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Ca²⁺-dependent activation of c-jun NH₂-terminal kinase in primary rabbit proximal tubule epithelial cells

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Abstract

Previous work from this laboratory demonstrated that arachidonic acid activates c-jun NH₂-terminal kinase (JNK) through oxidative intermediates in a Ca²⁺-independent manner (Cui X and Douglas JG. Arachidonic acid activates c-jun N-terminal kinase through NADPH oxidase in rabbit proximal tubular epithelial cells. *Proc Natl Acad Sci USA* 94: 3771–3776, 1997.). We now report that JNK can also be activated via a Ca²⁺-dependent mechanism by agents that increase the cytosolic Ca²⁺ concentration (Ca²⁺ ionophore A₂₃₁₈₇, Ca²⁺-ATPase inhibitor thapsigargin) or deplete intracellular Ca²⁺ stores [intracellular Ca²⁺ chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA)-AM]. The activation of JNK by BAPTA-AM occurs despite a decrease in cytosolic Ca²⁺ concentration as detected by the indicator dye fura 2, but appears to be related to Ca²⁺ metabolism, because modification of BAPTA with two methyl groups increases not only the chelation affinity for Ca²⁺, but also the potency for JNK activation. BAPTA-AM stimulates Ca²⁺ influx across the plasma membrane, and the resulting local Ca²⁺ increases are probably involved in activation of JNK because Ca²⁺ influx inhibitors (SKF-96365, nifedipine) and lowering of the free extracellular Ca²⁺ concentration with EGTA reduce the BAPTA-induced JNK activation.

Keywords

cytosolic calcium concentration; thapsigargin; 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid

The c-jun NH₂-terminal kinase (JNK) is a member of the mitogen-activated protein kinase (MAPK) superfamily. We recently reported for rabbit proximal tubule cells that this enzyme is activated by arachidonic acid in a Ca²⁺-independent manner, even though cytosolic Ca²⁺ concentrations ([Ca²⁺]_i) increased when arachidonic acid metabolites levels were raised (3,18). The conclusion that Ca²⁺ was not an important player in the activation of JNK by arachidonic acid was based on the observations that the presence of intracellular and extracellular Ca²⁺ chelators did not change the arachidonic acid-dependent activation. However, in many other cell types, JNK is activated in a Ca²⁺-dependent manner

(15,21,27,31,38). Therefore, the current experiments were designed to investigate the relationship between $[Ca^{2+}]_i$ and JNK activation in rabbit proximal tubule cells. Interestingly, we observed that the intracellular Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid (BAPTA)-AM activated JNK, similar to known elevators of $[Ca^{2+}]_i$, such as the Ca^{2+} ionophore A-23187 and the Ca^{2+} pump inhibitor thapsigargin. BAPTA-AM activation of JNK is also largely Ca^{2+} dependent, although in a paradoxical way because it results from chelation of cytosolic Ca^{2+} . This chelation causes store depletion and activation of “capacitative Ca^{2+} influx” (24,32,34) and, therefore, local increases of $[Ca^{2+}]_i$.

Materials and Methods

Materials

A₂₃₁₈₇, EGTA, thapsigargin, purified rabbit IgG, and anti-rabbit IgG agarose beads were purchased from Sigma Chemical (St. Louis, MO). Cell culture medium and additives were from GIBCO BRL (Gaithersburg, MD). BAPTA-AM, dimethyl BAPTA-AM, and fura 2-AM were from Molecular Probes (Eugene, OR). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[^3\text{H}]\text{arachidonic acid}$ were obtained from DuPont-NEN (Boston, MA). $^{45}\text{Ca}^{2+}\text{Cl}_2^-$ was from ICN Biomedicals (Aurora, OH). Rabbit anti-JNK1(FL) polyclonal antibody, which cross reacts with all three isoforms of JNK, recombinant activating factor-2 (ATF-2), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-phospho JNK, anti-phospho ATF-2, and anti-phospho *c-jun* antibodies were from New England Biolabs (Beverly, MA).

