The Journal of Neuroscience, April 15, 2000, 20(8):2860–2866

Calmodulin-Binding Peptide PEP-19 Modulates Activation of Calmodulin Kinase II *In Situ*

Roy A. Johanson,[1] Henry M. Sarau,[2] James J. Foley,[2] and J. Randall Slemmon[1]

Departments of [1]Protein Biochemistry and [2]Pulmonary Pharmacology, SmithKline Beecham Research and Development, King of Prussia, Pennsylvania 19406

PEP-19 is a 6 kDa polypeptide that is highly expressed in select populations of neurons that sometimes demonstrate resistance to degeneration. These include the granule cells of the hippocampus and the Purkinje cells of the cerebellum. Its only identified activity to date is that of binding apo-calmodulin. As a consequence, it has been demonstrated to act as an inhibitor of calmodulin-dependent neuronal nitric oxide synthase *in vitro*, although PEP-19 regulation of calmodulin-dependent enzymes has never been characterized in intact cells. The activation of the calmodulin-dependent enzyme calmodulin kinase II (CaM kinase II) was studied in PC12 cells that had been transfected so as to express physiological levels of PEP-19. The expression of PEP-19 yielded a stable phenotype that failed to activate CaM kinase II upon depolarization in high K^+. However, CaM kinase II could be fully activated when calcium influx was achieved with ATP. The effect of PEP-19 on CaM kinase II activation was not attributable to changes in the cellular expression of calmodulin. The cellular permeability of the transfected cells to calcium ions also appeared essentially unchanged. The results of this study demonstrated that PEP-19 can regulate CaM kinase II *in situ* in a manner that was dependent on the stimulus used to mobilize calcium. The selective nature of the regulation by PEP-19 suggests that its function is not to globally suppress calmodulin activity but rather change the manner in which different stimuli can access this activity.

Key words: PEP-19; calmodulin; calmodulin kinase II; calcium; regulation; enzyme assay; PC12; transfection

Calmodulin is a widespread and abundant transducer of calcium signaling in cells. The primary mechanism through which it accomplishes this is by complexing with calcium and then directly activating several types of intracellular enzymes, including kinases (Stull et al., 1986), phosphatases (Cohen, 1997), proteases (Harris et al., 1989), and adenyl cyclases (Taussig and Gilman, 1995). Calmodulin can also modulate the function of other types of proteins, including several cytoskeletal proteins, making it an important regulator of cellular restructuring (Landry et al., 1988; Pierce et al., 1989; Beckingham et al., 1998). Recent studies have also linked calmodulin to the regulation of ion channels, including those for NMDA (Leonard et al., 1999) and voltage-sensitive channels (Lee et al., 1999).

In addition to the direct action of calmodulin on cellular proteins, signaling through Ca^{+2}/calmodulin provides a crossover between calcium signaling and other receptor-mediated signaling cascades. An early example was the action of calmodulin on Ras-GTPase-activating proteins (Weissbach et al., 1994; Farnsworth et al., 1995; Joyal et al., 1997), which can place the downstream activation of Ras-dependent pathways under the control of intracellular calcium concentrations. A more recent example is the demonstration that Ca^{2+}/calmodulin-dependent phosphorylation of cAMP response element-binding protein works in concert with other cellular kinases, including extracellular signal-regulated kinase and protein kinase A to activate transcription (Finkbeiner et al., 1997; Chawla et al., 1998) (but see Impey et al., 1998).

Because of its widespread and sometimes opposing action in cells, it seemed likely that calmodulin might itself be regulated. This appeared even more likely when small intracellular peptides were identified that contained an IQ motif, which gave them a unique ability to bind calcium-poor calmodulin. These peptides had the capacity to bind calmodulin during conditions when cytosolic calcium levels were low and subsequently change its availability for activating targets once the cell was stimulated. The best characterized examples of such peptides are neuromodulin (Skene, 1989; Baudier et al., 1989; Liu and Storm, 1990; Coggins and Zwiers, 1991), neurogranin (Baudier et al., 1991; Watson et al., 1996; Gerendasy and Sutcliffe, 1997), and PEP-19 (Ziai et al., 1986; Slemmon et al., 1996). All of these have been demonstrated to inhibit calmodulin-dependent nitric oxide synthase *in vitro* by way of binding to calmodulin (Slemmon et al., 1996; Slemmon and Martzen, 1994; Martzen and Slemmon, 1995), suggesting that they could function to modulate calmodulin in cells.

To determine whether PEP-19 could regulate calmodulin activity in intact cells, it was transfected into pheochromocytoma (PC12) cells, and the activation of the Ca^{+2}/calmodulin-dependent enzyme calmodulin kinase II (CaM kinase II) (MacNicol et al., 1990) was studied. PEP-19 expression was found to inhibit the activation of CaM kinase II when PC12 cells were depolarized in high K^+. However, stimulating calcium influx through purinergic receptors resulted in normal levels of CaM kinase II activation, despite PEP-19 expression. Based on the present study, it appears that PEP-19 regulates calcium signaling

Received Oct. 28, 1999; revised Feb. 16, 2000; accepted Feb. 23, 2000.

This work and R.A.J. were supported by National Institute of Neurological Disorders and Stroke and National Institutes of Health Grant R01 NS33299 to J.R.S. We thank Dr. James I. Morgan (St. Jude's Children's Hospital, Memphis, TN) for supplying the rat PEP-19 cDNA and the antibodies against rat PEP-19.

Correspondence should be addressed to Dr. J. Randall Slemmon, Department of Protein Biochemistry, UE0433, Biopharmaceutical Research and Development, SmithKline Beecham Pharmaceuticals, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19406-0939. E-mail: j_randall_slemmon@sbphrd.com.

Copyright © 2000 Society for Neuroscience 0270-6474/00/202860-07$15.00/0

but that this regulation is dependent on the type of stimulus used to induce calcium influx.

