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Small Proteins that Modulate Calmodulin-Dependent Signal Transduction

Effects of PEP-19, Neuromodulin, and Neurogranin on Enzyme Activation and Cellular Homeostasis

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Abstract

Neuromodulin (GAP-43), neurogranin (RC3), and PEP-19 are small acid-stable proteins that bind calcium-poor calmodulin through a loosely conserved IQ-motif. Even though these proteins have been known for many years, much about their function in cells is not understood. It has recently become appreciated that calmodulin activity in cells is tightly controlled and that pools of otherwise free calmodulin are sequestered so as to restrict its availability for activating calcium/calmodulin-dependent enzymes. Neuromodulin, neurogranin, and PEP-19 appear to be major participants in this type of regulation. One way in which they do this is by providing localized increases in the concentration of calmodulin in cells so that the maximal level of target activation is increased. Additionally, they can function as calmodulin antagonists by directly inhibiting the association of calcium/calmodulin with enzymes and other proteins. Although neuromodulin, neurogranin, and PEP-19 were early representatives of the small IQ-motif-containing protein family, newer examples have come to light that expand the number of cellular systems through which the IQ-peptide/calmodulin interaction could regulate biological processes including gene transcription. It is the purpose of this review to examine the behavior of neuromodulin, neurogranin, and PEP-19 in paradigms that include both in vitro and *in situ* systems in order to summarize possible biological consequences that are linked to the expression of this type of protein. The use of protein:protein interaction chromatography is also examined in the recovery of a new calmodulin-binding peptide, CAP-19 (ratMBF1). Consistent with earlier predictions, at least one function of small IQ-motif proteins appears to be that they lessen the extent to which calcium-calmodulin-dependent enzymes become or stay activated. It also appears that these polypeptides can function to selectively inhibit activation of intracellular targets by some agonists

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while simultaneously permitting activation of these same targets by other agonists. Much of the mechanism for how this occurs is unknown, and possible explanations are examined. One of the biological consequences for a cell that expresses a calmodulin-regulatory protein could be an increased resistance to calcium-mediated toxicity. This possibility is examined for cells expressing PEP-19 and both anatomical and cell-biological data is described. The study of IQ-motif-containing small proteins has stimulated considerable thought as to how calcium signaling is refined in neurons. Current evidence suggests that signaling through calmodulin is not a fulminating and homogenous process but a spatially limited and highly regulated one. Data from studies on neuromodulin, neurogranin, and PEP-19 suggest that they play an important role in establishing some of the processes by which this regulation is accomplished.

Index Entries: IQ-motif; PEP-19; neuromodulin; GAP-43; neurogranin; RC3; CAP-19; mbf1; calcium signaling; calmodulin; regulation.

Calmodulin is a Principal Activator of Calcium-Dependent Targets

Calcium-dependent signal transduction in the nervous system is a primary means of linking external cellular stimuli with the activation of signaling molecules in the cell cytosol. The calcium required for activation to occur comes either directly from outside of the cell through ligand or voltage-gated channels or as a result of the liberation of internal stores. Both the amplitude and the frequency of the increases in intracellular calcium will then impact whether the signal will activate additional target proteins that are capable of propagating the signal into the nucleus *(1–3).* The resultant changes in gene expression can have significant impact on cellular homeostasis (e.g., *4,5*) as well as effects that are tightly linked to learning and memory *(6–8).*

The primary molecule in eukaryotic cells for executing calcium signaling is the heat-stable 17 kDa-protein, calmodulin. Calmodulin is associated with all subcellular compartments including the nucleus and plasma membrane *(9),* and it can be rapidly translocated upon stimulation *(2).* It exists in at least two different forms depending upon whether calcium is bound *(10).* The calcium-rich form is capable of activating a large number of proteins that carry out different functions. Early expectations concerning the manner in which this process was modulated assumed calcium influx was the only primary control on calmodulin activity. However, it is becoming appreciated that calmodulin-dependent activation of targets is likely regulated by a few different mechanisms. These include increasing the concentration of calmodulin in specific areas of the cytoplasm with calmodulin-binding proteins *(11–14),* selective changes in the activation of some calmodulin-dependent enzyme targets through phosphorylation of calmodulin *(15,16)* and tight regulation of calmodulin gene expression that appears to render calmodulin availability as a rate-limiting step in signal propagation *(17).* In the last case it was estimated that levels of free calmodulin were only about one-half the level of calmodulin-binding proteins in the cell. An additional regulatory mechanism may use small proteins that bind the calcium-poor form of calmodulin and directly regulate the activation of calcium/calmodulin-dependent enzymes.

One example of an endogenous neuronal protein that is capable of regulating calmodulin-dependent enzymes is PEP-19. This protein is a small, acid-soluble specie that can bind calmodulin through a loosely conserved IQ-motif. It is a member of a class of small calmodulin accessory proteins (ca. 6–25 kD) that are related by their abilities to bind calcium-poor calmodulin using this IQ-motif. However, outside of this domain, they show essentially no sequence homology. The most studied members of this protein family include Neuromodulin (GAP-43, B-50), Neurogranin (RC3), and PEP-19. Mounting evidence has

suggested that these proteins can have significant effects on cell signaling (for review, *see 18–20*) and the observation that the protein sequences are very different, outside of the calmodulin-binding domains, suggests that the regulation by each family member has been tailored to serve different types of functions. In an attempt to better understand how these regulators affect signal transduction, we have compared some of the properties of PEP-19 as a calmodulin-binding peptide to those of neuromodulin and neurogranin.

