plasmic extracts of ^{32}P-labeled cells that had been treated with IFN-γ for 3 min. A peptide corresponding to peptide 1 from whole cell extracts was detected (Fig. 5B). Immunoprecipitates of nuclear extracts contained little ^{32}P-labeled 91-kD protein after only 3 min of treatment (*16*).

^{32}P-labeling of peptide 1 was greatly diminished by the treatment of cells with staurosporine (Fig. 5B) whereas labeling of the phosphoserine-containing peptides was not affected. Staurosporine also blocked-binding of GAF to DNA (Fig. 4C); thus tyrosine phosphorylation appears to be the IFN-γ–dependent modification necessary for the activation of GAF.

These experiments indicate that the specificity of the cytoplasmic response to IFN-α and IFN-γ results from differential tyrosine phosphorylation of the 113-, 91- and 84-kD proteins (Fig. 6). Two different kinases may be required for the two different ligand-specific pathways (*7*). The mutant cell line 11.1 which does not respond to IFN-α, completely lacks the Tyk-2 mRNA and the Tyk-2 protein (*7*) but does activate genes in response to IFN-γ (*19*), suggesting that the response to IFN-γ must be mediated through another kinase. At present two other members (JAK1 and JAK2) (*20, 21*) are known of the same kinase family as Tyk2, one of which, JAK1 is increased in mRNA concentration in cells treated with IFN-γ (*22*).

The 91-kD protein can function in transcriptional activation in two different ways. In cells treated with IFN-α, the 91-kD protein participates in a high-affinity DNA binding complex for the ISRE (*23*) but does not itself contact DNA (*4*). Moreover, in cells treated with IFN-α most of the phosphorylated 91-kD protein is used in forming ISGF-3. In cells treated with IFN-γ the tyrosine phosphorylated 91-kD protein does bind to DNA, but at a different DNA element, the GAS. Other proteins have been described that participate in DNA-protein interaction either alone or in combination with other proteins (*24*) but those proteins have not been shown to be phosphorylated on tyrosine.

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Range of Messenger Action of Calcium Ion and Inositol 1,4,5-Trisphosphate

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The range of messenger action of a point source of Ca^{2+} or inositol 1,4,5-trisphosphate (IP_3) was determined from measurements of their diffusion coefficients in a cytosolic extract from *Xenopus laevis* oocytes. The diffusion coefficient (*D*) of [^{3}H]IP_3 injected into an extract was 283 μm^2/s. *D* for Ca^{2+} increased from 13 to 65 μm^2/s when the free calcium concentration was raised from about 90 nM to 1 μM. The slow diffusion of Ca^{2+} in the physiologic concentration range results from its binding to slowly mobile or immobile buffers. The calculated effective ranges of free Ca^{2+} before it is buffered, buffered Ca^{2+}, and IP_3 determined from their diffusion coefficients and lifetimes were 0.1 μm, 5 μm, and 24 μm, respectively. Thus, for a transient point source of messenger in cells smaller than 20 μm, IP_3 is a global messenger, whereas Ca^{2+} acts in restricted domains.

The transduction of many hormonal and sensory stimuli is mediated by transient increases in the concentration of intracellular free calcium ($[Ca^{2+}]_i$). Ca^{2+} influx into the cytosol can be induced by (i) opening of voltage-gated and receptor-operated Ca^{2+} channels in the plasma membrane; (ii) binding of receptors that activate the phosphoinositide cascade, which leads to the production of inositol 1,4,5-trisphosphate (IP_3) and the consequent opening of channels on internal Ca^{2+} stores; and (iii) the activation of ryanodine-receptor channels (*1*). The resulting increase in $[Ca^{2+}]_i$ is then detected by Ca^{2+} sensors that alter the activities of enzymes, pumps, and other targets. Many activated cells display repeated Ca^{2+} spikes or oscillations and Ca^{2+} waves (*1–3*). These macroscopic responses are produced by the spreading, amplification, and deactivation of localized increases in $[Ca^{2+}]_i$. Knowing the range of action of spatially localized impulses of Ca^{2+} and IP_3 is therefore fundamental to understanding Ca^{2+} signaling. Previous measurements of the diffusion constants of these messengers did not eliminate interfering processes such as sequestration, degradation, and messenger amplification; this was appreciated by the investigators (*4*).

