Calcium wave evoked by activation of endogenous or exogenously expressed receptors in *Xenopus* oocytes

(substance K receptors/calcium imaging/fura-2 fluorescence microscopy/acetylcholine receptors/angiotensin II receptors)

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ABSTRACT The mRNA encoding the cloned substance K receptor was microinjected into *Xenopus laevis* oocytes. After expression of the mRNA, Ca^{2+} was imaged in the oocytes with a digital imaging fluorescence microscopy system using the Ca^{2+} -sensitive dyes fura-2 and fluo-3. Application of substance K caused a dose-related wave of Ca^{2+} mobilization to spread from a focus and to elevate the Ca^{2+} concentration in the oocyte. Activation of endogenous muscarinic or angiotensin II receptors in noninjected oocytes evoked a similar response. The Ca^{2+} rise in oocytes induced by substance K was due to internal Ca^{2+} mobilization and was independent of external Ca^{2+} , since it occurred in Ca^{2+} -free medium fortified with 2 mM EGTA. The Ca^{2+} imaging was well correlated with ion current measurements of voltage-clamped oocytes. Imaging, in addition to detecting the spatial spread of Ca^{2+} across the cell, was at least as sensitive as voltage clamping and much faster when screening oocytes for the expression of receptor mRNAs that stimulate Ca^{2+} mobilization. While it is known that fertilization of *Xenopus* eggs causes a spreading wave of Ca^{2+} mobilization, we found that activation of either native or newly expressed receptors in oocytes causes a similar change in Ca^{2+} distribution.

Oocytes and eggs from *Xenopus laevis* have been extensively used in biological research both for developmental studies and for efficient mRNA translation and posttranslational processing of receptors and ion channels (1–3). When the translated mRNA produces a receptor or ion channel, it is usually biochemically functional, exhibiting the appropriate pharmacological and electrophysiological properties. A number of recent studies have dealt with *de novo* expression of neurotransmitter receptors, as detected by electrophysiological and biochemical methods (2–5), making the oocyte an excellent system to detect newly expressed ion channels or ion channel-linked receptors in cloning strategies.

The oocyte has a limited number of endogenous receptors. Stimulation of the endogenous muscarinic receptor, for example, results in chloride ion-mediated inward current and subsequent oscillations in membrane current. It is believed that these depolarizing chloride currents reflect oscillatory release of intracellular sequestered Ca^{2+} by inositol trisphosphate generated by agonist-induced inositol phospholipid hydrolysis (6). We now present direct evidence that stimulation of several endogenous receptors as well as a *de novo*-expressed receptor results in mobilization of intracellular Ca^{2+} , which is initially focal and eventually spreads across most of the cell.

Although Ca^{2+} is a well-established intracellular messenger, there are many questions concerning the kinetics and spatial localization of its release and effects. Such problems are now being approached by use of fluorescent indicators for

Ca^{2+} . In the present investigation, fura-2 (7) and the newly developed long-wavelength indicator fluo-3 (8) were used.

MATERIALS AND METHODS

Oocyte mRNA Microinjection. Adult *X. laevis* females were obtained from Carolina Biological (Burlington, NC), Xenopus I (Ann Arbor, MI), and Nasco (Fort Atkinson, WI). Oocytes were obtained by laparotomy at ice temperature. The oocytes were manually separated from the follicle and kept in modified Barth's solution [0.82 mM MgSO_4 /0.41 mM CaCl_2 /1 mM KCl/0.33 mM $\text{Ca}(\text{NO}_3)_2$ /88 mM NaCl/2.4 mM NaHCO_3 /10 mM Hepes/50 units of penicillin per ml/50 μg of streptomycin per ml/2.5 mM sodium pyruvate, pH 7.5] at 16°C–17°C. Injection and imaging were done at room temperature, which varied between 19°C and 22°C. Oocytes were injected with *in vitro*-transcribed mRNA encoding the cloned substance K receptor. The substance K receptor cDNA with its poly(dT-dA) tail and multiple restriction sites (9) was transferred to the plasmid pGEM 1 DNA (Promega Biotec); after plasmid linearization, mRNA was transcribed by T7 RNA polymerase in the presence of capping nucleotide. The mRNA (1–100 ng/ μl) was microinjected into stage V oocytes. The injection volume was 34 nl, delivered by a computerized *Xenopus* oocyte microinjection system with an air-actuated injection pipette (Atto Instruments, Potomac, MD). After 1–4 days, the oocytes were microinjected as described above with 34 nl of a solution (1 mM, pH 7.4) of either the sodium salt of fura-2 or fluo-3 (Molecular Probes) 1–3 hr before imaging. Substance K was from Peninsula Laboratories and other chemicals were from Sigma.

