In Situ Ca\textsuperscript{2+} Dependence for Activation of Ca\textsuperscript{2+}/Calmodulin-dependent Protein Kinase II in Vascular Smooth Muscle Cells* 

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S. Thomas Abraham, Holly Benscoter, Charles M. Schworer, and Harold A. Singer‡

From the Sigfried and Janet Weis Center for Research, Geisinger Clinic, Danville, Pennsylvania 17822

Activation of Ca\textsuperscript{2+}/calmodulin (CaM)-dependent protein kinase II (CaM kinase II) and development of the Ca\textsuperscript{2+}/CaM-independent (autonomous) form of the kinase was investigated in cultured vascular smooth muscle (VSM) cells. Within 15 s of ionomycin (1 \mu M) exposure 52.7 ± 4.4% of the kinase became autonomous, a response that was partially maintained for at least 10 min. This correlated with 32P phosphorylation of CaM kinase II \(\delta\)-subunits in situ and was abolished by pretreatment with the CaM kinase II inhibitor KN-93. The in situ Ca\textsuperscript{2+} dependence for generating autonomous CaM kinase II was determined in cells selectively permeabilized to Ca\textsuperscript{2+} and depleted of sarcoplasmic reticulum Ca\textsuperscript{2+} by pretreatment with thapsigargin. Analysis of the resulting curve revealed an EC\textsubscript{50} (concentration producing 50% of maximal response) of 692 ± 28 nM [Ca\textsuperscript{2+}], a maximum of 68 ± 2% of the total activity becoming autonomous reflecting nearly complete activation of CaM kinase II and a Hill slope of 3, indicating a highly cooperative process. Based on this dependence and measured [Ca\textsuperscript{2+}], responses in intact cells, increases in autonomous activity stimulated by angiotensin II, vaso-pressin and platelet-derived growth factor-BB (4.6-, 2-, and 1.7-fold, respectively) were unexpectedly high. In intact cells stimulated by ionomycin, the correlation between autonomous activity and [Ca\textsuperscript{2+}], resulted in a parallel curve with an EC\textsubscript{50} of 304 ± 23 nM [Ca\textsuperscript{2+}]. This apparent increase in Ca\textsuperscript{2+} sensitivity for generating autonomous activity in intact VSM cells was eliminated by thapsigargin pretreatment. We conclude that alteration of [Ca\textsuperscript{2+}], over a physiological range activates CaM kinase II in VSM and that this process is facilitated by release of Ca\textsuperscript{2+} from intracellular pools which initiates cooperative autophosphorylation and consequent generation of autonomous CaM kinase II activity.

Mobilization of intracellular free calcium ([Ca\textsuperscript{2+}]) following agonist-stimulated phospholipid hydrolysis is a major signal transduction pathway in vascular smooth muscle (VSM) cells (1). [Ca\textsuperscript{2+}], controls contractile activity in smooth muscle (2) as well as signaling pathways related to cell growth and differentiation (3, 4) and replication (5, 6). Calcium-mediated activation of a number of cellular enzymes requires the ubiquitous calcium-binding protein, calmodulin (CaM) (7). One such enzyme is Ca\textsuperscript{2+}/CaM-dependent protein kinase II (CaM kinase II), which is expressed in high amounts in the brain and in lesser amounts in most peripheral tissues (8, 9). CaM kinase II has been implicated in diverse Ca\textsuperscript{2+}-mediated processes, including neurotransmitter production and release (10, 11), regulation of smooth muscle myosin light chain kinase activity (12), VSM cell migration (13), oocyte fertilization (14), and gene expression (15, 16).

CaM kinase II is a large multimer (~600 kDa) composed of 8–10 individual kinase subunits of 54–60 kDa size. Four distinct kinase subunits (\(\alpha, \beta, \delta, \gamma\)) and a number of variants arising by alternative splicing have been cloned to date (9, 17, 18). A distinguishing feature of CaM kinase II is that it undergoes autophosphorylation in the presence of Ca\textsuperscript{2+}/CaM on a specific conserved threonine residue (Thr\textsuperscript{286} in the \(\alpha\)-subunit) which results in the generation of Ca\textsuperscript{2+}/CaM-independent (or "autonomous") kinase activity (9). 70–80% of the total Ca\textsuperscript{2+}/CaM-dependent kinase activity may become autonomous in vitro under optimal autophosphorylation conditions (19). Autophosphorylation on Thr\textsuperscript{286} has also been reported to result in a 1000-fold increase in the affinity of the kinase subunits for calmodulin (20). Thr\textsuperscript{286} autophosphorylation is predicted to be cooperative, since it has been shown to occur by an intersubunit intraholoenzyme reaction which requires that both the phosphorylating subunit and the substrate subunit have bound Ca\textsuperscript{2+}/CaM (21).

