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Physiology

A serum factor that activates the phosphatidylinositol phosphate signaling system in *Xenopus* oocytes

(blood serum/chloride channels/inositol 1,4,5-trisphosphate)

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ABSTRACT **Blood sera from many vertebrate species elicit large oscillatory chloride currents in oocytes from the frog *Xenopus laevis*. Rabbit serum was active at dilutions as great as one part in 10 million. Intracellularly applied serum was ineffective, and externally applied serum failed to trigger oscillatory currents when the intracellular level of ionized calcium was prevented from rising by loading the oocyte with EGTA. The serum also caused an increase of inositol 1,4,5-trisphosphate in the oocyte. We conclude that serum contains a factor which activates a membrane receptor that is coupled to the phosphatidylinositol second messenger system. The active factor is a protein with an apparent molecular mass of 60–70 kDa in gel permeation chromatography. Although the normal function of the serum factor is still unknown, it may have far-reaching implications, because it acts on the multifunctional phosphatidylinositol phosphate signaling system. Also, because of its great potency the serum factor and *Xenopus* oocytes are very useful for probing the operation of the phosphatidylinositol system.**

Several years ago, while trying to produce antibodies against neurotransmitter receptors expressed in oocytes, R.M. and C. B. Gundersen noticed that normal rabbit serum triggered an oscillatory chloride current in the membrane of *Xenopus laevis* oocytes (unpublished observation). This was an unwanted complication, but a very interesting one, because the response to serum was analogous to that produced by acetylcholine (AcCho) in native oocytes (1, 2). However, the response to serum could be obtained even in oocytes that did not respond to AcCho.

Xenopus follicles are known to possess many kinds of receptors for neurotransmitters and hormones (1–5), and blood serum contains hundreds of nutrients, hormones, and other factors that are of crucial importance to all cells (6–8). Therefore, we decided to investigate further the effect of serum on *Xenopus* oocytes, because this could allow us to identify the responsible factor and study its mode of action.

MATERIALS AND METHODS

Laboratory-reared *X. laevis* were obtained from Xenopus I (Ann Arbor, MI), Nasco (Fort Atkinson, WI), or Scientific Animals (Newark, NJ), and captured wild *X. laevis* were from Sullivan (Nashville, TN). Follicles containing oocytes at stages V and VI (9) were plucked from the ovaries and then "defolliculated" by treatment with collagenase (type 1, Sigma) at 80–630 units/ml in frog Ringer's solution for 0.5–2 hr to remove the follicular and epithelial cell layers surrounding the oocytes. In some experiments, the enveloping cell layers were manually peeled off, leaving only the vitelline membrane surrounding the oocyte (10). Oocytes were maintained at about 16°C in Barth's solution (11) in the presence of gentamicin (100 μg/ml) and nystatin (50 units/ml), and electrophysiological studies were performed usually during the first week of storage. Membrane currents were recorded with a standard two-electrode voltage clamp (2, 12), while oocytes were continually superfused with Ringer's solution at room temperature (22–25°C). The membrane was usually voltage clamped at −60 mV so as to be away from the equilibrium potentials of Na^+, K^+, Cl^-, and Ca^{2+} (2), and membrane currents were recorded in response to serum diluted in the superfusate.