MATERIALS AND METHODS

Materials. The Ca^{2+}/Calmodulin-Dependent Protein Kinase Assay System, Geneticin (G418), LipofectAMINE Reagent, LipofectAMINE PLUS, heat-inactivated fetal calf serum, and nonessential amino acids were from Life Technologies (Gaithersburg, MD). Polyvinylidene difluoride (PVDF) membrane (Immobilon P) was from Millipore (Bedford, MA). Peroxidase-linked goat anti-rabbit and goat anti-mouse antibodies, Supersignal Substrate (catalog #34080), and Immunopure Immobilized Protein A/G were from Pierce (Rockford, IL). The low molecular weight range electrophoresis protein standards were from Bio-Rad (Hercules, CA). Muscarine, ATP, carbachol (carbamylcholine chloride), heat-inactivated horse serum, and poly-L-lysine hydrobromide (catalog #P9155 or #P6282) were obtained from Sigma (St. Louis, MO). The expression vector pcDNA3 was from Invitrogen (Carlsbad, CA). Type I rat tail collagen was obtained from Collaborative Research (Bedford, MA). Mouse monoclonal anti-calmodulin antibody was from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-PEP-19 was kindly provided by James I. Morgan (St. Jude's Children's Hospital, Memphis, TN).

Cultured cells. Rat PC12 cells were cultured in DMEM containing high glucose (4500 mg/ml) and 2.2 gm/l sodium bicarbonate supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal calf serum, 2 mM L-glutamine,10 U/ml penicillin, and 20 μg/ml streptomycin. The cultures were maintained at 37°C in a humidified 95% air and 5% CO_2 incubator. When indicated, culture plates were coated using poly-L-lysine hydrobromide in tissue culture water at 0.025 mg/ml, rinsed with water, and air dried.

PEP-19 clone. Bluescript containing the cDNA for PEP-19 was kindly provided by James I. Morgan (Sangameswaran et al., 1989). The PEP-19 sequence was subcloned into pcDNA3.0 at the *Eco*RI restriction site. Clones containing a forward and a reverse insert of PEP-19 in the pcDNA3.0 were identified by cutting at the *Eco*RV site in the PEP-19 insert and the *Eco*RV site in the multiple cloning site and determining the size of the resulting fragments by agarose gel electrophoresis. Confirmation of the insert and orientation was obtained by sequencing through the gene and flanking plasmid sequence.

Transfection protocol. PC12 cells were plated at 200,000 cells per 35 mM well in six-well plates. Transfections were performed with Lipofectamine using a protocol based on the recommendations of the manufacturer. The cells were treated with a mixture of 2 μg of DNA and 15 μl of Lipofectamine in 1 ml of serum-free media per well for 5 hr, and then 3 ml of complete media was added to each well. After 3 d, the transfectants were selected by culturing in media containing 0.6 mg/ml G418 for 3 weeks. The stable transfectants were maintained with 0.3 mg/ml G418.

Western blots. SDS-PAGE and protein transfer to PVDF membranes were performed as described in detail previously (Slemmon and Martzen, 1994). The proteins were transferred electrophoretically onto Immobilon P membranes in 10 mM 3-(cyclohexylamino)propanesulfonic acid, pH 11.0, and 10% methanol. Carnation nonfat dried milk (5%) in 50 mM Tris, pH 7.2, and 150 mM NaCl was used to block the membranes. The primary antibody, rabbit polyclonal anti-PEP-19, or mouse monoclonal anti-CaM was incubated with the transfers overnight. The secondary antibody was goat anti-rabbit or anti-mouse conjugated with peroxidase, and the spots were detected by chemiluminescence using Supersignal Substrate and x-ray film. The positive protein bands were quantified by video imagery using an AlphaImager 2200 with Version 4.03 software (Alpha Innotech Corporation).

Immunoprecipitation of the calmodulin–PEP-19 complex. PEP-19-transfected (2.5×10^7) or transfection control PC12 cells were homogenized on ice in 1 ml of lysis buffer [50 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS), pH 7.3, containing 5 mM EDTA, 1 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM PMSF, and 0.1% Triton X-100] using 20 strokes in a ground-glass homogenizer. The homogenates were centrifuged at 10,000 × *g* for 5 min at 4°C. Twenty micrograms of anti-calmodulin monoclonal antibody or irrelevant monoclonal antibody were added to the supernatant, followed by an 18 hr incubation with gentle agitation at 4°C. The immunocomplex was recovered by incubating the samples with 40 μl each of a 1:1 suspension of Pierce Protein A/G-Agarose in homogenization buffer. The beads were recovered by centrifuging for 2 min in a microcentrifuge. The beads were washed twice with 1 ml of homogenization buffer and then one time with 50 mM MOPS, pH 7.3, containing 5 mM EDTA. Material bound to the beads was released by dilution into 1 ml of 0.1% trifluoroacetic acid in water. The sample was enriched for SDS-PAGE by chromatography on reverse-phase HPLC as described by Slemmon et al. (1996). Fractions that coeluted in the region in which PEP-19 was observed to elute were pooled and dried for subsequent analysis by Western blotting. The PEP-19 standard for HPLC was chromatographed after the tissue homogenates had been processed, and the column had not been exposed to PEP-19 before these analyses.

Assay of CaM kinase II activity. The enzymatic activity was assayed using the Ca^{2+}/Calmodulin-Dependent Protein Kinase Assay System according to the manufacturer's protocol. The kit used autocamtide 3 as the substrate (Hanson and Schulman, 1992). The cells in a 35 mm dish were lysed with 200 μl of extraction buffer with phosphatase inhibitors, quickly scraped, and homogenized by three strokes through a 0.5 ml repeat-pipetter barrel, and 20 μl of the homogenate was added to each vial for the final assay volume of 50 μl. Unless otherwise indicated, the final ATP concentration in the assays was 50 μM.

Stimulation of cells by ATP and high K^+. The cells were plated at high density (1.5×10^6 cells per well) on poly-L-lysine-coated six-well plates in medium buffered with 25 mM HEPES, pH 7.3, and incubated overnight. The media was removed, and the cells were stimulated with ATP in HEPES-buffered saline (HBS) (10 mM HEPES, pH 7.4, 5 mM glucose, 1 mM $MgCl_2$, 130 mM NaCl, 5 mM KCl, and 1 mM $CaCl_2$). When the cells were stimulated by depolarization, the HBS was modified by increasing the KCl concentration to 56 mM and reducing the NaCl to 79 mM (MacNicol et al., 1990). After the time indicated in each experiment, the stimulating buffer was rapidly removed, and the cells were immediately lysed with 200 μl of the extraction buffer for the CaM kinase II assay.