Autophosphorylation-dependent generation of autonomous activity and "calmodulin trapping" could result in CaM kinase II activity which in vivo would outlast transient increases in [Ca\textsuperscript{2+}], and enable the kinase to respond in a frequency-dependent manner to repetitive transient increases in [Ca\textsuperscript{2+}], (20, 21). However, previous in vitro studies indicated that at saturating calmodulin concentrations, CaM kinase II activation and phosphorylation of exogenous substrates required a relatively high concentration of free Ca\textsuperscript{2+} (K\textsubscript{a} > 700 nm) (22, 23). Given that: 1) inactive CaM kinase II has a low affinity for calmodulin, 2) CaM kinase II autophosphorylation is predicted to be a cooperative process requiring at least two activated subunits per holoenzyme, and 3) intact cells have phosphatase activities that are capable of reversing autophosphorylation, activation of CaM kinase II in vivo and subsequent autophosphorylation with generation of autonomous activity may be relatively insensitive to gradual or sustained small increases in activator Ca\textsuperscript{2+} which would be expected in response to many physiological stimuli.

CaM kinase II is present in cultured rat aortic VSM cells (about 1/30th the activity found in comparable brain extracts) and is comprised mainly of the \(\delta\)-subunit variant (18). In the present study, we assessed the ability of Ca\textsuperscript{2+}-mobilizing stimuli to activate CaM kinase II in VSM, resulting in the auto-
phosphorylation-dependent generation of the autonomous form of the kinase. The Ca\(^{2+}\) dependence for generating autonomous CaM kinase II activity was determined in cells that were made selectively permeable to Ca\(^{2+}\). Despite a relatively low sensitivity for [Ca\(^{2+}\)](21) in the development of autonomous CaM kinase II activity under these conditions (EC\(_{50}\) concentration producing 50% of maximal response) = 692 nM), all of the Ca\(^{2+}\)-mobilizing agents tested were able to significantly stimulate the development of autonomous CaM kinase II activity in intact cells indicating in situ activation of CaM kinase II. Our findings further suggest an important role for intracellular pools of Ca\(^{2+}\) in the activation of CaM kinase II and the subsequent initiation of cooperative autophosphorylation and generation of autonomous activity.

**EXPERIMENTAL PROCEDURES**

Materials—Angiotensin II and arginine vasopressin were obtained from Bachem California (Torrance, CA). Ionomycin, thapsigargin, and Fura-2/AM were from Calbiochem (La Jolla, CA). KN-93 was purchased from Seikagaku America (Rockville, MD); [\(\gamma\)-\(32\)P]adenosine triphosphate, (\(\beta\)-\(\gamma\)-\(32\)P)phosphoric acid, and [\(\alpha\]-\(32\)P]ATP were from DuPont NEN. All other materials were from standard commercial sources.

Cell Culture—VSM cells were dispersed from the medial layer of the thoracic aorta of Sprague-Dawley rats (150–250 g) using collagenase and elastase ( Worthington) according to the method of Geisfeier et al. (24). The cells were grown in an equal mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing antibiotic/antimycotic (Life Technologies, Inc.) and 10% fetal calf serum (Hyclone, Logan, UT) under standard culture conditions. Cells were subcultured weekly into 60- and 100-mm\(^2\) dishes and grown to confluence before use in the experiments. VSM cells from the third to tenth passage were used in this study.

