Spatial Patterns of Ca$^{2+}$ Signals Define Intracellular Distribution of a Signaling by Ca$^{2+}$/Calmodulin-dependent Protein Kinase II*

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Ca$^{2+}$ plays a central role in cell signaling, and Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) is a major mediator of Ca$^{2+}$ actions. The spatial distribution of intracellular Ca$^{2+}$ signaling is not homogenous, rather it is dynamically organized, and it has been speculated that spatial patterns of Ca$^{2+}$ signals may function as a form of cellular information transmitted to downstream molecules. To address this issue, we studied the intracellular distributions of the signalings by CaMKII and Ca$^{2+}$ in the same astrocytes. The former was visualized by monitoring site-specific phosphorylation of a cytoskeletal protein, vimentin, using site- and phosphorylation-specific antibodies, while the latter was examined by fura-2-based Ca$^{2+}$ microscopy. Local Ca$^{2+}$ signals induced vimentin phosphorylation by CaMKII localized in the same area. On the other hand, Ca$^{2+}$ waves in astrocytes induced global phosphorylation of vimentin by CaMKII. A small population of vimentin filaments highly phosphorylated by CaMKII underwent structural alteration into short filaments at electron microscopic level. These results indicate that CaMKII transmits spatial patterns of Ca$^{2+}$ signals to vimentin as cellular information. The possibility is discussed that spatial patterns of vimentin phosphorylation may be important for intracellular organization of vimentin filament networks.

Cell signaling is the fundamental strategy by which cells respond to extracellular stimuli. Intracellular distribution of cell signaling is considered to be an important factor affecting the manner in which cells respond to extracellular stimuli with spatial specificity (1, 2). Although little is known of the spatial aspect of cell signaling, that of Ca$^{2+}$ signaling visualized by Ca$^{2+}$-microscopy is presently the best characterized example. Numbers of reports have shown that intracellular Ca$^{2+}$ signals occur locally and globally (3–6). The intracellular distribution of Ca$^{2+}$ signaling in various types of cells was defined by the amplitude and direction of extracellular stimuli (7–10). Therefore, it has been speculated that spatial patterns of Ca$^{2+}$ signals might be transmitted, as a form of cellular information, by a downstream molecule that induces Ca$^{2+}$-dependent cellular responses (1–2, 6–10).

To address this issue, we visualized site-specific phosphorylation of vimentin by CaMKII$^1$ and Ca$^{2+}$ signaling in the same astrocytes. CaMKII is located downstream of Ca$^{2+}$ signaling and is thought to regulate various cellular responses (11, 12). Vimentin is an intermediate filament protein distributed widely in the cytoplasm (13, 14) and is phosphorylated by several protein kinases, including CaMKII, in vivo (15, 16). Therefore, vimentin can serve as a substrate for the examination of the cytoplasmic distribution of protein kinase activities (17, 18). Here we report that vimentin phosphorylation by CaMKII was induced locally and globally by Ca$^{2+}$ signaling. The intracellular area of the phosphorylation was precisely defined by that of Ca$^{2+}$ signaling.

**EXPERIMENTAL PROCEDURES**

Preparation of Antibodies, Peptides, and Proteins—Production of monoclonal antibodies YT33, TM50, 4A4, and MO82 was reported elsewhere (19–21). Vimentin peptides PV6 (Cys-Ser-Thr-Arg-Ser-Val-phosphoSer-Ser-Ser-Ser-Tyr-Arg), V6 (Cys-Ser-Thr-Arg-Ser-Val-Ser-Ser-Ser-Tyr-Arg), PV38 (Cys-Ser-Thr-Arg-Thr-Tyr-Phosphoser-Glu-Gly-Ser-Ala-Leu), and V38 (Cys-Ser-Thr-Arg-Thr-Tyr-Ser-Leu-Gly-Ser-Ala-Leu) were synthesized as described previously (21). A monoclonal antibody against PV6 (MO6) and a polyclonal antibody against PV38 (GK38) were produced following the methods described previously (21, 22). Then the specificity of MO6 and GK38 was checked by enzyme-linked immunosorbtent assay (21). MO6 bound to PV6 but not to the unphosphorylated peptide V6, while GK38 reacted with PV38 but not with the unphosphorylated form V38. Production of recombinant vimentin and vimentin phosphorylated by CaMKII was described previously (19). The affinity-purified antibody specific for both 50- and 60-kDa subunits of CaMKII (23) was provided by Drs. K. Fukunaga and E. Miyamoto (Kumamoto University).