Intraoocyte injections of serum or ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were achieved by pressure injection from glass micropipettes with 0.1- to 1.0-sec pulse duration at 138 kPa pressure (13). For intracellular injection the serum was dialyzed against 2 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes)/100 mM KCl, pH 7, and passed through a 0.2-μm-pore filter, and EGTA was injected in 50 mM aqueous solution buffered with 10 mM Hepes at pH 7.0. For focal extracellular application, pulses of serum were applied from a micropipette to a small area of the oocyte surface, and for bath application serum dilutions were made up freshly in Ringer's solution from a 10^3 dilution stock prepared every morning. The potency of highly diluted serum (dilutions $>10^4$) was detectably reduced within a few hours. Serum, plasma, and other biological fluids, from single donors or pooled, were either purchased from Sigma or prepared by us. For most of the experiments presented here we used type 100 normal rabbit serum (Diagnostic Biochemistry, San Diego), because it was the most potent of the serum samples tested so far. Serum was dialyzed against 1000-fold excess of frog Ringer's solution at 4°C over 7 days using Spectra Por 1 dialysis membranes (cut-off 6–8 kDa; Medical Industries, Los Angeles) with three daily changes. Human serum (10 μl) diluted 10^2-fold with 10 mM tris(hydroxymethyl)aminomethane·HCl, pH 7.6, was subjected to enzymatic digestion with 1 mg of trypsin (TPCK-treated; Worthington) or neuraminidase (Sigma) at 37°C for 24 hr. The tryptic digestion was terminated by the addition of 1 mg of soybean trypsin inhibitor (Sigma) prior to testing the digest on the oocytes. Trypsin alone elicited oscillatory currents in oocytes, but these currents were abolished by blocking the enzyme with the trypsin inhibitor. The inhibitor itself did not block the serum-induced oscillatory currents.

Quantitative inositol 1,4,5-trisphosphate ($InsP_3$) determinations were performed by using a radioligand-binding assay (Amersham). Three defolliculated oocytes were dropped into an Eppendorf vial containing 250 μl of rabbit serum at various dilutions. Two minutes later the samples were snap frozen in isopentane cooled by liquid nitrogen. To extract $InsP_3$, 60%

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Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; AcCho, acetylcholine; 5HT, serotonin (5-hydroxytryptamine).

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(wt/wt) perchloric acid was added to the frozen vials to a final concentration of 8.6% before they were thawed in a microwave oven (650 W) for 15 sec. The extraction was continued for 10 min on ice with occasional vigorous mixing and stopped by the addition of 750 mM Hepes and 5 M KOH that brought the sample pH back to neutral. Supernatants were harvested after a 5-min centrifugation at 13,000 rpm in an Eppendorf microcentrifuge, and samples were stored under liquid nitrogen until assayed. All other chemicals used in these experiments were obtained from Sigma.

RESULTS

Membrane Currents Elicited by Serum. When *Xenopus* follicles—that is, the oocytes still surrounded by the follicular and epithelial cells—were exposed to rabbit serum diluted 50- to 100-fold little or no oscillatory membrane current was seen. In contrast, when the oocytes were defolliculated large oscillatory currents were elicited even when the serum was greatly diluted (Figs. 1 and 2). In most respects the serum-induced currents resembled those elicited by activation of native muscarinic receptors (2) or by activation of AcCho, serotonin (5-hydroxytryptamine, 5HT), or glutamate receptors expressed in oocytes after injection of rat brain mRNA (14, 15). Like all those currents, the serum-induced current was also due to a movement of chloride ions with an equilibrium potential around −20 mV (Fig. 3*A*). The shape of the response to serum was also similar to that evoked by the various neurotransmitters. For instance, with highly diluted serum the response consisted of a few small oscillations that started a long time, sometimes more than 5 min, after the serum had been applied. But, with higher serum concentrations (10^4 to 10^3 dilution) the onset was more rapid and the response consisted of a large initial peak, followed by a transient secondary current rise superimposed on a slow smooth component (Figs. 1 and 4). In oocytes with high sensitivity, low concentrations of serum (10^4 or higher dilution) could be repeatedly applied without much loss in response, but, with higher concentrations, the oocyte desensitized and many minutes had to elapse before the response recovered fully.

