most consistent with the hypothesis that activation of the ISGF3α proteins occurred in a cellular fraction that contains plasma membranes.

In vivo activation of ISGF3α is associated with its translocation to the nucleus (*4*, *6*). We therefore determined whether or not activation of ISGF3α was directly coupled to its release from the membrane. After incubation with IFN-α for 30 min, the Mb fraction was sedimented at 15,000*g* for 15 min, and the supernatant from the membranes was added to the Sup fraction at 4°C (Fig. 3B). Under these conditions no ISGF3 was present. However, when the sedimented membranes were resuspended in buffer with the detergent NP-40, recentrifuged, and the supernatant then assayed, ISGF3 was formed. It can be inferred from this observation that release of the activating factor from the membranes is not directly coupled to the activation of the ISGF3α proteins; rather, it appears to require another reaction.

A variety of cofactors that are needed for well-described signaling systems were included in the incubation buffer of the cell-free system (Fig. 1). Because only the Mb fraction was necessary to activate ISGF3α in vitro, this provided an assay system to determine whether these cofactors were essential for the signaling process. Calcium had no effect on the activation of ISGF3. The removal of NaF, which inhibits serine-threonine phosphatases, was also without effect. Because the nonhydrolyzable guanosine triphosphate (GTP) analogue guanosine 5′-*O*-(3′-thiotriphosphate) (GTP-γ-S) was not required and the presence of guanosine 5′-*O*-(2-thiodiphosphate) (GDP-β-S, an inhibitor of GTP binding protein–mediated signaling) was without effect on activation of ISGF3α by IFN-α, guanine nucleotide binding proteins do not appear to be required. The absence of ATP or the substitution of a nonhydrolyzable analogue adenylyl (β,γ-methylene)-diphosphonate (AMP-PCP) for ATP inhibited activation of ISGF3α, as did the addition of staurosporine (50 nM), a protein kinase inhibitor. Lower concentrations of staurosporine, which specifically inhibit protein kinase C's, had no effect on formation of ISGF3 (*9*). These results confirm the results of several in vivo studies that indicated that Ca^{2+} is not needed for IFN-α signal transduction but that the activation of a protein kinase is required (*10*–*13*). However, the use of the in vitro system eliminates any secondary changes in cellular metabolism that these inhibitors might have caused in vivo.

Although much progress has been made in understanding the regulation of certain hormone-stimulated signaling systems, those systems that are activated by growth factors and cytokines have been more difficult to analyze, in part because it has not been possible to duplicate any rapid response in vitro. IFN-α–induced ISGF3 formation is a very rapid event mediated by interaction of this cytokine with its cell surface receptor (*1*). We observed formation of ISGF3 with IFN-α–treated membranes mixed with the Sup fraction at 4°C; maximal formation of the complex required less than 30 min. Furthermore, the addition of staurosporine (50 nM) to membranes after incubation with IFN-α (30 min, 30°C) but before mixing with the Sup did not inhibit the formation of ISGF3 (*8*). The most likely explanation of our results is that the ISGF3α proteins are membrane-associated and are rapidly activated in response to IFN-α. The activation of the factor in the membrane fraction appears not to be directly coupled to its release from the membrane fraction because only after the addition of detergent was a factor released that led to the formation of ISGF3. This suggests that another enzymatic reaction (for example the action of a protease or a lipase) may be needed to permit the association of ISGF3α with ISGF3γ.

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IP_3 Receptor: Localization to Plasma Membrane of T Cells and Cocapping with the T Cell Receptor

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Immune responses in lymphocytes require cellular accumulation of large amounts of calcium (Ca^{2+}) from extracellular sources. In the T cell tumor line Jurkat, receptors for the Ca^{2+}-releasing messenger inositol 1,4,5-trisphosphate (IP_3) were localized to the plasma membrane (PM). Capping of the T cell receptor–CD3 complex, which is associated with signal transduction, was accompanied by capping of IP_3 receptors. The IP_3 receptor on T cells appears to be responsible for the entry of Ca^{2+} that initiates proliferative responses.

Signal transduction in many cellular systems, initiated by neurotransmitters, hormones, or antigens, involves an initial rapid rise in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) followed by a slower plateau phase; the initial peak, but not the second phase, is independent of extracellular Ca^{2+} (*1*). The phosphoinositide (PI) second messenger system is responsible for the initial release of intracellular Ca^{2+} by the generation of IP_3, which releases Ca^{2+} from specific receptor proteins at intracellular sites that are presumably associated with the endoplasmic reticulum (ER) (*2*).