Like Neuromodulin and Neurogranin, PEP-19 is an Abundant Peptide in Brain

PEP-19 was discovered during an early effort in functional genomics that had been designed to identify markers of cerebellar development *(21).* It is a 6.7 kDa peptide that displayed high levels of expression in cerebellum. Subsequent localization demonstrated the Purkinje-cell neurons to be the source of the peptide in that region *(22–24).* As a neuronal marker, antisera to PEP-19 have been used to define biochemical similarities among different populations of neurons *(22)* and to investigate the organization of synaptic targets in the cochlear nucleus and superior olive *(24,25).* PEP-19 is also expressed in several other regions of the nervous system including the cortex, putamen, olfactory bulb, retina, substantia nigra, hippocampus *(22–24,26),* and dorsal-root ganglion *(27).* Within the hippocampus, it appears to be principally localized to granule-cell neurons in the dentate gyrus *(22,23).* In the trigeminal ganglion, PEP-19 appears to be expressed by myelinated nociceptors *(28),* suggesting a function for PEP-19 in pain transmission. Although PEP-19 is a primary translation product consisting of only 61 amino acids and the size of the messenger RNA is 0.6 kbp *(26),* the gene spans more than 30 kbp *(29).*

The abundance of PEP-19 in cerebellum and the dentate gyrus were of considerable interest because it could participate in the apparent resilience of these regions to some neurological diseases. In Alzheimer's disease (AD), the cerebellum is largely spared *(30).* The primary afferent neurons in this region are the Purkinje cells and they appear to express some of the highest levels of PEP-19 of any cells in the nervous system. In the dendate gyrus, the granule-cell neurons also display considerable PEP-19 immunoreactivity and are spared in the disease *(30).* By contrast, the PEP-19-negative pyramidal-cell neurons that are downstream of the granule cells in the perforant pathway displayed noticeable loss. Also in AD, PEP-19 expression in cerebellum demonstrated an upregulation based on analysis by high-performance liquid chromatography (HPLC) and this occurred in parallel with an increase in calmodulin expression *(31).* Because PEP-19 can bind calmodulin, and calcium/calmodulin activates several processes that are associated with neuronal death (e.g., *32,33),* a possible mechanism became apparent whereby this peptide could impart resistance to some types of cellular stress or insult.

In another example, it has recently been proposed that Huntington's disease (HD) might also cause an altered regulation of calcium/calmodulin-dependent signaling. The mutant gene product in this disease is a protein termed huntingtin. It demonstrates an increase in affinity for binding calmodulin when compared to wild-type huntingtin protein *(34).* As a result, the mutant protein may serve to cause an imbalance in the regulation of calcium homeostasis through an altered interaction with calmodulin. Consistent with this observation, structures affected in HD, including caudate nucleus, putamen, globus pallidus, and substantia nigra, show a large loss in PEP-19 expression *(23).* Thus, the ability of the mutant huntingtin protein to abnormally bind calmodulin may affect the expression levels of other calmodulin-binding proteins including PEP-19, thereby accelerating a process by which calcium homeostasis is lost. PEP-19 may serve an important prophylactic function in some types of neurons that can be