Enzymatic or manually defolliculated oocytes gave similar results and the serum consistently evoked membrane currents. However, the current amplitude varied greatly in different oocytes and, although oocytes from a given donor tended to give similar responses, there was considerable variation among donors. For example, the current generated

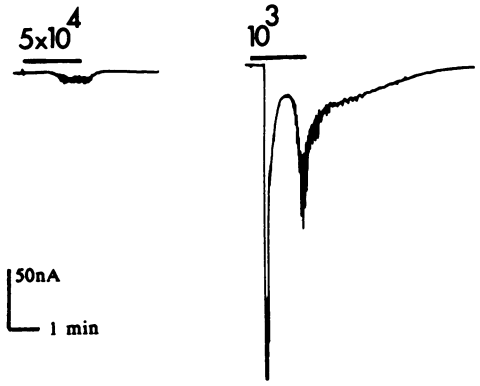

FIG. 1. Membrane currents induced by normal rabbit serum in a defolliculated *Xenopus* oocyte. In this and subsequent figures, serum was applied during the time indicated by the bars (not corrected for dead time) and downward deflections correspond to inward currents. Except for Fig. 3*A*, all records were obtained with the membrane potential clamped at −60 mV. Numbers above the bars indicate the dilution of serum.

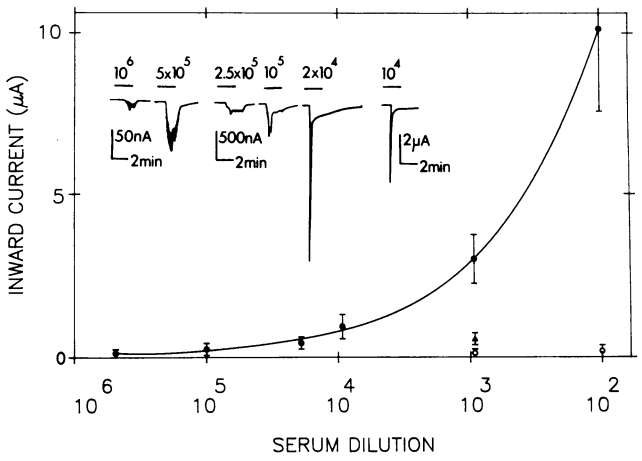

FIG. 2. Dose-response relation for serum-induced currents. ●, Data from oocytes recorded 1 day after defolliculation with collagenase. ▲, Data from oocytes 1 week after defolliculation. ○, Follicles (not treated with collagenase). (*Inset*) Sample records from one oocyte. All the oocytes derived from the same donor and the points show mean ± SEM of three to six oocytes.

by 10^3-fold diluted serum in 13 oocytes from five donors in January 1986 was 389 ± 519 nA (mean ± SD), while in September 1989 26 oocytes from seven different donors gave 2819 ± 2156 nA. The mean amplitude in one of these donors was 6050 ± 756 nA (n = 4), while in another it was 829 ± 405 nA (n = 4).

Dose-Response and Distribution of Serum Sensitivity. The serum concentration required to evoke a detectable current was greatly variable. Some oocytes responded to serum diluted 10^7-fold, while in others the threshold was at about 10^3 dilution. In a given oocyte the amplitude of the response increased as the serum concentration was increased (Fig. 2 *Inset*), and in double-logarithmic plots (not shown) the slope at low concentrations (10^6 to 10^4) was about 1.0, while the overall slope (10^6 to 10^2) was 0.97. The results illustrated in Fig. 2 derive from a single donor and show that in oocytes defolliculated by collagenase treatment serum can elicit currents of more than 10 μA. In contrast the follicles from the same donor (not treated with collagenase) were insensitive to serum. In this donor the serum-induced current was substantially diminished in oocytes examined 1 week after collagenase treatment, but in other cases the currents, although

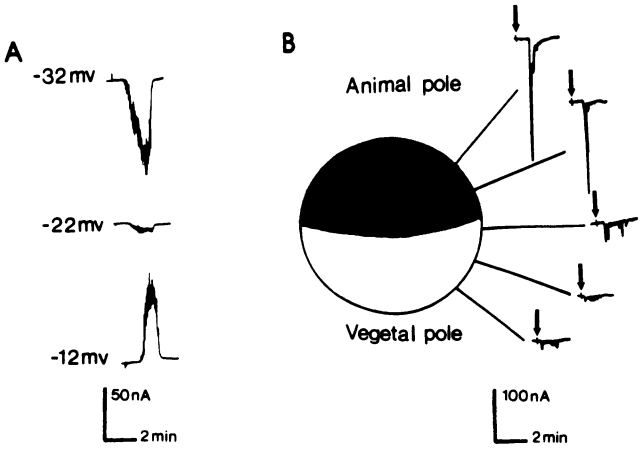

FIG. 3. (*A*) Reversal of the current elicited by serum diluted 5 × 10^3-fold. (*B*) Distribution of serum sensitivity. Membrane currents evoked by pulses of serum applied focally to the surface of an oocyte at the points indicated.

