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Biochemistry

Adrenocorticotropin receptors: Functional expression from rat adrenal mRNA in *Xenopus laevis* oocytes

(adrenal cortex/zona fasciculata/adenylate cyclase/cyclic AMP)

LAWRENCE M. MERTZ AND KEVIN J. CATT*

Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT The adrenocorticotropin (ACTH) receptor, which binds corticotropin and stimulates adenylate cyclase and steroidogenesis in adrenocortical cells, was expressed in *Xenopus laevis* oocytes microinjected with rat adrenal poly(A)$^+$ RNA. Expression of the ACTH receptor in individual stage 5 and 6 oocytes was monitored by radioimmunoassay of ligand-stimulated cAMP production. Injection of 5–40 ng of adrenal mRNA caused dose-dependent increases in ACTH-responsive cAMP production. These were detected at 48 h and reached a maximum 72 h after microinjection of 20–40 ng of adrenal mRNA. In response to 1 μM ACTH, total cAMP production increased within 2.5 min and reached half-maximal and maximal levels (5-fold greater than basal) at 10 and 75 min, respectively, and then remained elevated for up to 5 h. Extracellular cAMP levels were much lower but showed prominent linear increases from almost undetectable levels, with 70- and 150-fold increases evident at 1 and 2 h, respectively. The half-maximal concentration (ED_{50}) for stimulation of cAMP formation was 5×10^{-8} M ACTH-(1–24); the ED_{50} for ACTH-(1–17) was 5×10^{-7} M, and no response was observed with ACTH-(1–10). Size fractionation of rat adrenal poly(A)$^+$ RNA by sucrose density-gradient centrifugation revealed that mRNA encoding the ACTH receptor was present in the 1.1- to 2.0-kilobase fraction. These data indicate that ACTH receptors can be expressed from adrenal mRNA in *Xenopus* oocytes and are fully functional in terms of ligand specificity and signal generation. The extracellular cAMP response to ACTH is a sensitive and convenient index of receptor expression. This system should permit more complete characterization and expression cloning of the ACTH receptor.

Adrenocorticotropin (ACTH) is released from pituitary corticotrophs and regulates glucocorticoid production through binding to specific plasma membrane receptors in the zona fasciculata cells of the adrenal cortex (1, 2). The interaction of ACTH with its adrenal receptors activates adenylate cyclase (3) via a GTP-dependent mechanism (4), leading to an increase of cAMP-dependent protein kinase activity (5). These signaling events stimulate production of a labile protein that activates the mitochondrial cholesterol side-chain cleavage complex (6, 7) and increases glucocorticoid biosynthesis (8). ACTH has also been implicated in the maintenance of adrenocortical steroidogenic capacity (9) as well as in promoting ACTH receptor expression in adrenal cells (10). Despite its primary importance in adrenal steroid production, little is known about the molecular structure of the ACTH receptor.

The *Xenopus laevis* oocyte expression system has proven to be a powerful tool in the study of mammalian receptor structure and function. Such studies are performed by microinjecting poly(A)$^+$ RNA prepared from mammalian target tissues and analyzing the coupling of the expressed mammalian receptor to signaling systems in the oocyte (11, 12). This approach has been used to characterize and clone several Ca^{2+}-mobilizing receptors (13, 14), but it has not been widely applied to receptors that activate adenylate cyclase. To facilitate the characterization and cloning of the ACTH receptor, we have performed functional expression of the mammalian adrenocortical receptor in the *X. laevis* oocyte. The expressed ACTH receptor mediates signal transduction through adenylate cyclase activation and is encoded by a 1.1- to 2.0-kilobase (kb) mRNA, which can be functionally identified in frog oocytes.

MATERIALS AND METHODS

Materials. Synthetic ACTH-(1–24) (Cortrosyn) was kindly provided by Organon. ACTH-(1–17) and ACTH-(1–10) were purchased from Pharmacia. ^{125}I-labeled adenosine 3′,5′-cyclic phosphoric acid, 2′-*O*-succinyl-(iodotyrosine methyl ester) for use in cAMP radioimmunoassays was purchased from Hazelton Biotechnologies (Vienna, VA). 3-Isobutyl-methylxanthine (IBMX), forskolin, and all other reagents were from Sigma.