Cell Preparation and Drug Application—Primary cultured astrocytes (type 1 astrocytes) were prepared from the cerebral cortices of newborn rats as described previously (19). Two days before the experiments, astrocytes were subcultured on collagen (type 1, Sigma)-coated glass coverslips attached to silicon walls (Heraeus Flexiperm Disc) for the measurement of intracellular free Ca$^{2+}$ concentration (Ca$^{2+}$i). Then they were differentiected into process-bearing astrocytes by incubation with 250 μM dibutyryl cAMP in serum-free Eagle’s minimal essential medium. Ionomycin or prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) dissolved in HEPES-buffered Krebs-Ringer solution (containing the following [in mM]: NaCl, 115; KCl, 5.4; CaCl$_2$, 2; MgCl$_2$, 0.8; glucose, 13.8; Hepes, 20 (pH 7.4)) were bath applied, or locally applied using a micropipette (Sterile Femtotips, Eppendorf) equipped with Transjector 5246 (Eppendorf) by pressure (30 kPa). The flow rate of the locally applied solution was 0.05–0.1 μl/h.

Ca$^{2+}$i Measurements—The [Ca$^{2+}$i] of cultured astrocytes was measured as described elsewhere (8, 24). Briefly, the cells were incubated

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** The abbreviations used are: CaMKII, Ca$^{2+}$/calmodulin-dependent protein kinase II; PBS, phosphate-buffered saline; PGF$_{2\alpha}$, prostaglandin F$_{2\alpha}$

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with 10 μM fura-2/AM in the Hepes-buffered Krebs-Ringer solution for 1 h and washed with the solution for 30 min. Cells on a coverslip were placed on the stage of an Olympus IMT-2 inverted microscope. Fluorescence images were obtained with a Hamamatsu CCD camera C2400 and stored in a digital image processor Argus-50. [Ca\textsuperscript{2+}] was calculated from the ratio of the fluorescence intensities obtained with excitations at 340 nm and 380 nm on a pixel basis.

**Immunocytochemistry**—Cells were fixed with 3% formaldehyde in phosphate-buffered saline (PBS) for 10 min, followed by treatment with −20 °C methanol for 10 min. They were incubated with MO6 (3 μg/ml), YT33 (3 μg/ml), GK38 (14 μg/ml), TM50 (3 μg/ml), 4A4 (1 μg/ml), or MO82 (0.2 μg/ml) diluted in PBS for 2 h, followed by incubation with fluorescein isothiocyanate-conjugated anti-mouse antibodies (BioSource) diluted 1:100 for PBS by 1 h. Then the samples were examined with a fluorescent microscope (Olympus). For double immunostaining with MO82 and anti-vimentin antibody, fixed cells were incubated with a fluorescent microscope (Olympus). For double immunostaining with MO82 and anti-vimentin antibody, fixed cells were incubated with biotinylated anti-mouse IgG (Vector Laboratories Inc.) diluted 1:300 in PBS for 1 h, followed by the incubation with streptavidin-Texas Red (Amersham Corp.) diluted 1:300 in PBS for 1 h. On the other hand, vimentin immunoreactivity was visualized by incubation with fluorescein isothiocyanate-conjugated anti-goat antibodies (BioSource) diluted 1:300 in PBS for 1 h. Then the double-stained samples were examined with a confocal microscope (Olympus, LSM-GB200). Immunofluorescent localization of CaMKII in astrocytes was done using an affinity-purified antibody specific for both 50- and 60-kDa subunits of CaMKII (23) as described previously (28).

**Western Blotting**—Proteins or lysate of 1.8 × 10⁶ astrocytes were loaded in the lanes, resolved by SDS-polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore). Then the blots were incubated with 2 ng/ml MO82 or MO6 (0.2 μg/ml), GK38 (14 μg/ml), TM50 (3 μg/ml), 4A4 (1 μg/ml), or MO82 (0.2 μg/ml) diluted in PBS for 2 h, followed by incubation with fluorescein isothiocyanate-conjugated anti-mouse antibodies (BioSource) diluted 1:100 for PBS by 1 h. Then the double-stained samples were examined with a confocal microscope (Olympus, LSM-GB200).

