 1B in 10 µM Yb3+). The increase in IAC occurred even though this current had clearly reached a stable maximum value previous to Ln3+ exposure.

To further explore the biphasic effect of Ln3+s on IAC current amplitude, IAC was allowed to reach a stable maximum value before the AZF cells were superfused with Ln3+ (50 µM) for 10 min. Ln3+ was then washed from the chamber with control saline (Fig. 2). Under these conditions, Yb3+ and La3+ produced similar effects. The initial rapid inhibition (Fig. 2, A and B, trace 3) was followed by a gradual increase in current amplitude that persisted for a period of minutes (Fig. 2, A and B, trace 4). Subsequent superfusion of control saline immediately reversed inhibition, and IAC rapidly increased to a value significantly greater than that reached before Ln3+ application (Fig. 2, trace 5). In the two experiments illustrated in Fig. 2, IAC increased by 66 and 63%, respectively. Overall, a 10-min exposure to 50 µM Yb3+ or La3+ followed by washing increased IAC amplitude significantly in 6 of 11 and 5 of 7 cells, respectively.

The increases in IAC current amplitude observed after exposure to Ln3+s and subsequent wash with control saline suggested that, in addition to their inhibitory action, Ln3+s produced a secondary enhancement of IAC by a mechanism that persists in their absence. We considered the possibility that the Ln3+s...
reversed the effects of an endogenous inhibitory sub-
stance that may have been present in the serum-sup-
plemented culture medium.

$\text{Ln}^{3+}$s reverse ACTH-mediated inhibition of $I_{AC}$. The peptide hormone ACTH inhibits $I_{AC}$ half-maximally at a concentration of 5.4 pM (22). The requirement for $\text{Ca}^{2+}$ in ACTH binding and inhibition (6, 10), combined with the high affinity of $\text{Ln}^{3+}$s for some $\text{Ca}^{2+}$-binding sites, raised the possibility that $\text{Ln}^{3+}$-stimulated enhancement of $I_{AC}$ current occurred through interaction of the $\text{Ln}^{3+}$ with a $\text{Ca}^{2+}$-binding site located on the ACTH receptor.

To test this possibility, we studied the effects of $\text{Ln}^{3+}$s on reversal of ACTH-mediated inhibition of $I_{AC}$. In the experiment illustrated in Fig. 3A, $I_{AC}$ increased to a stable maximum value after ~20 min of whole cell recording (trace 2). Superfusion of 200 pM ACTH produced near complete inhibition of $I_{AC}$ (trace 3) by a mechanism that was not measurably reversed by a 10-min exposure to control saline. Subsequent superfu-
sion with saline containing 50 $\mu$M $\text{LaCl}_3$ produced a
slow reversal of the ACTH-mediated inhibition (trace 4), an effect that was partially masked in time and extent by the separate inhibitory action of $\text{La}^{3+}$ on $I_{AC}$. A second wash with control saline reversed the $\text{La}^{3+}$ inhibition, at which time $I_{AC}$ was restored to 84% of its original maximum value (trace 5).

In the experiment illustrated in Fig. 3B, 200 pM ACTH produced total inhibition of $I_{AC}$ (trace 3), and this effect was completely reversed by a 20-min exposure to 50 $\mu$M $\text{YbCl}_3$, followed by a 3-min wash in control saline (trace 4). Subsequent superfusion of 200 pM ACTH again produced inhibition of $I_{AC}$, but this effect was not measurably reversed by pro-
longed washing in control saline (trace 5). In four similar experiments (3 with $\text{La}^{3+}$, 1 with $\text{Yb}^{3+}$), super-
fusion of cells with 50 $\mu$M $\text{Ln}^{3+}$ after ACTH inhibition restored $I_{AC}$ to 91 ± 6% of its control value.

