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CaM kinase II-dependent activation of tyrosine kinases and ERK1/2 in vascular smooth muscle

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Ginnan, Roman, and Harold A. Singer. CaM kinase II-dependent activation of tyrosine kinases and ERK1/2 in vascular smooth muscle. *Am J Physiol Cell Physiol* 282: C754–C761, 2002. First published November 27, 2001; 10.1152/ajpcell.00335.2001.—In vascular smooth muscle (VSM) and many other cells, G protein receptor-coupled activation of mitogen-activated protein kinases has been linked, in part, to increases in free intracellular Ca^{2+}. Previously, we demonstrated that ionomycin-, angiotensin II-, and thrombin-induced activation of extracellular signal-regulated kinase (ERK)1/2 in VSM cells was attenuated by pretreatment with KN-93, a selective inhibitor of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase II). In the present study, we show that the Ca^{2+}-dependent pathway leading to activation of ERK1/2 is preceded by nonreceptor proline-rich tyrosine kinase (PYK2) activation and epidermal growth factor (EGF) receptor tyrosine phosphorylation and is attenuated by inhibitors of *src* family kinases or the EGF receptor tyrosine kinase. Furthermore, we demonstrate that pretreatment with KN-93 or a CaM kinase II inhibitor peptide inhibits Ca^{2+}-dependent PYK2 activation and EGF receptor tyrosine phosphorylation in response to ionomycin, ATP, and platelet-derived growth factor but has no effect on phorbol 12,13-dibutyrate- or EGF-induced responses. The results implicate CaM kinase II as an intermediate in the Ca^{2+}/calmodulin-dependent activation of PYK2.

calcium; calcium/calmodulin-dependent protein kinase; proline-rich tyrosine kinase; extracellular signal-regulated kinase 1/2; epidermal growth factor receptor transactivation; *src*; vascular smooth muscle cells; KN-93; autoinhibitory peptide

MITOGEN-ACTIVATED PROTEIN (MAP) kinases such as extracellular signal-regulated kinase (ERK)1/2 are involved in the regulation of essential cellular processes, including gene expression and cell proliferation (5, 23). Signaling pathways involving ERK1/2 are triggered by diverse cellular stimuli, including receptor tyrosine kinase-coupled growth factors (25), G protein-coupled receptor (GPCR) agonists (8), mechanical stimuli (19), and integrin-dependent cell/matrix interactions (7). Although the signaling cascade initiated by receptor tyrosine kinase-coupled growth factors such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) is understood in considerable molecular detail, the signaling pathways by which GPCR ligands activate ERK1/2 are not fully understood.

A number of proximal signaling pathways, including G protein βγ-subunits (26), protein kinase C, and/or Ca^{2+} (4), may converge on the ERK signaling cascade. The nonreceptor tyrosine kinases, proline-rich tyrosine kinase (PYK2) and *src*, have been proposed to be points of convergence for Ca^{2+}- and protein kinase C-dependent pathways leading to ERK1/2 activation (6). PYK2 and *src* may act via a pathway involving tyrosine phosphorylation and activation of the EGF receptor, scaffolding of adaptor proteins and guanine nucleotide exchange factors (SHC/GRB2/*sos*), and consequent *ras*-dependent activation of the well-described protein kinase cascade, culminating in ERK1/2 activation (10, 12). Alternatively, PYK2 and *src* may lead to activation of a protease that produces heparin/EGF from extracellular matrix that then acts as a ligand for the EGF receptor (20). Also, *src* has been proposed to directly activate the SHC/GRB2/*sos* complex (12). In all of these models, the proximal events that couple increases in free intracellular Ca^{2+} or protein kinase C to PYK2/*src* activation are not known.

In vascular smooth muscle (VSM), ERK1/2 activation in response to angiotensin II (ANG II), thrombin, or ATP appears to involve both Ca^{2+} and protein kinase C-dependent mechanisms (2, 4, 6). We previously reported that activation of ERK1/2 in cultured rat aortic VSM in response to receptor and nonreceptor Ca^{2+}-mobilizing stimuli (ANG II, thrombin, and ionomycin) is temporally preceded by activation of the multifunctional serine/threonine kinase Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) (2). Inhibition of CaM kinase II by pretreatment with a calmodulin antagonist (calmidazolium) or a CaM kinase II inhibitor (KN-93) attenuated ERK1/2 activation in response to these stimuli. This pharmacological evidence, along with limited molecular studies, suggests that CaM kinase II, like protein kinase C, is an intermediate in GPCR-mediated activation of ERK1/2 in VSM cells. However, these studies provided no insight into the mechanisms by which CaM kinase II could activate the ERK signaling pathway.

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In the present study, we used the Ca^{2+} ionophore ionomycin to selectively stimulate Ca^{2+}-dependent signaling pathways in VSM cells independent of other GPCR pathways involving either activated G protein βγ-subunits or protein kinase C and determined the effects of inhibitors of tyrosine kinases and CaM kinase II on the activation of PYK2, the EGF receptor, and ERK1/2. The results indicate that Ca^{2+}-dependent activation of ERK1/2 in VSM cells is completely dependent on PYK2/*src* activation and transactivation of EGF receptor. Pretreatment with the CaM kinase II inhibitors, KN-93 or autoinhibitory peptide (AIP), attenuates PYK2 and EGF receptor activation in response to Ca^{2+}-mobilizing stimuli in VSM, supporting a role for CaM kinase II in the activation of the nonreceptor tyrosine kinases PYK2 and *src*.

