Calexcitin interaction with neuronal ryanodine receptors

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Calexcitin (CE), a Ca^{2+} - and GTP-binding protein, which is phosphorylated during memory consolidation, is shown here to co-purify with ryanodine receptors (RyRs) and bind to RyRs in a calcium-dependent manner. Nanomolar concentrations of CE released up to 46% of the ^{45}Ca label from microsomes preloaded with $^{45}\text{CaCl}_2$. This release was Ca^{2+} -dependent and was blocked by antibodies against the RyR or CE, by the RyR inhibitor dantrolene, and by a seven-amino-acid peptide fragment corresponding to positions 4689–4697 of the RyR, but not by heparin, an $\text{Ins}(1,4,5)\text{P}_3$ -receptor antagonist. Anti-CE antibodies, in the absence of added CE, also blocked Ca^{2+} release elicited by ryanodine, suggesting that the CE and ryanodine binding sites

were in relative proximity. Calcium imaging with bis-fura-2 after loading CE into hippocampal CA1 pyramidal cells in hippocampal slices revealed slow, local calcium transients independent of membrane depolarization. Calexcitin also released Ca^{2+} from liposomes into which purified RyR had been incorporated, indicating that CE binding can be a proximate cause of Ca^{2+} release. These results indicated that CE bound to RyRs and suggest that CE may be an endogenous modulator of the neuronal RyR.

Key words: calcium-binding proteins, calcium channels, liposomes, memory, squid.

INTRODUCTION

Transient Ca^{2+} signals through voltage-dependent membrane channels and membrane receptor-mediated release of Ca^{2+} from intracellular stores are critical for hormone secretion [1], synaptic transmission [2], learning [3] and cellular models of learning, such as long-term potentiation and long-term depression [4–6]. Although the initial Ca^{2+} signal in most of these systems is Ca^{2+} influx through membrane channels (such as the *N*-methyl-D-aspartate receptors and voltage-gated channels), calcium release from intracellular stores, such as the endoplasmic reticulum (ER), can greatly amplify the signal. Of the two predominant pathways for Ca^{2+} release, the $\text{Ins}(1,4,5)\text{P}_3$ -receptor pathway is primarily involved in calcium release induced by neurotransmitter receptors [7], whereas the ryanodine receptor (RyR) is the principal mediator of calcium-induced Ca^{2+} release [8–14]. The RyR can be blocked in a Ca^{2+} -dependent manner by calmodulin (CaM) [9–11], or activated in sea urchin oocytes by adenine nucleotides such as cADP ribose [9–12].

Calexcitin (CE), a 22 kDa Ca^{2+} - and GTP-binding protein originally isolated from the sea snail *Hermisenda*, can reproduce many of the effects of associative conditioning when micro-injected into invertebrate neurons, cerebellar Purkinje cells [15] or hippocampal pyramidal cells (M. K. Sun, personal communication), including changes in axonal transport [16] and inhibition of K^+ channels [15,17,18], which lead to increased membrane excitability. There is considerable evidence that intracellular- Ca^{2+} levels are also changed after learning [3,19]. Therefore the interaction of CE with RyRs was investigated. The experiments described in the present work demonstrate that CE binds to the RyR in a Ca^{2+} -dependent manner and releases

calcium from microsomes in a manner consistent with activation of the RyR.

MATERIALS AND METHODS

Antibodies

Peptide Ac-DVNDTSGDNIIDKHEYSTC-NH₂, corresponding to residues 115–133 of CE, conjugated to keyhole-limpet haemocyanin, was used to raise polyclonal antibodies in two rabbits. The antibody was effective only against non-denatured CE, therefore all Western blotting was performed using non-denaturing gels. Antibodies against FK506-binding protein and RyR were purchased from Santa Cruz Biotechnology and Upstate Biotechnology respectively.

Squid optic-lobe extract

Squid optic lobes (0.5 g) were homogenized by sonication in 4 ml of *Hermisenda* buffer [10 mM Tris/HCl, pH 7.4/50 mM NaF/1 mM EDTA/1 mM EGTA/1 mM PMSF containing 20 µg/ml leupeptin, 50 µg/ml pepstatin and 1% (v/v) CHAPS]. The sample was centrifuged at 100 000 *g* for 10 min (Beckman TL-100 ultracentrifuge) and the supernatant was saved. Triton X-100 (0.2% v/v) was used instead of CHAPS in some experiments.

CE–Sephacrose affinity chromatography

CE–Sephacrose was prepared by directly coupling cloned CE with CNBr-activated Sepharose 4B (Pharmacia LKB) and was equilibrated with buffer A [25 mM Tris/HCl/150 mM NaCl (TBS) containing 1 mM PMSF, 1 mM CaCl_2 and 1% (v/v) CHAPS] (column vol., 0.2 ml). The CHAPS extract (0.4 ml) from squid

Abbreviations used: CE, calexcitin; ER, endoplasmic reticulum; RyR, ryanodine receptor; BCIP, 5-bromo-4-chloroindol-3-yl phosphate; NBT, Nitro Blue Tetrazolium; PKC, protein kinase C; CaM, calmodulin.

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optic lobe was applied to a 1-cm³ column and was eluted with buffer A containing 0–2 M NaCl, followed by buffer A containing 2 M NaCl but without calcium, and then by buffer A containing 2 M NaCl and 1 mM EGTA. Fractions were desalted and concentrated by ultrafiltration (Centricon-3). Each fraction was 2.5 column volumes. Samples (10–40 μ l) containing up to 50 μ g of protein were boiled for 2 min in SDS sample buffer [4% (w/v) SDS/0.125 M Tris/HCl, pH 6.8/20% (v/v) glycerol/10% (v/v) 2-mercaptoethanol/0.0025% Bromophenol Blue], subjected to electrophoresis on a 4–20% polyacrylamide gradient gel, blotted on to nitrocellulose and stained with colloidal gold (Aurodyne; Amersham Corp.).

Non-denaturing PAGE and Western-blot analysis

Samples were mixed with equal volumes of sample buffer [50% (v/v) glycerol/0.5 M Tris/HCl (pH 8.0)/5% (v/v) CHAPS/0.1% (w/v) Bromophenol Blue], separated by non-denaturing PAGE (4–20% acrylamide gradient), blotted on to nitrocellulose and stained with Nitro Blue Tetrazolium (NBT)/5-bromo-4-chloroindol-3-yl phosphate (BCIP).