Electrophysiology. For electrophysiological recordings, oocytes were placed in a 14- μl Teflon bath and perfused with amphibian Ringer's solution (116 mM NaCl/2 mM KCl/1.8 mM CaCl_2 /5 mM Tris-HCl, pH 7.5); drugs were dissolved and applied in this medium. Cells were voltage-clamped at a holding potential of –50 mV with two electrodes filled with 3 M KCl (2–3 M Ω) using a W-P Instruments (New Haven, CT) M-707 microprobe system and S-7050 voltage patch module.

Fluorescence Imaging. Oocytes that had been injected 1–3 days before with either vehicle or the *in vitro*-transcribed mRNA corresponding to the cloned substance K receptor were then injected with 34 nl of either 1 mM fura-2 or fluo-3 1–3 hr before imaging. Oocytes were imaged using an Atto-fluor digital microscopy system (Atto Instruments). The system consisted of a Zeiss ICM-405 inverted epifluorescence microscope with all quartz optics. A 100 W mercury burner served as the source for excitation. Fura-2 was excited at two wavelength pairs of either 340 nm or 380 nm for experiments in Figs. 2 and 3 or at 334 nm and 390 nm for experiments shown in Figs. 4 and 7 A and C using 10-nm bandpass interference filters, which were alternately selected by the computer-controlled excitation and shutter control unit. A 485-nm interference filter with a 20-nm bandpass

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(Zeiss) was used for fluo-3 excitation. Zeiss dichromatic beam splitters were used for fura-2 (FT-395) and fluo-3 (FT-510). Emission of both dyes was monitored with an intensified CCD camera whose sensitivity was set for each wavelength and then switched to that sensitivity by the computer just before each wavelength of excitation was selected. A 510-nm long-pass emission filter was used to select emission fluorescence >500 nm. The video signals were digitized to 8-bit resolution (256 shades of gray; 512×512 pixels per frame) in real time, and in most cases images were captured after a 60-msec shutter opening of the excitation source and one 30-msec video frame. In some cases five video frames were averaged. In addition to automatic capture and disk storage of the 340-nm and 380-nm image pairs and/or ratioed images (in the case of fura-2), the system also continuously calculated and graphically displayed the

mean intensity of a 64-pixel box area (or larger) of the oocyte image. The outline of the box is seen on the images. In some experiments with fura-2, the instrument was operated in the calibrated mode, in which background subtraction and correlation to Ca^{2+} standards were performed in real time, such that Ca^{2+} concentration within the selected box area was displayed in graphical form versus time (see ref. 10 for equation). A data file of the pixel intensities and Ca^{2+} concentration was saved by the computer. The oocytes were viewed through no. 1 glass coverslip dishes or glass six-well slides in the case of fura-2 or in 96-well plastic multiwell dishes for imaging with fluo-3. Drugs, dissolved in modified Barth's solution, were applied while imaging each oocyte. The volume was sufficient to completely bathe the oocyte and achieve the desired concentration immediately upon addition. A Zeiss 6.3 power Neofluor (numerical aperture,

FIG. 1. Substance K-triggered Ca^{2+} wave in a *Xenopus* oocyte previously injected with substance K receptor mRNA. Time sequence images of fluo-3 fluorescence after challenge with $1 \mu\text{M}$ substance K. The oocyte was previously injected with substance K receptor mRNA and fluo-3 as described. Time after addition of substance K was as follows (left to right): top row, 43, 67, 86 sec; middle row, 121, 149, 220 sec; bottom row, 289, 320, 394 sec. Color scale at bottom depicts pixel intensity of 0 (pink) to 255 (red). Basal background fluorescence of the oocyte was 25% of peak fluorescence and was subtracted from each image.