Oocyte Isolation. Adult female *X. laevis* frogs (Xenopus I, Ann Arbor, MI) were maintained and used as the oocyte source as described (11). To obtain ovaries, frogs were anesthetized with 0.2% (wt/vol) 3-aminobenzoate and lobes of the ovary were removed and placed in modified Barth's solution (82.5 mM NaCl/2.5 mM KCl/1 mM each $MgCl_2$, $CaCl_2$, and NaH_2PO_4/5 mM Hepes, pH 8.0) supplemented with penicillin (10 units/ml), streptomycin (10 mg per ml of stock), and gentamycin sulfate (50 mg per ml of stock) to final concentrations of 0.1% and 0.2% (vol/vol), respectively. Stage 5 and 6 follicular oocytes were manually removed from ovarian lobes with jeweler's forceps and a dissecting microscope. Isolated oocytes were maintained in modified Barth's solution at 18°C and viable oocytes were transferred to fresh medium every 16 h.

Adrenal Poly(A)$^+$ RNA Isolation and Oocyte Injection. Rat adrenal glands were rapidly frozen in liquid N_2 immediately after removal from male Sprague–Dawley rats (250–350 g; Sprague–Dawley; Charles River Breeding Laboratories). RNA was extracted and purified as described (11). Poly(A)$^+$ RNA was prepared by column chromatography on oligo(dT)-cellulose (Collaborative Research) and size-fractionated by centrifugation through continuous gradients of sucrose (10–30%; wt/vol) as described (15). Before its application to the sucrose gradient, the poly(A)$^+$ RNA (85–100 μg) was heated

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Abbreviations: ACTH, corticotropin; IBMX, isobutylmethylxanthine.

*To whom reprint requests should be addressed at: Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Building 10, Room B1L-400, Bethesda, MD 20892.

to 70°C for 15 min and then placed on ice to promote disruption of secondary structure. Stage 5 and 6 oocytes were injected (33 nl per oocyte) with poly(A)$^+$ RNA dissolved in 1 mM EDTA using a computerized pressure-controlled microinjector (Atto Instruments, Potomac, MD). Control oocytes were microinjected with 1 mM EDTA. A Flaming/Brown micropipette puller (model P-87; Sutter Instruments, San Rafael, CA) was used to construct micropipettes with an external tip diameter of 200–300 μm.

Assay of ACTH Receptor Expression. Microinjected or control oocytes were individually incubated in 0.1 ml of modified Barth's solution containing 1 mM IBMX and the appropriate test substance. To terminate incubations, tubes were placed in dry ice and rapidly thawed; the oocytes were then homogenized directly in the incubation medium. Homogenates were heated at 100°C for 10 min followed by centrifugation at 700 × *g* for 10 min; the cAMP content of the supernatant from each oocyte was individually measured by a direct radioimmunoassay, which had a detection limit of 1–2 fmol (16). When extracellular cAMP levels were measured from individual oocytes, the oocytes were removed from the medium just prior to freezing the samples.

RESULTS

The appearance of ACTH-responsive cAMP production in oocytes microinjected with increasing amounts of adrenal poly(A)$^+$ RNA is shown in Fig. 1. Significant ACTH receptor expression was first observed 48 h after mRNA microinjection, with 2- and 2.5-fold increases in ACTH-stimulated cAMP production in oocytes injected with 20 and 40 ng of mRNA, respectively. Maximal increases in cAMP formation (4- to 5-fold over basal levels) were observed by 72 h in oocytes injected with 20–40 ng of adrenal mRNA. Similar responses were obtained in oocytes from which the follicular cell layer was manually removed after collagenase treatment (2 mg/ml; data not shown). A decrease in ACTH-induced cAMP responses was frequently observed by 96 h, regardless of the amount of mRNA injected (data not shown). This decline was associated with decreased viability of the oocyte. The reason for the inability of higher doses of microinjected mRNA (>40 ng) to support maximal expression is not known, but it might be due to increased expression of translation products that exert toxic effects on the oocyte.

