Tyrosine Kinase Inhibitors Block Sperm-Induced Egg Activation in Xenopus laevis

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Fertilization of Xenopus laevis eggs triggers a wave of increased \([Ca^{2+}]_{\text{i}}\). The exact signal transduction pathway culminating in this \([Ca^{2+}]_{\text{i}}\) wave remains unknown. To determine whether increases in tyrosine kinase activity are part of this pathway, we microinjected tyrosine kinase inhibitors into unfertilized eggs. Upon fertilization, signs of activation were monitored, such as fertilization envelope liftoff and the \([Ca^{2+}]_{\text{i}}\) wave (for eggs microinjected with lavendustin A). Various concentrations of lavendustin A and tyrphostin B46 were microinjected, as well as inactive forms of these compounds (lavendustin B and tyrphostin A1) to provide negative controls. Peptide A, a 20-amino-acid peptide derived from the SH2 region of pp60\(^{v-src}\) tyrosine kinase, was also microinjected. Peptide A inhibits tyrosine kinase activity but not PKA or PKG activity. Dose-response curves for lavendustin A, tyrphostin B46, and peptide A show clear inhibition of vitelline envelope liftoff by these three compounds. Confocal imaging of eggs coinjected with lavendustin A and Oregon Green–dextran showed that the \([Ca^{2+}]_{\text{i}}\) wave was inhibited under normal insemination conditions but that the block of the \([Ca^{2+}]_{\text{i}}\) wave could be overcome with very high sperm densities. A phenomenon of small local \([Ca^{2+}]_{\text{i}}\) increases termed “hot spots” seen in lavendustin A containing eggs is also described. Since this inhibition of egg activation by tyrosine kinase inhibitors can be overcome by \([Ca^{2+}]_{\text{i}}\) microinjection, the inhibitors must act on a step in the signal transduction cascade that is upstream of the \([Ca^{2+}]_{\text{i}}\) Wave. © 1999 Academic Press

INTRODUCTION

The early events of fertilization in Xenopus have been studied extensively and include rapid depolarization of the egg membrane (the fertilization potential), a wave of increased \([Ca^{2+}]_{\text{i}}\), cortical granule exocytosis, vitelline envelope (VE) liftoff, and cortical contraction (see Nuccitelli, 1991, for review). The underlying mechanisms generating these interesting phenomena have been the focus of much study but remain incompletely understood. Here we study the signal pathway used by the sperm to initiate the \([Ca^{2+}]_{\text{i}}\) wave that traverses the egg at fertilization. The \([Ca^{2+}]_{\text{i}}\) wave triggers the wave of cortical granule exocytosis resulting in vitelline envelope liftoff, as well as the release of cell cycle arrest (Kline, 1988). In several species the activity of tyrosine kinase-linked pathways changes dramatically during fertilization (Kinsey, 1997). Since activation of tyrosine kinases (especially the Src-related tyrosine kinases, see Thomas and Brugge, 1997 for review) can result in cell proliferation and since fertilization changes tyrosine kinase activity, we chose to determine whether this pathway is physiologically important in the activation of Xenopus eggs.

The majority of evidence indicating a substantive role for tyrosine kinase activity in egg activation comes from experiments with sea urchin gametes (Kinsey, 1997). Plasma membrane-associated tyrosine kinase activity is increased after fertilization (Ribot et al., 1984), and specific proteins increase their phosphotyrosine content, sometimes rapidly (Moore and Kinsey, 1995; Kinsey, 1995; Ribot et al., 1984; Peaucellier et al., 1988). Increased tyrosine kinase activity can occur within 1 min of fertilization (Abassi and Foltz, 1994), and specific proteins increase their phosphotyrosine content, sometimes rapidly (Moore and Kinsey, 1995; Kinsey, 1995; Ribot et al., 1984; Peaucellier et al., 1988). Increased tyrosine kinase activity can occur within 1 min of fertilization (Abassi and Foltz, 1994; Peaucellier et al., 1988; Ciapa and Epel, 1991), and these increases can be duplicated by application of \(Ca^{2+}\) ionophore (Kinsey 1984). The Src-related tyrosine kinases such as Fyn, and Abl, have been specifically localized in sea urchin eggs (Kinsey, 1996; Moore and Kinsey, 1994; Walker et al., 1996). In sea urchin eggs, overall increases in tyrosine kinase activity (and phosphorylation of a 350-kDa protein) can be inhibited with genistein (a well-known tyrosine kinase inhibitor) but not erbstatin or tyrphostin B42, and