0.2) objective was used. Images presented are of a single oocyte per figure. Some of the experiments are not presented in a statistical format; however, they have been repeated at least 10 times over a period of 10 months. Statistical results are reported as the mean $\pm$ SEM.

RESULTS

Application of substance K to oocytes previously injected with substance K receptor mRNA resulted in elevated Ca^{2+} concentration as detected by either fluo-3 imaging, as shown in Fig. 1, or fura-2 imaging, as shown in Fig. 2. Ca^{2+} mobilization was initiated from a focus in or near the animal pole of the oocyte. Subsequently, a wave of increased free Ca^{2+} spread to involve most of the oocyte. When Ca^{2+} was imaged with fluo-3, there was an increase in emission fluorescence at wavelengths >510 nm. Ca^{2+} imaging with fura-2 at emission >510 nm with excitation at 340 nm and 380 nm resulted in an increase in the 340 nm/380 nm ratio. This was a result of an increase in fluorescence at 340 nm and a decrease at 380 nm. There never was a response to substance K in uninjected or in vehicle-injected oocytes when measured with either fura-2 or fluo-3.

The rate of spread varied greatly between oocytes. In some cases, the response started in seconds and, in others, there was a delay of several minutes before uniformly elevated Ca^{2+} concentration was observed. While it was found that fluo-3 was a more sensitive Ca^{2+} indicator than fura-2, the latter dye was used to calculate the Ca^{2+} concentration. Thus, by use of fura-2 and its calibration with Ca-EGTA standards, we found that the basal Ca^{2+} concentration in oocytes varied between 50 and 90 nM as shown in Figs. 3 and 4. This is similar to the resting level of free Ca^{2+} in oocytes determined by the photoprotein aequorin (11). Application of 1 μM substance K caused Ca^{2+} to increase to 225 nM as shown in Fig. 3. Even though there was some variation in the basal concentration of Ca^{2+} between oocytes, the dose-related mobilization of intracellular free Ca^{2+} could be demonstrated by using fura-2 as shown in Fig. 4. It can be seen that the rate and maximal Ca^{2+} response is related to the concentration of substance K applied, with 1 μM substance K causing a 4- to 5-fold increase in intracellular Ca^{2+} after 2-3 min. Doses of substance K as low as 1 nM caused significant but small changes when monitored with fura-2. As shown in Fig. 4, the control free Ca^{2+} was 84.8 ± 1.66 nM and the level between 125 and 145 sec after 1 nM substance K was 92.3 ± 0.76 nM, a small but detectable change significant at $P < 0.002$. Fluo-3 was able to detect presumably even smaller

FIG. 2. Fura-2 ratio images (340-nm image/380-nm image) of a substance K receptor mRNA-injected *Xenopus* oocyte stimulated with substance K as in Fig. 1. (Upper Left) Nine sec; (Upper Right) 30 sec; (Lower Left) 79 sec; (Lower Right) 120 sec after substance K.

FIG. 3. Analysis of time course of Ca^{2+} concentration change in the oocyte shown in Fig. 2. The 64-pixel area shown by the red box in Fig. 2 was continuously monitored and the average pixel intensity values at 340 nm, 380 nm, and the 340 nm/380 nm ratio is shown. The right scale is the corresponding Ca^{2+} concentrations determined from Ca-EGTA-fura-2 standards.

changes in Ca^{2+} mobilization, since detectable responses were observed with doses of substance K as low as 0.1 nM in some oocytes as shown in Fig. 5B.

In parallel with the Ca^{2+} imaging, we voltage-clamped the oocytes; the results obtained were consistent with the imaging data in that all the above agonists evoked responses that are believed to depend on inositol trisphosphate-mediated release of intracellular Ca^{2+} , which in turn activates chloride channels (2, 4-6). The number of agonist-responsive oocytes detected electrophysiologically was similar to that found by imaging. As shown in Fig. 5A, in the same group of oocytes that were used to obtain the dose-response curves to substance K in Fig. 4, there were detectable current changes to doses of substance K as low as 1 nM with much larger currents at doses of 10 nM and higher. A comparison was made of the responsiveness between voltage clamping and fura-2 detectable Ca^{2+} changes. Oocytes were tested in groups of five by electrophysiology and imaging and both gave responses at doses of 0.01, 0.1, and 1 μM substance K. By imaging with fluo-3, 5/5 responded at 1 nM and 2/5 responded at 0.1 nM. With voltage clamping, 4/5 responded at 1 nM and 0/5 responded at 0.1 nM. Similar electrophys-