Phage-display plasmid library screening

CE was desalted using a Sephadex G-25 spin column (5 Prime 3 Prime) equilibrated with TBS. Polystyrene tissue-culture plates were coated with CE (83 μ g) in 0.1 M NaHCO₃ and used to screen 2×10^{11} phage from a combinatorial library of random peptide heptamers (New England Biolabs). Bound phage were eluted with 1 ml of 0.2 M glycine, pH 2.2, containing 1 mg/ml BSA, then neutralized with Tris/HCl, pH 9.0, amplified in *E. coli* NM522 cells and precipitated with 20% poly(ethylene glycol)-8000/2.5 M NaCl. The screening and amplification of bound phage was repeated three times and 20 plaques were selected for sequencing.

Purification of CE

Cloned squid optic-lobe CE containing a His₉ leader sequence was expressed in BL21(DE3) cells and purified by repeated affinity chromatography on Ni²⁺-charged His-Bind columns (Novagen) [15]. In a previous study, CE was designated 'cp20' [15].

To avoid potentially confounding effects of CE on Ca²⁺-ATPase, in most of the experiments described in the present work, a variant form of CE, which lacks the normal C-terminal GTP-binding site, was used. This variant rendered the CE independent of exogenous GTP and almost eliminated the effect of CE on Ca²⁺-ATPase. Binding of the normal form to RyR was found to be similar to that of the shortened variant form in all experiments.

Iodination

Purified CE or RyRs were iodinated by incubation with an Iodo-Bead (Pierce Chemical Co.) in 0.2 ml TBS containing 1% (w/v) SDS and 1.0 mCi of carrier-free Na¹²⁵I at 25 °C for 10 min. The labelled protein was isolated by repeated ultrafiltration (Centricon-3 filtration unit), followed by chromatography on Sephadex G-25 in a spin column.

Phosphorylation of CE

Purified, cloned CE was phosphorylated using a modified procedure described by Nelson and Alkon [20]. CE (50 μ g) was incubated at 37 °C in the presence of 20 mM Hepes, pH

7.4/1 mM EDTA/2 mM EGTA/10 mM MgCl₂/1.2 mM Ca²⁺ containing 20 μ g/ml 1,2-dioctanoyl-*sn*-glycerol, 100 μ g/ml phosphatidyl-L-serine, 10 μ g/ml leupeptin, 0.1 mM PMSF and 0.5 unit protein kinase C (PKC)- α . The phosphorylation was initiated by adding ATP to the reaction mixture to a final concentration of 100 μ M. The reaction was terminated after 30 min by applying the reaction mixture to a His-Bind column, through which the phosphorylated CE was separated from PKC and the rest of reaction mixture. The eluted phosphorylated CE was desalted and concentrated using the Millipore Ultrafree-MC filter unit. The CE concentration was determined using Bio-Rad dye-binding Protein Reagent (Bio-Rad) and the sample was used immediately or stored overnight at –20 °C and used the next day. To confirm the phosphorylation of CE, a small amount of the protein was phosphorylated in a separate tube using the same conditions, except that 1 μ Ci [γ -³²P]ATP was present in the reaction mixture. After separation on a His-Bind column, the phosphorylated CE was subjected to SDS/PAGE, blotted on to PVDF and autoradiographed. Using these conditions, the PKC catalytic subunit had much lower activity toward CE than the holoenzyme.

Fluorescence spectroscopy

CE (6 μ g) was incubated at room temperature for 1 h in 50 μ l 0.1 M NaHCO₃ (pH 9.0) with 100 μ g eosin isothiocyanate in 10 μ l DMSO, followed by incubation for 1 h with 1 M Tris/HCl (pH 8.5). Excess reagent was removed by repeated passage through Sephadex G-25 spin columns equilibrated with TBS. Uncorrected spectra of CE samples in 3.5-ml silica cuvettes were obtained (Spex Fluorolog-2 DM3000 spectrofluorimeter) using excitation and emission slit widths calibrated to give a band-pass of 2 nm.

Purification of RyR

RyR was purified from squid optic-lobe ER fractions, corresponding in density to the terminal cisternae of muscle, by solubilization in 1.25% (v/v) CHAPS and affinity chromatography on spermine-agarose gel [21]. Fractions binding [³H]-ryanodine were concentrated by ultrafiltration (Centricon-100). This step also removed any contaminating low-molecular-mass proteins. In some experiments, RyRs were purified by a sucrose-gradient method [22].

Liposomes

Squid optic-lobe RyR (18.6 μ g), which had been purified to apparent homogeneity by spermine-agarose affinity chromatography, was sonicated for 1 s in 1 ml of buffer L [50 mM NaCl/50 mM KCl/20 mM Tris/HCl (pH 7.4)] containing 8 mg/ml phosphatidylcholine, 1% (v/v) CHAPS and 10 mM CaCl₂. The suspension was dialysed for 48 h against buffer L containing 2 mM 2-mercaptoethanol, 1 μ g leupeptin and 120 μ M CaCl₂. Liposomes were collected by centrifugation at 150 000 *g* for 90 min, resuspended in 0.2 ml of buffer L with a Teflon/glass homogenizer (without sonication) and used immediately. Liposomes containing Ba²⁺ instead of Ca²⁺ were prepared similarly. Liposomes were not reused after a measurement was completed, and were discarded after 48 h.

Release of Ca²⁺ from liposomes

Liposomes (10 μ l) were diluted to 3.0 ml in degassed buffer L in a quartz cuvette containing 1.67 μ M Calcium Green 5N, with a final external calcium concentration ([CaCl₂]_o) of 200 nM. Flu-

orescence readings were obtained immediately after the addition of liposomes. CE was desalted on Sephadex G-25 spin columns before addition and fluorescence emission intensity was monitored at 530 nm (excitation = 488 nm).

Ryanodine-binding activity

Purified RyRs (10 μ g) were preincubated with 0.1 mg soybean lecithin for 5 min at room temperature and mixed with incubation solution (final concentration: 0.2 M KCl/10 mM Na/Hepes, pH 7.2/1 mM EGTA/100 μ M free Ca^{2+}) in a total volume of 20 μ l. [^3H]Ryanodine (215 nM, 0.2 μ Ci) was added and, after 120 min at 30 °C, samples were diluted ten times with ice-cold buffer B [10% (w/v) poly(ethylene glycol)-8000/0.3 M NaCl/19 mM Mops, pH 7.4] and incubated on ice for 15 min. Samples were then filtered through GF/B glass-fibre filters, washed with 1 ml of buffer B and radioactivity was estimated by scintillation spectroscopy.

