Activation of ERK by Ca^{2+} store depletion in rat liver epithelial cells

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Maloney, Judith A., Oxana M. Tsygankova, Lijun Yang, Qiuyang Li, Agnieszka Szot, Kemal Baysal, and John R. Williamson. Activation of ERK by Ca^{2+} store depletion in rat liver epithelial cells. *Am. J. Physiol.* 276 (*Cell Physiol.* 45): C221–C230, 1999.—In rat liver epithelial (WB) cells, Ca^{2+} pool depletion induced by two independent methods resulted in activation of extracellular signal-regulated protein kinase (ERK). In the first method, Ca^{2+} pool depletion by thapsigargin increased the activity of ERK, even when rise in cytosolic Ca^{2+} was blocked with the Ca^{2+} chelator BAPTA-AM. For the second method, addition of extracellular EGTA at a concentration shown to deplete intracellular Ca^{2+} pools also increased ERK activity. In each instance, ERK activation, as measured by an immunocomplex kinase assay, was greatly reduced by the tyrosine kinase inhibitor genistein, suggesting that Ca^{2+} store depletion increased ERK activity through a tyrosine kinase pathway. The intracellular Ca^{2+}-releasing agent thapsigargin increased Fyn activity, which was unaffected by BAPTA-AM pretreatment, suggesting that Fyn activity was unaffected by increased cytosolic free Ca^{2+}. Furthermore, depletion of intracellular Ca^{2+} with EGTA caused inactivation of protein phosphatase 2A and protein tyrosine phosphatases. ANG II-induced activations of Fyn, Raf-1, and ERK were augmented in cells pretreated with BAPTA-AM, but ANG II-induced expression of the dual-specificity phosphatase mitogen-activated protein kinase phosphatase-1 was blocked by BAPTA-AM pretreatment. Together these results indicate that ERK activity is regulated by the balance of phosphorylation vs. dephosphorylation reactions in intact cells and that the amount of Ca^{2+} stored in intracellular pools plays an important role in this regulation.

Raf-1; mitogen-activated protein kinase; Fyn; protein phosphatase

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VARIOUS GROWTH FACTORS AND G protein-linked agonists initiate the release of Ca^{2+} from intracellular stores by generating the second messenger D-*myo*-inositol 1,4,5-trisphosphate. An influx of extracellular Ca^{2+} also occurs, but the mechanism has not yet been fully elucidated. Recently, the capacitive Ca^{2+} entry model has been proposed to explain the hormone-induced activation of Ca^{2+} influx (36). This model predicts that depletion of intracellular Ca^{2+} pools opens store-operated Ca^{2+} channels in the plasma membrane. This has been suggested to occur via the activation of one or more tyrosine kinases (26, 41). Conversely, Ca^{2+} store repletion may activate tyrosine phosphatases (42, 51). These observations imply that the degree of filling of the hormone-sensitive Ca^{2+} pools is involved in the regulation of protein tyrosine phosphorylation within the cell.

The extracellular signal-regulated protein kinase (ERK) cascade is an important signaling pathway involved in cell growth and development that is also controlled by the activity of tyrosine kinases and phosphatases. Receptor and nonreceptor tyrosine kinases constitute initiating events of the ERK signaling cascade (1, 32). These tyrosine kinases are involved in the phosphorylation of the adaptor protein Shc. A second adaptor protein, growth factor receptor-bound protein 2 (GRB2), binds to tyrosine phosphorylated Shc through its SH2 domain (38), bringing the guanine nucleotide exchange factor son of sevenless to the membrane, where it causes activation of Ras (27). Ras, in turn, activates Raf-1, a serine/threonine kinase (31), which phosphorylates and activates the dual-specificity protein kinase mitogen-activated protein kinase (MAP kinase) kinase (MEK) (25). MEK, by phosphorylating ERK on tyrosine and threonine residues, is the direct upstream activator of ERK (12). Although the activation of Src kinases is believed to be the major upstream pathway for Ras-dependent activation of ERK, previous studies have also demonstrated the involvement of Ca^{2+} in this pathway (11, 14, 37).

ERK is inactivated by protein phosphatases in the cell. There are several classes of phosphatases, including serine/threonine phosphatases, tyrosine phosphatases, and dual-specificity phosphatases, some of which have been shown to directly dephosphorylate and inactivate ERK in vitro (3, 22, 40). Furthermore, activation of ERK in intact cells occurs either when protein phosphatase 2A (PP-2A) and protein phosphatase 1 (PP-1) are inhibited with okadaic acid (4, 10) or when protein tyrosine phosphatases (PTPases) are inhibited with pervanadate (56). These findings indicate that phosphatases are involved in the suppression of constitutive ERK activity. In contrast, the dual-specificity phosphatases, such as MAP kinase phosphatase-1 (MKP-1), are the products of immediate-early genes that are induced following mitogenic stimulation and contribute to negative feedback regulation. Changes in cellular Ca^{2+} have been implicated in the induction of MKP-1, but factors controlling the activity of these phosphatases have not been extensively characterized.

The present study was undertaken to investigate the Ca^{2+} dependence of ERK activation in rat liver epithelial (WB) cells. A novel mechanism for the regulation of the ERK pathway is described in which Ca^{2+} pool

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depletion inactivates various protein phosphatases, resulting in an activation of the tyrosine kinase Fyn and an increase of ERK activity in WB cells. The potential significance of this form of Ca^{2+} regulation was confirmed by showing that pretreatment of WB cells with the intracellular Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA)-AM augmented the activation of Fyn, Raf-1, and ERK by ANG II and blocked the induction of MKP-1.

MATERIALS AND METHODS

Materials. The monoclonal antibody directed against $p42^{ERK}$ and ERK substrate peptide were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-ACTIVE-MAP kinase was from Promega (Madison, WI). Antibodies to Raf-1 and MKP-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antiserum to Fyn was a generous gift of A. Y. Tsygankov (Temple University, Philadelphia, PA). Thapsigargin and okadaic acid were purchased from LC Laboratories (Woburn, MA). Genistein and ionomycin were from Calbiochem (La Jolla, CA), and EGTA was purchased from Sigma Chemical (St. Louis, MO). Radioisotope-labeled ATP ([γ-^{32}P]ATP) was obtained from Amersham. Fura 2-AM and BAPTA-AM were from Molecular Probes (Eugene, OR). A plasmid encoding an inactive glutathione *S*-transferase (GST)-MEK-1 was provided by Michael J. Weber (University of Virginia, Charlottesville, VA).

