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Hormonal activation of ionic currents in follicle-enclosed *Xenopus* oocytes

(ovarian follicle/gonadotropin/follicle-stimulating hormone/potassium conductance/cAMP)

R. M. WOODWARD AND R. MILEDI

Laboratory of Cellular and Molecular Neurobiology, Department of Psychobiology, University of California, Irvine, CA 92717

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ABSTRACT **Membrane currents were recorded, using the voltage clamp technique, from *Xenopus laevis* oocytes still surrounded by their enveloping follicular and epithelial cells. Exposure of the follicles to mammalian gonadotropins elicited a current generated largely by an increase in membrane K^+ conductance. The gonadotropin response resembled responses elicited by adenosine and catecholamines in the same follicle, but was not blocked by purinergic or catecholaminergic antagonists. The gonadotropin-induced currents were potentiated by the adenylate cyclase activator forskolin and by phosphodiesterase inhibitors; similar currents were elicited in the same follicle by intraoocyte injection of cAMP, which indicates a role for this second messenger in the response mechanism. Gonadotropin responses were either abolished or substantially reduced after treatments that remove the ovarian epithelial and follicular cells. Our experiments suggest that the gonadotropin receptors, and the K^+ channels they regulate, reside in the follicular cells.**

Xenopus laevis oocytes have been used to investigate neurotransmitter receptors because their membrane already contains some types of receptors (1, 2) and many other receptors can be induced by injecting the oocytes with brain mRNA (3–5). It seemed to us that *Xenopus* oocytes might also be used to investigate the mode of action of hormone receptors, either induced in the oocyte or already present in the native ovarian follicle.

In *Xenopus*, pituitary gonadotropins are considered to be involved in the regulation of various aspects of ovarian physiology (6–10). Details of the mechanisms by which gonadotropins exert their effects remain unclear, but in the mammalian ovary it appears that one facet of gonadotropin action involves receptor-mediated stimulation of adenylate cyclase (11). In response to catecholamines and purinergic agonists, *Xenopus* follicles generate membrane currents due to an increase in K^+ conductance (1, 2, 12); and cAMP seems to act as a second messenger in the generation of this response (refs. 13–15; and unpublished results). We reasoned that if gonadotropins increased the synthesis of cAMP in *Xenopus* follicles, then it might be possible to monitor the gonadotropin–follicle interaction electrophysiologically.

MATERIALS AND METHODS

Follicles were manually isolated from the ovaries of adult female *Xenopus* obtained from three sources: laboratory reared animals from Xenopus I (Ann Arbor, MI) or from Nasco (Fort Atkinson, WI) and captured, wild females from Sullivan (Nashville, TN). Electrophysiology was usually performed over the first 3 days of storage using methods described (2). Except where indicated, membrane currents were recorded from the voltage clamped oocyte still surrounded by its follicular envelope, theca, and ovarian epithelia (an ovarian follicle) (16). Crude pituitary preparations of porcine (p) follicle-stimulating hormone (FSH), and human chorionic gonadotropin (hCG), isolated from urine, were purchased from Calbiochem and Sigma, respectively. High-purity (iodination grade) samples of human (h) FSH and luteinizing hormone (hLH) were obtained from Peninsular Laboratories (Belmont, CA). All hormone preparations were initially made up as concentrated stocks (0.04–3.0 mg/ml) in 10 mM Hepes (pH 7.0), distributed into aliquots, and stored at −20°C. Before use the preparation was diluted in frog Ringer's solution containing either 2 or 5 mM KCl (2). Forskolin was purchased from Calbiochem, SCH 23390 was from Research Biochemicals (Wayland, MA), and the potassium liquid ion exchanger IE 190 was from World Precision Instruments (New Haven, CT). All other drugs and enzymes were purchased from Sigma.

Intraoocyte application of hormones and drugs was made by pneumatic pressure injection from micropipettes (17). We used pFSH at ≈3.0 mg/ml, cAMP (100 mM) or EGTA (50 mM) in 10 mM Hepes, filtered and pH adjusted to pH 7.0 with KOH. Dialysis of pFSH was carried out using Spectra/por 3 dialysis tubing with molecular weight cut off 3.5 kDa; 0.4 ml of a stock solution (3.0 mg/ml) was dialyzed against 4 liters of Ringer's solution for 9 hr. Maturation studies were carried out on batches of 30 follicles, stages V and VI (18), in 2 ml of Barths saline (2) plus gonadotropin at 1.0–10 μg/ml. Germinal vesicle breakdown was scored by white-spot formation (19).