Cell culture

Proximal tubule epithelial cells were isolated from New Zealand White rabbits as previously described (18). They were maintained in modified DMEM:F-12 (1:1) media supplemented with 5% fetal calf serum (FCS), 5 $\mu\text{g}/\text{ml}$ insulin, 5 $\mu\text{g}/\text{ml}$ transferrin, 0.5 μM hydrocortisone, 350 $\mu\text{g}/\text{ml}$ L-glutamine, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Subconfluent monolayers of first-passage cells were employed for experiments.

Immunoprecipitation and immune complex kinase assay for JNK

Cells were serum deprived for 16–18 h before any experiment. The kinase assay was conducted as previously described (3). In brief, after an experimental treatment, cells were washed twice with ice-cold Dulbecco's PBS and were lysed on ice by adding 0.3 ml of lysis buffer [50 mM Tris (pH 7.2), 1% (vol/vol) Triton X-100, 1 mM Na_3VO_4 , 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 25 $\mu\text{g}/\text{ml}$ leupeptin, and 10 $\mu\text{g}/\text{ml}$ aprotinin]. The samples were centrifuged at 14,000 g for 10 min. Protein content in the supernatants was determined by the BCA Protein Assay according to the manufacturer's instructions (Pierce, Rockford, IL). Two hundred micrograms of lysate protein in a total volume of 800 μl was precleared with 1 μg of nonimmune rabbit IgG and 30 μl of goat anti-rabbit IgG agarose beads on a rotating plate for 1.5 h at 4°C. After centrifugation at 10,000 g for 10 min, the supernatant was mixed with 1 μg of anti-JNK1(FL) polyclonal antibody and 25 μl of goat anti-rabbit IgG agarose beads on a rotating plate overnight at 4°C. The beads were pelleted and washed twice with lysis buffer and once with the kinase assay buffer [20 mM HEPES, pH 7.6, 20 mM MgCl_2 , 25 mM β -glycerol phosphate, 0.1 mM Na_3VO_4 , and 2 mM dithiothreitol]. The kinase assay was carried out at 30°C for 15 min in 30 μl of assay buffer containing 0.5 μg of ATF-2, 20 μM ATP, and 2 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The reaction was terminated by addition of Laemmli's sample buffer followed by boiling for 5 min. The samples were resolved by 10% SDS-PAGE followed by staining with Coomassie brilliant blue to check for protein loading. The gel was dried, and the incorporation of ^{32}P was

visualized by autoradiography. Gel slices of the 69-kDa ATF-2 bands were also cut out, and the radioactivity was measured by liquid scintillation counting.

Immunoblot assay for phospho-JNK, phospho-ATF-2, and phospho-c-jun

Cell lysates were prepared as described above, following treatments indicated in the figure legends. Fifteen micrograms of total cell lysate protein were subjected to SDS-PAGE and then transferred to a polyvinylidene difluoride membrane by electroblotting at 200 mA for 1.5 h. The membrane was incubated overnight at 4°C with 5% nonfat milk dissolved in Tris-saline buffer containing 0.1% (vol/vol) Tween 20 (TTBS), followed by three washes with TTBS. The membrane was then incubated with primary antibodies overnight at 4°C, followed by six washes and another incubation with 1:2,000 dilution of HRP-conjugated goat anti-rabbit antibody at room temperature for 1 h. After extensive washes, the immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). The exposure autoradiograph is analyzed by the OS-Scan Image Analysis System to obtain the densitometry data.

Cytosolic Ca^{2+} determination

$[\text{Ca}^{2+}]_i$ was measured as previously described (13). Briefly, cells were grown on collagen-coated glass coverslips and loaded with the fluorescent Ca^{2+} indicator dye fura 2. The coverslip was then mounted in a Sykes-Moore chamber (Bellco, Vineland, NJ) and constantly perfused with Ringer buffer. Under the microscope, cells were alternately illuminated through epifluorescent optics with light at 340 and 380 nm. Light emitted >510 nm was captured by an intensified charge-coupled device camera. The video images were digitized and analyzed with Image-1/FL software (Universal Imaging, Media, PA). Images at the two wavelengths were each acquired for one-half second, and then the ratio image (340/380) was calculated with acquisition of ratio images every 5 s. Free $[\text{Ca}^{2+}]_i$ was derived from the following equation (9): $[\text{Ca}^{2+}]_i = K_d \times \beta \times (R - R_{\min}) / (R_{\max} - R)$ where $K_d = 224$ nM, β is the ratio of fluorescence at 380 nm with low (10 mM EGTA) and high (1.2 mM Ca^{2+}) free Ca^{2+} , R is the ratio of fluorescence with excitation at 340 and 380 nm, and $R_{\min}$ and $R_{\max}$ are the fluorescence ratios with low and high free Ca^{2+} . Experiments were carried out at 37°C.

