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Degradation of a Calcium Influx Factor (CIF) Can Be Blocked by Phosphatase Inhibitors or Chelation of Ca^{2+}*

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In many cell types, depletion of Ca^{2+} stores causes activation of Ca^{2+} influx by a mechanism whose molecular basis remains unclear. We recently described a new messenger that is released by empty Ca^{2+} stores and that activates Ca^{2+} influx in heterologous cells (Randriamampita, C. & Tsien, R. Y. (1993) *Nature* 364, 809–814). This factor, provisionally named CIF (for Ca^{2+} influx factor), seems to be a small nonprotein factor possessing a phosphate group. Meanwhile Parekh *et al.* reported that okadaic acid, an inhibitor of protein phosphatases 1 and 2A, potentiates Ca^{2+} influx in *Xenopus* oocytes (Parekh, A. B., Terlau, H. & Stühmer, W. (1993) *Nature* 364, 814–818). A link between these two observations is presented in this paper. We show that in astrocytoma cells, okadaic acid and cyclosporin A (an inhibitor of calcineurin) both potentiate the Ca^{2+} elevations due to low doses of CIF, thapsigargin, or carbachol. In lymphocytes, okadaic acid potentiates the Ca^{2+} elevations due to low doses of phytohemagglutinin and increases the amount of extractable CIF. CIF degradation can be observed in cell-free homogenates of lymphocytes and is prevented by the above phosphatase inhibitors, an effect that can at least partly explain their potentiation of Ca^{2+} influx. CIF degradation is also prevented by lowering free Ca^{2+} concentrations, which could be a feedback mechanism to enhance Ca^{2+} influx when cells are depleted of Ca^{2+}.

In many cell types, depletion of intracellular Ca^{2+} stores causes compensatory influx of Ca^{2+} across the plasma membrane. The mechanisms that link these two events have been the subject of widespread speculation and intensive investigation (1–5). Recently, two very different lines of experiments have suggested that empty Ca^{2+} stores may release a diffusible messenger that transmits a signal to the plasma membrane to activate influx pathways. We recently showed that Jurkat lymphocytes contain a soluble factor of low molecular weight that can induce Ca^{2+} influx when applied extracellularly to various other mammalian cell types (6). This factor does not match any of a wide variety of known messengers, but seems to contain phosphate and a periodate-sensitive group. Until its chemical structure is completely defined, it is provisionally referred to as CIF[1] (for Ca^{2+} influx factor). CIF is stored in digitonin-resistant organelles of resting cells and is released into the cytoplasm upon stimulation, although pathways for synthesis and breakdown of CIF presumably also exist. Meanwhile, Parekh *et al.* (7) reported that in *Xenopus* oocytes, depletion of intracellular Ca^{2+} stores caused Ca^{2+} influx, measured indirectly as a Ca^{2+}-activated Cl^- current. If the patch of plasma membrane was ripped off the oocyte, the current immediately disappeared, but could be reactivated if the patch was reinserted into the oocyte cytoplasm, as if the cytoplasm contained a soluble ligand that activated the influx. The current could be enhanced by okadaic acid (OA), an inhibitor usually considered specific for protein phosphatases 1 and 2A (8).

The enhancement found by Parekh *et al.* (7) might be attributed to an effect of OA on various stages between the emptying of the Ca^{2+} stores and the activation of the Ca^{2+}-activated Cl^- channels. To decide whether something like CIF is involved in this effect, an obvious experiment is to test in intact mammalian cells whether OA and other phosphatase inhibitors can enhance Ca^{2+} entry, activated either by depletion of endogenous Ca^{2+} stores or by application of exogenous CIF. If so, then one could test more directly *in vitro* the tempting hypothesis that cells possess enzymes that degrade CIF, but are directly or indirectly inhibited by OA. Inhibition of such enzymes could then lead to an increase in CIF concentration, which would provide a mechanism for potentiating Ca^{2+} influx, but would not exclude other possible effects of OA. Attempts to prevent breakdown could give valuable hints about the metabolism of CIF even while its detailed structure remains the subject of separate investigations.