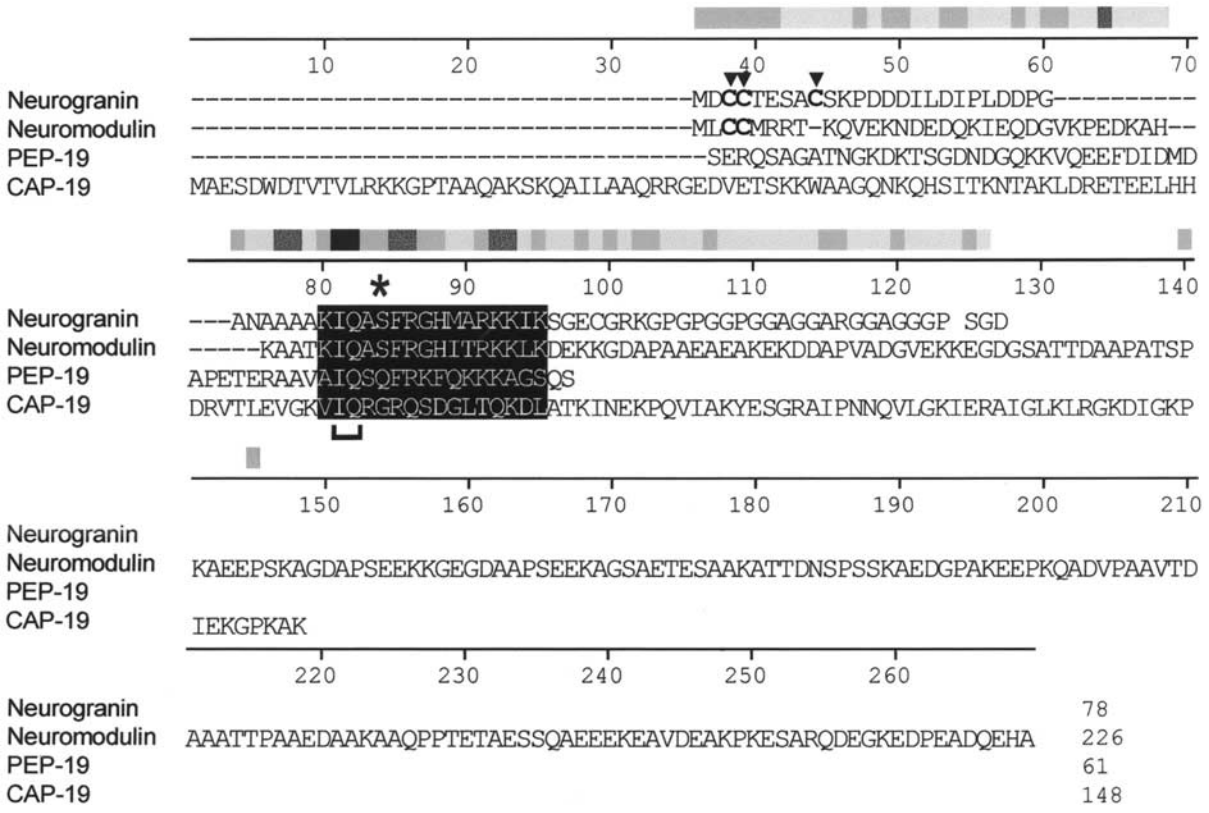

Fig. 1. Comparison of the peptide sequences for neurogranin, neuromodulin, PEP-19, and CAP-19. Alignments were made with Lasergene using the IQ motifs as a set point (black box). Neuromodulin and neurogranin show the most homology because their IQ-motifs are the most similar and they both possess cysteine residues within their amino termini (▼). Outside of these similarities, all of the proteins appear to be very different. The "*" above the sequences highlights the serine residue which is a substrate for pkC in neuromodulin and neurogranin. All four of these proteins are acid-soluble.

lost as part of the pathology of this disease or other disease processes.

Outside of the IQ-Motif, Neuromodulin, Neurogranin and PEP-19 are Dissimilar

A comparison of the sequences for PEP-19, neurogranin, neuromodulin, and a new IQ-peptide, CAP-19 *(35)*, center on the IQ-motifs (Fig. 1). All of these proteins can be purified from the nervous system based on their relative abundance, solubility in perchloric acid, and efficient capture on calmodulin-affinity chromatography in the absence of calcium. They also separate well upon reverse-phase (RP)-HPLC *(35–37)*. Neuromodulin and neurogranin have identical IQ-motifs over nine amino acids, whereas both PEP-19 and CAP-19 share only the IQ dipeptide. All of the IQ motifs show a number of lysine and arginine residues that are important for calmodulin binding *(38,39)*. Several important differences between these polypeptides have been reported. One of the most interesting is that the IQ motifs in neuromodulin and neurogranin are not only the most conserved motifs but they are also excellent substrates for phos-

phorylation by protein kinase C (PKC) *(40–42).* PEP-19 is not phosphorylated by PKC mixed isoforms in vitro *(39)* and we have failed to recognize any phosphoPEP-19 by mass spectrometric analysis of purified protein. This is in spite of the purification of PEP-19 being carried out under denaturing conditions and without the aid of calmodulin-affinity chromatography (personal observation). Additionally, phosphoneurogranin and phosphoneuromodulin are efficiently dephosphorylated by the calmodulin-dependent phosphatase, calcineurin *(43).* Phosphorylated IQ sequences do not bind calmodulin, thereby conferring a control step on calmodulin binding for neuromodulin and neurogranin *(13,42).* In contrast, the ability of PEP-19 to bind calmodulin is apparently not regulated by a phosphorylation cycle on the PEP-19 protein *(39).*

Another important difference between PEP-19 and neuromodulin and neurogranin is the presence of two cysteine residues near the amino terminus in the later two. These are in part responsible for trafficking the proteins to membranes after fatty acylation *(44–46)* and they can activate Go in their nonacylated form *(47–48).* Thus, they appear to participate in a regulatory cycle that bridges the propagation of G-protein-coupled receptor signaling with the sequestration of calmodulin. Neither CAP-19 nor PEP-19 contain any cysteine residues within their sequences, thereby conferring a regulation of calmodulin that is independent of this mode of membrane trafficking.

Neuromodulin, Neurogranin, and PEP-19 Inhibit In Vitro Activation of Nitric Oxide Synthase, an Example of a Calcium/Calmodulin-Dependent Enzyme

One of the earlier enigmas concerning the possible function of these polypeptides centered on how a protein that binds calcium-poor calmodulin with low micromolar affinity could affect the activation of an enzyme that binds calcium-rich calmodulin with low nanomolar affinity? It was plausible that as soon as cellular levels of calcium rose, calmodulin would be rapidly transferred from neuromodulin to the enzyme target with little effect on the subsequent activation. It was in part this expectation that suggested that a major role for these IQ proteins would be to localize calmodulin where it was needed for subsequent activation of targets upon cellular stimulation. Indeed, this is likely a key element of the biology of these small proteins (e.g., *11,13,14),* but their interaction with calmodulin could also directly affect the activation of calcium/calmodulin-dependent enzymes. Because cerebellar Purkinje cells express neuromodulin, PEP-19, and nitric oxide synthase (NOS) *(49)* it was suggested that IQ-motif polypeptides could directly affect activation of the synthase. It was also of interest that NOS activity is upregulated in PEP-19-rich Purkinje cells after injury *(50),* since this would be consistent with neuromodulin or PEP-19 having a role in reversing changes associated with cellular degeneration.

