The Role of pp60c-src in the Regulation of Calcium Entry via Store-operated Calcium Channels*

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In many cell types, G protein-coupled receptors stimulate a transient Ca2+ release from internal stores followed by a sustained, capacitative Ca2+ entry, which is mediated by store-operated channels (SOCs). Although it is clear that SOCs are activated by depletion of internal Ca2+ stores, the mechanism for this process is not well understood. Previously, we have reported that inhibitors of tyrosine kinase activity block the bradykinin- and thapsigargin-stimulated Ca2+ entry in fibroblasts, suggesting that a tyrosine kinase activity may be involved in relaying the message from the empty internal Ca2+ stores to the plasma membrane Ca2+ channel (1). This hypothesis is based on our observations that inhibitors of tyrosine kinase activity (such as genistein and tyrphostin) block the plateau phase of the BK-induced Ca2+ response, whereas an inactive analog of genistein (diadezin) or inhibitors of serine/threonine kinases have no effect (5). The tyrosine kinase activity appears to lie downstream from the empty Ca2+ pool because tyrosine kinase inhibitors also block the Ca2+ entry stimulated by thapsigargin (5), an agent that directly empties the Ca2+ pools by inhibiting the ATPase that pumps Ca2+ into the internal stores.

To investigate the identity of the tyrosine kinase involved in regulating SOCs, we first had to identify the tyrosine kinases activated in response to BK stimulation. We recently reported (6) that in fibroblasts BK stimulates the tyrosine kinase activity of pp60c-src (c-src). With this information in hand, we turned to the investigation of whether this tyrosine kinase is involved in the regulation of SOCs. To test for the involvement of c-src in the regulation of SOCs, we chose to utilize fibroblast lines that do not express the c-src protein tyrosine kinase as a result of gene disruption by homologous recombination (7). To investigate the regulation of SOCs by c-src, the intracellular Ca2+ stores of either wild type cells or Src− cells were emptied by various methods in a nominally Ca2+-free buffer, and the influx of Ca2+ was monitored following addition of Ca2+ to the external medium.

Most cultured fibroblasts respond to the peptide hormone bradykinin with a biphasic elevation of intracellular Ca2+ concentration. The initial peak of the Ca2+ response is due to Ca2+ release from inositol trisphosphate-sensitive stores, whereas the longer duration, plateau phase is due to Ca2+ influx from the extracellular medium. Our previous studies have demonstrated that the BK-stimulated Ca2+ influx is via a "capacitative" Ca2+ pathway (1) similar to the one first described in pancreatic acinar cells by Putney (2). The physiological importance of capacitative Ca2+ entry is suggested by the resulting primary immunodeficiency associated with defective T cell proliferation in patients whose lymphocytes have low capacitative Ca2+ entry following T cell receptor stimulation (3, 4). Putney hypothesized that the mechanism for opening this type of Ca2+ channel involves a signal transduction process in which the "fill state" of the internal Ca2+ stores is sensed and a message is sent to open plasma membrane Ca2+ channels, if the stores are empty. Subsequent studies from a number of different laboratories have supported the basic concepts of this hypothesis, although the exact mechanism for regulation of these capacitative Ca2+ channels, or store-operated channels (SOCs), is still an area of active investigation.

Based upon previous results from our laboratory, we have proposed that a tyrosine kinase activity is involved in relaying the message from the empty internal Ca2+ stores to the plasma membrane Ca2+ channel (5). This hypothesis is based on our observations that inhibitors of tyrosine kinase activity (such as genistein and tyrphostin) block the plateau phase of the BK-induced Ca2+ response, whereas an inactive analog of genistein (diadezin) or inhibitors of serine/threonine kinases have no effect (5). The tyrosine kinase activity appears to lie downstream from the empty Ca2+ pool because tyrosine kinase inhibitors also block the Ca2+ entry stimulated by thapsigargin (5), an agent that directly empties the Ca2+ pools by inhibiting the ATPase that pumps Ca2+ into the internal stores.