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this treatment can cause polyspermy (Moore and Kinsey, 1995). In sea urchins, tyrosine kinase inhibition primarily interferes with later developmental events such as gastrulation rather than earlier events such as fertilization envelope liftoff (Moore and Kinsey, 1995; Livingston et al., 1998). In many of these studies tyrosine kinase inhibitors (having unknown permeabilities) were applied to the outside of the egg making the actual intracellular concentration uncertain.

In Xenopus, the src family of nonreceptor tyrosine kinases has been implicated in early fertilization events (p57, Steele, 1989b; Sato, et al., 1996). Src-like protein tyrosine kinases such as c-src (Shartl and Branechow, 1984), Fyn (Steel and al., 1990), and c-Yes (Steele et al., 1989a), have been cloned from Xenopus oocytes. The p57 src-like tyrosine kinase activity isolated from the particulate fraction of Xenopus laevis oocytes (Sato et al., 1996) of p57 is low in unfertilized eggs but increases dramatically upon fertilization (Sato et al., 1996). In addition, approximately 10% of p57 enzyme activity translocates from membrane to cytosol and the phosphotyrosine content of p57 increases within 3–5 min postinsemination suggesting that p57 activity has an important role in fertilization (Sato et al., 1996). This value of 3–5 min is that reported as 1 min postinsemination including the sample processing time (K.-I. Sato, personal communication). Because of the general importance of tyrosine kinases to cell biology, a variety of inhibitors have been developed. Among these, genistein, tyrphostins, and lavendustin A act as competitive inhibitors with ATP for the phosphate acceptor site (Agbotounou et al., 1994; Akiyama and Ogawara, 1991; Akiyama et al., 1987; Anafi, 1992; Hsu et al., 1991; Kovalenko et al., 1997; Onoda et al., 1989). Since the action of lavendustin A and the tyrphostins appears similar, peptide A was also used to inhibit tyrosine kinase activity. Peptide A has a mechanism of action different from that of tyrphostins or lavendustin A (Sato et al., 1990). This peptide fragment prevents autophosphorylation and appears to decrease kinase activity via steric actions (Fukami et al., 1993). Based upon recent crystallographic data, a mechanism of Src family tyrosine kinase activation involving intramolecular rearrangements has been described (Xu et al., 1997; Sicheri et al., 1997; Pawson, 1997). Peptide A has the potential to interfere with associations within the Src tyrosine kinase perhaps preventing the complex rearrangements required for Src activation (autophosphorylation).

In determining whether tyrosine kinase activity is required for the early events of fertilization, we determined the dose–response characteristics of three tyrosine kinase inhibitors (lavendustin A, tyrphostin B46, and peptide A) microinjected into unfertilized frog eggs at known concentrations. We find that these inhibitors block the early events of fertilization (FE liftoff) dose-dependently. Further studies of one of these inhibitors, lavendustin A, showed that the Ca^{2+} increases seen at fertilization are blocked by lavendustin A, but that this block can be overcome with increased amounts of sperm.

**MATERIALS AND METHODS**

**Collection of Eggs**

Wild-type female X. laevis were injected with 300–500 IU of human chorionic gonadotropin (hCG) in the evening before egg collection. Nine to 12 h after injection, eggs were obtained from females by exerting gentle abdominal pressure. Eggs were deposited into F1 solution (in mM, 41.25 NaCl, 1.75 KCl, 0.5 NaHPO<sub>4</sub>, 1.9 NaOH, 2.5 Hepes, 0.063 MgCl<sub>2</sub>, 0.25 CaCl<sub>2</sub>, pH 7.8) for microinjection.

