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RC3/Neurogranin, a Postsynaptic Calpacitin for Setting the Response Threshold to Calcium Influxes

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Abstract

In this review, we attempt to cover the descriptive, biochemical and molecular biological work that has contributed to our current knowledge about RC3/neurogranin function and its role in dendritic spine development, long-term potentiation, long-term depression, learning, and memory. Based on the data reviewed here, we propose that RC3, GAP-43, and the small cerebellum-enriched peptide, PEP-19, belong to a protein family that we have named the calpacitins. Membership in this family is based on sequence homology and, we believe, a common biochemical function. We propose a model wherein RC3 and GAP-43 regulate calmodulin availability in dendritic spines and axons, respectively, and calmodulin regulates their ability to amplify the mobilization of Ca^{2+} in response to metabotropic glutamate receptor stimulation. PEP-19 may serve a similar function in the cerebellum, although biochemical characterization of this molecule has lagged behind that of RC3 and GAP-43. We suggest that these molecules release CaM rapidly in response to large influxes of Ca^{2+} and slowly in response to small increases. This nonlinear response is analogous to the behavior of a capacitor, hence the name calpacitin. Since CaM regulates the ability of RC3 to amplify the effects of metabotropic glutamate receptor agonists, this activity must, necessarily, exhibit nonlinear kinetics as well. The capacitance of the system is regulated by phosphorylation by protein kinase C, which abrogates interactions between calmodulin and RC3 or GAP-43. We further propose that the ratio of phosphorylated to unphosphorylated RC3 determines the sliding LTP/LTD threshold in concert with Ca^{2+}/calmodulin-dependent kinase II. Finally, we suggest that the close association between RC3 and a subset of mitochondria serves to couple energy production with the synthetic events that accompany dendritic spine development and remodeling.

Index Entries: RC3; neurogranin; GAP-43; neuromodulin; PEP-19; calpactin; long-term potentiation; long-term depression; dendritic spine; calmodulin; protein kinase C; mitochondria.

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Introduction

RC3 was originally identified in a subtractive hybridization study (Watson et al., 1990) designed to isolate mRNAs enriched in the rat forebrain but absent from the cerebellum, and its name indicated, simply, that it was rat cortex-enriched cDNA clone number 3. In the same study, the mouse homolog was cloned and the sequence of its encoded protein proved to be identical to that of rat. The homologous bovine RC3 protein, called neurogranin, was independently purified by Baudier et al. (1991) based on its affinity for calmodulin (CaM) and its ability to act as a substrate for protein kinase C (PKC) in hippocampal slices. RC3 cDNA homologs have since been cloned from goat (Piosik et al., 1995) and canary libraries (1996: J. M. George, S. M. Siepka, H. Jin, C. C. Holloway and D.F. Clayton; accession number 1709258) and, in the latter case, called canarigranin. The protein was also identified on several other occasions so, in addition to the above designations, it has appeared in the literature as B-50 immunoreactive C kinase substrates (BICKS) (Coggins et al., 1995) and P17 (Klann et al., 1992).

RC3 bears intriguing biochemical similarities to GAP-43 (also known as neuromodulin, b50, P-57, or F1), a protein associated with axonal growth cone development and maturation (for reviews, *see* Coggins and Zwiers, 1991; Gispen et al., 1991; Liu and Storm, 1990; Skene, 1989; Strittmatter et al., 1992). However, RC3 is only observed in dendrites, dendritic spines, and cell bodies, whereas GAP-43 is primarily located in axons. Interest in RC3 was aroused by its coincident expression with synaptogenesis and data from several laboratories, suggesting that it is involved in Ca^{2+}/CaM- and PKC-dependent cascades that guide dendritic spine development and remodeling, as well as long-term potentiation (LTP) and long-term depression (LTD). In this review we will present a model that predicts that RC3 phosphorylation determines the LTP/LTD threshold in conjunction with Ca^{2+}/CaM-dependent kinase II (CaMKII) and that RC3-mediated elaboration of dendrites, dendritic spines, and the activity-dependent remodeling of postsynaptic structures are the subcellular morphological correlates of its involvement in LTP and LTD. One goal of this review is to argue that RC3 and GAP-43 perform similar biochemical functions, albeit, on opposite sides of the synapse and, thus, are members of a protein family; here referred to as calpacitins. As this is the first review dedicated entirely to this intriguing protein, we will begin with a summary of the descriptive experiments that have led to the present state of knowledge.

Genomics

The rat RC3 cDNA, isolated by subtractive hybridization, had the potential to encode a 78-amino acid product (Watson et al., 1990). Antisera were raised against peptides based on the sequence of two different regions of the putative translation product and used to detect RC3 protein in forebrain extracts and by immunocytochemistry. The mouse homolog was then cloned and used to map the gene to the proximal region of chromosome 9 (Danielson et al., 1994). The mouse locus has been designated *Pss1* for postsynaptic spine 1. A 14-kb genomic rat clone (Sato et al., 1995) and a 12-kb human genomic clone (Martinez de Arrieta et al., 1997), each containing the entire RC3 gene, have since been cloned and their sequences determined. The human gene was mapped to chromosome 11q24, a region that is syntenic with the mouse locus *Pss1*.

Northern blots of rat and mouse forebrain revealed 1.0-kb and 1.4-kb mRNAs postulated to be derived by alternative polyadenylation of a single 1.4-kb transcript (Watson et al., 1990). Only a single 1.3-kb mRNA was observed in human brain tissue (Martinez de Arrieta et al., 1997). Primer extension analysis suggested that the rat mRNA has multiple start sites. As with many neuron-specific genes, the rat RC3 promoter does not contain a TATA box, but contains numerous putative transcription factor binding sites including; AP-1, AP-2, c-mos, SRE, and NF-E1 (Iniguez et al., 1994; Sato et al.,

1995). Additionally, it contains putative response elements for retinoids and glucacortacoids that are not conserved in the human gene (Martinez de Arrieta et al., 1997).

The structures of the mouse and human genes are similar. Both contain four exons and three introns. In each case, the first splice site falls within the codon of the fifth amino acid. This may have functional significance since the first intron within the human and rat genomic GAP-43 clones also interrupts the coding region close to the N-terminus, in this case within the codon specifying the 10th amino acid. The first 10 amino acids of GAP-43 are able to elicit some of the biological activities that RC3 and GAP-43 have in common (described below). Since exons often represent functionally or structurally distinct domains within their encoded proteins, the first five amino acids of RC3 might have a specific function or structure and, as we will discuss in greater detail below, the function may be similar to that of the N-terminus of GAP-43. Furthermore, cysteines 3 and 4 are conserved between RC3 and GAP-43 (Fig. 1), lending support the belief that the N-terminus of RC3 is functionally important.

Alternatively, the first intron of RC3 and GAP-43 may interrupt the coding sequence close to the N-terminus of the translation product because the intron contains sequences that must be close to the promoter or 5'-untranslated sequences. Vaeselow et al. (1994) reported that, in addition to the GAP-43 promoter and 5' untranslated region, sequences from the first intron of GAP-43 were required to drive faithful expression of a reporter gene in transgenic mice. Their data suggested that the first intron contained sequences necessary to maintain neuronal specificity, implying that the intron exerts a silencer effect on the promoter in cells other than neurons. In unpublished studies performed in collaboration with Luis De Lecea, we expressed the lacZ reporter from the RC3 promoter without intron 1 in transgenic mice, but were unable to obtain expression that mimicked the anatomical distribution of RC3, suggesting that the promoter, by itself, was insufficient to direct forebrain-specific neuronal expression, perhaps because intronic sequences are required.

Comparison of the translation products of all known species of RC3 indicates a high degree of conservation (Fig. 1). The sequence of the bovine homolog was determined by direct sequencing of the protein, a method much more error prone than nucleotide sequencing. Since the authors reported that the N-terminus was capped and difficult to sequence (Baudier et al., 1991), the two N-terminal omissions between the bovine sequence and those of other species are probably artifacts. Similarly, bovine Cys9 should probably be lysine and the carboxyl-terminal glycine should probably be an aspartate, since these are conserved in all other mammals investigated and even in the canary. Based on these assumptions, the bovine sequence would be identical to the sequence of its nearest relative, the goat.