MATERIALS AND METHODS

Cell Culture and $[Ca^{2+}]_i$ Measurements—1321N1 astrocytoma cells and Jurkat lymphocytes were grown as described previously (6). Astrocytoma cells were plated on coverslips 1 day before experiments. Astrocytoma and Jurkat cells were loaded with 300 and 500 nM fura-2/AM, respectively, for 30 min. The usual medium for $[Ca^{2+}]_i$ measurements was Dulbecco's phosphate-buffered saline (D-PBS) with 1 mM $CaCl_2$, 11 mM glucose, and 20 mM HEPES. $[Ca^{2+}]_i$ measurements were performed with an IMSTAR imaging system (9) and are averages of 10 cells in the same field, sampled at 5-s intervals. Integrated responses were measured as the area of the average $[Ca^{2+}]_i$ increase during the 6–8 min after stimulation. For histograms, individual values correspond to different dishes. To pool results from different experiments, averages were expressed as percent of control values. Averages are given ±S.E. Values have been statistically compared using Student's t test with $p < 0.05$ as the criterion for significance.

Chemicals—Okadaic acid (Na^+ salt), 1-norokadaone, and calyculin A were from LC Laboratories; fura-2/AM was from Molecular Probes, Inc.; carbachol, PHA, and D-PBS were from Sigma; thapsigargin was from Calbiochem; and cyclosporin A was from Sandoz.

Cell Extract Preparation—For experiments presented in Fig. 1, cell extract was prepared by acidic treatment of unstimulated Jurkat cells as described previously (6), except that the pH of D-PBS was adjusted with 300 mM NaOH or HCl, fatty acid-free albumin was not added, and treatment with hexokinase was omitted because astrocytoma cells were insensitive to ATP. The extract was passed through a small reverse-phase silica cartridge (C_{18} SPICE, Rainin Instrument Co. Inc.) for a

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[1] The abbreviations used are: CIF, calcium influx factor; OA, okadaic acid; D-PBS, Dulbecco's phosphate-buffered saline; PHA, phytohemagglutinin; CsA, cyclosporin A.

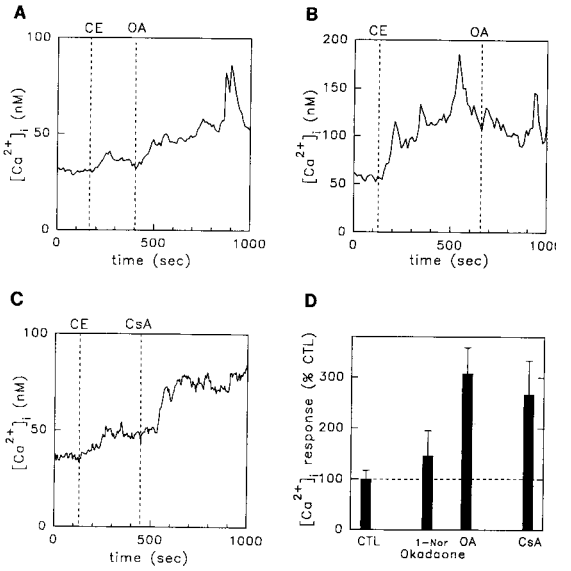

FIG. 1. **Protein phosphatase inhibitors potentiate the $[Ca^{2+}]_i$ response to cell extract.** *A–C*, Jurkat cell extract was added (1:20 dilution in *A*, 1:2 in *B*, and 1:12 in *C*) to fura-2-loaded astrocytoma cells where indicated by the *dashed lines* marked *CE*. 50 nM OA (*A*), 100 nM OA (*B*), or 100 nM CsA (*C*) was added a few minutes later (*second dashed lines*). *D*, astrocytoma cells were stimulated with submaximal doses of Jurkat extract (diluted 4–8 times) either under control conditions (*CTL*; no pretreatment) or after 3 min of incubation with 500 nM 1-norokadaone, 100 nM OA, or 1 μM CsA, each normalized to its respective control. For each condition, values (integrated from 0 to 6–8 min after cell extract addition) correspond to the mean of six to eight individual dishes resulting from three independent experiments.