Cell culture. WB cells are an epithelial cell line that was originally isolated from the liver of an adult Fisher rat (48). The cells were plated onto 100-mm tissue culture plates and incubated in Richter's improved essential medium containing L-glutamine and insulin (Irvine Scientific, Santa Ana, CA) plus 10% fetal bovine serum until confluent. Cells were made quiescent by incubation for 24 h in Richter's medium without serum before the start of the experiment. Cells were used between *passages 20* and *40*.

Western blotting. Serum-starved WB cells were treated with or without agonist as indicated. Cells were scraped into lysis buffer containing 10 mM Tris·HCl (pH 7.5), 100 mM NaCl, 1% Triton X-100, 2 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin. Cell lysates were obtained by solubilization of the cells for 30–60 min in lysis buffer, and insoluble material was removed by centrifugation at 16,000 rpm for 5 min at 4°C. The protein concentration in the supernatant was measured by the method of Bradford (7) using BSA as a standard. Equivalent amounts of protein were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Equal protein loading and the efficiency of protein transfer were assessed by staining the nitrocellulose membranes with Ponceau S. Nitrocellulose membranes were blocked with 3% (wt/vol) BSA in PBS containing 0.1% Tween 20 (PBST) for 1 h and then incubated with an antibody directed against MKP-1, $p42^{ERK}$, or phosphorylated MAP kinase. Nitrocellulose membranes were washed three times with PBST and then incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min. Protein bands were visualized by enhanced chemiluminescence (Amersham). For the gel shift assay, ERK activation was determined by the appearance of a slower migrating band in gel electrophoresis due to phosphorylation of specific threonine and tyrosine residues.

Immune complex ERK assay. After stimulation, WB cells were solubilized with lysis buffer, and precleared cell lysates containing equal amounts of protein were incubated with polyclonal anti-ERK2 antibody for 1 h on ice, followed by an incubation with protein A-agarose with rotation for 1 h at 4°C. Preimmune serum was used as the negative control. Immunoprecipitates were washed once with lysis buffer, twice with modified RIPA buffer [10 mM MOPS (pH 7.0), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 2 mM EDTA, 50 mM NaF, and 1 mM Na_3VO_4] and twice with kinase buffer [10 mM PIPES (pH 7.0) and 10 mM $MgCl_2$]. The reactions were carried out by addition of 5 µM [γ-^{32}P]ATP (10 Ci/mmol) to 5 µg/ml myelin basic protein (MBP) in the kinase buffer at 30°C for 15 min and stopped by heating at 95°C for 5 min after the addition of Laemmli sample buffer. The kinase assay samples were subjected to 15% SDS-PAGE, followed by gel drying and exposure to X-ray film at −86°C. The results were analyzed by densitometry, with the intensity of the autoradiograms kept in the linear range of exposures.

Raf-1 assay. Stimulation of WB cells and immunoprecipitation of Raf-1 were carried out as described above using an antibody against Raf-1. An irrelevant rabbit antibody was used as a negative control. Immunoprecipitates were washed three times with lysis buffer and then twice with kinase buffer. The reaction was initiated by addition of 5 µM [γ-^{32}P]ATP (10 Ci/mmol) to 10 µg/ml GST-MEK in the kinase buffer at 30°C for 10 min and stopped by heating at 95°C for 5 min after the addition of Laemmli sample buffer. The kinase assay samples were subjected to 10% SDS-PAGE and analyzed as described for the ERK assay.

Fyn kinase assay. Fyn was immunoprecipitated from WB cell lysates prepared as described above using anti-Fyn serum. The antibody was raised in rabbits against the unique region of murine $p60^{fyn}$ (amino acids 6–84) and does not recognize other Src family kinases (9, 49). Immunoprecipitates were washed as described for the Raf-1 assay, and the kinase reaction was initiated by addition of 5 µM [γ-^{32}P]ATP (10 Ci/mmol) to 2 µg acid-denatured enolase in kinase buffer at 30°C for 5 min. The assay samples were subjected to 10% SDS-PAGE and analyzed as described above.

Phosphatase activity assay. PP-2A activity was measured in whole cell lysates of WB cells using the serine/threonine phosphatase assay system (Promega). PTPase activity was similarly measured with the tyrosine phosphatase assay system (Promega).

Briefly, WB cells were grown to confluence, starved for 20–24 h, and incubated with 3 mM EGTA for up to 30 min. Cells were washed twice in ice-cold Tris-buffered saline and lysed in 200 µl of lysis buffer containing 25 mM Tris·HCl (pH 7.5), 2 mM EDTA, 10 mM β-mercaptoethanol, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1% Triton X-100, and 10% glycerol. Cell lysates were incubated for 30 min on ice and then centrifuged at 100,000 *g* for 1 h at 4°C to remove particulate matter. Free phosphate was removed by loading 200 µl of the cell lysate onto a Sephadex G-25 spin column, followed by centrifugation at 600 *g* for 5 min at 4°C. The protein concentration was adjusted to 1 mg/ml, and aliquots were kept frozen at −80°C until use. PP-2A activity was measured by incubating the cell lysate (5 µg protein) in 50 mM imidazole (pH 7.2), 0.2 mM EGTA, 0.02% β-mercaptoethanol, 0.1 mg/ml BSA, 1 mM sodium vanadate, and 0.1 mM phosphoserine peptide substrate in a total volume of 50 µl at 30°C for 15 min. Incubations were started by addition of the cell lysate and terminated by addition of 50 µl of a molybdate dye additive. The absorbance was read at 620 nm with an ELISA plate reader. PTPase activity was similarly assayed except that the reaction buffer contained 50 mM imidazole (pH 7.2),

1 mM EDTA, 0.02% β-mercaptoethanol, 0.1 mg/ml BSA, 50 mM NaF, and 0.1 mM phosphotyrosine peptide substrate (Glu-Asn-Asp-pTyr-Ile-Asn-Ala-Leu) (13).

Measurement of DNA synthesis. DNA synthesis was measured by an immunocytochemical method, which assays for the incorporation of bromodeoxyuridine (BrdU) into nuclei (50). WB cells were plated onto coverslips in six-well plates, and the cells were grown to 80% confluence before being serum starved for 24 h. The cells were incubated with okadaic acid or sodium vanadate for 24 h, and the incorporation of BrdU was measured by using a commercial kit from Calbiochem (Cambridge, MA).

Measurement of cytosolic free Ca^{2+}. Intracellular Ca^{2+} was measured in single cells using either fluorescence video microscopy with digital imaging analysis (Compix) or fluorescence microscopy of single cells as described previously (5).

Statistical analysis. Statistical analysis was performed by Student's *t*-test using SigmaSTAT software. Data are expressed as means ± SE. Differences with a *P* of 0.05 were considered statistically significant.