We measured the diffusion coefficients of Ca^{2+} and IP_3 in a cytosolic extract from *Xenopus* oocytes (*5*). Measurement of IP_3 diffusion requires inhibition of its degradation, which typically occurs in ~1 s (*6*). Degradation was blocked by chelating divalent cations, which are required for activity by the 5′-phosphomonoesterase and IP_3-kinase (Fig. 1A) (*7*). To avoid sequestration of Ca^{2+} by internal stores, which would affect the measured diffusion coefficient for Ca^{2+}, we added thapsigargin to inhibit Ca^{2+} pumps; hexoki-

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nase and glucose to lower the adenosine triphosphate (ATP) concentration; and carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) to block mitochondrial sequestration (Fig. 1B). Excess cytoplasmic Ca^{2+} that leaked into the cytosol was removed by passing the extract through a column to remove Ca^{2+} (*5, 8*). The concentration of free Ca^{2+} in this preparation was ~100 nM (*9*). The effectiveness of this treatment was shown by the finding that less than 3% of added $^{45}Ca^{2+}$ was sequestered (*10*).

$[^3H]IP_3$ or $^{45}Ca^{2+}$ was layered on top of a cytosolic extract contained in a thin tube and allowed to diffuse for various periods of time (*11*). The tube was then frozen and sliced, and the concentration of labeled messenger in each slice was determined (*11, 12*) (Fig. 2, A and B). The measured *D* values, which were nearly independent of the incubation time, were 38 ± 11 $\mu m^2/s$ (n = 9) for Ca^{2+} and 283 ± 53 $\mu m^2/s$ (n = 6) for IP_3. The effective viscosity of the cytosolic extract was determined by measuring the diffusion of $^{22}Na^+$ and ^{125}I-labeled immunoglobulin G (IgG) (Fig. 2C). The measured *D* values of 790 ± 127 $\mu m^2/s$ (n = 4) and 27 ± 8 $\mu m^2/s$ (n = 4) for Na^+ and IgG, respectively, are close to those measured in muscle cells or predicted by theory for a medium of twice the viscosity of water (*13, 14*). IP_3 diffused faster than Ca^{2+} (Fig. 2D). The *D* values were 35 $\mu m^2/s$ for Ca^{2+} and 268 $\mu m^2/s$ for IP_3. For comparison, the calculated *D* values for unbound Ca^{2+} and IP_3 in a medium with twice the viscosity of water are 370 and 250 $\mu m^2/s$, respectively (*13, 15*).

The *D* value for Ca^{2+} depended on the concentration of Ca^{2+} added to the cytosol (Fig. 3). Decreasing the concentration of diffusing $^{45}Ca^{2+}$ by a factor of ten decreased the *D* of Ca^{2+} to 13 ± 8 $\mu m^2/s$ (n = 4) (*16*). Conversely, *D* increased to 200 $\mu m^2/s$ if 1 mM $CaCl_2$ was added to the extract before the diffusion experiment was done. To determine the relationship between added and free Ca^{2+}, the free Ca^{2+} con-

Table 1. Estimated range and time scale of messenger action of Ca^{2+} and inositol 1,4,5-trisphosphate.

Messenger	Diffusion coefficient ($\mu m^2/s$)	Time scale (s)	Range (μm)
Calcium			
Free ion	223	0.00003	0.1
Buffered	13	1	5
Inositol 1,4,5-trisphosphate	280	1	24

