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A novel Ca^{2+} indicator protein using FRET and calpain-sensitive linker

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Abstract

Here, we report the properties of a FRET-based calcium indicator protein. We constructed a tandem fusion protein, named F2C, of ECFP and EYFP combined with calpain-sensitive sequences of α-spectrin, with N-terminal palmitoylation signal of GAP-43. It was previously reported that calpain cleaved a similar ECFP–EYFP fusion protein linked by a calpain-sensitive sequence of α-spectrin (fodrin). Unexpectedly, F2C was not cleaved by calpain, but demonstrated properties of a Ca^{2+} indicator when transiently infected in Purkinje cells of rat primary cerebellar culture or in the brainstem neurons infected in vivo using Sindbis virus encoding F2C. The emission ratio of 480 nm/535 nm was repeatedly increased when the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was raised. F2C had a Ca^{2+} sensitivity with an apparent dissociation constant (K_d for Ca^{2+}) of 150 nM, and demonstrated kinetics that paralleled Fura-2 when $[Ca^{2+}]_i$ was measured simultaneously. These properties of F2C are useful to be a Ca^{2+} indicator.

Keywords: FRET; Calcium; Calcium probe; Calpain; Fura-2; ECFP; EYFP; GAP-43; Fodrin

Intracellular Ca^{2+} plays important roles in many neuronal functions, such as release of neurotransmitters at synapses, activation of ion channels in the cell membrane, and regulation of a number of cytoplasmic enzymes; these are mainly induced by transient increases in cytoplasmic Ca^{2+} concentration. Cytoplasmic concentration of free Ca^{2+} has been measured by loading chemically synthesized Ca^{2+} chelators such as Fura-2 [1]. Recently, several Ca^{2+} probes have been developed genetically, based on GFP: Ca^{2+} probes using fluorescence resonance energy transfer (FRET) technique such as Cameleon [2,3] and FIP-CB_{SM} [4,5], or using circular permutated fluorescent protein such as Camgaroo [6,7] and Pericam [8], or using a conformational change of its protein such as G-CaMP [9] were developed. These indicators contain calmodulin (CaM) or a combination of CaM and CaM-binding domain of myosin light chain kinase (M13). Presently, there is no indicator which has a calpain-sensitive sequence. Calpain is a Ca^{2+}-activated proteolytic enzyme [10] found in a large variety of mammalian cells.

To visualize the synaptic activity, we attempted to use the FRET technique. Vanderklish et al. [11] have reported experiments by using the FRET method to mark the active synapse visually. They designed a fusion protein of enhanced cyan- (ECFP) and enhanced yellow- (EYFP) fluorescent protein by using a linker of the calpain-sensitive sequence [12], and a sequence of Shaker PDZ cognate domain at the C-terminal in order to target the protein to the postsynaptic domain. This fusion protein was reported to be cleaved in a Ca^{2+}-sensitive manner, and the FRET was disrupted permanently when the linker peptide was cleaved. We adopted this idea that Ca^{2+}-dependent proteases cleave the linker sequence and change the emission ratio permanently; however, we intended to target the fusion protein to the presynaptic membrane by conjugating it with the N-terminal palmitoylation signal of growth-associated protein-43 (GAP-43) [13].

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We constructed a fusion protein, F2C, encoding the following sequences in tandem, palmitoylation signal, ECFP, and two identical sequences of calpain-sensitive site of α-spectrin and EYFP, expecting to permanently label the presynaptic terminal of high activities. The Sindbis virus expression system was used for transient expression of F2C. But surprisingly, this fusion protein was not cleaved by calpain; that on the contrary, it repeatedly demonstrated responses to the rise of intracellular Ca^{2+} concentration. By Ca^{2+} calibration and fluorescence measurements carried out simultaneously with Fura-2, it turned out that F2C acted as a Ca^{2+} indicator. Here, we report the properties of a newly developed FRET-Ca^{2+} probe which has two calpain-sensitive sequences in tandem, targeted to the cell membrane and having many features useful in sensing the intracellular Ca^{2+} concentration.