Figure 1 aligns the protein sequences of RC3, GAP-43, and PEP-19 for all of the reported species. The PEP-19 peptides (Slemmon et al., 1996) are small cerebellum-enriched, CaM-binding proteins that appear to be members of the calpacitin family. The alignment indicates that GAP-43 and RC3 are closely related to each other, at least at their N-termini, and both are related, although more distantly, to PEP-19. The PKC phosphorylation site within RC3 and GAP-43 is indicated along with the minimal CaM-binding domain of GAP-43 according to Alexander et al. (1988) and Chapman et al. (1991a). Additionally, we have indicated the IQ domain; IQXXXRGXXXR, a motif described by Espriafico et al. (1992) that has been found in a growing subset of CaM-binding proteins. Two other regions that include the IQ motif have also been demarcated: one covers the sequence of a peptide that was shown to act as an efficient and specific PKC substrate (Gonzalez et al., 1993; Chen et al., 1993) and the second covers a more extensive region that, according to structural studies (described below), is involved in CaM binding (Gerendasy et al., 1995a) and possibly membrane-PKC interactions.

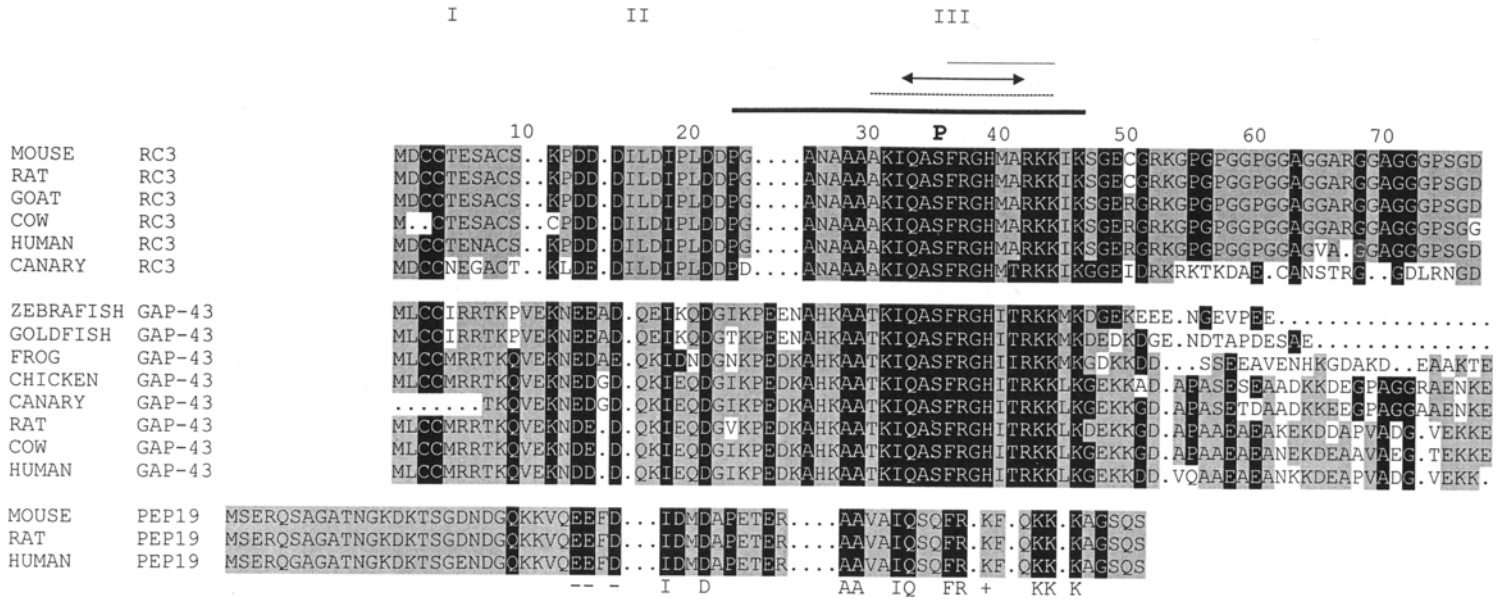


Fig. 1. The calpacitin family: multiple alignment of the protein sequences of RC3, GAP-43, and PEP19 for all reported species. The following sequences, which were obtained from the NCBI peptide database, were aligned by eye: mouse RC3 (Watson et al., 1990), rat RC3 (Watson et al., 1990; Huang et al., 1993; Sato et al., 1995), goat RC3 (Piosik et al., 1995), cow RC3 or neurogranin (Baudier et al., 1991), human RC3 (Martinez de Arrieta et al., 1997; Mertsalov et al., 1996), canary RC3 or canarigranin (George, J. M., Siepka, S. M., Jin, H., Holloway, C. C. and Clayton, D. F.; accession number 1709258), zebrafish GAP-43 (Lin, Y. P. and Fishman, M. C.; accession number 441159), goldfish GAP-43 (LaBate and Skene, 1989), frog GAP-43 (Shain et al., 1995), chicken GAP-43 (Baizer et al., 1990), canary GAP-43 (Clayton, D. F., Jin, H., Siepka, S. M., Mello, C. V. and George, J. M.; Simpson, H. B., Siepka, S. M., and George, J. M.; accession number 1658499), rat GAP-43 (Basi et al., 1987; Sangameswaran et al., 1987), cow GAP-43 (Wakim et al., 1987), human GAP-43 (Neve et al., 1987; Kosik et al., 1988; Ng et al., 1988; Nielander et al., 1993), mouse PEP19 (Chen and Orr, 1990), rat PEP19 (Ziai et al., 1986; Sangameswaran et al., 1989; Sangameswaran and Morgan, 1993), and human PEP19 (Chen et al., 1996); Cabin, D., Gardiner, K., and Reeves, R.; Chen, H. M. and Antonarakis, S. E.; accession numbers 1568615, 1352732). Those amino acids that are conserved between three or more species within a group have been lightly shaded. Dark shading has been applied to all amino acids that are conserved in two or more groups of the entire calpacitin family. A consensus sequence is displayed underneath the multiple alignment. It contains amino acids that are completely conserved within and among all three groups of the family. Additionally, we have indicated completely conserved acidic (-) and basic (+) residues. Three sequence domains that may be functionally important are numbered at the top of the figure. Also indicated are the minimal CaM-binding sequence of GAP-43 (·····) (Alexander et al., 1988; Chapman et al., 1991); the IQ motif (↔) (Espriafico, et al., 1992), a region of RC3 from which a highly efficient and specific peptide PKC substrate was derived (-------) (Chen et al., 1993; Gonzalez et al., 1993); the residues that form the α-helix when RC3 binds to CaM in the absence of Ca^{2+} (—) (Gerendasy et al., 1995). Serine 36, the target of PKC phosphorylation, is also indicated (P) (Baudier et al., 1991; Gerendasy et al., 1994).

Comparison of the sequences reveals three conserved domains, that we have numbered. RC3 and GAP-43 share N-terminal domain I containing a pair of conserved cysteines. Domain II contains four acidic residues that are conserved in all members of the calpacitin family. Additionally, GAP-43 and PEP-19 share a fifth acidic residue. All three members also contain a conserved isoleucine. RC3 and GAP-43 share a lysine in this domain as well. Domain III is conserved among all three members of the family. It includes, and extends beyond, the region that interacts with CaM, PKC and, as discussed below, lipids. The IQ motif is contained within this region. PEP-19 is not a PKC substrate, probably because the target serine of PKC phosphorylation in RC3 and GAP-43 is shifted to the left by one amino acid in PEP-19 and separated from Phe37 (based on numbering in RC3) by the polar amino acid glutamine. Our studies (Gerendasy et al., 1994b) and those of Chen et al. (1993) suggest that Phe37 is important for RC3·PKC interactions.

Distribution and Developmental Onset

Anatomical Studies

RC3 was shown to be neuron specific by *in situ* hybridization (Watson et al., 1990). These studies indicated that rat RC3 mRNA is completely absent from the cerebellum, but is abundant in various thalamic and hypothalamic nuclei, several regions of the olfactory bulb and tubercle, the medial amygdaloid nucleus and pontine nucleus. It is also highly abundant in the caudate putamen, the CA1 and CA3 regions of the hippocampus, the dentate gyrus, the primary olfactory cortex, the somatomotor cortex, and the frontal cortex.

According to immunohistochemical analyses (Represa et al., 1990), the anatomical distribution of RC3 protein is more restricted than that of the mRNA. The protein is particularly abundant in the frontal parietal cortex, granular cells of the dentate gyrus, apical dendrites of pyramidal cells of the CA1 and CA3 regions of the hippocampus, and in the striatal cortex. It is slightly less abundant in the caudate putamen and piriform cortex, and almost completely absent from the thalmus and hindbrain. In cases in which cell polarity could be delineated, the protein, like the mRNA, was only observed in dendrites and the perikarya.