In lymphocytes, the phase of elevated $[Ca^{2+}]_i$ derived from external sources is more prolonged than in most other types of cells and leads to proliferation (*3*). The mechanisms responsible for this receptor-mediated influx of extracellular Ca^{2+} have not been clarified. Inositol 1,3,4,5-tetrakisphosphate (IP_4) has been suggested as a mediator (*4*). Alternatively, IP_3 may mediate this Ca^{2+} entry either by communication of the IP_3-responsive ER vesicles with the plasma membrane (PM) or by direct actions of IP_3 at receptors located on the PM (*1*, *5*).

In Purkinje cells of the cerebellum, IP_3 receptors (IP_3R) are concentrated in discrete components of the ER; there is no evidence for the receptor on the PM, as

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revealed by immunohistochemistry and electron microscopy (6). In lymphocytes, however, patch clamp recordings revealed IP_3-sensitive Ca^{2+} channels in the PM (7, 8). Subcellular fractionation also suggests an association of the IP_3R with the PM in liver and adrenal tissues (9). In neurons, IP_3Rs in the PM may constitute only a small fraction of the total IP_3Rs, whereas in lymphocytes IP_3Rs in the PM may be more abundant and could be related to capping of the T cell receptor CD3 complex (10) and Ca^{2+} entry.

To determine whether affinity-purified goat antibodies to the IP_3R in nervous tissue (anti-IP_3R) (6, 11) could recognize a similar protein in lymphocytes, we analyzed Jurkat cells and crude thymus membranes by protein immunoblot. As in cerebellar preparations, a single 260-kD band was detected, reflecting the presence of authentic IP_3R protein (Fig. 1).

In intact cells, proteins in the PM can be selectively labeled with ^{125}I (12). Jurkat cells were iodinated with lactoperoxidase, the proteins separated by sialic acid–specific lectin column chromatography, and the samples immunodepleted with anti-IP_3R. A discrete 260-kD band, whose mobility was the same as that of the IP_3R and which was depleted by immunoprecipitation with anti-IP_3R, was iodinated (Fig. 2); thus, the protein was associated with the PM. The majority of the ER IP_3R is cytoplasmic, with membrane-spanning loop regions in the lumen of the ER (2). A similar structure presumably exists in the IP_3R of the PM, with only a small region of the protein exposed extracellularly. Accordingly, the number of tyrosine residues available for iodination may be few, which could account for the faintness of the surface-labeled band. Spectrin, a prominent protein of the membrane skeleton underlying the plasma membrane, was not labeled by this procedure; however, when the cells were permeabilized with methanol, prominent labeling of spectrin was evident (13).

Immunohistochemical examination of Jurkat cells revealed a selective association of IP_3R immunoreactivity with the PM (Fig. 3A). If a limited amount of antibody enters the cell, then IP_3R immunoreactivity might reflect ER that was adherent to the inside surface of the PM. However, the cells were impermeable because they excluded the dye tryptan blue (tryptan blue is excluded by intact living cells). In contrast, when we permeabilized the Jurkat cells with methanol, clumps of IP_3R immunoreactivity were apparent in the cell and also in association with the PM (Fig. 3D).

Surface receptor patching and capping are initial events of signal transduction in lymphocytes during which numerous proteins migrate to a single pole of the cell after stimulation with antigen or other proliferative stimuli (14). Elements of the ER do not participate in the capping response; however, some cytoskeletal elements associated with the PM do cap (15). In resting lymphocytes, staining for both the IP_3R and CD3 complex was uniformly distributed along the cell surface (Fig. 3, A, B, and E). Double immunofluorescent labeling was used to examine the distribution of the IP_3R and CD3 complex during capping. Patching and capping of Jurkat cells, initiated by exposure to concanavalin A (Con A), resulted in the comigration of IP_3R and CD3 complex immunoreactivity to a single pole of the lymphocytes (Fig. 3, E to J). Similar

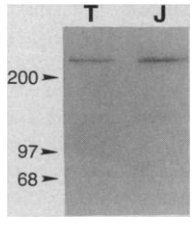


Fig. 1. Protein immunoblot detection of IP_3R with affinity-purified goat anti-IP_3R. Lane T, thymus homogenates; Lane J, Jurkat homogenates. Molecular weights are shown at the left (in kilodaltons).