Suppression of ACTH-mediated $I_{AC}$ inhibition by $\text{Ln}^{3+}$s and $\text{Ni}^{2+}$. In addition to reversing the ACTH-
mediated inhibition of $I_{AC}$, pretreatment of cells with
Ln$^{3+}$s produced a concentration-dependent suppression of $I_{AC}$ inhibition by ACTH. In the experiment illustrated in Fig. 4A, cells were superfused with either 10 (left) or 50 μM (right) La$^{3+}$ before cells were superfused with La$^{3+}$ and ACTH (200 pM) in combination. At a concentration of 10 μM, La$^{3+}$ failed to alter the complete inhibition of $I_{AC}$ by ACTH (left). However, at a concentration of 50 μM, La$^{3+}$ completely prevented $I_{AC}$ inhibition by ACTH (right).

Overall, in the presence of 10 μM La$^{3+}$, ACTH (200 pM) inhibited $I_{AC}$ by 95 ± 3% ($n = 2$) compared with the control value of 96 ± 2% ($n = 6$). In contrast, ACTH (200 pM) was completely ineffective in the presence of 50 μM La$^{3+}$, inhibiting $I_{AC}$ by only 2 ± 1% ($n = 9$) (Fig. 4B).

Nearly identical results were obtained when Yb$^{3+}$ replaced La$^{3+}$ in a series of similar experiments. At a concentration of 10 μM, Yb$^{3+}$ failed to significantly alter inhibition of $I_{AC}$ by ACTH. In contrast, raising the concentration of Yb$^{3+}$ to 50 μM rendered ACTH ineffective in suppressing $I_{AC}$ (Fig. 4B). The suppression of ACTH-mediated $I_{AC}$ inhibition by 50 μM La$^{3+}$ was not affected by raising the ACTH concentration to 2 nM or 20 nM in three experiments (data not shown).

Antagonism of ACTH inhibition of $I_{AC}$ by Ni$^{2+}$. The divalent cation Ni$^{2+}$ blocks T-type Ca$^{2+}$ channels and voltage-gated Kv1.4 K$^+$ channels in AZF cells with respective IC$_{50}$ values of 5.7 and 467 μM (24, 25). By comparison, at concentrations ≤500 μM, Ni$^{2+}$ was ineffective as an inhibitor of $I_{AC}$ K$^+$ current. However, 500 μM Ni$^{2+}$ nearly eliminated $I_{AC}$ inhibition by ACTH (200 pM).

In the experiments illustrated in Fig. 5A, $I_{AC}$ was allowed to reach a stable maximum amplitude before cells were superfused with either 50 μM (left) or 500 μM (right) Ni$^{2+}$, followed by saline containing the divalent cation and ACTH in combination. Under these conditions, 50 μM Ni$^{2+}$ failed to blunt the nearly complete inhibition of $I_{AC}$ by ACTH. In contrast, in the presence of 500 μM Ni$^{2+}$, ACTH was completely ineffective.

Overall, 50 μM Ni$^{2+}$ minimally reduced $I_{AC}$ inhibition by ACTH from 96 ± 2% ($n = 6$) to 87 ± 3% ($n = 5$). By comparison, in the presence of 500 μM Ni$^{2+}$,
ACTH produced no measurable inhibition of $I_{AC}$ ($n = 7$) (Fig. 5B).

La$^{3+}$ and Ni$^{2+}$ do not alter $I_{AC}$ inhibition by ANG II. In addition to ACTH, $I_{AC}$ K$^+$ channels are potently inhibited by the peptide hormone ANG II with an IC$_{50}$ of 150 pM (22, 23). Experiments with ANG II showed that the block of ACTH-mediated $I_{AC}$ inhibition by La$^{3+}$ and Ni$^{2+}$ was specific and not a generalized effect on peptide hormone receptors.

In the experiment illustrated in Fig. 6A (left), $I_{AC}$ was allowed to reach a stable amplitude before the cell was sequentially superfused with saline containing La$^{3+}$ (50 µM) alone followed by La$^{3+}$ plus ACTH (200 pM) and, finally, La$^{3+}$ plus ANG II (10 nM). In the presence of 50 µM La$^{3+}$, ACTH was completely ineffective at inhibiting $I_{AC}$, whereas ANG II inhibited $I_{AC}$ almost completely.