Trace amounts of mRNA are detectable by Northern blot in the rat forebrain on embryonic d 18 (E18) but abundant expression does not occur until postnatal d 1 (P1), reaching a maximum approx 10–15 d after birth (Watson et al., 1990). Immunoblots and immunohistochemistry place peak protein expression at P20 and P14, respectively (Alvarez-Bolado et al., 1996; Represa et al., 1990). This developmental period is characterized by rapid dendritic growth and the formation of more than 80% of cortical synapses (Uylings et al., 1990; Blue and Parnavelas, 1983). Expression then decreases to adult levels, but RC3 remains abundant throughout life. Thus, an increase in RC3 protein and mRNA concentrations coincides with the onset of synaptogenesis in rats and mice, and its expression occurs in those regions, excepting the cerebellum, that exhibit a high degree of neuroplasticity. Interestingly, RC3 expression peaks prenatally in goats where, as in humans, synaptogenesis occurs before birth (Piosik et al., 1995).

Alverez-Bolado et al. (1996) performed a comprehensive survey of RC3 protein expression during the development of the rat telencephalon. Immunoreactivity could be detected as early as E18 in the amygdalar primordium and in the piriform cortex where it is confined until P1, at which point expression increases dramatically and appears in the olfactory cortex, isocortex, subiculum, hippocampus, and striatum. During the first postnatal week, immunoreactivity was also observed in the cell bodies of presumptive layers 4 and 5 of the somatosensory cortex and layers 2 and 5 in the anterior cingulate and agranular insular cortices. By the end of wk 1, immunoreactvity begins to decrease in cell bodies and to appear in the neuropil, suggesting a dendritic migra-

tion that culminates during synaptogenesis and thereafter remains constant in the adult. Between P1 and P10, Iniguez et al. (1996) showed that RC3 mRNA was most abundant in layers II/III and V of the cerebral cortex and CA fields of the hippocampus. From P10 to P15, RC3 mRNA decreased in layer V and increased in layer VI, the retrosplenial cortex, the caudate-putamen, and the dentate gyrus. Expression in the caudate followed a lateral to medial gradient. The developmental pattern of RC3 expression and the time course over which it accumulates in dendrites are very similar to the expression pattern of the γ isoform of PKC (Yoshida et al., 1988; Hashimoto et al., 1988; Sposi et al., 1989) suggesting that RC3 may be phosphorylated by this particular isoform.

Subcellular Studies

In situ hybridization histochemical studies revealed that RC3 mRNA is most abundant in the somata, but that significant quantities are found in dendrites (Landry et al., 1994). Additionally, Chicurel et al. (1993) detected both the 1.0- and 1.4-kb forms of RC3 mRNA in synaptosomal preparations enriched in hippocampal dendritic spines. This suggests that the majority of RC3 is translated in the perikaryal cytoplasm and transported into the dendrites, but that translation may also be regulated locally at the base of individual spines.

Immunoelectron microscopy of the neostriatum (Watson et al., 1992), cerebral cortex, and hippocampus (Neuner-Jehle et al., 1996) of adult rats indicates that RC3 protein exists in the perinuclear and dendritic cytosol. It accumulates in dendritic spines, often in close association with postsynaptic densities and subsynaptic membranes. This ultrastructural assignment is intriguing because RC3 is a particularly abundant PKC substrate that interacts with CaM in vitro (Baudier et al., 1989, 1991; Deloulme et al., 1991; Gerendasy et al., 1994a,b, 1995a). Both PKC and CaM are required for the induction of (LTP) (Abeliovich et al., 1993; Chetkovich and Sweatt, 1993; Klann et al., 1993; Lester and Bramham, 1993; Angenstein et al., 1994; Hvalby et al., 1994; Powell et al., 1994; Suzuki, 1994; Fukunaga et al., 1995; Wang and Kelly, 1995), and a postsynaptic PKC substrate that interacted with CaM would be expected to be important in this regard. Electron-dense RC3 immunoreactivity has also been observed in the vicinity of trans-Golgi vesicles and mitochondrial membranes (Neuner-Jehle et al., 1996) and the implications of these observations will be discussed below.

Subcellular fractionation studies are consistent with immunoelectron microscopy studies (Watson et al., 1994). They confirm that RC3 is predominantly cytosolic but that smaller amounts can be found in membrane-enriched microsomal and synaptosomal fractions. Immunohistochemical staining indicates that RC3 associates closely with postsynaptic densities in forebrain neurons, but detergent solubilization studies suggest the association is loose, at best. One possible explanation is that the association is mediated through low-affinity interactions with CaM or lipids. As discussed below, the RC3·CaM dissociation constant is relatively large and would probably not permit copurification of complexes dependent on such interactions. Its association with lipids, although significant, is also relatively weak.

Is Neurogranin Really Granular?

Except for in the striatum RC3 immunoreactive material exhibits a punctate or granular intracellular distribution (hence the name neurogranin) (Baudier et al., 1991; Watson et al., 1992; Neuner-Jehle et al., 1996). We also observed such a pattern in primary hippocampal cell cultures (Fig. 2). Since RC3 is only 78 amino acids long, highly abundant, and relatively unstructured and flexible (*see below*), it may be subject to antibody-induced aggregation by some antisera but not others, even in fixed preparations. Therefore, attaching biological significance to this phenomenon may be premature. On the other hand, clusters of RC3 protein could reflect an underlying, but yet unrecognized, ultrastructural feature.

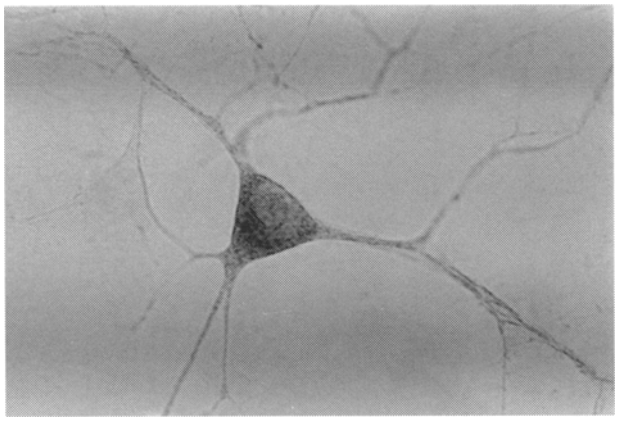


Fig. 2. Micrograph of a single primary cultured hippocampal pyramidal cell stained with anti-RC3 polyclonal antibody. In collaboration with Donna Gruol we isolated hippocampal cells from embryonic rat brains and cultured them for 2 wk. They were then fixed, incubated with purified anti-RC3 rabbit IgG antibodies, washed, and then stained with goat anti-IgG antibodies conjugated to horse radish peroxidase. This ×100 photomicrograph was created using bright-field optics. At this stage of development, only a few cells were mature enough to express RC3. However, those neurons that exhibited RC3 immunoreactivity did so quite intensely as is evident here. Immunoreactivity was highly punctate or granular and was visible in dendrites and the parikaryal cytoplasm.

Regulation of RC3 Expression

Hypothyroidism and RC3 Expression

Northern blot analysis indicated that that RC3 mRNA levels were two- to threefold lower in neonatal hypothyroid rats (P15) than in euthyroid controls, and that this effect could be reversed by the administration of thyroid hormone (Iniguez et al., 1993). A developmental analysis of the effects of thyroid hormone on RC3 expression indicated that thyroid hormone influenced the final concentration of RC3, but not its developmental pattern of expression (Iniguez et al., 1993,1996). At P15, hypothyroid rats exhibited decreased expression within layer VI of the cerebral cortex, the retrosplenial cortex, the dentate gyrus, and caudate; mRNA expression actually increased slightly in layer V. Postnatal d 15 includes the "critical period" in rodent brain development during which hypothyroidism exerts its most debilitating effect. In humans, the "critical period" occurs perinatally and hypothyroidism during this interval can result in severe mental retardation commonly known as cretinism. Iniguez et al. (1992) demonstrated that RC3 mRNA is also reversibly decreased in adult hypothyroid rats. Since RC3 is one of a small, select group of neuron-specific molecules whose expression is regulated by thyroid hormone in the postcritical period of development, it may be a molecular correlate of those symptoms experienced by adult hypothyroid humans. These include listlessness, mild memory loss, and learning deficits. All of these symptoms are reversible by thyroid hormone replacement therapy.

Piosik et al. (1995) reasoned that the goat was a better model for human brain development than rodents because synaptogenesis occurs prenatally in this species. They compared RC3 expression in the forebrains of euthyroid goats with an inbred, congenitally hypothyroid strain. In euthyroid goats, RC3 mRNA exhibits a similar anatomical distribution to that of rats, but differs in that its expression is first observed at E90 and increases threefold to adult levels by P1. RC3 mRNA levels were approx 75% lower in the striatum of the hypothyroid lines but, unlike rats, the diminution was restricted to the striatum. The lower level of striatal RC3 expression was rapidly reversed by treatment with thyroid hormone.