FIG. 4. Dose and time response to substance K in substance K receptor mRNA-injected oocytes stimulated with 1 nM (-9), 10 nM (-8), 100 nM (-7), and 1 μM (-6) substance K. Ca^{2+} was determined by fura-2 imaging as described. Ca^{2+} concentration was determined in the fura-2 calibration mode of the instrument.

FIG. 5. (A) Electrophysiological response to 0.1, 1, and 10 nM substance K in substance K receptor mRNA-injected oocytes. (B) Fluo-3 imaging response to 0.1 nM substance K in a substance K receptor mRNA-injected oocyte. The ordinate is the mean pixel intensity of a 15-pixel square box positioned on the oocyte. This graph was automatically scaled to this small range of 10 intensity units because of the small change observed during the experiment. The maximal intensity range for the system is 256 intensity units.

iological and imaging responses were also observed in uninjected oocytes stimulated with agonists for endogenous receptors that are coupled to inositol phospholipid hydrolysis with mobilization of intracellular Ca^{2+} (11). In noninjected oocytes from some toad oocyte donors, 100 μM carbachol (Fig. 6) and 0.1 μM angiotensin II (Fig. 7A) induced responses similar to that observed with substance K receptor mRNA-injected oocytes stimulated by substance K.

Thus, several agonists that interacted with both endogenous and exogenous receptors appeared to elicit an identical type of response. Because the response to substance K was largest and most reproducible, substance K receptor mRNA-injected oocytes were studied to further characterize the response. Fig. 7B illustrates the increase in intracellular free Ca^{2+} after addition of substance K as studied in near real time (20 images per sec) in a fluo-3-injected oocyte. The increase in Ca^{2+} concentration was rapid but was not accompanied by oscillations in the time frame studied. The source of increased Ca^{2+} observed after addition of substance K was investigated by stimulating substance K receptor mRNA-injected oocytes with 1 μM substance K in the presence of

FIG. 6. Carbachol-triggered Ca^{2+} wave in a noninjected *Xenopus* oocyte. The oocyte was injected with fluo-3 before imaging. Times after 100 μM carbachol are as follows: (Upper Left) 9 sec; (Upper Right) 18 sec; (Lower Left) 29 sec; (Lower Right) 63 sec.

modified Barth's solution in which the CaCl_2 and $\text{Ca}(\text{NO}_3)_2$ was replaced with 2 mM EGTA to effectively reduce extracellular Ca^{2+} to <10 nM. As shown in Fig. 7C, the response to 1 μM substance K remained in the absence of extracellular Ca^{2+} . In six oocytes tested in the presence of EGTA, Ca^{2+} determined with fura-2 imaging increased from a basal value of 50.8 ± 3.58 nM to 153 ± 22.7 nM after stimulation by substance K. However, intracellular injection of either EGTA, fluo-3, or fura-2 to a calculated intra-oocyte concentration of 0.5 mM abolished the increase in fluorescence stimulated by substance K (data not shown).

DISCUSSION

The present study shows that the activation of either neurotransmitter or hormone receptors in *Xenopus* oocytes causes a focal intracellular Ca^{2+} release, which is followed by a spreading Ca^{2+} wave. Since it has been shown that acetylcholine and serotonin, acting via the inositol phospholipid-coupled M1 and 1C receptor, respectively, can induce fertilization events in *Xenopus* eggs (12), it is likely that Ca^{2+} mobilization underlies such events. In the oocyte, however, the function of Ca^{2+} mobilizing receptors remains unclear. The maturation of the oocyte induced by progesterone is reported not to depend on an increase in cytosolic Ca^{2+} (11), and thus receptor-induced oocyte maturation may have a different pathway(s) for signal initiation.