Ni$^{2+}$ also selectively suppressed $I_{AC}$ inhibition by ACTH. In the experiment illustrated in Fig. 6A (right), ACTH (200 pM) inhibited $I_{AC}$ by ~10% in the presence of Ni$^{2+}$ (500 µM), whereas subsequent superfusion with ANG II (10 nM) produced complete inhibition of this current.

Overall, ACTH (200 pM) inhibited $I_{AC}$ by 96 ± 2% ($n = 6$) under control conditions, but only by 2 ± 1% ($n = 10$) and 3 ± 3% ($n = 7$), in the presence of 50 µM La$^{3+}$ and 500 µM Ni$^{2+}$, respectively (Fig. 6B). In contrast, ANG II was equally effective at inhibiting $I_{AC}$ in the presence of La$^{3+}$ and Ni$^{2+}$ as it was in control saline (Fig. 6B).

La$^{3+}$ and Ni$^{2+}$ do not affect inhibition of $I_{AC}$ by cAMP. La$^{3+}$ and Ni$^{2+}$ could potentially act at several different locations in the signaling pathway to block ACTH-mediated inhibition of $I_{AC}$. If the site of action of the metals was proximal to the activation of adenylate cyclase, inhibition of $I_{AC}$ by cAMP should not be affected. Accordingly, it was discovered that La$^{3+}$ and Ni$^{2+}$ were ineffective at preventing inhibition of $I_{AC}$ by the membrane-permeable cAMP analog 8-(4-chlorophenylthio)-cAMP (8-pcpt-cAMP).
In the experiment illustrated in Fig. 7A, the cell was preexposed to saline containing La$^{3+}$ before sequential superfusion of saline containing La$^{3+}$ (50 μM) and ACTH (200 pM) or 8-pcpt-cAMP (500 μM). In this experiment, La$^{3+}$ reduced $I_{AC}$ amplitude by 47%, whereas ACTH failed to produce any further inhibition in the presence of La$^{3+}$. The subsequent superfusion of 8-pcpt-cAMP inhibited $I_{AC}$ completely. As shown in Fig. 7B, 8-pcpt-cAMP inhibited $I_{AC}$ by 84 ± 2% ($n$ = 3) in the presence of 50 μM La$^{3+}$ compared with 74 ± 7% ($n$ = 3) in control saline.

Ca$^{2+}$ antagonizes effects of La$^{3+}$ and Ni$^{2+}$. The continued effectiveness of 8-pcpt-cAMP in inhibiting $I_{AC}$ in the presence of La$^{3+}$ indicated that this cation blocks ACTH action at a step that precedes the activation of adenylate cyclase. In view of the requirement for Ca$^{2+}$ in ACTH binding and the previously mentioned requirement for Ca$^{2+}$ in ACTH-mediated inhibition of $I_{AC}$, it is possible that La$^{3+}$ could antagonize the binding of this peptide by occupying a specific Ca$^{2+}$-binding site associated with the ACTH receptor. If so, then raising extracellular Ca$^{2+}$ should competitively antagonize the La$^{3+}$ block of $I_{AC}$ inhibition by ACTH.

When extracellular Ca$^{2+}$ was raised from 2 to 10 mM, La$^{3+}$ (50 μM) was completely ineffective at suppressing ACTH-mediated inhibition of $I_{AC}$. In the experiment illustrated in Fig. 8A, the cell was superfused with control saline containing 10 mM Ca$^{2+}$. In this high-Ca$^{2+}$-containing external solution, La$^{3+}$ failed to blunt the complete inhibition of $I_{AC}$ by 200 pM ACTH. Overall, in saline containing 10 mM Ca$^{2+}$ and 50 μM La$^{3+}$, ACTH (200 pM) inhibited $I_{AC}$ by 96 ± 4% ($n$ = 3), a value not significantly different from that produced by ACTH alone in standard (2 mM Ca$^{2+}$) saline. By comparison, in standard saline containing 50 μM La$^{3+}$, ACTH was far less effective, inhibiting $I_{AC}$ by only 2 ± 1% ($n$ = 9) (Fig. 4A).