EXPERIMENTAL PROCEDURES

Cell culture. VSM cells were dispersed from the medial layer of thoracic aortas of Sprague-Dawley rats weighing 200–300 g as described earlier (14), cultured in DMEM/F-12 medium containing 10% fetal bovine serum (Hyclone) at 37°C and 5% CO_2, and split twice a week. Cells from *passages 3–10* were used in the experiments. Confluent cultures were growth arrested for 16–24 h in DMEM/F-12 containing 0.4% serum. One hour before the experiments, the 0.4% serum-containing medium was removed from the cells and replaced with Hanks' balanced salt solution (HBSS) supplemented with Ca^{2+}/Mg^{2+} and 10 mM HEPES, pH 7.4.

Cell lysate preparation. VSM cells were pretreated with inhibitors for 30 min before being exposed to stimulants for the indicated times. Reactions were stopped by transferring the dishes to ice, removing the HBSS, and adding ice-cold lysis buffer [in mM: 25 MOPS, pH 8.6, 1.5 EGTA, 125 NaCl, 50 NaF, 50 sodium pyrophosphate, 1 dithiothreitol, and 0.1 phenylmethylsulfonyl fluoride, with 0.5% NP-40 and 0.2 U/ml aprotinin (1 ml/100 mm dish, 0.3 ml/60 mm dish)]. The cells were then scraped and collected into 1.5-ml microcentrifuge tubes and stored at −20°C.

Permeabilization of smooth muscle cells. AIP, a CaM kinase II-specific inhibitor peptide (16), was introduced into the VSM cells by reversible permeabilization, as described previously (17). Briefly, cells were growth arrested for 24 h in serum-free media followed by a series of 2-min incubations in PBS to reduce their temperature to 4°C. The cells were then incubated in a permeabilization buffer (20 mmol/l HEPES, pH 7.4, 10 mmol/l EGTA, 140 mmol/l KCl, 50 μg/ml saponin, 5 mmol/l NaN_3, and 5 mmol/l oxalic acid dipotassium salt) containing 10 μM CaM kinase II inhibitor peptide (myristoylated-AIP, autocamtide-2-related inhibitory peptide; Biomol Research Labs, Plymouth Meeting, PA) for 10 min on ice. The cells were then washed with PBS (4°C) three times and allowed to recover in PBS for an additional 20 min at 4°C. After incubation at room temperature in PBS for 2 min, the cells were placed in HBSS supplemented with Ca^{2+}/Mg^{2+} and 10 mmol/l HEPES, pH 7.4, and placed in the incubator (37°C) for 15 min before treatment with agonists.

Immunoprecipitations and Western blotting. Cell lysates were thawed and cleared by centrifugation at 10,000 *g*. The supernatants were transferred to fresh microcentrifuge tubes and incubated with primary antibody for 90 min with rocking at 4°C. Washed protein A beads (25 μg; Pierce) were then added and incubated at 4°C with continued rocking overnight. The beads were recovered by centrifugation and washed three times with lysis buffer. Immunoprecipitated proteins were solubilized in 3× gel loading buffer and heated for 5 min at 95°C. The sample was cleared of beads by brief centrifugation, and the supernatant was loaded onto SDS-PAGE gels, transferred to either Nitro plus or PVDF-plus membranes (MSI). The membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) containing either 5% nonfat dry milk or 3% BSA. After being blocked, the membranes were incubated with the primary antibody for 1 h at room temperature or overnight at 4°C, washed three times with TBST, and incubated with horseradish peroxidase-conjugated secondary antibody (1:1,500 dilution; Amersham) for 1 h at room temperature, followed by another three TBST washes. The membranes were developed using enhanced chemiluminescence substrate (Amersham) followed by exposure to ECL Hyper film (Amersham).

Antibodies. Antibody to the phosphorylated sequence of CaM kinase II was obtained through the services of HTI Bio-Products (Ramona, CA). Antigen peptide was $_{283}$HRQET-(P)VDCLKKF$_{294}$, corresponding to the Thr^{287} site in the δ_2-CaM kinase II subunit. The antibody was specific and reacted minimally with either unphosphorylated CaM kinase II or other phosphorylated sequences from the kinase. Production and specificity of the antibody for detection of δ_2-subunits of CaM kinase II has been documented in previous publications (18, 27). The polyclonal antibody specific for PYK2 and the monoclonal antibody specific for tyrosine-phosphorylated protein (4G10) were purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies specific for the phosphorylated PYK2 were purchased from Biosource International (Camarillo, CA). The polyclonal antibody specific for the EGF receptor was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal antibodies for the phosphorylated and unphosphorylated ERK1/2 were purchased from New England Biolabs (Beverly, MA).

Materials. All tissue culture media were purchased from GIBCO BRL (Rockville, MD) unless otherwise specified. Tissue culture dishes and disposable plastic pipettes were purchased from Fisher Scientific (Pittsburgh, PA). KN-93 and KN-92 were purchased from Seikagaku America (Falmouth, MA). Ionomycin, phorbol 12,13-dibutyrate, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2), AG-1478, and AG-1296 were obtained from Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma (St. Louis, MO).