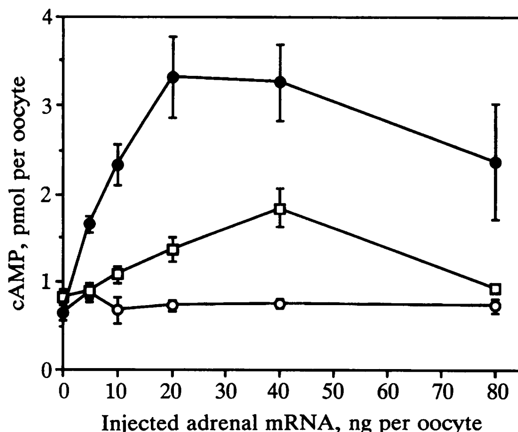

FIG. 1. ACTH-stimulated cAMP responses in adrenal mRNA-injected oocytes. Oocytes were microinjected with various amounts of rat adrenal mRNA and then incubated for either 48 (□) or 72 (○ and ●) h. The oocytes were then individually incubated with 1 μM ACTH for 1 h and subsequent total cAMP accumulation was measured by radioimmunoassay. Basal cAMP levels are also shown (○). Data points represent the sample mean ± SEM (n = 10). Where no bar appears, the error is within the symbol size.

We observed an accelerated loss of oocytes injected with 80 ng of mRNA over the 72-h incubation period. During maximal ACTH receptor expression, we could not detect any significant effect of ACTH on intracellular Ca^{2+} levels in the adrenal mRNA-injected oocytes, as judged by use of the microinjected Ca^{2+}-specific photoprotein aequorin. In contrast, exposure of the mRNA-injected oocytes to angiotensin II (0.5 μM) resulted in 3- to 5-fold increases in light emission, similar to, but somewhat lower than, those described by Sandberg *et al.* (12). This difference results from our use of poly(A)$^+$ RNA from the whole adrenal gland, whereas Sandberg *et al.* (12) used poly(A)$^+$ RNA isolated from the adrenal zona glomerulosa, which represents a more enriched source of mRNA encoding the angiotensin II receptor (subtype AT_1). More detailed studies on ACTH receptor expression were performed 72 h after microinjection of oocytes with 20 ng of rat adrenal poly(A)$^+$ RNA.

To better define the kinetics of ACTH-induced signal generation, we examined the temporal characteristics of cAMP production over a 5-h period of ACTH (1 μM) stimulation. The results in Fig. 2 indicate that the appearance of cAMP was rapid, with appreciable increases during the first 2.5 min (Fig. 2 *Inset*); half-maximal (2.5-fold) and maximal (5-fold) increases were apparent at 10 and 75 min, respectively, after addition of ACTH. Maximal levels of cAMP were maintained throughout the remainder of the 5-h incubation period. Control oocytes injected with vehicle (1 mM EDTA) did not respond to ACTH throughout the incubation period.

Endogenous adenylate cyclase-coupled receptors on *Xenopus* ovarian follicular cells, including the β-adrenergic receptor, require costimulation with low doses of forskolin (10–20 μM) in addition to the receptor agonist to generate detectable cAMP accumulation (17, 18). To further evaluate the possible presence of endogenous ACTH receptors in the follicular oocyte, we treated vehicle-injected oocytes with ACTH (1 μM) and forskolin (20 μM). Such treatment did not cause significant increases in cAMP production (data not shown) over those observed in similar oocytes treated with forskolin alone, consistent with the absence of endogenous ACTH receptors or others native to the oocyte that interact with ACTH and activate adenylate cyclase.