The question of oscillatory Ca^{2+} signals and their relationship to the oscillatory chloride currents seen upon receptor activation in oocytes remains an open question. In some of our imaging experiments, the Ca^{2+} concentration has an oscillatory behavior. However, this is not seen in all oocytes examined. Furthermore, the time period of chloride current oscillation is sufficiently slow (≈ 5 cycles per min) and thus the Ca^{2+} oscillation should be easily detected by fluo-3 imaging in which Ca^{2+} imaging at 20 data points per sec has been performed, as shown in Fig. 7B. However, no significant oscillation was seen. This is in contrast to rapid oscillations observed in muscle (13) and other cells (14). Because there are also periods of electrophysiological quiescence, it will be necessary to simultaneously monitor the electrical and optical activity of individual oocytes to clarify the relationship between Ca^{2+} and chloride oscillations.

It is quite clear from our experiments with medium containing EGTA, which effectively chelates free extracellular Ca^{2+} to <10 nM, that the response to substance K in oocytes injected with substance K receptor mRNA does not require

FIG. 7. (A) Time course of intracellular Ca^{2+} concentration response to $0.1 \mu\text{M}$ angiotensin II in a noninjected *Xenopus* oocyte. Ca^{2+} concentration was determined in the fura-2-calibrated mode of the instrument. (B) Time course of Ca^{2+} change as measured by fluo-3 fluorescence in a substance K receptor mRNA-injected oocyte after application of $1 \mu\text{M}$ substance K. The ordinate is the mean pixel intensity (0–255 scale) of a 64-pixel box positioned on the oocyte. Substance K was applied at 12 sec. (C) Time course of $1 \mu\text{M}$ substance K-stimulated Ca^{2+} mobilization in substance K receptor mRNA-injected oocytes in the presence of 2 mM EGTA. Ca^{2+} concentration was determined in the fura-2-calibrated mode of the instrument.

extracellular Ca^{2+} . Therefore, we conclude that the imaged changes in oocyte Ca^{2+} concentration after receptor activation mainly reflect the mobilization of intracellular Ca^{2+} —most likely through activation of the inositol phospholipid signal transduction pathway.

Whatever the ultimate function of inositol phospholipid-coupled receptors in the oocyte, their *de novo* expression has made the cloning of the substance K (9) and serotonin 1C (15) receptors possible. It is to be expected that a number of other receptors will be cloned by the use of cDNA expression vectors. Screening of oocytes or transfected cells for *de novo*-expressed receptors could be greatly facilitated by Ca^{2+} imaging of oocytes. The imaging method is sensitive and specific since we have validated it with two different Ca^{2+} -sensitive dyes, fura-2 and fluo-3. The method is quite rapid so that hundreds of oocytes can be screened per day. This is in contrast to laborious electrophysiological methods, which require a long equilibration period for each oocyte. Recently, a change in Ca^{2+} after translation of heterologous mRNA has been detected by measuring aequorin-mediated light emission in albino oocytes (16); however, these investigators did not perform spatial imaging of the Ca^{2+} signal as we have done with fura-2 and fluo-3. It is possible that the present methodology can be used to detect eukaryotic cells transfected with genes that either directly or indirectly generate a Ca^{2+} signal. Fluo-3 may be more advantageous than fura-2, because fluo-3-loaded cells can be imaged by using excitation energy in the visible region of the spectrum, making screening in any kind of plastic or other ultraviolet light opaque dishes possible. This could be especially important if transfected cells in cultures are to be screened. In addition, because fluo-3 is a more sensitive probe than fura-2 (7), smaller changes in Ca^{2+} should be more readily detected with less sophisticated equipment. In fact, our experiments demonstrated that fluo-3 could detect smaller changes in Ca^{2+} than fura-2. However, for quantitative free intracellular Ca^{2+} measurements, fura-2 is preferable because the ratio method renders the determination independent of differences in dye distribution within the cell.

Several important and interesting questions are brought to light by the present studies. Why does receptor activation lead to a focal and delayed spread of Ca^{2+} across the oocyte? Is there some mechanism that prevents further foci of initiation once the spread starts from one point? Why does it start from a specific part of the cell? Clearly the sequence of activation is a most fundamental process, probably occurring in other cell types, which are, however, more difficult to detect and to study. There is an opportunity to investigate the mechanism for localized control of intracellular Ca^{2+} concentration as a result of activating endogenous or exogenously expressed receptors in the *Xenopus* oocyte.

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