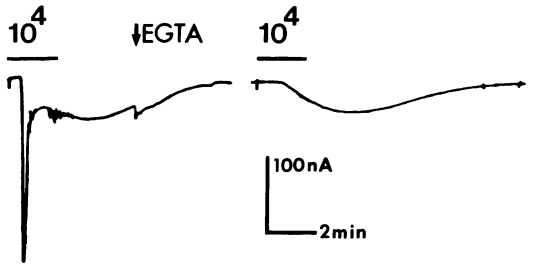

FIG. 4. Dependence of the serum-induced currents on intracellular Ca^{2+}. After serum had been applied an EGTA-filled pipette was inserted into the oocyte (arrow) and EGTA was injected into the oocyte. Several minutes later, the serum failed to produce the initial sharp peak and current oscillations.

reduced, were still quite large in oocytes kept for longer periods.

To determine if the whole oocyte surface was sensitive to serum, brief pulses of serum were applied focally, by pressure ejection from a micropipette. While responses to serum could be obtained all over the oocyte's surface, the currents generated were much larger in the animal hemisphere (Fig. 3*B*). The lower sensitivity was near the vegetal pole, where the currents generated by similar pulses of serum were about 1/15th of those in the animal side. We also noticed that even though the responses in the animal hemisphere were larger their latency—i.e., the time elapsed between serum application and onset of the response—was longer than that of the currents elicited in the vegetal hemisphere (Fig. 3*B*; see also ref. 16). In contrast, when increasing doses of serum were applied in the bath, or focally, the latency of the response decreased as the current amplitude increased (cf. Fig. 1).

No oscillatory currents were generated when pulses of serum were applied intracellularly, in either the animal or vegetal hemispheres. At some spots, where an extracellular pulse of serum evoked a current of hundreds of nanoamperes, doses of serum 5 times larger, applied intracellularly, were without effect. Moreover, the responses reappeared as soon as the micropipette was withdrawn and serum was again released extracellularly onto the oocyte surface.

Ca^{2+} Dependence of the Serum Response. It is known that the oscillatory currents elicited in oocytes by AcCho, 5HT, and other neurotransmitters are carried through Cl^- channels which are opened by a rise in the intracellular level of free Ca^{2+} (12, 17). Those currents were abolished when the intraoocyte level of Ca^{2+} was prevented from rising by previously loading the oocytes with the Ca^{2+}-chelating agent EGTA (18). The oscillatory currents elicited by serum were similarly abolished after loading the oocytes with EGTA. In some cases, there remained a "smooth" Cl^- current, which started later than the original current peak (Fig. 4), but even this smooth current disappeared when more EGTA was injected.

In contrast to its dependence on intracellular Ca^{2+}, the oscillatory current elicited by serum did not require the presence of Ca^{2+} in the extracellular fluid. Large currents were still elicited when Ca^{2+}-free serum was applied to oocytes superfused with a solution in which the free Ca^{2+} was reduced to less than 1 μM by omitting Ca^{2+} and adding 5 mM EGTA and 2 mM Mg^{2+}. The possibility that serum opened oscillatory Ca^{2+} channels is further ruled out because the serum still evoked oscillatory currents when the bathing fluid contained 5 mM Mn^{2+} or 2 mM Cd^{2+} to block Ca^{2+} channels. In contrast, the current underlying the smooth slow component of the serum-induced response is blocked by Mn^{2+} and Cd^{2+} (see also ref. 19).