Materials and methods

Primary culture of cerebellar Purkinje cells. Purkinje cells were cultured as reported [14,15]. In short, cerebella were dissected from Wistar rat fetuses on approximately embryonic day 20 and freed of meninges. The cerebella were incubated at 20 °C for 4 min in 1% trypsin (Invitrogen, CA, USA)/0.05% DNase (Sigma, MO, USA) solution, which contained 137 mM NaCl, 5 mM KCl, 7 mM Na_2PO_4, and 25 mM Hepes (pH 7.2). After washing with Ca–Mg-free Hanks' balanced salt solution (Invitrogen) three times, the tissue was dissociated by trituration with a fire-polished Pasteur pipette in Ca-free Hanks' balanced salt solution containing 0.05% DNase and 12 mM $MgSO_4$. The cell suspension was centrifuged at 180*g* at room temperature and the pelleted cells were resuspended at a concentration of 10^6 cells/ml in a defined medium, which facilitates the survival of neurons [14,16]. Two milliliters of this cell suspension was plated on a Petri dish containing several pieces of heat-sterilized coverslips coated with 0.01% poly-D-lysine (Sigma). This cell culture was incubated at 37 °C, 5% CO_2. Cells on a coverslip were used for infection with Sindbis virus. Purkinje cells showed action potentials and robust synaptic responses during culture for at least 9 weeks.

In vivo injection of Sindbis virus F2C and slice preparations. Young Wistar rats (9–10 days old) were anesthetized with chloral hydrate (0.3 mg/g body weight). Then, the head of the rat was fixed on a stereotaxis stage by using a pair of ear bars and a nose clamp (SR-5N, Narishige, Tokyo, Japan). The head skin was sagittally incised and a small hole was made through the skull to expose the cerebellum. A micropipette was inserted through the cerebellum, and a suspension of Sindbis virus-F2C as described below was injected (0.5–1 μl) into the brainstem by applying a gentle positive pressure to a micropipette by mouth. The incision of the skin was then sewn closed and the rat was returned to the mother after recovery from anesthesia.

Two days after the injection, coronal slices (200–300 μm) of the brainstem about the height of cochlear nuclei were made. Rats were deeply anesthetized by diethyl ether and decapitated, and the brainstem was quickly removed. The block of brainstem was cooled in an ice-cold 35 mM glucose saline (35 GS, concentrations in mM: 130 NaCl, 4.5 KCl, 2 $CaCl_2$, 5 Pipes-Na, and 35 glucose, pH 7.4) saturated with 100% O_2 and then embedded in a 4% agarose gel (Low gelling temperature, Nakalai Tesque, Kyoto, Japan) prepared with the 35 GS. Brain slices were made by a tissue slicer (Pro-1, Dosaka, Kyoto, Japan) in the ice-cooled 35 GS. These slices were preincubated in an oxygenated high-glucose artificial cerebro-spinal fluid (HG-ACSF) at 37 °C for at least 1 h before conducting the imaging experiments. HG-ACSF contained (mM): 75 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, and 100 glucose.

Gene construction. The FRET protein (named F2C, Fig. 1A) was designed as the fusion protein of N-terminal palmitoylation signal of GAP-43 (palmitoylation signal sequence is a gift from Dr. Kaneko, Graduate School of Medicine, Kyoto University), enhanced CFP, a calpain-sensitive sequence of α-spectrin, and enhanced YFP. The calpain-sensitive sequence of α-spectrin encoded the amino acid sequence GSGSGQQEVYGMMPRDGSG and was the same as that reported by Vanderklish et al. The cDNA of ECFP and EYFP was amplified by the polymerase chain reaction (PCR) with PfuTurbo (Stratagene, CA, USA) from pECFP-N1 (Clontech, CA, USA) and pEYFP-C1 (Clontech) as a template, respectively. All PCR products were verified by sequencing (BigDye Terminator Cycle Sequencing, Applied Biosystems, CA, USA). F2C has two identical calpain-sensitive sequences of α-spectrin (Fig. 1A), intending to make it easier for calpain or other proteins to interact with. This construct was digested with *Xba*I and *Eco*RV, and ligated into multiple cloning sites of pSinRep5 (pSindbis-F2C).

Virus production. The production of Sindbis virus encoding F2C protein (Sindbis-F2C) was performed according to manufacturer's instructions of the Sindbis Expression System (Invitrogen), as follows. The capped transcript of recombinant RNA was synthesized from the pSindbis-F2C which contained the construct of F2C. Sindbis viral particles were obtained by co-transfecting baby hamster kidney (BHK) cells electrophoretically with the capped recombinant RNA transcript and DH (26S) helper RNA encoding the structural protein. The viral particles in the culture supernatant were concentrated by centrifugation (6000*g*, 16 h, 4 °C). The virus was stored in aliquots at −80 °C until use. The resulting Sindbis virus was replication-deficient and had the least chance of production of parent viral particles in the infected cells [17]. Cultured cells and slices were transiently infected with Sindbis-F2C and experiments were performed 24–48 h later.