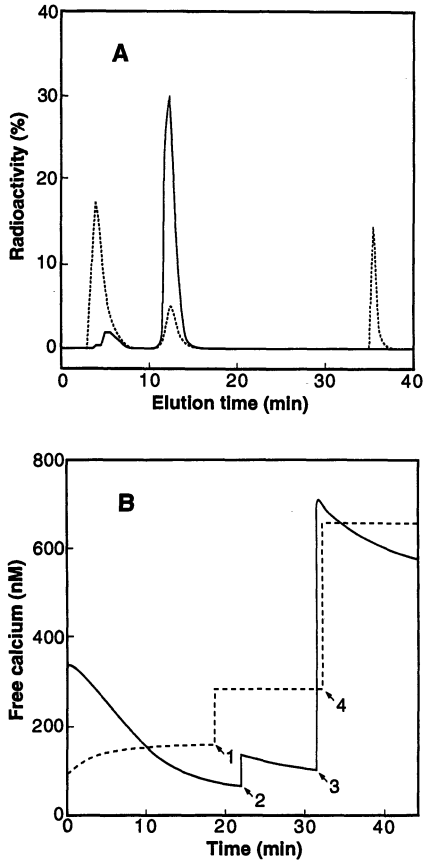

Fig. 1. Extent of IP_3 breakdown and Ca^{2+} uptake during the diffusion experiments. (**A**) Cytosolic extract (50 μl) was incubated with 100 nM $[^3H]IP_3$ in the presence (solid line) or absence (dashed line) of 30 mM EDTA for 60 min at room temperature (*26*). Only 8% of the IP_3 in the sample with EDTA was metabolized as measured by HPLC. [^{3}H]inositol 1-phosphate, [^{3}H]inositol 1,4-bisphosphate, [^{3}H]inositol 1,4,5-trisphosphate, and [^{3}H]inositol 1,3,4,5-tetrakisphosphate were eluted at 4, 5, 13, and 36 min, respectively. Units on the *y* axis indicate the percentage of the total amount of radioactivity added to the extract. (**B**) At time 0, cytoplasm (35 μl) was added to a cuvette containing 2 ml of XB buffer (4) with 0.25 μM fluo-3, 1 mM Mg^{2+}, and either 1 mM ATP (solid line) or 40 μM thapsigargin and 1 μM FCCP (dashed line), and the mixture was stirred. Ca^{2+} was added to the solutions at times marked by the arrows (*27*).