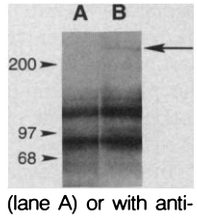


Fig. 2. Immunoprecipitation of ^{125}I-labeled plasma membrane IP_3R by anti-IP_3R. Arrowhead marks the position of immunoprecipitated IP_3R. Immunoprecipitation from Jurkat lymphocytes with nonspecific goat IgG (lane A) or with anti-IP_3R (lane B). Molecular weights are shown at the left (in kilodaltons).

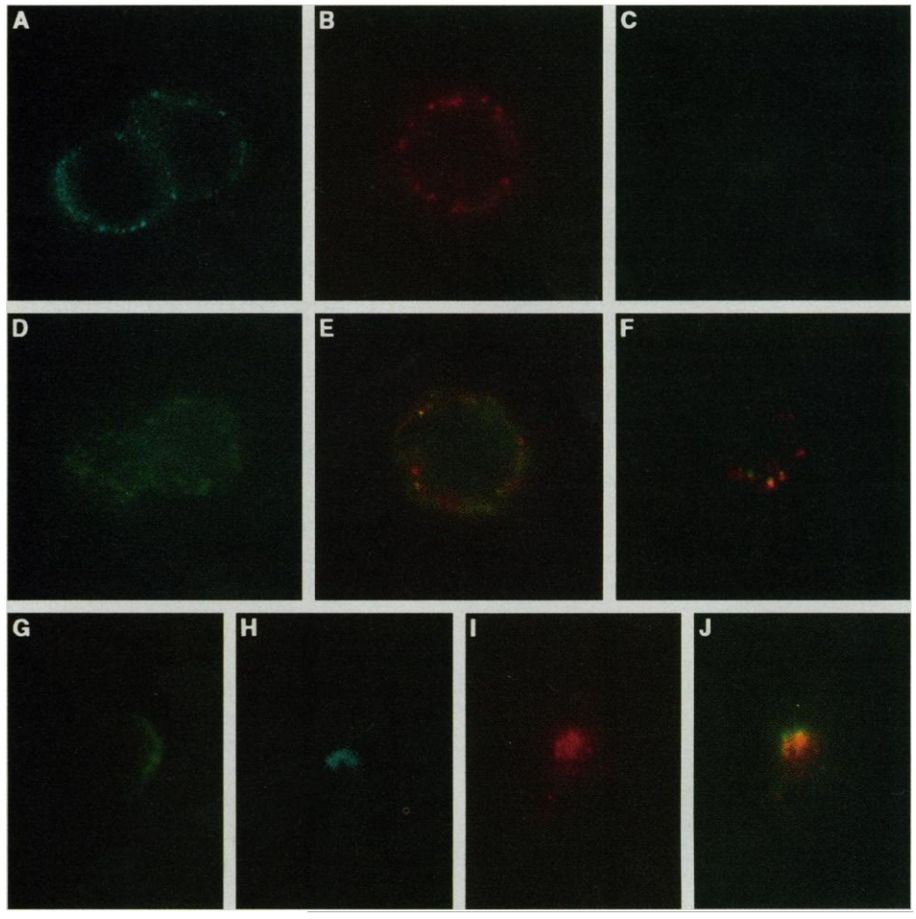
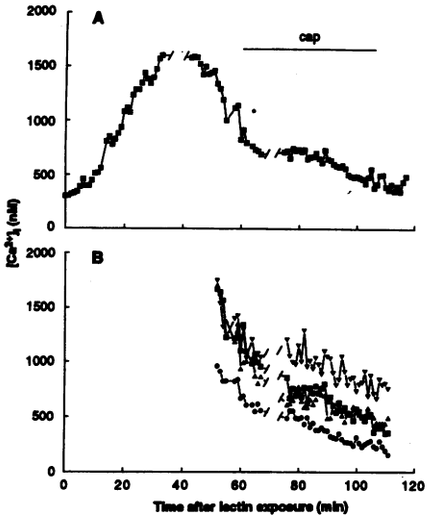