RESULTS

Membrane-Current Response to Gonadotropins. To determine whether the hormones generated membrane currents, the membrane potential was routinely voltage clamped at −60 mV, so as to be away from the equilibrium potential of Na, K, Cl, and Ca ions (2). Exposure of freshly isolated follicles to gonadotropins almost invariably generated an outward, hyperpolarizing current (Fig. 1). Follicles were usually screened for responses to pFSH and hCG at concentrations of 2–10 μg/ml. In the more sensitive follicles, responses to pFSH were first detectable at 0.5 μg/ml and had peak amplitudes of more than 750 nA with pFSH at 10 μg/ml. Of >250 follicles isolated from 46 donors and tested with gonadotropins only follicles from 1 donor (3 follicles) failed to give an appreciable response. However, considerable variation in response size and time course occurred between follicles from different donors, as well as among follicles isolated from the same ovary. The onset of the response was also highly variable. Depending on the sensitivity of the donor, and the concentration of hormone applied, we record-

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Abbreviations: hCG, human chorionic gonadotropin; FSH, follicle-stimulating hormone; NE, norepinephrine; p, porcine; h, human; LH, luteinizing hormone.

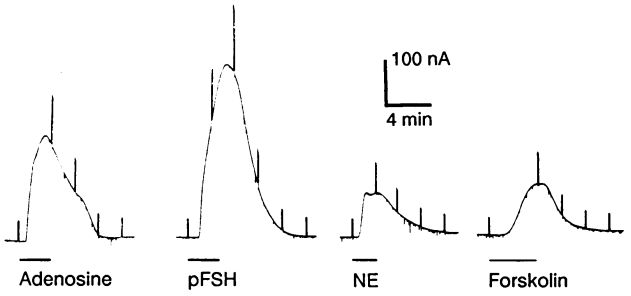

FIG. 1. Native responses recorded from a single follicle with the oocyte voltage clamped at −60 mV. Adenosine at 0.1 mM, pFSH at 5.0 μg/ml, 0.1 mM NE, or 0.5 μM forskolin was applied as indicated, and in the displayed sequence with additional 5- to 10-min periods of washing between exposures not shown. During each exposure, the clamp potential was periodically pulsed to −50 mV to monitor changes in membrane conductance. The small downward deflections correspond to spontaneous oscillatory activity present in this preparation. The dead time of the perfusion system was about 20 sec.

ed latencies of 10 sec to 3 min and rise times of 1–6 min. Extended exposure to hormone resulted in desensitization, and again the rate of desensitization and the time required to wash out the response showed considerable variation. In general gonadotropin responses desensitized less rapidly than norepinephrine (NE) responses in the same follicle. Repeated exposure to gonadotropins often resulted in a reduction in response size even following extended periods of wash, and freshly isolated follicles became progressively insensitive to gonadotropin over the first week of storage. Occasionally when using pFSH at 10 μg/ml, we observed small inward currents preceding the much larger outward current. This inward current was not seen using lower concentrations of hormone, and we have not yet investigated its ionic basis.

High-purity hFSH and hLH were tested on 25 follicles from five donors. In these experiments responses to hFSH could be detected at 100 ng/ml (≈2.6 nM), whereas the currents elicited by 1.0 μg/ml were >250 nA; and hLH was consistently less potent than hFSH. In many instances the same follicle was also exposed to NE, forskolin, or adenosine affording a comparison with the gonadotropin response (Fig. 1). So far we have not discerned any clear pattern, between donors, in the relative sizes of the different responses, but there is a large degree of independent variation. In four donors (a total of 51 follicles), we specifically looked at the size of the gonadotropin response (hCG or pFSH) in relation to the follicle diameter or stage of development (18). In each donor small responses were first detected in stage IV follicles; this low level of response then increased substantially in stage V and VI follicles. Sensitivity to NE followed a similar pattern to that of gonadotropin (Fig. 2), though their relative sensitivities could vary drastically among different donors.