$^{45}\text{Ca}^{2+}$ uptake

Cells were grown in 24-well culture plates to subconfluence. Serum was removed from the culture medium 1 day before experiments. To measure Ca^{2+} uptake, the old medium was completely removed and cells incubated at room temperature (25°C) with new medium containing $^{45}\text{Ca}^{2+}$ (1 $\mu\text{Ci}/\text{well}$, 6.7 Ci/mol CaCl_2) in the presence or absence of BAPTA-AM or dimethyl BAPTA-AM for 1–20 min, respectively. To terminate $^{45}\text{Ca}^{2+}$ uptake, the cells were washed three times with ice-cold sucrose solution. The cells were then lysed with 0.1 N NaOH/1% SDS, and the $^{45}\text{Ca}^{2+}$ counts remaining in each well were determined by scintillation counting.

Arachidonic acid release

Cells were subcultured into 12-well plates to subconfluence and labeled with 0.5 $\mu\text{Ci}/\text{ml}$ per well [^3H]arachidonic acid for 3 h before treatment. The wells were washed three times with medium containing 5% fatty acid-free FCS to remove free, labeled arachidonic acid. After treatment of cells, the medium was removed and the released arachidonic acid was determined by scintillation counting. Each data point is the average from at least three wells.

Data analysis

For fura 2 experiments, fluorescence data from at least 12 (*n*) cells were converted to $[Ca^{2+}]_i$ and shown as the mean in the figures and given as mean $\pm$ SE in RESULTS. $^{45}Ca^{2+}$ uptake data are expressed as percentage of control without treatment, whereby *n* represents the number of wells. JNK activity is quantitated by counts per minute from kinase assays or by densitometry units from immunoblots (see Fig. 4B) and is expressed relative to that in unstimulated cells. Data are given as means $\pm$ SE of *n* assays. The effects of putative inhibitors for JNK were quantified by setting the stimulated portion of JNK activity (total stimulated activity minus baseline activity) to 100% and calculating the decrease brought about by putative inhibitors. Thus 100% inhibition indicates that the experimental drug completely suppressed any effect of the stimulatory agent present simultaneously. Significance between differently treated groups was determined by unpaired two-tail Student's *t*-test.

Results

To delineate the relationship between changes in $[Ca^{2+}]_i$ and JNK activation, a variety of manipulations were employed to change $[Ca^{2+}]_i$. The Ca^{2+} ionophore A-23187 (1 μ M) induced a significant increment in $[Ca^{2+}]_i$ from a basal level of 87 ± 18 nM to a peak of $1,353 \pm 576$ nM (*n* = 12) within 5 min, which was followed by a slow return to about 258 nM by 13 min (a level that was 3-fold higher than the basal one). Although significant JNK activation could be observed as early as 5 min, maximum activation of $443 \pm 55\%$ (*n* = 3, *P* < 0.01, relative to basal level) occurred at 15 min (Fig. 1A). Similarly, thapsigargin (1 μ M) induced a transient Ca^{2+} elevation with a peak value of $1,153 \pm 382$ nM (*n* = 12), followed by return to a plateau concentration that was twofold higher than the basal one. This treatment also activated JNK with a maximum activation of $462 \pm 87\%$ (*n* = 6, *P* < 0.01) at 15 min (Fig. 1B).