Intracellular free Ca^{2+} determination. The PC12 cells were plated at 5×10^5 cells per well onto black-masked 96-well plates (Polyfiltronics; Packard, Meridian, CT) that had been coated with 0.125 ml of rat tail collagen per well for 1 hr (0.1 mg/ml in sterile 0.02N acetic acid), followed by rinsing with H_2O and then Dulbecco's PBS. Cells were stimulated with 100 μM ATP or depolarizing buffer containing 56 mM KCl (MacNicol et al., 1990). Calcium mobilization studies were conducted using Fluo 3-loaded PC12 cells and a microtiter plate-based assay, using FLIPR (Molecular Devices, Sunnyvale, CA) (Schroeder and Neagle, 1996). After allowing the cells to adhere to the microtiter plates overnight, growth media was removed and replaced with 1 μM Fluo-3 AM fluorescent indicator dye (Molecular Probes, Eugene, OR) in HBSS with 10 mM HEPES, 200 μM $CaCl_2$, 0.1% BSA, and 2.5 mM probenecid. After incubation for 1 hr (37°C, 5% CO_2) cells were washed three times with the same buffer. At the initiation of the experiment, fluorescence is read every 1 sec for 1 min and then every 3 sec for the following 1 min. Cells were stimulated with 100 μM ATP or depolarizing buffer containing 56 mM KCl after 10 sec, and fluorescence was monitored.

RESULTS

Expression of PEP-19

The PC12 cells were stably transfected with plasmids containing the gene for rat PEP-19. Transfection controls were prepared using a reversed insert of the PEP-19 cDNA. In addition to the use of pooled stable-transfected PC12 cells, isolated subclones of both the PEP-19-expressing and transfection control were established by limiting dilution of the bulk-transfected cell populations. The expression of PEP-19 was confirmed and quantified on Western blots (Fig. 1*A*). Video imagery and integration of the band intensities were used to measure the amounts of PEP-19 in the cell samples and in known amounts of PEP-19 protein loaded on separate lanes of the same gel (data not shown). Purification of the PEP-19 protein used as standard was described by Slemmon et al. (1996). It showed only one major specie on reverse-phase HPLC and yielded a single mass of 6759 on matrix-assisted laser desorption ionization mass spectrometry (data not shown). Therefore, the three bands that can be observed on electrophoresis in SDS appear to be an artifact of the technique and not processed forms of PEP-19. The estimated amount of PEP-19 expressed in the bulk transfectants was 5×10^5 molecules per cell, and the amounts in the subcloned PEP-19-transfected PC12 cells (subclone 1 and subclone 2) were 1.2×10^6 and 1.1×10^6 molecules per cell, respectively. No PEP-19 expression was de-

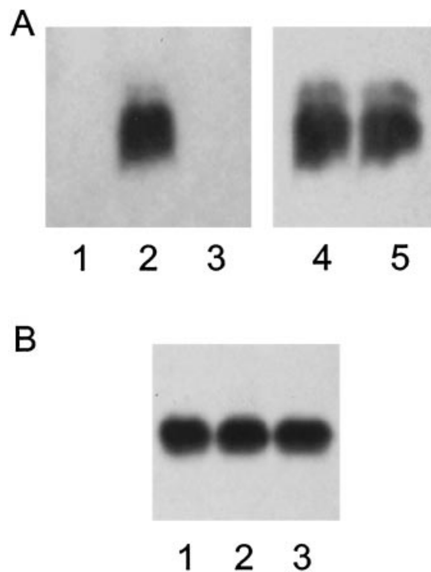

Figure 1. Expression of PEP-19 and calmodulin in PC12 cells. Western blots of control and PEP-19-transfected PC12 cells were performed as described in Materials and Methods. *A,* Expression of PEP-19 in PC12 cells. Samples consisting of 2.5×10^5 cells per lane were separated on electrophoresis and analyzed using rabbit anti-PEP-19 antisera. Bands were detected using chemiluminescence with goat anti-rabbit peroxidase conjugate as the secondary antibody. *Lane 1*, Control (wild-type) PC12 cells. *Lane 2*, PEP-19 bulk-selected transfectant PC12 cells. *Lane 3*, Reverse-orientation cDNA control-transfected PC12 cells. *Lane 4*, PEP-19-transfected PC12 cells, subclone 1. *Lane 5*, PEP-19-transfected PC12 cells, subclone 2. PC12 cells do not normally express PEP-19, but strong expression could be achieved after transfection. *B*, Levels of calmodulin in control and transfected PC12 cells. The sample loads were 1×10^5 cells per lane. The blocked membrane was exposed overnight to mouse anti-CaM monoclonal antibody diluted 1500:1 and detected using goat anti-mouse peroxidase conjugate and chemiluminescence. The samples are as follows. *Lane 1*, Control (wild-type) PC12 cells. *Lane 2*, PEP-19-transfected PC12 cells. *Lane 3*, Reverse-cDNA transfection control PC12 cells. Calmodulin levels appeared unchanged in the different PC12 cell lines.

tected in either the wild-type PC12 or in the transfection controls (Figs. 1*A*, 2). Several efforts to express PEP-19 mutant peptides possessing changes in the calmodulin-binding domain yielded clones with only ~10% of the expression observed with wild-type PEP-19 (data not shown) and were therefore not used for this study.

Western blots for calmodulin in the wild-type PC12 cells, PEP-19-expressing PC12 cells, and transfection control PC12 cells (Fig. 1*B*) showed no measurable differences in calmodulin levels. By comparison with standards using video imagery and integration, the calmodulin levels were estimated to be $\sim 0.5–1 \times 10^7$ molecules per cell.