RESULTS

Ca^{2+}-dependent activation of ERK1/2 is attenuated by inhibitors of CaM kinase II. Once activated by Ca^{2+}/calmodulin, CaM kinase II subunits rapidly autophosphorylate on a conserved threonine residue (Thr^{287} in the δ-subunit) (1). Phosphorylation of Thr^{287} results in partial activation of the kinase, even in the absence of Ca^{2+}/calmodulin. Thus either Ca^{2+}/calmodulin-independent (autonomous) activity or the degree of Thr^{287} phosphorylation can be used as an index of CaM kinase II activation in situ. Previous work in this lab established the in situ Ca^{2+} dependency for CaM kinase II activation (by assaying generation of autonomous activity) in VSM cells by using ionomycin, a Ca^{2+} ionophore, as a stimulus (1). A selective inhibitor of CaM kinase II, KN-93, attenuated this activation in situ with an IC_{50} of 14 μM and maximal inhibition at 30 μM (2). Figure 1*A* shows Western blots of VSM cell lysates using an antibody that specifically recognizes the pep-

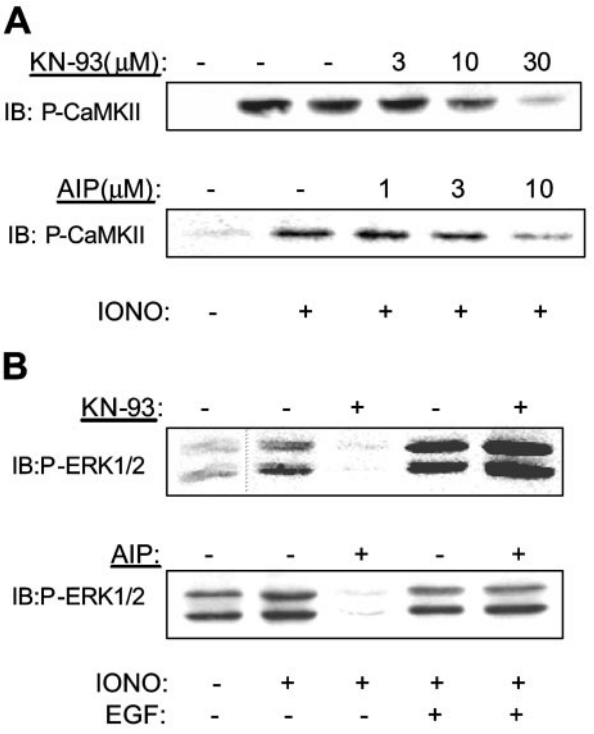

Fig. 1. Activation of Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase II) and extracellular signal-regulated kinase (ERK)1/2 in vascular smooth muscle (VSM) cells. *A*: cells were treated with 3–30 μM KN-93 or 1–10 μM CaM kinase II autoinhibitory peptide (AIP) before addition of 0.5 μM ionomycin (IONO) as indicated. Equivalent protein loadings of each lysate were immunoblotted (IB) with an antibody specific for the autophosphorylated (Thr^{287}) form of CaM kinase II (P-CaMKII). *B*: cells treated with 30 μM KN-93 or 10 μM AIP were stimulated with ionomycin or 5 ng/ml epidermal growth factor (EGF) and immunoblotted for active ERK1/2 (P-ERK1/2). Both CaM kinase II inhibitors blocked ionomycin-induced ERK1/2 activation, but neither affected EGF-induced activation.

tide sequence in CaM kinase II containing phosphorylated Thr^{287} (27). Both KN-93 and a peptide (AIP) modeled on the CaM kinase II autoinhibitory domain, the latter introduced into the cells by reversible permeabilization, were found to inhibit ionomycin-stimulated Thr^{287} autophosphorylation, confirming their effectiveness as inhibitors of CaM kinase II activity in the VSM cells. Ionomycin-induced activation of ERK1/2 was blocked by both KN-93 and AIP (Fig. 1*B*), consistent with previous findings (2) that implicated CaM kinase II as an intermediate in the Ca^{2+}-dependent ERK1/2 signaling pathway. In contrast, EGF-induced ERK1/2 activation was unaffected by pretreatment with KN-93 or AIP.

Tyrosine kinases are intermediates in the Ca^{2+}-dependent activation of ERK1/2. Activation of ERK1/2 in response to GPCR agonists depends on activation of the nonreceptor tyrosine kinases, PYK2 and *src*, which results in the tyrosine phosphorylation and activation of EGF receptors (11, 12). To determine if the Ca^{2+}-dependent activation of ERK1/2 specifically required activation of *src* and the EGF receptor tyrosine kinase, VSM cells were pretreated with PP2, a *src* family-selective tyrosine kinase inhibitor, or AG-1478, a selective EGF receptor tyrosine kinase inhibitor. Both PP2 (Fig. 2, *B* and *C*) and AG-1478 (Fig. 2) inhibited ERK1/2 activation in response to addition of ionomycin. Transactivation of PDGF receptor following stimulation with GPCR agonists has also been linked to ERK1/2 activation (15). However, pretreatment of VSM cells with the PDGF receptor tyrosine kinase inhibitor AG-1296, which inhibited control PDGF-induced activation of ERK1/2, had no effect on ionomycin-induced activation of ERK1/2 (Fig. 2*A*). To rule out nonspecific effects of these tyrosine kinase inhibitors on CaM kinase II activation, the effects of AG-1478 and PP2 on CaM kinase II autophosphorylation were determined. Neither drug inhibited the peak activation-