As with the β-adrenergic response, the gonadotropin-induced current was effectively abolished by procedures that remove the oocyte's follicular envelope. Treatment with collagenase (2, 17), or manual removal of ovarian epithelia and incubation in Ca^{2+}-free, EDTA-containing medium (20), invariably either greatly reduced or entirely abolished the gonadotropin response. For example, in one donor the response to pFSH at 5.0 μg/ml in seven follicles was 201 ± 92 nA (mean ± SD) and in six collagenase-treated oocytes the response was 0.3 ± 0.7 nA. Intraoocyte pressure injections of pFSH (≈0.5 nl of pFSH at 3.0 mg/ml) failed to elicit the membrane current up to 5 min after the injection, whereas the same pressure pulses generated responses when the pipette tip was close to the outside of the follicle, both before and after the internal injections.

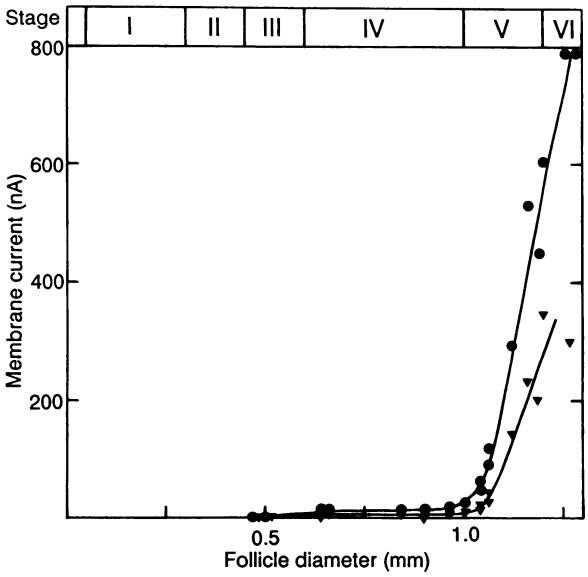

FIG. 2. Relation between follicle diameter or stage and peak amplitude of the responses to pFSH at 5 μg/ml (▼) or 0.1 mM NE (●). All follicles were isolated from the same donor and were screened on the day of isolation. In most cases follicles were first exposed to pFSH, washed for 15–30 min, and then exposed to NE.

Ionic Basis of the Gonadotropin-Induced Current. Like the catecholamine and adenosine responses, the gonadotropin current is generated by an increase in membrane conductance (Fig. 1). To determine if the current was carried by K^+, the intraoocyte K^+ activity was first rechecked with a K^+-selective electrode and confirmed (see ref. 2) to be ≈120 mM (six follicles were tested; the mean was 120 mM; the SD was 17.2 mM). In 5 mM KCl/Ringer's solution, the gonadotropin current, as well as the currents generated by forskolin and NE in the same follicle, all reversed at −80 mV (Fig. 3), close to the predicted reversal potential for K^+. Furthermore the reversal potential of gonadotropin currents in follicles bathed in Ringer's solution containing various concentrations of K^+ (1–10 mM) obeyed a Nernstian relationship (2, 11), and the current was blocked by 20 mM tetraethylammonium bromide or 1.0 mM $BaCl_2$. In some donors where the responses desensitized slowly, the amplitude of the gonadotropin current at various membrane potentials was determined by stepping the voltage clamp potential briefly to different

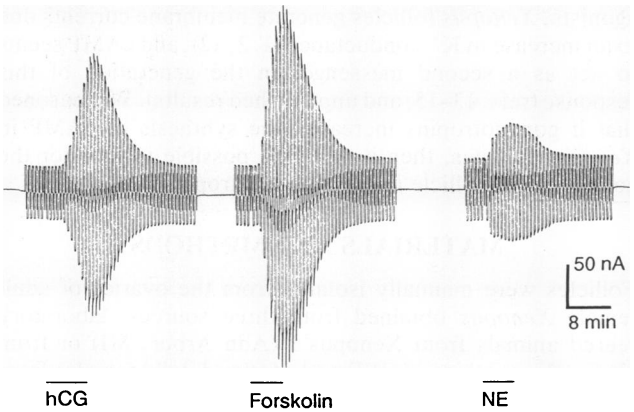

FIG. 3. Reversal potentials for currents induced by hCG at 5.0 μg/ml, 1 μM forskolin, or 0.1 mM NE. The follicle was voltage clamped at −82 mV, close to the predicted reversal potential for K^+ in 5 mM KCl/Ringer's solution, and the command potential was pulsed for 10 sec alternately to −67 and −97 mV at a frequency of 0.05 Hz.

levels, before, during, and after the response. The voltage dependence of the K^+ currents elicited by the different gonadotropin preparations (pFSH, hCG, hFSH, and hLH) were all fairly linear over holding potentials of −120 to 0 mV, indistinguishable from each other and from currents generated by NE and adenosine in the same follicle.