RyR overlay experiments

Nitrocellulose membranes were blocked with BSA, incubated in TBS containing various concentrations of CaCl_2 , and squid optic-lobe microsomal CHAPS extract (4 μ l) or purified RyR (20 μ g) was overlaid on to the membrane. The membrane was incubated at 23 °C for 30 min, washed with TBS and a polyclonal antibody against rat brain RyR was added (3 μ g per ml of TBS). The membrane was then incubated for 45 min at 23 °C, washed with TBS and an alkaline-phosphatase-conjugated second antibody was added. After 45 min, the membrane was washed again and stained with BCIP and NBT. All solutions contained the same fixed concentration of CaCl_2 .

Computer analysis

Curve fitting and parameter estimation by non-linear regression were done using nplot. Gel quantification and molecular-mass estimation were done using tnmage. nplot and tnmage are computer programs written in-house.

Isolation of microsomes for Ca^{2+} -release experiments

The microsomal fractions were prepared according to [23]. Optic lobe tissues (1 g) were homogenized in 9 ml of precooled buffer C (0.32 M sucrose/5 mM Hepes, pH 7.4/0.1 mM PMSF) in a Teflon/glass homogenizer. The homogenate was diluted to 10% of wet weight of the tissue in buffer C and centrifuged at 900 g for 5 min. The supernatant was preserved and the pellet was washed once by resuspending it in buffer C and centrifuging at 900 g for 5 min. The supernatants from these two steps were combined and centrifuged at 17000 g for 15 min. The resulting supernatant from this step was subjected to centrifugation at 100000 g for 30 min. The pellet (microsomal fractions) was harvested and resuspended in a small volume of buffer C. The microsomal tissues were either used immediately or were stored at -80 °C and used within one week.

Ca^{2+} -loading and -release assay

Calcium uptake and release were measured using a rapid filtration method [24,25]. Reactions were performed at 37 °C and in a total volume of 50–100 μ l. $^{45}\text{Ca}^{2+}$ (20 μ M) was preincubated for 5 min in the reaction mixture (20 mM Mops, pH 7.2/100 mM KCl/20 mM NaCl/3.5 mM MgCl_2 /3 mM ATP/20 mM phosphocreatine/1 mM glucose 6-phosphate/5 mM NaN_3 and 10 units/ml creatine kinase). Ca^{2+} uptake was initiated by adding microsomal preparation to the reaction mixture to a final concentration

of 50 μ g protein/ml. Ca^{2+} fluxes across microsomes were detected by measuring changes in intramicrosomal $^{45}\text{Ca}^{2+}$ content. The reaction mixture (5–10 μ l) was pipetted, in duplicate, on to the cellulose nitrate membrane discs (0.2 μ m pore diam.), which were rapidly washed twice in buffer C and rinsed with 1 mM EGTA in buffer C. The membranes were air-dried and radioactivity was measured (Beckman LS-3801 scintillation counter).

Ca^{2+} -release measurement using a Ca^{2+} indicator (Calcium Green 5N)

Microsomal fractions (final concentration 50 μ g/ml) were added to 3 ml of reaction mixture (20 μ M Ca^{2+} /20 mM Mops/100 mM KCl/20 mM NaCl/3.5 mM MgCl_2 /3 mM ATP/20 mM phosphocreatine/1 mM glucose 6-phosphate/5 mM NaN_3 containing 10 units/ml creatine kinase) in a quartz cuvette. Calcium Green 5N (final concentration 1.67 μ M) was added and the emission intensity at 530 nm was measured (excitation, 488 nm).

Ca^{2+} imaging

Transverse hippocampal slices (400 μ m) taken from rats on postnatal day 7 were maintained as described previously [26]. Slices ($\approx$ 200 μ m), cultured for 6–10 days, were transferred to an immersion-type slice chamber and continuously perfused (2 ml/min) with artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 2 mM KCl, 1.3 mM MgSO_4 , 1.24 mM KH_2PO_4 , 17.6 mM NaHCO_3 , 2.5 mM CaCl_2 and 10 mM D-glucose, adjusted with sucrose to 310–320 mOsm, at room temperature and gassed with CO_2 /air (19:1). Whole-cell recordings from the soma of CA1 pyramidal neurons, identified by infrared differential-interference contrast (DIC) optics, were performed with unpolished patch electrodes containing 120 mM caesium gluconate, 10 mM Na/Hepes, 17.5 mM CsCl, 10 mM NaCl, 2 mM Mg-ATP, 0.2 mM Na-GTP, 5 mM QX-314 (lidocaine *N*-ethylbromide quaternary salt), 0.5 mM bis-fura-2 (penta-potassium salt) (K_d 525 nM; Molecular Probes), pH 7.2, 280–290 mOsm (5–8 M Ω , final resistance). Intact or heat-inactivated CE (0.64–0.71 μ g/ml) was included in the intracellular solution immediately before patching. Calcium imaging and simultaneous whole-cell recordings were performed [27] using a fixed-stage, upright microscope (Axioskop FS; Zeiss) with a water immersion objective (Achromplan 63X, 0.9NA; Zeiss). Bis-fura-2 fluorescence was excited at 357 nm (which was the isosbestic point in our optical configuration) and 380 nm light (12 nm band width) using a galvanometric scanner-mounted grating (Polychrome I; Till Photonics), and the emission at 510 nm was imaged with a 12-bit digital cooled charge-coupled device (CCD) camera system (PXL-37; Photometrics). The digital CCD camera, polychromator and intracellular amplifier [Axoclamp-2B micro-electrode amplifier (Axon Instruments Inc.) using an ITC-16 data acquisition unit (InstruTech Corp.)] were controlled by custom-developed software in a 120 MHz PowerPC computer (Macintosh), which allowed simultaneous on-line analysis of optical and electrophysiological data (T. Inoue, University of Tokyo, Tokyo, Japan). Free cytosolic Ca^{2+} concentration was determined from background-corrected images using the isosbestic ratio method [28]. Neurons were maintained slightly hyperpolarized (-70 to -80 mV) in the current-clamp mode with small DC current (-0.1 to -0.5 nA) to prevent spontaneous firing of Ca^{2+} spikes. Na^+ -dependent action potentials were blocked intracellularly by QX-314. K^+ channels were blocked intracellularly by Cs $^+$. Electrophysiological data was digitized at 10 kHz for analysis and display.