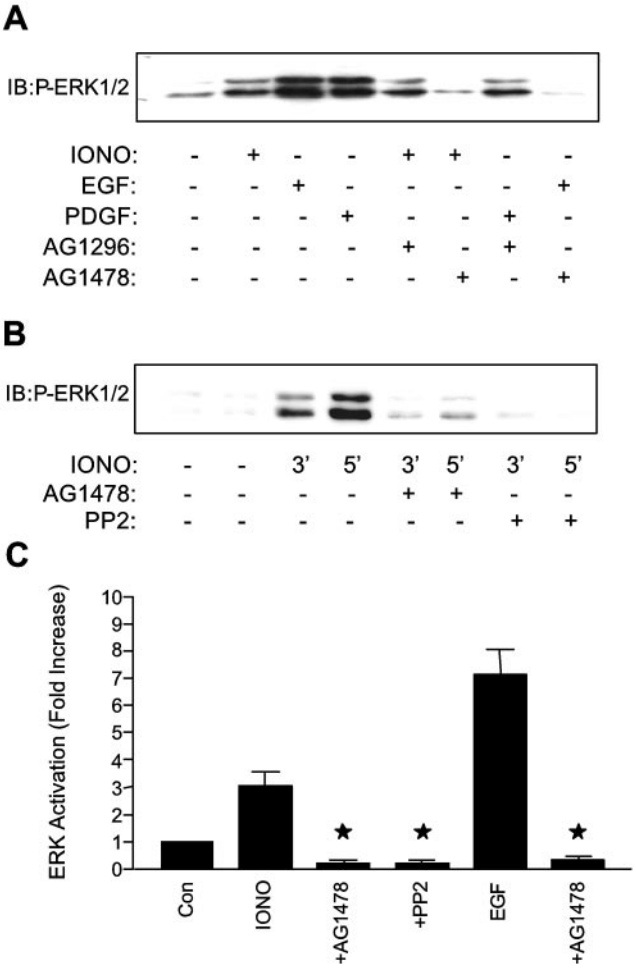

Fig. 2. Tyrosine kinase intermediates in Ca^{2+}-dependent ERK1/2 activation. *A*: VSM cells were pretreated with an EGF receptor tyrosine kinase inhibitor (1 μM AG-1478) or a platelet-derived growth factor (PGDF) receptor tyrosine kinase inhibitor (10 μM AG-1296) before addition of 0.5 μM ionomycin, 5 ng/ml EGF, or 40 ng/ml PDGF. Equal protein loadings of lysates were immunoblotted with the antibody specific for active ERK1/2. AG-1478 inhibited ionomycin-induced activation of ERK1/2, but AG-1296 had no effect. *B*: cells were pretreated with 1 μM AG-1478 or an *src* family kinase inhibitor (10 μM PP2) for 30 min before addition of ionomycin. *C*: quantification of ionomycin- and EGF-induced ERK1/2 activation. Both AG-1478 and PP2 inhibited ionomycin-induced activation of ERK1/2. Con, control. Values are means ± SE; $n = 3$; $^*P < 0.05$.

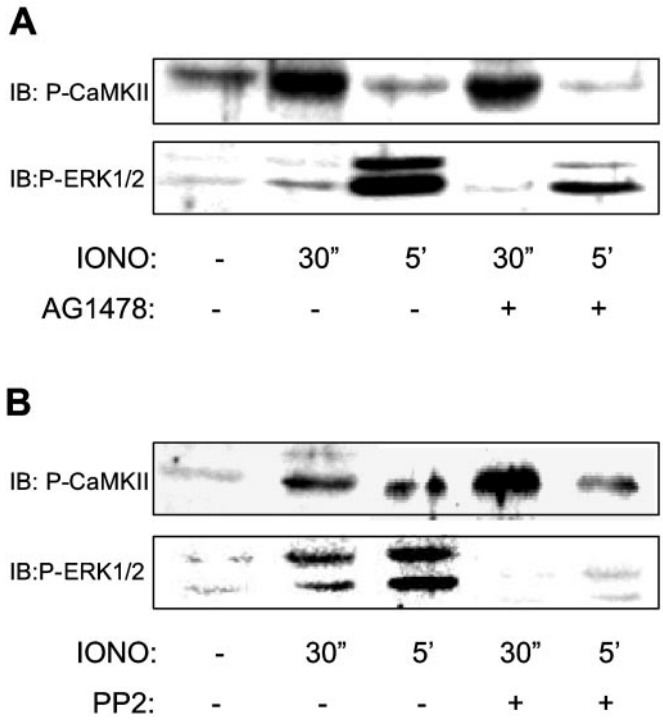

Fig. 3. Tyrosine kinase inhibitors do not block CaM kinase II activation. After treatment with 1 μM AG-1478 (*A*), or 10 μM PP2 (*B*), VSM cells were treated with 0.5 μm ionomycin. Equal protein loadings of lysates were immunoblotted with antibody specific for autophosphorylated CaM kinase II or active ERK1/2. The tyrosine kinase inhibitors had no significant effect on CaM kinase II activation.

dependent autophosphorylation of the kinase after 30-s stimulation with ionomycin (Fig. 3).

Ca^{2+}-dependent activation of the EGF receptor is inhibited by KN-93, Ca^{2+} chelators, and inhibitors of src family kinases. Inhibition of ionomycin-stimulated ERK1/2 activation by AG-1478 implicates the EGF receptor tyrosine kinase as an intermediate in the Ca^{2+}-dependent signaling pathway. As shown in Fig. 4, this was confirmed by directly documenting tyrosine phosphorylation of the EGF receptor in response to addition of ionomycin. Tyrosine phosphorylation of the EGF receptor was maximal 3 min after ionomycin treatment (Fig. 4*A*), temporally lagging peak CaM kinase II activation at 30 s (Fig. 3) but preceding peak ERK1/2 activation at 5 min (Fig. 2*B*). KN-93 blocked ionomycin-stimulated EGF receptor tyrosine phosphorylation but had no effect on phosphorylation stimulated by EGF binding. Activation of CaM kinase II is dependent on increases in intracellular Ca^{2+}. Chelation of free intracellular Ca^{2+} with 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA), introduced into the cells by addition of the cell-permeable BAPTA-AM, prevented the ionomycin-dependent activation of CaM kinase II (data not shown) and EGF receptor (Fig. 4*B*), confirming the Ca^{2+} dependence of the response.