SDS–PAGE, Western blot hybridization. For cell lysate, cells were pelleted and homogenized in EGTA (10 mM EGTA-Na, 10 mM Hepes-K, and 150 mM NaCl) or Ca-EGTA buffer (10 mM $CaCl_2$, 10 mM EGTA-Na, 10 mM Hepes-K, and 150 mM NaCl). Ca^{2+} concentration was finally adjusted to 20 μM. Cell lysates were incubated at 30 °C for 30 or 60 min. In some experiments, cell lysates were incubated with a purified μ-calpain (calpain I, Calbiochem, CA, USA) at 30 °C for 30 or 60 min. These cell lysates were centrifuged at 20,000*g* for 20 min and the supernatant was fractionated. The supernatants were loaded onto SDS–PAGE gels. Western blotting was carried out according to the method of Towbin et al. [18] using mouse anti-GFP for detecting F2C cleavage (dilution rate 1:1000, MBL, Nagoya, Japan) and anti-PKC-α for confirming calpain activity (dilution rate 1:500, Upstate, NY, USA).

Image analysis. The primary culture of cerebellum was incubated at 37 °C, 5% CO_2. The cells on a coverslip were infected with Sindbis-F2C 24 h before the incubation with Fura-2/AM (Molecular Probes, Eugene, OR, USA). This coverslip was transferred to a new dish filled with an external solution (ACSF, artificial cerebro-spinal fluid; 155 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 17 mM glucose, and 5 mM KOH, and adjusted to pH 7.4). Fura-2/AM was added to a final concentration of 20 μM, and the solution was further incubated for 30 min at 37 °C. Fura-2/AM (10 mM) stock was dissolved into DMSO. Cerebella cultures were transferred under the upright microscope equipped with a cooled CCD camera and imaging was done (ORCA-ER on Aquacosmos, Hamamatsu Photonics, Hamamatsu, Japan). For Fura-2 imaging, excitation light wavelengths at 340 nm (10 nm band width 100% transmission) and at 380 nm (10 nm band width 100% transmission) were alternately applied, and fluorescence was captured at 510 nm and a longer wavelength. When F2C fluorescence was measured simultaneously, excitation light at 440 nm (10 nm bandwidth 100% transmission) was applied and the fluorescence at the wavelength longer than 510 nm was captured. This