Electron Microscopy—Immunogold localization using MO82 was done as described previously (19). For standard electron microscopy, cells were fixed in 2% glutaraldehyde and 1 mM MgCl₂ in 0.1 M cacodylate buffer for 30 min, followed by postfixation with 1% OsO₄ in the same buffer on ice for 1 h. The cells were dehydrated with ethanol and embedded in Epon 812. Thin sections were mounted on grids, doubly stained with uranyl acetate and lead citrate, and observed under an electron microscope (JEM1200EX).

**RESULTS AND DISCUSSION**

**Visualization of a Signaling by CaMKII**—For visualization of CaMKII signaling, we monitored the site-specific phosphorylation of the cytoskeletal protein vimentin. Ser\textsuperscript{38} and Ser\textsuperscript{82} of vimentin are identified as the two major in vitro phosphorylation sites of vimentin by CaMKII, while Ser\textsuperscript{6}, Ser\textsuperscript{23}, Ser\textsuperscript{33}, and Ser\textsuperscript{50} were phosphorylated by CaMKII but by other kinases (15) (Table I). We recently developed monoclonal antibodies YT33, TM50, 4A4, and MO82 that recognize the site-specific phosphorylation of vimentin at Ser\textsuperscript{33}, Ser\textsuperscript{23}, Ser\textsuperscript{50}, and Ser\textsuperscript{82}, respectively (19–21, 27) (Table I). In addition, we produced a monoclonal antibody MO6 and a polyclonal antibody GK38 that recognize the phosphorylation of vimentin at Ser\textsuperscript{6} and Ser\textsuperscript{38}, respectively (Table I), as described under “Experimental Procedures.” Consistent with the in vitro CaMKII phosphorylation sites, Western blotting analysis showed that GK38 and MO82 reacted with vimentin phosphorylated by CaMKII but not with nonphosphorylated vimentin (Fig. 1, A and B). On the other hand, MO6, YT33, TM50, and 4A4 did not recognize vimentin phosphorylated by CaMKII (data not shown).
Spatial Signaling from Ca\textsuperscript{2+} via CaMKII to Vimentin

Cultured astrocytes differentiated by dibutylryl cAMP were used to detect CaMKII activity. Previous studies have demonstrated the existence of CaMKII in astrocytes (26, 28), and CaMKII activated by Ca\textsuperscript{2+} was shown to phosphorylate vimentin in these cells (19, 26). Furthermore, they display local and global Ca\textsuperscript{2+} in signaling to neurotransmitters (8, 29).

**In vivo** phosphorylation of vimentin at Ser\textsuperscript{4}, Ser\textsuperscript{6}, Ser\textsuperscript{33}, Ser\textsuperscript{38}, Ser\textsuperscript{50}, Ser\textsuperscript{52}, and Ser\textsuperscript{82} were immunocytochemically visualized using antibodies MO6, YT33, GK38, TM50, 4A4 and MO82, respectively. When [Ca\textsuperscript{2+}], of astrocytes was elevated by incubation of the cells with 1 \( \mu \)M ionomycin for 10 min, the phosphorylation of vimentin at Ser\textsuperscript{38} and Ser\textsuperscript{82} remarkably increased (Fig. 1, C–F) but those of Ser\textsuperscript{4}, Ser\textsuperscript{6}, Ser\textsuperscript{33}, and Ser\textsuperscript{50} did not (Fig. 1, G–J). Elevations in the levels of phosphorylation at Ser\textsuperscript{38} and Ser\textsuperscript{82} were further confirmed by Western blotting analysis using GK38 and MO82 (Fig. 2). Thus, the sites of vimentin phosphorylated by [Ca\textsuperscript{2+}], elevation completely overlapped with the **in vitro** phosphorylation sites by CaMKII (Table I). These results indicate that the phosphorylation of vimentin at Ser\textsuperscript{38} and Ser\textsuperscript{82} detected the vimentin phosphorylation by CaMKII.

We also located CaMKII in differentiated astrocytes. The affinity-purified antibody specific for 50- and 60-kDa subunits of CaMKII (23) immunostained astrocytes as described previously (26). Both the cell bodies and processes showed diffuse immunoreactivity, indicating that CaMKII is distributed throughout the cytoplasm of differentiated astrocytes (Fig. 1, K and L).

