Microinjection of a 19-kDa Guanine Nucleotide-binding Protein Inhibits Maturation of Xenopus Oocytes*

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ADP-ribosylation factors (ARFs) are 19-21-kDa proteins purified from bovine brain that bind guanosine 5'-triphosphate (GTP). They exhibit GTP-dependent activity as activators of cholera toxin-catalyzed ADP-ribosylation of the α-subunit of the stimulatory guanine nucleotide-binding protein of the adenyl cyclase system (Gs). ARF, which interacts directly with the catalytic subunit of cholera toxin, has no known physiologic role. Intracellular microinjection of ARF was employed to investigate the role of ARF on progestosterone- and insulin-stimulated maturation of Xenopus oocytes. Maturation was inhibited by injection of ARF 3-8 h before exposure of oocytes to progesterone or insulin. ARF inhibition was dependent on progesterone concentration but not on insulin concentration. Inhibition was enhanced by concomitant injection of GTP and to a greater extent by guanosine 5'-O-(thiotriphosphate) (GTPγS), which, in the absence of ARF, inhibited somewhat at early time points. The demonstration of this effect of ARF on both progesterone- and insulin-stimulated oocyte maturation may provide a clue to the physiologic role of this guanine nucleotide-binding protein.

A membrane protein termed ARF, for ADP-ribosylation factor, was initially purified based on its activity as a cofactor for cholera toxin-catalyzed ADP-ribosylation of Gαs, the α-subunit of the stimulatory guanine nucleotide-binding protein of the adenyl cyclase system (1). This ARF activity was GTP-dependent, and it was later shown that ARF is a GTP-binding protein (2). Subsequently, two other very similar ARF proteins were purified from bovine brain cytosol (3). Both membrane and soluble ARF proteins apparently interact directly with cholera toxin in a GTP-dependent manner thereby increasing its catalytic activity (3, 4). Thus, they stimulate toxin-catalyzed ADP-ribosylation of arginine, other simple guanidine compounds, and several proteins unrelated to Gαs (3, 4). They also enhance auto-ADP-ribosylation of the toxin Aβ protein (3, 4).

The physiological role of the ARF proteins, which are found in many tissues and are present in high concentration in brain (5), is unknown. Comparison of deduced amino acid sequences of ARF and the ras protooncogene product c-Ha-ras p21 reveals limited similarities confined to regions of ras p21 (5).

Both of these proteins bind GTP (2, 8, 9), and ras p21 exhibits GTPase activity (8, 9) which has thus far not been detected with ARF (2). It has been shown that injection of ras p21 into Xenopus oocytes induces maturation (10), whereas injection of monoclonal antibody against ras p21 prevents insulin- but not progesterone-induced maturation (11, 12). To determine whether ARF might have functional effects like those of ras p21 in Xenopus oocytes, the studies reported here were initiated.

EXPERIMENTAL PROCEDURES

Oocytes were harvested from 2-2.5-year-old virgin female Xenopus laevis toads (Nasco, Inc., Fort Atkinson, WI) using tricaine anesthesia, 3-5 days after injection of 25-35 IU human chorionic gonadotropin (Sigma) into the dorsal lymph sac. After incubation in calcium-free OR-2 (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 5 mM Hepes, pH 7.6) containing collagenase (Sigma, type IA), 2.5 mg/ml, for 5-7 h at 20 °C, oocytes to be stimulated by progesterone were washed twice at 20 °C in ND-96 (96 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl2, 5 mM Hepes, pH 7.6) containing 2.5 mM pyruvate, penicillin (100 units/ml), and streptomycin (100 μg/ml), and incubated overnight in the same medium. Oocytes to be stimulated by insulin were washed twice and incubated overnight in modified OR-2 (83 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 1 mM MgCl2, 5 mM Hepes, pH 7.6) containing 2.5 mM pyruvate, penicillin (100 units/ml), and streptomycin (100 μg/ml), and incubated overnight in the same medium. Oocytes to be stimulated by insulin were washed twice and incubated overnight in modified OR-2 (83 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 5 mM Hepes, pH 7.6) containing 2.5 mM pyruvate, penicillin (100 units/ml), and streptomycin (100 μg/ml), and incubated overnight in the same medium.