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The absence of c-src produced a significant decrease in the Ca2+ influx in response to both bradykinin and thapsigargin. This could be reversed by expression of c-src in Src− cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—The wt and src−/src− (Src−) cell lines were derived from spontaneous immortalization of mouse embryo fibroblasts prepared from either wild type mice or mice homozygous for a disruption in the c-src gene. These nonclonal cell populations were kindly provided by Philippe Soriano (Fred Hutchinson Cancer Center, Seattle, WA). The chicken c-src plasmid was kindly provided by David Shalloway (8). The plasmid carrying hygromycin resistance was obtained from Invitrogen (San Diego, CA). Avian-sarcoma virus and Moloney- Moloney were from Upstate Biotechnology, Inc. (Lake Placid, NY). Moloney- anti-c-src antibodies (mAb 327) were obtained from Oncogene Sciences (Uniondale, NY). Horseradish peroxidase-labeled secondary antibodies

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1 The abbreviations used are: BK, bradykinin; SOC, store-operated calcium channels; wt, wild type; Src−, fibroblasts from c-src knock-out mice; TG, thapsigargin; mAb, monoclonal antibody; HHBSS, HEPES-buffered Hanks’ balanced salt solution.
were purchased from Promega (Madison, WI). Protein assay kits and ECL reagents were obtained from Pierce.

Cell Culture—Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin in a 5% CO2 incubator. For measurements of intracellular calcium concentration, cells were plated onto coverslips 2–5 days prior to experiments. For Western blotting, the cells were plated onto 100-mm dishes.

Western Blot Analysis—Cell lysates were prepared by treating cells with 0.5% SDS/8 M urea. The lysates were freeze-thawed three times to reduce viscosity. The protein concentration was determined by the BCA method (Pierce). The protein samples were mixed with equal volumes of 2× SDS sample buffer (1× = 62.5 mM Tris, pH 6.8, 1% SDS, 0.001% pyroin-Y, 10% glycerol, 5 mM 2-mercaptoethanol), boiled for 3 min, and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes, and nonspecific binding sites were blocked by incubating the membranes in TBS (20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM KCl) containing 3% bovine serum albumin and 0.2% Nonidet P-40 for 1 h at room temperature. Antibodies at a concentration of 1 μg/ml were added, and the blot was incubated for 16 h at 4°C. The membrane was washed with TBS/0.2% Nonidet P-40 for 40 min with horseradish peroxidase-labeled secondary antibody. The blot was washed five times, and the immunostaining was detected by enhanced chemiluminescence.

Image Analysis—Cells were loaded with 5 μM fura-2 acetoxyethyl ester in HEPES-buffered Hanks’ balanced salt solution (HHBSS) + 1 mg/ml bovine serum albumin + 0.025% Pluronic F127 detergent for 30 min at room temperature and incubated without fura-2 acetoxyethyl ester in HHBSS for 30 min, and the intracellular [Ca2+]i was monitored as described previously (1). All traces represent the average response of 300–400 cells on a nearly confluent coverslip. Although data shown here are from nearly confluent coverslips, measurements on lower density coverslips showed that there was no density dependence of the results. Cells perfused in “nominally” Ca2+-free HHBSS prepared as described previously (1) are still tightly adherent and can be vigorously perfused for extended time periods.

Establishment of Src Fibroblasts Stably Expressing Chicken c-src—Fibroblasts deficient in c-src were cotransfected with chicken c-src and a plasmid carrying hygromycin resistance (pCEN4). After 2 days, cells were cultured in the presence of 250 μg/ml hygromycin for 24 h. The selection pressure was removed, and cells were cultured in normal medium. A week later cells were reselected with 150 μg/ml hygromycin for 2 days. Approximately 200 clones survived. All of the surviving clones were harvested, mixed together, and expanded to generate a heterogeneous population of cells expressing chicken c-src. Cells were periodically put under selection pressure.

RESULTS AND DISCUSSION

BK-stimulated Ca2+ Entry in wt Fibroblasts and Src Fibroblasts—For the initial studies, cells were challenged with 100 nM BK in a nominally Ca2+-free external medium. The Ca2+ peak under these conditions (Fig. 1) is due to the emptying of inositol triphosphate-sensitive intracellular stores (1). The difference in the height of initial peaks in individual experiments generally is due to the fact that some fibroblasts in the field had delayed response to BK, thereby producing a wider, lower peak under these conditions (Fig. 1) is due to the emptying of intracellular Ca2+ stores. When the [Ca2+]i of a cell is not pumped by the Ca2+-ATPase, the reduced Ca2+ entry observed in Src- cells is not due to an increased Ca2+ pump activity but is the result of decreased capacitative Ca2+ influx. This observation suggests that c-src may be involved in the regulation of Ca2+ entry in response to BK.