Assuming the average diameter of a PC12 cell to be 10 μm, the average PEP-19 concentration (assuming no specific subcellular localization) would be minimally 3 μM, whereas the concentration of calmodulin would be ~4–10 μM. Indeed, PEP-19 expression could exceed 4 μM at localized sites. Therefore, PEP-19 expression in the transfected cells paralleled physiological levels (Slemmon et al., 1996), and calmodulin was within the expected concentration range (Klee and Vanaman, 1982).

Interaction of PEP-19 with calmodulin

To confirm the ability of PEP-19 to bind the calmodulin in PC12 cells, calmodulin was immunoprecipitated by treating the cell homogenates with mouse anti-calmodulin antibody. Western blots using anti-PEP-19 antibody showed that PEP-19 specifically coprecipitated with the calmodulin in the cell line that expressed PEP-19 (Fig. 2). A control immunoprecipitation of the PEP-19-containing cell homogenate with an irrelevant monoclonal antibody was negative (Fig. 2). These results demonstrate the interaction of PEP-19 with calmodulin from PC12 cells.

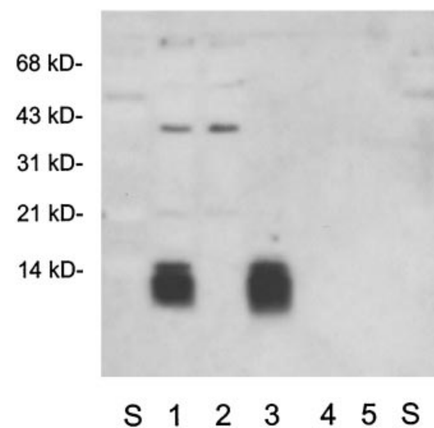

Figure 2. Immunoprecipitation of the calmodulin–PEP-19 complex. Calmodulin and the proteins that were associated with it were immunoprecipitated as described in Materials and Methods. The presence of PEP-19 in the transfected PC12 cells and in the immunoprecipitates of the transfected cells was determined by Western blot analysis. The samples are as follows. *Lane 1*, Crude lysate of PC12 cells transfected with PEP-19. *Lane 2*, Crude lysate of PC12 cells transfected with reversed insert of PEP-19. *Lane 3*, Immunoprecipitate of calmodulin from PC12 cells transfected with PEP-19. *Lane 4*, Immunoprecipitate of calmodulin from PC12 cells transfected with reversed cDNA for PEP-19. *Lane 5*, Control immunoprecipitation using an irrelevant antibody from crude lysates of PC12 cells transfected with PEP-19. Protein standards were run in the outside *lanes* labeled *S*. PEP-19 could be coprecipitated with calmodulin from PC12 cells.

Modulation of CaM kinase II by PEP-19

CaM kinase II activity in PC12 cells has been characterized extensively (MacNicol et al., 1990; MacNicol and Schulman, 1992a,b). Resting cells demonstrate a calcium-independent basal activity of CaM kinase II (referred to as autonomous activity) that is ~5–10% of the total activity that can be obtained with saturating levels of calcium and calmodulin. When PC12 cells were depolarized in 56 mM K^+, the wild-type cells and pooled stable transfectant control cells showed a twofold to threefold increase in the calcium-independent CaM kinase II activity. In contrast, the pooled stable transfectant cells that expressed PEP-19 did not show an increase in calcium-independent CaM kinase II activity (Fig. 3). Additionally, two subclones derived from the PEP-19-expressing pooled stable transfectants also showed the same failure to activate CaM kinase II after depolarization (Fig. 3). The subclones from the pooled stable transfectant control cells showed the same level of calcium-independent CaM kinase II activity as the wild-type PC12 cells after depolarization (Fig. 3).

To test for whether the changed response was universal or limited to specific signaling pathways, the cells were stimulated with 200 μM ATP. The peak response of calcium-independent CaM kinase II activity in the PEP-19-expressing cells was not significantly different from the responses by the wild-type and the nonexpressing control transfectants (Fig. 4). However, a trend toward a more rapid decrease in calcium-independent CaM ki-

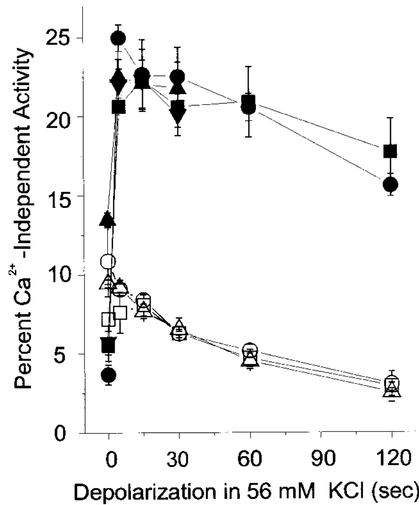

Figure 3. Effect of PEP-19 expression on CaM kinase II activation. The PC12 cells in individual 35 mm wells were stimulated by exposure to the high K^+ depolarizing buffer for the number of seconds indicated and then immediately assayed for calcium-independent CaM kinase II activity (see Materials and Methods). Calcium-independent CaM kinase II activity was defined as the calcium-independent enzyme activity in the cell lysate expressed as a percent of the total enzyme activity obtained in the presence of saturating calcium and calmodulin. The PC12 cell lines tested were as follows: ●, control (wild-type); ■, control bulk-transfected; ▲, transfection control, subclone 1; ▼, transfection control, subclone 2; ○, PEP-19 bulk-transfected; □, PEP-19-expressing subclone 1; △, PEP-19-expressing subclone 2. Each point represents the mean of three determinations, and the error bars are SD. CaM kinase II in PEP-19-expressing cells failed to activate after depolarization.