Kinase Assay—Cells were stimulated with various Ca\(^{2+}\)-mobilizing agents at 37°C in HEPES-buffered balanced salt solution (HBSS, pH 7.4) while still attached to the culture dish. At appropriate times the reaction was stopped by aspiration of the media and addition of ice-cold lysis buffer containing an equal mixture of Ca\(^{2+}\)–Mg\(^{2+}\)-free HBSS (Life Technologies, Inc.) and Buffer A. Buffer A was composed of 50 mM MOPS (pH 7.4), 2 mM EGTA, 100 mM sodium fluoride, 100 mM sodium pyrophosphate, 2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.4 unit/ml aprotinin and 1% Nonidet P-40. After 5–10 min on ice, crude lysates were aspirated and centrifuged at 17,000 \(\times\) g for 10 min. The resulting supernatant was kept on ice until the kinase assay was performed. Protein content in the lysates was determined using the Bio-Rad Protein reagent with bovine serum albumin as the standard. Total CaM kinase II activity in the lysate was assayed in 25 \(\mu\)l containing 10 mM MOPS (pH 7.4), 10 mM EGTA, 4 mM calcium chloride, 400 mM calf thymus, 0.2 mM \(\gamma\)-\(32\)P]ATP (400–1000 cpm/pmol), 20 \(\mu\)M autacamide-2 (KKALRQETVDAL, Ref. 19) as substrate, and 0.5–2.5 \(\mu\)g of lysozyme protein. To determine the Ca\(^{2+}\)-CaM-independent (autonomous) activity in the same lysates, Ca\(^{2+}\) and CaM were omitted from the kinase assay mixture. The reactions were carried out at 30°C in a shaking incubator for 3 min and terminated by precipitation of the phosphorylated peptide on Whatman P-81 paper (Whatman). The papers were rinsed thoroughly in 75 mM phosphoric acid, and the adherent radioactive content was quantified in a ReadySafe LS mixture (Amer sham Corp.) by liquid scintillation counting. Kinase activity was expressed as nanomoles of P, transferred to the substrate/mg lysozyme protein. Autonomous (Ca\(^{2+}\)/CaM-independent) kinase activity was expressed as a percent of total CaM/CaM-dependent activity from the same samples.

\[\text{[\(\beta\)-\(\gamma\)-\(32\)P]P}_{4}\text{O} \] Labeling and Immunoprecipitation of CaM Kinase II—Cellular ATP pools were labeled for 16 h by addition of 100–200 \(\mu\)Ci/ml [\(\beta\)-\(\gamma\)-\(32\)P]orthophosphoric acid in phosphate-free medium under standard culture conditions. Cells were equilibrated at 37°C in HEPES-buffered HBSS before stimulation with \(1 \mu\text{M}\) thapsigargin for 10 min, at which time the medium was aspirated and cells were disrupted with ice-cold lysis buffer. Equivalent amounts of lysate protein were incubated with 3 \(\mu\)l of 4% CH-DELTAR anti-serum precomplexed to protein A-agarose beads (18) for 2 h at 4°C unless indicated otherwise. Bead-immune complexes were washed twice with 500 \(\mu\)l of lysis buffer and solubilized in SDS sample buffer. Proteins were resolved electrophoretically on 8% SDS-polyacrylamide gels, transferred electrophoretically onto Immobilon membranes (Millipore), and visualized by autoradiography.

\[125\text{-Ca}\] Overlay—Immunoprecipitated lysate proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon membranes as indicated above. Membranes were incubated for 1 h with \(125\text{-Ca}\) (1 \(\mu\)Ci/5 ml) at room temperature in a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl (Tris-buffered saline), 1 mM Ca\(^{2+}\) and 5% non-fat dry milk. Following thorough rinsing of membranes with Tris-buffered saline, membranes were probed with CaM-binding proteins in immunoprecipitated samples were identified by exposing membranes to x-ray film for up to 72 h. Control overlay procedures were performed under identical conditions except that 1 mM Ca\(^{2+}\) was replaced with 1 mM EGTA.

**RESULTS**

CaM Kinase II Activation and Autophosphorylation in Situ—The synthetic peptide autacamide-2 has been reported to be a relatively specific substrate for CaM kinase II compared with other multifunctional kinases such as protein kinase C and CaM-dependent protein kinase (19). In representative experiments, total Ca\(^{2+}/\text{CaM-dependent autacamide-2 kinase activity in cell lysates from unstimulated VSM cells was found to be 8.81 ± 0.74 nmol/min/mg protein, while Ca\(^{2+}/\text{CaM-independent activity in the same samples was 0.87 ± 0.14 nmol/min/mg of protein (n ≥ 10). A hallmark of CaM kinase II is activation-dependent autophosphorylation leading to development of Ca\(^{2+}/\text{CaM-independent or autonomous kinase ac-
activity (9). Exposure of intact VSM cells to the Ca\textsuperscript{2+} ionophore ionomycin (1 \mu M) resulted in a rapid increase in the level of Ca\textsuperscript{2+}/CaM-independent autokinase-2 kinase activity assayed in vitro, from 11.4 \pm 1.9% of the total (Ca\textsuperscript{2+}/CaM-dependent) kinase activity at rest to a peak value (15 s) of 52.7 \pm 4.4% of total activity, a response which was partly sustained for the duration of the 10 min exposure to the ionophore (Fig. 1A).