Although we have demonstrated that BK-induced Ca2+ entry is significantly suppressed in Src fibroblasts, there does appear to be capacitative Ca2+ entry in Src cells. This residual Ca2+ influx may be due to either regulation by other tyrosine kinases or by regulation by a tyrosine kinase-independent mechanism. It has been shown that other c-src tyrosine kinase family members (e.g. c-fyn and c-yes) have some overlapping activities with c-src, which suggests the possibility that c-fyn or c-yes could also participate in the regulation of SOCs. This possibility is supported by experiments using genistein, a tyrosine kinase inhibitor that further suppressed the residual capacitative Ca2+ entry in Src cells (data not shown). Thapsigargin-stimulated Ca2+ Entry in wt Fibroblasts and Src Fibroblasts—To exclude the possibility that c-src might mediate an increase in intracellular Ca2+ stores, we have also examined capacitative Ca2+ influx in Src cells challenged with thapsigargin (TG), a potent Ca2+-ATPase inhibitor (Fig. 2). Following TG-induced Ca2+ store depletion, Ca2+ influx was initiated by the addition of Ca2+ to the extracellular solution. As a measure of Ca2+ influx, we compared the slope of the initial rise (10–20 s) of [Ca2+]i, in both cells lines.
Src- and wt fibroblasts. As was the case for BK, thapsigargin-induced Ca\textsuperscript{2+} influx is significantly lower in fibroblasts deficient in c-src than that measured in wt fibroblasts (30 ± 15% of the slope observed in wt cells, n = 7; value significantly different from wt value based on a t test, p < 0.005), suggesting that the effect of c-src in the regulation of SOCs is downstream from the depletion of intracellular Ca\textsuperscript{2+} stores. The fact that Ca\textsuperscript{2+} entry is lower in Src\textsuperscript{2-} fibroblasts in response to both BK and TG could mean either that c-src is involved in regulation of Ca\textsuperscript{2+} entry following store depletion or that the level of Ca\textsuperscript{2+} entry was coincidentally lower in the independently derived Src\textsuperscript{2-} cell lines. To distinguish between these possibilities, we established Src\textsuperscript{2-} fibroblasts stably expressing chicken c-src (B) were resolved on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membrane was immunoblotted with mAb 327 (A) or an anti-avian src antibody (B). The blot is representative of three experiments.

Stable Transfection of c-src into Src\textsuperscript{2-} fibroblasts—To express c-src in Src\textsuperscript{2-} fibroblasts, we chose chicken c-src, because this src protein was shown to have little or no transforming activity when overexpressed in NIH3T3 fibroblasts, compared with v-src and mouse c-src (8–11). We did not want to transform the cells, because transformation very often results in the overexpression or hyperphosphorylation of proteins involved in important signaling processes. The expression of c-src in Src\textsuperscript{2-} fibroblasts posed some technical difficulties because these cells are difficult to transfect transiently. Therefore, we generated a population of cells that stably expresses the protein (see “Experimental Procedures”). The expression of chicken c-src was detected by immunostaining and also by Western blotting using the avian
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specific anti-c-src antibodies (Fig. 3). Western blots performed with mAb 327 demonstrated a 3–4-fold higher level of staining in the c-src transfected cells compared with wt cells (data not shown), although without knowing the relative specificity of mAb 327 for chicken versus mouse c-src, it is not clear whether this represents a 3–4-fold difference in protein expression.

BK- and Thapsigargin-stimulated Ca\(^{2+}\) Entry in Src- and Fibroblasts Stably Expressing Chicken c-src—When capacitative Ca\(^{2+}\) entry was monitored following Ca\(^{2+}\) pool depletion with BK, it was observed that stable expression of chicken c-src significantly enhanced the capacitative Ca\(^{2+}\) influx (Fig. 4). The expression of chicken c-src resulted in a 2.8 ± 0.2-fold increase in the initial slopes of the Ca\(^{2+}\) traces at the addition of external Ca\(^{2+}\) (value is significantly higher than Src- value based on a t test, p < 0.001). In addition, when intracellular Ca\(^{2+}\) stores were depleted by treating cells with thapsigargin (1 μM), the capacitative Ca\(^{2+}\) influx was greatly enhanced in the cells expressing chicken c-src (2.7 ± 0.6-fold increase, n = 9; value is significantly higher than Src- value based on a t test, p < 0.01) (Fig. 5). Although the initial BK-stimulated release of Ca\(^{2+}\) was frequently higher in the cells transfected with chicken c-src than in the Src- cells, this does not explain the higher Ca\(^{2+}\) influx in the transfected cells. First, although measurements of the initial peak in chicken c-src transfected cells varied widely in height between individual coverslips, there was no correlation between first peak height and size of Ca\(^{2+}\) influx. Second, the STG-stimulated peaks frequently had the opposite relationship (although this was not the case in Fig. 5, the first peak height was often higher in Src- fibroblasts than in the cells transfected with chicken c-src), and Ca\(^{2+}\) influxes were still higher than in Src- cells. Therefore, these observations strongly support our proposal that c-src is indeed involved in the regulation of calcium entry via store-operated calcium channels.