RESULTS

The major receptor for CE, determined by binding of protein extracts from squid optic-lobe homogenates to CE–Sephacryl 4B, was a protein of $M_r > 500$ which co-migrated with native purified RyR (Figures 1A and 1B). Several other minor protein bands, most noticeably bands of M_r 43, 49 and 56, were also

Figure 1 Binding of CE to RyRs

(A) Calyculin-binding proteins. Squid optic-lobe Triton X-100 extract was applied to a calyculin–Sephacryl 4B affinity column and washed with increasing concentrations of NaCl. Fractions were desalted, separated by SDS/PAGE (4–20% gradient gel), blotted on to nitrocellulose and stained with colloidal gold. Proteins of M_r 42.1, 49.1, 56.2 and > 500 were retained. In other experiments, the lower molecular-mass bands were occasionally more prominent than those shown here. The M_r of the high molecular-mass band could not be determined accurately but its position was identical to that of purified RyR. (B) Non-denaturing Western blot of RyR in squid optic-lobe extract. (C) Non-denaturing Western blot of CE-like immunoreactivity in rat cerebellum ER fraction. (D–F) Binding of 125 I-labelled CE to RyR. 125 I-labelled CE (24000 d.p.m.) was incubated with (D) squid-muscle sarcoplasmic reticulum, (E) optic-lobe microsomal fractions or (F) alone, separated by non-denaturing gradient PAGE, blotted on to nitrocellulose and subjected to autoradiography. The high molecular-mass band (shown by an arrowhead on the right) co-migrated with RyR. The positions of native ferritin (Fer) (M_r 500000) and the top of the running gel are indicated. (G) 125 I-labelled cloned CE was overlaid on a nitrocellulose blot from a non-denaturing polyacrylamide gel of RyR. The blot was washed extensively and subjected to autoradiography. The single band co-migrated with RyR. (H) CHAPS-solubilized RyR purified by the sucrose gradient method [22] was subjected to non-denaturing PAGE, blotted and stained with anti-CE antibody. Intense CE immunoreactivity can be seen co-migrating with RyR, which was not observed when RyR was purified by harsher methods. (I–K) Purified squid optic-lobe RyR was separated by non-denaturing PAGE, blotted on to nitrocellulose and overlaid with (I) cloned CE, (J) microsomal CHAPS extract or (K) buffer alone, and incubated with affinity-purified polyclonal antibody against CE in the presence of $20 \mu\text{M}$ Ca^{2+} . Lanes overlaid with cloned CE or with extracts containing native CE showed a single band that co-migrated with RyR ($M_r > 500$). (L) Non-denaturing Western blot of CE in squid optic-lobe homogenate. (M–O) Squid optic-lobe homogenate was separated by non-denaturing PAGE, blotted on to nitrocellulose and overlaid with purified RyR (M), microsomal CHAPS extract (unpurified RyR) (N), or buffer alone (O) and incubated with affinity-purified polyclonal antibody against RyR in the presence of $20 \mu\text{M}$ Ca^{2+} . Lanes overlaid with RyR or with extracts containing native RyR showed a single band that co-migrated with CE (M_r 22, shown with an arrowhead). A Western blot using anti-CE antibody is shown for comparison (L).

Figure 2 Binding of RyRs to immobilized CE

(A) Squid optic-lobe CHAPS extract was applied to a CE–Sephacryl 4B affinity column and treated as described in the Materials and methods section. All buffers contained 1% CHAPS. Ryanodine binding activity was not released from the column by 2 M NaCl but was eluted when Ca^{2+} was removed. (B) Binding of purified RyR to calyculin–Sephacryl 4B. Purified 125 I-labelled RyR was applied to a CE–Sephacryl 4B affinity column and treated as described in the Materials and methods section. 125 I-labelled RyR eluted from the column when Ca^{2+} was removed, indicating that intermediate proteins were not required for binding or to confer calcium dependence. Control experiments where CNBr-activated Sepharose was bound to BSA or to a control peptide showed no calcium-dependent retention of 125 I-label, indicating that the RyR was retained by the CE and not by the Sepharose itself. (C) Cloned CE was bound to nitrocellulose membranes, blocked with BSA, overlaid with purified RyR and the RyR that remained bound was stained with a polyclonal antibody against RyR; the indicated Ca^{2+} concentrations were maintained throughout. Other proteins tested gave no signal (the error bars indicate the S.E., $n = 4$; * $P < 0.025$, two-tailed Student's t test).

Figure 3 Dot blots of CE–RyR binding

Equal amounts (50 ng) of cloned CE or other proteins were spotted on to a nitrocellulose membrane, blocked with BSA and overlaid with purified RyR, in the presence of 100 nM Ca^{2+} (A), $1 \mu\text{M}$ Ca^{2+} (B), $10 \mu\text{M}$ Ca^{2+} (C), $100 \mu\text{M}$ Ca^{2+} (D), 1 mM Ca^{2+} (E), 1 mM Ca^{2+} + 1 mM GTP (F), 1 mM Ca^{2+} + 1 mM ATP (G) or 1 mM Ca^{2+} + squid optic lobe cytosol (H). Filters were stained with anti-RyR antibody; Ca^{2+} and other solutes were maintained at a fixed concentration until stained with NTB/BCIP. Only the CE sample bound RyR under these conditions. Similar results were obtained using squid optic-lobe CHAPS extract instead of purified RyR.