In view of the great similarities between the responses to gonadotropins and those to catecholamines, adenosine, and the hyperpolarizing response to acteylcholine (2), it was necessary to ascertain that the gonadotropin responses were not due to the activation of any of the neurotransmitter receptors that are known to be present in native follicles. This could occur if neurotransmitters were contaminating all our gonadotropin preparations, if gonadotropin was acting directly on these receptors, or if the neurotransmitters were released from the follicle in response to gonadotropin. The following considerations appear to obviate these possibilities: (*i*) The response was not significantly altered after stock pFSH was dialyzed and, furthermore, stock gonadotropin was inactivated by 10 min of boiling or overnight incubation with trypsin (hCG at 1.0 mg/ml; trypsin at 0.05 mg/ml). (*ii*) As mentioned above, there was a large and independent variation between donors in the size of the responses to the different agonists. (*iii*) The gonadotropin responses persisted relatively unchanged in the presence of antagonists, applied individually or in mixtures, that completely abolished the native responses to neurotransmitters. The antagonists used were as follows: 0.1 mM propanolol, β-adrenergic (2); 10 μM atropine, muscarinic (2); 1 μM SCH 23390, a selective blocker of the native response to dopamine (unpublished results); 0.5 μM methysergide, a blocker for the rarely seen native response to serotonin (unpublished results); and 0.1 mM theophylline, purinergic (11).

Gonadotropin Current and cAMP. Thus, it seems very likely that gonadotropin acts on specific receptors that generate a K^+ current like that elicited by catecholamines and adenosine. To see if cAMP was similarly implicated in the gonadotropin responses, we examined, in the same follicle, the current–voltage relation of currents generated by hFSH, by the adenylate cyclase activator forskolin, and by cAMP pressure-injected into the oocyte. The generated currents were all similar (Fig. 4). Moreover, the gonadotropin responses were potentiated with forskolin and with the phosphodiesterase blockers theophylline (Fig. 5*A*) and 3-isobutyl-1-methylxanthine. In follicles showing a high sensitivity to gonadotropin, responses could be detected with pFSH between 5 and 10 ng/ml following pretreatment with 0.5 mM 3-isobutyl-1-methylxanthine. We have also observed that after brief exposure to gonadotropin (pFSH or hCG) the membrane current returns to the basal level but nonetheless the follicle remains in an activated state. This can be shown using phosphodiesterase blockers (2–5 mM theophylline, 0.1–0.5 mM 3-isobutyl-1-methylxanthine), or 0.1 μM forskolin, and the effect is not obvious following responses to NE (Fig. 6). At present we do not know whether this reflects a slower wash out of gonadotropins or, as seems more likely, a difference in the mechanisms of the NE and gonadotropin responses. As described for the gonadotropin response, the residual activation was not blocked by antagonists.

All this suggests that cAMP is involved in generating the gonadotropin-induced current. When we looked for the involvement of Ca^{2+} in the gonadotropin response, we found that the response still persisted in Ca^{2+}-free Ringer's solution containing 1.0 mM EGTA and 10 mM $MgCl_2$. Moreover, the current was not greatly altered by the intraoocyte injection of EGTA, the internal EGTA-Ca^{2+} chelating activity being