Experiments were carried out to determine if these increases in Ca\textsuperscript{2+}/CaM-independent autokinase-2 kinase activity correlated with phosphorylation of CaM kinase II subunits and to confirm that the activity measured was in fact due to CaM kinase II. Ionomycin stimulation of cells with \textsuperscript{32}P-labeled ATP confirmed that the activity measured was in fact due to CaM kinase II. Each point is the mean \pm S.E. of five independent determinations.

\textbf{Immunoprecipitation with CK2-DELTA antibody}.

Immunoprecipitation with CK2-DELTA antibody depleted Ca\textsuperscript{2+}/CaM-dependent and -independent automokinase-2 activity by 80–90% from lysates of ionomycin-stimulated cells, confirming the specificity of the automokinase-2 substrate based assay for CaM kinase II and indicating that the stimulated Ca\textsuperscript{2+}/CaM-independent activity was due to autonomous CaM kinase II (Fig. 2).

While Ca\textsuperscript{2+}/CaM-independent automokinase-2 kinase activity was low in resting cells, the CK2-DELTA antibody also immunoprecipitated 60% of this activity, consistent with a small amount of CaM kinase II autophosphorylation which was observed in unstimulated cells (Fig. 1B). The residual Ca\textsuperscript{2+}/CaM-dependent activity remaining following immunoprecipitation could be due to other kinases such as CaM kinase IV which do not undergo autophosphorylation-dependent transition to a Ca\textsuperscript{2+}/CaM-independent form (27). Further confirmation that Ca\textsuperscript{2+}/CaM-independent automokinase-2 kinase activity in the VSM cell lysates was due to autonomous CaM kinase II was obtained by pretreating the cells with KN-93, a CaM kinase II inhibitor which interferes with calmodulin binding to the kinase subunit (10). In the experiments shown in Fig. 3, stimulation of VSM cells for 15 s with ionomycin (1 \mu M) resulted in a 7.9-fold increase in Ca\textsuperscript{2+}/CaM-independent activity without altering total CaM kinase II activity in cells. Pretreatment of cells with 30 \mu M KN-93 completely prevented the ionomycin-induced increase in Ca\textsuperscript{2+}/CaM-independent kinase activity. Based on the experiments shown in Figs. 1–3 we concluded that automokinase-2 kinase activity was indicative of CaM kinase II in the VSM cell lysates and that Ca\textsuperscript{2+}/CaM-independent kinase activity was due to the autophosphorylated or autonomous form of the kinase.

\textbf{In Situ Ca\textsuperscript{2+}}.

Dependence for Generating Autonomous CaM Kinase II—Vascular smooth muscle cells were loaded with the fluorescent Ca\textsuperscript{2+} indicator Fura-2, depleted of Ca\textsuperscript{2+} by incubating with thapsigargin (1 \mu M) media in Ca\textsuperscript{2+}-free HBSS containing 2 mM EGTA, and selectively permeabilized to Ca\textsuperscript{2+} by addition of ionomycin (1 \mu M). Stepped increases in free [Ca\textsuperscript{2+}], were obtained by cumulative additions of CaCl\textsubscript{2} to the media (Fig. 4, inset) and quantified using the Fura-2 fluorescence. After each step change in [Ca\textsuperscript{2+}], a 40-\mu l aliquot of the cell suspension was removed (indicated by the vertical deflections in the tracing) and assayed for Ca\textsuperscript{2+}/CaM-dependent and -independent CaM kinase II activity. The resulting data established the in situ Ca\textsuperscript{2+} dependence for the generation of autonomous CaM kinase II activity (Fig. 4). Nonlinear curve fitting

\textbf{FIG. 1.} A, time course for the activation of CaM kinase II by 1 \mu M ionomycin. Each point is the mean \pm S.E. of five independent determinations. B, time course of CaM kinase II phosphorylation in cells labeled with \textsuperscript{32}P-\textsuperscript{32}P, exposed to ionomycin and immunoprecipitated with CK2-DELTA antibody. Radiolabeled proteins were separated by 8% SDS-polyacrylamide gel electrophoresis, and molecular mass marker positions are indicated to the left. The 52-kDa position marks the major CaM kinase II subunit immunoprecipitated in VSM cells. "Overlay" lane indicates cell proteins that were immunoprecipitated with CK2-DELTA antibody, separated by electrophoresis and identified by an \textsuperscript{125}I-CaM overlay technique (see "Experimental Procedures").