Raising extracellular Ca$^{2+}$ from 2 to 10 mM also rendered Ni$^{2+}$ (500 μM) ineffective at suppressing...
ACTH-mediated inhibition of $I_{AC}$ (Fig. 8B). In saline containing 10 mM CaCl$_2$ and 500 µM NiCl$_2$, ACTH (200 pM) inhibited $I_{AC}$ by 96.5 ± 2.5% ($n = 3$).

**DISCUSSION**

In this study, it was discovered that Ln$^{3+}$s produce dual effects on K$^+$ current through $I_{AC}$ background K$^+$ channels in AZF cells. In addition to directly inhibiting $I_{AC}$, the Ln$^{3+}$s block and reverse ACTH-mediated inhibition of these channels. These opposing actions are mediated through distinct binding sites associated with $I_{AC}$ K$^+$ channels and ACTH receptors. Most interestingly, Ln$^{3+}$s appear to uncouple ACTH receptors from $I_{AC}$ K$^+$ channel inhibition by competitively antagonizing the binding of Ca$^{2+}$ to a specific site. By displacing Ca$^{2+}$ from this site, which is required for ACTH binding or receptor activation, Ln$^{3+}$s act as potent noncompetitive ACTH antagonists. The divalent cation Ni$^{2+}$ antagonized the actions of ACTH in a similar fashion but was ~10-fold less potent than the Ln$^{3+}$s.

**Block of $I_{AC}$ K$^+$ channels by Ln$^{3+}$s.** The results in this study show that Ln$^{3+}$s inhibit $I_{AC}$ K$^+$ channels through a mechanism that differs in at least two fundamental respects from their inhibition of voltage-gated bKv1.4 K$^+$ channels. Specifically, Ln$^{3+}$s reduced bKv1.4 K$^+$ current by altering voltage-dependent gating and kinetic parameters (11). Because $I_{AC}$ channels exhibit little or no voltage dependence, the inhibition of these channels by La$^{3+}$ and Yb$^{3+}$ must have occurred through a different mechanism, probably by pore occlusion.

Second, with regard to potency, Yb$^{3+}$ and La$^{3+}$ inhibited $I_{AC}$ K$^+$ channels with almost identical potency, despite the marked difference in ionic radii (33). By comparison, Ln$^{3+}$s inhibit bKv1.4 K$^+$ channels with a potency that varies inversely with ionic radius (11). Because both of these Ln$^{3+}$s at low concentrations...
inhibited bKv1.4 current by actions on gating, we were unable to determine whether these agents also inhibit K⁺ permeation through voltage-gated channels. In this regard, voltage-gated K⁺ channels and background K⁺ channels have distinct pore structures and exhibit different pharmacology (20, 29).

The molecular identity of the $I_{\text{AC}}$ channel is unknown, but it displays several properties of background K⁺ channels, which are structurally characterized by four membrane-spanning regions and two pore domains. In this regard, several members of this family of background K⁺ channels are blocked by the Ln³⁺/Gd³⁺ with potency similar to what we observed for the block of $I_{\text{AC}}$ by Ln³⁺/Gd³⁺ (20, 29).

**Ln³⁺ inhibition of ACTH action.** The studies demonstrating the reversal and block of ACTH-mediated inhibition of $I_{\text{AC}}$ K⁺ channels by Ln³⁺/Gd³⁺ suggest a model in which these cations displace Ca²⁺ from a specific site on the ACTH receptor, allosterically producing a marked reduction in the affinity of the receptor for ACTH. The results of our study are consistent with this model, given well established quantitative measurements relating ACTH receptor activation to $I_{\text{AC}}$ inhibition and assuming only that the potent inhibitory action of ACTH on $I_{\text{AC}}$ channels requires the continued occupancy of the receptor by this peptide.