The sequence of the rat RC3 promoter contains response elements for retinoic acid and steroid (glucocorticoids/progesterone) hormone receptors, but none for thyroid hormone (Iniguez et al., 1994). Thyroid hormone also has no effect on reporter gene expression when the reporter gene is fused to various deletion constructs of the RC3 promoter region and transfected into either neuroblastoma Neuro-2a (Iniguez et al., 1994) or 293 cells (Sato et al., 1995), suggesting that thyroid-mediated regulation of RC3 levels is secondary to other regu-

latory events. However, reporter gene expression was modulated by retinoic acid and/or dexamethasone when the appropriate RC3 promoter deletion construct was cotransfected into Neuro-2a cells along with retinoic acid or glucocorticoid receptor expression vectors. The 5′ untranslated region of human RC3 contains no response elements for retinoic acid and steroid receptors (Martinez de Arrieta et al., 1997). One avenue through which thyroid hormone could influence RC3 expression levels is by affecting PKC levels and/or activity. Sato et al. (1995) demonstrated that a reporter gene fused to the RC3 promoter was upregulated by phorbol 12-myristate 13-acetate (PMA) in cultured 293 cells and that the effect was amplified by the cotransfection of genes encoding several different PKC isoforms that are activated by PMA but not the PMA-insensitive isoform PKCζ. In some systems, inhibitors or activators of PKC have been shown, respectively, to inhibit or mimic the effects of thyroid hormone (Lin et al., 1996a,b; Petcoff and Platt, 1992) suggesting that thyroid hormone can, in some instances, exert its effects through the modulation of PKC levels or activity.

Decreased numbers and altered distributions of dendritic spines have been well documented in hypothyroid rats both during development and in the adult animal, and these deficits can be reversed in the adult by hormone replacement. In addition to changes in hippocampal morphology (Gould et al., 1991), hypothyroidism causes reduced dendritic spine density of hippocampal pyramidal cells in the adult rat (Gould et al., 1990), decreased arborization of dendritic fields of granular and pyramidal cells of the rat hippocampus (Rami et al., 1986), and delayed synaptogenesis in the rat dentate gyrus (Rami and Rabie, 1990). Examination of mossy fiber connections to the CA3 region of hypothyroid rats also reveals fewer synapses, fewer synaptic boutons, and shorter postsynaptic densities (Madeira and Paula-Barbosa, 1993) than in controls. Additionally, Ruiz-Marcos et al. (1988) have shown that thyroidectomized adult rats exhibit a rapid decrease in apical spines along the shafts of pyramidal cells of layer V of the visual area of the cerebral cortex.

When L-thyroxine was administered to euthyroid, neonatal DBA mice (a particularly unintelligent strain of mouse with a small hippocampus), the mice become more adept at working- and reference-memory tasks in a radial maze (Crusio and Schwegler, 1991; Schwegler et al., 1991). The amount of thyroxine administered had previously been shown to induce hyperplasia of the intra- and infrapyramidal mossy fiber terminal fields. Unfortunately RC3 levels have not been examined in this system. Nevertheless, reversible changes in RC3 expression appear to correlate with reversible changes in dendritic spine density, and altered abilities to remember and learn. Additionally, decreases in RC3 expression during the critical period of brain development correlate with the irreversible deficit known as cretinism in humans, but this condition is more difficult to attribute to RC3 levels since many developmental systems are affected by hypothyroidism during this period.

Sleep Deprivation and RC3 Expression

Not long after RC3 cDNA was cloned in a study in which cerebellar mRNA sequences were subtracted from forebrain mRNA sequences, Rhyner et al. (1990) independently isolated RC3 cDNA clones using a subtraction paradigm designed to identify rat forebrain mRNA affected by sleep deprivation. These workers found that RC3 mRNA concentrations peaked at night with the onset of the rodent active period and then slowly decreased, although statistical significance could not be established in the first 12 h. After 24 h of sleep deprivation, RC3 mRNA levels were significantly lower (approx 50% of controls). A closer look, using RNase protection assays, showed that RC3 mRNA decreased by 34% in the subcortical forebrain and midbrain areas but was unaffected in the cerebral cortex or hippocampus (Neuner-Jehle et al., 1995). However, Western blots indicated that the protein levels were 37% lower in the cerebral cortex but unaffected

in the other areas, suggesting that translation of RC3 mRNA can be influenced by the physiological state of the rat and that regulation at the level of translation may be as important as regulation at the level of transcription, if not more so. This notion is reinforced by the observations, cited above, that RC3 protein expression is more anatomically restricted than the expression of its mRNA. A decrease in RC3 protein in the cortex may correlate with the deterioration of mental functions that accompany sleep deprivation in humans and this could be related to spine density or function.

Dendrite Elaboration: Cause or Effect?

Changes in RC3 levels and developmentally programmed accumulation of RC3 protein in dendrites and dendritic spines during synaptogenesis and observations that hormonally induced changes in RC3 expression tend to coincide with changes in dendritic spine density as well as deficits in memory and learning suggest a causal relationship. However, since RC3 protein accumulates in dendritic spines, one cannot ascertain formally whether decreases in spine number lead to decreases in RC3 concentrations or alternatively, a decrease in RC3 protein causes spine loss. While RC3 protein accumulates in spines and dendrites, its mRNA is most abundant in the cell soma, thus a decrease in spine density should not affect mRNA levels significantly. This implies that changes in RC3 mRNA cause changes in spine density and distribution. The argument is strengthened by the biochemical similarities between RC3 and GAP-43. The latter has been clearly implicated in axon development, presynaptic events that accompany synaptogenesis (Strittmatter et al., 1992), neurite extension (Ramakers et al., 1991; Aigner and Caroni, 1995; Aigner et al., 1995; Strittmatter et al., 1995), terminal arborization (Moya et al., 1988; Caroni and Becker, 1992), growth cone formation (Strittmatter et al., 1994a,b) and LTP (Lovinger et al., 1985; Gianotti et al., 1992; Meberg et al., 1993; Schaechter and Benowitz, 1993; Luo and Vallano, 1995; Pasinelli et al., 1995; Ramakers et al., 1995), as well as modulation of neurotransmitter release (Dekker et al., 1991; De Graan and Gispen, 1993; Ivins et al., 1993; Hens et al., 1995; De Graan et al., 1994).

Similarities Between RC3 and GAP-43

RC3 and GAP-43 share several characteristics that suggest similar biochemical activities for both proteins on opposite sides of the synapse. GAP-43 is an extremely abundant axonal growth cone protein that can be induced in adults by axonal injury (Woolf et al., 1990; Doster et al., 1991; Plantinga et al., 1993; Meyer et al., 1994). It is expressed in all neurons before birth, but persists postnatally in the forebrain where a high degree of plasticity is maintained throughout life (Benowitz et al., 1988). It associates tightly with the cytoplasmic face of axonal growth-cone membranes (Perrone-Bizzozero et al., 1988; Liu et al., 1991, 1993, 1994; Apel and Storm, 1992; De Graan et al., 1993), preferentially interacts with CaM in the absence of Ca^{2+} (in low salt), and serves as a substrate for PKC (Alexander et al., 1987, 1988; Apel et al., 1990; Chapman et al., 1991a,b; De Graan et al., 1993; Gerendasy et al., 1994b). Like RC3, GAP-43 is able to act in hippocampal slices as a Ca^{2+}-dependent phosphorylation substrate for PKC (Baudier et al., 1991; De Graan and Gispen, 1993; De Graan et al., 1994; Ramakers et al., 1995). Both molecules are unusual in that they are soluble in perchloric acid, a quality that has been used to facilitate their purification (Baudier et al., 1989, 1991). While RC3 is only 7.4 kDa, it migrates as though it were 14–18 kDa depending on the polyacrylamide concentration in the gel (Baudier et al., 1991). GAP-43 is 23.6 kDa but behaves as though it were 43–57 kDa. Velocity centrifugation (Masure et al., 1986) and gel filtration studies (Huang et al., 1993) indicate that both molecules are rod shaped. Like GAP-43,

RC3 binds to CaM more tightly when Ca^{2-} is low (Baudier et al., 1991; Deloulme et al., 1991; Gerendasy et al., 1994a,b, 1995a). Only one other protein, the Igloo protein of *Drosophila* neurons, is known to preferentially bind CaM in the absence of Ca^{2+} (Neel and Young, 1994). Igloo contains three regions that are homologous to the overlapping PKC-recognition and CaM-binding domains of RC3 and GAP-43. Other proteins have been described that bind to CaM regardless of ambient Ca^{2-}, but these interactions are either Ca^{2+}-independent or stronger when Ca^{2+} is present (Dasgupta et al., 1989; Ladant, 1988; Sharma and Wang, 1986). Phosphorylation of RC3 or GAP-43 by PKC abrogates all detectable interactions between these proteins and CaM (Chapman et al., 1991b; Gerendasy et al., 1994a, 1995a).