\textbf{FIG. 2.} Immunodepletion of automokinase-2 kinase activity from VSM cell lysates with the CK2-DELTA CaM kinase II antibody. Ca\textsuperscript{2+}/CaM-independent kinase activity (Indep. Activity) was measured in lysates from unstimulated cells (Rest; n = 3) and cells stimulated with ionomycin (1 \mu M for 15 s; Iono; n = 3). Ca\textsuperscript{2+}/CaM-dependent kinase activity (Total Activity; n = 6) was measured in lysates from Rest and Iono-stimulated cells. Lysates were divided and incubated with preimmune serum (left panel) or CK2-DELTA antiserum (right panel) for 1 h at 4°C prior to loading the immune complexes with protein A-agarose beads. Values are mean \pm S.E.
Autonomous CaM Kinase II Activity Stimulated by Physiological Agonists in VSM Cells—Several physiological stimuli were evaluated for their ability to increase [Ca2+]i, and autonomous CaM kinase II activity in intact VSM cells. The average resting [Ca2+]i in the intact cells was determined to be 100–150 nM. Angiotensin II (0.1 μM) characteristically stimulated rapid transient increases in [Ca2+]i of about 200–300 nM as depicted in Fig. 5A. Vasopressin (0.1 μM) also stimulated rapid increases in [Ca2+]i (Fig. 5B), although in these cells the response was generally smaller than that obtained with angiotensin II. Platelet-derived growth factor (PDGF-BB; 40 ng/ml) stimulated slower and relatively small increases in average [Ca2+]i (Fig. 5C), whereas 1 μM ionomycin elicited a rapid somewhat transient increase in [Ca2+]i which was partially sustained (Fig. 5D). By quantitatively comparing these [Ca2+]i responses with the [Ca2+]i dependence for generating autonomous CaM kinase II shown in Fig. 4, it would not be expected that resting levels of [Ca2+]i would support significant autonomous CaM kinase II activity or that addition of these receptor agonists would substantially increase autonomous activity. In contrast to this prediction, resting levels of autonomous activity in the intact VSM cells ranged from 4.4 to 12.5% of total activity, and each stimulus significantly increased autonomous CaM kinase II activity with kinetics which paralleled increases in [Ca2+]i (Fig. 5). In the case of angiotensin II or vasopressin, transient increases in autonomous CaM kinase II activity to 41 ± 4% (n = 5) and 22.4 ± 2.2% (n = 3) of total activity, respectively, were reached within 15 s (Figs. 5, A and B). PDGF also increased autonomous CaM kinase II activity, but at a slower rate and to a lesser extent (14.7 ± 1.1% autonomous activity at 60 s), as compared with angiotensin II and vasopressin (Fig. 5C). Angiotensin II-induced increases in autonomous CaM kinase II activity were concentration-dependent with an estimated EC50 of 6 nM (data not shown), which is similar to the reported dissociation constant of the agonist for its receptor (29, 30).

Role of Intracellular Ca2+ Pools in the Generation of Autonomous Activity—Increasing [Ca2+]i by stimulating intact Fura-2-loaded VSM cells with various concentrations of ionomycin (10 nM to 10 μM) in normal Ca2+-containing HBSS (Fig. 6A, inset) resulted in a second apparent Ca2+ dependence for the

**Fig. 3. Effect of KN-93 on CaM kinase II activity from ionomycin-stimulated VSM cells.** Cells were treated with vehicle or 30 μM KN-93 for 1 h before exposure to 1 μM ionomycin for 15 s. Closed bars indicate mean ± S.E. (three experiments) of total and Ca2+/CaM-independent kinase activity in cell lysates, respectively. ** indicates independent activity that is statistically different from the independent activity in control cells (p < 0.001).