We also compared ACTH-responsive cAMP formation in the adrenal mRNA-injected oocyte with that observed when mRNA encoding the human β_2-adrenergic receptor (19) was microinjected (5 ng of mRNA per oocyte) into stage 5 and 6 oocytes. These data (Fig. 2*B*) indicate that stimulation of the expressed β_2-adrenergic receptor with a maximal dose of 100 μM isoproterenol for 30 min elicits a 5-fold increase in cAMP levels, similar to that achieved by 1 μM ACTH in the adrenal mRNA-injected oocytes (Fig. 2*A*). No responses to the β_2-adrenergic agonist were observed in control (uninjected) oocytes since, as discussed above, the endogenous system requires costimulation with forskolin to detect increases in cAMP accumulation.

To determine the extent of cAMP release vs. intracellular accumulation within the oocyte, we analyzed the medium content of cAMP during adenylate cyclase activation in both forskolin-stimulated control (noninjected) oocytes and ACTH-treated oocytes previously microinjected with rat adrenal mRNA (20 ng per oocyte). As shown in Fig. 3, treatment of follicular oocytes with 25 or 100 μM forskolin in medium containing 1 mM IBMX caused saturable increases in intracellular cAMP with maximal levels at 30 min, followed by a significant decrease during continued incubation (Fig. 3*A*). Unlike the intracellular cAMP levels, the extracellular accumulation of cAMP increased linearly from almost undetectable amounts throughout the 2-h incubation (Fig. 3*B*).

The temporal patterns of intra- and extracellular cAMP accumulation in 1 μM ACTH-stimulated oocytes that were

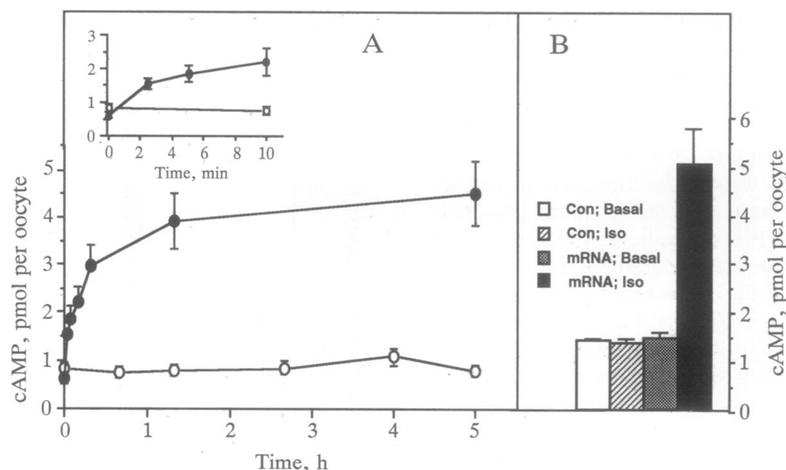

FIG. 2. (*A*) Time course of ACTH-stimulated cAMP formation in adrenal mRNA-injected oocytes. Oocytes were microinjected with 1 mM EDTA alone (control; ○) or 1 mM EDTA containing 20 ng of adrenal mRNA (●) and after 72 h were stimulated with 1 μM ACTH for the times shown during a 5-h incubation period. (*Inset*) Early phase of cAMP accumulation during ACTH stimulation. (*B*) Oocytes were microinjected with 5 ng of mRNA encoding the human β_2-adrenergic receptor (mRNA) and after 48 h were stimulated with 100 μM isoproterenol (Iso) for 30 min (Con, uninjected oocytes; Basal, basal cAMP accumulation at the end of the 30-min incubation). Data points represent the sample mean ± SEM (*A*, $n = 8$; *B*, $n = 10$). Where no bar appears, the error is within the symbol size.

microinjected with rat adrenal mRNA (20 ng per oocyte) 72 h earlier are shown in Fig. 3 *C* and *D*. Also shown are ACTH-treated control (noninjected) oocytes as well as mRNA-injected oocytes that were incubated in medium not supplemented with 1 mM IBMX during ACTH stimulation. The production of both intra- and extracellular cAMP by ACTH treatment qualitatively, but not quantitatively, paralleled the characteristics of forskolin-stimulated accumulation. As shown in Fig. 3*D*, robust increases of 70- and 150-fold (over basal values) of extracellular cAMP were