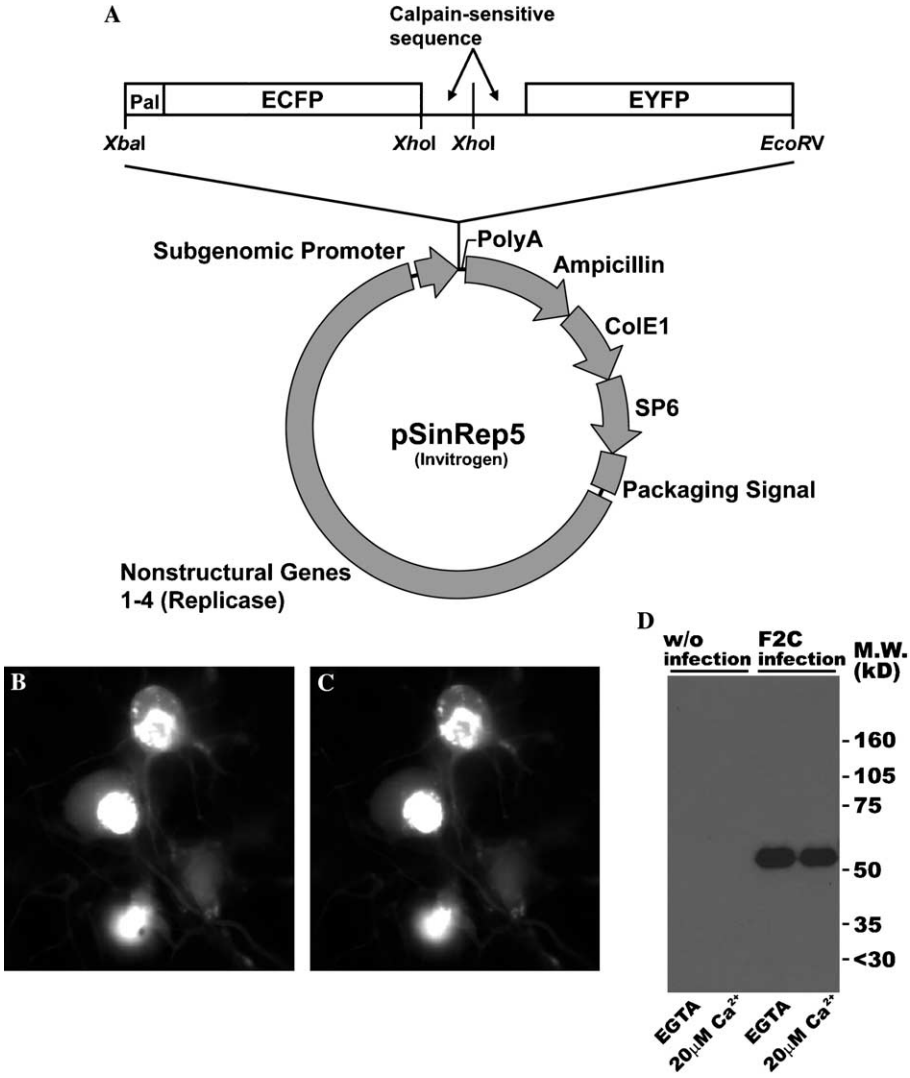

Fig. 1. Schematic structures of new Ca^{2+} probe, F2C. (A) Construct of pSindbis-F2C. Donor (ECFP) and acceptor (EYFP) were combined with the sequence of calpain-sensitive site of α-spectrin. (B,C) The fluorescence image of Purkinje cells expressing F2C; 480 nm emission for (B) and 535 nm emission for (C), excitation through 440 nm and dichroic mirror at 455 nm. (D) Western blotting of F2C. Primary culture cells infected with Sindbis-F2C were homogenized and separated by SDS–PAGE, followed by blotting onto PVDF membrane. The membrane was incubated with anti-GFP antibody, as described under Materials and methods.

fluorescence corresponds with EYFP. When only FRET was measured from brain slice preparations or from cerebella cultures, neurons were excited at 440 nm (20 nm bandwidth 60% transmission, XF1071 Omega Optical) with a dichroic mirror at 455 nm (XF2034 Omega Optical), and fluorescences were monitored by a cooled CCD camera mounted on a double view mirror system (Aquacosmos/FRET W-View, Hamamatsu Photonics) through 480 nm (30 nm bandwidth 75% transmission, XF3075 Omega Optical) for ECFP and 535 nm (25 nm bandwidth 70% transmission, XF3079 Omega Optical +50% ND filter) for EYFP.

Calpain inhibitor. To test the inhibition of calpain activities, calpain inhibitor-1 (ALLN, Calbiochem) and calpain inhibitor-2 (ALLM, Calbiochem) were adopted, and Fura-2 fluorescence and EYFP fluorescence measurements were similarly conducted. The primary cultures of cerebellum were preincubated in ACSF containing ALLN (100 μM in DMSO), ALLM (50 μM in DMSO), or a combination of both for 1 h. The same concentration of DMSO was dissolved into ACSF as a control.

Results

Expression of F2C protein and Western blotting analysis

When F2C was expressed in a primary culture of rat cerebellum, Purkinje cell infected with Sindbis-F2C showed brighter fluorescence (Figs. 1B and C) in the cell body than in the dendrite. Figs. 1B and C showed fluorescence of ECFP and EYFP. The fusion proteins would be associated with cell membranes by palmitoylation signal, but were highly localized in the cell nuclei. This might indicate aggregated fusion proteins as described by Furuta et al. [19]. When fluorometry was conducted, regions of interest were set excluding those regions within the nucleus.

SDS–PAGE and Western blotting analysis revealed that this construct gave rise to a 61.2 kDa fusion protein. Anti-GFP antibody recognized ECFP and/or EYFP and its signal was detected when primary cultures were infected with Sindbis-F2C, while the signal was not detected when primary cultures were not infected (Fig. 1D). The μ-calpain required some micromolar level of calcium for activation. When the cell lysate was homogenized in Ca-EGTA buffer (Ca^{2+} concentration of Ca-EGTA buffer was 20 μM), fragmented F2C was not detected (Fig. 1D), while fragments of PKC-α were detected by anti-PKC-α as a control of digestion by calpain (data not shown). Furthermore, F2C was incubated with the purified μ-calpain (Calbiochem) in Ca-EGTA buffer, but fragmented F2C signals were also not detected (data not shown). These experiments indicate that F2C fusion protein was not cleaved by calpain.

Application of F2C in vitro and in vivo

Typical responses of the fluorescence ratio (CFP/YFP) in FRET measurement and individual CFP and YFP emissions are illustrated in Figs. 2A and B for cerebellar Purkinje cells in a 14 day culture (14 DIV), and in Figs. 2C and D for cochlear nucleus neuron in a brain slice prepared from P11 rat, 2 days after injection of Sindbis-F2C.