**Fig. 4. Autonomous CaM kinase II activity as a function of free intracellular Ca2+ ([Ca2+]i) in VSM cells.** Fura-2-loaded cells were depleted of intracellular Ca2+ and permeabilized with ionomycin before increasing [Ca2+]i by addition of CaCl2 to the suspension (inset). After each CaCl2 addition the cell suspension in the cuvette was sampled (vertical deflections) and cell lysates were prepared and assayed for kinase activity. The scatter plot of open triangles is from 12 independent experiments and the sigmoidal (logistic function) curve was obtained by nonlinear regression analysis (r2 = 0.915, see “Experimental Procedures”). The dashed lines above and below the curve mark the 95% confidence interval for the curve.
generation of autonomous CaM kinase II activity in situ (Fig. 6A, open circles, n = 8). Curve fitting analysis predicted a sigmoidal relationship for Ca\textsuperscript{2+}-induced activation with an EC\textsubscript{50} of 304 ± 23 nM and 67 ± 5% of the kinase becoming autonomous at saturating [Ca\textsuperscript{2+}]\text{\textsubscript{i}} (goodness of fit r\textsuperscript{2} = 0.849). Analysis of variance predicted that the EC\textsubscript{50} obtained with this protocol was statistically different from that of the curve in Fig. 3 (F\textsubscript{(4,139)} = 56.600, p < 0.001). The apparent Ca\textsuperscript{2+} dependence obtained with this protocol was quantitatively consistent with the increases in [Ca\textsuperscript{2+}]\text{\textsubscript{i}} and autonomous CaM kinase II activity observed in intact VSM cells in response to addition of the physiological stimuli shown in Fig. 5.

In order to explain the apparent Ca\textsuperscript{2+} sensitivity for generating autonomous CaM kinase II in intact VSM cells, we considered the possibility that average [Ca\textsuperscript{2+}]\text{\textsubscript{i}}, calculated using the Fura-2 technique did not reflect locally high [Ca\textsuperscript{2+}]\text{\textsubscript{i}}, produced in response to ionomycin and peptide agonists that release intracellular pools of Ca\textsuperscript{2+} (3, 30, 31), which could support the cooperative autophosphorylation of CaM kinase II. To test this hypothesis, intact cells in normal Ca\textsuperscript{2+} HBSS were pre-treated with 1 \textmu M thapsigargin to deplete intracellular Ca\textsuperscript{2+} pools and the protocol shown in Fig. 6A was repeated using ionomycin as a stimulus. The resulting apparent Ca\textsuperscript{2+} dependence (Fig. 6B; goodness of fit r\textsuperscript{2} = 0.940) was significantly to the right of the curve obtained from VSM cells with intact intracellular pools of Ca\textsuperscript{2+} (EC\textsubscript{50} = 616 ± 37 nM; F\textsubscript{(4,62)} = 29.278, p < 0.001, n = 8), although the maximal increase in autonomous activity, Hill slope, and the total Ca\textsuperscript{2+}/CaM-dependent kinase activity were not significantly altered by thapsigargin treatment (Fig. 6B). The apparent [Ca\textsuperscript{2+}]\text{\textsubscript{i}} dependence for generating autonomous CaM kinase II activity described by this data set was not significantly different from that obtained using the Ca\textsuperscript{2+} step protocol shown in Fig. 4. A 10-min treatment of VSM cells with 1 \textmu M thapsigargin also abolished a 3-fold increase in autonomous activity elicited by 0.1 \textmu M angiotensin II (n = 3, data not shown). These results are consistent with the contribution of intracellular Ca\textsuperscript{2+} pools in the activation of CaM kinase II and the cooperative generation of autonomous kinase activity.

**DISCUSSION**

The present study was undertaken as a first approach toward assessing the activation of CaM kinase II in intact VSM cells. Ca\textsuperscript{2+}/CaM-independent CaM kinase II activity (autonomous activity) was measured in VSM cell lysates for two reasons: 1) generation of autonomous activity provides an index of CaM kinase II activation in the intact cell prior to lysis, and 2) the appearance of autonomous CaM kinase II activity in situ has been hypothesized to be of functional significance. Evidence that the ionomycin-induced Ca\textsuperscript{2+}/CaM-independent autcamtide-2 kinase activity was due to autophosphorylation-dependent generation of autonomous CaM kinase II includes: 1) the activity was efficiently immunoprecipitated with a CaM kinase II \(\delta\)-subunit-specific antibody; 2)
the onset correlated temporally with increased in situ \(^{32}\)P phosphorylation of CaM kinase II; and 3) formation of autonomous activity was inhibited with KN-93, a reported CaM kinase II inhibitor which acts by preventing Ca\(^{2+}\)/CaM binding to the subunit. While the ionomycin-induced increase in autonomous CaM kinase II was somewhat transient with a peak at 15 s, phosphorylation of CaM kinase II \(\delta\)-subunits subunits was maximal within 15–30 s and sustained for the duration of the stimulus. This apparent dissociation between subunit phosphorylation and autonomous activity at later time points may be
due to phosphorylation on additional serine/threonine sites (9) accompanied by dephosphorylation of Thr286. The functional significance of these additional phosphorylation events is largely unknown.