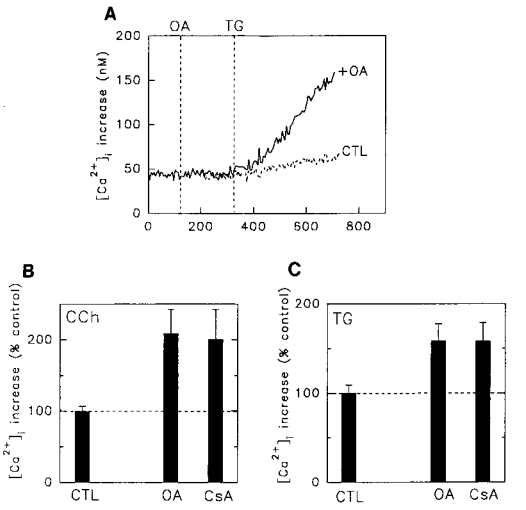

FIG. 2. **Protein phosphatase inhibitors potentiate the $[Ca^{2+}]_i$ responses to low doses of carbachol and thapsigargin.** Fura-2-loaded astrocytoma cells were stimulated with 1 nM thapsigargin (*TG*; *A* and *C*) or 1 μM carbachol (*CCh*; *B*) either under control conditions (*CTL*; no pretreatment) or after 3 min of incubation with 100 nM OA or 1 μM CsA. An example of such experiment is presented in *A*. Pooled results resulting from three independent experiments are shown in *B* and *C*. Individual response magnitudes have been normalized to the control. For each condition, values have been integrated from 0 to 6–8 min after cell extract addition and correspond to the mean of 10–12 individual dishes.

quick cleanup before pH neutralization and 4–8-fold dilution into the medium containing astrocytoma cells.

For *in vitro* degradation experiments, Jurkat lymphocytes (0.3-ml packed volume) were stimulated with PHA (20 μg/ml) for 10 min in 1 ml of the following medium: 145 mM NaCl, 5 mM KCl, 5 mM HEPES, 1 mM $MgCl_2$, and no added Ca^{2+}. The cells were then sonicated for 2.5 min (Deltasonic 011C) and finally centrifuged. The pellet was discarded, and the supernatant was isolated, aliquoted, and kept at room temperature (22–25 °C) for different periods of time to allow degradation of CIF activity. Incubations were terminated by rapid freezing. In some runs, okadaic acid (500 nM), cyclosporin A (CsA; 1 μM), or EGTA (1 mM) was added to the samples at the beginning of the incubation period. (The OA and CsA concentrations were set higher than in the intact cell experiments because the sonicated homogenates were derived from much more concentrated cell suspensions.) The same inhibitor concentrations were then added to other aliquots at the end of the incubation period, just before freezing, so that each pair of samples would have the same final composition of additives. For $[Ca^{2+}]_i$ measurements, 150 μl of extract were rapidly thawed and added to astrocytoma cells in 450 μl of standard medium with 1 mM Ca^{2+}.

RESULTS

Phosphatase Inhibitors Potentiate $[Ca^{2+}]_i$ Increases Induced by Low Doses of CIF—The effect of OA was first tested with cell extract, which had previously been shown to induce only Ca^{2+} influx in astrocytoma cells (6). We used threshold concentrations of cell extract, adjusted to give no or only small $[Ca^{2+}]_i$ increases by themselves. The addition of OA (50 nM) then produced an immediate further increase in $[Ca^{2+}]_i$ (Fig. 1*A*). This effect resulted from a potentiation of divalent cation entry when assayed by accelerated influx of Mn^{2+} (data not shown). OA alone (up to 500 nM) did not affect the basal $[Ca^{2+}]_i$ level (data not shown). On the other hand, higher sustained $[Ca^{2+}]_i$ increases produced by larger concentrations of cell extract were not augmented by 100 nM okadaic acid (Fig. 1*B*).

OA has been described as a specific inhibitor of protein phosphatases 1 (EC_{50} = 20 nM) and 2A (EC_{50} = 0.2 nM), the EC_{50} for protein phosphatase 2C being much higher (5 μM) (8). Several structurally unrelated phosphatase inhibitors with different specificities were tested on the response to threshold concentrations of CIF. CsA (100 nM), whose complex with cyclophilin specifically inhibits calcineurin (protein phosphatase 2B) (10), was comparable to OA (Fig. 1*C*) at potentiating $[Ca^{2+}]_i$ increases. Calyculin A (50 nM), which inhibits protein phosphatase 1 as well as protein phosphatase 2A (11), was also as effective as OA (data not shown). However, this concentration of calyculin A seemed to be highly toxic because the cells developed blebs within a few minutes. For this reason, calyculin A was not further studied.

The potentiating effects of these inhibitors were equally evident if OA (100 nM) or CsA (1 μM) was added 3 min before the submaximal dose of Jurkat extract. 1-Norokadaone, a structural analogue of OA unable to inhibit protein phosphatases (12), was also tested as a control for nonspecific effects. Fig. 1*D* shows that OA and CsA amplified the $[Ca^{2+}]_i$ response by a factor of ~3. Both were statistically different from the control without inhibitors. 1-Norokadaone even at 500 nM did not produce a statistically significant potentiation.