In Fig. 2A, the fluorescence ratio sharply increased when KCl was increased from the basal level of 5 mM (the timing and concentration of KCl are indicated by

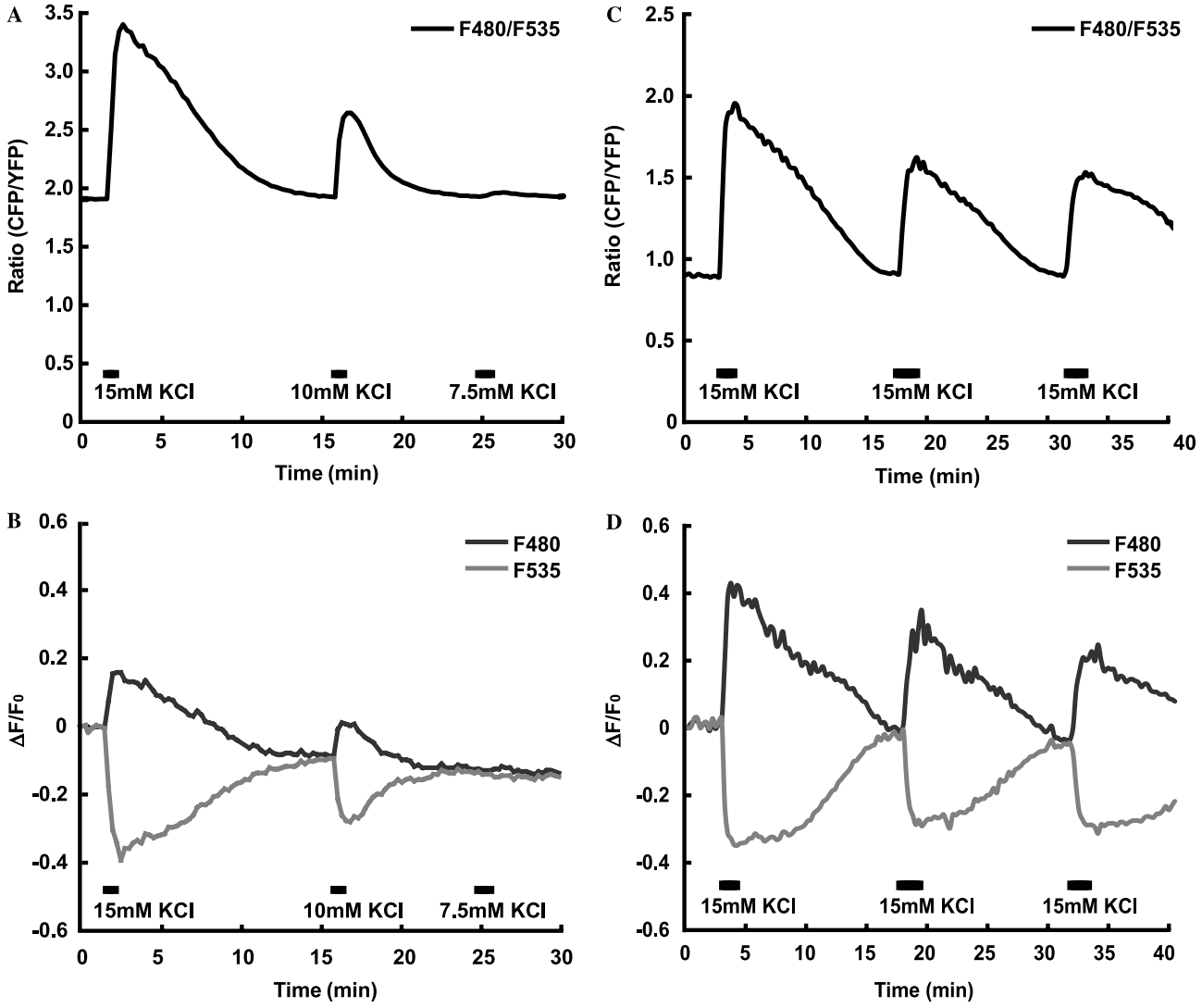

Fig. 2. The measurement of FRET ratios in Purkinje cells in culture and in auditory neurons in brain slices. The time course of fluorescence intensities was measured in single Purkinje cells (A,B) and in a single cochlear nucleus cell (C,D) expressing F2C. Small black bars on abscissa indicate the timing of application of KCl to the external solution. FRET ratios were repeatedly changed when various concentrations of KCl were added (A,C). (B,D) show reciprocal changes of fluorescence from CFP (F480 nm) and YFP (F535 nm). The fluorescent intensities for F480 and F535 are plotted after normalization by the initial fluorescence intensity F_0 (B,D). F_0 is defined as the average intensity of the first five measurements.