It was previously estimated that the free \([Ca^{2+}]_i\) for half-maximal activation of CaM kinase II in vitro under conditions of saturating CaM was relatively high, in the range of 700-2000 nM (22, 23). Because generation of autonomous CaM kinase II activity results from intersubunit phosphorylation between activated \((Ca^{2+}/CaM \text{ bound})\) subunits (9, 27), a similar \(Ca^{2+}\) dependence would be predicted for generation of autonomous activity in vivo. Other factors in intact cells could act to further decrease the apparent \(Ca^{2+}\) sensitivity for CaM kinase II activation and generation of autonomous activity, such as limiting concentrations of free calmodulin and/or protein phosphatase activities that are capable of reversing CaM kinase II autophosphorylation. The calculated EC_{50} of 692 nM \(Ca^{2+}\) in selectively permeabilized VSM cells is the first direct estimation of the in situ \(Ca^{2+}\) dependence for generating autonomous CaM kinase II activity. Significantly, a positive Hill slope of 3 for the relationship indicates that \(Ca^{2+}\)-dependent generation of autonomous CaM kinase II activity in situ is a highly cooperative process, consistent with in vitro data indicating cooperativity in the intersubunit intraholoenzyme autophosphorylation reaction (21). This value also provides an estimate of the \(Ca^{2+}\) sensitivity for activation of CaM kinase II, which is a prerequisite for generating autonomous activity. Tansey et al. (32) reported a similar half-maximal \([Ca^{2+}]_i\) (500 nM) for phosphorylation and desensitization of myosin light chain kinase in permeabilized bovine tracheal smooth muscle cells, a process thought to be mediated by CaM kinase II.

Under optimal autophosphorylation conditions in vitro, we have found that autonomous activity of the CaM kinase II holoenzyme composed of recombinant \(\delta_2\)-subunits could reach as much as 70% of the total \(Ca^{2+}/CaM\)-dependent activity. Similar maximal levels of independent activity have been observed by others using various purified and recombinant forms of CaM kinase II (19). To our knowledge, it is not known to what extent the difference in maximal autonomous activity compared with total activity reflects the stoichiometry of holoenzyme autophosphorylation, specifically on Thr286 in the subunits, and/or whether autophosphorylated kinase has a lower activity for substrates than \(Ca^{2+}/CaM\)-activated kinase. Therefore, as a marker of \(Ca^{2+}/CaM\)-dependent activation of CaM kinase II in vivo, the level of autonomous activity may actually underestimate the full extent of CaM kinase II activation. Similar maximal levels of autonomous activity were also generated in the selectively permeabilized VSM cells (Fig. 4), or transiently in cells with intact \(Ca^{2+}\) pools in response to ionomycin stimulation (Figs. 1A and 6A), indicating a near complete activation of kinase subunits in situ. This suggests that in VSM cells under these conditions, free CaM is not a limiting factor and protein phosphatases do not significantly antagonize the maximal development of autonomous activity.

Comparable increases in autonomous CaM kinase II activity in situ were reported previously only in KCl-depolarized PC12 cells (33). In most other studies where this approach has been used to assess CaM kinase II activation, small increases in autonomous activity (2-fold or less) were reported in response to stimuli which raise \([Ca^{2+}]_i\) (34, 35), indicating either lower levels of CaM kinase II activation and/or incomplete control of CaM kinase II subunit dephosphorylation during cell lysis.

Average resting VSM cell \([Ca^{2+}]_i\) was found to be between 100 and 150 nM, and the physiological agonists angiotensin II, vasopressin, and PDGF produced transient increases in \([Ca^{2+}]_i\) to values which were typically in a range of 200-400 nM. These results are similar both qualitatively and quantitatively to previously published data obtained in VSM cells (31). Based on the quantitative relationship between \([Ca^{2+}]_i\) and generation of autonomous CaM kinase II activity (Fig. 4), these levels of \([Ca^{2+}]_i\) should have produced minimal autonomous CaM kinase II activity. However, a small amount of \(Ca^{2+}/CaM\)-independent activity was measurable in lysates from unstimulated intact cells (about 3-12% of the total activity of which about 60% was immunoprecipitated with the CaM kinase II antibody), and all of the stimuli tested produced significant transient increases in autonomous CaM kinase II activity, with angiotensin II producing the largest increases, to as much as 40% of the total kinase activity.