**Sperm Handling and Scoring of Fertilization**

Wild-type X. laevis males were anesthetized by placing them in an ice-water slurry for 45–90 min until they were unresponsive to stimuli. The males were decapitated with surgical scissors and these testes were removed by dissection. Testes were stored in OR2 at 4°C until use. Sperm were released from the testes tissue by macerating the tissue with scissors and triturating the macerate with a pipet in 1 ml of F1 solution. The sperm solutions were stored on ice until used. In these experiments, the amount of sperm in the macerate was “normalized” for equivalent fertilizability by dilution. This entailed diluting the macerate to varying degrees and then using each dilution to inseminate groups of control eggs. The highest dilution of macerate still giving 100% fertilization was used for that experiment. This dilution procedure prevents obscuring inhibitory effects by excessive sperm addition. In the imaging experiments requiring increased sperm density, the concentrated macerate was used (as indicated in the text). The dilution method to adjust density was used instead of using the optical density of the macerate because the dilution method normalizes for fertilizing activity of the macerate rather than simply sperm number.

Insemination was achieved by adding 1 drop of sperm macerate (diluted as described above) to 1 ml of F1 in which the egg resides. Eggs were considered fertilized if FE liftoff occurred at the egg surface. This was always confirmed with observations of the sperm entry spot, cortical contraction, and rotation. Some form of cleavage was usually observed but consisted of multiple furrows with different axis of contraction resulting in a grossly disorganized egg surface. This made observations of the sperm entry spot(s) difficult and rarely conclusive as to number so the number of entering sperm was unknown. This pattern of grossly disorganized cleavage has traditionally been attributed to a polyspermic egg where several sperm enter, each acting as a cleavage center. We also are attributing this abnormal pattern to polyspermy, although we do not have direct evidence demonstrating polyspermy.

**Prick Activation**

Eggs injected with a final concentration of 1 μM lavendustin A were “prick-activated” in classic fashion, penetrating the egg with a microinjection needle while in Ca^{2+} containing medium (F1). Prick activation allows local Ca^{2+} entry to occur at the site of prickling resulting in egg activation. A microinjection needle containing 2 μM free Ca^{2+} EGTA buffer (MaxChelator, HTTP://www.stanford.edu/~cpatton) was used for prickling the egg. The needle was advanced approximately 50 μm into the egg. 5 nl of buffer was expelled (to ensure activation), and the needle was withdrawn. The egg was then monitored as described above to determine activation status.

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Microinjection

Microinjection was accomplished using methods described in Nuccitelli et al., (1993). Briefly, eggs were placed in “Ca²⁺-free” OR2 (in mM: 82.5 NaCl, 2 KCl, 5 Hepes, 20 M gCl₂, 0.1 EGTA, pH 7.4) containing 10 mM chlorobutanol (Sigma, St Louis, MO) and allowed to equilibrate for several minutes. The injection pipet was pulled to a 1-µm tip and beveled to a 30° angle to form an 8- to 10-µm oval-shaped opening. The pipet was then filled with oil (Fluorinert, Sigma, No. F-9880) and a small bolus of Hg was inserted into the Fluorinert column well away from the injectate (to prevent sharp pressure transients). The pipet was then calibrated by measuring the diameter of a drop of injectate expelled into Fluorinert (to form a sphere due to hydrophobic interactions) using an eyepiece reticule and calculating the volume of a sphere having that diameter. The injectate-oil interface within the pipet is easily visible because it moves down the pipet as the injectate is expelled. The volume of injectate represented by a given interfacial movement was then related to the volume of the spherical drop of expelled injectate. This procedure was performed to calibrate each pipet. After injecting 4.2 nl, the egg was allowed to heal in F1 for at least 15 min, also allowing time for the injected compounds to diffuse throughout the egg.

Inhibitors and Dyes

Except for peptide A, all inhibitors and their inactive controls used in this study were obtained from CalBiochem (San Diego, CA) and were dissolved in 100% DMSO. Peptide A was obtained from CalBiochem or Biomol (Plymouth Meeting, PA). Peptide A was synthesized using FMOC chemistry and subsequently HPLC purified on a reverse-phase C-18 column using acetonitrile:trifluoroacetic acid (TFA) as eluent. A single peak was observed indicating no contamination and the sample was lyophilized to remove the acetonitrile:TFA solvent. The peptide identity and purity was confirmed using mass spectroscopy. A peptide control was considered unnecessary given this purification scheme. To inject peptide A it was dissolved in “injection buffer” consisting of (in mM) 10 NaCl, 105 KCl, 10 Hepes, pH 7.3.