Phosphatase Inhibitors Potentiate $[Ca^{2+}]_i$ Increases Induced by Low Concentrations of Carbachol or Thapsigargin Acting on Astrocytes or Phytohemagglutinin on Lymphocytes—If conventional agonists that deplete intracellular Ca^{2+} stores use endogenous CIF to trigger Ca^{2+} entry, those latter responses should also be enhanced by OA and CsA. Low concentrations of carbachol (1 μM) or thapsigargin (1 nM) only partially emptied the Ca^{2+} stores; the subsequent sustained $[Ca^{2+}]_i$ increases were likewise submaximal. The effects of phosphatase inhibitors were tested in experiments similar to those presented in Fig. 1*C*. As presented in Fig. 2*A*, the response to 1 nM thapsigargin (*dashed line*) was increased by 3 min of incubation with OA (*solid line*). The $[Ca^{2+}]_i$ increases induced by the above low doses of carbachol (Fig. 2*B*) or thapsigargin (Fig. 2*C*) were clearly potentiated by OA (100 nM) and CsA (1 μM). These values were statistically different from the control. OA and CsA did not affect the responses to high concentrations of carbachol or thapsigargin (data not shown). Contrary to what has been

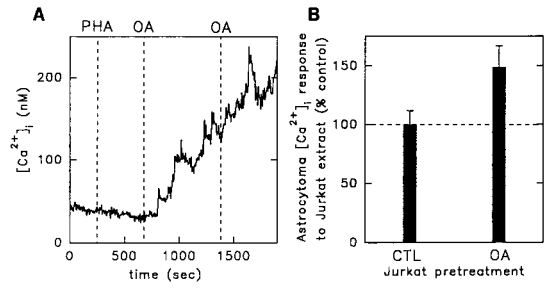

FIG. 3. **Okadaic acid increases CIF concentration in PHA-stimulated Jurkat T cells.** *A*, fura-2-loaded Jurkat cells were stimulated with 5 μg/ml PHA (*first dashed line*). OA (100 nM each) was added twice (*second* and *third dashed lines*). *B*, acidic extract was prepared from Jurkat T cells stimulated with 5 μg/ml PHA in the absence (control (*CTL*)) or presence of 500 nM OA for 15 min. The same amount of OA was added to the control cells just before centrifugation to equalize OA carryover. $[Ca^{2+}]_i$ elevations measured in fura-2-loaded astrocytoma cells were normalized to the mean of the control. Each point corresponds to the mean of seven dishes from two independent experiments. The difference is statistically significant to $p < 0.05$ (Student's t test).

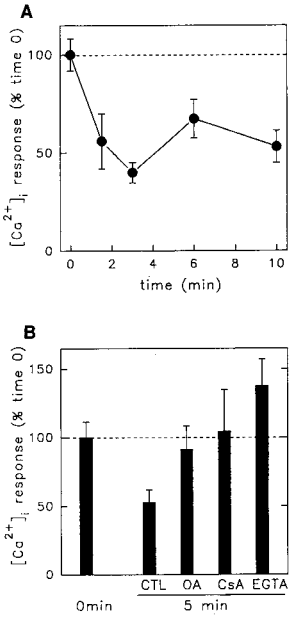

FIG. 4. **Time course and pharmacology of CIF degradation *in vitro*.** *A*, cell extract was prepared from sonicated Jurkat lymphocytes and kept for variable times (shown on the *abscissa*) at room temperature. Degradation was stopped by freezing. Each ordinate is the mean of 12–27 $[Ca^{2+}]_i$ increases in astrocytoma cells in response to equal dilutions of the incubated extract from three to six independent experiments. Values have been normalized to those obtained with no added incubation time before freezing. *B*, cell extract activity was measured for 0 or 5 min of incubation under control conditions (*CTL*) or for 5 min with 500 nM OA, 1 μM CsA, or 1 mM EGTA. Values have been normalized as described for *A*. Each bar corresponds to the mean of 7–18 dishes from two to three independent experiments.

reported for long-term treatment of HeLa cells with high doses of OA (13), we never observed OA to inhibit Ca^{2+} influx in astrocytes.

Such potentiations of threshold stimuli are not restricted to astrocytoma cells. As shown in Fig. 3*A*, responses of Jurkat lymphocytes to a low dose of phytohemagglutinin (5 μg/ml), a classic phosphoinositide-mobilizing agonist, are greatly potentiated by subsequent addition of 100 nM OA. This dose of OA by itself did not elevate $[Ca^{2+}]_i$ above resting levels (data not shown).