Fig. 3. Localization and topographical distribution of the IP_3R and CD3 complex surface antigens, as shown by immunofluorescence. Jurkat lymphocytes in Hanks basic salt solution, 1 mM Hepes, and FBS (2%) were stained with (**A**) affinity-purified rabbit anti-IP_3R and goat anti-rabbit fluorescein isothiocyanate (FITC) and (**B**) mouse monoclonal anti-CD3 and goat anti-mouse Texas Red. (**C**) Jurkat lymphocytes were stained with anti-IP_3R that had been blocked with an excess of purified IP_3R. (**D**) Permeabilized Jurkat cells (100% methanol, 5 min) were stained with anti-IP_3R. (E to J) Redistribution of IP_3R and CD3 complex antigens during the capping of surface receptors in Jurkat lymphocytes. (**E**) Double exposure showing fluorescence staining pattern of surface IP_3R (FITC/green) and CD3 complex (Texas Red). (**F**) Coincidental patching of IP_3R and CD3 complex after 30 min of stimulation with Con A. (**G**) Patching of IP_3R after 30 min of stimulation with Con A. (**H**) Capping of IP_3R after 60 min of stimulation with Con A. (**I**) Capping of CD3 complex after 60 min of stimulation with Con A. (**J**) Co-capping of IP_3R and CD3 complex after 60 min of stimulation with Con A.

immunohistochemical results were seen in T lymphocytes isolated from blood and lymph nodes.

Inositol 1,4,5-trisphosphate receptor localized in the cap may serve as a principal entry point for Ca^{2+} in proliferating lymphocytes. We measured the spatial distribution of $[Ca^{2+}]_i$ in mature peripheral T lymphocytes with the use of the Ca^{2+}-sensitive fluorescent indicator fura-2 and microscope-based digital video imaging methods (*16*) (Fig. 4). If IP_3Rs in the PM are responsible for the influx of Ca^{2+} during cap formation, then the $[Ca^{2+}]_i$ in the cap should be higher than in the rest of the cell. After exposure of the cells to lectin, the $[Ca^{2+}]_i$ rose sharply to five times the resting concentration, declined to a plateau at twice the resting concentration for 0.5 hour, and then fell to the resting concentration after 2 hours. At the start of the phase of sustained $[Ca^{2+}]_i$ elevation, a prominent cap formed at the pole of the lymphocyte where the $[Ca^{2+}]_i$ exceeded the concentration in other areas of the lymphocyte, and was maintained until the end of the recording period (Fig. 4B). Before the development of the cap, three of four subareas (*17*) in a single examined cell contained a similar $[Ca^{2+}]$ (Fig. 4B). By the start of the formation of the cap, the $[Ca^{2+}]$ in the cap region exceeded the concentration in the other three regions. The concentration of Ca^{2+} in all regions of the cell declined in parallel over the next 40 min, but the $[Ca^{2+}]$ in the cap region was always higher than in any other part of the cell. Pseudo-colored images of the $[Ca^{2+}]$ in a lymphocyte at various times after exposure to con A showed a higher $[Ca^{2+}]$ in the cap than in other parts of the cell, which persisted even while the mean $[Ca^{2+}]$ in the cell declined (Fig. 4, G and H). In six other con A–stimulated lymphocytes, the relative time course of $[Ca^{2+}]_i$ elevation and cap formation were similar. T cells stimulated with anti-CD3 displayed a similar distribution of $[Ca^{2+}]$.

In summary, several lines of evidence indicate that the IP_3R in Jurkat T cell lymphocytes is localized to the PM. Surface iodination labels the IP_3R, which is immunodepleted by anti-IP_3R after isolation by a sialic acid–specific lectin. Immunohistochemical analysis shows localization of the receptors to the PM and capping after stimulation with con A. Subcellular fractionation indicates localization of the Jurkat cell IP_3R to the PM (*18*). In thymus or PM fractions of Jurkat lymphocytes, IP_3R