BAPTA-AM is widely used as an agent that is converted intracellularly to the Ca^{2+} chelator BAPTA. Interestingly, BAPTA-AM activated JNK in renal proximal tubule epithelial cells. As shown in a time series experiment in Fig. 2A, JNK activity was induced by 5 min and peaked at 30 min ($290 \pm 31\%$, *n* = 16, *P* < 0.01); the activity returned to the basal level after 2 h. To assess whether BAPTA-AM indeed chelated cytosolic Ca^{2+} , $[Ca^{2+}]_i$ was monitored continuously by fura 2 fluorescence. BAPTA-AM decreased $[Ca^{2+}]_i$ from basal 101 ± 10 nM (*n* = 12) to 66 ± 7 nM (*n* = 12) at 5 min (a 35% decrease) when cells were bathed in a medium containing normal $[Ca^{2+}]$, i.e., 1.05 mM. $[Ca^{2+}]_i$ eventually normalized at ~ 1 h during continuous exposure to BAPTA-AM.

To evaluate JNK activation in intact cells, the cell lysate was probed for the phosphorylated (activated) form of JNK and its downstream signaling products, i.e., phospho-ATF-2 and phospho-*c-jun* (Fig. 2B). Equal sample loading was verified by stripping the membrane and immunoblotting for total JNK protein with anti JNK(FL) antibody (data not shown). BAPTA-AM clearly activated JNK by these criteria (Fig. 2B).

Although BAPTA-AM is a Ca^{2+} chelator, the activation of JNK is not necessarily related to this property. To resolve this question, we compared the effects of BAPTA-AM and dimethyl BAPTA-AM, reasoning that, if the BAPTA-AM effect was related to Ca^{2+} chelation, then dimethyl BAPTA-AM should be more potent because it has a higher affinity for Ca^{2+} (K_d : 40 nM vs. 160 nM, Fig. 3A). Although both analogs gave a similar time course of JNK activation, dimethyl BAPTA-AM activation of JNK was either more intensive at the same concentration, or was effective at a lower concentration than that of BAPTA-AM as determined by both vitro kinase assay (Fig. 3B) and immunoblot for the

phosphorylated forms of JNK (Fig. 3C). Therefore, it is likely that JNK activation by BAPTA-AM is somehow related to cytosolic Ca^{2+} chelation or cellular Ca^{2+} metabolism.

Although it is possible that JNK is activated by both cytosolic $[\text{Ca}^{2+}]$ increases or decreases, both essentially representing stress situations, paradoxical activation of a number of Ca^{2+} -dependent processes by chelation of cytosolic Ca^{2+} has been shown before, e.g., for exocytosis in chromaffin cells and Ca^{2+} -dependent inhibition of adenylyl cyclase (6,7). The paradox is explained by depletion of endoplasmatic Ca^{2+} stores that, in turn, results in activation of plasma membrane Ca^{2+} channels (usually termed activation of “ Ca^{2+} release-activated channels” or “capacitative Ca^{2+} entry”) and therefore highly localized increases in $[\text{Ca}^{2+}]$. Localized $[\text{Ca}^{2+}]$ increases around channel openings would not have been detected with the fura 2 method employed in this study because of insufficient temporal and spatial resolution (about 5 s and 5 μm , respectively).

To assess whether BAPTA-AM activates JNK by a Ca^{2+} -dependent mechanism, similar to A-23187 and thapsigargin, we measured to what extent JNK activation is dependent on Ca^{2+} influx. Cells were stimulated with BAPTA-AM or thapsigargin in the absence or presence of Ca^{2+} influx inhibitors or an extracellular Ca^{2+} chelator. As shown in Fig. 4A, SKF-96365, a general Ca^{2+} influx inhibitor (19), and nifedipine, an L-type Ca^{2+} channel blocker, significantly reduced BAPTA-AM-induced JNK activation by 62% and 99.5%, respectively. Both drugs together brought BAPTA-AM-induced JNK activation to below the basal level. In comparison, JNK activation induced by thapsigargin was inhibited by SKF-96365 and nifedipine by 87% and 90%, respectively. Furthermore, chelation of extracellular Ca^{2+} with 3 mM EGTA decreased both BAPTA-AM- and thapsigargin-induced JNK activation by 62% ($n = 3$) and 94% ($n = 3$), respectively (Fig. 4B). These results indicate that localized Ca^{2+} influx and $[\text{Ca}^{2+}]$ increases, and not bulk cytosolic $[\text{Ca}^{2+}]$, represent a major pathway for JNK activation in the case of BAPTA-AM and thapsigargin.