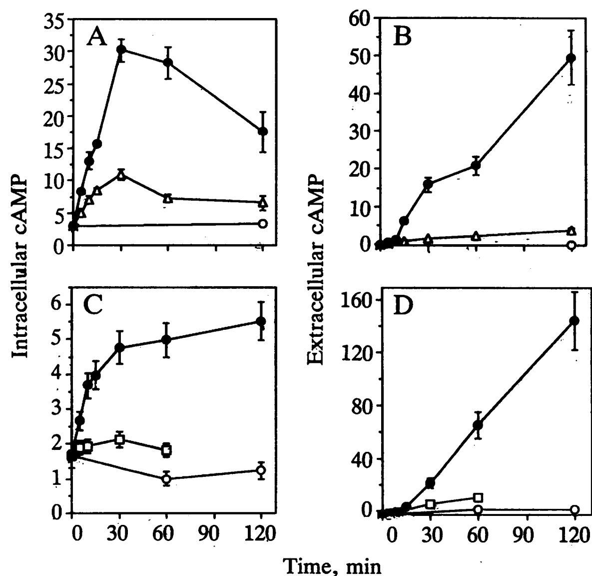

FIG. 3. Intracellular (*A* and *C*) and extracellular (*B* and *D*) cAMP accumulation in control and adrenal mRNA-injected oocytes. (*A* and *B*) Control (noninjected) follicular oocytes were incubated in medium alone (○) or in medium containing either 25 (△) or 100 (●) μM forskolin over a 2-h period. (*C* and *D*) Adrenal mRNA-injected oocytes (20 ng per oocyte) were stimulated with 1 μM ACTH in medium alone (□) or in medium containing 1 mM IBMX (●). ○, Vehicle-injected (1 mM EDTA) oocytes treated with 1 μM ACTH. Note the different numeric scales on the ordinates (data in *A*–*C* are expressed as pmol per oocyte; data in *D* are expressed as fmol per oocyte). All data points represent sample mean ± SEM (*A* and *B*, $n = 5$; *C* and *D*, $n = 10$). Where no bar appears, the error is within the symbol size.

observed 1 and 2 h, respectively, after addition of agonist. ACTH stimulation of mRNA-injected oocytes incubated in the absence of IBMX also caused significant elevations of intracellular and extracellular cAMP, but the extent of this accumulation in either compartment during the 1-h incubation was less than that observed with oocytes stimulated in the presence of IBMX.

The functional properties of the adrenocortical ACTH receptor were also examined by analyzing the dose dependence of total cAMP formation (sum of intra- and extracellular accumulation) in adrenal mRNA-injected oocytes treated with ACTH-(1–24), ACTH-(1–17), a truncated polypeptide with an altered positive charge contribution at its C terminus, and ACTH-(1–10), a peptide corresponding to the N terminus of ACTH. The concentration–response curves for these peptides are shown in Fig. 4. Accumulation of cAMP in the adrenal mRNA-injected oocyte was dose dependently elevated by ACTH-(1–24) and ACTH-(1–17), but it was not stimulated by ACTH-(1–10). ACTH-(1–24) stimulated cAMP formation with an ED_{50} of $\approx 5 \times 10^{-8}$ M, whereas the ED_{50} value for ACTH-(1–17) was at least an order of magnitude ($ED_{50} = 5 \times 10^{-7}$ M) less potent. ACTH-(1–17)