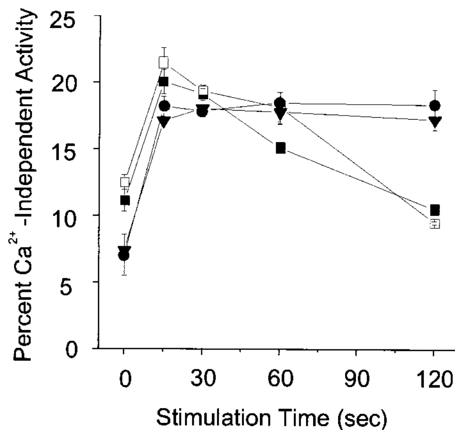

Figure 4. CaM kinase II responses after ATP stimulation. The cells in each 35 mm well were stimulated with 200 μM ATP in HBS for the number of seconds indicated and then immediately assayed for calcium-independent CaM kinase II activity (see Materials and Methods). The ATP concentration in these CaM kinase II assays was 450 μM. The 0 sec time point was measured using a 10 sec exposure to HBS without ATP. The cell lines tested were as follow: ●, control (wild-type) PC12; ▼, transfection control; ■, PEP-19-expressing PC12, subclone 1; □, PEP-19-expressing PC12, subclone 2. Each point represents the mean of three determinations, and the error bars are SD. Although maximal activation of CaM kinase II was similar in the different PC12 cell lines, the PEP-19-expressing cells showed a more rapid return toward basal activity.

nase II activity in the PEP-19-expressing cells after 2 min of ATP stimulation was apparent. The absolute difference can vary between experiments, but the trend suggests that PEP-19 may of had an effect on how long CaM kinase II was active.

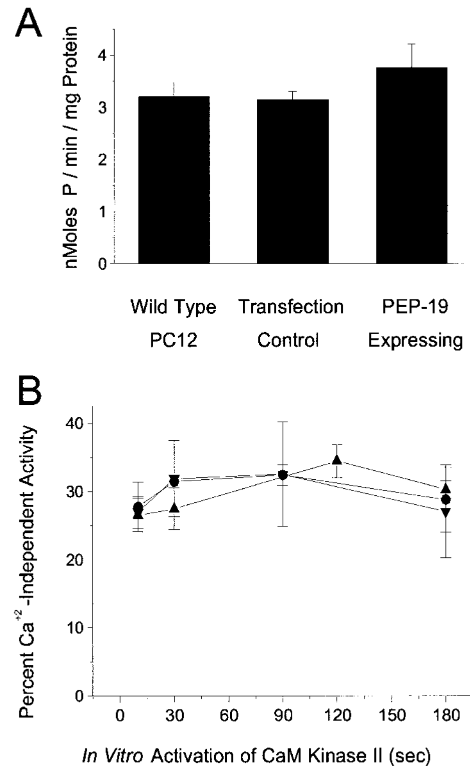

Figure 5. Specific activity of CaM kinase II and maximal *in vitro* activation. *A*, The specific activity of total CaM kinase II in wild-type, transfection control, and pooled transfectant PEP-19-expressing PC12 cells was compared. The enzyme levels were similar, although PEP-19-expressing PC12 cells showed a modest 16% elevation in activity that was significant at the $p = 0.05$ level. Each bar represents the mean of four determinations, and the error bars are SD. *B*, Percent calcium-independent CaM kinase II activity in the PC12 cells after *in vitro* stimulation. Homogenates were stimulated with 3 μM calmodulin–400 μM $CaCl_2$ for the time indicated and then diluted 2.5-fold into the enzyme assay, which contained either excess 2 mM EGTA or 2 mM $CaCl_2$. The cell lines were as follows: ●, control (wild-type) PC12; ▲, PEP-19-expressing PC12; ▼, transfection control PC12. Each point represents four determinations, and the error bars are SD. No differences were observed for the amount of calcium-independent activity that could be generated in the different PC12 cells.

The decrease in the calcium-dependent activation of CaM kinase II in the PEP-19-expressing cells is not a result of less total CaM kinase II activity in these cells. When the total Ca^{2+}/calmodulin-stimulated CaM kinase II activity was determined, the specific activity in the PEP-19-expressing transfected cells was actually slightly higher than the activity in the wild-type and transfection control cells (Fig. 5*A*). The 16% increase in the PEP-19-expressing PC12 cells was significant at the 0.05 level and may reflect compensatory responses by the cell for the losses in CaM kinase II stimulation.

To assess the maximal level of calcium-independent enzyme activity that could be attained, CaM kinase II was activated *in vitro* with excess calcium and calmodulin and then assayed immediately (Fig. 5*B*). Activated enzyme assayed in excess EGTA to remove free calcium yielded ~27–35% of the activity seen when the sample was assayed in the presence of calcium. Because the stimulation of intact PC12 cells generated ~22–25% calcium-independent CaM kinase II (Fig. 3), it appeared that ~70–75% of the calcium-independent CaM kinase II activity present in the PC12 cells is stimulated after depolarization or stimulation with

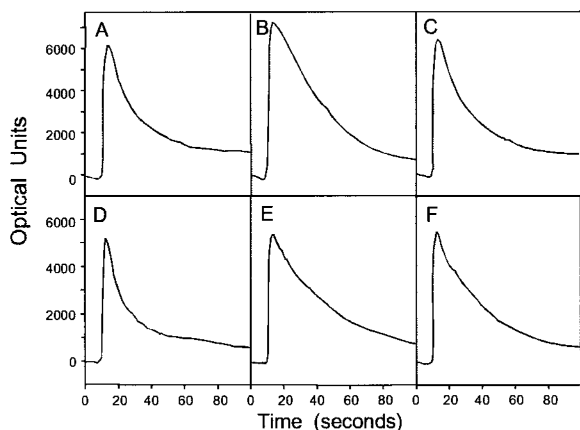

Figure 6. Measurement of intracellular free Ca^{2+} transients after stimulation of PC12 cells. PC12 cells in 96-well black-walled plates were stimulated with 56 mM KCl depolarization buffer or with 100 μM ATP. The intracellular calcium transients obtained by FLIPR analysis after depolarization in high K^+ as described in Materials and Methods were as follows: *A*, PC12 wild-type cells; *B*, PEP-19-expressing PC12; *C*, transfection control. The free calcium transients after stimulation with 100 μM ATP were as follows: *D*, wild-type PC12 cells; *E*, PEP-19-expressing PC12 cells; *F*, transfection control PC12 cells. Calcium mobilization maximums appeared similar in the different PC12 cell lines with both high K^+ and ATP stimulation. Although the decay of the signal sometimes appeared to be different, we were unable to observe a consistent difference across multiple determinations.

agonist. This indicated that the majority of the available CaM kinase II was activated by both types of stimuli.