A 10,000 MW dextran conjugate of Oregon Green (Molecular Probes, Eugene, OR) was dissolved in injection buffer. Assuming a 450-nl free cytoplasmic volume, the final concentration of Oregon Green in the egg was 90 µM. At least 15 min was allowed for diffusion of Oregon Green to occur. This allows the dye to diffuse to uniform brightness within the egg. We assume that this time was also appropriate for the diffusion of the smaller-molecular-weight inhibitors.

Imaging

Oregon Green was imaged using the 488-nm line of an Ar/Kr laser on a Zeiss LSM 410 confocal microscope for excitation. A 488/568 dichroic filter and 515 long-pass filter were used to filter the emission signal. The image contrast and brightness were optimized with an algorithm that was part of the LSM software (Zeiss) to optimize the signal/noise ratio. The confocal image used here is a 40–100-µm-thick optical section of the egg, and the sperm may contact the egg at any point to generate a Ca²⁺ wave. Therefore, most waves traverse the egg at an angle to the confocal image and it was not possible to accurately calculate Ca²⁺ wave velocity from these experiments. The angle at which the wave passed through the plane of observation varied widely, frustrating these attempts.

RESULTS

Previous investigations in other systems, primarily sea urchin, have indicated that increases in tyrosine kinase activity may underlie the early events of fertilization. To determine if this is the case in Xenopus, we have microinjected tyrosine kinase inhibitors to see if the early events of fertilization (signal by FE liftoff, sperm entry spot, and/or Ca²⁺ increases) are affected. Microinjection of a calibrated volume of injectate was performed instead of bath application because of the low permeability of Xenopus egg membranes and to allow the precise cytoplasmic inhibitor concentration to be known.

Genistein

High concentrations of genistein (above 1 µM) did inhibit sperm-induced egg activation, but not in a dose-dependent manner. Instead, genistein gave approximately 30% inhibition of fertilization at all concentrations tested except for 1 µM (0% inhibition). Genistein was microinjected to give a final cytoplasmic concentration of 1 µM, 100 µM, 1 mM, and 100 mM. Sperm entry spots were only observed in fertilizing eggs. Since the inhibition was not dose-dependent (it was nonspecific), experiments with genistein were not continued.

Tyrphostins

Tyrphostin B46 inhibited FE liftoff in a dose-dependent manner. Tyrphostin B46 was microinjected to give final concentrations of 50, 100, 300, and 600 µM and 1 mM. The

FIG. 1. Inhibition of sperm-induced egg activation in eggs injected with tyrphostin B46. The number of eggs injected is indicated next to the corresponding data point in parentheses.
curve fitted to the data shown in Fig. 1 is a logistic dose-response equation with a correlation coefficient ($r^2$) of 0.999. Using this equation, the IC$_{50}$ for tyrphostin B46 is 400 μM. Values for the IC$_{50}$ of tyrphostins (taken as a class) can vary from 0.8 to 100 μM (using the same assay) simply due to structural differences (Anafi et al., 1992). IC$_{50}$ values specific for tyrphostin B46 range from 1 to 30 μM (EGF-R autophosphorylation, Gazit et al., 1991; K562 cell growth, Kaur et al., 1994; HER1/HER2, Osherov et al., 1993; EGF-R, Sion-Vardy et al., 1995; viral replication, Yura et al., 1996). The IC$_{50}$ of 400 μM obtained in this study indicates that tyrphostin B46 has a low affinity for the kinase(s) operative during Xenopus fertilization, or that it is acting nonspecifically. However, the negative control, an inactive tyrphostin (A1), was microinjected at concentrations of 100 μM ($n = 8$) and 1 mM ($n = 15$) without inhibiting fertilization. Sperm entry spots were not observed in nonfertilizing eggs and were observed in fertilizing eggs. Microinjection of solvent (DMSO) at the same level (1% v/v, 0.13 μM) did not inhibit fertilization ($n = 19$).