A significant factor in interpreting these data was that the in situ \(Ca^{2+}\) dependence curve for generating autonomous CaM kinase II resulted from experiments using cells which were depleted of intracellular \(Ca^{2+}\) and which required \(Ca^{2+}\) influx from the extracellular medium to activate the kinase. In the case of experiments examining agonist-stimulated increases in CaM kinase II activity, intracellular pools of \(Ca^{2+}\) were intact and the stimuli used (angiotensin II, vasopressin, and PDGF) are known to mobilize \(Ca^{2+}\) from inositol trisphosphate-sensitive intracellular pools (36-38). Ionomycin was useful in determining the effect of intracellular \(Ca^{2+}\) pools on the apparent \(Ca^{2+}\) dependence for generating autonomous CaM kinase II activity, since it is able to mobilize \(Ca^{2+}\) from both intracellular and extracellular pools to elevate \([Ca^{2+}]_i\) (3). The relationship between ionomycin-induced increases in \([Ca^{2+}]_i\), and autonomous CaM kinase II activity in cells with intact \([Ca^{2+}]_i\), pools predicted an apparent EC_{50} for \([Ca^{2+}]_i\), of 304 nM for the generation of autonomous kinase activity. This apparent \(Ca^{2+}\) sensitivity was consistent with the physiological agonist-induced increases in \([Ca^{2+}]_i\), and autonomous kinase activity. Prior depletion of intracellular \(Ca^{2+}\) pools with thapsigargin markedly decreased the apparent \(Ca^{2+}\) sensitivity for ionomycin-induced generation of autonomous CaM kinase II activity and blocked angiotensin II-induced transients in \([Ca^{2+}]_i\), and generation of autonomous activity. At least three factors could contribute to the observed differences in \(Ca^{2+}\) sensitivities for generating autonomous CaM kinase II in cells with or without intact pools of intracellular \(Ca^{2+}\). 1) In the protocol using ionomycin-permeabilized and \(Ca^{2+}\)-depleted cells, \(Ca^{2+}\) added to the extracellular medium might be expected to equilibrate uniformly through the cytoplasm allowing Fura-2 to faithfully report \([Ca^{2+}]_i\). This protocol should then result in a reasonable estimation of the \(Ca^{2+}\) sensitivity for activation of CaM kinase II and generation of autonomous activity. In cells with intact intracellular \(Ca^{2+}\) pools, locally high \([Ca^{2+}]_i\), resulting from sarcoplasmic reticulum release in response to ionomycin or physiological stimuli may contribute to an average \([Ca^{2+}]_i\), reported by Fura-2. Several studies have documented spatial inhomogeneities in \([Ca^{2+}]_i\), using microscopic imaging of single cell \(Ca^{2+}\)-transients (39, 40). Models of \(Ca^{2+}\) signaling in VSM, which take into account restricted diffusional spaces underlying the plasma membrane and surrounding sarcoplasmic reticulum, suggest that micromolar concentrations of \([Ca^{2+}]_i\) could develop and persist for short periods of time in these spaces in response to \(Ca^{2+}\)-mobilizing stimuli (41). 2) Assuming that locally high concentrations of \(Ca^{2+}\) are produced in intact cells, a second factor contributing to the observed variable \(Ca^{2+}\) sensitivity is the cooperative nature of the process for generating autonomous CaM kinase II activity. 3) CaM kinase II itself could be

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localized in discrete subcellular regions in close proximity to the sarcoplasmic reticulum and released Ca$^{2+}$. In this case, the effects of locally high [Ca$^{2+}$], and a cooperative process for generating autonomous activity would be maximized by CaM kinase II holoenzyme and the requirement for intersubunit autophosphorylation on Thr$^{286}$ to generate autonomous activity. Locally high concentrations of [Ca$^{2+}$] in intact VSM cells, produced by agonists that release Ca$^{2+}$ from intracellular pools, result in the cooperative generation of autonomous CaM kinase II activity over a range of average cell [Ca$^{2+}$] of 0.1–1 μM.

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