The primary amino acid sequence of RC3 contains a highly conserved stretch of residues that are homologous to a region near the amino terminus of GAP-43 that contains its site of phosphorylation by PKC and its CaM-binding domain (domain III in Fig. 1) (Baudier et al., 1991). This region contains a motif that has been termed the IQ domain (Espreafico et al., 1992) that is homologous to the CaM-binding domains of several other proteins, including Igloo. Other proteins belonging to the IQ-domain family proteins include p68 RNA helicase (Buelt et al., 1994), p140 Ras-GRF (Buchsbaum et al., 1996), calcium-vector protein (Petrova et al., 1996), all known myosins (Espreafico et al., 1992), IQGAP1 (Hart et al., 1996) and PEP-19 (Slemmon et al., 1996).

RC3 and GAP-43 share a pair of cysteines at their N-termini. In the case of GAP-43 these two cysteines can be palmitylated (Skene and Virag, 1989) and this is probably responsible for axonal targeting and its tight association with the inner leaf of the growth cone membrane (Zuber et al., 1989; Liu et al., 1991, 1993, 1994). RC3, on the other hand appears to reside primarily in the cytosol suggesting that it is not palmitylated (Watson et al., 1994). Like RC3, GAP-43 associates with Golgi membranes. Mutating the N-terminal cysteines of GAP-43 prevents this association and also interferes with axonal targeting. Whereas RC3 also associates with Golgi vesicles (Neuner-Jehle et al., 1996), it is targeted to dendrites. Mutagenesis experiments to determine exactly which N-terminal amino acids cause the two proteins to partition to opposite compartments might prove interesting and may also elucidate the role of cysteine acylation in this process. RC3 contains a putative casein kinase II recognition domain N-terminal to its PKC phosphorylation site, whereas GAP-43 contains several such sequences C-terminal to its PKC-recognition domain. However, only GAP-43 acts as a substrate for casein kinase in vitro and CaM inhibits the reaction (Apel et al., 1991; Huang et al., 1993). Additionally, both molecules are able to act as substrates for ADP-ribosyltransferase (Coggins et al., 1993a,b).

Both RC3 and GAP-43 are affected by nitric oxide (NO) (Hess et al., 1993; Mahoney et al., 1996; Sheu et al., 1996). In GAP-43, NO appears to modify the N-terminal pair of cysteines, thereby preventing long-chain fatty acylation. This may be responsible for NO-induced collapse of axonal growth-cones, presumably by interfering with GAP-43's interaction with growth-cone membranes or its transport into axons. Oxidation of RC3 by NO or other oxidants attenuates its interaction with CaM, and its ability to serve as a substrate for PKC. Mahoney et al. (1996) substituted each of the four cysteines in rat RC3, individually and in combinations, with serine or glycine and examined oxidant-induced formation of intramolecular disulfide bonds in vitro. Their data indicated that Cys51 pairs with one of the other three cysteines, Cys3, Cys4, or Cys9, and suggested this altered the conformation of the PKC-recognition/CaM-binding domain that lies between Cys51 and the other three cysteines. The biological relevance of this reaction, however, is somewhat questionable since Cys51 is probably not conserved in cows and definitely not in goats or humans. If NO modulates RC3 activity, we expect it would use the same mechanism as that used in the case of GAP-43. Since RC3 is predominantly cytosolic, it is probably not highly palmitylated, which

could either mean that NO-induced regulation is not applicable to RC3 in vivo or that Cys2 and Cys3 are constitutively modified by NO in the majority of RC3 molecules and, therefore, only available for long-chain fatty acylation in a small minority of the molecules. This could explain why small amounts of RC3 are observed to be associated with microsomal and synaptosomal membrane fractions. The observation that RC3 derived from membrane fractions of the rat forebrain exhibits a slower mobility then that of the cytosolic fraction when resolved by SDS-PAGE is consistent with the hypothesis that the membrane-bound fraction is modified in some way (Houbre et al., 1991).

Some of the most compelling data concerning GAP-43 function has been derived from knockout and transgenic mice. The retinal axons of GAP-43 knockout mice fail to navigate past the optic chiasm for 6 d, supporting the hypothesis that GAP-43 amplifies path-finding signals to the growth cone (Strittmatter et al., 1995). Aigner et al. (1995) created transgenic mice that overexpressed wild-type GAP-43 or one of two sequence variants. In one case, Ser42 was changed to an alanine, thus preventing phosphorylation but not CaM binding, and in the other case Ser 42 was changed to aspartate, which prevents CaM binding. GAP-43 overexpressors exhibited spontaneous nerve sprouting at neuromuscular junctions and in the terminal field of the hippocampal mossy fibers. Expression of the Ser42Ala variant caused a decrease in sprouting compared to controls. The Ser42Asp expressors exhibited no obvious phenotype, suggesting that the phenotypes observed in these mice were, in some way, dependent on interactions between GAP-43 and CaM. Given the biochemical similarities between RC3 and GAP-43, these findings suggest that RC3 may play a role in dendritic spine development and postsynaptic aspects of forebrain neuroplasticity. Unfortunately, the previously mentioned difficulties in targeting RC3 expression to the appropriate population of neurons have, thus far, precluded a direct test of this notion.

PEP-19 also deserves comparison with RC3. It is a 61-amino acid, postnatal onset, neuron-specific peptide that is highly enriched in the Purkinje cells of the cerebellum, a highly neuroplastic region where RC3 is not expressed, and, to a lesser extent, the olfactory bulb (Ziai et al., 1988; Sangameswaran et al., 1989; Sangameswaran and Morgan, 1993; Chen et al., 1996). Immunoreactive material can also be detected in the caudate putamen (Ziai et al., 1988). Its onset of expression coincides with the formation of synaptic contacts onto Purkinje cells and it contains two domains that are conserved within RC3 and GAP-43 (Sangameswaran et al., 1989; Sangameswaran and Morgan, 1993; Chen et al., 1996). Like RC3, the majority of its sequence is dedicated to its interaction with CaM, but it differs in that it cannot be phosphorylated by PKC. It also differs in that its expression is not restricted to any one area of the neuron (Ziai et al., 1988). PEP-19 may fulfill some of the functions that RC3 performs in the forebrain.

The observations discussed above along with many that are touched upon below suggest that GAP-43 and RC3 have similar biochemical roles on opposite sides of the synapse. Their biochemical and physiological similarities make it almost impossible to discuss one without mentioning the other. Whereas this review concentrates on the function and importance of RC3 to dendritic spine development and postsynaptic components of LTP and LTD, we will, by necessity, make frequent reference to GAP-43, which has been implicated in presynaptic components of these same phenomena.

Biochemical and Biophysical Characterization of RC3

Expression, Mutagenesis, and Purification of the Recombinant Protein

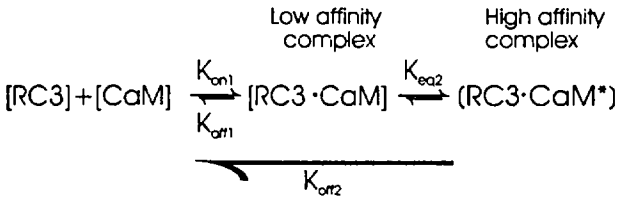
To lay the ground work for biochemical and biophysical analysis of RC3 and GAP-43 the protein-coding regions of their respective genes were amplified by PCR and cloned into a bacterial expression vector (Gerendasy et al.,

1994b). Key residues were then mutagenized. Recombinant RC3, GAP-43, and variants Ser36Asp (S36D), Ser36Lys (S36K), Ser36Ala (S36A), and Phe37Trp (F37W) were purified to near homogeneity based on their solubility in perchloric acid and their ability to interact, to varying degrees, with CaM-sepharose. In the presence of low Ca^{2+} and physiological salt concentrations, recombinant RC3 interacted with CaM-sepharose more strongly than recombinant GAP-43. The S36D variant did not interact with CaM-sepharose and was, therefore, purified by gel filtration. Antisera were raised against purified recombinant RC3, which have been suitable for histological and biochemical studies. Recombinant (Gerendasy et al., 1994b) and native (Huang et al., 1993) RC3 serve as PKC substrates in vitro, but none of the Ser36 variants served as a PKC substrate, confirming that residue as the phosphorylation target site.