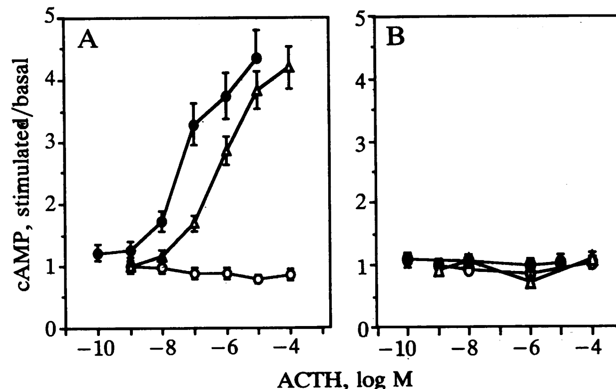

FIG. 4. Effects of ACTH peptides on cAMP accumulation in mRNA-injected oocytes. Total (intracellular and extracellular) cAMP accumulation was measured in adrenal mRNA-injected (20 ng of mRNA per oocyte) (*A*) and control (noninjected) (*B*) follicular oocytes stimulated for 1 h with increasing doses of either ACTH-(1–24) (●), ACTH-(1–17) (△), or ACTH-(1–10) (○). All data points represent mean ± SEM of individual oocytes ($n = 18$).

was able to evoke maximal increases in cAMP accumulation at concentrations above 10^{-5} M. Control uninjected oocytes did not respond to any of the ACTH polypeptides tested throughout the concentration range of 10^{-10}–10^{-4} M (Fig. 4*B*).

To determine the approximate size of the mRNA that encodes the ACTH receptor, we size-fractionated rat adrenal poly(A)$^+$ RNA on a continuous sucrose gradient (10–30%; wt/vol). The data shown in Fig. 5*A* indicate the amounts of poly(A)$^+$ RNA obtained in the sucrose gradient fractions, and the autoradiograph shown in Fig. 5*B* reveals the various size ranges of the fractions shown in Fig. 5*A*. Poly(A)$^+$ RNA from each fraction was injected into oocytes (20 ng per oocyte) and 48 and 72 h later the oocytes were incubated with 1 μM ACTH for 1 h. We quantitated extracellular cAMP levels after the 1-h stimulation.

As shown in Fig. 6, expression of functional ACTH receptors was observed in oocytes microinjected with poly(A)$^+$ RNA from fraction 4 of the sucrose gradient. A small amount of activity was also observed in oocytes injected with poly(A)$^+$ RNA from fraction 5. The activity observed in oocytes injected with poly(A)$^+$ RNA from fraction 4 was significantly enhanced (≈3-fold, 72 h after microinjection) over that observed in oocytes microinjected with 20 ng of total poly(A)$^+$ RNA. Such enrichment would be expected since equal amounts of total and size-enriched material were injected into the oocytes. Based on the size distribution of fraction 4 and its relationship to that in fraction 3, which does not appear to contain ACTH receptor mRNA, the approximate size of the mRNA that encodes the functional ACTH receptor is between 1.1 and 2.0 kb. The presence of mRNA within this size range in fraction 5 probably accounts for the small amount of ACTH receptor expression observed in oocytes injected with this fraction.

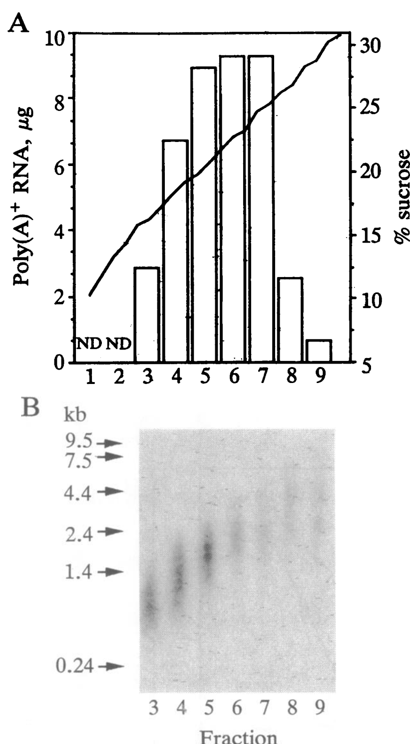

FIG. 5. Size fractionation of adrenal poly(A)$^+$ RNA by sucrose density-gradient centrifugation. (*A*) Rat adrenal poly(A)$^+$ RNA was sedimented through a sucrose gradient (10–30%; wt/vol) and separated into nine fractions. The concentration of RNA in each fraction was determined by measuring the optical density at 260 nm. (*B*) Sizes of the poly(A)$^+$ RNA in each fraction were determined by electrophoresis through an agarose gel containing formaldehyde followed by blotting onto a Nytran immobilization membrane (Schleicher & Schuell). Poly(A)$^+$ RNA on the membrane was visualized by hybridization with 5′-end-labeled ^{32}P-labeled oligo(dT)$_{15}$ followed by autoradiography. ND, not detectable.