To verify that BAPTA-AM and dimethyl BAPTA-AM also increase Ca^{2+} influx across the plasma membrane of proximal tubule cells, as predicted by the capacitative Ca^{2+} entry mechanism, cellular $^{45}\text{Ca}^{2+}$ uptake was measured. As shown in Fig. 5A, exposure to BAPTA-AM (10 μM) increased $^{45}\text{Ca}^{2+}$ uptake significantly at 20 min. Dimethyl BAPTA-AM caused an even higher Ca^{2+} influx that was significant at both 1 min and 20 min. The greater efficacy of dimethyl BAPTA-AM compared with BAPTA-AM is again in line with its greater chelation affinity for Ca^{2+} , greater store depletion, and hence greater activation of capacitative Ca^{2+} entry. The dimethyl BAPTA-AM-induced $^{45}\text{Ca}^{2+}$ uptake was blocked by SKF-96365 and nifedipine by 89% and 66%, respectively (Fig. 5B), indicating that specific Ca^{2+} entry pathways had been activated.

Our previous data (3,18) demonstrated that arachidonic acid induced JNK activation by a Ca^{2+} -independent mechanism. To investigate whether BAPTA-AM and thapsigargin involve arachidonic acid as a mediator in the activation of JNK, we measured the release of arachidonic acid after exposure to BAPTA-AM and thapsigargin. As shown in Fig. 6, whereas thapsigargin significantly increased free arachidonic acid, BAPTA-AM had only a moderate, statistically nonsignificant effect.

Discussion

JNK is an important signal molecule associated with mitogenesis and apoptosis and is activated especially during stress-induced injury (2,14,23,36,37). Therefore, a detailed understanding of its activation mechanisms is of considerable interest. In many cell types, JNK activation has been correlated with elevated $[\text{Ca}^{2+}]_i$ and is thus considered to be “ Ca^{2+} dependent” (15,21,27,31,38). However, no consensus has emerged from many different

types of experiments that elevation of $[Ca^{2+}]_i$ is the direct cause of JNK activation. In addition, the Ca^{2+} ionophore A-23187 has been found to be insufficient to activate JNK in several cell types (8,15,31).

The complexity of intracellular Ca^{2+} metabolism in regulating cellular function has become obvious with the increased resolution provided by modern electrophysiological and fluorescence-imaging technology (33). It is now recognized that many cellular functions are normally regulated by localized and not global cytosolic increases of $[Ca^{2+}]_i$ (30). Local, short-lived $[Ca^{2+}]_i$ increases can be sufficient to act as signal transducers. For example, Lau et al. (16) have demonstrated by photolysis of caged Ca^{2+} that a local transient $[Ca^{2+}]_i$ elevation is sufficient to induce new filopodia in neuronal growth cones. Another important aspect of intracellular Ca^{2+} metabolism is the phenomenon of Ca^{2+} -induced Ca^{2+} release that serves to amplify localized increases of $[Ca^{2+}]_i$ so that relatively high $[Ca^{2+}]_i$ concentrations can be achieved for short periods of time (1,4,10,25,26). For example, in excitable cells, such as cardiac myocytes and neurons, Ca^{2+} -induced Ca^{2+} release from the internal stores is triggered by a transient microdomain of high $[Ca^{2+}]_i$ beneath an open L-type Ca^{2+} channel (17,35).

On this background, the paradoxical, Ca^{2+} -influx-dependent activation of JNK by BAPTA-AM and dimethyl BAPTA can be explained in rabbit proximal tubule cells. BAPTA-AM activates “capacitative Ca^{2+} influx” in this cell type (that could be inhibited by either SKF-96365 or nifedipine, see $^{45}Ca^{2+}$ data) and other epithelial cells, measured usually as electrical Ca^{2+} current (I_{CRAC} , I = current; CRAC = Ca^{2+} release-activated channel) (34). Although the phenomenon of capacitative Ca^{2+} entry is well established for many cell types, including epithelial cells, the signal transduction pathway leading from store depletion to plasma membrane Ca^{2+} channel activation is not (24,32). Paradoxical activation of Ca^{2+} -dependent processes through store depletion and local $[Ca^{2+}]_i$ increases would be expected to be highly variable depending on cell type and on the kinetic and binding properties of the cytosolic Ca^{2+} chelator employed, because rates and extent of store depletion, magnitude of Ca^{2+} stores, efficiency of signal transduction, or extent of activatable Ca^{2+} channels in the plasma membrane may be different. Thus variability of Ca^{2+} -dependent JNK activation with cell type is consistent with indirect activation through capacitative Ca^{2+} entry. In quantitative terms, a minimum of 62% of JNK activation by BAPTA-AM is explained with Ca^{2+} entry based on the inhibition data by chelation of extracellular (62%) or the SKF inhibitor (62%). The mechanism that results in the residual JNK activation by BAPTA-AM after chelation of extracellular Ca^{2+} is not understood, however, it is also nifedipine sensitive.