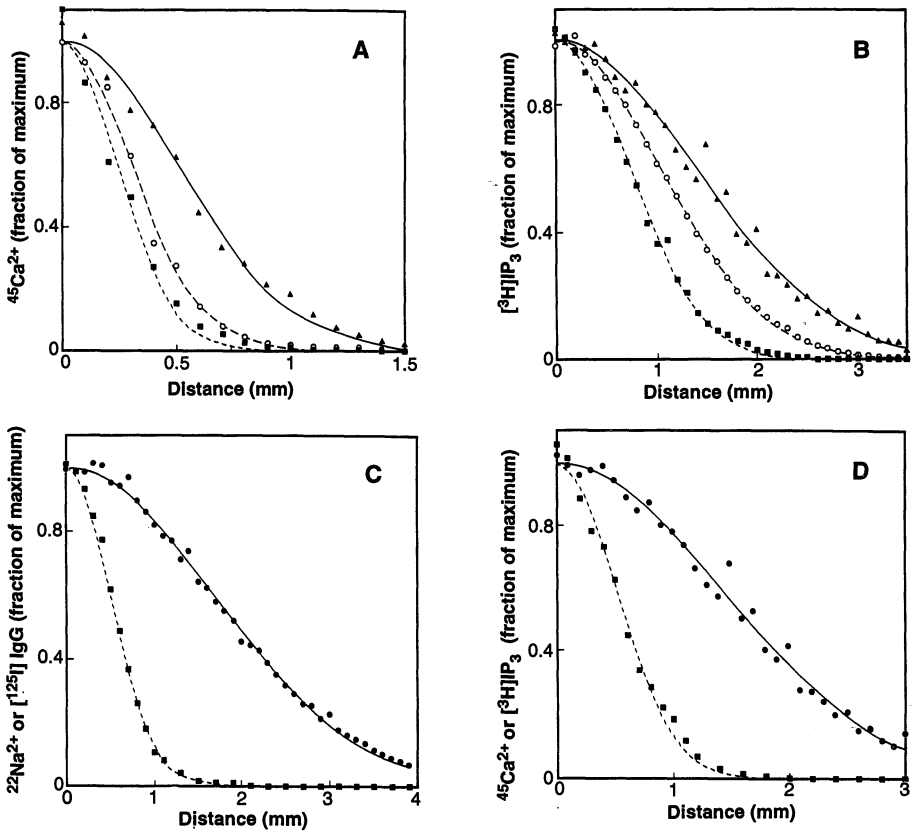

Fig. 2. Measurement of *D* for Ca^{2+} and IP_3 in cytosol from *Xenopus* oocytes. (**A**) $^{45}CaCl_2$ (44 μM) was allowed to diffuse in extract with inactive stores in the presence of 1 mM $MgCl_2$, 40 μM thapsigargin, and 1 μM FCCP for 15 (squares), 30 (circles), or 60 (triangles) min. The *D* values were 37, 25, and 35 $\mu m^2/s$, respectively. Data were normalized for plotting by setting the value of the first time point equal to one. The lines are fits of the data to Fick's Law (*12*). (**B**) $[^3H]IP_3$ (1.5 μM) was allowed to diffuse for 15 (squares), 30 (circles), or 60 (triangles) min in extract containing 30 mM EDTA. *D* was 295, 274, and 268 $\mu m^2/s$, respectively. (**C**) $^{22}NaCl$ (4.5 μM) or ^{125}I-labeled IgG (20 μg/ml, rabbit antibody to mouse IgG) was allowed to diffuse for 30 min ($^{22}Na^+$, circles) or 60 min (^{125}I-labeled IgG, squares). The extract for IgG but not Na^+ diffusion contained 10 mM EDTA to decrease proteolysis of the IgG. (**D**) In 1 hour, IP_3 (circles) diffused much farther than $^{45}CaCl_2$ (squares).

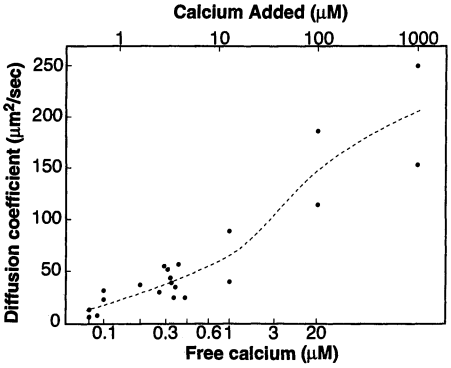

Fig. 3. Dependence of D for Ca^{2+} on the concentration of free Ca^{2+} and the amount of added Ca^{2+}. The concentration of free Ca^{2+} was determined as described in the text. The average concentration of added Ca^{2+} was determined as described (*28*). The concentration of $^{45}Ca^{2+}$ was calculated from the amount loaded and the volume over which it diffused. The dashed line was drawn empirically.

centration was measured fluorimetrically (*9*). Addition of 13 μM or 100 μM Ca^{2+} gave free Ca^{2+} concentrations of 1 and 20 μM, respectively. As the Ca^{2+} concentration was increased, D became larger because a smaller proportion of the Ca^{2+} was bound. The measurements also suggest that the dissociation constants of Ca^{2+} buffer sites range from less than 1 μM to more than 10 μM. In contrast, decreasing the amount of IP_3 by a factor of ten did not alter the D of IP_3 (*16*); thus, IP_3 is not appreciably bound to buffers.

The diffusion coefficient of the messenger that propagates Ca^{2+} waves can be estimated from the relationship $D = v\lambda$, where v is the velocity of the wave, and λ is the length of the concentration gradient at the wave front (*17*). D has also been estimated from the slope of the relationship between the curvature and velocity of circular waves (*2*). These experimental approaches indicate that D of the propagating messenger is between 300 and 600 $\mu m^2/s$. These limits make IP_3, but not Ca^{2+}, an attractive candidate for the mobile messenger in calcium wave propagation (*18, 19*).