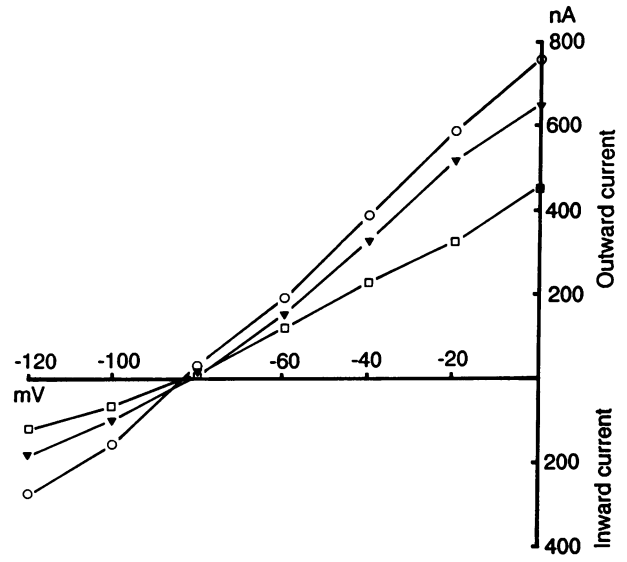

FIG. 4. Current–voltage relationships of the responses generated by hFSH at 1 μg/ml (▼), 1 μM forskolin (□), or an intraoocyte injection of ≈2.0 pmol of cAMP (○) in one follicle bathed in 5 mM KCl/Ringer's solution.

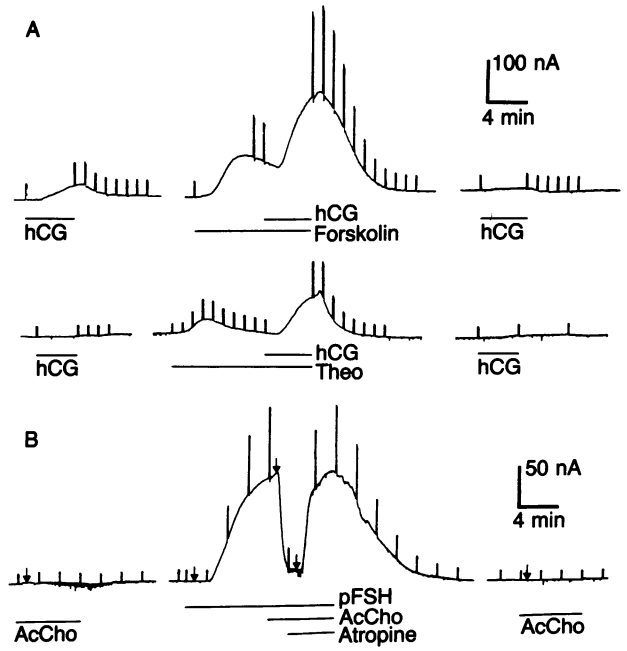

FIG. 5. (*A*) Potentiation of gonadotropin responses after preincubation with either forskolin or theophylline. A single follicle, bathed in 5 mM KCl/Ringer's solution, was voltage clamped at −60 mV and pulsed as in Fig. 1. Intervals of ≈10 min for washes elapsed after the initial exposures to hCG (1 μg/ml). Similarly a 30-min interval for washing elapsed after the record showing the application of 0.5 μM forskolin and 5.0 mM theophylline (Theo). The perfusion dead time in this experiment was about 25 sec. (*B*) Reduction of an established gonadotropin response by 10 μM acetylcholine. The follicle was voltage clamped at −60 mV, and membrane conductance was monitored by command pulses to −50 mV. An initial exposure to 10 μM acetylcholine (AcCho) elicited only a small oscillatory inward current (downward deflections) with little change in basal membrane conductance. The same concentration of acetylcholine was nevertheless able to reduce the K^+ conductance generated by pFSH, an effect that was itself largely reversed by the subsequent introduction of 1 μM atropine. A 20-min interval of washing has been removed from the record following the first exposure to acetylcholine and after washout of the pFSH (5 μg/ml) response. The dead time of the perfusion system is indicated by arrows, and the experiment was performed in 5 mM KCl/Ringer's solution.