Intracellular free Ca^{2+} transients after stimulation

The intracellular free Ca^{2+} responses after depolarization by high K^+ or stimulation by 200 μM ATP stimulation are shown in Figure 6. No observable differences were apparent in the levels of free calcium mobilization in the three cell lines in response to either depolarization in high K^+ or ATP stimulation. Therefore, it appeared that the effect of PEP-19 on CaM kinase II activation was not a result of a block on calcium flux.

DISCUSSION

Calmodulin is a widespread transducer of intracellular calcium signaling, and it shows very few changes in its amino acid sequence from simple to complex organisms (Klee and Vanaman, 1982). This lack of diversity is surprising in light of how calmodulin often impacts different types of signaling simultaneously, such as phosphorylation and dephosphorylation (Klee, 1991). Therefore, it would not have been unexpected for calmodulin to have evolved into a number of functionally distinct isoforms that bind calcium with different affinities or recognize different target enzymes. How then is diverse signaling through this regulator directed so that many targets can be controlled in a differential manner? One way appears to be through the different affinities that targets can have for calmodulin, but this may not always be sufficient for the rapid or reversible action observed in most calcium signaling cascades. Additionally, the activation of calmodulin may be controlled by peptides that stabilize the inactive form. The best characterized examples to date for such peptides are neuromodulin, neurogranin, and PEP-19 (Gerendasy and Sutcliffe, 1997). The ability of these peptides to bind calmodulin *in vitro* is well studied, but there is very little information that suggests how this may function in intact cells. The results of this study demonstrated that PEP-19 is a regulator of CaM kinase II *in situ* and that this regulation is selective because it does not impact all of the pathways by which the enzyme becomes activated.

The effect of PEP-19 expression on the activation of CaM kinase II in PC12 cells was dependent on how calcium was mobilized. Whereas the cells expressing PEP-19 displayed a tight inhibition of CaM kinase II activation upon depolarization in high K^+, the mobilization of calcium through ATP in these same cells showed normal enzyme activation. Therefore, PEP-19 expression does not inhibit all CaM kinase II activation within the cell, but rather its regulation appeared restricted to specific signaling pathways. It was also apparent from the results that PEP-19 expression yielded a robust and stable phenotype in regards to its inhibition of CaM kinase II in PC12 cells upon depolarization, because both pooled stable transfectants and several clonal lines yielded an identical result. Although PEP-19 expression showed little effect on the activation of CaM kinase II after stimulation with ATP, there was a noticeable deactivation after 2 min. Hence, in addition to inhibiting enzyme activation in some situations, PEP-19 may also have the ability to hasten enzyme deactivation. This last possibility will require further studies to adequately characterize the effect.

The reason why PEP-19 selectively inhibits the activation of CaM kinase II associated with voltage-sensitive calcium influx but not ATP stimulation is unknown. There could be different pools of CaM kinase II that are differentially activated by high K^+ and ATP. However, this seems less likely because the majority of the available CaM kinase II is activated after stimulation with ATP or high K^+. PEP-19 may inhibit the ability of CaM kinase II to perform calmodulin trapping (Meyer et al., 1992; Rich and Schulman, 1998). It could increase the off rate of trapped calmodulin when calcium levels are low, thereby inhibiting enzyme activation. It could also hasten the deactivation of the enzyme by a similar process. A more speculative alternative would require that purinergic receptors activate a different set of kinases and phosphatases than stimulation by depolarization. For PEP-19 to inhibit CaM kinase II activation upon depolarization, it could either impede calmodulin-dependent phosphorylation of the enzyme or promote phosphatase activity that returns activated CaM kinase II to its resting state. In contrast, purinergic receptors could fail to sufficiently induce the inhibitory phosphatase activity such that the equilibrium would favor the generation of active CaM kinase II.

The direct regulation of calmodulin itself may also be responsible for changes in its activity. At least six phosphorylation sites on calmodulin, which include serine, threonine, and tyrosine residues, have been demonstrated in Chinese hamster ovary cells after stimulation of the insulin receptor (Joyal et al., 1997). Neurogranin and neuromodulin can be phosphorylated within the IQ motifs by protein kinase C (Baudier et al., 1989, 1991), which results in the inhibition of calmodulin binding. The IQ motif in PEP-19 is not a protein kinase C substrate, and this mechanism does not pertain (Slemmon et al., 1996). However, directly phosphorylating calmodulin may interrupt PEP-19 binding and result in the activation of CaM kinase II. Because there are multiple potential mechanisms, understanding how PEP-19 differentially affects CaM kinase II activation as a function of stimulus must await additional studies.

The ability of PEP-19 to alter intracellular calcium signaling is consistent with its presence in neurons that can display resistance to some forms of degeneration. It is highly expressed by granule cells in the dentate gyrus (Sangameswaran et al., 1989). These cells resist insult both from ischemic damage (Kirino, 1982) and

exposure to excitatory amino acids (Mattson and Kater, 1989). PEP-19 is also abundant in Purkinje cells of the cerebellum (Berrebi et al., 1991), a brain region that is generally resistant to neurodegenerative disease (West et al., 1994). PEP-19 has the capacity for providing cellular resistance to degeneration by directly modulating CaM kinase II because this enzyme has been shown to mediate signal transduction in apoptosis (Wright et al., 1997). Alternatively, PEP-19 may affect the activation of other calmodulin targets that are linked to cell death, such as nitric oxide synthase (Samdani et al., 1997). In support of a role for PEP-19 in neuronal protection, recent results have shown that PEP-19 expression in PC12 cells reduces the levels of apoptosis observed after staurosporine treatment or UV irradiation (J. A. Erhardt, R. A. Johanson, J. R. Slemmon, and X. Wang, unpublished observations). In contrast, Huntington's disease tissue has been shown to express lowered levels of PEP-19 (Utal et al., 1998), which may be a contributing factor to the dysfunction that leads to the pathophysiology of that disease. Understanding how PEP-19 serves to alter Ca^{+2}/calmodulin signaling in the nervous system may prove a valuable tool for identifying targets that can be antagonized in an effort to offer protection against degeneration.