Although there have been indications that BAPTA-AM may induce JNK activation in other cell types, either no specific data were presented or the effect was suggested as being nonspecific (27,38). Interestingly, the thapsigargin data suggest that JNK activation in this case also occurs largely through the capacitative Ca^{2+} entry pathway, although store depletion occurs through a different mechanism, i.e., inhibition of the Ca^{2+} -ATPase in the endoplasmic reticulum. Differences in cytosolic $[Ca^{2+}]_i$ between BAPTA-AM and thapsigargin can actually explain differences between the two agents in the duration of JNK activation. A comparison of Fig. 1B and Fig. 2A indicates a shorter duration of JNK activation by thapsigargin compared with BAPTA. High $[Ca^{2+}]_i$ with thapsigargin has been shown to inactivate I_{CRAC} (11,22,29) and would thus explain a shorter duration of JNK activation. Our finding of prolongation of JNK activation by BAPTA-AM is analogous to several electrophysiology observations demonstrating that inclusion of BAPTA in the patch pipette attenuates the inactivation of I_{CRAC} that normally occurs in the high $[Ca^{2+}]_i$ situation with thapsigargin alone (11,28–30). The nature of the channels activated by store depletion is not known at the moment. Although nifedipine is known as an inhibitor of voltage-

dependent L-type channels, it should be recognized that the concentration of 10 μM used in the studies is high and could block other types of channels.

Previous observations from our laboratory demonstrated that arachidonic acid and the cytochrome *P*-450 products mediate the Ca^{2+} -dependent activation of p42 and p44 MAPK via the tyrosine kinase-Shc/Grb2/Sos-Ras pathway (5,12). JNK activation appears to be more complex with at least a Ca^{2+} -dependent and a Ca^{2+} -independent step. These steps may be in the same pathway, although parallel pathways of activation cannot be excluded. Cytosolic phospholipase A_2 has been shown to be activated by thapsigargin via either increased $[\text{Ca}^{2+}]_i$ or local $[\text{Ca}^{2+}]$ (20). BAPTA-AM activation of JNK could also involve arachidonic acid release (Fig. 6), even though our experiments could not show statistical significance for the increase. To clarify this point, another study is required.

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Fig. 1.

Time course of *c-jun* NH₂-terminal kinase (JNK) activation and its relationship to elevation of cytosolic Ca²⁺ concentration ([Ca²⁺]_i) by the Ca²⁺ ionophore A-23187 and the Ca²⁺-ATPase inhibitor thapsigargin. *A*: effects of A-23187. Rabbit proximal tubular epithelial cells were serum starved overnight and treated with 1 μM A-23187 for the length of time indicated. Cell lysates containing 200 μg protein were immunoprecipitated with anti-JNK1 polyclonal antibody, and the in vitro kinase assay was carried out as described in MATERIALS AND METHODS. Equal protein loading was confirmed by staining the gel with Coomassie brilliant blue before drying and detection of ³²P on X-ray film. The autoradiograph is representative of 3 similar results. [Ca²⁺]_i was monitored by fura 2 fluorescence as described in MATERIALS AND METHODS. *B*: effects of thapsigargin. Cells were treated and analyzed as in *A*, except that 1 μM thapsigargin replaced A-23187. pATF-2; phosphorylated ATF-2 detected by ³²P incorporation.

Fig. 2.