Serum Activates the Phosphatidylinositol (PtdIns) Second Messenger System. As already mentioned, the serum-induced currents are similar to those elicited by AcCho, 5HT, and glutamate in oocytes injected with brain mRNA (14, 15). The currents induced by those neurotransmitters are all mediated by the same receptor-channel coupling mechanism (20, 21), one in which receptor activation leads to the production of $InsP_3$, which causes the release of Ca^{2+} from intracellular stores, and this Ca^{2+} in turn opens the Cl^- channels (13, 22–24). If the serum factor were acting on a receptor linked to the same receptor-channel coupling system, then one would expect interactions when serum and a neurotransmitter are applied simultaneously to an oocyte in which the neurotransmitter receptors are expressed (4, 20, 21). This is illustrated in Fig. 5, which shows that a subthreshold dose of serum greatly potentiates the action of a subthreshold dose of 5HT, and vice versa. However, the serum factor and 5HT act on different receptors, because the potentiating effect and the responses to 5HT were blocked by methysergide, a 5HT receptor antagonist, which had no effect on the serum response.

To determine directly if serum action involved PtdIns phosphate hydrolysis, we measured the content of $InsP_3$ in oocytes exposed to various concentrations of serum. The radioligand assay system that we used gave variable results. A similar variability was found by others (L. D. Smith, personal communication). Nonetheless, a large increase in $InsP_3$ content was seen with serum diluted 10^4-fold, and an increase was detected even when the serum was diluted 10^5 times (Fig. 6). We also measured the membrane current elicited by serum in the same batch of oocytes, and these results indicated that there is no linear correlation between $InsP_3$ content and serum-induced Cl^- current. This would be expected if the serum response, like that to neurotransmitters, includes some nonlinear steps and feedbacks in the receptor-channel coupling mechanism.

Animal Species Distribution and Preliminary Characterization of the Serum Factor. To see if the serum factor is present in animals other than rabbits, we tested serum samples from 18 different species. All were able to evoke oscillatory Cl^- currents in *Xenopus* oocytes. However, their potency varied appreciably, and Fig. 7 illustrates responses to nine sera arranged according to their potency in one particular oocyte. It is interesting that sera obtained from *Xenopus* donors elicited Cl^- currents in their own oocytes.

In addition to blood serum, a number of other biological fluids were found to evoke oscillatory activity. These included mouse hybridoma ascites fluid, human and rat cerebrospinal fluid, and human amniotic fluid. However, human saliva, urine, and milk, as well as cows' milk, failed to elicit oscillatory currents at dilutions of 10^1 to 10^4, in oocytes that responded to 5×10^4 diluted rabbit serum.

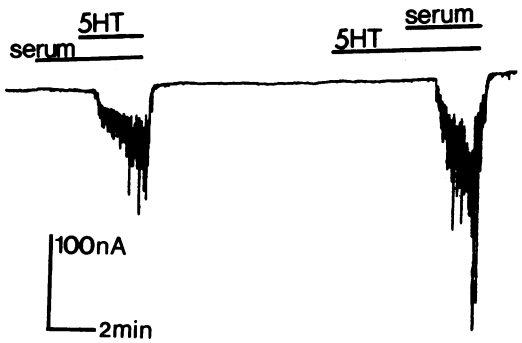

FIG. 5. Interactions between serum and 5HT. A near-threshold concentration of serum (10^5 dilution) was applied to an oocyte, first alone and then together with a subthreshold concentration of 5HT (10^{-9} M). After a few minutes, the order was reversed.