RESULTS

Ca^{2+} store depletion activates ERK in WB cells. It has been suggested that an increase in intracellular free Ca^{2+} is involved in the activation of the ERK pathway, but the mechanism has not been clarified (39). Thapsigargin, an endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor, has been extensively used to produce an increase in cytosolic free Ca^{2+} without the generation of other second messengers. The mechanism of its action is to block Ca^{2+} uptake into the ER, which gradually becomes depleted of Ca^{2+} due to an unopposed Ca^{2+} leak (47). To elucidate the role of Ca^{2+} in ERK activation, WB cells were treated with thapsigargin for up to 30 min, and the activation of immunoprecipitated ERK2 was assessed by three separate assays. For the first, ERK activity was measured by the incorporation of ^{32}P from [γ-^{32}P]ATP into MBP (Fig. 1*A*). ERK activation is associated with phosphorylation of the enzyme on threonine and tyrosine residues, which causes an increase in its electrophoretic mobility. Therefore, ERK activity was also evaluated by the gel shift assay (Fig. 1*B*). For the third method, measurements were made of the amount of phosphorylated ERK in cell lysates, as detected by an antibody directed against the phosphorylated forms of ERK1 and ERK2 (Fig. 1*C*). All three methods demonstrated that treatment of WB cells with thapsigargin caused an increase in ERK activity. Maximum activity varied between 15 and 30 min for the different experiments shown in Fig. 1 and was considerably slower than the rise in intracellular free Ca^{2+} produced by thapsigargin stimulation. This was maximal at ~1 min and gradually declined to basal Ca^{2+} levels by 15 min (Fig. 2*A*). Occasionally, as in Fig. 2*B*, maximum ERK activity after thapsigargin addition occurred before 15 min. Densitometric quantitation of ERK activity measured in four experiments similar to Fig. 1*A* showed a three- to fourfold increase in ERK activity after 15 or 30 min. These data suggest that an increase in cytosolic free Ca^{2+} per se may not be directly responsible for the thapsigargin-induced increase in ERK activity.

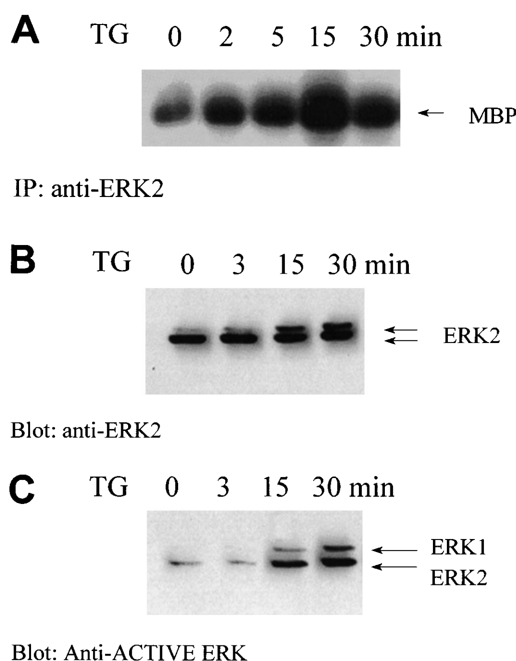

Fig. 1. Effect of thapsigargin on activation of extracellular signal-regulated protein kinase (ERK) in WB cells. ERK activity was assessed in serum-starved WB cells by 3 different methods after stimulation with 2 μM thapsigargin (TG). ERK2 was immunoprecipitated (IP) with an anti-ERK2 anti-serum, and immune complex kinase assays were performed as described in MATERIALS AND METHODS (*A*). Correspondingly, cell lysates were collected, and equal amounts of protein were separated by SDS-PAGE, immunoblotted with either a monoclonal antibody raised against $p42^{ERK}$ protein (*B*) or an anti-phospho-ERK antibody (*C*), and visualized with enhanced chemiluminescence (ECL). Data are representative of 2–4 independent experiments. MBP, myelin basic protein.

The ER Ca^{2+} pools have been shown to be extensively emptied after 10–15 min of thapsigargin treatment (33). Therefore, to determine whether ER Ca^{2+} depletion as opposed to a rise in cytosolic Ca^{2+} causes an activation of the ERK pathway, cells were pretreated with 5 μM BAPTA-AM before thapsigargin stimulation. BAPTA-AM is a membrane-permeant Ca^{2+} chelator that is hydrolyzed by intracellular esterases to the free acid Ca^{2+}-binding form, thereby producing a powerful Ca^{2+} buffer. As shown in Fig. 2*A*, a 30-min incubation of single fura 2-loaded cells with 5 μM BAPTA-AM caused a complete inhibition of the thapsigargin-induced increase in intracellular Ca^{2+} without affecting resting Ca^{2+} levels. Further experiments showed that thapsigargin increased cytosolic free Ca^{2+} to peak values that were 496 ± 84 nM (n = 7) above basal, whereas with BAPTA present the cytosolic free Ca^{2+} level was 64 ± 3 nM (n = 8) after 15 min of thapsigargin stimulation compared with 72 ± 13 nM (n = 7) for resting control cells. These data indicate that the increased Ca^{2+} influx due to Ca^{2+} store depletion did not overcome the buffering capacity of intracellular BAPTA. Figure 2*B* compares the effect of thapsigargin on ERK activation in control cells and cells treated with 5 μM BAPTA-AM for 30 min before the addition of thapsigargin. This experiment shows that the presence of cytosolic BAPTA did not cause a decrease in the

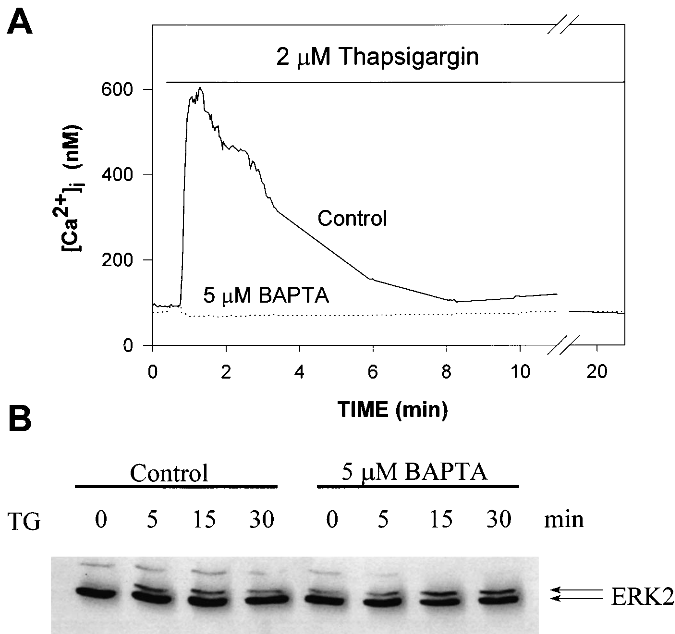

Fig. 2. Effect of BAPTA on thapsigargin-induced increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and ERK activation in WB cells. Serum-starved WB cells were pretreated with or without 5 μM BAPTA for 30 min before stimulation with 2 μM thapsigargin. $[Ca^{2+}]_i$ was measured in fura 2-loaded cells (*A*). Each trace is from a single cell in a field of cells and represents a typical response. ERK activation was assessed by gel shift assay (*B*). Cell lysates were collected, and equal amounts of protein were separated by SDS-PAGE, immunoblotted with an anti-ERK antibody, and visualized with ECL. Data are representative of 3 independent experiments.

thapsigargin-induced activation of ERK compared with thapsigargin treatment alone. These data indicate that the rise in cytosolic free Ca^{2+} is unlikely to be responsible for the thapsigargin-induced activation of ERK in WB cells.