These results show that the response to threshold concentrations of CIF, either exogenously applied or endogenously released from the Ca^{2+} stores, is synergistically potentiated by protein phosphatase inhibitors. Two main explanations can be proposed, which are not mutually exclusive. Either OA and CsA enhance the sensitivity to CIF of its targets, for example the Ca^{2+} channels, or they directly increase CIF concentration, for example by inhibiting its degradation in the cytoplasm. To begin to discriminate between these two possibilities, experiments were designed to test whether OA affected CIF concentrations in intact cells and in cell-free homogenates. Such experiments were performed with Jurkat lymphocytes because such nonadherent cells were much easier than astrocytoma cells to culture in the necessary large quantity and high density.

Okadaic Acid Induces an Increase in CIF Concentration in Stimulated Jurkat Lymphocytes—To test whether OA can increase the amount of CIF activity extractable from intact cells, the lymphocytes (0.3-ml packed volume) were stimulated for 15 min with 5 μg/ml PHA in 1 ml of Ca^{2+}-free medium in the presence or absence of 500 nM OA. To equalize any carryover of OA onto the astrocytoma cells used to assay CIF, the same concentration of OA was added to the control lymphocytes for 1–2 min before both samples were centrifuged. Each pellet was then resuspended as usual in 1 ml of acid to disrupt the cells and to stop metabolism. The extracts were neutralized after 15 min and assayed for CIF activity. As shown in Fig. 3*B*, the CIF content was statistically higher in cells stimulated in the presence of okadaic acid compared with the control. A greater difference between both conditions might have been obtained by using larger doses of PHA or OA to compensate for the very high cell density and probable depletion of PHA and OA from the initial incubation medium. Nevertheless, this result provides evidence that OA at least modestly increases the amount of CIF in weakly stimulated intact cells. The possibility that OA may inhibit CIF degradation was more directly investigated by *in vitro* experiments.

Phosphatase Inhibitors Inhibit Degradation of CIF in Vitro—To see whether CIF can be degraded by cellular constituents, cell extracts were prepared by ultrasonic disruption of lymphocytes that had been stimulated for 10 min with PHA in a medium lacking inorganic phosphate and Ca^{2+}. Sonication, unlike our usual acidic extraction, should preserve the activity of enzymes that might synthesize or degrade CIF. The extract from sonicated cells was held for varying periods at room temperature and then rapidly frozen and eventually assayed on astrocytes. As shown in Fig. 4*A*, the activity initially present in the cell extract rapidly decreased upon standing, showing that CIF can be degraded under these *in vitro* conditions. Most of the decline took place in the first 3 min after extract isolation, after which the activity then leveled off around 50% of its initial value. The time to reach plateau varied in different experiments from 1.5 to 3 min.

The pharmacology of the degradation (Fig. 4*B*) was then investigated using the above protocol, but with drugs added just before *versus* after room temperature incubation for 5 min. This mode of comparison ensured that the same amount of drug was carried over into the assay on the responder astrocytes. As in Fig. 4*A*, ~50% of the initial activity remained after 5 min at room temperature with no inhibitors added. However, the presence of OA (500 nM) or CsA (1 μM) during incubation completely prevented net degradation. Because calcineurin has been reported to be sensitive to Ca^{2+} and calmodulin (8), the effect of Ca^{2+} deprivation on CIF degradation was also tested. For all the *in vitro* experiments presented above, Ca^{2+} was not buffered and was probably several μM. The addition of EGTA (1 mM) at the beginning of the incubation also completely abolished the run-down of CIF activity. The activities left after 5 min of incubation in the presence of OA, CsA, or EGTA were all

statistically different from the incubation without additives, but similar to the initial activity before incubation.

DISCUSSION

The aim of this study was to determine whether phosphatases control Ca^{2+} influx in mammalian cells and are implicated in metabolic inactivation of a putative messenger mediating Ca^{2+} influx. Our results show that protein phosphatase inhibitors potentiate Ca^{2+} influx during submaximal stimulation via conventional receptors in the plasma membrane, by thapsigargin blockade of endoplasmic reticulum pumps, or by direct application of CIF. CIF assays examining the effects of phosphatase inhibitors in intact cells or sonicated homogenates suggest that this potentiation can be at least partly explained as an increase in CIF concentration due to prevention of CIF degradation, which is also Ca^{2+}-dependent. We doubt that phosphatase inhibitors act mainly by stimulating CIF synthesis because it would be surprising if such stimulation so exactly canceled the degradation observed under control conditions. However, we cannot exclude that mechanisms other than protection of CIF contribute in intact cells to the potentiation of the $[Ca^{2+}]_i$ response. Furthermore, because CIF activity was assayed in a crude cell extract rather than as a purified single species, the present results cannot yet exclude the possibility that these modulations involve more than one interacting molecular species in the extract.