In vitro studies with NOS demonstrated that even though this enzyme binds calcium-rich calmodulin with an EC_{50} of 10 nM *(51)* and neuromodulin, neurogranin, and PEP-19 bind calcium-poor calmodulin with EC_{50} values ranging from 0.23–6 μ*M,* depending upon which peptide and the ionic strength *(13,39,42),* physiological levels of these polypeptides significantly impeded the activation of the enzyme. In the case of neuromodulin and neurogranin, regulation was lost after phosphorylation with PKC, since this renders them unable to bind calmodulin. These polypeptides may be able to effectively regulate enzyme activation because they bind calmodulin before the calcium concentration rises. As a result, they would interfere with the establishment of the calcium-calmodulin-enzyme complex. Consistent with proposed models, they could function by noncompetitively sequestering calmodulin so that it is not

available to some enzymes after calcium concentrations rise. This would suggest that these IQ-motif polypeptides not only serve to localize large concentrations of calmodulin within neurons, but that they could also directly inhibit the activation of calmodulin targets under specific conditions *(36)*.

Figure 2 shows a comparison of the inhibition of neuronal NOS by neuromodulin, neurogranin, and PEP-19. The effect of increasing concentrations of protein demonstrated that over a low-μ*M* range all three polypeptides acted as inhibitors of NOS. Neuromodulin and neurogranin functioned most similarly, which is consistent with the greater similarity of their IQ motifs. By contrast, PEP-19 required an order-of-magnitude greater concentration to achieve a similar effect. Consistent with the relative affinities seen in the peptide titration curves, panel B demonstrates how they are able to shift the activation of NOS over a physiological range of calcium. The effect of both neurogranin and neuromodulin are very pronounced, with a noticeable effect by PEP-19. In this in vitro model system, even PEP-19 was able to decrease the activation of enzyme at moderate levels of calcium by one-half.

Although the levels of proteins and calcium used in vitro could be made to estimate levels found in vivo, this type of experiment suffers from an inability to reliably duplicate in vivo conditions. However, the in vitro studies were able to demonstrate that these IQ-motif-containing polypeptides do have the physical capacity to directly inhibit calmodulin-dependent target activation. These results were consistent with extensive studies on neurogranin (RC3) from which the authors suggested a model whereby the response threshold to calcium influx could be modulated by neurogranin in such a way as to impede target activation in a calcium concentration-dependent manner (reviewed in *20*). It became apparent from these mechanistic studies that neuromodulin, neurogranin, and PEP-19 may carry out the regulation of calmodulin target activation in vivo. However, what was not well-understood was how this regulation would be manifested.

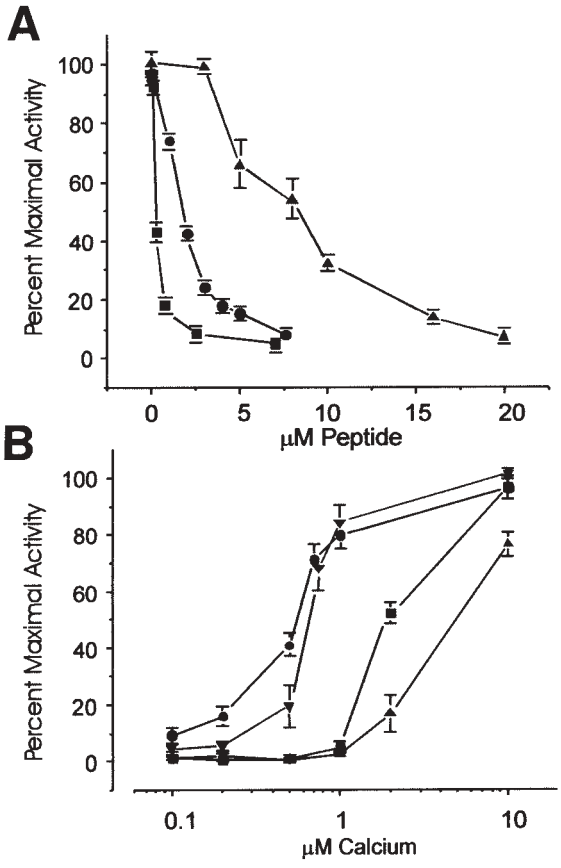

Fig. 2. Neuromodulin, neurogranin, and PEP-19 function to regulate calmodulin-dependent NOS in vitro. **(A)** Enzyme activity is inhibited in a concentration-dependent manner with increasing amounts of protein. Naturally occurring levels of these proteins are estimated to be in the low to medium micromolar range. ●, neuromodulin; ■, neurogranin; ▲, PEP-19. **(B)** The activation of enzyme by increasing amounts of calcium is right-shifted in the presence of protein. ●, no added protein; ■, 1 μ*M* neuromodulin; ▲, 1 μ*M* neurogranin; ▼, 4 μ*M* PEP-19. All three proteins functioned as enzyme regulators in vitro, suggesting a similar process could occur in cellular signaling.

PEP-19 Expression has Differential Effects on the Activation of CaM Kinase II in PC12 Cells

Functional studies of IQ-motif polypeptides in cultured cells has exploited PEP-19 because it

has fewer apparent associations with additional cell-signaling pathways. It neither possess the G-protein interaction domain or the PKC phosphorylation sites found in neuromodulin or neurogranin. This provides an advantage for interpreting experimental data, although PEP-19 displays the lowest affinity for calmodulin of the three polypeptides. As a result, PEP-19 may have a less profound effect on signaling in vivo.