Figure 4 Promotion of Ca^{2+} release from microsomes by CE

(A) Dose response of CE on Ca^{2+} release. Percentage Ca^{2+} release was monitored 10 min after CE at the concentrations shown was added to $^{45}\text{Ca}^{2+}$ -loaded microsomes. Samples containing 15–300 nM CE from showed a 24–46% reduction in intramicrosomal $^{45}\text{Ca}^{2+}$. A significant (*) dose effect was found using one-way ANOVA [$F(5,18) = 7/98$, $P = 0.004$]. Values are the means $\pm$ S.E., $n = 4$. (B) Time course of the effect of CE on Ca^{2+} release. Intact and heat-inactivated CE was added to $^{45}\text{Ca}^{2+}$ -loaded microsomes to a final concentration of 30 nM (total $[\text{Ca}^{2+}] = 20 \mu\text{M}$), and Ca^{2+} release was measured at various times after the addition of CE. Controls (○) contained CE buffer in place of CE. Addition of heat-denatured CE or buffer alone did not cause Ca^{2+} release. The error bars indicate S.E., $n = 4$ –17, $*P < 0.001$ (two-way ANOVA). CE was added to the reaction mixture 5 min after Ca^{2+} loading. Intramicrosomal $^{45}\text{Ca}^{2+}$ was measured 5 min after addition of CE. The 100% value represents 27 ± 1.69 nmol Ca^{2+} /mg of protein. (C) Effect of Ca^{2+} concentration on CE-induced Ca^{2+} release. The CaCl_2 concentrations indicated were used in reaction mixtures which also contained $0.1 \mu\text{M}$ $^{45}\text{Ca}^{2+}$. CE was added to Ca^{2+} -loaded microsomes for 5 min and Ca^{2+} release was measured 5 min after the addition of CE. The error bars indicate S.E., $n = 3$, $*P = 0.001$ (one-way ANOVA). (D) Effect of dantrolene and anti-RyR antibody on CE-modified Ca^{2+} release. Dantrolene ($10 \mu\text{M}$) or anti-RyR antibody (1:100) was incubated with microsomes during Ca^{2+} loading before the addition of CE and Ca^{2+} release was monitored. The error bars indicate S.E., $n = 3$. (E) Effects of heparin and co-presence of $\text{Ins}(1,4,5)\text{P}_3$ (IP_3) and CE on Ca^{2+} release. Heparin (5 mg/ml) was incubated with microsomes during Ca^{2+} loading followed by the addition of CE. The effects of CE (30 nM) alone, $\text{Ins}(1,4,5)\text{P}_3$ ($10 \mu\text{M}$) alone or CE and $\text{Ins}(1,4,5)\text{P}_3$ together (CE + IP_3) were also monitored. (F) Effect of anti-CE antibody on ryanodine (RyA)-stimulated Ca^{2+} release. Microsomal fractions, or microsomes to which anti-CE antibody was added immediately before Ca^{2+} loading, were loaded with $^{45}\text{Ca}^{2+}$ for 10 min, ryanodine ($20 \mu\text{M}$) was added and $^{45}\text{Ca}^{2+}$ release was measured at the times indicated. The addition of anti-CE antibody alone did not release $^{45}\text{Ca}^{2+}$ (results not shown). The error bars indicate S.E., $n = 3$. (G) Effect of phosphorylated CE on Ca^{2+} mobilization in comparison with non-phosphorylated CE. CE was phosphorylated by PKC as described in the Materials and methods section. The intramicrosomal $^{45}\text{Ca}^{2+}$ was measured 10 and 20 min after CE addition. The error bars indicate S.E., $n = 3$, $*P = 0.0001$ (one-way ANOVA).

observed. When purified ^{125}I -labelled CE was incubated with squid optic-lobe or muscle ER extracts and separated by non-denaturing PAGE, a significant portion of the ^{125}I co-migrated with native RyR (Figures 1D and 1E). Samples incubated without ER extract showed only a band of M_r 22 from the labelled CE (Figure 1F).

When purified ^{125}I -labelled CE was overlaid on a blot derived from a 4–20% acrylamide gel of purified native RyR, a single faint band was observed that co-migrated with native RyR (Figure 1G). Moreover, RyR purified with a sucrose-gradient method [22], used previously to isolate and record Ca^{2+} -release channels after incorporation into artificial lipid bilayers, when

Figure 5 Putative CE-binding region of RyR

(A) Fluorescence quenching of CE–eosin emission by peptide derived from the RyR sequence. CE–eosin conjugate (75 nM) was mixed with increasing amounts of peptide (Ac-LLPNTQSFNCA-amide) in PBS containing 20 μ M CaCl_2 , and fluorescence emission spectra were monitored between 530–560 nm using an excitation wavelength of 516 nm. Quenching by the peptide was also observed with Rhodamine–CE (results not shown). In a separate experiment, no photobleaching of CE–eosin was detected over the same time period. (B) Titration of the peptide with CE–eosin. Values are percentages of unquenched emission intensity at 540 nm taken from the spectra in (A). A K_d of 15 μ M was calculated from these data. (C) Sequence of putative CE-binding region of RyR. The peptide sequence, based on that obtained by screening a peptide phage-display library, was aligned with type 2 rat brain RyR peptide sequence starting at position 4688. The relative residue positions of the CE-binding site and known CaM-binding sites on the RyR [34] are shown on the right.

subjected to non-denaturing PAGE, showed a strong CE-immunoreactive band which co-migrated with native RyR (Figure 1H). This indicated that CE co-purified with RyR when

isolated using gentle conditions. This band was not observed when RyR was purified by spermine–agarose affinity chromatography, in which harsher elution conditions were used, indicating that the band was not the result of antibody cross-reactivity with RyR itself. FK560-binding protein was also detected in these fractions (results not shown).

An overlay experiment, in which RyR (purified by affinity chromatography) was subjected to non-denaturing electrophoresis, blotted on to nitrocellulose, overlaid with cloned CE or squid optic-lobe ER CHAPS extract, and then stained with anti-CE antibody in the presence of a fixed concentration of Ca^{2+} , also demonstrated specific binding of CE to RyR (Figures 1I and 1J). No band was observed when the CE was omitted (Figure 1K), indicating that the staining was not due to antibody cross-reactivity or non-specific binding.

In the reverse overlay experiment, in which purified RyR or squid optic-lobe ER extract was overlaid on to electrophoretic blots of cloned CE and then stained with anti-RyR antibody, bands co-migrating with native CE (M_r 22) were observed (Figures 1L, 1M and 1N), which were absent when RyR was omitted (Figure 1O). Similar results were obtained on autoradiograms from ^{125}I -labelled purified RyR overlaid on to blots of either crude or purified CE (results not shown).

^3H Ryanodine binding activity and ^{125}I -labelled purified RyR were also specifically retained on CE–Sephacryl in a calcium-dependent manner (Figures 2A and 2B). In contrast, high salt concentrations did not elute RyR from CE–Sephacryl (Figure 2C). The purified ^{125}I -labelled RyR was eluted in an unusually broad peak, suggesting that the ^{125}I -labelling interfered, to some extent, with its ability to dissociate from the CE column.

To test the Ca^{2+} dependence of the CE–RyR interaction, cloned CE was bound to nitrocellulose, overlaid with purified RyR and stained with antibody against RyR; the $[\text{Ca}^{2+}]$ was kept constant throughout the procedure. Specific binding to CE was observed but several other proteins similar to CE gave no signal (Figure 3). Addition of squid optic-lobe supernatant, cADP ribose or CaM during the overlay step did not affect the binding of RyR to CE, indicating that binding did not require soluble factors other than calcium. Binding was strongly dependent on Ca^{2+} and was half-maximal at ≈ 500 nM Ca^{2+} , which was comparable with the K_d of CE for Ca^{2+} (400 nM) [15], and decreased at high Ca^{2+} concentrations (> 0.1 mM) (Figure 2C). Previous results showed that no additional binding of CE to Ca^{2+} and no change in secondary structure in CE were observed above the K_m [15,29]. Thus the decreased binding at high Ca^{2+} concentrations is likely to be due to an effect on the RyR rather than on CE.