Within this framework, superfusing AZF cells with saline containing Ln³⁺/Gd³⁺s rapidly reversed ACTH-mediated inhibition of $I_{\text{AC}}$, when normal saline did not, because replacement of Ca²⁺ with Ln³⁺ lowered the affinity of the receptor for ACTH, leading to the peptide’s rapid dissociation. Similarly, ACTH was ineffective at inhibiting $I_{\text{AC}}$ in experiments where cells were preexposed to Ln³⁺/Gd³⁺s, because Ln³⁺/Gd³⁺-bound receptors have a low affinity for ACTH.

This model also explains the novel biphasic effect of Ln³⁺ in which rapid $I_{\text{AC}}$ inhibition was often followed by a slower increase in $I_{\text{AC}}$ amplitude. Presumably, the serum-supplemented culture medium contained sufficient ACTH to partially inhibit $I_{\text{AC}}$ current. This inhibition was unmasked only when the residual ACTH dissociated in the presence of Ln³⁺/Gd³⁺. Values are means ± SE of the indicated no. of separate determinations.

**Quantitative aspects of effects of Ln³⁺ on ACTH binding.** Results from previous studies indicated that ACTH must occupy only a small fraction of ACTH receptors to completely inhibit $I_{\text{AC}}$ K⁺ current (3, 5, 22). Specifically, ACTH binds to a single type of ACTH receptor on AZF cells with a dissociation constant ($K_d$)
of ~1.5 nM (3, 5), whereas $I_{AC}$ is inhibited half-maximally by ACTH at 5.4 mM (32). Substitution of these values into an equation of the Langmuir absorption isotherm form indicates that activation of only 0.4% of ACTH receptors would result in 50% inhibition of $I_{AC}$. Thus the observed effectiveness of 50 μM La$^{3+}$ or Yb$^{3+}$ in completely blocking ACTH inhibition of $I_{AC}$ indicates that, at this concentration, ≥99.5% of ACTH receptors are occupied by Ln$^{3+}$ and cannot be activated by ACTH.

This information indicates that Ln$^{3+}$s bind with high affinity to the ACTH receptor-associated site. Specifically, for Ln$^{3+}$s to occupy 99.5% of binding sites at a concentration of 50 μM, the $K_{d8}$ must be <2.5 × 10$^{-7}$ M. This binding affinity greatly exceeds that of Ca$^{2+}$ for the same site. Accordingly, Ln$^{3+}$s have been shown to bind to Ca$^{2+}$ sites on proteins with 10$^{-7}$- to 10$^{-5}$-fold higher affinity than Ca$^{2+}$ itself (16). It is clear from these data that the dual effects of Ln$^{3+}$s on $I_{AC}$ are mediated through interaction with two sites whose binding affinities differ by several orders of magnitude. Raising the external Ca$^{2+}$ concentration from 2 to 10 mM prevented the Ln$^{3+}$-mediated block of $I_{AC}$ inhibition by ACTH. This observation is consistent with competition between Ca$^{2+}$ and Ln$^{3+}$ for a common site on the ACTH receptor. Even though Ln$^{3+}$s interact with a specific binding site associated with the ACTH receptor. The observation that Ln$^{3+}$s act as potent noncompetitive antagonists of ACTH in AZF cells.

Our findings are consistent with and extend those of a previous study that demonstrated an obligatory role for Ca$^{2+}$ in ACTH binding and steroidogenesis. Specifically, Cheitlin et al. (6) showed that Ca$^{2+}$ at millimolar concentrations was required for optimum ACTH binding to rat AZF receptors and that removal of all external Ca$^{2+}$ markedly accelerated ACTH dissociation from its receptor, reducing the $t_{1/2}$ from 32 to 3.5 min. Although this study demonstrated a requirement for Ca$^{2+}$ in ACTH binding, it did not provide evidence for a specific Ca$^{2+}$-binding site that is linked to the ACTH receptor. For example, according to surface potential theory, Ca$^{2+}$ at millimolar concentrations could influence ACTH binding by screening negative surface charges on the membrane and electrostatically influencing the interaction of ACTH with its receptor.