Stage VI (see Ref. 13) oocytes were selected, transferred to plastic dishes containing 6 ml of the appropriate medium (≈250 oocytes/dish), and kept overnight at 20 °C with gentle agitation. Next morning, damaged oocytes were discarded, and oocytes, with or without injection as indicated for each experiment, were distributed to plastic dishes containing 6 ml of medium. Progesteron (Sigma) or insulin (regular Insulin, U-100, Lilly) was added as described for each experiment, and at the indicated times thereafter oocytes were inspected to evaluate appearance of the maturation spot, signifying germinal vesicle breakdown (GVBD). The number of oocytes with GVBD was recorded and percent GVBD calculated. Oocytes with equivocal morphologic changes at the end of each experiment were fixed in 10% trichloroacetic acid and dissected to verify the status of the germinal vesicle. Progesteron solutions were prepared from ethanol stock; ethanol concentration in the incubation medium was 0.01%. Insulin was added directly to the incubation medium to yield the indicated final concentration. A pressure injection system and micropipette with outside tip diameter of 12-15 μm were used for microinjection. Solutions of injected proteins and nucleotides were prepared in 10 mM NaCl, 10 mM Hepes, pH 7.5.

ARF was purified from bovine brain as described for the preparation of sARFII in Ref. 3. Where indicated, proteins were incubated with guanine nucleotide for 50 min at 20 °C before injection. For calculation of concentrations of ARF solutions a M, of 19,000 was assumed. Injected volume was 50 nl for progesterone- and 35-40 nl for insulin-treated oocytes. If injected ARF proteins were uniformly distributed in total cell water (approximately 500 nl/oocyte), intracellular concentrations would be 7-10% of those injected. Data are means plus or minus standard error of the mean for values from two or more replicate dishes containing the indicated number of oocytes. When

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1 The abbreviations used are: ARF, ADP-ribosylation; GTPγS, guanosine 5'-O-(thiotriphosphate); Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; GVBD, germinal vesicle breakdown.
RESULTS AND DISCUSSION

When oocyte maturation, as determined by GVBD, was induced by addition of 10 nM progesterone to the incubation medium, maturation of a group of oocytes was usually complete within 7 h (Fig. 1), although there was some variation between oocytes harvested from different toads and from the same toad at different times. Concentrations of progesterone greater than 10 nM caused more rapid maturation, and concentrations less than 10 nM failed to induce maturation of 90–100% of oocytes in 7 h (Fig. 1).

Injection of ARF into oocytes 6 h before addition of 10–50 nM progesterone inhibited GVBD, and inhibition was dependent on the concentration of injected ARF (Fig. 2). Inhibition was evident as early as 4 h after addition of progesterone and persisted for the duration of the experiment although diminishing with time (18 h). At all observation times, inhibition of GVBD in oocytes injected with 10, 20, or 30 μM ARF (9.5, 19, and 29 ng/oocyte, respectively) was significant (p < 0.001); 3 μM ARF had no measurable effect (Fig. 2). Inhibition of GVBD produced by 10 μM ARF was less than that produced by 20 or 30 μM ARF at times between 5 and 18 h (p < 0.001) as well as at 4 h (p < 0.05) after progesterone was added; 20 μM ARF was less inhibitory than 30 μM ARF at 7 (p < 0.06), 8, and 18 h (p < 0.001) after progesterone.