Time course of JNK activation and its relationship to depression of [Ca²⁺]_i by the intracellular Ca²⁺ chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA)-AM. *A*: effect of BAPTA-AM on [Ca²⁺]_i and JNK measured by an in vitro kinase assay. Cells were treated and analyzed as in Fig. 1A, except that 10 μM BAPTA-AM replaced A-23187. *B*: effect of BAPTA-AM on JNK activity in intact cells measured as amount of phosphorylated form of JNK as well as its substrates. Total cell lysates containing 15 μg protein were resolved by 10% SDS-PAGE, electroblotted onto polyvinylidene difluoride membranes, and immunostained with antibodies against the phosphorylated forms of the indicated proteins as described in MATERIALS AND METHODS. Equal protein loading was confirmed by staining the membrane with Ponceau Red (data not shown). *Left lane*: no stimulation. *Right lane*: stimulation for 30 min with 10 μM BAPTA-AM. *p46JNK, phosphorylated (Thr183/Tyr185) JNK, the p54JNK is phosphorylated identically as p46JNK (data not shown); *c-Jun, phosphorylated (Ser63) c-Jun; *ATF-2, phosphorylated (Thr71) ATF-2. The figure is representative of results from 3 independent experiments.

Fig. 3. Correlation of Ca^{2+} -chelating affinity with JNK activation potency. *A*: structure and Ca^{2+} dissociation constants for BAPTA and dimethyl BAPTA. *B*: time course of JNK activation by BAPTA-AM and dimethyl BAPTA-AM. Quiescent cells were treated with 10 μM BAPTA-AM or dimethyl BAPTA-AM for the times indicated. JNK activity was measured by the in vitro kinase assay. *C*: dose-response data for JNK activation by BAPTA-AM and dimethyl BAPTA-AM at 30 min. JNK activity was measured by immunoblot with anti-phospho JNK antibody. Results from 1 series of experiments (out of 2) are shown; the results of the 2 series were similar.

Fig. 4. Effect of inhibition of Ca^{2+} influx on BAPTA-AM- and thapsigargin-dependent activation of JNK. **A:** effect of SKF-96365 and nifedipine. Cells were serum starved overnight and pretreated with 10 μM SKF-96365 or 10 μM nifedipine for 20 min, before exposure to 10 μM BAPTA-AM for 30 min or 1 μM thapsigargin for 15 min. JNK activity was measured by the in vitro kinase assay. Data are presented as percentage of unstimulated JNK activity (=100%). Significance was determined between stimulation in the presence or absence of each inhibitor. * $P < 0.05$. **B:** effect of removal of free, extracellular Ca^{2+} with EGTA. Cells were stimulated with 10 μM BAPTA-AM for 30 min or 1 μM thapsigargin for 15 min in the presence or absence of 3 mM EGTA in the medium. Data shown is a representative of in vitro kinase assay.

Fig. 5.

Effect of intracellular Ca^{2+} chelation and Ca^{2+} -transport inhibitors on $^{45}\text{Ca}^{2+}$ uptake. **A:** effect of BAPTA-AM and dimethyl BAPTA-AM. Quiescent cells were exposed to $^{45}\text{Ca}^{2+}$ (1 $\mu\text{Ci}/\text{well}$, 6.7 Ci/mol CaCl_2) in the presence or absence of 10 μM of BAPTA-AM or dimethyl BAPTA-AM for 1 to 20 min, respectively. The cells were washed, and the remaining $^{45}\text{Ca}^{2+}$ in each well was determined by scintillation counting techniques. Significance was determined between control and BAPTA-AM or dimethyl BAPTA-AM. $*P < 0.05$, $**P < 0.01$. **B:** effect of transport inhibitors on dimethyl BAPTA-AM-stimulated $^{45}\text{Ca}^{2+}$ uptake. The data are expressed relative to uptake by unstimulated cells. Cells were serum starved overnight and pretreated with 10 μM SKF-96365 or 10 μM nifedipine for 20 min before exposure to 10 μM dimethyl BAPTA-AM for 20 min. $^{45}\text{Ca}^{2+}$ uptake was measured as described **A**. The data are from 2 to 5 independent experiments.

Fig. 6. Effect of BAPTA-AM and thapsigargin on arachidonic release. Quiescent cells were labeled with [^3H]arachidonic acid for 3 h, followed by stimulation with 10 μM BAPTA-AM or 1 μM thapsigargin for 5 min.