Because Ca^{2+} store depletion is difficult to measure in the presence of BAPTA, an alternative approach was taken to examine the effect of Ca^{2+} depletion on ERK activity. The addition of the extracellular Ca^{2+} chelator EGTA is expected to deplete intracellular Ca^{2+} stores as Ca^{2+} is gradually lost from the cell by the plasma membrane Ca^{2+}-ATPase. The effect of EGTA on the amount of stored Ca^{2+} was measured in single fura 2-loaded WB cells exposed to EGTA. EGTA (3 mM) caused a gradual decrease in the level of cytosolic free Ca^{2+} over 5 min (Fig. 3*A*). Moreover, pretreatment of the cells for 15 min with 3 mM EGTA caused a large depletion of the Ca^{2+} pools, as demonstrated by the reduced Ca^{2+} transient that was observed after addition of either thapsigargin (Fig. 3*B*) or the Ca^{2+} ionophore ionomycin (Fig. 3*C*). These data indicate that exposing cells to 3 mM EGTA for 15 min not only prevents extracellular Ca^{2+} influx but also depletes both cytosolic Ca^{2+} and intracellular Ca^{2+} stores. Intracellular Ca^{2+} depletion with excess EGTA, however, did not adversely affect cell viability for up to 1 h of treatment, as indicated morphologically and by staining with trypan blue. Assessment of ERK activity by the gel shift assay after the addition of 3 mM EGTA showed a time-dependent activation of ERK2 (Fig. 3*D*).

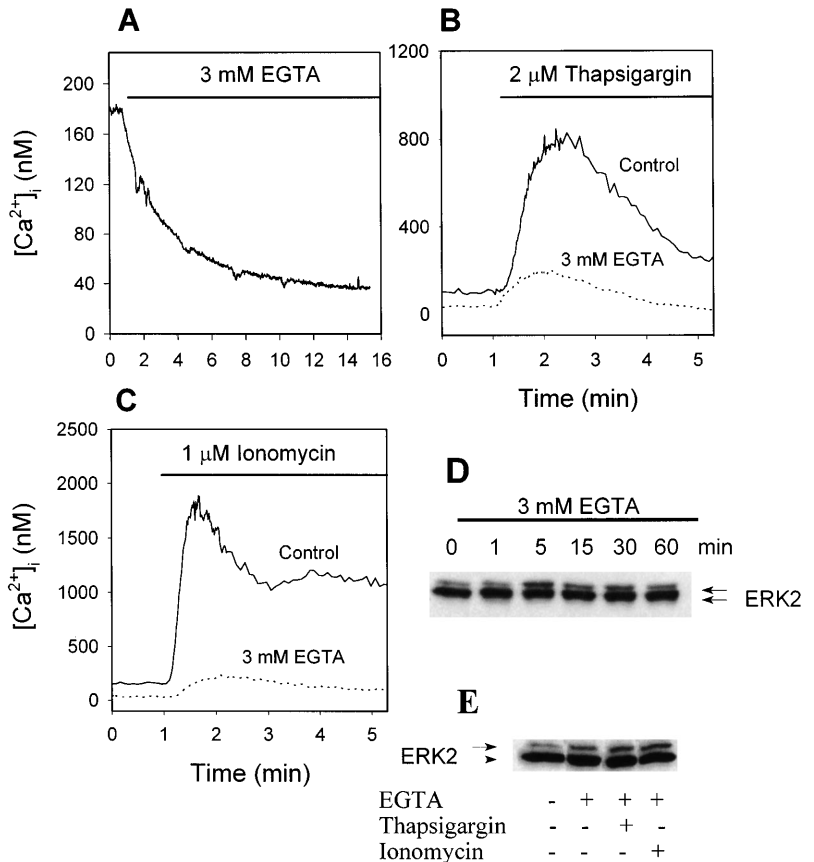

Fig. 3. Time course of effect of EGTA on $[Ca^{2+}]_i$ and ERK activation in WB cells. Change in basal Ca^{2+} levels due to 3 mM EGTA was assessed in fura 2-loaded WB cells (*A*). Subsequently, cells were stimulated with either 2 μM thapsigargin (*B*) or 1 μM ionomycin (*C*) to determine amount of Ca^{2+} remaining in intracellular stores. Each trace is from a single cell and represents a typical response. ERK activation was assessed by gel shift assay in WB cells treated for up to 60 min with 3 mM EGTA (*D*) or pretreated with EGTA for 15 min and then stimulated with ionomycin or thapsigargin (*E*). Cell lysates were collected, and equal amounts of protein were separated by SDS-PAGE, immunoblotted with an anti-ERK antibody, and visualized with ECL. Similar results were observed in 3 or more independent experiments.

The activity peaked at 5 min and remained elevated for up to 60 min.

When WB cells were pretreated with EGTA and stimulated for 5 min with either 2 µg/ml thapsigargin or 10 µM ionomycin, a further activation of ERK was observed, as shown by the gel shift assay in Fig. 3*E*. This effect was confirmed by the ERK kinase assay, which showed that, in the presence of EGTA, thapsigargin and ionomycin increased ERK activity by 2.4 ± 0.5-fold and 3.2 ± 0.6-fold over the EGTA-treated control, respectively. This effect occurred subsequent to the small increases in Ca^{2+} observed 1–2 min after thapsigargin or ionomycin addition to the cells in the presence of EGTA (Fig. 3, *B* and *C*), suggesting that it is the further depletion of the Ca^{2+} stores by these agents that caused the increased ERK activity in EGTA-pretreated cells. Taken together, these data indicate that Ca^{2+} pool depletion, as opposed to an increase in cytosolic free Ca^{2+}, is responsible for activation of the ERK pathway in WB cells.