The effect of PEP-19 expression in PC12 cells on the activation of calmodulin kinase II is summarized in Fig. 3 (for details, refer to *52*). PEP-19 functioned as a conditional regulator. When cells were depolarized in high potassium (3A), the presence of PEP-19 clearly blocked the activation of the kinase. A higher resting level of activated kinase was apparent in PEP-19-expressing cells, which may be a compensatory mechanism resulting from the altered calmodulin activity in the cells. Conversely, when calmodulin kinase II was activated through purinergic receptors (3B), PEP-19 did not have a noticeable effect on the activation of the kinase. The time-course and amplitude for the activation of the enzyme was indistinguishable in PEP-19-positive and negative cells. PEP-19 expression had no detectable effect on the levels of calmodulin in the cells or the time-course and amplitude for the subsequent increase in cytosolic calcium concentration after stimulation. At longer time intervals after stimulation by agonist PEP-19-expressing cells demonstrated an accelerated deactivation of kinase that mirrored the decline in free cytosolic calcium. This has suggested that PEP-19, and other IQ-motif polypeptides, may serve a function in the deactivation of enzymes, in addition to their differential effects on enzyme activation.

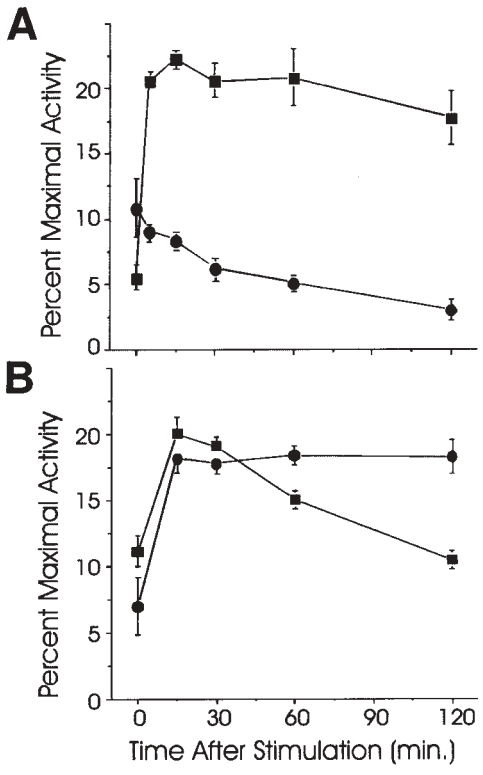

Fig. 3. Conditional regulation of calmodulin kinase II activation in pooled-stable transfectant PC12 cells. **(A)** activation of enzyme after depolarization of cells in 56 m*M* potassium chloride. ●, PEP-19 expressing PC12 cells. ■, PEP-19-negative PC12 cells that had been transformed using PEP-19 vector in the reverse orientation (*see 52* for details of cell preparation and culture). **(B)** activation of the same PC12 cell lines shown in panel (A) with 0.5 m*M* adenosine triphosphate. ●, PEP-19 negative cells; ■, PEP-19-expressing cells. PEP-19 expression failed to block activation of the enzyme, but it did hasten the decline of activated enzyme after stimulation. PEP-19 functioned as a conditional regulator of calmodulin kinase II activation, which depended on the stimulus used to activate the enzyme.

How is PEP-19 Able to Confer Differential Regulation?

The mechanism by which PEP-19 controls the activation of calmodulin kinase II after one type of stimulus but not another is not yet clear. Figure 4 summarizes a scheme by which PEP-19 could regulate target activation. There is likely to be a key step in the release of PEP-19 from calmodulin that purinergic receptors are capable of stimulating (shown by a "?" in the figure). In the case of neuromodulin or neurogranin, this could be activation of PKC, since this enzyme efficiently phosphorylates the IQ motif and interrupts binding to calmodulin. However, there is no evidence that PEP-19 becomes phos-