Several points still remain unclear. First, the molecular targets of OA and CsA are not identified. These drugs have been reported to be specific inhibitors of protein phosphatases (8, 14). However, current evidence indicates that CIF is a small nonprotein factor. Several possibilities can be proposed to reconcile those two points. 1) Protein phosphatases could dephosphorylate and activate a CIF-degrading enzyme, which might or might not itself be a phosphatase. Because OA and CsA are not supposed to act on the same protein phosphatase, one would presume that the CIF-degrading enzyme is sensitive to protein phosphatase 1 (or protein phosphatase 2A) *and* to protein phosphatase 2B. A more complex cascade can also be proposed where this enzyme would be dephosphorylated only by protein phosphatase 1 and where protein phosphatase 2B would modulate the activity of protein phosphatase 1 by dephosphorylating inhibitor-1. 2) Dephosphorylations of nonprotein substrates by protein phosphatases 1 and 2B have been reported (15). However, these observations result from *in vitro* experiments, not yet in intact cells. 3) Finally, it is possible that OA as well as CsA are able to inhibit enzymes or phosphatases other than protein phosphatases. Although other defined phosphatases so far tested are insensitive to these agents, one cannot exclude that such a phosphatase could exist. Whatever are the relevant immediate targets of OA and CsA, our results in intact cells suggest that enzymes involved in CIF degradation are active in unstimulated cells or at least at resting $[Ca^{2+}]_i$ levels.

CIF degradation can be demonstrated in a cell-free homogenate, always appearing within the first 3 min after extraction and never proceeding beyond 60% reduction of the initial activity. Because the degradation can be blocked by OA, CsA, or EGTA, it probably results from an enzymatic process and not from a spontaneous breakdown of CIF, which is stable in the absence of proteins (6). The inability to degrade more than 60% of the initial activity could be due to run-down of the enzyme(s) involved after cell disruption, so that CIF molecules that have not been metabolized in the first few minutes will never be. Because no reducing agents were added to the extract, one possible inactivation mechanism could be oxidation. Another possibility is that some fresh synthesis of CIF takes place in parallel to CIF degradation. Two observations argue in favor of this latter hypothesis. In the presence of EGTA, the activity after 5 min of incubation seemed to be even greater than at time 0, as if the concentration of CIF had increased. Even without inhibitors, CIF activity seems to increase slightly after its initial decline. This augmentation varied from one experiment to another and may reflect a delayed appearance of fresh CIF in the extract. The pharmacology of the synthesis has not been yet established. In intact stimulated cells, Ca^{2+} influx can last for minutes or hours, suggesting that if degradation events take place, they have to be balanced by the appearance of CIF in the cytoplasm. Under our cell-free conditions, with enough Ca^{2+} to allow microsomal stores to refill, degradation is presumably somewhat favored compared with synthesis.

In these *in vitro* experiments, ~2–3 min were required for a 50% loss of activity. This value appears to be high compared with shorter values that have been reported in the literature for the decline of the Ca^{2+} influx after Ca^{2+} store refilling (*e.g.* see Ref. 16). Perhaps our *in vitro* conditions are not optimal for enzymatic degradation. Simple dilution in preparing the homogenate would tend to slow enzymatic reactions. However, CIF degradation by phosphatases might not be the only mechanism to remove CIF from the cytoplasm when Ca^{2+} stores are allowed to refill. Reuptake of CIF into its organellar storage sites (perhaps the endoplasmic reticulum) may also contribute. Phosphatase action may be more important during cell stimulation for adjusting the concentration of CIF and the amplitude of Ca^{2+} influx especially for submaximal conditions of stimulation. Indeed, with saturating doses of carbachol, thapsigargin, or cell extract, the Ca^{2+} response was not modified by protein phosphatase inhibitors. Furthermore, because CIF degradation seems to be very Ca^{2+}-dependent, phosphatases may play a role in negative feedback induced by $[Ca^{2+}]_i$ on Ca^{2+} influx, which has been observed in many cell types with a time course compatible to the degradation observed here (17–20).

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