As reported previously (3), *src* and *src* family kinases mediate GPCR-dependent activation of PYK2 and transactivation of the EGF receptor. To determine *src*'s role in the Ca^{2+}-dependent activation of these tyrosine kinases, VSM cells were treated with the selective inhibitor of *src* family kinases, PP2. At 10 μM, PP2 blocked ionomycin-dependent activation of both PYK2 and the EGF receptor, strongly suggesting that *src* or an *src*-like kinase mediates the CaM kinase II-dependent activation of PYK2 and the subsequent transactivation of the EGF receptor (Fig. 5). It should be noted, however, that PP2 also partially inhibits the EGF-stimulated EGF receptor activation (data not shown). This may indicate that PP2 has a nonspecific effect on the EGF receptor tyrosine kinase or that *src* or an *src*-like kinase may have some role in the ligand-dependent activation of the receptor tyrosine kinase.

Activation of PYK2 is dependent on CaM kinase II. Previous studies have implicated the nonreceptor tyrosine kinase PYK2 in the Ca^{2+}-dependent pathway leading to EGF receptor transactivation and ERK1/2 activation. Addition of ionomycin to VSM cells resulted in the rapid (within 1–3 min) tyrosine phosphorylation of PYK2 (Fig. 6*A*) that has been previously shown to correlate with PYK2 activation (22). Pretreatment of the cells with the CaM kinase II inhibitors KN-93 or

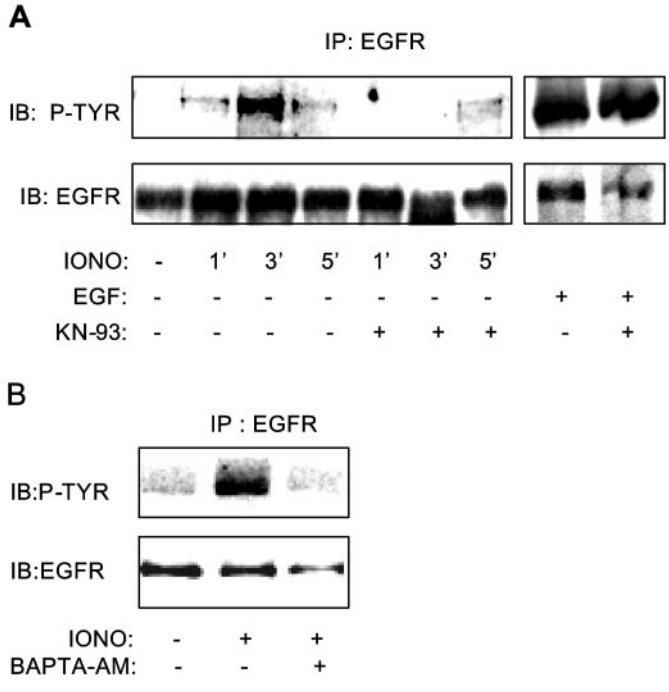

Fig. 4. Calcium and CaM kinase II-dependent activation of the EGF receptor. *A*: VSM cells were pretreated with the CaM kinase II inhibitor KN-93 (30 μM) for 30 min before addition of 0.5 μM ionomycin for the indicated times or 5 ng/ml EGF for 3 min. Lysates were immunoprecipitated with polyclonal antibody specific for the EGF receptor (IP:EGFR). Immunoprecipitated proteins were immunoblotted with a monoclonal antibody that detects protein tyrosine phosphorylation (P-TYR) and then stripped and reprobed with the EGF receptor-specific antibody. *B*: cells treated with 50 μM 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA)-AM before ionomycin treatment were immunoprecipitated with an EGF receptor antibody and immunoblotted for P-TYR and EGF receptor as described above. KN-93 and Ca^{2+} chelation inhibited ionomycin-induced activation of the EGF receptor but had no effect on EGF-induced activation.

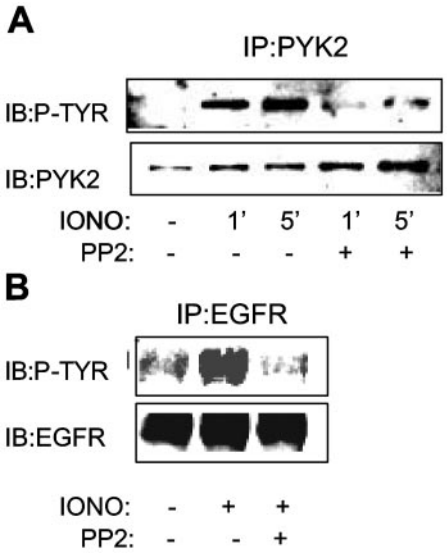

Fig. 5. *src*-dependent activation of nonreceptor proline-rich tyrosine kinase (PYK2) and the EGF receptor. VSM cells were pretreated with 10 μM PP2 for 30 min before stimulation with 0.5 μm ionomycin for 3 min. The cell lysates were then immunoprecipitated with anti-PYK2 (*A*) or anti-EGF receptor (*B*) and immunoblotted with anti-P-TYR. The immunoblots were then reprobed to insure equal immunoprecipitation of PYK2 and EGF receptor, respectively. PP2, the *src* family kinase inhibitor, attenuated the Ca^{2+}-dependent activation of PYK2 and EGF receptor.

AIP (Fig. 6) significantly attenuated ionomycin-induced PYK2 tyrosine phosphorylation. Chelation of intracellular free Ca^{2+} with BAPTA (Fig. 7*A*) also inhibited the ionomycin-dependent activation of PYK2. The physiological stimuli ATP and PDGF have also been shown to cause an increase in intracellular Ca^{2+} as well as to activate CaM kinase II in VSM cells (27, 28). Treatment with these agonists resulted in the rapid activation of PYK2 in the VSM cells, responses that were partly inhibited by pretreatment with KN-93 (Fig. 7*B*).