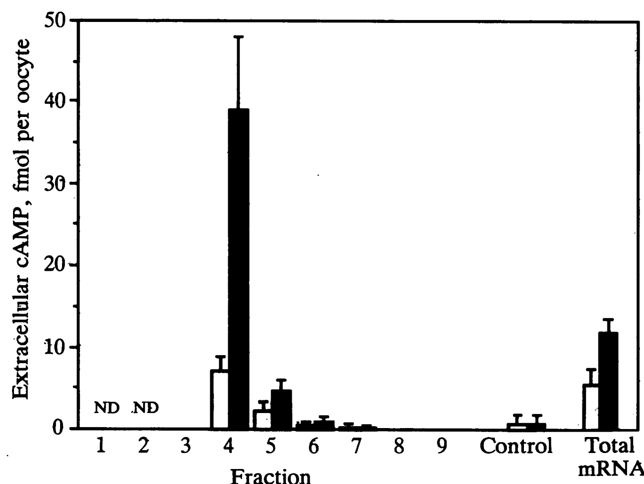

FIG. 6. ACTH receptor expression in oocytes microinjected with poly(A)$^+$ RNA fractions obtained from sucrose density-gradient centrifugation (see Fig. 5). After injection with 20 ng of either total or the sized fractions of poly(A)$^+$ RNA, oocytes were incubated for either 48 (□) or 72 (■) h. To assess ACTH receptor expression, oocytes were incubated with 1 μM ACTH for 1 h and the amount of cAMP released into the medium was measured by radioimmunoassay. Control values represent extracellular cAMP levels of control (uninjected) oocytes incubated with 1 μM ACTH for 1 h. Values shown represent the sample mean ± SEM of extracellular cAMP measurements from individual oocytes (n = 15). ND, not determined.

DISCUSSION

The ACTH receptor still awaits complete characterization, despite the abundance of information about the biological importance of its ligand. When expressed in the oocyte, this receptor retains its ability to transduce hormone binding into adenylate cyclase activation, the mechanism by which it controls glucocorticoid production in the adrenal zona fasciculata. Although ACTH receptor signaling has been suggested to involve intracellular Ca^{2+} in the past, the expressed ACTH receptor does not evoke detectable changes in oocyte Ca^{2+} levels. It cannot be ruled out, however, that this receptor or one akin to it may activate the Ca^{2+} signaling system in the adrenal and/or other tissues but to a minor degree that is below the detection limit of the aequorin method. A role for extracellular Ca^{2+} in the binding of ACTH to the adenylate cyclase-stimulating receptor has been suggested (20). We observed that stimulation of adrenal mRNA-injected oocytes with 1 μM ACTH in Ca^{2+}-free medium or 1 mM EGTA-containing medium resulted in 50% and 100% inhibition, respectively, of cAMP accumulation. Such removal of external Ca^{2+} had no significant effect on the ability of 100 μM forskolin to enhance cAMP formation (unpublished observation) with respect to the accumulation observed in oocytes incubated in Ca^{2+}-containing medium.