The RC3 sequence contains two potential sites of phosphorylation: Ser36, which is phosphorylated by PKC in vivo and in vitro and Ser10, which lies in a putative casein kinase II recognition domain. To date, the only other kinase that has been shown to phosphorylate RC3 is phosphorylase kinase, which phosphorylates Ser36, albeit less efficiently than PKC (Paudel et al., 1993). This kinase is also able to phosphorylate the PKC target in GAP-43. Neither we (unpublished) nor others (Huang et al., 1993) were able to phosphorylate RC3 with casein kinase II. Other kinases that have been tried unsuccessfully include: cAMP- and cGMP-dependent kinases, Ca^{2+}/CaM-dependent kinase II, myosin light-chain kinase and casein kinase I (Huang et al., 1993).

Kinetic and Structural Studies of RC3·CaM Interactions

The tryptophan replacement in F37W permits one to monitor its interaction with CaM by fluorescence spectroscopy, and thus, in competition experiments, to measure the affinities of other sequence variants at various salt and calcium concentrations by competition, using F37W as a reporter (Gerendasy et al., 1994a).



Eq. 1. Kinetic analysis suggested a formal representation where the rate constants K_{on1} and K_{off1} (these are the rate components of equilibrium constant K_{eq1} which is not shown) determine the concentration of low affinity and K_{eq2} governs the portion of molecules involved in the high affinity interaction. The structural data described below indicate that the CaM-binding domains of RC3 and GAP-43 assume an α-helical conformation (designated by an asterisk in the equilibrium equation) solely in the absence of Ca^{2+}, suggesting that the binding mode is determined by the conformational state of RC3.

Distinct biophysical characteristics were thereby assigned to each of the sequence variants. These analyses indicated that, under physiological ionic conditions, S36A exhibited a higher affinity for CaM than the wild-type species when Ca^{2+} was absent, but a similar affinity in its presence. F37W displayed a higher affinity for CaM in the presence and absence of Ca^{2+}, and the serine to aspartate substitution abolished all detectable interactions regardless of Ca^{2-} concentrations. A kinetic analysis of the data indicated that RC3 cycles between two affinity forms. When [Ca^{2+}] increased, only the lower affinity form was detected. Nevertheless, complete abrogation of the interaction required phosphorylation of RC3, as mimicked by S36D. Using similar techniques, Chapman et al. (1991a,b) demonstrated that GAP-43 and CaM also interact via two different modes depending on the presence or absence of Ca^{2+} and that phosphorylation abolishes these interactions. Further analysis suggested the hypothesis that at low [Ca^{2+}], RC3, and CaM bind as a low affinity complex that undergoes a transition to a high affinity form. A Ca^{2+} influx eliminates the high affinity complex, but the low affinity complex releases free Ca^{2+}/CaM slowly (K_{off1} in Eq. 1). However, rapid dissociation occurs when [Ca^{2+}] rises

rapidly (K_{off2}). Thus, RC3 may serve a capacitor-like function for CaM, releasing Ca^{2+}/CaM gradually or in a rapid pulse, depending on the size and duration of a Ca^{2+} flux.

The structural basis of the two RC3·CaM binding modes were explored along with possible mechanisms underlying the unusual ability of RC3 and GAP-43 to preferentially bind to CaM in the absence of Ca^{2+} (Gerendasy et al., 1995a). Interactions between CaM and purified, recombinant RC3, the previously characterized sequence variants, GAP-43, and each of nine synthetic peptides that collectively spanned the entire sequence of RC3 were monitored by circular dichroism spectroscopy (CD) using a tandem cuvet. In each case RC3, one of the variants, GAP-43, or one of the peptides was placed in one compartment of the cuvet while an equimolar concentration of CaM was placed in the other. A spectrum was obtained before and after mixing the contents of the two chambers in the presence or absence of Ca^{2+}, allowing detection of changes in spectra that resulted from molecular interactions between the components in the two chambers. During the course of these experiments, salt concentrations were varied and the effect of trifluoroethanol (TFE), an organic solvent known for its ability to stabilize the α-helical conformations in peptides or proteins that have such a propensity, was examined. The data produced strong evidence that an α-helix was stabilized within the CaM-binding domains of RC3 and GAP-43 upon binding to CaM when and only when Ca^{2+} was absent and that the effect occurred within a physiological ionic environment. Thus, the high affinity RC3·CaM complex was characterized by the formation of an α helix within RC3. In the presence of Ca^{2+}, RC3, and GAP-43 appear to maintain their natural conformation which, in an aqueous environment, is relatively flexible and unstructured, consisting mainly of random coils.

Through the use of overlapping peptides derived from the sequence of RC3 CaM-binding domain was mapped and determined to be considerably larger than the IQ domain originally proposed (Espreafico et al., 1992). This may explain the high degree of amino acid sequence conservation. Sequence variants that would be expected to interfere with the formation of an amphiphilic α helix would also be expected to decrease the strength of the domain's interaction with CaM, as was observed with peptides containing amino acid substitutions derived from PEP-19 (Slemmon et al., 1996). Thus, structural constraints may be responsible for the high degree of sequence conservation within domain III of the calpacitin family

In the case of GAP-43, and also for the aqueous conformation of a peptide derived from RC3, the CD results have been confirmed by nuclear magnetic resonance (NMR) (Zuber et al., 1989; Zhang et al., 1994; Urbauer et al., 1995; Wertz et al., 1996). Masure et al. (1986) concluded that GAP-43 is rod shaped based on its sedimentation rate during velocity centrifugation and that its CD spectrum indicated a predominantly random-coiled configuration with very little α helicity (1%). Gel filtration chromatography suggested that RC3 is also rod shaped (Huang et al., 1993). Based on CD spectra of peptides corresponding to the sequence of RC3, its N-terminus (residues 1–30) appears to be somewhat more structured than its C-terminus (residues 50–78), which is similar in sequence to collagen and forms a random coil conformation. The distinctive CD spectrum generated by the triple helix conformation of collagen was not evident in any of the spectra. The central region (residues 30–50) is unstructured but has a strong helical propensity that is stabilized by CaM. Thus, RC3 and GAP-43 are rod shaped, but quite fluid within their CaM binding domains. Stabilization of a helix within this region could decrease the freedom of motion at the ends as well as within the immediate binding domain resulting in a stiffer, more pronounced rod-like structure when Ca^{2-} levels are low. This structure and the less organized form may correspond to the two affinity states of RC3 which were inferred by fluorescence emission spectroscopy studies.

In the presence of physiological salt concentrations (150 m*M* KCl), GAP-43 exhibits the

same affinity for CaM regardless of Ca^{2+} concentrations (Alexander et al., 1987, 1988) and the identification of two binding modes by Chapman et al. (1991a,b) required careful observations. PEP-19 appears to bind to CaM with equal affinity in the absence of salt regardless of the presence of Ca^{2+} (Slemmon et al., 1996). The effect of KCl on PEP-19·CaM interaction has not been investigated. RC3, on the other hand clearly exhibits a higher affinity for CaM in the absence of Ca^{2+}, even at fairly high salt concentrations (Gerendasy et al., 1994a,b). CD data indicated that only the Ca^{2+}-free form of CaM induced a change in conformation in both RC3 and GAP-43, and this change was evident in GAP-43 even in the presence of physiological salt concentrations (Gerendasy et al., 1995a). Therefore, whereas the affinity of GAP-43 for CaM appears to be Ca^{2+}-independent, CaM affects its conformation in a Ca^{2+}-sensitive manner just as it does RC3. Since the CaM-binding domain of PEP-19 is homologous to those of RC3 and GAP-43, we suspect that CaM induces a Ca^{2+}-sensitive conformational change in this molecule as well.

Does RC3 Interact with CaM In Vivo?

When Baudier and colleagues (1991) initially purified neurogranin (RC3) from bovine brain, the yields suggested that RC3 was much less abundant than GAP-43, which is one of the most abundant CaM-binding proteins in the brain. Cimler et al. (1985) estimated that GAP-43 comprised up to 0.5% of the total protein in cell-body-rich regions of the bovine brain based on radioimmune assays and photoaffinity labeling with azido-^{125}I-CaM. The low yields of neurogranin obtained contrasted with our observation that RC3 was an abundant dendritic protein, based on Northern and Western blots, *in situ* mRNA hybridization studies (Watson et al., 1990), and immunohistochemical light and electron microscopic studies (Watson et al., 1992). Modified purification protocols, however, yield nearly equivalent amounts of RC3 and GAP-43; 2.0 and 2.5 mg/100 g bovine brain tissue, respectively (Slemmon and Flood, 1992; Slemmon and Martzen, 1994). Since RC3 is anatomically more restricted than GAP-43 and has a smaller molecular mass, local concentrations of RC3 are even higher than those of GAP-43. The dissociation constant for the GAP-43·CaM complex in low Ca^{2+} was determined to be 3.4×10^{-6} at physiological salt concentrations. Since the concentration of CaM is believed to be approx 2 μM, Cimler et al., (1985) concluded that a significant portion of CaM would be sequestered by GAP-43 when Ca^{2+} levels were low. This consideration has even greater validity for RC3, which is more concentrated and binds to CaM more tightly than GAP-43 under physiological conditions. Moreover, RC3 resides in the cytosol of the dendritic spine and is loosely associated with postsynaptic densities (Watson et al., 1992, 1994), permitting easy access to CaM. Finally, RC3·CaM and GAP-43·CaM interactions are specific, obey a strict stoichiometry of 1:1 (Gerendasy et al., 1994a) and elicit a Ca^{2+}-sensitive conformational change in both molecules (Gerendasy et al., 1995a). The concentration of PEP-19 in Purkinje cells has been estimated to as high as 40 μM (Slemmon et al., 1996). Since it also exhibits a micromolar affinity for CaM, the concentration is more than sufficient to sequester most of the CaM, although the K_d of its interaction has not been determined in the presence of physiological salt concentrations.