The range of action of a spatially localized impulse of IP_3 or Ca^{2+} can be approximated by the equation $s \simeq (2D\tau)^{1/2}$, where τ is the time scale of messenger action (Table 1) (*20*). The value of τ for IP_3 was determined by its degradation time of about 1 s in rat basophilic leukemia cells and smooth muscle cells (*6*). The measured D for IP_3 was 280 $\mu m^2/s$; hence, s is ~24 μm. For free Ca^{2+}, τ is given by the time needed to bind to buffers, $(k_{on}c_b)^{-1}$, where k_{on} is the on-rate of a typical calcium buffer, 10^8 M^{-1} s^{-1} (*21*), and c_b is the concentration of Ca^{2+} buffers, about 300 μM (*22*). These values give a τ of 3×10^{-5} s for free Ca^{2+}. The measured D was 223 $\mu m^2/s$, and so s is ~0.1 μm. For buffered Ca^{2+}, τ is determined by the time for sequestration into stores, about 1 s (*1*). Because D was 13 $\mu m^2/s$, s for buffered Ca^{2+} is ~5 μm.

The rapid buffering of Ca^{2+} makes it a localized messenger for effector systems that require high concentrations of Ca^{2+} for activation. Such effectors must be less than ~0.5 μm from a calcium source, such as a voltage-gated calcium channel on the plasma membrane. For example, synaptotagmin, a calcium sensor in synaptic vesicle membranes (*23*), is activated by high (~10 μM) but not moderate (~1 μM) $[Ca^{2+}]_i$. The effect of buffering is to markedly lower the concentration of free Ca^{2+} and slow its diffusion. This creates a second domain with a much lower peak concentration of Ca^{2+} and a range of about 5 μm. Effectors with high affinity for Ca^{2+}, such as those stimulated by calmodulin, can be activated in this domain (*24*). In contrast, IP_3 has a much larger domain of messenger action because it is virtually unbuffered and has a lifetime of ~1 s. The ~24-μm range of IP_3 indicates that it serves as a global messenger in most cells. IP_3 could be a localized messenger with a 1-μm range only if it were degraded 1000 times more rapidly than has been observed (*25*).

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9. To determine the approximate concentration of free Ca^{2+}, 0.5 μM rhod-2 or 5 μM fura-2 was added together with 1 mM $MgCl_2$, 40 μM thapsigargin, and 1 μM FCCP to a concentrated extract with inactive stores. Portions of this extract received either 10 mM EGTA, 10 mM EGTA with 10 mM Ca^{2+}, 2.8 mM Ca^{2+}, 0.28 mM Ca^{2+}, or an equal amount of XB buffer. This was done to ensure that the concentrations of cytosol and Ca^{2+} indicator were the same in all experiments. The cytoplasmic extract was placed in a cuvette covered with black tape such that only a 60 μl volume was illuminated regardless of the total volume of sample. The fluorescence (f) of the sample was measured before and after multiple additions of more cytoplasm containing either 2.8 mM or 0.28 mM Ca^{2+}. The minimal fluorescence (f_{min}) of the indicator was determined from cytoplasm containing 10 mM EGTA and the maximal fluorescence (f_{max}) from cytoplasm containing 10 mM EGTA and 10 mM Ca^{2+}. The fluorescence of rhod-2 in buffer containing 10 mM EGTA and 10 mM Ca^{2+} was virtually identical to that of rhod-2 in buffer containing 100 μM Ca^{2+}. Thus, the observed fluorescence was in fact f_{max}. For rhod-2 measurements the quantity $(f - f_{min})/(f_{max} - f)$ was converted to the concentration of free Ca^{2+} with a calibration curve measured in cytoplasm containing various amounts of Ca^{2+} in which free Ca^{2+} was buffered by 50 mM EGTA. The measurements of free Ca^{2+} with rhod-2 were made on two batches of Ca^{2+}-depleted cytoplasm. The average of these measurements defined the relationship between the free Ca^{2+} concentration and the concentration of added Ca^{2+}. Calculations of the concentration of free Ca^{2+} in cytoplasm from fura-2 fluorescence ratios were done with the published K_d (250 nM) for Ca^{2+} [G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J. Biol. Chem.* **260**, 3440

(1985)] and were similar to those obtained with rhod-2 (K_d = 1 μM). Much higher concentrations of fura-2 than those of rhod-2 were required because fura-2 is excited in the ultraviolet and emits in the blue, where the background fluorescence is much higher. Consequently, rhod-2 was used in most experiments.