PEP-19 expression in the nervous system is selective. Thus, it is notable that two of the neuronal subtypes that express the highest levels of PEP-19, cerebellar Purkinje cells and hippocampal granule cells, are both in neurophysiological pathways in which long-term potentiation (LTP) and long-term depression (LTD) have been characterized extensively. Cerebellar Purkinje cells undergo LTD (Daniel et al., 1998), and hippocampal granule cell neurons are upstream participants in LTD and LTP as part of the perforant pathway through the hippocampus (Eccles, 1983). Consistent with a role for PEP-19, several Ca^{2+}/calmodulin-dependent enzymes are required to propagate LTP and LTD, including CaM kinase II (Pettit et al., 1994; Mayford et al., 1995; Giese et al., 1998), neuronal nitric oxide synthase (Lev-Ram et al., 1997), calcineurin (Mulkey et al., 1994), and adenyl cyclase type I (Weisskopf et al., 1994). Additionally, PEP-19 has already been characterized as a regulator of neuronal nitric oxide synthase *in vitro* (Slemmon et al., 1996). Consequently, regulation of calmodulin-dependent signaling through PEP-19 could affect the establishment of LTP or LTD.

That the expression of PEP-19 did not alter the levels of calmodulin in the cell is significant. First, it indicated that PEP-19 expression did not change the activation of CaM kinase II by decreasing the levels of calmodulin. It also demonstrated that the micromolar expression of a peptide that binds the inactive form of calmodulin need not cause an increase in calmodulin expression. An alternate possibility had been that the sequestration of calmodulin would be counteracted by increased gene expression for calmodulin. Although not well understood, an increase in the expression of calmodulin-dependent target proteins may cause an increase in the expression of calmodulin, because tissues such as brain contain the highest levels of calmodulin-dependent proteins and they also express the highest levels of calmodulin (Klee and Vanaman, 1982). The regulation of calmodulin through peptides such as PEP-19 might provide a mechanism by which the cell can avoid a compensatory increase in calmodulin expression that could negate the original action of the regulator.

The results of this study demonstrated that PEP-19 is able to affect the activation of calmodulin-dependent targets within intact cells. They further demonstrated that the regulation was conditional on the type of stimulus used. As a consequence, calmodulin-binding peptides such as PEP-19 may play an important role in determining when specific signaling pathways can activate Ca^{2+}/calmodulin-dependent enzymes. Such a function could have broad significance in the pathophysiology of nervous system disease and a potential role in learning and memory.

REFERENCES

Baudier J, Bronner C, Kligman D, Cole RD (1989) Protein kinase C substrates from bovine brain. Purification and characterization of neuromodulin, a neuron-specific calmodulin-binding protein. J Biol Chem 264:1824–1828.

Baudier J, Deloulme JC, Dorsselear AV, Black D, Matthes HWD (1991) Purification and characterization of a brain specific protein kinase C substrate, neurogranin (p17). J Biol Chem 266:229–237.

Beckingham K, Lu AQ, Andruss BF (1998) Calcium-binding proteins and development. Biometals 11:359–373.

Berrebi AS, Oberdick J, Sangameswaran L, Christakos S, Morgan JI, Mugnaini E (1991) Cerebellar Purkinje cell markers are expressed in retinal bipolar neurons. J Comp Neurol 308:630–649.

Chawla S, Hardingham GE, Quinn DR, Bading H (1998) CBP: a signal-regulated transcriptional coactivator controlled by nuclear calcium and CaM kinase IV. Science 281:1505–1509.

Coggins PJ, Zwiers H (1991) B-50 (GAP-43): biochemistry and functional neurochemistry of a neuron-specific phosphoprotein. J Neurochem 56:1095–1106.

Cohen PTW (1997) Novel protein serine/threonine phosphatases: variety is the spice of life. Trends Biol Sci 22:245–251.

Daniel H, Levenes C, Crépel F (1998) Cellular mechanisms of cerebellar LTD. Trends Neurosci 21:401–407.

Eccles JC (1983) Calcium in long-term potentiation as a model for memory. Neuroscience 10:1071–1081.

Farnsworth CL, Freshney NW, Rosen LB, Ghosh A, Greenberg ME, Feig LA (1995) Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF. Nature 376:524–527.

Finkbeiner S, Tavazoie SF, Maloratsky A, Jacobs KM, Harris KM, Greenberg ME (1997) CREB: a major mediator of neuronal neurotrophin responses. Neuron 19:1031–1047.

Gerendasy DD, Sutcliffe JG (1997) RC3/Neurogranin, a postsynaptic Calpacitin for setting the response threshold to calcium influxes. Mol Neurobiol 15:131–163.

Giese KP, Fedorov NB, Filipkowski RK, Silva AJ (1998) Autophosphorylation at Thr^{286} of the α calcium-calmodulin kinase II in LTP and learning. Science 279:870–873.

Hanson PI, Schulman H (1992) Inhibitory autophosphorylation of multifunctional Ca^{+2}/calmodulin-dependent protein kinase analyzed by site-directed mutagenesis. J Biol Chem 267:17216–17224.

Harris AS, Croall DE, Morrow JS (1989) Calmodulin regulates fodrin susceptibility to cleavage by calcium-dependent protease I. J Biol Chem 264:17401–17408.

Impey S, Obrietan K, Wong ST, Poser S, Yano S, Wayman G, Deloulme JC, Chan G, Storm DR (1998) Cross talk between ERK and PKA is required for C^{+2} simulation of CREB-dependent transcription and ERK nuclear translocation. Neuron 21:869–883.

Joyal JL, Annan RS, Ho Y-D, Huddleston ME, Carr SA, Hart MJ, Sacks DB (1997) Calmodulin modulates the interaction between IQGAP1 and Cdc42. J Biol Chem 272:15419–15425.

Kirino T (1982) Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 239:57–69.

Klee CB (1991) Concerted regulation of protein phosphorylation and dephosphorylation by calmodulin. Neurochem Res 16:1059–1065.

Klee CB, Vanaman TC (1982) Calmodulin. Adv Protein Chem 35:213–321.