Phorbol ester activators of protein kinase C have also been shown to activate PYK2 (22). Pretreatment with KN-93 (Fig. 8*A*) or AIP (data not shown) had no effect on phorbol 12,13-dibutyrate-induced activation of PYK2 (Fig. 8*B*) or ERK1/2 (2). These experiments document the relative specificities of the CaM kinase II inhibitors that are restricted to a Ca^{2+}-dependent pathway proximal to the activation of PYK2. Overall, the results implicate CaM kinase II as an intermediate in the Ca^{2+}/calmodulin-dependent activation of PYK2 and provide a pathway for Ca^{2+}-dependent activation of ERK1/2, independent of protein kinase C or by direct coupling through GPCRs (Fig. 9).

DISCUSSION

Agonists for growth factor receptors and GPCR (8), membrane depolarization (28), and mechanosensitive mechanisms (19) are among the diverse stimuli that have been reported to result in an activation of MAP kinases, including ERK1/2. In the case of stimuli acting through GPCR, there appear to be at least three general pathways leading to ERK1/2 activation; a G protein βγ-subunit-dependent pathway, a protein kinase C-dependent pathway, and a Ca^{2+}-dependent pathway. Elucidation of the precise mechanisms involved for any one of these or the relative importance of each for a given agonist is complicated by the fact that the pathways may be redundant and activated in both an agonist- and cell type-specific manner. For example, in VSM cells and cardiac fibroblasts, ANG II-induced ERK1/2 activation occurs through redundant protein kinase C- and Ca^{2+}-dependent pathways. Inhibition of both pathways is required to block ANG II-stimulated

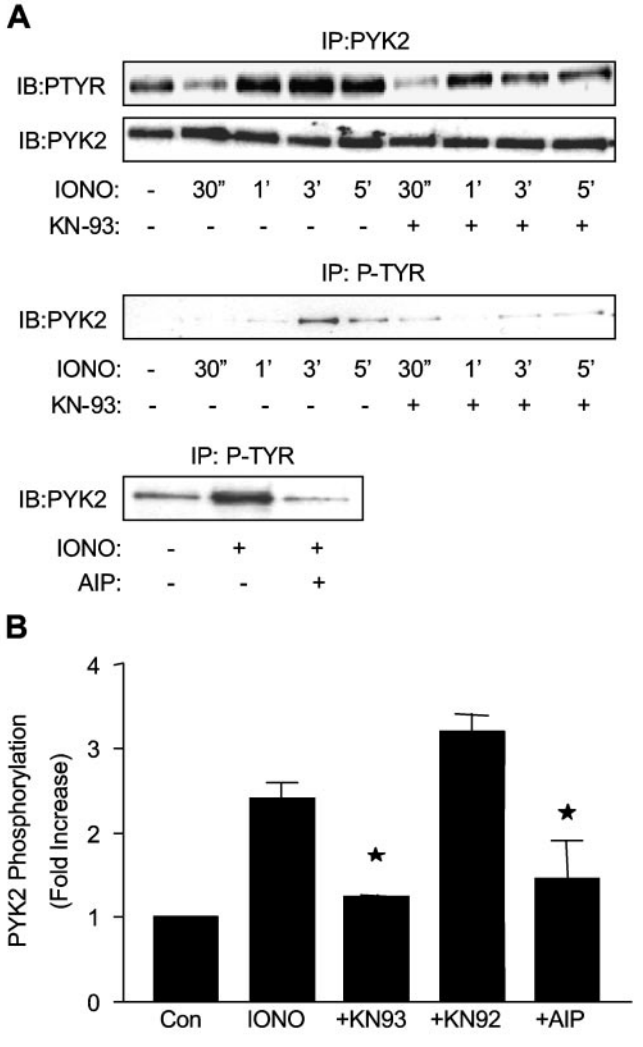

Fig. 6. CaM kinase II dependent activation of PYK2. *A*, *top*: VSM cells were pretreated with 30 μM KN-93 before treatment with 0.5 μM ionomycin for the indicated times. Lysates were immunoprecipitated with polyclonal antibody specific for PYK2 and immunoblotted with anti-P-TYR antibody, stripped, and reprobed with anti-PYK2. *Middle*: conversely, lysates were immunoprecipitated with anti-P-TYR, and the precipitated proteins were immunoblotted with anti-PYK2. *Bottom*: cells were pretreated with 10 μM AIP before treatment with ionomycin. *B*: PYK2 activation in cells pretreated with KN-93 (30 μM), an inactive analog of KN-93 (KN-92, 30 μM), or 10 μM AIP before treatment with ionomycin for 3 min. Cell lysates were immunoprecipitated with a PYK2 antibody and immunoblotted with anti-phosphotyrosine antibody. The immunoblots were quantified by scanning densitometry. Values are means ± SE; $n = 3$; $^*P < 0.05$.