Lavendustin

Lavendustin A, having similar action to the tyrphostins, was also tested to avoid artifacts possible when using a single inhibitor. Lavendustin A, but not lavendustin B, also inhibited FE liftoff in a dose-dependent fashion (the effects on \([Ca^{2+}]_i\), are described below). Lavendustin A was the most potent inhibitor used in these experiments, having a calculated IC$_{50}$ of 11 nM. For comparison, reported IC$_{50}$ values for lavendustin A range from 11 nM to 18 μM, with most values in the low (10–100) nM range (pp60$^{527}$, Agbotounou et al., 1994; VEGF angiogenesis, Hu and Fan 1995; EGF-R, Onoda et al., 1989; Src kinase activity, O’Dell et al., 1991; NMDA-stimulated NO production, Rodriguez et al., 1994). The dose-response curve obtained with lavendustin A is shown in Fig. 2. The data are fit with a logistic dose-response curve ($r^2 = 0.991$). Lavendustin A was injected to yield final cytoplasmic concentrations of 0.1, 1, 10, 20, 50, 100, and 200 nM and 1 μM. Sperm entry spots were observed in fertilizing eggs and were not observed in nonfertilizing eggs.

Lavendustin B was used as an inactive control for lavendustin A since it is 100 times less active than lavendustin A (Onoda et al., 1989). Microinjection of lavendustin B at cytoplasmic concentrations of 100 nM ($n = 5$), 200 nM ($n = 5$), or 1 μM ($n = 6$) did not result in inhibition of fertilization (0%) and sperm entry spots were observed in these eggs. In contrast, these concentrations of lavendustin A resulted in 100% inhibition. Lavendustin A was dissolved in DM SO and solvent control injections gave no inhibitory effect (see above).

Peptide A

We also tested a very different tyrosine kinase inhibitor, peptide A. Peptide A, being a small peptide, is chemically and mechanistically distinct from the hydrophobic tyrphostins and lavendustins (see Discussion). Peptide A microinjections resulted in dose-dependent inhibition of FE liftoff. Peptide A was microinjected into eggs to give final

![FIG. 2. Inhibition of sperm-induced egg activation in eggs injected with various concentrations of lavendustin A. The number of eggs injected for each data point is shown in parentheses next to that point.](image_url)
concentrations of 6 nM, 40 nM, 200 nM, and 4 μM. The curve shown in Fig. 3 is generated using a logistic dose-response equation and fits the data with an $r^2 = 0.994$. The inhibition of fertilization by peptide A has a calculated IC$_{50}$ of 180 nM. This IC$_{50}$ is much less than those reported in the literature of 8 μM (Fukami et al., 1993) and 7.5 μM (Sato et al., 1990) obtained via kinase activity assay of pp60$^{{\text{src}}}$. Again, sperm entry spots were observed in fertilizing eggs and not in nonfertilizing eggs.

### Ca$^{2+}$ Imaging with Low Sperm Concentrations

Since lavendustin A was the most potent tyrosine kinase inhibitor, further experiments were performed to determine the effect of tyrosine kinase inhibitors on the sperm-induced Ca$^{2+}$ wave. To this end eggs containing Oregon Green and lavendustin A were inseminated with sperm diluted to the minimum needed for 100% fertilization (see Materials and Methods) while imaging with a confocal microscope. Eggs containing 1 μM lavendustin A (n = 7) did not increase their Ca$^{2+}$ levels above baseline in any visible part of the egg (a central section of the egg having a thickness of 100 μm) after insemination. This result confirms that lack of VE liftoff was coincident with a lack of [Ca$^{2+}$] changes within the egg (Fig. 4). Lavendustin B-injected eggs injected at 200 nM (n = 5) and 1 μM (n = 6) responded to similar insemination with normal Ca$^{2+}$ waves.