Genistein sensitivity of ERK activation induced by Ca^{2+} pool depletion. Recent studies suggest that depletion of the intracellular Ca^{2+} pool leads to an activation of tyrosine kinases that stimulate Ca^{2+} influx, although the mechanism remains unknown (26, 41). To determine whether the activation of ERK by Ca^{2+} store depletion involved a tyrosine kinase pathway, WB cells were pretreated with 100 µM genistein for 30 min before the addition of 3 mM EGTA. Figure 4*A* shows that genistein almost completely inhibited the EGTA-induced activation of ERK, indicating that intracellular Ca^{2+} depletion is associated with the activation of a tyrosine kinase upstream of ERK in the ERK pathway. Also, the increased activity of ERK induced by thapsigargin in the presence or absence of BAPTA was inhibited by genistein (Fig. 4*B*). Genistein also blocked the increased ERK activity observed in cells treated with BAPTA alone. Similar results were obtained when ERK activation was measured by the gel shift assay and by the amount of phosphorylated, activated ERK in immunoblots (data not shown). These findings support the conclusion that the activation of ERK induced by Ca^{2+} pool depletion takes place through a tyrosine kinase-sensitive pathway in WB cells.

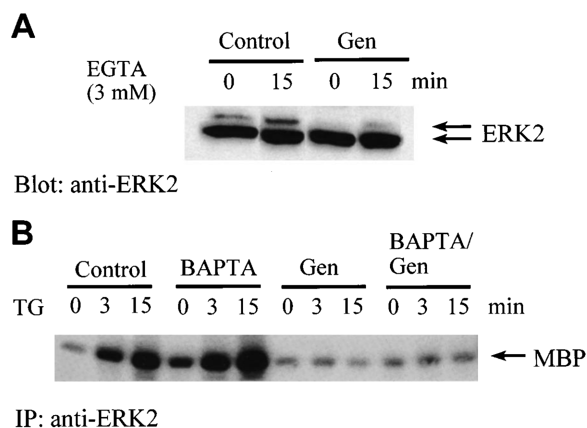

Fig. 4. Effect of genistein (Gen) on ERK activation induced by Ca^{2+} store depletion. ERK activation was determined in serum-starved WB cells pretreated for 30 min with 100 µM genistein or 0.2% DMSO (vehicle control) and treated by addition of either 3 mM EGTA for 15 min (*A*) or 2 µM thapsigargin for indicated times in presence or absence of 5 µM BAPTA (*B*). Cell lysates were collected, and equal amounts of protein were separated by SDS-PAGE, immunoblotted with a monoclonal antibody raised against $p42^{ERK}$ protein, and visualized with ECL (*A*). Alternatively, ERK2 was immunoprecipitated with an anti-ERK2 anti-serum, and immune complex kinase assays were performed as described in MATERIALS AND METHODS (*B*). Results are representative of 3 independent experiments.

Ca^{2+} pool depletion increases the activity of Fyn and Raf-1. Fyn and Yes, two members of the Src family of tyrosine kinases, have previously been shown to be upstream kinases of the ERK pathway in WB cells (50). Because Ca^{2+} pool depletion with thapsigargin increased ERK activity in a genistein-sensitive manner, the activation of Fyn and Yes was examined in WB cells treated with 2 µM thapsigargin for various times. These Src family kinases were immunoprecipitated and subjected to an in vitro kinase assay using enolase as the substrate. Figure 5*A* shows that thapsigargin caused a rapid threefold activation of Fyn, and Fig. 5*B* shows that the thapsigargin-induced increase in Fyn activity was unaffected by buffering the intracellular free Ca^{2+} with BAPTA, although BAPTA alone caused a twofold activation of Fyn. In contrast, thapsigargin had no effect on the activity of Yes in WB cells (unpublished observations). These results suggest that there is a specificity of function between Fyn and Yes and that only Fyn tyrosine kinase is activated by depletion of the thapsigargin-sensitive Ca^{2+} pool.

Raf-1 is a serine/threonine kinase upstream of ERK and downstream of Fyn. Assessment of the effect of thapsigargin on Raf-1 activity with inactive GST-MEK as substrate showed that the activity of Raf-1 increased within 1 min of thapsigargin addition and remained significantly ($P < 0.05$) elevated after 15 min (data not shown). Furthermore, pretreatment of WB cells with PD-98059, a specific MEK inhibitor, completely blocked the thapsigargin-induced activation of ERK (data not shown). These data suggest that Ca^{2+} pool depletion activates a protein kinase pathway involving Fyn, Raf-1, MEK, and ERK.

Ca^{2+} depletion increases PP-2A and PTPase activities in WB cells. Intracellular Ca^{2+} pools have been suggested to regulate the activity of protein phosphatases within the cell (42, 51). Treatment of WB cells with okadaic acid, an inhibitor of the serine/threonine phosphatases PP-2A and PP-1, and vanadate, a tyrosine phosphatase inhibitor, caused an increase in ERK activity in WB cells (data not shown). Moreover, 10 nM okadaic acid or 10 µM vanadate caused a twofold increase of DNA synthesis (Table 1). These results suggest that one or more phosphatases contribute to maintenance of a low basal level of ERK activity and cell proliferation in the absence of growth factors. The possibility that Ca^{2+} depletion might also influence the overall phosphatase activity in the cytosol was evaluated by measuring the activities of PP-2A and PTPase in cells treated with 3 mM EGTA (cf. Fig. 3). Ca^{2+} depletion resulted in a time-dependent decrease in the activity of PP-2A, as measured by phosphate loss from