Landry J, Crete P, Lamarche S, Chretien P (1988) Activation of Ca^{+2}-dependent processes during heat shock: role in cell thermoresistance. Radiat Res 113:426–436.

Lee A, Wong ST, Gallagher D, Li B, Storm DR, Scheuer T, Catterall WA (1999) Ca^{+2}/calmodulin binds to and modulates P/Q-type calcium channels. Nature 399:155–159.

Leonard AS, Lim IA, Hemsworth DE, Horne MC, Hell JW (1999) Calcium/calmodulin-dependent protein kinase II is associated with the *N*-methyl-D-aspartate receptor. Proc Natl Acad Sci USA 96:3239–3244.

Lev-Ram V, Jiang T, Wood J, Lawrence DS, Tsien RY (1997) Synergies and coincidence requirements between NO, cGMP, and Ca^{2+} in the induction of cerebellar long-term depression. Neuron 18:1025–1038.

Liu Y, Storm DR (1990) Regulation of free calmodulin levels by neuromodulin: neuron growth and regeneration. Trends Pharmacol Sci 11:107–111.

MacNicol M, Schulman H (1992a) Cross-talk between protein kinase C and multifunctional Ca^{2+}/calmodulin-dependent protein kinase. J Biol Chem 267:12197–12201.

MacNicol M, Schulman H (1992b) Multiple Ca^{2+} signaling pathways converge on CaM kinase in PC12 cells. FEBS Lett 304:237–240.

MacNicol M, Jefferson AB, Schulman H (1990) Ca^{2+}/calmodulin kinase is activated by the phosphatidylinositol signaling pathway and becomes Ca^{2+}-independent in PC12 Cells. J Biol Chem 265:18055–18058.

Martzen MR, Slemmon JR (1995) The dendritic peptide neurogranin can regulate a calmodulin-dependent target. J Neurochem 64:92–100.

Mattson MP, Kater SB (1989) Development and selective neurodegeneration in cell cultures from different hippocampal regions. Brain Res 490:110–125.

Mayford M, Wang J, Kandel ER, O'Dell TJ (1995) CamKII regulates frequency-response function of hippocampal synapses for the production of both LTD and LTP. Cell 81:891–904.

Meyer T, Hanson PI, Stryer L, Schulman H (1992) Calmodulin trapping by calcium-calmodulin-dependent protein kinase. Science 256:1199–1202.

Mulkey RM, Endo S, Shenollkar S, Malenka RC (1994) Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. Nature 369:486–488.

Pettit DL, Perlman S, Malinow R (1994) Potentiated transmission and prevention of further LTP by increased CaMKII activity in postsynaptic hippocampal slice neurons. Science 266:1881–1885.

Pierce SK, Politis AD, Cronkite DH, Roland LM, Smith Jr LH (1989) Evidence of calmodulin involvement in cell volume recovery following hypo-osmotic stress. Cell Calcium 10:159–169.

Rich RC, Schulman H (1998) Substrate-directed function of calmodulin in autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II. J Biol Chem 273:28424–28429.

Samdani AF, Dawson TM, Dawson VL (1997) Nitric oxide synthase in models of focal ischemia. Stroke 28:1283–1288.

Sangameswaran L, Hempstead J, Morgan JI (1989) Molecular cloning of a neuron-specific transcript and its regulation during normal and aberrant cerebellar development. Proc Natl Acad Sci USA 86:5651–5655.

Schroeder KS, Neagle BD (1996) FLIPR: A new instrument for accurate, high throughput optical screening. J Biomol Screening 1:75–80.

Skene JHP (1989) Axonal growth-associated proteins. Annu Rev Neurosci 12:127–156.

Slemmon JR, Martzen MR (1994) Neuromodulin (GAP-43) can regulate a calmodulin-dependent target *in vitro*. Biochemistry 33:5653–5660.

Slemmon JR, Morgan JI, Fullerton SM, Danho W, Hilbush BS, Wengenack TM (1996) Camstatins are peptide antagonists of calmodulin based upon a conserved structural motif in PEP-19, neurogranin and neuromodulin. J Biol Chem 271:15911–15917.

Stull JT, Nunnally MH, Michnoff CH (1986) Calmodulin-dependent protein kinases. In: The enzymes, Vol 17, Control by phosphorylation (Boyer PD, Krebs EG, eds), pp 113–166. New York: Academic.

Taussig R, Gilman AG (1995) Mammalian membrane-bound adenyl cyclases. J Biol Chem 270:1–4.

Utal AK, Stopka AL, Roy M, Coleman PD (1998) PEP-19 immunohistochemistry defines the basal ganglia and associated structures in the adult human brain, and is dramatically reduced in Huntington's disease. Neuroscience 86:1055–1063.

Watson JB, Margulies JE, Coulter II PM, Gerendasy DD, Sutcliffe JG, Cohen RW (1996) Functional studies of single-site variants in the calmodulin-binding domain of RC3/neurogranin in *Xenopus* oocytes. Neurosci Lett 219:183–186.

Weissbach L, Settleman J, Kalady MF, Snijders AJ, Murthy AE, Yan Y-X, Bernards A (1994) Identification of a human RasGAP-related protein containing calmodulin-binding motifs. J Biol Chem 269:20517–20521.

Weisskopf MG, Castillo PE, Zalutsky RA, Nicoll RA (1994) Mediation of hippocampal mossy fiber long-term potentiation by cyclic AMP. Science 265:1878–1882.

West MJ, Coleman PD, Flood DG, Troncoso JC (1994) Differences in the pattern of hippocampal neuronal loss in normal ageing and Alzheimer's disease. Lancet 344:769–772.

Wright SC, Schellenberger U, Ji L, Wang H, Larrick JW (1997) Calmodulin-dependent protein kinase II mediates signal transduction in apoptosis. FASEB J 11:843–849.

Ziai R, Pan Y-CE, Hulmes JD, Sangameswaran L, Morgan JI (1986) Isolation, sequence, and developmental profile of a brain-specific polypeptide, PEP-19. Proc Natl Acad Sci USA 83:8420–8423.