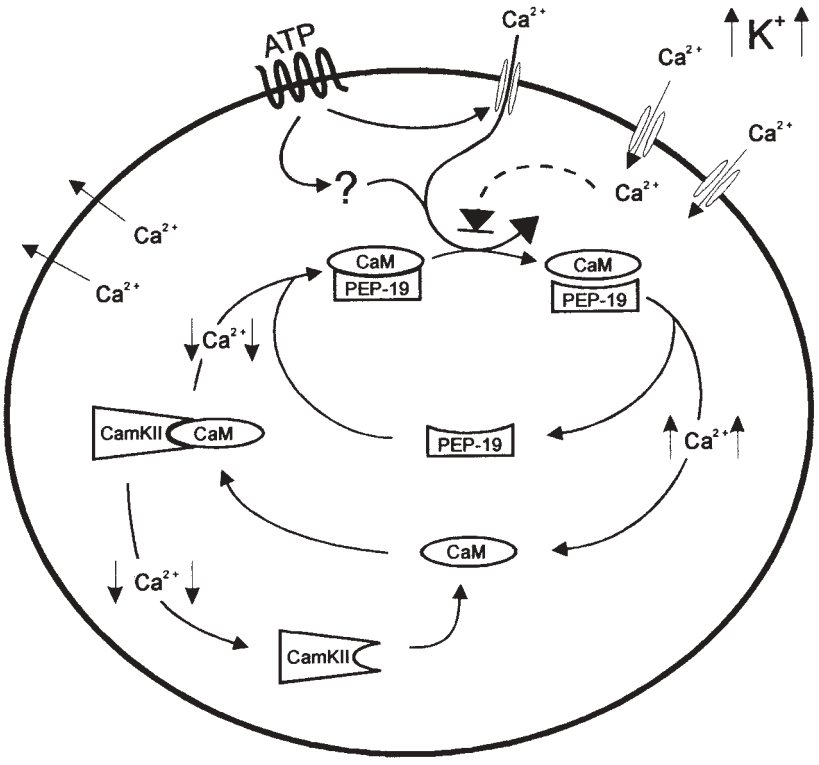

Fig. 4. Proposed model for the effect of PEP-19 on calmodulin kinase II activation in PC12 cells. Mobilization of calcium by depolarization in 56 m*M* potassium chloride normally causes maximal activation of calmodulin kinase II (dashed line). However, in the presence of PEP-19, this activation is blocked (blocked arrowhead). Calcium influx after depolarization is not sufficient to liberate calmodulin from PEP-19. In contrast, activation of purinergic receptors and the resultant calcium influx ($\uparrow Ca^{2+} \uparrow$) are sufficient to activate calmodulin kinase II, even in the presence of PEP-19. This has suggested that calmodulin is liberated from PEP-19 by an unknown mechanism (indicated by "?"), which is mediated through the activation of purinergic receptors but not by depolarization alone. Such a function for PEP-19 would serve to uncouple selected extracellular stimuli from their normal intracellular signaling pathways, while leaving the signaling of other stimuli intact. Proposed mechanisms for imparting selective activation may include phosphorylation on calmodulin (i.e., *14,15*) or structural changes to the IQ-motif in PEP-19 *(20).* PEP-19 may also serve to accelerate the deactivation of calmodulin kinase II as intracellular calcium levels decline ($\downarrow Ca^{2+} \downarrow$) by releasing "trapped" calmodulin *(65,66).*

phorylated. One possible alternative for reversing the binding of PEP-19 to calmodulin may involve the generation of phosphocalmodulin, since this could be the equivalent of phosphorylating PEP-19. In vivo phosphorylation of calmodulin is carried out by both cytosolic and receptor-linked kinases *(53–55)*, so that a number of signaling pathways might affect such a process. An immediate need is to determine if the phosphorylation of calmodulin on any of its sites affects binding to PEP-19.

An additional mechanism for regulating PEP-19 binding to calmodulin is suggested by Gerendasy et al. based on studies with neurogranin (RC3) and neuromodulin (GAP-43) (reviewed in *20*). The calmodulin-binding domain of neuromodulin adopts an apparent α-helical structure in the absence of calcium and this form binds calmodulin with more avidity than the relaxed structure. If this same property is inherent in the calmodulin-binding domain of PEP-19, then any modification that interferes with the formation

of the α-helix could reverse the inhibition of PEP-19 on the activation of calmodulin kinase II. Thusly, activation of purinergic receptors could promote a process that relaxes the α-helical conformation of the binding site in PEP-19. This would result in the release of calmodulin from PEP-19 and the subsequent activation of the enzyme. Calcium mobilization by depolarization may not affect the α-helical structure in PEP-19 and therefore not interfere with its inhibition of enzyme activation.

Calmodulin is Phosphorylated in PC12 Cells

Preliminary data on the phosphorylation state of calmodulin from PC12 cells after stimulation is consistent with a role for phosphocalmodulin in the action of PEP-19. Because calmodulin is a relatively abundant and heat-stable protein and it separates well on RP-HPLC, it can be sufficiently purified from cultured cells for analysis on LC/MS (Fig. 5). In addition to the expected mass of calmodulin at 16791, an additional mass at 17192 is observed to be enriched in PC12 cells that have been stimulated with ATP (*see 52* for details). This exactly corresponds to a mass-shift expected from the addition of five phosphates. Additional studies are needed in order to better estimate the amount of calmodulin that is phosphorylated, but current estimations indicate that the ratio of 17192/16791 calmodulin (phosphocalmodulin/calmodulin) approximately doubles, relative to cells that were not stimulated. Based on the current results, the process denoted by the "?" in Fig. 4 may include phosphorylation of calmodulin. However, this possibility will require further investigation.

It has been known for several years that calmodulin is phosphorylated in vivo. Thr-79, Ser-81, and Ser-101 have been found to be phosphorylated in calmodulin isolated from rat liver (e.g., *53*). It has also been demonstrated that the insulin-receptor kinase is responsible for phosphorylating calmodulin on Tyr-99 and Tyr-138 *(55).* Additional studies have implicated the epidermal growth-factor receptor (EGFR) in the phosphorylation of calmodulin on primarily tyrosine residues *(56).* Therefore, it is apparent that sufficient sites of phosphorylation are present on calmodulin in