Squid optic-lobe microsomes showed rapid Ca^{2+} uptake, which was dependent on Ca^{2+} -ATPase activity and was inhibited by 100 nM thapsigargin (results not shown).

To determine whether CE could release Ca^{2+} from microsomes, cloned CE was added to the reaction mixture 5 min after Ca^{2+} was loaded and intramicrosomal Ca^{2+} was measured 2 min later. At concentrations ranging from 15–300 nM, CE released a significant proportion (24–46%) of $^{45}\text{Ca}^{2+}$ from microsomes (Figure 4A). Half-maximal activation was observed at 14 nM CE.

In a time-course experiment, application of CE (30 nM) triggered a rapid Ca^{2+} release from microsomes. This Ca^{2+} -releasing effect lasted for more than 20 min and the intramicrosomal $^{45}\text{Ca}^{2+}$ content returned to control levels when assayed at 30 min after initiation of the reaction. Addition of heat-denatured CE into the reaction mixture did not cause calcium release (Figure 4B). The effect of CE was no longer seen at 30 min following Ca^{2+} loading, possibly due to phos-

Figure 6 Induction of slow Ca^{2+} transients in hippocampal CA1 pyramidal neurons by CE

Bis-fura-2 ratiometric imaging revealed highly localized (arrows), slow Ca^{2+} transients in neurons loaded with intact CE (left panels), but not in cells dialysed with heat-inactivated CE (right panels). Each neuron is shown in a pseudocolour spatial map of Ca^{2+} concentration (upper panels), obtained by taking ratios of the image frames at the isosbestic point (357 nm); the images at 380 nm illustrate the location of the slow Ca^{2+} transients at their peak during a sequence of images. The grayscale fluorescence images (middle panels), taken at the isosbestic point to show the neuronal morphology and regions of interest (outlined in colours matching the traces in the bottom panels; regions outside the neurons were used for background correction) which were used to obtain numerical values for the time-based plots, illustrate the dynamics of the slow and fast Ca^{2+} transients. The maps show only the peak of the slow Ca^{2+} transients in the CE-loaded neurons (left panels) and the resting levels in the inactivated CE-dialysed cells (right panels). Faster Ca^{2+} transients during membrane depolarization showed a widespread distribution (shown by the simultaneous increase in all regions of interest plotted against time), and therefore are not represented in the pseudocolour Ca^{2+} -concentration maps. Acquisition rate was 0.25 Hz. Scale bars = 10 μm .

phorylation by endogenous kinases or to the adaptation effect frequently observed with the RyR. Ca^{2+} release was not the result of inhibition of Ca^{2+} uptake. In a separate series of experiments (W. Q. Zhao, T. J. Nelson, D. Siew and D. L. Alkon, unpublished work), it was shown that CE had no effect on microsomal Ca^{2+} -ATPase activity between 0–20 min.

At longer times, intramicrosomal Ca^{2+} content began to return to normal levels (results not shown). As with RyR binding, Ca^{2+} release induced by CE showed a bell-shaped Ca^{2+} -dependence curve, with significant Ca^{2+} release only occurring between 1 and 100 μM (Figure 4C), when tested in the absence of ATP-regenerating agents. However, with an ATP-regenerating system (phosphocreatine and creatine kinase), the decrease in Ca^{2+} release at high Ca^{2+} concentrations was not observed (results not shown).

The RyR inhibitor dantrolene (10 μM) and anti-RyR antibody, when incubated with microsomal fractions during Ca^{2+} loading, completely blocked CE-stimulated Ca^{2+} release (Figure 4D), whereas the $\text{Ins}(1,4,5)\text{P}_3$ -inhibitor heparin (5 μM) [30] had no effect (Figure 4E). $\text{Ins}(1,4,5)\text{P}_3$ alone (10 μM) caused 25–37% Ca^{2+} release from microsomes (Figure 4C), and addition of CE

produced an additional release which was approximately additive to the Ca^{2+} release produced by CE alone (Figure 4E), indicating that CE activates the RyR but not the $\text{Ins}(1,4,5)\text{P}_3$ receptor. Ca^{2+} measurements using the Ca^{2+} indicator Calcium Green 5N confirmed the $^{45}\text{Ca}^{2+}$ results, showing a cumulative Ca^{2+} release with successive addition of $\text{Ins}(1,4,5)\text{P}_3$ or CE [10 μM $\text{Ins}(1,4,5)\text{P}_3$, 148 000 photons/s; 30 nM CE, 120 000 photons/s] [30].

In the absence of CE, ryanodine released similar amounts of Ca^{2+} from squid optic-lobe microsomes at 10, 20 and 200 μM . These concentrations are similar to those used by other investigators in different species [31]. The effect of ryanodine was abolished by adding affinity-purified antibody against CE to the reaction mixture (Figure 4F). The response to $\text{Ins}(1,4,5)\text{P}_3$ was not affected by these treatments.

To test the effect of phosphorylation, CE phosphorylated by PKC was applied to the Ca^{2+} -loaded microsomes, and intramicrosomal $^{45}\text{Ca}^{2+}$ was measured at 10 and 20 min post-reaction. In contrast to non-phosphorylated CE, phosphorylated CE did not release Ca^{2+} from microsomes. After longer incubation times (> 20 min), phosphorylated CE instead caused an increase in Ca^{2+} uptake, to 145% of the initial control level (Figure 4G).

Figure 7 CE- and ryanodine-induced Ca^{2+} release from RyR liposomes

(A) SDS/PAGE (4–20% gradient) gel of purified squid opt-lobe RyR used for liposomal incorporation. (B) Ca^{2+} release from liposomes by cumulative additions of cloned non-phosphorylated CE. RyR was incorporated into liposomes containing 10 mM $[Ca^{2+}]_i$. A liposomal suspension containing 0.2 μ M external $CaCl_2$ was used and fluorescence produced by Ca^{2+} binding to Calcium Green (0.167 μ M) was monitored. This concentration of Ca^{2+} is close to the K_d of Ca^{2+} for CE but is subthreshold for channel opening. CE was added at 90, 135 and 210 s (final concentrations, 0.1, 0.2 and 0.3 nM respectively) after the addition of liposomes. (C) Liposomes (Lip.) depleted of internal Ca^{2+} by preincubation for 1 min with 40 mM caffeine did not release additional Ca^{2+} when CE (0.3 nM) was added. (D) The addition of 0.3 nM CE to liposomes (Lip.) treated with 10 μ M Ruthenium Red (Ru Red) had no effect on Ca^{2+} release. The calibration of $[Ca^{2+}]_i$ was corrected to account for the slight quenching effect caused by Ruthenium Red (4.8% at 10 μ M). (E) Effect of BSA. Fluorescence from liposomes (Lip.) alone was monitored for 80 s, BSA (1 μ g) was added and monitoring was continued for a further 40 s. (F) Effect of phosphorylated cloned CE (Phos. CE) added at 20 s (final concentration 0.10 nM). (G) CE (0.3 nM) did not release Ba^{2+} from liposomes loaded with Ba^{2+} instead of Ca^{2+} . Conditions were similar to those described in (A), except 200 nM Ba^{2+} was present instead of Ca^{2+} and liposomes contained Ba^{2+} instead of Ca^{2+} . (H) Release