Potential Regulation of CaM-Activated Proteins by RC3

Since RC3 is extremely abundant in regions rich in CaM, there is a strong possibility that it can alter the activity of other CaM-dependent proteins. Martzen and Slemmon (1995) have explored the possibility in vitro. As a test case they examined the effect of RC3 on the activity of CaM-dependent nitric oxide synthase (NOS). RC3 at 0.2 μM was sufficient to shift the

concentration of Ca^{2+} required to achieve 50% maximal velocity of NOS from 0.55 to 1.0 μ*M*. A concentration of 1.0 μ*M* RC3 effectively shifted the required concentration to 10 μ*M*. These Ca^{2+} concentrations are consistent with the range that is believed to exist in the dendritic spine (Connor et al., 1994). Under conditions required to obtain 80% maximal velocity of NOS activity, RC3 showed inhibitory activity with an EC_{50} of 0.45 μ*M*, which is far below the concentration of RC3 in the spine. Phosphorylation of RC3 with PKC completely reversed inhibition of NOS activity. These results are startling when one considers that the NOS·CaM complex has a dissociation constant (K_d) of only 10 μ*M* (Bredt and Snyder, 1990) compared to the μ*M* dissociation constant of the RC3·CaM complex. They imply that RC3 would have even greater influences on the activities of CaMKII and CaM-dependent adenylate cyclase, two enzymes that are unquestionably involved in postsynaptic events associated with LTP, learning and memory, and which bind CaM with K_ds of 50–100 n*M* and 15 n*M*, respectively (Meyer et al., 1992). Similar experiments performed with GAP-43 and PEP-19 indicate that these can also alter the Ca^{2+} requirements of NOS (Slemmon and Martzen, 1994; Slemmon et al., 1996).

RC3, GAP-43, and PEP-19 bind to CaM with micromolar affinities. Therefore, their abilities to drastically alter the Ca^{2+} requirements of a Ca^{2+}/CaM-dependent enzyme that binds CaM with a nanomolar affinity suggest that more is involved than mere competition. Since proteins that bind Ca^{2+}/CaM stabilize the Ca^{2+}-containing form of CaM and thereby increase CaM's affinity for Ca^{2+}, we suspect that RC3, GAP-43, and PEP-19 stabilize the Ca^{2+}-free form of CaM, thereby lowering its affinity for Ca^{2+}. This would shift the concentration of Ca^{2+} required to activate many Ca^{2+}/CaM-dependent enzymes, giving RC3, GAP-43, and PEP-19 greater leverage over Ca^{2+}-dependent signaling systems than their simple affinities might imply. NMR studies of the interaction between CaM and a peptide derived from the CaM-binding domain of GAP-43 indicate that CaM maintains its free apo state when bound to the peptide (Urbauer et al., 1995). However the peptide did not include all of the CaM-binding domain as determined by CD (Gerendasy et al., 1995a) and a tryptophan was substituted for Phe41, weakening any analogies one might draw from these experiments to the in vivo behavior of GAP-43 and related proteins.

Interactions Involving RC3, PKC, and Phospholipids

PKC isoforms can be categorized according to whether they require Ca^{2-}, diacylglycerol (DAG) and/or phosphatidyl serine (PS) for activity (for reviews, *see* Nishizuka, 1988, 1992; Huang, 1990). Four groups have been described: group A contains the "conventional" PKCs consisting of α, βI, βII, and γ; group B, δ, ε, η, θ; group C, PKCs ζ and ι; and group D which contains only PKC μ. Group A PKCs are activated by Ca^{2+}, PS and DAG. Group B PKCs are Ca^{2+}-independent but activated by PS and DAG. Group C PKCs are Ca^{2+}-independent, and DAG-independent but requires PS. Group D or PKC μ has the characteristics of group B PKCs except that it contains a signal sequence and transmembrane domain.

Addition of Ca^{2+}, DAG and phospholipid to hippocampal homogenates enhances RC3 phosphorylation and this can be inhibited by a selective peptide inhibitor of PKC (Klann et al., 1992). Similar cofactor dependencies are also evident in completely reconstituted systems using purified PKCα, β,or γ (Huang et al., 1993). Thus, RC3 is a substrate for the group A PKCs although these data do not rule out the possibility that it is able to serve as a substrate for isoforms belonging to other groups. The kinetic parameters of RC3 as a substrate for this group have been compared (Huang et al., 1993) and the K_m values for each of the kinases were similar, although V_{max} was greatest for PKCα. Using V_{max}/K_m as an index of efficacy, the authors concluded that RC3 was a better substrate for PKCα than the other isoforms.

Nevertheless, the anatomical and subcellular pattern of RC3 expression and its developmental onset match those of the neuron-specific γ isoform of PKC (Represa et al., 1990), implying that this isoform phosphorylates RC3. Whereas precise colocalization studies have not been performed, both are synthesized soon after birth reaching a maximal level around postnatal d 15 in the dendritic processes of the mouse cerebral cortex, hippocampus, striatum, and amygdala and both accumulate in dendrites (Hashimoto et al., 1988; Yoshida et al., 1988; Sposi et al., 1989). However, unlike RC3, the γ isoform is also found in the cerebellum.

If RC3 is a substrate for PKCγ , the electrophysiological and behavioral phenotype of PKCγ knockout mice created by Abeliovich et al. (1993) should provide information concerning the function of RC3. Such mice exhibit greatly diminished LTP, but normal LTD and paired-pulse facilitation. Unexpectedly, normal LTP can be induced if its induction is preceded by a low-frequency stimulus. The mice also exhibit mild deficits in spatial and contextual learning as determined by their ability to perform in the hidden-platform Morris water maze and context-dependent fear conditioning tasks. The authors conclude that PKCγ is not part of the machinery that produces LTP but that it is a key regulatory component. Interestingly, we observed a compensatory twofold upregulation of PKCα in PKCγ knockout mice (Gerendasy, 1997), perhaps providing an explanation as to why the phenotype was milder than expected.

Translocation of PKC from cytosolic to membrane fractions after various learning paradigms or after induction of LTP has been well documented (Bank et al., 1989; Angenstein et al., 1994), so the biochemical analysis of neuronal substrates such as RC3 or GAP-43 would not be complete without investigating their interactions with lipids and membranes. Houbre et al. (1991) studied interactions between biochemically purified GAP-43, RC3 and multilammelar vesicles (MLV) of different lipid compositions and found that both molecules bound only to membranes containing acidic lipids such as phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, or phosphatidic acid. This suggests that basic amino acids play a role in RC3 and GAP-43 lipid interactions. However, there was always a population of up to 30%, depending on the preparation, that failed to bind, indicating that some molecules were modified in a way that prevented interactions with membranes. It was further shown that phosphorylation of GAP-43 inhibited its interaction with MLVs significantly. Nonetheless, phosphorylation is probably not responsible for the previous observation because RC3 and GAP-43 were originally purified based on their ability to bind CaM-sepharose and this would have weeded out the phosphorylated forms.

Phosphorylated GAP-43 binds MLVs less effectively, thus one can infer that the negatively charged phosphate moiety interfered with interactions between neighboring positively charged amino acids and the MLVs. The most likely suspects are the conserved lysines in domain III that are essential for CaM-binding (Alexander et al., 1988; Apel and Storm, 1992; Gerendasy et al., 1995a). This hypothesis was supported by the observation that CaM also interferes with GAP-43·MLV interactions. Collectively, these data suggested a model, in which Ca^{2+} causes RC3 to dissociate from CaM, allowing RC3 to associate with the membrane. There, it is phosphorylated by PKC, whereupon it loses its affinity for the membrane and returns to the cytosol.