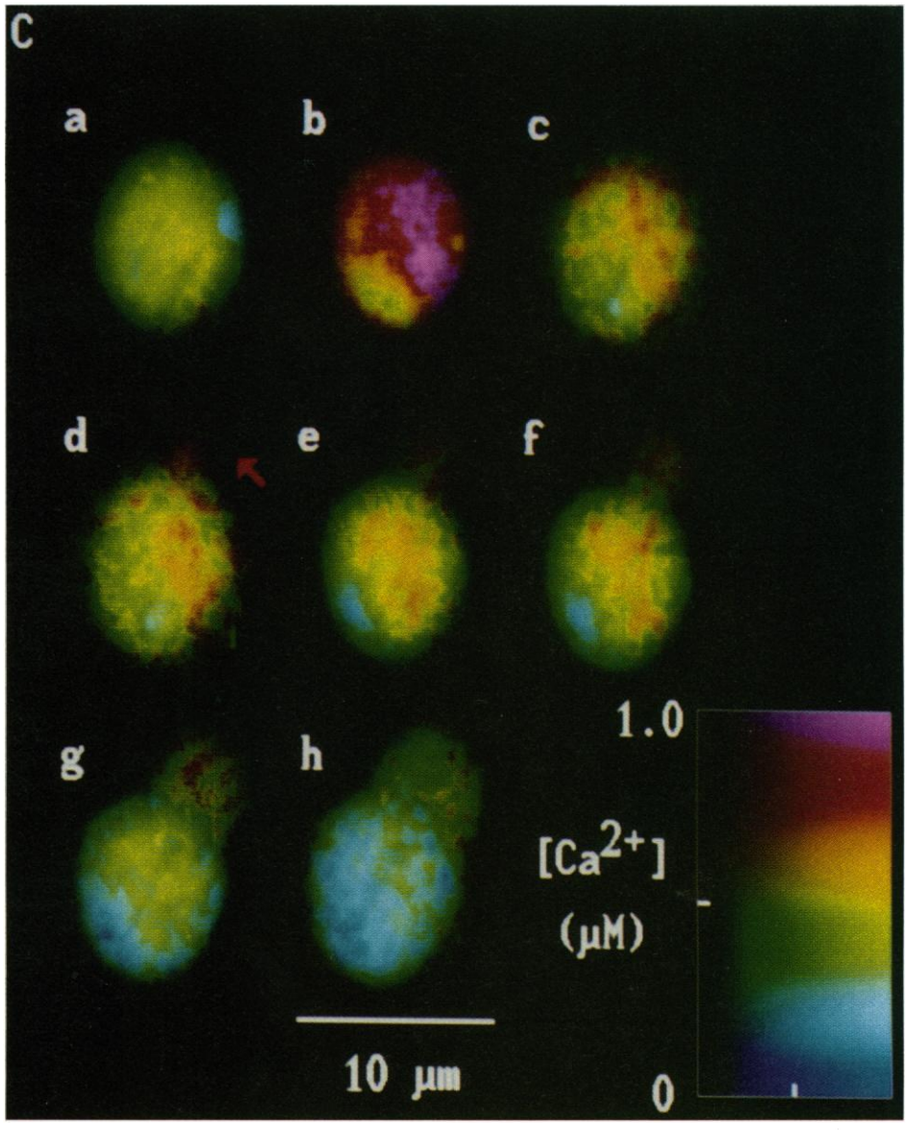


Fig. 4. (**A**) Time course of elevation of $[Ca^{2+}]$ in a single peripheral human T lymphocyte. Con A (15 μg/ml) was added to the bath at time zero. Each point represents the mean $[Ca^{2+}]$ determined by averaging all pixels in the calculated $[Ca^{2+}]$ image of the cell whose 358-nm fluorescence intensity image was above a given threshold. The horizontal line (labeled "cap") indicates the time during which a cap was evident in the 358-nm fluorescence image of this cell. (**B**) $[Ca^{2+}]$ in four regions of the same cell as in (A) monitored just before and during cap formation (*17*); (▼) (top line) subarea encompassing the cap region; (■, ▲, ●) subareas encompassing regions of the cell outside the cap. (**C**) Pseudo-colored, intensity-coded $[Ca^{2+}]$ images in a Con A–stimulated lymphocyte during different stages of cap formation. Ca, 7 min after addition of Con A; Cb, 29 min after stimulation with Con A; Cc, 42 min after stimulation with Con A; Cd, 43 min after stimulation with Con A; arrowhead indicates formation of cap; Ce, Cf, Cg, Ch, 44, 46, 51, and 55 min, respectively, after stimulation with Con A. The color and intensity scale indicates $[Ca^{2+}]$ vertically and the relative concentration of fura-2 horizontally (both scales are linear).

contains substantial amounts of sialic acid (*18*), a sugar selectively associated with proteins of the PM that does not occur in proteins of intracellular membranes (*19*).