Briefly, 300 nM $^{45}Ca^{2+}$ or 38 μM $^{45}Ca^{2+}$ was added to 50 μl of cytoplasm with 1 mM $MgCl_2$, 40 μM thapsigargin, and 1 μM FCCP. The higher Ca^{2+} concentration was selected to mimic the conditions at the injection site during diffusion experiments and the lower concentration to mimic that near the edge of a region of diffusing Ca^{2+}. The cytoplasm was incubated at room temperature for 30, 40, or 60 min, after which a portion (15 μl) was removed, and the amount of radioactivity in the portion was counted. The Ca^{2+} ionophore A23187 (10 μM) was added to another portion (15 μl) as a measure of nonspecific binding in the filter assay, and a final portion (15 μl) was used to determine uptake into stores. For the filter assay, 100 μl of sample (15 μl of cytoplasm and 85 μl of cold XB) was loaded onto a Whatman GF/A glass microfiber filter that had been washed with cold XB (5 ml). The filter was then washed with cold XB (5 ml) and counted. Uptake of $^{45}Ca^{2+}$ by the cytoplasm with inactive stores was less than 3% of the total amount added for both Ca^{2+} concentrations and for all time points.

For the diffusion experiments, the cytoplasm was thawed and then mixed with the indicated agents and either [^{3}H]sucrose or $^{22}Na^+$. The latter were used to correct for variations in slice width when sectioning the tube. For $^{45}Ca^{2+}$, ^{125}I-labeled IgG, and $^{22}Na^+$, 0.17 μM [^{3}H]sucrose was added and with [^{3}H]IP_3, we added 0.23 μM $^{22}Na^+$. The cytoplasm was loaded into a 20-mm Silastic tube (Dow Corning) of internal diameter 1.5 mm and one end of the tube was plugged with a pipette tip filled with Sylgard (Dow Corning). The tube was placed in a vertical position in a custom-built microtome and equilibrated in a temperature-stabilized room (20°C) for 45 min. The vertical geometry was used to prevent mixing caused by density differences between the messenger solution and the cytoplasm. Just before injection of the diffusing substance, the upper 2 mm of tubing with cytoplasm was removed with a razor blade. The radioactive messenger (~0.2 μl) was applied to the cytoplasm, which was then overlaid with 0.5 μl of mineral oil. After the indicated diffusion time, the cytosol was rapidly frozen with powdered dry ice. The microtome was placed on a block of dry ice and the tube was sectioned at 100-μm intervals.

The concentration of the messenger in each slice was fit to the solution of Fick's Law: $C(x,t) = M(Dt\pi)^{-1/2}e^{-x^2/4Dt}$, where $C(x,t)$ is the concentration of the diffusing substance at distance x and time t, M is the total amount of diffusing substance, and D is the diffusion coefficient [J. Crank, *The Mathematics of Diffusion* (Clarendon, Oxford, ed. 2, 1975), pp. 12–13, 326–327]. In using this equation we make three simplifying assumptions: (i) the binding reaction to buffers is very rapid compared to diffusion, so an equilibrium exists between bound and free messenger; (ii) the buffers are immobile; and (iii) the concentration of bound messenger is proportional to the concentration of free messenger. These assumptions are necessary because data that would give the concentration of buffer, on and off rates for messenger, and the diffusion coefficients of the messenger-bound buffers are not available. Because the concentrations of messengers used in the diffusion experiments were in the range of their physiologic values, the measured diffusion coefficients reflect the average time the messenger spends bound to buffers and the average diffusion properties of those buffers.