To ensure that the block of sperm-induced egg activation seen in lavendustin A and not lavendustin B-injected eggs was not due to variability in eggs or injectors, additional paired injections were undertaken (Fig. 4). This consisted of microinjecting three eggs with lavendustin A and three with lavendustin B where all six came from the same frog on the same day and were injected by the same microinjector. This experiment was repeated (on a different day with a different frog). In the case of lavendustin B injections grossly normal Ca$^{2+}$ waves were observed (Fig. 4) with no suggestion of hot spots. No Ca$^{2+}$ events were observed in the lavendustin A injected eggs (Fig. 4).

### Ca$^{2+}$ Imaging with High Concentrations of Sperm

Since 1 μM lavendustin A blocks increases in [Ca$^{2+}$], the question of whether increasing the number of sperm used in insemination could overcome this blockage arose. Eggs were again injected with 1 μM lavendustin A but now inseminated with a very high sperm density ($\sim 1 \times 10^9$/ml, n = 17). The inability of normally inseminated eggs to increase [Ca$^{2+}$] could be overcome with high sperm densities. Among the 17 eggs observed under these conditions, grossly normal waves were seen in 6/17, slower waves (precise velocities were not calculable using these techniques) were seen in 5/17 eggs and no Ca$^{2+}$ increase was seen in 6/17 eggs. In the subset of these eggs in which any Ca$^{2+}$ increase occurred, localized Ca$^{2+}$ rises that were small (approximately 100-200 μm wide), transient (approximately 1 min in duration), and nonpropagating were observed. We refer to these events as “Ca$^{2+}$ hot spots” because of their similarity to the phenomenon of the same name first described by Nuccitelli et al., (1993). An example Ca$^{2+}$ hot spot from a high density inseminated egg is shown in Fig. 5A. Ca$^{2+}$ hot spots were observed in 3 of 11 high-density inseminated eggs in which [Ca$^{2+}$] increases occurred. This may underestimate the frequency of these isolated, localized events as some may escape detection since the entire egg cannot be visualized simultaneously (a propagating Ca$^{2+}$ wave however would always be detected).

Using 100 nM lavendustin A (compared to 1 μM, n = 5 total), which completely blocks activation, and inseminating at high density, a similar distribution of Ca$^{2+}$ events was observed. A grossly normal wave occurred in 1/5 eggs, a noticeably slower [Ca$^{2+}$] wave was observed in 2/5 eggs, and no Ca$^{2+}$ increase occurred in 2/5 eggs. In 2 of the 3 eggs which increased [Ca$^{2+}$], hot spots were observed. In one of these eggs, approximately 15–20 hot spots were seen with no Ca$^{2+}$ wave ever occurring. An example of two hot spots from this egg is shown in Fig. 5B. These Ca$^{2+}$ hot spots, as well as those seen in eggs injected with 1 μM lavendustin A, did not originate from the same location twice and so are not due to repetitive stimulus or a “pacemaker.”

### The Effect of the Lavendustins on the Time to First Ca$^{2+}$ Rise

The postinsemination time required for eggs to increase [Ca$^{2+}$] (either hot spot or wave) was determined for control (Oregon Green injected), lavendustin A-injected (high sperm density insemination), and lavendustin B-injected eggs (normal sperm insemination). Using a t-test comparison of the Oregon Green-injected eggs to those injected with lavendustin A or B, no statistically significant difference ($\alpha = 0.05$) between control (average 395 s, SEM ± 54 s), lavendustin A (average 390 s, SEM ± 80 s), and lavendustin B (average 478 s, SEM ± 170 s) was seen.

### Prick Activation of Lavendustin A-Injected Eggs

Eggs injected with 1 μM lavendustin A (sufficient for 100% fertilization block) were 100% prick-activatable (n = 6). Control eggs (not injected) also were 100% prick-activatable (n = 6). This indicates that the level in the pathway that lavendustin A acts upon is prior to (upstream of) the Ca$^{2+}$ increase seen at fertilization since introduction of Ca$^{2+}$ by pricking substitutes for this increase.