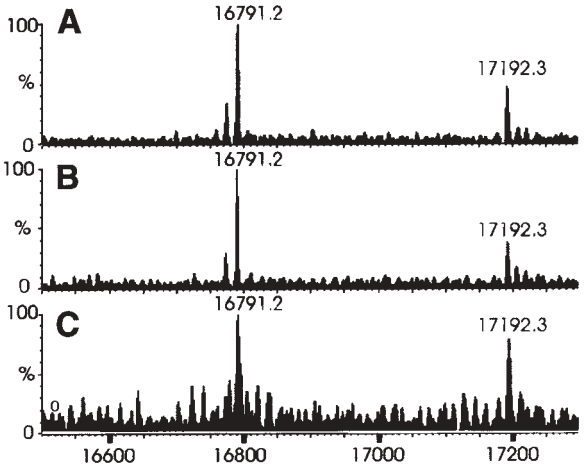

Fig. 5. Analysis of calmodulin from PC12 cells on liquid chromatography/electrospray/time-of-flight mass spectroscopy (LC/ES/MS). 1.5×10^7 cells were harvested into 50 m*M* phosphoric acid, centrifuged at 30,000g for 30 min at 4°C. The supernatant was made pH 7.0 with 6 N sodium hydroxide, placed in boiling water for 5 min. and re-centrifuged. The resultant supernatant was applied to a 1 × 25 cm C18 RP-HPLC column and developed in 0.1% trifluoracetic acid in water using acetonitrile as the mobile phase (gradient from 20–60% over 60 min.). A calmodulin peak was collected based upon elution time from a known standard of bovine calmodulin. The sample was lyophilized and an aliquot was analyzed on LC/ES/MS (LCT, MicroMass, Beverly MA) using a 2.1 × 50 mm RP column and the same solvent system (0.3 mL/min, Vydac, The Separations Group, Hisperia CA). The bovine calmodulin standard yielded a single mass of 16791, consistent with the rat calmodulin from PC12 cells. **(A)** Cells treated with normal HEPES-buffered saline for 20 s. **(B)** Cells depolarized in high potassium buffer for 20 s. **(C)** Cells depolarized in 0.5 m*M* adenosine triphosphate for 20 s. Details of the cell treatment followed that described in *(52).* These data indicate that the phosphorylation state of calmodulin appears to change after stimulation with some agonists. This may underlie some of the selectivity observed on the activation of calmodulin kinase II in cells that express PEP-19.

order to account for the mass shift observed in calmodulin that had been isolated from PC12 cells. A change in affinity of phosphocalmodulin for its target enzymes has also been reported (e.g., *15,16,53*), which further suggests that the PEP-19 interaction with calmodulin could be regulated in a similar manner.

PEP-19 Expression Makes PC12 Cells More Resistant to Degeneration

The expression profile of PEP-19 in the nervous system has suggested that it may participate in processes that can make cells more resistant to insult. Additionally, the ability of PEP-19 to modulate signaling through calmodulin further suggested that such a process could include the amelioration of cell death that results from increases in intracellular calcium and the subsequent activation of calmodulin. Erhardt et al. *(57)* have tested this hypothesis using the well-characterized PC12 cell model. PC12 cells are known to undergo cell death that is accompanied by an early rise in intracellular calcium concentration after exposure to staurosporine *(58)*. Cell death by this insult is known to require calcium since staurosporine-mediated death can be blocked by the addition of BAPTA in order to remove free calcium. Additionally, hippocampal neurons have also been shown to be protected from staurosporine-mediated cell death by sequestering free calcium with the calcium-binding protein, calbindin *(59)*. Similarly, PEP-19 could protect cells by inhibiting calcium signaling that occurs through calcium/calmodulin after exposure to staurosporine.

The effect of PEP-19 expression on cell death induced by staurosporine is demonstrated in Fig. 6. Using intracellular lactate dehydrogenase (LDH) release as a marker for loss of plasma-membrane integrity, cell death was observed to be significantly lower in PC12 cells expressing PEP-19 following 24-h exposure to staurosporine. Similarly, DEVD cleavage activity associated with apoptotic proteases (indicative of activation of caspase-3-like enzymes) was significantly diminished in PEP-19-expressing

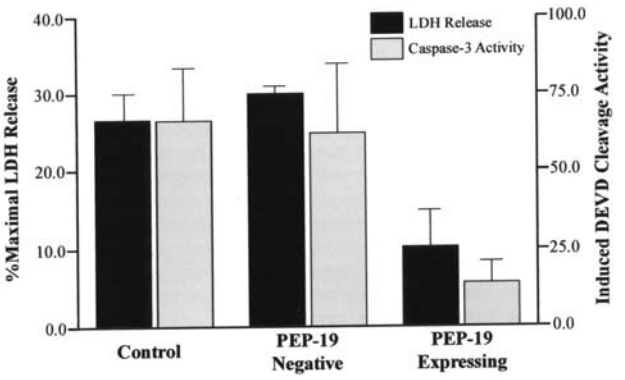

Fig. 6. Inhibition of cell death in pooled-stable transfectant PC12 cells. PC12 cell lines were exposed to 100 nM staurosporine for 24 h, and plasma-membrane disruption was measured via release of intracellular lactate dehydrogenase activity. The DEVD cleavage activity (a measure of apoptotic caspase-3-like proteases) of cell lysates following the same treatment was also determined with a fluorescent DEVD substrate (DEVD-AMC). Groups were as follows: Control PC12 (parental nontransfected PC12 cell line); PEP-19 negative (pooled stable PC12 cells transfected with PEP-19 vector in reverse orientation); PEP-19 expressing (pooled stable PC12 cells transfected with PEP-19 expressing vector). This information is consistent with the potential role of PEP-19 in protection from calcium-dependent cellular degeneration.

PC12 cells. These results demonstrated the ability of PEP-19 to impart resistance in cells to calcium-dependent death. Which enzymes/protein targets of calmodulin are involved in the death-signaling pathways will need to be the topic of future research.