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16. The lowest injected concentrations of IP_3 (150 nM) and Ca^{2+} (4.4 μM) are comparable to or less than the peak concentrations occurring in cells [G. M. Burgess, J. S. Mckinney, R. F. Irvine, J. W. Putney, *Biochem. J.* **232**, 237 (1985); S. Palmer, K. T. Hughes, D. Y. Lee, M. J. O. Wakelam, *Cell. Signalling* **1**, 147 (1989)].
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18. We tested whether the fluorescent Ca^{2+} indicator used in these experiments would appreciably alter Ca^{2+} diffusion [F. Sala and A. Hernandez-Cruz, *Biophys. J.* **57**, 313 (1990); L. A. Blatter and W. G. Weir, *ibid.* **58**, 1491 (1990); M. P. Timmerman and C. C. Ashley, *FEBS Lett.* **209**, 1 (1986)]. In the presence of 50 or 100 μM fura-2, D for Ca^{2+} increased from 38 μm^2/s to 75 μm^2/s (n = 2) and 100 μm^2/s (n = 2), respectively. Thus, fura-2 accelerated the diffusion of Ca^{2+} by pulling Ca^{2+} away from less mobile buffers. The acceleration of Ca^{2+} diffusion by fura-2 is insufficient to bring it into the range of the estimated diffusion coefficient of the propagating messenger in the generation of Ca^{2+} waves (D ~ 300 to 600 μm^2/s).
19. Two conditions must be met for a fluorescent indicator to report the diffusion coefficient of the propagating messenger without perturbing the process. The indicator must contribute only a small percentage of the total calcium buffering capacity. A much more restrictive condition is that the diffusion of Ca^{2+} must not be enhanced by the presence of the Ca^{2+} indicator. Specifically, $D_i f_i$ must be much less than $D_f f_f + \Sigma D_j f_j$, where D_i is the diffusion coefficient of the indicator and f_i is the mole fraction of the Ca^{2+}-bound indicator with respect to all Ca^{2+}-bound species that are in rapid equilibrium with free Ca^{2+}. D_f and D_j are the diffusion coefficients of free Ca^{2+} and Ca^{2+}-bound buffers, and f_f and f_j are the mole fractions of free Ca^{2+} and Ca^{2+}-bound buffers.
20. We have chosen a spatially localized impulse to illustrate the range of messenger action because of its conceptual simplicity and physiological relevance. The model assumes a small impulse of Ca^{2+} in a previously unstimulated cell with a resting $[Ca^{2+}]_i$ of ~100 nM. The concentration profile would be altered if one assumes persistent local production of IP_3 or sustained local release of Ca^{2+}.
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26. Trifluoroacetic acid (400 μl of a 10% solution) was added to the cytosol after the indicated time. The mixture was centrifuged at 150,000*g* for 20 min. The supernatant (350 μl) was removed and dried. The samples were resuspended in buffer A [50 mM tetrabutylammonium hydrogen sulfate, 40 mM KH_2PO_4 (pH 3.5)], mixed, filtered (0.2 μm), and injected onto a C18 high-pressure liquid chromatography column (Alltech). The ^{3}H-labeled inositol phosphates were eluted with a gradient of buffer A and acetonitrile. This protocol was modified from those described by J. A. Shayman and D. M. BeMent [*Biochem. Biophys. Res. Commun.* **151**, 114 (1988)] and J. C. Sulpice, C. Bachelot, P. Gascard, and F. Giraud [in *Methods in Inositide Research*, R. F. Irvine, Ed. (Raven, New York, 1990), pp. 45–63].
27. Ca^{2+} was added to the approximate final concentration of: 1, 200 nM; 2, 200 nM; 3, 2 μM; and 4, 600 nM. Additions 3 and 4 were made to bring the final free Ca^{2+} concentration to approximately 600 nM. The free Ca^{2+} concentration was calculated with the published K_d of fluo-3 for Ca^{2+} [A. Minta, J. Kao, R. Y. Tsien, *J. Biol. Chem.* **264**, 8171 (1989)].
28. When the amount of Ca^{2+} added was less than 10 μM, the only source of exogenous Ca^{2+} was the $^{45}Ca^{2+}$ used to measure diffusion. When the amount of Ca^{2+} added was greater than 10 μM, 0.1 μl of 44 μM $^{45}Ca^{2+}$ was added to cytoplasm to which nonradioactive Ca^{2+} had already been added.

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