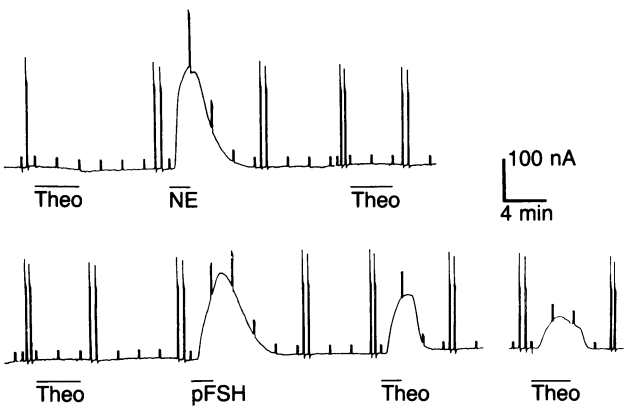

FIG. 6. Residual "activation" effect following exposure to pFSH in a single follicle. The follicle shows no response to 5.0 mM theophylline (Theo) either before or after generating a response to 0.1 mM NE. In contrast, after washing out the response to pFSH (5 μg/ml), reexposure to 5.0 mM theophylline generates a substantial K^+ conductance, an effect that in this case could still be detected after 1 hr of washing (data not shown). The follicle was voltage clamped at −60 mV and periodically pulsed to either −50 mV or 0 mV; command pulses during responses were always to −50 mV. The second exposure to 5.0 mM theophylline has been reproduced twice to facilitate comparisons, and a further 12-min interval of washing has been removed from the record before the fourth exposure to 5.0 mM theophylline.

monitored by its abolition of the Ca^{2+}-dependent transient outward current (21).

Acetylcholine acting through muscarinic receptors potently reduces the K^+ conductance generated by β-adrenergic agonists and adenosine (refs. 14, 22, and 33; and unpublished results). Acetylcholine also reduced the gonadotropin-induced current either upon prior exposure or, as shown in Fig. 5*B*, when acetylcholine was applied during an established gonadotropin response. As reported (2, 23), we observed no currents in response to 1–20 μM progesterone over the first 20 min of exposure nor did the presence of progesterone prevent expression of the gonadotropin response over this period. However, epithelium-covered oocytes that were stimulated to maturity with progesterone *in vitro* lost their gonadotropin sensitivity—an effect also observed with the catecholamine responses (2). *In vitro* maturation studies were carried out on follicles from 18 donors, and follicles from 15 of these donors showed substantial levels of oocyte maturation (60–100%) when exposed to hCG at 1.0–10 μg/ml. Our preparations of FSH had little ability to promote maturation, though in a few cases high concentrations of pFSH (50 μg/ml) did have some activity.

DISCUSSION

Our experiments suggest that in *Xenopus* follicles gonadotropins combine with specific receptors that are not accessible from within the oocyte. This leads to activation of adenylate cyclase and a local increase in cAMP, one consequence of which is the opening of membrane channels permeable to K^+. In anurans, gonadotropins are considered to affect oogenesis and maturation mainly by the control of steroidogenesis in the ovarian soma (6, 24–26), and oocytes divested of their follicular envelope, though sensitive to progesterone, fail to respond to gonadotropins in *in vitro* assays of maturation or amino acid uptake (8, 19, 20, 27).

The precise location, within the follicle, of the gonadotropin receptor, adenylate cyclase, or K^+ channels remains to be determined. However, our experiments show that treatments designed to remove the follicular cell layer subsequently prevent any significant expression of the response. Gap junction contacts between oocyte and follicular cells occur in *Xenopus*, and these contacts allow both the passage of low molecular weight dyes and a degree of electrical coupling (refs. 9, 28, and 29; and unpublished data). Furthermore, adenylate cyclase activity has been reported in follicular tissue when separated from the oocyte (30), and K^+ currents generated by intraoocyte injections of cAMP are substantially reduced following treatment of follicles with collagenase (unpublished results). It therefore seems likely that the gonadotropin response is partly or entirely follicular in origin. In this case the extent to which we are able to monitor this current by voltage clamping the oocyte would depend on the degree of coupling between oocyte and follicular cells. Similarly, agents that modulate the hormonal responses could be acting at the level of the gap junctions.

Due to the uncertainties of working between species, defining the possible physiological significance of the gonadotropin-induced current and the true relative potencies of FSH and LH will depend on the availability of high-purity anuran hormones (31, 32). The different gonadotropin preparations used in this work all generated responses with apparently the same ionic basis and underlying mechanism. However, while hCG was able to mature follicle-enclosed oocytes at concentrations that induced membrane currents, preparations of pFSH, though eliciting similar membrane responses, were consistently less active in inducing maturation (see also ref. 19).

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