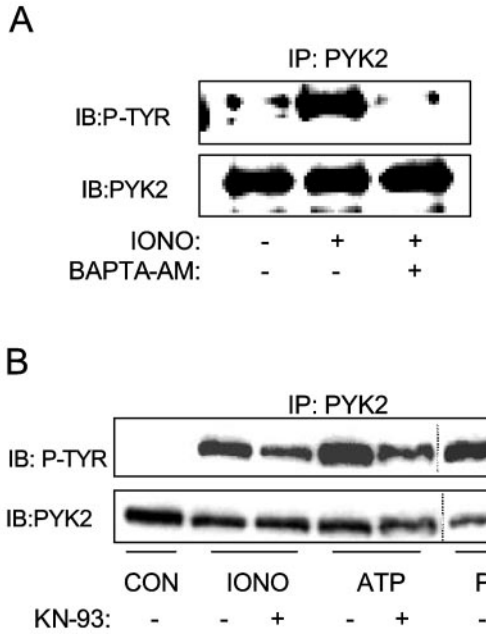

Fig. 7. Effects of Ca^{2+} chelation and KN-93 on agonist-dependent PYK2 activation. *A*: VSM cells were pretreated with 50 μM BAPTA-AM for 1 h before stimulation with 0.5 μM ionomycin. Cell lysates were immunoprecipitated with anti-PYK2 and immunoblotted with anti-P-TYR and reprobed with anti-PYK2 to insure equal PYK2 immunoprecipitation. *B*: cells were treated with 0.5 μM ionomycin, 50 μM ATP, or 40 ng/ml PDGF with or without pretreatment with 30 μM KN-93. Cell lysates were immunoprecipitated with a PYK2-specific antibody, and precipitated proteins were immunoblotted with anti-P-TYR, stripped, and reprobed with anti-PYK2 to compare protein loadings. Ca^{2+} depletion and treatment with the specific CaM kinase II inhibitor KN-93 attenuated the Ca^{2+}- and agonist-stimulated PYK2 activation, respectively.

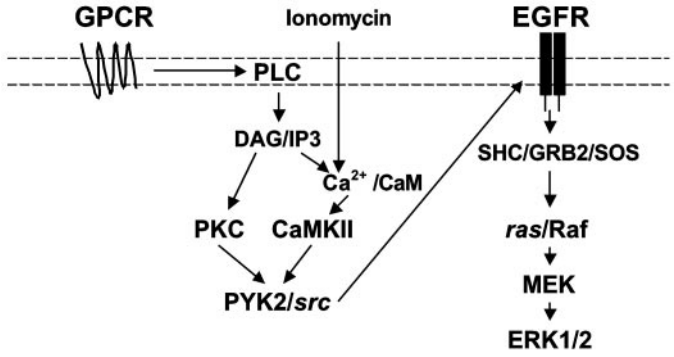

Fig. 9. Simplified model of Ca^{2+}-dependent activation of ERK1/2 in VSM cells. This model illustrates a pathway by which activation of PYK2 and *src* family kinases occurs in a CaM kinase II-dependent manner as a result of an increase in intracellular Ca^{2+}. GPCR, G protein-coupled receptor; PLC, phospholipase C; DAG, diacylglycerol; IP3: inositol trisphosphate; PKC, protein kinase C; CaMKII: CaM kinase II; Ras, p21^{ras}; Raf, *raf* kinase; MEK, mitogen-activated protein kinase kinase; SHC/GRB2/SOS, scaffolding of adaptor proteins and guanine nucleotide exchange factors.

ERK1/2 activation (4). To specifically study the Ca^{2+}-dependent pathways leading to ERK1/2 activation in VSM cells, we have relied on the Ca^{2+} ionophore, ionomycin, to selectively activate these pathways independent of heterotrimeric G protein activation or protein kinase C. Results from previous studies have implicated the multifunctional serine/threonine kinase CaM kinase II as an intermediate in the Ca^{2+}-dependent pathway leading to ERK1/2 (2). In the present study, we have narrowed down the likely site of CaM kinase II involvement to a point proximal to activation of the nonreceptor tyrosine kinases PYK2 and *src*.

There is a substantial body of evidence implicating growth factor receptors, such as the EGF receptor, as intermediates in the activation of ERK1/2 following GPCR activation in a number of cell types, including VSM (9, 10, 12, 13, 15, 22). This circuitous pathway requires activation of PYK2 and *src* or *src* family kinases to initiate EGF receptor "transactivation" (10) or to stimulate a protease that releases latent EGF from the extracellular matrix (9, 13, 20, 21) with consequent EGF receptor activation leading to *ras*-dependent activation of the MAP kinase signaling cascade. The results shown here, indicating essentially complete inhibition of ionomycin-stimulated ERK activation with an

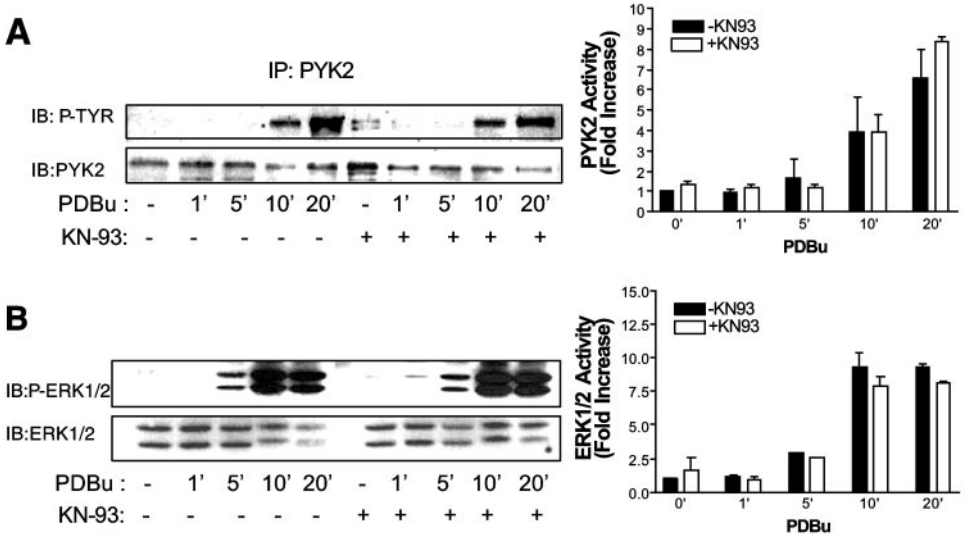

Fig. 8. Effect of CaM kinase II inhibitors on protein kinase C-dependent PYK2 stimulation. *A*: cells were treated with 0.3 μM phorbol 12,13-dibutyrate (PDBu) from 0 to 20 min with or without 30 min pretreatment with 30 μM KN-93, immunoprecipitated with anti-PYK2 antibody, and immunoblotted with anti-P-TYR or anti-PYK2 antibodies. *B*: equal protein loads of lysates from cells treated as described were immunoblotted with antibody against active ERK1/2 and reprobed for total ERK1/2. PDBu-dependent activation of PYK2 and ERK1/2 was not inhibited by treatment with KN-93.