The present study provides evidence that Ln$^{3+}$s interact with a specific binding site associated with the ACTH receptor. The observation that the addition of Ln$^{3+}$s at micromolar concentrations is as effective as Ca$^{2+}$ removal in blocking ACTH inhibition of $I_{AC}$ suggests a common mechanism involving displacement of Ca$^{2+}$ from a specific site.

With regard to the specificity of the Ln$^{3+}$-binding site, our experimental design did not detect a difference in potency between La$^{3+}$ and Yb$^{3+}$, which span nearly the entire range of ionic radii for these elements (33). These results are in contrast to the large size-dependent differences in potency observed for Ln$^{3+}$ inhibition of ion channels and indicate significant differences in these binding sites (11, 24).
Comparison with Ln$^{3+}$ effects in other biological systems. The actions of Ln$^{3+}$'s in AZF cells parallel their effects in other biological systems. Despite their high affinity for Ca$^{2+}$-binding sites, the Ln$^{3+}$'s have no known biological function. Indeed, when Ln$^{3+}$'s interact with Ca$^{2+}$-specific and nonspecific sites on proteins, ranging from enzymes to receptors to ion channels, their action is generally inhibitory (12, 19, 24). In one exceptional example, terbium (Tb$^{3+}$) has been shown to enhance insulin binding to its receptor by high-affinity interaction with a Ca$^{2+}$-binding site (37).

Although it appears likely that Ln$^{3+}$s antagonize ACTH binding to its receptor through interaction with a Ca$^{2+}$-binding site, other possibilities cannot be ruled out. For example, it is possible that Ln$^{3+}$s do not inhibit ACTH binding to its receptor, but instead interfere with subsequent steps required for the production of cAMP. Furthermore, a direct interaction of Ln$^{3+}$s with ACTH seems unlikely but cannot be excluded.

The number of Ca$^{2+}$-dependent G protein-coupled receptors whose activity could be modulated by Ln$^{3+}$s as well as divalent cations is unknown. ACTH receptors belong to the melanocortin receptor subfamily that includes multiple receptors for melanocyte-stimulating hormone (26). It is possible that Ln$^{3+}$s and Ni$^{2+}$ would also inhibit hormone binding to all of these receptors through interaction with Ca$^{2+}$-binding sites.

Effect of Ni$^{2+}$ on ACTH response. Even though Ni$^{2+}$ failed to inhibit IAC K$^+$ channels, it suppressed ACTH inhibition of IAC, although it was 10-fold less potent than Ln$^{3+}$ in this respect. Thus the effects of Ni$^{2+}$ on IAC were mediated through a single site associated with the ACTH receptor. The inhibitory effects of Ni$^{2+}$ on voltage-gated K$^+$ channels occur through actions on gating rather than permeation (11, 35). Apparently, these binding sites are missing on non-voltage-gated background channels such as IAC.

It is likely that Ni$^{2+}$ and Ln$^{3+}$s inhibit ACTH receptor activation through interaction with a common binding site. Ni$^{2+}$ and Ln$^{3+}$s inhibit a number of ion channels through Ca$^{2+}$-binding sites. Invariably, the Ln$^{3+}$s are 10- to 100-fold more potent in this respect (11, 19, 24).

Use of Ln$^{3+}$s and Ni$^{2+}$ as Ca$^{2+}$ antagonists. Ln$^{3+}$s and Ni$^{2+}$ and other divalent cations have been used as Ca$^{2+}$ antagonists in a range of studies to explore the role of voltage-gated Ca$^{2+}$ entry in peptide hormone-stimulated secretion (4, 17). These include studies of ACTH-stimulated cortisol secretion (9, 21, 31, 32, 34). Results of our study indicate that interpretation of these experiments is complicated by the inhibitory actions of these metal cations on ACTH receptors. Finally, they also suggest that Ln$^{3+}$s as well as divalent cations may produce toxic actions on the adrenal gland through inhibitory actions on ACTH binding. Toxic effects of metals on adrenal steroidogenesis have been reported (28).

REFERENCES