**DISCUSSION**

In sea urchin eggs postfertilization plasma membrane-associated tyrosine kinase activity has been shown to increase (Kinsey, 1984; Ribot et al., 1984; Peaucellier et al., 1988; Ciapa and Epel, 1991), and, in certain cases, increase persistently through early development (Dasgupta and Garbers, 1983). Tyrosine kinase inhibitors such as genistein,
tyrphostins, and erbstatin have been used in sea urchin eggs to block tyrosine kinase activity (Moore and Kinsey, 1995). The effects of genistein appear to be on later events in development such as cleavage, although polyspermy is sometimes seen (Moore and Kinsey, 1995; Livingston et al., 1998). In the present study, we report that three tyrosine kinase inhibitors block early events of fertilization in Xenopus.

A physiological role for tyrosine kinase activity in starfish and Xenopus (Shilling et al., 1994; Yim et al., 1994) egg

![Figure 4. Calcium wave observed in eggs injected with 1 µM lavendustin A (Lav A) or 1 µM lavendustin B (Control). Both eggs also were injected with 90 µM Oregon Green to visualize Ca²⁺. These eggs were injected in parallel by the same microinjector on the same day with the same reagents. The respective column titles denote the same time series with the control egg shown in column 1 and 3, while the lavendustin A-injected egg is shown in columns 2 and 4.](image-url)
activation has been demonstrated. In starfish oocytes, mRNA encoding the extracellular PDGF receptor domain and the cytosolic FGF receptor domain was injected into oocytes (Shilling et al., 1994). Exposing these eggs to PDGF induced them to activate similarly to normally fertilized eggs (Shilling et al., 1994). In *Xenopus*, similar experiments using mRNA encoding the human EGF receptor were performed (Yim et al., 1994). Application of EGF also caused these eggs to activate similarly to normally fertilized eggs (Yim et al., 1994). In other studies, EGFR mRNA was also injected into *Xenopus* oocytes (Opresko and Wiley, 1990). The application of EGF to these matured oocytes resulted in Ca\(^{2+}\) release from the egg (suggesting intracellular Ca\(^{2+}\) release), receptor autophosphorylation, and receptor down-regulation (Opresko and Wiley, 1990). These experiments taken together indicate a competent tyrosine kinase pathway in these eggs.

The microinjection of tyrosine kinase inhibitors was undertaken to extend the data described above by demonstrating that inhibition of tyrosine kinase activity can disrupt normal fertilization. One of these, Peptide A, is a synthetic peptide sequence corresponding to residues 137–157 within the SH2 domain of pp60\(^{src}\) (Sato et al., 1990). Peptide A specifically inhibits src-family tyrosine kinase activity (Fukami et al., 1993). The crystalline solutions of Src and Hck have provided data for a model of activation via intramolecular rearrangement (Sicheri et al., 1997; Xu et al., 1997). Src family proteins have two halves, one catalytic and one regulatory (containing SH2 and SH3 domains). One area of contact between these halves contains the Peptide A sequence (Xu et al., 1997). Based on the rearrangements seen in Src activation, the inhibitory mechanism of Peptide A might involve substitution of Peptide A for the normal contact region between the halves of Src, sterically hindering molecular rearrangements needed for Src activation. It is important to note that Peptide A is not a competitive phosphorylation substrate, since pp60\(^{src}\) cannot tyrosine phosphorylate Peptide A even at 1 mM Peptide A concentration (Sato et al., 1990). In contrast to Peptide A, tyrphostins act as competitive inhibitors with ATP for the ATP binding site; however, their behavior is more complex than that of a simple competitive inhibitor (Kovalenko et al., 1997). Lavendustin A also competes with ATP for the ATP binding site and kinetic evidence also supports multiple binding sites for this inhibitor (Anafi, 1992; Kavalenko et al., 1997), allowing for the precise actions of tyrphostins and lavendustin A (Onoda et al., 1989; Agbotounou et al., 1994), to be dependent on the activation state of the