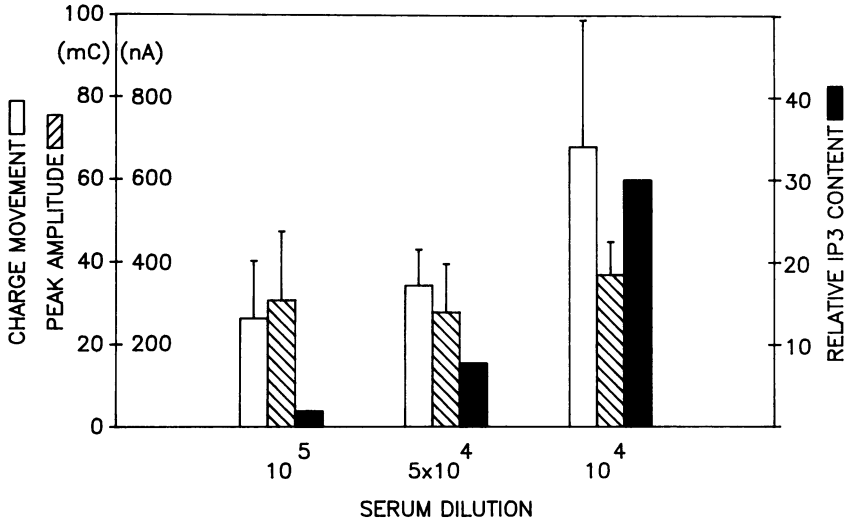

FIG. 6. InsP_3 content and Cl^- currents in oocytes exposed to serum. Open bars show the net charge movement in millicoulombs (mean ± SD, $n = 5$) elicited by sequential application of increasing serum dilutions (10^5 to 10^4). Hatched bars show the peak current amplitudes (mean ± SD). Filled bars represent the increase in InsP_3 (IP3) content over the resting level in oocytes derived from the same donor.

We have partially purified the factor responsible for the activity in rabbit and human sera. Details of the purification will be presented elsewhere, but a few points are pertinent here. The active factor was nondialyzable (>8 kDa) and was sensitive to trypsin: a 1-hr digestion abolished its activity. In contrast, longer treatment with neuraminidase (4 hr at 37°C) did not alter the activity. To exclude the possibility that the effect was due to proteolytic activity present in serum, a number of protease inhibitors were added to serum. Soybean trypsin inhibitor (1 mg/ml), leupeptin (10 μg/ml), and aprotinin (2.2 units/ml) when added to serum did not significantly alter its potency to evoke oscillatory currents. Moreover, serum was also treated with phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate, two potent enzyme inhibitors (100 mM), and its potency in evoking the currents was maintained. The sensitivity to trypsin and other proteases (unpublished data) indicates that the active factor is probably a protein; nevertheless, the factor was very resistant to a number of denaturing conditions, such as 8 M urea, 6 M guanidinium thiocyanate, pH 2–11, and even 1% sodium dodecyl sulfate. To exclude the possibility that the factor is a protein-bound lipid, serum was extracted with organic solvents, including methanol, ethanol, chloroform, and diethyl ether, without markedly reducing its potency to evoke oscillatory currents. The factor was moderately heat resistant: a 30-min boiling water bath treatment reduced, but did not abolish, the activity. Finally, when serum was fractionated by gel permeation chromatography the activity was eluted under the major albumin peak, with an apparent molecular mass of 60–70 kDa.

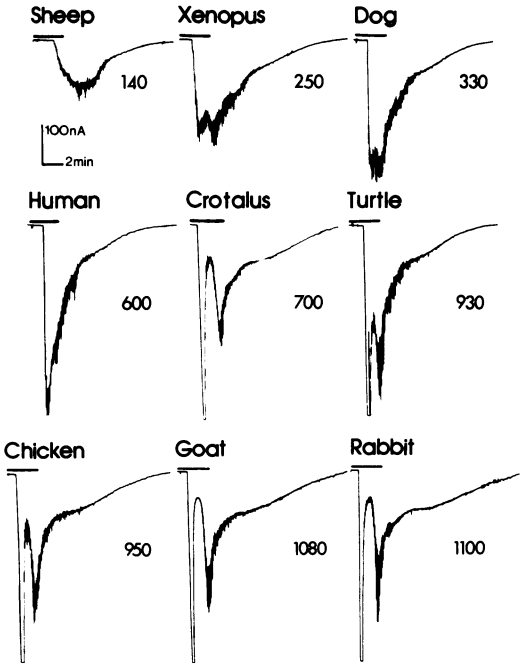

FIG. 7. Membrane currents evoked by sera from different animal species. All sera were diluted 10^3-fold and tested on a single oocyte. The numbers indicate the peak amplitudes of the responses (in nA), sometimes truncated in the records. *Crotalus viridis*, rattlesnake; turtle was *Chrysemys scripta*.