bars in the figure). If the F2C fusion protein was cleaved at the calpain-sensitive sequence by μ- or m-calpain, the fluorescence ratio (CFP/YFP) should be maintained at a high level because YFP emission would be decreased; however, contrary to the expectation, the ratio decreased rapidly. The CFP/YFP ratio changed 1.78-fold when cells were exposed to 15 mM KCl, 1.39-fold by 10 mM, and 1.03-fold by 7.5 mM. The emission fluorescence measured at F480 and F535 changed reciprocally. In rat brainstem slices, a FRET ratio changed reversibly when neurons were exposed to high KCl solution (Fig. 2C). Fifteen millimolar KCl was applied three times and the fluorescence ratio increased rapidly each time; also, the fluorescences measured at 480 and 535 nm changed reciprocally. In all these measurements, the fluorescence intensities returned to the original level after some time. These results were different from the observation made by Vanderklish et al. [11] where the YFP/CFP FRET ratio was decreased and maintained when calpain was activated by glutamatergic agonists.

Fluorescence properties of F2C as the intracellular Ca^{2+} indicator

The kinetics and sensitivity to Ca^{2+} of F2C were compared with those of Fura-2. Figs. 3A and B show the time course of ratio changes of Fura-2 emission (F340/F380, Fig. 3A) and F2C (F535, Fig. 3B). Fluorescence of three wavelengths was measured by exposure time of 112 ms for each, and images were sampled at the time interval of 896 ms. When KCl was added to the recording chamber, it produced changes in fluorescence. Fluorescence of Fura-2 and F2C was changed almost simultaneously. The rise times (20–80%) of F535 are plotted versus the rise times of F340/F380 in Fig. 3C, and the result shows a quasi-linear relationship ($n = 240$ cells), indicating that Ca^{2+} responses of F2C and Fura-2 were of almost the same speed. The time course of fluorescence changes is compared in Fig. 3D by plotting F535 ($\Delta F/F_0$) versus F340/F380 at the corresponding time; open circles represent the rising phase of the response and filled circles show the falling phase of the responses. Both the rising phase and the falling phase followed an overlapping trajectory. A slight downward curvature of the plot might reflect lower K_d for F2C than for Fura-2. The peak Ca^{2+} concentration induced by K^+ stimulation was about 0.5 μM from the basal level of near 0.05 μM when calibrated using Fura-2 signals (see legend of Fig. 3). Furthermore, data from 10 cells are plotted together for the rising phase (Fig. 3E) and for the falling phase (Fig. 3F). These plots are fitted by the following equation: $[Ca^{2+}]_i = K^*(\Delta F/F_0 - \Delta F/F_{0max})\ /(\Delta F/F_{0min} - \Delta F/F_0)$; $[Ca^{2+}]_i$ was estimated by Fura-2 ratio and $\Delta F/F_{0max} = -0.025$, $\Delta F/F_{0min} = -0.63$, and $K^* = 0.19$ were calculated by the maximum likelihood method. The goodness of fit was significant (correlation coefficient = 0.98). Fig. 3 shows that the dynamic range of F2C overlapped with that of Fura-2. The titration of F2C indicated an apparent K_d for Ca^{2+} of 150 nM at pH 7.4.

Cerebellar cultures were preincubated by calpain inhibitors (ALLN 100 μM and ALLM 50 μM), and K stimulation was similarly applied. The reciprocal changes of F340 and F380 Fura-2 fluorescences and the reduction of F535 EYFP fluorescence were similarly induced. The percentage of F535 EFYP maximum fluorescence change to Fura-2 maximum ratio change was not different from the control without calpain inhibitors (ALLN, ALLM, ALLN + ALLM; $p > 0.28$) (Table 1); this indicated that calpain inhibitors were not effective.

Discussion

We have designed a Ca^{2+} reporter protein, F2C, which is constructed to be a calpain-sensitive product. F2C was expected to be cleaved by calpain because of the presence of a calpain-sensitive linker sequence of α-spectrin [11], and a maintained high emission ratio of CFP/YFP was expected after activation of calpain by the rise of intracellular Ca^{2+} concentration. However, as Figs. 2A and C indicate, the emission ratio was decreased gradually after a quick increase. Western blotting analysis revealed that F2C was not cleaved by endogenous or purified μ-calpain, which is ubiquitously and constitutively expressed in mammalian cells [10]. These data indicate that μ-calpain does not cleave F2C in vitro. These phenomena