An association of the IP_3R with the PM may not be restricted to lymphocytes. We have demonstrated the presence of sialic acid in highly purified preparations of IP_3R from olfactory cilia, although concentrations are less than in those from preparations of lymphocytes (*18*). In olfactory cilia, immunohistochemical techniques that use confocal and electron microscopy reveal the presence of IP_3R localized to the PM (*20*). Localization of the IP_3R to liver and adrenal tissues has also been suggested by subcellular fractionation studies (*8*).

In lymphocytes, IP_3R in the PM may account for the entry of extracellular Ca^{2+} after proliferative stimuli. Depletion of internal Ca^{2+} stores might influence Ca^{2+} entry (*21*) by influencing IP_3-gated Ca^{2+} channels in the PM. No evidence has been obtained in lymphocytes for other mechanisms of Ca^{2+} entry, such as voltage-dependent Ca^{2+} channels (*8*, *22*) and ryanodine receptors. Because proteins in the PM do not also occur in intracellular membranes (and vice versa) (*23*), IP_3R in the PM may differ structurally from IP_3R in the ER, as suggested by their different inositol phosphate specificities and sugar content (*18*). Alternative splicing (*24*) and multiple genes (*25*) may account for this diversity.

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16. Human or rat T lymphocytes were obtained by Ficoll-Hypaque density centrifugation and placed in culture medium [RPMI 1640 containing heat-inactivated fetal bovine serum (FBS) (10%) supplemented with streptomycin (100 μg/ml), Fungizone (0.25 μg/ml), and con A (0.25 μg/ml)]. T lymphocytes were loaded with 2 μM Fura-2-AM (Molecular Probes) [2 mM stock solution in dimethyl sulfoxide (DMSO)] by incubation for 30 min at 25°C in 145 mM NaCl, 5 mM KCl, 0.1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM glucose, and 15 mM Hepes (pH 7.5). After being washed with buffer, the cells were stimulated with the addition of con A (15 μg/ml). Fluorescence images at 380 (F_{380}) and 358 nm (F_{358}) provided the $[Ca^{2+}]_i$ [G. Grynkiewicz, M. Poenie, R. Tsien, *J. Biol. Chem.* **260**, 3440 (1985)]. Out-of-focus fluorescence was removed numerically in order to improve the spatial resolution of the Ca^{2+} images [K. R. Castleman, *Digital Image Processing* (Prentice-Hall, Englewood Cliffs, NJ, 1979); D. A. Agard, *Annu. Rev. Biophys. Bioeng.* **13**, 191 (1984); J. R. Monck, A. F. Oberhauser, T. J. Keating, J. M. Fernadez, *J. Cell Biol.* **116**, 745 (1992)]. The in-focus and defocused objective point spread functions were determined [Y. Hiraoka, J. W. Sedat, D. A. Agard, *Biophys. J.* **57**, 325 (1990)]. The emitted fluorescence of the beads was measured at 510 nm with the same apparatus and filters as used in the fura-2 imaging.
17. To determine the $[Ca^{2+}]$ in discrete regions of a single cell, we selected rectangular, nonoverlapping subareas on the 358-nm fluorescence intensity image. Pseudo-colored images were encoded for intensity (proportional to the concentration of fura-2 in the cell) [R. Y. Tsien and A. T. Harootunian, *Cell Calcium* **11**, 93 (1990)]. During the time the cap was evident, the mean (±SEM) $[Ca^{2+}]$ was 1011 ± 33 nM in the cap region and 767 ± 48 nM, 732 ± 45 nM, and 440 ± 30 nM in three subareas outside the cap region (Fig. 4B). The $[Ca^{2+}]$ in the cap region was significantly higher ($P < 0.001$) than in the two regions of intermediate $[Ca^{2+}]$. The region directly opposite the cap had a consistently lower $[Ca^{2+}]$ than any other region throughout the experiment. The sustained phase of increased $[Ca^{2+}]_i$ was abolished with the addition of EGTA (10 mM), and there was no evidence for regions of locally elevated $[Ca^{2+}]$. Removal of EGTA restored the elevated $[Ca^{2+}]_i$, which demonstrated the dependence of elevated $[Ca^{2+}]_i$ on external Ca^{2+}. The mean concentration of fura-2 in the cap region was 54.6% of the concentration in regions outside the cap. In all cells, the mean elevation of $[Ca^{2+}]$ in the cap region was 36.5 ± 6.4% (mean ± SEM, $n = 7$, $P < 0.001$) greater than in regions outside the cap.
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