The BK-induced capacitative Ca\(^{2+}\) entry in c-src transfected cells was 78 ± 11% of the signal observed in the wild type cells. In the case of TG-induced Ca\(^{2+}\) entry we observed 82 ± 18% restoration. Immunostaining of hygromycin-selected cells showed that we could detect strong expression of avian c-src in 25% of the cells, intermediate expression in 30% of the cells, and little or no chicken c-src staining in 45% of the cells (data not shown). Thus, because of the selection method used, not all of the cells in this heterogenous population express chicken c-src. Cells were selected based on their expression of an antibiotic resistance gene marker; thus some percentage of cells express the antibiotic resistance gene and no c-src or low levels of c-src.

Even though these data support the hypothesis that c-src may play an important role in the regulation of Ca\(^{2+}\) entry via store-operated calcium channels, we cannot exclude the possibility that c-src might be acting via a longer term mechanism, such as by controlling the level of expression of SOCs. However, our previous data (5) indicate that tyrosine kinase inhibitors reduce capacitative Ca\(^{2+}\) influx within a matter of minutes of their addition, a finding that supports the involvement of tyrosine kinases in a short term, regulatory role. However, based on the observation that the SH2 and SH3 domains but not the kinase activity of c-src are important for the effect of c-src on the rate of cell spreading on fibronectin (12), we cannot rule out the possibility that the c-src effect we observe might be independent of the src kinase domain. In the future, we plan to express c-src constructs with mutations in the kinase, SH2, and SH3 domains in Src- cells to determine in more detail the importance of these domains in the regulation of Ca\(^{2+}\) entry.

Although the exact mechanism for c-src regulation of capacitative Ca\(^{2+}\) entry is not known, it appears that the decrease in Ca\(^{2+}\) entry in Src- cells is not the result of a reduction in the membrane potential and therefore a decrease in the driving force for Ca\(^{2+}\) entry. Hyperpolarization of Src- and wild type cells, by the addition of valinomycin in a 6 mM K\(^+\) medium, did not alter the fact that wild type cells had a dramatically higher level of Ca\(^{2+}\) entry than those measured in Src- cells (data not shown).

There is considerable evidence that protein phosphorylation can regulate ion channels. Ion channels are known to be regulated and directly phosphorylated by a number of serine/threonine kinases, such as protein kinase C, calmodulin-dependent kinase, and cyclic AMP-dependent protein kinase (13). Some recent studies suggest that tyrosine phosphorylation can also regulate channel activities. For example, tyrosine phosphorylation regulates the channel activities of N-methyl-D-aspartate receptor (14), a brain- and heart-specific delayed rectifier-type potassium channel (15), and a voltage-dependent n-type K+ channel (Kv1.3) (16). A recently cloned focal adhesion kinase family member, PYK2, directly tyrosine phosphorylates a potassium channel (Kv1.2) and inhibits currents elicited by phorbol myristyl acetate (17). In regard to the regulation of SOCs, several studies suggest that serine and threonine phosphorylation may inhibit capacitative Ca\(^{2+}\) entry (18, 19), and a number of studies utilizing tyrosine kinase inhibitors (5, 20–23) support our initial proposal (5) for a role of tyrosine kinases in the activation of SOCs. Further studies are required to determine whether the effect of c-src on the regulation of capacitative Ca\(^{2+}\) entry is a direct effect on the store-operated Ca\(^{2+}\) channel, as seen for hKv1.5 and N-methyl-D-aspartate channels, or an indirect effect via other c-src substrates.

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