Fig. 3. Correlated fluorescence changes of F2C with Fura-2. Time courses of the emission ratio of Fura-2/AM (A) and the F535 fluorescence intensity of F2C (B). Sampling time interval of each fluorescence was 112 ms. (A) The emission ratio of F340/F380. Filled triangles indicate the timing of application of 10 mM KCl to the external solution. The scale in the right indicates the intracellular Ca^{2+} concentration, estimated by the following calibration equation: $[Ca^{2+}]_i = 0.65(R - R_{min})/(R_{max} - R)$; R = F340/F380. $R_{max} = 2.38$ and $R_{min} = 0.58$ were measured in Fura-2 loaded Purkinje cells; membrane was perforated by 1 μM ionomycin after equilibration with normal ACSF (2 mM $CaCl_2$) for R_{max} and 0 mM $CaCl_2$, 10 mM EGTA ACSF for R_{min}. (B) The fluorescent intensity (F535) was normalized by the initial fluorescence F_0. Excitation was 440 nm. (C) A correlation of 20–80% rise and fall time between F535 and F340/F380. These plots included experiments performed in various recording conditions; 0.5, 1.0, and 2.0 mM $CaCl_2$ in the external medium and Ca^{2+} responses were induced by application of KCl at concentrations of 10, 12.5, and 15 mM. Twenty to eighty percent rise time was measured and is plotted for individual cells ($n = 240$). (D–F) $\Delta F/F_0$ (F535) is plotted against F340/F380 at corresponding time. Open circles indicate the rising phase of the fluorescence response, and filled circles the falling phase. D indicates representative plots from 1 cell. (E,F) The rising phase and the falling phase from 10 cells, respectively. The solid line in (E,F) is drawn by the following equation: $[Ca^{2+}]_i = 0.19(\Delta F/F_0 + 0.025)/(-0.63 - \Delta F/F_0)$. See details in the text. The scale at the bottom (D) indicates the intracellular Ca^{2+} concentration estimated from Fura-2 ratios.

disagree with the report by Vanderklish et al. They reported that the fusion protein, named YSCS, and which had the calpain cleavage site of α-spectrin, was cleaved to fragments of 27 kDa cleavage products corresponding to the molecular masses of monomeric EYFP and ECFP. Although proteolytic separation of YSCS occurred rapidly and completely, F2C was not cleaved for an incubation time of 60 min by endogenous or purified μ-calpain and calcium (20 μM). The YSCS has an EYFP in the N-terminal