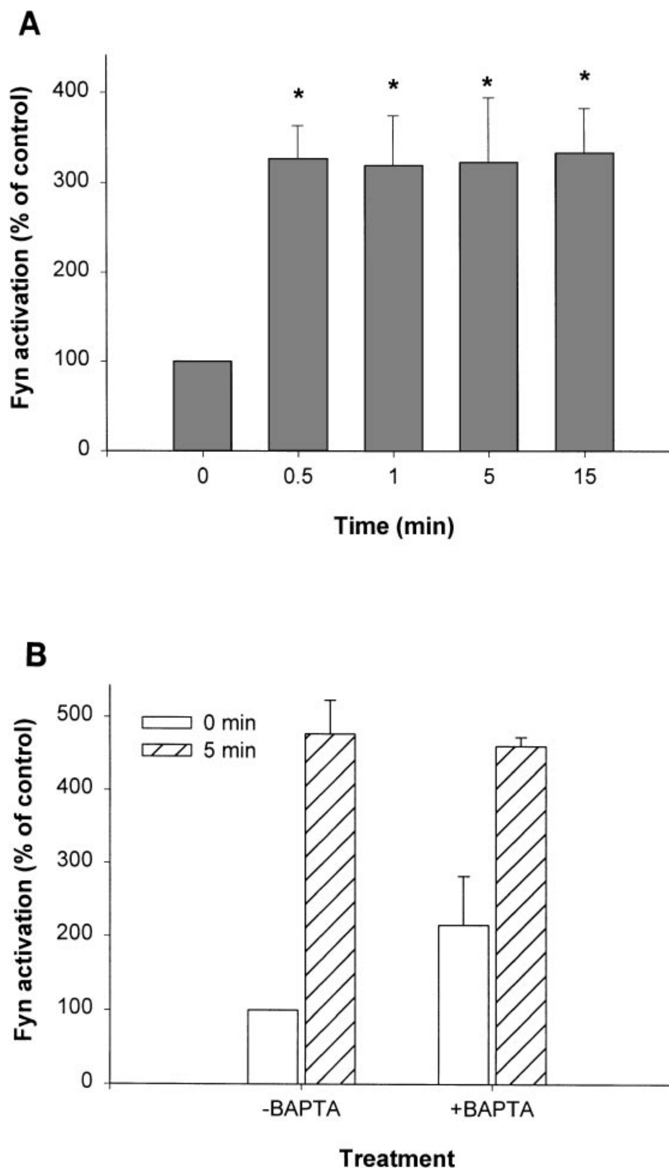

Fig. 5. Effect of BAPTA on activation of Fyn by thapsigargin in WB cells. *A*: activity of Fyn was measured in cells treated with 2 μM thapsigargin for various times up to 15 min. *B*: cells were pretreated for 30 min with 5 μM BAPTA-AM before addition of thapsigargin for 5 min. Immune complex kinase assays were performed as described in MATERIALS AND METHODS. Data are means ± SE of 4 independent experiments. * $P < 0.05$ vs. *time 0* value.

a phosphoserine peptide substrate (Fig. 6, *bottom*). Inhibition was maximal at 5 min, and phosphatase activity remained decreased for up to 30 min. The peptide substrate used was not a substrate for PP-1, and addition of okadaic acid to the reaction mixture fully blocked the phosphatase activity, indicating it was due to PP-2A (data not shown). Additionally, there was a similar time-dependent decrease in PTPase activity in WB cells treated for up to 30 min with EGTA (Fig. 6, *top*). These data suggest that intracellular Ca^{2+} depletion results in the inactivation of both PP-2A and PTPases, two groups of phosphatases that are known to dephosphorylate a variety of proteins, including the Src family kinase members MEK and ERK.

Table 1. *Effects of okadaic acid and sodium vanadate on BrdU incorporation into WB cells*

Phosphatase Inhibitor Concentration	%BrdU-Stained Cells	
	Okadaic acid (nM)	Vanadate (μM)
0	8.5 ± 0.22	7.9 ± 0.71
0.5	11.6 ± 0.86*	8.7 ± 0.90
1.0	11.9 ± 1.05*	9.9 ± 1.09
10.0	16.6 ± 0.56*	17.2 ± 1.90*

Values are means ± SE; $n = 4$–6 for each group. BrdU, bromodeoxyuridine. * $P < 0.05$ vs. corresponding control.

Intracellular Ca^{2+} chelation augments ANG II-induced activation of the ERK pathway and represses expression of MKP-1 by ANG II. Mobilization of Ca^{2+} from the ER is a well-established response to numerous hormones and growth factors. Therefore, the Ca^{2+} chelator BAPTA was used to investigate the involvement of Ca^{2+} in the ERK pathway initiated by the G protein-coupled receptor agonist ANG II. Pretreatment of cells with BAPTA-AM for 15 min caused a greatly augmented activation of ERK by ANG II, which was

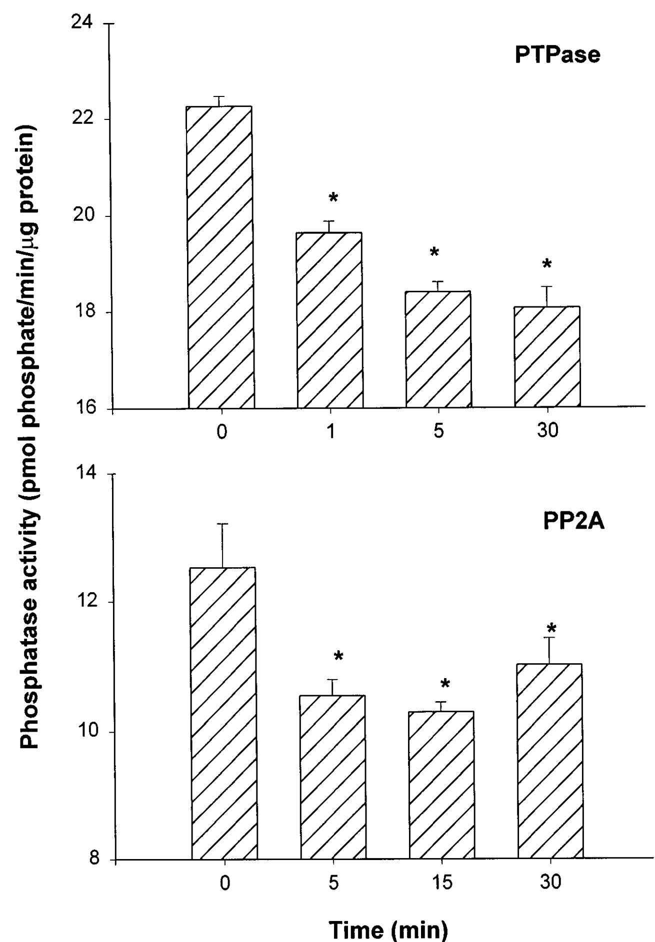

Fig. 6. Effect of 3 mM EGTA on protein tyrosine phosphatase (PTPase; *top*) and protein phosphatase 2A (PP-2A; *bottom*) activity in WB cells. PTPase and PP-2A activities were measured in whole cell lysates from quiescent WB cells treated with 3 mM EGTA for different times up to 30 min at 37°C. Results are means ± SE from 4 independent experiments. * $P \leq 0.05$ vs. *time 0* value.

statistically significant after 30 and 60 min (Fig. 7*A*), but completely blocked the rise in intracellular free Ca^{2+} normally obtained with ANG II (54). Pretreatment of the cells with BAPTA also augmented the ANG II-induced activation of Fyn and Raf-1, even after 30 s (Fig. 7, *B* and *C*). Similarly, Graves et al. (21) have shown that the ANG II-induced activation of ERK and $p90^{RSK}$ was augmented in GN4 cells pretreated with similar concentrations of BAPTA. The probable reason for this effect is that buffering the cytosolic Ca^{2+} with BAPTA leads to a diminished refilling of the Ca^{2+} pools in these cells after stimulation with ANG II, thereby causing a greater Ca^{2+} depletion relative to conditions with ANG II and no BAPTA.