The ED_{50} value of ACTH-(1–24) for cAMP formation in receptor-expressing oocytes was at least 1 order of magnitude higher than that observed in rat adrenocortical cells (21). This may result from differences in receptor number, efficiency of ACTH receptor–effector coupling, and also in ACTH receptor posttranslational processing between the native and oocyte expression systems. A difference in plasma membrane microenvironment between the mammalian and amphibian systems could also be a factor. Alterations in cou-

pling efficiency could result from different stoichiometric relationships between the GTP-binding protein (G_s) and/or adenylate cyclase present in the oocyte expression system, as compared to the amounts of these components present in the adrenal cortex. The ligand-binding specificity of the ACTH receptor (21) is retained in the oocyte, as indicated by the relative potencies with which the different ACTH ligands promote adenylate cyclase activation (Fig. 4). The importance of basic residues 15–18 of ACTH in receptor function (21, 22) appears to be maintained in the oocyte, based on the reduced potency of ACTH-(1–17) and the inability of ACTH-(1–10) to activate the receptor.

These studies have also demonstrated a marked and progressive increase in cAMP release into the extracellular medium during agonist stimulation. Although the release mechanism is unknown, it is possible that adenylate cyclase may itself expel cAMP, since its proposed structure resembles one commonly assigned to membrane transporters and channels (23). The extrusion rate of cAMP from the oocyte, like intracellular cAMP accumulation, is dramatically enhanced by phosphodiesterase inhibition but is not solely a result of it, since detectable secretion was also observed in ACTH-treated adrenal mRNA-injected oocytes stimulated in the absence of IBMX. The ability of forskolin to evoke much greater cAMP accumulation in both intra- and extracellular compartments probably reflects its ability to stimulate adenylate cyclase activity not only in the oocyte but also in other cell types present in the follicular oocyte, such as steroidogenic follicular cells, which possess greater amounts of forskolin-sensitive adenylate cyclase activity than the defolliculated oocyte (17). Nevertheless, the oocyte itself possesses sufficient amounts of adenylate cyclase and G_s to mediate hormone-induced cAMP responses, since the human β_2-adrenergic receptor can be functionally expressed in the oocyte as shown by Kobilka *et al.* (19) and in this study.

Release of cAMP into the medium provides a sensitive and convenient index of the functional status of ACTH and/or other adenylate cyclase-coupled receptors expressed in the oocyte. Our observations on cAMP release differ from those of Horiuchi *et al.* (24), who found less marked increases in extracellular cAMP when examining parathyroid hormone receptor expression in oocytes injected with mRNA from the rat osteogenic cell line UMR 106. This is probably due to a much lower and transient enhancement of intracellular cAMP accumulation in response to parathyroid hormone receptor agonists in their study; in our mRNA-injected oocytes, ACTH stimulation led to a 5-fold increase in intracellular cAMP levels that was maximal by 30 min and remained elevated thereafter. This prolonged increase in intracellular cAMP accumulation is responsible for the marked and progressive increase in extracellular cAMP accumulation in ACTH-stimulated oocytes.

Application of the screening method of extracellular cAMP quantitation to detect ACTH receptor expression from size-fractionated preparations of adrenal poly(A)$^+$ RNA revealed that the size of the mRNA that encodes the adenylate cyclase-activating receptor is between 1.1 and 2.0 kb. Previous attempts to identify the ACTH receptor by photoaffinity labeling and disuccinimidylsuberate crosslinking studies have yielded candidates of 40 (25) and 43 kDa (26), respectively, in bovine adrenocortical membranes, whereas other studies using mouse adrenal Y-1 and rat adrenocortical cells have revealed candidates of 83 (27) and 100 kDa (28), respectively. In relation to the approximate size of the ACTH receptor mRNA, the 40- and 43-kDa species are potential translation products since their mRNAs would be at least 1.1–1.2 kb. The other candidates of 83 and 100 kDa would require larger mRNAs of at least 2.3 and 2.7 kb, respectively.

Functional reconstitution of the ACTH receptor in the oocyte provides an expression system that can be used to clone the ACTH receptor by screening adrenal cDNA libraries. The attractiveness of this system for receptor cloning lies in its use of a functional assay to detect ACTH receptor expression, since receptor antibodies and amino acid sequence data are not available and ACTH receptor-binding techniques have proven to be difficult in the past. Furthermore, the receptor can be more readily characterized in terms of its ability to couple with either endogenous or coexpressed signal transduction components in the oocyte.

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