DISCUSSION

The central finding in this work is that blood sera from many different vertebrate animals contain a factor that is very potent in activating the PtdIns phosphate intracellular signaling system and triggering oscillatory Cl^- currents in *X. laevis* oocytes. The factor is a protein that is an isoform of albumin, or a similar macromolecule (G.T. and R.M., unpublished data).

In *Xenopus* oocytes, various neurotransmitters and divalent cations also elicit Cl^- currents, which are mediated by the same PtdIns phosphate receptor-channel coupling system (21, 25). However, the serum factor probably acts on special receptors, because it elicits currents in oocytes that do not respond to the other agents. Thus, it seems that the oocyte plasma membrane has a receptor which is activated by the serum factor and which is also linked to the PtdIns phosphate messenger system. The serum factor receptors are activated when the serum is externally applied and, similar to neurotransmitter receptors (26), they fail to be activated by intracellular applications. However, it is still possible that there are also intracellular serum factor receptors, which could have gone undetected because they are not coupled to the Cl^- channel gating mechanism.

The current elicited by serum has three main components: a large initial peak, subsequent oscillatory currents, and a slow smooth component. Furthermore, the serum, like 5HT and other neurotransmitters, induces a membrane state in

which hyperpolarization leads to a transient inward Cl^- current consequent to an influx of Ca^{2+} through Ca^{2+} channels (27–29). The currents elicited by serum are all due to the opening of Ca^{2+}-dependent Cl^- channels, and are all abolished by chelating intracellular Ca^{2+} with EGTA. However, the smooth current component appears to be more sensitive to Ca^{2+}, since it remains after the oscillations have been abolished by increasing the intracellular Ca^{2+} buffering with EGTA. These observations may be explained in several ways. For example, the oocyte membrane may have multiple types of Ca^{2+}-dependent Cl^- channels with different affinities for Ca^{2+} (30), or the external Ca^{2+} responsible for the smooth current may enter very close to the Cl^- channels, or even through the Cl^- channels themselves (31), reducing the efficiency of Ca^{2+} sequestration by EGTA.

Functional serum receptors occur all over the oocyte surface, but, since the size and latency of the current response differed in the animal and vegetal hemispheres (see also refs. 16 and 32), it seems likely that the receptors are not uniformly distributed. However, it should be noted that even a uniform distribution of serum receptors could lead to different regions giving responses of different sizes, because the sensitivity to intracellular Ca^{2+} and to $InsP_3$ is higher in the animal hemisphere (16, 17, 32).

Another question raised by our experiments concerns the comparative inability of serum to evoke oscillatory currents in follicles, in contrast to its great potency in defolliculated oocytes. Since the serum factor is a macromolecule, the enveloping epithelial and follicular cells might be expected to provide a barrier to its diffusion to the receptors located in the oocyte plasma membrane. However, this does not seem to be the full explanation, because the epithelial cell envelope was open at the point where the follicle had been attached to the ovary, and the follicular cells do not completely cover the oocyte (10, 33). Thus, it is probable that defolliculation of the oocyte, either manually or by collagenase treatment, leads to a greatly increased sensitivity to the serum factor. This could occur in various ways. For example, it could be that the serum acts on proteins associated with the follicular cell–oocyte gap junctions (34, 35), and that those proteins become more accessible, or activated, when the follicular cells are removed. Further work, including experiments with labeled serum factor and single-channel recordings, will be necessary to provide answers to some of the questions posed above.

The serum factor that we describe is a protein that seems to be present in all vertebrate species. We presume it may play many important roles in animal life because it activates the multifunctional PtdIns phosphate intracellular signaling system (22, 36–38). We already know that the serum factor retards the multiplication of malignant cells and causes neurite retraction in pheochromocytoma cells (39), and it seems likely that it may also be involved in other processes.

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