EGF receptor tyrosine kinase inhibitor (AG-1478) or a *src* family tyrosine kinase inhibitor (PP2), suggest a requirement for these intermediate tyrosine kinases in the Ca^{2+}-dependent pathways leading to ERK1/2 activation in VSM cells. However, because of potential specificity problems with the tyrosine kinase inhibitor, it is still not possible to conclude with certainty which *src* family tyrosine kinases are involved or whether they act proximal to, coincident with, and/or distally to PYK2 activation.

In VSM cells, transactivation of both the PDGF and EGF receptors has been implicated in ANG II-dependent activation of ERK1/2 (10, 15). However, in the present study we found that treatment with AG-1296, a selective PDGF receptor tyrosine kinase inhibitor, had no effect on ionomycin-stimulated ERK1/2. Together, these results suggest that even within the same cell type there can be differences in signaling pathways, possibly due to the source of the cells, differences in primary cell culture, or relative abundance or differential cellular compartmentalization of receptor types. It is also possible that PDGF receptor transactivation, although not Ca^{2+} dependent, may be activated by alternative pathways stimulated by GPCR agonists, for example involving protein kinase C-dependent activation of PYK2/*src*.

Because the primary approach used in this study was pharmacological, we were careful to document the intended effect of the CaM kinase II inhibitors on CaM kinase II activation and to establish their relative specificity by demonstrating a lack of effect of the inhibitors on steps downstream of CaM kinase II or on pathways independent of Ca^{2+}/calmodulin. Two chemically and mechanistically distinct inhibitors of CaM kinase II were used in the studies with similar results. KN-93 prevents activation of CaM kinase II by interfering with calmodulin-dependent activation of the kinase (24). On the other hand, AIP is a peptide modeled on the autoinhibitory domain of CaM kinase II and appears to act as a competitive inhibitor of CaM kinase II substrates (16). Treatment of VSM cells with either drug inhibited ionomycin-stimulated CaM kinase II and ERK1/2 activation with similar potencies. Although maximal concentrations of KN-93 and AIP strongly inhibited Ca^{2+}-dependent activation of PYK2, EGF receptor tyrosine kinase, and ERK1/2, no effects were observed on the same responses stimulated by phorbol 12,13-dibutyrate or EGF. KN-92, the inactive analog of KN-93, also had no effect on ionomycin-stimulated PYK2 activation. Conversely, the two tyrosine kinase inhibitors (AG-1478 and PP2), which effectively blocked ionomycin-stimulated ERK1/2 activation, had no effect on activation of CaM kinase II. Collectively, these controls support the conclusions that are based on the specificity of these drugs.

Recently, it was reported that membrane depolarization stimulated phosphorylation and activation of PYK2, the EGF receptor, and ERK1/2 in PC12 cells, and, on the basis of experiments that used a calmodulin antagonist (W-7) and a CaM kinase II inhibitor (KN-62), it was concluded that these responses were CaM kinase II dependent (17). Interestingly, activation of these responses by either bradykinin or ionomycin was unaffected by the CaM kinase II inhibitors. It is possible to rationalize the lack of effect of the inhibitors on bradykinin-induced responses on the basis of activation of redundant signaling pathways, for example, involving protein kinase C. However, without a direct demonstration of the efficacy of the inhibitors on activation of CaM kinase II in the PC12 cells, it is difficult to reconcile their lack of effect on ionomycin-induced EGF receptor activation. Previously, we reported that the activation of CaM kinase II in VSM cells in response to ionomycin was due to a release of Ca^{2+} from intracellular pools (14). Activation of CaM kinase II in PC12 cells by KCl is due primarily to influx of Ca^{2+} due to membrane depolarization. Thus another possible explanation for the results in PC12 cells may be a relative selectivity of the inhibitors used for CaM kinase II activated via Ca^{2+} influx as opposed to release of intracellular Ca^{2+}.

Activated *src* has been shown to physically associate with both PYK2 (10) and EGF receptor (12). However, a number of molecular studies using active and dominant-negative PYK2 constructs have implicated PYK2 activation as a proximal step in the transactivation of the EGF receptor (3, 28). The data presented in this study support a model of Ca^{2+}/calmodulin-dependent ERK1/2 activation that is dependent on a CaM kinase II at a step proximal to activation of PYK2 and *src* and, consequently, the EGF receptor tyrosine kinase (Fig. 9). Although the exact mechanisms by which CaM kinase II leads to activation of nonreceptor tyrosine kinases such as PYK2 or *src* family kinases remains to be determined, the model potentially explains the Ca^{2+}/calmodulin dependency for PYK2 activation and suggests that CaM kinase II and protein kinase C may act via a common substrate or set of substrates proximal to PYK2 and *src* family kinases. The requirement for PYK2 and *src* family kinases in the GPCR-induced transactivation of the EGF receptors has recently been established using genetic (knockout) approaches (3). Importantly, these studies also established the requirement for *src* family kinases in the GPCR-induced activation of PYK2. This suggests that future studies aimed at elucidating the intermediate steps that couple activation of CaM kinase II, and possibly protein kinase C, to PYK2 might be directed toward proteins known to be involved in regulating *src* family kinases.

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