ARF inhibition was dependent on the concentration of progesterone used to stimulate GVBD and on the time elapsed between ARF injection and addition of progesterone (Fig. 3). GVBD stimulated by 20 nM progesterone was only slightly inhibited by 30 μM ARF whether injected 1 or 5 h before stimulation, and inhibition was negligible when ARF was injected 12 or 15 h before stimulation (Fig. 3). Significant inhibition was produced by injection of 30 μM ARF, 3 (p < 0.03), or 8 (p < 0.006), but not 15 h before addition of 10 nM progesterone. These findings would be consistent with the conclusion that either active ARF itself or responsiveness to ARF does not persist 15 h after injection.

With oocytes harvested from the same toads at a different time of year, 24 μM ARF caused 75% inhibition of GVBD stimulated by 50 nM progesterone (p < 0.002). It appears that there is a seasonal variation in oocyte sensitivity to progesterone and the greatest inhibitory effect of ARF is seen when the concentration of progesterone used to stimulate maturation is near the minimum necessary to cause a full response of 90–100% GVBD within 7 h (Fig. 1).

As ARF activity (when assessed by its ability to activate cholera toxin) requires the presence of GTP or a GTP analogue (4), the effects of guanine nucleotides on GVBD were investigated (Fig. 4). Injection of ovalbumin alone or in combination with GDP or GTP had no effect on progesterone-stimulated GVBD, nor did injection of boiled ARF. GTPγS (plus ovalbumin) produced slight but significant inhibition of GVBD stimulated by 4, 5, 6 (p < 0.001), and 7 h (p < 0.05) after addition of progesterone, but not at 8 nor 18 h. Inhibition of GVBD by ARF was not altered by concomitant injection of GDP (Fig. 4). ARF inhibition (with or without GDP) was significant (p < 0.001) at all times compared to ovalbumin with or without GDP or GTP, or boiled ARF, and ranged from 32 to 79%. Inhibition was not significant compared to ovalbumin plus GTPγS at 4 and 5 h. Coinjection of GTP significantly

FIG. 2. Inhibition of progesterone-stimulated GVBD by prior injection of oocytes with ARF. Oocytes were injected with 30 μM ovalbumin (Sigma) or ARF at the indicated concentration. Progesterone (10 nM) was added 6 h after cells were injected and GVBD was evaluated at 4 (open circle), 5 (filled circle), 6 (open triangle), 7 (filled triangle), 8 (open square), and 18 h (filled square). Percentage inhibition of GVBD at each time is based on the difference between oocytes injected with ovalbumin and those injected with ARF. For each experimental condition there were four dishes, each with 20–25 oocytes.

FIG. 3. Inhibition of GVBD as a function of time between injection of ARF and addition of progesterone. Oocytes were injected with 30 μM ovalbumin or ARF. 10 (filled figures) or 20 nM (open figures) progesterone was added at the indicated times thereafter. GVBD was evaluated, 0 h (circle), 2 (square), and 8 h (triangle) after progesterone was added. For each experimental condition there were three dishes, each with 20–25 oocytes.

FIG. 1. GVBD in un.injected oocytes as a function of time after progesterone was added. Progesterone, 5 (open circle), 10 (filled circle), 50 or 100 (filled triangle), or 500 nM (open square) was added, and the percentage GVBD was evaluated at the indicated time thereafter. For each experimental condition there were two dishes, each with 10–15 oocytes.
of GVBD was 59% ($p < 0.03$) with 7 μM insulin, and 52% ($p < 0.02$) with 1 μM insulin. In this regard (as in some others) insulin stimulation of maturation appears to differ from progesterone stimulation, since ARF inhibition of the latter was best demonstrated with low progesterone concentrations. It is possible that the inhibitory effect of ARF on GVBD induced by these relatively low concentrations of progesterone (which were nonetheless sufficient to cause a brisk and complete oocyte response) could result from an ARF-induced increase in progesterone uptake or metabolism. Since ARF also inhibits insulin-stimulated GVBD, however, its effects on oocyte maturation are presumably not due solely to increased removal or inactivation of progesterone. It seems more likely that ARF inhibits both progesterone- and insulin-induced GVBD by acting at a site in the maturation pathway that is common to both.