Screening of a peptide phage display library with cloned CE to search for possible CE-binding sequences yielded a peptide (LPNTQSP) that had significant similarity (5–7 amino acids) to a site at position 4689–4697 of the type 2 RyR. The transcription of this isoform, which in brain is predominantly localized in the CA1, CA3, CA4 and dentate gyrus regions of the hippocampus [13], increases after rat water-maze training [32]. A peptide containing this sequence, and surrounding amino acids from the type 2 RyR (Ac-LLPNTQSFPNC-NH₂) [33], quenched the fluorescence of eosin-labelled CE in the presence of 20 μ M Ca²⁺ with an apparent affinity constant of 15 μ M, indicating an interaction with CE (Figures 5A and 5B). The quenching was not due to an inner filter effect, since the peptide had no detectable absorbance at the excitation or emission wavelength maxima (520 and 540 nm respectively). A peptide corresponding to the nearby CaM-binding site YQQKLLNYFARNC [34], although it bound to CaM (results not shown), and variety of other, unrelated peptides, including YSRLDYS, ARLWTEYFVIIDC, glutathione, leupeptin and RIFKLSRHSKGLQIC, also showed no evidence of binding to CE. Preincubating the peptide ($\geq 100 \mu$ M) with CE in the presence of 20 μ M Ca²⁺ also blocked the Ca²⁺ release from microsomes produced by CE by $\geq 85 \pm 5\%$ ($n = 4$). These results are consistent with a binding site for CE at position 4689–4697, which is in close proximity to, but does not overlap, the CaM-binding site at position 4534–4552 (Figure 5C). The moderate affinity, however, suggests significant differences in amino-acid sequence between the squid and rat brain RyR. Further experiments are therefore required to definitively establish this as the CE binding site.

If CE and RyR do interact *in vivo*, increased CE availability would be expected to produce transient increases in [Ca²⁺]_i in living neurons under conditions in which Ca²⁺ influx from extracellular sources does not occur. Previous studies have shown that, although the DNA sequence of mammalian and squid CE may be significantly different, the functionality, immunoreactivity and tertiary structure of CE appear to be highly conserved in several vertebrate species, including rat [15,18] (Figure 1C). Therefore because of the relevance of intracellular Ca²⁺ signals in models of synaptic plasticity in the rodent hippocampus, and the relative ease of culturing slices from hippocampus, we tested the effects of CE on intracellular Ca²⁺ dynamics in individual CA1 pyramidal neurons from rat hippocampal slices. A preliminary calcium-imaging result from this experiment has been published previously in a review paper [35], which also refers to a few of the results described here.

Simultaneous whole-cell recordings and Ca²⁺ imaging were obtained from CA1 pyramidal neurons in rat hippocampal slice cultures with patch pipettes containing 0.64–0.71 μ g/ml of intact or heat-inactivated CE and the Ca²⁺-indicator dye bis-fura-2, with 5 mM QX-314 to block Na⁺ channels. Intracellular K⁺ was replaced with Cs⁺ to block K⁺ currents and a small DC current was used in order to prevent spontaneous firing of Ca²⁺ spikes. Image acquisition was started immediately after the whole-cell configuration was achieved (break-in, $t = 0$), allowing for cytoplasmic dialysis with CE and bis-fura-2. Slow Ca²⁺ transients of 232 ± 22 nM in amplitude (range 189–314 nM), with rise times of 12–24 s and durations of 20–60 s, were observed only in neurons dialysed with intact CE, and after 20–25 min from break-in (Figure 6; $n = 6$ neurons). These slow transients were inde-

pendent of membrane potential changes, occurred in highly localized regions ($\approx 10 \mu$ m) of the proximal dendrites or in perinuclear areas of the somata, and were never observed in neurons dialysed with heat-inactivated CE ($n = 5$ neurons; $P < 6 \times 10^{-6}$, one-tailed Student's t test). However, faster Ca²⁺ transients (rise time < 4 s) associated with spontaneous membrane depolarizations (-50 to -30 mV), most likely due to Ca²⁺ spikes, were observed in all neurons (Figure 6; $n = 11$). Although the duration of these fast spontaneous Ca²⁺ transients was typically shorter in neurons dialysed with heat-inactivated CE, there were no significant differences in the amplitude between neurons (581 ± 197 nM for intact CE in dendrites versus 394 ± 90 nM for heat-inactivated CE). Similarly, no differences were observed in the membrane-input resistance, resting Ca²⁺ levels or Ca²⁺ transients induced by depolarizing current pulses to the soma via the patch pipette ($1.3 \pm 0.4 \mu$ M versus $1.1 \pm 0.03 \mu$ M in dendrites; $0.9 \pm 0.2 \mu$ M versus $0.8 \pm 0.05 \mu$ M in somata).

These slow Ca²⁺ transients elicited by CE occurred in the absence of functional K⁺ and Na⁺ channels and without activation of voltage-dependent Ca²⁺ channels, indicating that CE can also promote Ca²⁺ release from intracellular stores *in vivo*.

Preliminary experiments showed that these slow Ca²⁺ transients also occurred when Ca²⁺ influx was prevented by voltage-clamping CA1 neurons at their resting potential in the presence of Cd²⁺ and tetrodotoxin to block all voltage-gated Ca²⁺ channel activity and synaptic transmission respectively. Based on the *in vitro* binding and Ca²⁺-release biochemistry described above, the Ca²⁺ transients most likely are mediated by CE–RyR interactions.