**FIG. 5.** Eggs injected with lavendustin A and inseminated at high density. (A) An egg injected with 90 \(\mu\)M Oregon Green and 1 \(\mu\)M lavendustin A. Insemination is with a high sperm density. A Ca\(^{2+}\) “hot spot” and slow wave develop in this series. The arrows in the first panel indicate the respective origins. The time from insemination is shown in the upper left of each panel. The hot spot starts at 408 s after insemination and ends 42 s later. The slow wave starts at 630 s after insemination and ends at approximately 1020 s (taking about 6 min to cross the egg). Blue indicates basal Ca\(^{2+}\) levels, greens indicate two- to fourfold increases from basal, and red indicates five- to sixfold increases (of pixel intensity). Egg diameter is 1200 \(\mu\)m. (B) An egg injected with 90 \(\mu\)M Oregon Green and 100 nM lavendustin A. The panels are taken at 15-s intervals and the sequence shows two distinct hot spots (out of many). The white arrows in the first panel indicate the origin of the two hot spots (spot 1 and spot 2). Blue indicates basal levels of Ca\(^{2+}\), green indicates two- to fourfold elevation, and red indicates five- to sixfold elevation.
receptor. Irrespective of mechanistic action, the tyr-
phostins, lavendustin A, and peptide A (among others) are
useful inhibitors of many tyrosine kinases.

Using the tyrosine kinase inhibitors lavendustin A, tyr-
phostin B46, and peptide A (but not genistein), we have
demonstrated a dose-dependent inhibition of early activa-
tion events by these compounds. These inhibitory dose-
response relations demonstrate that tyrosine kinase activ-
ity plays a role in early fertilization events that lead to Ca2+
release in Xenopus. The inhibitory ability of peptide A
suggests that a member of the src family of nonreceptor
tyrosine kinases may be involved in this signaling pathway.

**Ca2+ Hot Spots**

Reports of transient, nonpropagating increases of Ca2+
during fertilization have been presented previously (Eck-
berg and Miller, 1995; Nuccitelli et al., 1993). In previous
work with Xenopus, Ca2+ hot spots have been observed in
heparin-injected eggs as a result of blocking the IP3 receptor
(Nuccitelli et al., 1993). These Ca2+ hot spots are qualita-
tively similar to those observed in lavendustin A-injected
eggs. An interesting difference is that the Ca2+ hot spots
seen in lavendustin A-injected eggs are of shorter duration
than those seen in heparin-injected eggs (Nuccitelli et al.,
1993). Ca2+ hot spots are not seen in lavendustin B-injected
eggs. If a similar mechanism underlies the hot spots when
heparin or lavendustin A is present, then this would imply
a tyrosine kinase(s) involvement in IP3 generation. The
fact that eggs can be prick-activated when containing 1 μM
lavendustin A also supports this idea that a tyrosine kinase
is involved upstream of the Ca2+ changes seen at fertiliza-
tion.

To image Ca2+ changes we used 90 μM Oregon Green-
dextran, the level of which might be expected to signifi-
cantly buffer intracellular Ca2+ (Kline, 1988). This buffering
is a probable explanation for the observed Ca2+ hot spots.
However, we do not think that this is a likely explanation
because fura-2 has been previously used in Xenopus eggs at
50–100 μM with only slight Ca2+ buffering (Larabell and
Nuccitelli, 1992). Other work in our laboratory shows that
90 μM Oregon Green does not alter the Ca2+ wave and has
never caused Ca2+ hot spots to occur (n = 70, Fontanilla and
Nuccitelli, in press). The fact that eggs injected with
lavendustin B also show a normal wave (with the same
amount of Oregon Green, Fig. 4) also indicates that buffer-
ing does not affect the wave or cause hot spots.

**SUMMARY**

In these experiments, we have demonstrated, using ty-
rosine kinase inhibitors, that tyrosine kinase activity is
essential for the early events of fertilization in Xenopus.
Tyrosine kinase inhibitors were microinjected at known
cytoplasmic concentrations and their effect on egg activate-
dation was determined. Three of the four compounds tested
inhibited these events in a dose-dependent fashion. We
have directly shown that tyrosine kinase activity is essen-
tial to VE liftoff and to normal Ca2+ wave generation. In
addition, we have shown that tyrosine kinases act upstream
of the Ca2+ events seen at fertilization (i.e., the Ca2+ wave)
and that disruption of this activity abolishes these Ca2+
events. Interestingly, unique changes (hot spots) in Ca2+
levels can occur under conditions of greatly increased
sperm density, although tyrosine kinase activity is inhib-
ited.

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