MKP-1 is a dual-specificity phosphatase that selectively dephosphorylates ERK, thereby terminating ERK activity after growth factor stimulation (46). In quiescent cells, there is normally a very low expression level of MKP-1. After growth factor stimulation, the expression of MKP-1 is rapidly induced. It has been suggested that a rise in intracellular Ca^{2+} is required for MKP-1 induction (43, 55). Therefore, we investigated the Ca^{2+} dependency of the expression of MKP-1 induced by ANG II. Cells were pretreated with BAPTA-AM for 30 min before stimulation with 1 μM ANG II. Figure 8 shows that BAPTA completely inhibited the ANG II-induced expression of MKP-1, thereby contributing to an enhancement of ERK phosphorylation and activation. The apparent Ca^{2+} dependency for MKP-1 induction, therefore, may be more related to a rise in intracellular free Ca^{2+} than to a depletion of the intracellular Ca^{2+} pool.

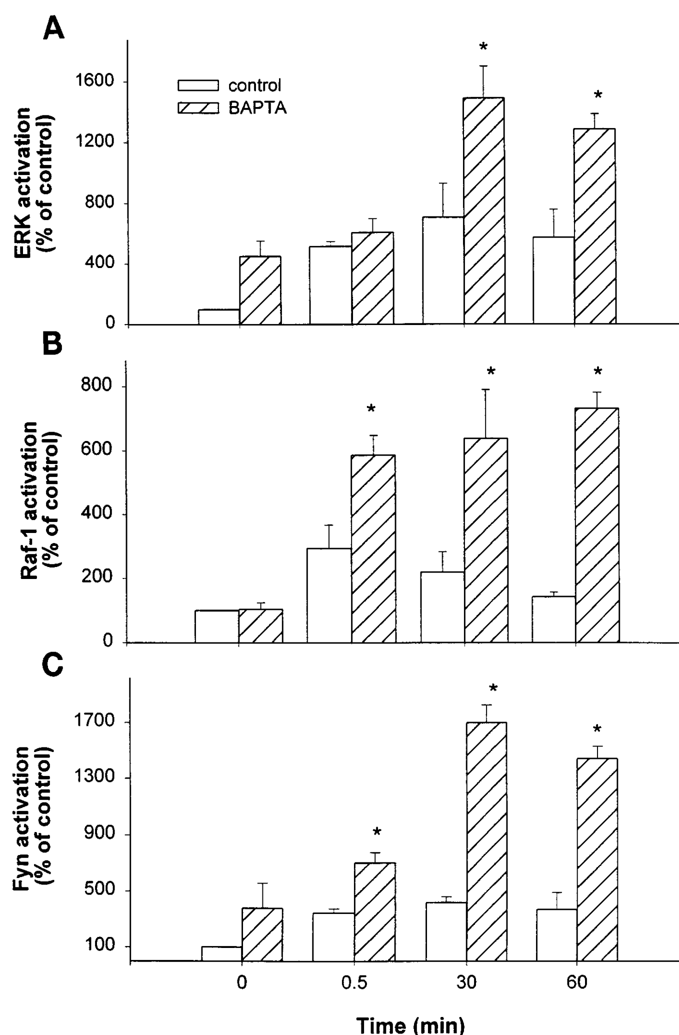

Fig. 7. Effect of BAPTA on ANG II-induced activation of ERK, Raf-1, and Fyn in WB cells. ERK (*A*), Raf-1 (*B*), and Fyn (*C*) activities were measured in serum-starved WB cells pretreated for 15 min with 25 μM BAPTA or 0.2% DMSO (vehicle control) and stimulated with 1 μM ANG II for various time intervals. Immune complex kinase assays were performed as described in MATERIALS AND METHODS. Autoradiograms were quantitated by densitometric scanning, and results are expressed as percent of control. Results are means ± SE from 3 independent experiments. * $P < 0.05$ vs. *time 0* value.

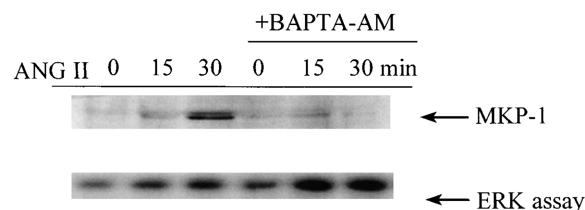

Fig. 8. Effect of BAPTA on ANG II-induced expression of MAP kinase phosphatase-1 (MKP-1) in WB cells. Expression of MKP-1 protein was determined by Western blot analysis in serum-starved WB cells pretreated for 30 min with 25 μM BAPTA or 0.2% DMSO (vehicle control) and stimulated with 1 μM ANG II for various times. Cell lysates were collected, and equal amounts of protein were separated by SDS-PAGE, immunoblotted with anti-MKP-1 antibody, and visualized with ECL.

DISCUSSION

The amount of Ca^{2+} within the ER has been shown to affect several cellular functions, including translation, folding, processing and assembly of proteins (30), protein synthesis (15, 29), cell proliferation (20), Ca^{2+}-independent PLA_2 ($iPLA_2$) activity (53), and Ca^{2+} influx (6). In the present study with WB cells, regulation of the ERK pathway was investigated after depletion of the ER Ca^{2+} pools by two different methods. In the first method, Ca^{2+} stores were depleted with thapsigargin, which produced an increase in ERK activity. This was not due to a rise in cytosolic free Ca^{2+}, since similar findings were obtained when the thapsigargin-induced increase in cytosolic free Ca^{2+} was blocked by introducing the Ca^{2+} chelator BAPTA into the cells. In the second method, intracellular Ca^{2+} was depleted by the addition of excess EGTA to the medium. This caused the depletion of cellular Ca^{2+} pools as well as a fall in cytosolic free Ca^{2+}. Because ERK was also activated under these conditions, it is evident that Ca^{2+} store emptying as opposed to an elevation of cytosolic Ca^{2+} was responsible for the increase in ERK activity in WB cells under these conditions (Figs. 2–4).