Identifying Other Small IQ-Polypeptides that Link Calcium/Calmodulin Regulation to Cell Signaling

Neuromodulin, neurogranin, and PEP-19 have continued to generate interest because they appear linked to multiple signal-transduction pathways, including activation of targets through calcium/calmodulin. Although these

peptides are often thought of as a family, they actually have very little primary structural basis for this assignment. Additionally, the IQ motif is very difficult to use as a basis for finding other similar polypeptides due to its small size and degenerate coding sequence. In spite of this, it is necessary that we continue to find related polypeptides both because of the potentially important functions they serve and to better understand the function of the currently known examples. Fortunately, the two properties that define this polypeptide class (interaction with calmodulin in the absence of calcium and solubility in acid), can be employed to probe for new class members. As an example of this strategy, CAP-19 (for 19 kDal-calmodulin-associated protein) was identified from bovine brain as containing an IQ-motif using affinity chromatography on calmodulin-Sepharose, RP-HPLC and a screen that employed gas-phase Edman sequencing *(35).* Data from the protein sequence was then used to recover a corresponding cDNA from a rat-brain library. Like PEP-19, neuromodulin, and neurogranin, CAP-19 is a relatively abundant polypeptide in the central nervous system (CNS) that appears localized to neurons in this region *(35).* It is soluble in perchloric acid, relatively small in size, and binds calmodulin through an IQ motif. However, as can be seen in Fig. 1, there is considerable difference between this motif in CAP-19 and other IQ-proteins. Also similar to neurogranin and PEP-19, CAP-19 is expressed late in development and persists into adulthood.

As with other IQ-motif-containing peptides, the in vivo dynamics of the CAP-19-calmodulin interaction is not well-understood. However, an important homology for CAP-19 has emerged that indicates its biological function. CAP-19 is 63% identical to the multiple protein bridging factor 1 from silkworm (MBF1, *B. mori*). This protein functions as a transciptional coactivator that bridges between the TATA box-binding protein and the nuclear hormone receptor FTZ-F1 *(60).* Recently, new data has indicated that hMBF1 (human MBF1) has similar activity to the silkworm homolog, demonstrating functional conservation of the factor between imvertebrates and higher eukaryotes *(61).* However, confirmation that hMBF1 bound to calmodulin in a manner similar to the bovine protein was only recently described for the protein from human umbilical-vein endothelial cells (EDF-1; *62*). This study also described how treatment of cells with TPA (an activator of PKC) caused the localization of EDF-1 to shift to the nucleus. This is a plausible explanation for why the study by Smith et al. *(35)* failed to visualize nuclear CAP-19 (ratEDF-1 or ratMBF-1), since the brain sections used had not been stimulated. Interestingly, EDF-1 (hMBF1) had been identified in endothelial cells as a novel gene product that downregulated during differentiation *(63).* It is still unkown if the original protein from silkworm (MBF1) binds calmodulin. The region around the IQ motif in CAP-19 and silkworm MBF1 contained some of the lowest homology between the two proteins. In particular, the IQ motif became IM in the silkworm sequence *(35).* It will be of considerable interest to determine if eukaryotes added a calmodulin-binding capability to a protein that was otherwise functionally conserved from invertebrates. The rapid accumulation of data concerning CAP-19 has made it a good example of how a functional genomics (Proteomics) approach can be exploited in the quest to catagorize additional polypeptides with IQ motifs that recognize calmodulin.

Conclusion

Neuromodulin, neurogranin, and PEP-19 have been known for over 15 years, but even now our understanding of how this type of polypeptide functions in vivo is very limited. Because these proteins appear to have a role in regulating calcium signals through an interaction with calmodulin, they may tailor signaling pathways in order to change the way in which neurons respond to agonists. One manner in which this could be accomplished is by the differential regulation of opposing enzyme activities that both become activated by calmodulin during the same stimulus. For example, during

concerted regulation of protein phosphorylation and dephosphorylation by calmodulin *(64)*, PEP-19 may preferentially accelerate the deactivation of calmodulin kinase II by aiding in the removal of calmodulin. Because of the subsequent reduction in kinase activity, the catalytic cycle would reflect a change towards more phosphatase activity from calcineurin. This process would also be dependent on the distribution of phosphocalmodulin forms, since they may interact with PEP-19 in a differential fashion or display different activities towards calmodulin kinase II and calcineurin *(15,16)*.

Small IQ-motif-containing proteins also have roles outside of calcium/calmodulin signaling. Working in concert with the calmodulin-binding domains on these polypeptides are additional regions that activate other signaling molecules or control the localization of the peptides. Neuromodulin and neurogranin are already known to work in this fashion, but little is known about how the amino terminal region of PEP-19 controls its function. The role of calmodulin binding in the function of CAP-19 is also not understood. However, the presence of functional domains on these polypeptides in addition to the calmodulin-binding site strongly suggests that one important role is the linking of calmodulin activity to other actions in cells.

The study of IQ-motif-containing polypeptides has stimulated considerable thought as to how calcium signaling is refined in neurons. We are beginning to appreciate that signaling through calmodulin is not a fulminating and homogenous process but rather a limited and highly regulated one. As the study of these peptides continues, we can look forward to a clearer understanding of how calcium/calmodulin signaling systems can be tailored in order to meet the needs of specific cell types.

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