When insulin was used as the stimulating hormone, GTPγS (plus ovalbumin) was inhibitory at 9.0 and 10.5 h, but inhibition was not significant at later times (Fig. 5). GTP (plus ovalbumin) did not cause significant inhibition of GVBD at any time. Inhibition by ARF (or ARF plus GTP, which was not different) was significant (compared to ovalbumin with our without GTP) at all times after 6.25 h with inhibition ranging from 45 to 100% ($p < 0.05$). With ARF plus GTPγS, inhibition was greater than in any other ARF-injected group at 13.25 and 16.5 h ($p < 0.05$). Inhibition of GVBD in ARF-injected oocytes decreased with time; however, 96 h after insulin addition, inhibition by ARF or ARF plus GTPγS (relative to oocytes injected with ovalbumin with or without nucleotide) was still 36–46% ($p < 0.05$).

Whereas injection of 9.5–19 ng of ARF/oocyte, as described above, caused significant inhibition of hormone-stimulated maturation, injection of ARF (or ovalbumin) had no discernible effects for at least 72 h on oocytes not exposed to hormone (data not shown). These nanogram amounts of ARF represent less than 0.02% of the total cellular protein, assumed to be approximately 280 μg/oocyte (14). Higher concentrations of ras p21 (10–50 ng/oocyte) have been reported to stimulate oocyte maturation (10–12, 15). ARF appears to have an effect opposite to that described for Ha-ras p21. This may suggest that two distinct classes of low molecular weight GTP-binding proteins exist, each with opposing effects. It is not clear whether other endogenous ARF- or ras-like GTP-binding proteins may affect oocyte maturation; conceivably, ARF or ras is mimicking the action of other GTP-binding proteins.

In un.injected oocytes, a small amount of immune-reactive ARF was detected in the soluble fraction by immunoblot analysis using anti-ARF polyclonal antibodies (Fig. 6). This assay was capable of detecting 25–50 ng of bovine ARF/lane. After ARF injection, additional soluble immune-reactive ARF was apparent, forming a doublet with endogenous ARF. This suggests that endogenous ARF in the oocyte has electrophoretic mobility similar to that of bovine sARFII (3) which differs slightly from sARFII. Immune-reactive ARF seen immediately following injection of 50 nl of 30 μM ARF/oocyte remained present through 15 h (Fig. 6). No ARF was detected in membrane fractions of oocytes either before or after injection (data not shown). The failure of ARF to inhibit GVBD when injected 15 h before progesterone stimulation and the decrease in inhibition of both progesterone- and insulin-stimulated maturation by ARF with time may be due to the disappearance of an active form with no change in immune-reactive ARF or to desensitization of the oocyte, although alternative explanations have not been excluded.

The heterotrimeric guanine nucleotide-binding proteins are components of several transmembrane signaling systems,
linking cell surface receptors with their intracellular effectors. These so-called “G-proteins” are functionally regulated by guanine nucleotides and possess GTPase activity, GTP binding causes activation that is terminated by GTP hydrolysis, and nonhydrolyzable GTP analogues such as GTPγS cause persistent activation (see Ref. 16 for review). Similarly, ras were homogenized for preparation of immunoblots. Positions of molecular size standards (kDa) are indicated on the left.

Microinjection of sARFII. Either before (time 0) or at the indicated times after microinjection of 50 nl of 30 μM ARF, groups of 25 oocytes were homogenized for preparation of immunoblots. Positions of molecular size standards (kDa) are indicated on the left.

FIG. 6. Immunoblot of oocyte supernatant before and after microinjection of sARFII. Either before (time 0) or at the indicated times after microinjection of 50 nl of 30 μM ARF, groups of 25 oocytes were homogenized for preparation of immunoblots. Positions of molecular size standards (kDa) are indicated on the left.

ACKNOWLEDGMENTS—We thank Dr. Carolyn J. Smith for helpful discussions and Barbara Mihalko for expert secretarial assistance.

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