In most electrically nonexcitable cells, Ca^{2+} store depletion stimulates Ca^{2+} influx via the activation of store-operated Ca^{2+} channels (26, 41). Furthermore, under a number of conditions, Ca^{2+} influx has been shown to be sensitive to the tyrosine kinase inhibitor genistein (6, 44, 51). This finding has led to the hypothesis that the release of sequestered Ca^{2+} acti-

vates a tyrosine kinase, whereas repletion of Ca^{2+} stores activates one or more phosphatases (42, 51). Moreover, it has also been demonstrated that activation of store-operated Ca^{2+} channels and the resultant Ca^{2+} influx are governed by the status of Ca^{2+} within the ER (23). In WB cells, we observed that the Ca^{2+} depletion-induced activation of ERK was sensitive to inhibition by genistein (Fig. 4). Furthermore, data presented here suggest that the tyrosine kinase Fyn may be specifically involved in the activation of ERK under conditions of Ca^{2+} store depletion, since its activity was increased after thapsigargin treatment of WB cells and since this activity was unaffected by pretreatment of the cells with the Ca^{2+} chelator BAPTA (Fig. 5). This coincides with the fact that Fyn is an upstream component of the ERK pathway initiated by ANG II in WB cells (50). A decreased activity of two groups of phosphatases, PP-2A and PTPases, was also observed after treatment of the cells with EGTA (Fig. 6). Therefore, it would seem that signals transmitted by depleted Ca^{2+} stores to enhance ERK activity involve the activation of the protein tyrosine kinase Fyn and the inactivation of at least two types of protein phosphatases, PP-2A and PTPase. The involvement of both Fyn and these phosphatases in the ERK pathway may contribute to the delayed activation that was observed following Ca^{2+} pool depletion.

Although it has been suggested that intracellular Ca^{2+} stores are involved in the regulation of cellular phosphatases, the present study extends this concept by demonstrating a reduction in PP-2A and PTPase activity by Ca^{2+} store depletion. However, the mechanism by which the degree of filling of the Ca^{2+} stores regulates the phosphatase activities remains unclear. A possible mechanism is suggested by the fact that one of the main PTPases, PTP-1B, contains a COOH-terminal hydrophobic segment that targets it to the ER (17). Agents that increase intracellular Ca^{2+} promote the cleavage of PTP-1B by calpain, resulting in its translocation to the cytosol and subsequent activation (17). In turn, depletion of stored Ca^{2+} may also affect the activity of PTP-1B by promoting its retention in the ER.

Protein phosphatases generally have a negative effect on cell proliferation and appear to be active in resting cells, thereby providing a mechanism for the maintenance of growth arrest by limiting the activities of growth-promoting kinases (2). This is in agreement with the results showing that inhibition of phosphatase activity with okadaic acid and/or vanadate caused an increase in the rate of DNA synthesis (Table 1). Because an inactivation of PP-2A and PTPases occurs with intracellular Ca^{2+} depletion, one or more phosphatases that are normally involved in the maintenance of a low basal level of ERK activity in quiescent cells probably become inhibited. Pervanadate has been shown to cause an activation of ERK as well as of the upstream kinases MEK and Raf-1 (56). Furthermore, pervanadate induced tyrosine phosphorylation of the epidermal growth factor (EGF) receptor and its association with GRB2. This indicates that pervanadate-sensitive phosphatases are interacting with upstream components of the ERK pathway, possibly also causing an activation of Src family kinases, whereas okadaic acid-sensitive phosphatases may directly dephosphorylate MEK and/or ERK (56).

Control of tyrosine kinases and phosphatases by the amount of stored Ca^{2+} may also be critical for regulating ERK activity following hormonal stimulation. Previously, the involvement of Ca^{2+} in the ERK pathway initiated by various agonists in different cells has been shown to be dependent on the cell characteristics and the agonist used (16, 18). For example, in cardiac myocytes, ANG II-induced activation of ERK appeared to be dependent on a rise in cytosolic Ca^{2+} (39), whereas carbachol-induced activation of ERK was minimally affected by BAPTA or EGTA pretreatment in Rat 1a fibroblasts (34). The present study suggests that intracellular Ca^{2+} pool content may play a novel role in the hormonal regulation of the ERK pathway. Under resting conditions, ERK activity appears to be restrained by phosphatases that are constitutively active in the quiescent cell. When stimulated with ANG II, intracellular Ca^{2+} is mobilized, resulting in Ca^{2+} store depletion. This normally is a transient phenomenon, since the release of stored Ca^{2+} initiates Ca^{2+} influx, which leads to a partial refilling of the Ca^{2+} pools (26, 41). It is proposed that the initial reduction of Ca^{2+} in the ER pool activates Fyn and inactivates PP-2A and PTPases, both of which may contribute to the activation of ERK observed after ANG II stimulation. Subsequently, the Ca^{2+} pools are refilled and PP-2A and PTPases are reactivated. Concurrently, the initial transient rise in intracellular free Ca^{2+} induces the expression of MKP-1. Together, these events affect the duration of activated ERK, as a new steady state is reached between the activities of the various protein kinases and phosphatases affecting different steps in the ERK pathway.

ERK is directly involved in the initiation of mitogenesis as a consequence of the phosphorylation and activation of a variety of transcription factors. However, although the depletion of stored Ca^{2+} activates ERK, this alone is insufficient for the induction of mitogenesis (20). In cells in which there is a prolonged elevation of cytosolic Ca^{2+}, thapsigargin induces apoptosis (19). In contrast, prolonged intracellular Ca^{2+} pool depletion induced by thapsigargin caused an arrest of DDT_1 MF-2 smooth muscle cells in a G_0-like state, which could only be reversed by the synthesis of new functional Ca^{2+} pump protein and subsequent replenishment of the Ca^{2+} pools (52). In our hands, thapsigargin completely blocked EGF-induced BrdU incorporation in WB cells (data not shown), indicating that functioning (replete) Ca^{2+} pools are an absolute requirement for cells to proceed through the cell cycle. Depletion of Ca^{2+} stores has been implicated in the regulation of gene transcription and translation (8, 35). Ca^{2+} store depletion has been shown to activate double-stranded RNA-dependent/regulated protein kinase (PKR), which in turn phosphorylates and activates eukaryotic initiation factor 2α, resulting in the arrest of translation at initiation (35, 45). Conversely, PKR has also been shown to activate the transcription factor

nuclear factor-κB (24). Additionally, depletion of Ca^{2+} pools increases the expression of genes that encode select resident ER proteins, such as glucose-regulated protein 78 and calreticulin (28, 29). This indicates that Ca^{2+} pools are important for both the inhibition and stimulation of protein synthesis. Therefore, the amount of Ca^{2+} within the ER may help to maintain a precise control over ERK activity and consequently over protein synthesis and mitogenesis under different conditions of either cellular stress or proliferation.

In summary, it has been established that depletion of Ca^{2+} stores, even in the absence of an elevation of cytosolic free Ca^{2+}, is sufficient to induce the activation of ERK in WB cells. This apparently occurs via the activation of Fyn, and possibly via the inactivation of both serine/threonine and/or tyrosine phosphatases. However, the mechanism whereby such diverse systems as Ca^{2+} channels, protein kinases, phosphatases, and phospholipases such as $iPLA_2$ respond to the Ca^{2+} content of intracellular Ca^{2+} stores remains elusive.

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