To test whether the observed Ca²⁺ release from microsomes and hippocampal neurons could be caused by a direct effect of CE on the calcium channel, calcium-containing liposomes were prepared from RyR that had been purified to apparent homogeneity by spermine–agarose chromatography followed by ultrafiltration (Figure 7A). This RyR preparation contained no immunodetectable FK560-binding protein or CE (results not shown). The addition of 0.1 nM CE to CaCl₂-containing liposomes, suspended in buffer L containing 0.167 μ M Calcium Green and 200 nM CaCl₂, produced a rapid release of Ca²⁺ (Figure 7B). This CE concentration represented approx. 0.37 mol CE/mol RyR monomer. A second addition of CE released additional Ca²⁺, however, subsequent additions above 1:1 stoichiometric levels had no further effect. CE treated with Factor Xa to remove the histidine purification tag produced similar results (results not shown). CE did not release Ca²⁺ from liposomes that had been depleted of Ca²⁺ by preincubation with 40 mM caffeine for 1 min (Figure 7C). The RyR blocking agent Ruthenium Red (10 μ M) [36] also completely blocked the effect of CE (Figure 7D). At lower concentrations of Ruthenium Red, CE produced slow release of Ca²⁺ (results not shown). The addition of other proteins or peptides, including BSA and leupeptin, had no effect (Figure 7E). CE that had been phosphorylated with PKC released little or no Ca²⁺ from liposomes (Figure 7F).

In our conditions, Ba²⁺ reacted with Calcium Green with approx. 40% the fluorescence intensity of Ca²⁺. When external Ca²⁺ was replaced with Ba²⁺, CE did not release any detectable Ba²⁺ from RyR-liposomes filled with Ba²⁺ instead of Ca²⁺ (Figure 7G), which was consistent with the finding described above, that

of Ba²⁺ from Ba²⁺-filled liposomes by CE (0.3 nM) in the presence of 200 nM Ca²⁺. The Ba²⁺ signal intensity was $\approx 40\%$ that of Ca²⁺. Buffer conditions were the same as those described in (A), except that liposomes contained Ba²⁺ instead of Ca²⁺. Fluorescence units are calibrated from known additions of Ca²⁺ or Ba²⁺. A constant background fluorescence signal of $\approx 150\,000$ c.p.s. from Calcium Green alone was subtracted from these data. The Calcium Green signal with liposomes present was stable for at least 20 min. CE alone produced no change in fluorescence in the absence of liposomes.

Ca^{2+} is required for CE binding to RyR. Ba^{2+} itself does not induce calcium-induced Ca^{2+} release through the RyR but passes readily through the RyR [37]. A control experiment showed that Ba^{2+} -containing liposomes could function normally, releasing Ba^{2+} when 0.3 nM CE was added in the presence of 200 nM external Ca^{2+} (Figure 7H).

DISCUSSION

The results of the present study show that CE binds to the RyR, promotes Ca^{2+} release in microsomes and can induce Ca^{2+} transients in neuronal cells. Earlier results by other investigators using RyR purified by density-gradient centrifugation and incorporated into artificial lipid bilayers, also showed Ca^{2+} -channel activity in the absence of added CE [22]. As shown above, however, RyR purified using a sucrose-gradient method also can contain bound CE (Figure 1H). Because of the large size of the RyR tetramer, a 1:1 molar ratio of CE to receptor would be less than 1% of the total protein and thus could easily have been overlooked in the absence of a specific antibody.

Although CE may not be essential for channel function, CE released Ca^{2+} from liposomes incorporating RyR in the absence of other detectable proteins, suggesting that binding of CE to RyR can be a proximate cause of the Ca^{2+} release. Because CE is found exclusively in neurons, CE thus may be an endogenous modulator of neuronal RyR function.

Apart from CE, a few other proteins have been found previously to bind to the RyR in muscle sarcoplasmic reticulum [38,39]. Most of these proteins bind constitutively to the RyR and are not known to be involved in signalling pathways. For example, triadin is involved in the functional coupling between the calcium-binding protein calsequestrin and the RyR [38]. Reportedly CaM also binds preferentially to the RyR at low concentrations of Ca^{2+} . The predominant effect of CaM in isolation is one of inhibition and addition of exogenous CaM has no effect on calcium-induced Ca^{2+} release [10]. In contrast, exogenous CE has a strong activating effect on calcium release even in the absence of exogenous cADP ribose, CaM or other factors. This suggests that the endogenous CE in the microsomes may exist at substoichiometric concentrations at the ER membrane.

CE that had been phosphorylated by PKC was unable to release Ca^{2+} from microsomes or RyR-containing liposomes. This suggests that endogenous CE in particulate fractions, the preponderance of which is found in a phosphorylated state [18,20], is less able to mediate activation of the RyR until it becomes dephosphorylated by endogenous phosphatases. It also helps to explain why exogenous CE promoted Ca^{2+} release from microsomes, despite the presence of excess endogenous CE. In rat brain, RyRs are located in both microsomes and synaptosomes [14], which are rich in calcium-dependent protein phosphatases. Subcellular fractionation experiments showed that CE also is most abundant in rough and smooth ER, synaptosomes as well as ribosomes (T. J. Nelson, unpublished work). The finding that anti-CE antibody can block Ca^{2+} release by ryanodine in microsomes suggests that microsomal RyR normally contains bound CE, and that binding of this RyR-CE complex to antibody can hinder channel opening. If so, this CE would remain inactive, by virtue of its phosphorylated state, until activated by dephosphorylation or displaced by dephosphorylated CE.

The presence of endogenous phosphorylated CE may also explain why higher CE concentrations were required in microsomes compared with liposomes. Alternatively, the higher con-

centration needed in microsomes may reflect a higher RyR concentration in the microsomal preparation.

In contrast to the Ca^{2+} -releasing effect of dephosphorylated CE, CE phosphorylated by PKC promoted Ca^{2+} uptake in microsomes, whereas it had no effect in liposomes. It is possible that phosphorylated CE may play a positive regulatory role in activation of the ER Ca^{2+} -ATPase that in turn increases Ca^{2+} uptake. This hypothesis is currently under investigation.

In addition to type II RyR, type I and type III RyRs are also found in the mammalian and invertebrate nervous systems [8]. In both mammals and invertebrates, all three types appear to have similar pharmacological properties with regard to Ca^{2+} dependency and effects of ryanodine [40]. It is not known whether CE has any subtype-specific effects. Thus other RyR subtypes could also be involved.

Previous results in this laboratory have shown that associative conditioning markedly increases type II RyR mRNA [32]. Because of the positive feedback that could result from interactions between increased RyR, elevated Ca^{2+} and CE, the increased number of RyR-binding Ca^{2+} channels after learning may produce a disproportionately greater release of Ca^{2+} in response to depolarizing signals, and thereby contribute to memory consolidation and other long-term signalling-induced neuronal responses.

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