**Local and Global Signaling of CaMKII Induced by Ca\textsuperscript{2+} Signals**—Ser\textsuperscript{82} is at present the only known in **vitro** phosphorylation site specific to CaMKII (Table I) and the Ca\textsuperscript{2+}-induced vimentin phosphorylation at Ser\textsuperscript{82} was inhibited by a specific inhibitor of CaMKII, KN-62 (19). Therefore in the following studies, we monitored the phosphorylation of Ser\textsuperscript{82} to visualize CaMKII signaling. Ca\textsuperscript{2+} signaling in astrocytes was induced by PGF\textsubscript{2\alpha}, PGF\textsubscript{2\beta}, binds to FP-receptors on astrocytes and induces phosphatidylinositol 4,5-bisphosphate hydrolysis and intracellular Ca\textsuperscript{2+} mobilization (30). [Ca\textsuperscript{2+}], of astrocytes was measured using fura-2-based digital imaging Ca\textsuperscript{2+} microscopy, then they were fixed and immunostained with MO82.

When 10 \( \mu \)M PGF\textsubscript{2\alpha} was locally applied using a micropipette for 15 s near the end of a process of an astrocyte, [Ca\textsuperscript{2+}], was elevated from the basal level (about 100 nM) to about 600 nM in the process but not in the cell body or in other processes (Fig. 3A, a and b). [Ca\textsuperscript{2+}], then decreased to the basal level within 4 min (Fig. 3A, c). The [Ca\textsuperscript{2+}], increase did not appreciably spread beyond the boundary seen in Fig. 3A, b, throughout the period. Activation of CaMKII monitored by the phosphorylation at Ser\textsuperscript{82} localized only in the process where [Ca\textsuperscript{2+}], had been elevated (Fig. 3A, d, arrowheads). We also observed local CaMKII activations that were similarly defined by the area of Ca\textsuperscript{2+} signals in five other experiments. Propagation of intracellular Ca\textsuperscript{2+} waves has been observed in astrocytes (8, 29). Consistent with reports that Ca\textsuperscript{2+} waves often initiate when cells receive stimuli strong enough to induce sustained [Ca\textsuperscript{2+}], elevation in a localized area (8, 31), sustained PGF\textsubscript{2\alpha}-induced [Ca\textsuperscript{2+}], elevation propagated from a process to the cell body and then to the rest of the cell in the form of waves (Fig. 3B, a–c).

In this case, vimentin phosphorylation by CaMKII was evoked throughout the cell (Fig. 3B, d). The data above show a good spatial correlation between Ca\textsuperscript{2+} signaling and vimentin phosphorylation by CaMKII. Next, astrocytes were double-immunostained by MO82 and an anti-vimentin antibody, then examined by confocal microscopy. Vimentin phosphorylation by CaMKII occurred locally and globally, defined by the area of Ca\textsuperscript{2+} signaling (Fig. 3C, a, b, d, and e). On the other hand, vimentin was localized diffusely throughout the cells (Fig. 3C, c and f). Furthermore, CaMKII immunoreactivity was observed diffusely throughout the cells (Fig. 1, K and L). These data demonstrate that local and global phosphorylation of vimentin by CaMKII was not due to local and global intracellular distribution of vimentin or CaMKII, thereby indicating that the spatial patterns of Ca\textsuperscript{2+} signaling were indeed transmitted by CaMKII to vimentin.