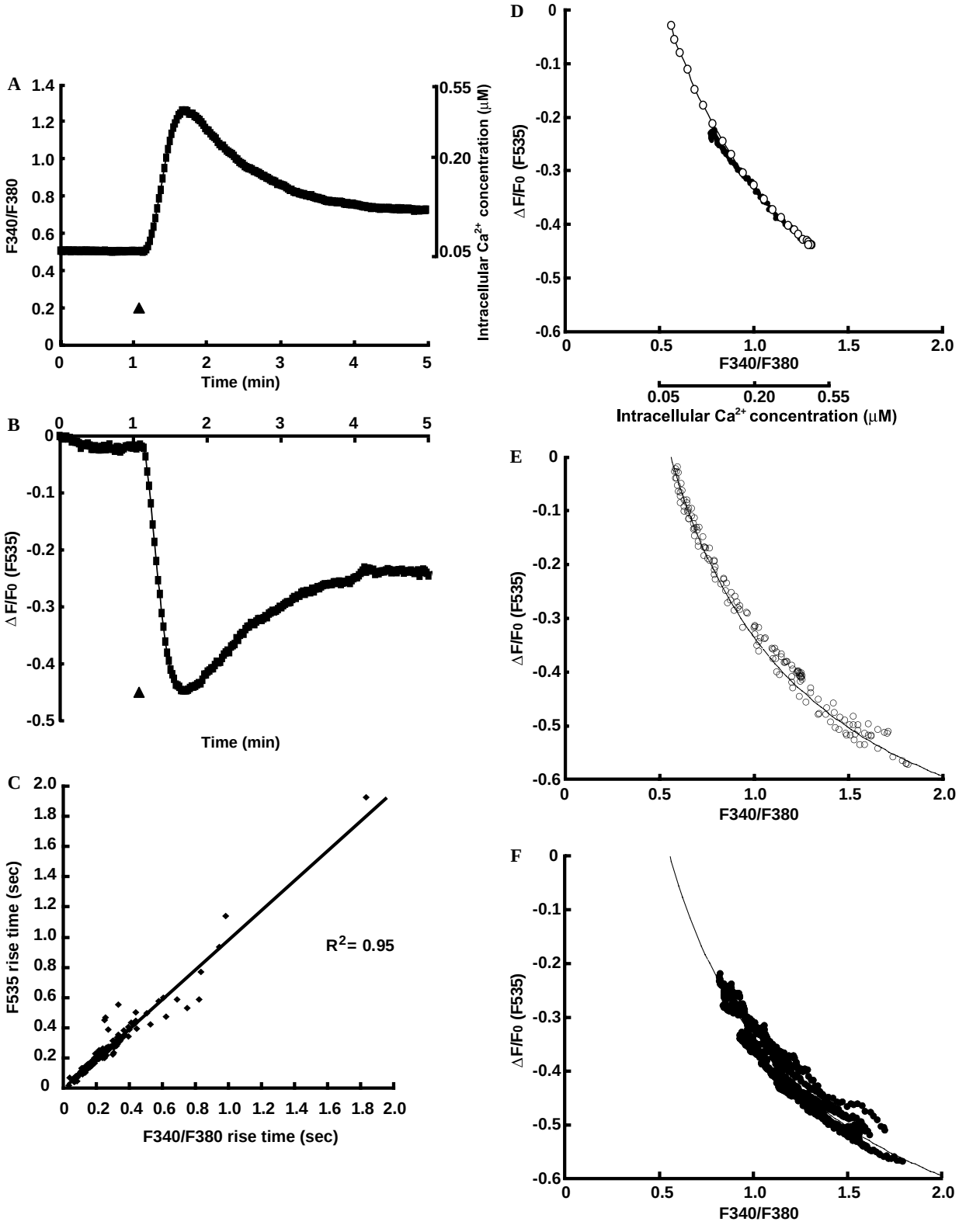

Table 1
Effect of calpain inhibitors

	Control	ALLN	ALLM	ALLN + ALLM
Mean ± SEM (%)	36.2 ± 1.1	37.1 ± 2.1	35.9 ± 1.4	38.4 ± 1.6
n	10	10	10	10

n, the number of cells.

and an ECFP in the C-terminal of the protein. On the other hand, F2C has an ECFP in the N-terminal and an EYFP in the C-terminal of the protein, so the sequences of the two fluorescent proteins are inverse to each other. Furthermore, F2C has a palmitoylation signal [19] in its N-terminal but YSCS has a Shaker PDZ cognate domain in the C-terminal. These differences may generate the unexpected response of F2C that is apparently resistive to the cleavage by μ-calpain but has obtained the sensitivity to $[Ca^{2+}]_i$. We constructed a variant which has one calpain-sensitive sequence as a linker (F1C). The cell lysate expressing F1C was prepared and incubated with 20 μM Ca^{2+} and μ-calpain, and then Western blotting analysis was performed. This experiment showed that F1C was not cleaved by μ-calpain either (data not shown). Fluorescent image analysis showed that the Ca^{2+}-sensitive response of F1C was reversible. Thus, the linker structure may not affect the behavior of this protein as a Ca^{2+} sensor. Moreover, calpain inhibitors (ALLN and ALLM) were not effective in eliminating the F2C response. These might indicate that the calpain-sensitive sites of F2C apparently behaved differently from our expectation and changed the level of FRET by some structural reorganization of the protein without being cleaved at the calpain-sensitive site. This idea is consistent with the results of Western blotting analysis.

Fig. 3D is the representative trace indicating that responses of F2C to Ca^{2+} correlate well to those of Fura-2. Traces of the rising phase and the falling phase corresponded particularly well. The correlation curve was bent slightly downwards; this might reflect the difference of apparent K_d values between two Ca^{2+} indicators. The apparent K_d for Ca^{2+} of Fura-2 is 224 nM [1] and that of F2C is 150 nM. The 20–80% rise times of F535 were well correlated with those of F340/F380 (Fig. 3C). The coefficient of determination (R^2) was 0.95 ($p < 0.0001$). These data indicate that F2C can be an intracellular Ca^{2+} indicator as equi-potent as Fura-2.

There are many Ca^{2+} indicators constructed using the FRET technique, such as Cameleon [2,3], or using a conformational change of its protein such as G-CaMP [9]. These proteins use CaM or a combination of CaM and M13, so F2C using calpain-sensitive sequences is a new type of indicator of intracellular Ca^{2+}. Fura-2 is a chelator of Ca^{2+} and its concentration affects the kinetics of intracellular Ca^{2+} dynamics [20]. Because of the absence of a Ca^{2+}-sensitive site in F2C primary amino acid sequence such as the EF-hand structure, F2C may not bind to free calcium directly. This implies that the absence of Ca^{2+}-binding structure might allow this protein a unique property of sensing the intracellular Ca^{2+} concentration without affecting the physiological kinetics of the intracellular Ca^{2+} dynamics, even though detailed Ca^{2+} sensing mechanism of F2C is still unclear.

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