**Electron Microscopic Analysis of the Vimentin Filaments Phosphorylated by CaMKII**—We noted here that a small population of vimentin filaments in the processes of ionomycin- or PGF\textsubscript{2\alpha}-stimulated astrocytes underwent structural alteration into partial granular aggregates, but not in unstimulated cells. Fig. 4 is an electron microscopic analysis of the CaMKII-phosphorylated vimentin filaments in astrocytes. Vimentin filaments in most of glial processes formed thick bundles running along glial processes (Fig. 4A), as reported previously (32). On the other hand, vimentin filaments in the aggregates were fragmented into short filaments running in random directions and thin glial processes appeared to form a varicosity there (Fig. 4B). When examined by immunoelectron microscopy, the density of MO82 immunoreactive gold particles was higher in the aggregates of vimentin filaments (Fig. 4, C and D) compared with those on the filaments in other regions (Fig. 4, C and E). We counted the number of the gold particles per micrometer of vimentin filaments in Fig. 4C. The mean density of the particles in the aggregates of vimentin filaments was 11.7 particles/\( \mu \)m of filament, while that in the other regions was 2.9 particles/\( \mu \)m of filament. Similar data were obtained in three other samples. These data suggest that the filament reorganization occurs when the level of phosphorylation by CaMKII is very high. It is unclear whether the structural alteration observed here is a typical change of filament structure under control of cell signaling. Because the population of the fragmented filaments was very low, more minute and coordinated alteration of the filament dynamics not detectable by microscopy may predominate. However, these findings are consistent...
with in vitro data that vimentin filaments disassembled when phosphorylated by CaMKII (15). The possibility that organization of intracellular vimentin filament networks is regulated by local and global phosphorylation by CaMKII would need to be considered.

In conclusion, we visualized CaMKII signaling by monitoring the site-specific phosphorylation of vimentin and showed that the spatial patterns of Ca\(^{2+}\) signaling defined the intracellular distribution of vimentin phosphorylated by CaMKII. These results suggest that the spatial patterns of Ca\(^{2+}\) signaling were transmitted via CaMKII to vimentin, as a type of spatial information. Although the population was very low, the structural change of vimentin filaments observed here raises the possibility that spatial signaling from Ca\(^{2+}\) via CaMKII to vimentin may regulate the dynamics of vimentin filaments in astrocytes.

**FIG. 3.** Local and global signaling of CaMKII defined by the area of Ca\(^{2+}\) signals. A, local Ca\(^{2+}\) signaling evokes localized signaling of CaMKII. a–c, [Ca\(^{2+}\)]\(_i\) in an astrocyte before (a), and at 30 s (b) and 4 min (c) after the local application of 10 \(\mu\)M PGF\(_{2\alpha}\) for 15 s. The arrow in a indicates the site of PGF\(_{2\alpha}\) application. The arrowheads in b indicate the process that showed Ca\(^{2+}\) signaling, d, vimentin phosphorylation at Ser\(^{82}\) by CaMKII in the same astrocyte in a–c. The photograph is magnified to present the area indicated by a rectangle in b. The cell was fixed at 5 min after the [Ca\(^{2+}\)]\(_i\) measurement in c and immunostained by MO82. The arrowheads indicate the process that showed CaMKII signaling. Bar, 20 \(\mu\)m. B, Ca\(^{2+}\) wave evokes global signaling of CaMKII. a–c, [Ca\(^{2+}\)]\(_i\) in an astrocyte before (a), and at 30 s (b) and 90 s (c) after the local application of 10 \(\mu\)M PGF\(_{2\alpha}\) for 15 s. The arrowhead in a indicates the site of PGF\(_{2\alpha}\) application. d, vimentin phosphorylation at Ser\(^{82}\) by CaMKII in the same astrocyte in a–c. The cell was fixed at 5 min after the [Ca\(^{2+}\)]\(_i\) measurement in c and immunostained by MO82. The arrowheads indicate the process that showed CaMKII signaling. Bar, 20 \(\mu\)m.

**FIG. 4.** Electron microscopic analysis of vimentin filaments in partial granular aggregates. A and B, standard electron micrographs of vimentin filaments in a glial process usually observed (A) and those in granular aggregates (B). C–E, immunogold localization of the MO82 epitope in a glial process containing an aggregate of vimentin filaments (C) and photographs at higher magnification (D and E) of the areas in C. Arrows in D indicate filaments running in random directions. Note that the density of the gold particles is higher on the fiber aggregate (D) compared with those on the filaments in the flanking region (E). The asterisks in D and E indicate areas corresponding to those indicated by the asterisks in C. Astrocytes were stimulated by 1 \(\mu\)M ionomycin for 5 min, and electron micrographs were taken as described under “Experimental Procedures.” Bars, 500 nm.
with spatial specificity. CaMKII phosphorylates a wide range of cellular proteins as well as vimentin (11, 12), therefore a population of CaMKII activity that could not be monitored by phosphorylation of vimentin might exist. Spatial signaling from Ca\textsuperscript{2+} via CaMKII to other substrates needs to be addressed in the future studies.

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