A large region of domain III is able to form an amphiphilic α-helix (Fig. 1) (Gerendasy et al., 1995a), so there was a possibility that interplay between the hydrophobic side of the helix and the interior of the membrane could be involved in RC3·membrane interactions, along with the previously described electrostatic contacts. Wertz et al. (1996) and Chang et al. (1997) utilized NMR, electron paramagnetic spin, and CD spectroscopy to examine this question. In the former case, interactions between the peptide and lipid micelles or membranes was examined. Their peptide consisted of GAP-43 residues KIQAASFRGHITRKKLKG (homolo-

gous to RC3 residues 32–49 in Fig. 1). They did not observe an α helix but did observe slight penetration of the peptide into the surface of the membrane. In the latter case, SDS-micelle-bound structures were examined using a peptide derived from the sequence of residues 28–43 of bovine RC3 (residues 30–43 in all other species investigated). They observed an α helix and the hydrophobic side appeared to penetrate into the center of the micelles. These experiments are difficult to interpret because neither peptide included all of the residues required for the stable formation of an α helix and, in the second case one questions the biological relevance of SDS-micelles. Thus, in our opinion, the possibility that lipids stabilize an α helix in RC3 has not been resolved.

Recently, Lu and Chen (1997) discovered that a peptide based on the sequence of RC3 residues 28–43 binds selectively to phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) with a 1:1 stoichiometry and a K_d of 2 μm. CD spectroscopy revealed that binding caused the peptide to become more structured, however the tell-tale spectrum of a helix was not evident. They also demonstrated that $PI(3,4,5)P_3$ bound selectively to recombinant RC3 and that its presence enhanced RC3 phosphorylation by PKC. The authors speculated that $PI(3,4,5)P_3$ recruits PKC substrates such as RC3 and concentrates them at the membrane interface to facilitate their phosphorylation. This may also be the mechanism by which RC3 associates with the postsynaptic density and subsynaptic membranes. Since RC3 binds to $PI(3,4,5)P_3$ with a micromolar affinity, one would expect it to be washed away by detergents commonly used to isolate postsynaptic densities.

Dephosphorylation of RC3

Identification of candidate phosphatases is difficult in vitro because they tend to exhibit broad specificities. Calcineurin is abundant in nervous tissue and is dependent on Ca^{2+} and CaM for its activity, thus it is a good candidate for involvement in Ca^{2+}-driven second messenger cascades. Mulkey et al. (1994) presented evidence that dephosphorylation of inhibitor 1 by calcineurin and consequent activation of protein phosphatase 1 was required for induction of LTD. Since the phosphorylation states of RC3 and GAP-43 decrease after LTD induction (*see below*), this pathway may be relevant. Alternatively, calcineurin could dephosphorylate these proteins directly. Liu and Storm (1989) demonstrated that calcineurin can dephosphorylate GAP-43 and Seki et al. (1995) showed that, of the three isoforms, calcineurin-1 acted with the most favorable kinetics on phosphorylated RC3 (based on V_{max}/K_m). Protein phosphatases 1 and 2A are also able to dephosphorylate both molecules, therefore, these experiments do not indicate whether calcineurin acts directly on RC3 and GAP-43 or, alternatively through protein phosphatase-1. However Martzen and Slemmon (1995) reported that they were unable to dephosphorylate RC3 with calcineurin under conditions that were previously reported to work for GAP-43. In this case a commercial preparation of calcineurin was used and it is unclear whether dephosphorylation of the positive control, GAP-43, was carried out side by side with RC3.

RC3 and LTP

After inducing LTP in the CA1 region of the hippocampus, Klann et al. (1992,1993) observed increased *post hoc* phosphorylation of a 17 kDa PKC substrate (P17) in hippocampal extracts. They later determined that this protein was RC3 based on its immunological cross-reactivity. Using a back-phosphorylation assay, these workers demonstrated that the level of phosphorylated RC3 increased 45 min after the induction of LTP and that its phosphorylation was dependent on the NMDA receptor and persistent PKC activation (Chen et al., 1994, 1997). These findings were consistent with other *in situ* phosphorylation studies that also indicated that RC3 and GAP-43 are phosphorylated when LTP is induced (Gianotti et al., 1992; Ramakers et al., 1995). A critical role

for RC3 in LTP can be inferred from experiments performed by Fedorov et al. (1995), who demonstrated that a monoclonal antibody against the CaM-binding/PKC-recognition domain of RC3 and GAP-43 could prevent LTP in the CA1 region of the hippocampus when introduced into the postsynaptic neuron through a whole-cell clamp pipet. The antibody had previously been shown to inhibit phosphorylation, dephosphorylation and CaM-binding in GAP-43 (Hens et al., 1995).

The time course over which RC3 is phosphorylated and dephosphorylated is interesting and unexpected (for a review of the following work, *see* Ramakers et al., 1997). Ramakers et al. (1995) performed quantitative immunoprecipitation of RC3 from labeled hippocampal slices 10, 30, 60, and 120 min after inducing LTP. Increased phosphorylation (78%) was only observed at the 60-min time point. Phosphorylation was NMDA-dependent and did not occur in those slices that failed to produce LTP. Additionally, the degree of RC3 phosphorylation was strongly correlated with the increase of the slope of the EPSP in each slice. De Graan et al. (1996) also demonstrated that LTD caused dephosphorylation of RC3 10 min after induction, with recovery to baseline at the 30-min time point. For technical reasons they were unable to measure phosphorylation levels between $t = 0$ and 10 min in either group of experiments, so one cannot rule out the possibility that RC3 is phosphorylated or dephosphorylated immediately following induction of LTP or LTD, respectively. In a separate pharmacological study, these workers reported that RC3 was phosphorylated *in situ* after stimulating metabotropic glutamate receptors (Ramakers et al., 1997). The increase in phosphorylation occurred in the first minute and lasted at least 15 min more, suggesting that RC3 phosphorylation is coupled to the activation of the metabotropic glutamate receptor and that RC3 may be phosphorylated immediately after induction of LTP and then dephosphorylated and rephosphorylated an hour later.

Rodriguez-Sanchez et al. (1997) examined glutamate-induced stimulation of RC3 phosphorylation in primary hippocampal cell cultures. Hippocampal neurons from 18-d-old rat embryos were cultured for 10 d, preincubated with $^{32}PO_4$ and then exposed to various combinations of glutamate agonists and antagonists for 30 s. The state of RC3 phosphorylation was then analyzed by immunoprecipitating perchloric acid-soluble material from cell homogenates. The ionotropic receptor agonists kainate and NMDA caused the greatest increase in RC3 phosphorylation and selective activation of metabotropic glutamate receptors had a more moderate effect. Although informative, these data should be interpreted cautiously. The cell cultures were fairly immature, so RC3 expression was very low. Also, phosphorylation of RC3 increased when the NMDA receptor was selectively stimulated in resting cells. Under these conditions one would expect Mg^{2+} to block the influx of Ca^{2+}. These data are difficult to reconcile with the previously cited data of Ramakers et al. (1997) and suggest that immature hippocampal cultures do not behave the same as mature neurons *in situ*.

Mobilization of Internal Ca^{2+} Stores by RC3 and GAP-43

Cohen et al. (1993) demonstrated that phospho-RC3 increased the mobilization of internal stores of Ca^{2+} in *Xenopus* oocytes after stimulation of endogenous muscarinic receptors. Calcium mobilization was detected by measuring inward Cl^- currents. These Ca^{2+}-dependent currents are proportional to inositol trisphosphate (IP_3) concentrations within the oocyte. The muscarinic receptor is coupled to phospholipase Cβ through the α subunit of the GTP-binding protein G_0 (Moriarty et al., 1990). When activated, the phospholipase cleaves phosphotidyl inositol triphosphate (PIP_3) to form IP_3 and DAG. When RC3 RNA was injected into oocytes, agonist-induced Cl^- currents increased two- to threefold. The increase was augmented by phorbol esters, even in Ca^{2+}-free media, and prevented by the protein

kinase inhibitor H-7. When Ser36, the site of PKC phosphorylation, was substituted with glycine, inward Cl^- currents were not enhanced.

In a different set of experiments, mRNA encoding the $5HT_{2C}$ receptor was coinjected with RC3, S36A, S36G, F37W, or S36D mRNA (Watson et al., 1996). Biophysical characterization of S36A, F37W, and S36D had revealed that S36A interacts with CaM in a Ca^{2+}-sensitive manner like the wild-type protein but with a greater affinity than the wild-type species when Ca^{2+} is absent (Gerendasy et al., 1994a). F37W spends more time in the high affinity helical state than the wild-type protein and binds to CaM much more tightly regardless of Ca^{2+} levels. The S36D variant did not interact appreciably with CaM. Of the three variants, only F37W can serve as a PKC substrate, although less efficiently than the wild-type protein (Gerendasy et al., 1994b). As in the prior experiments, RC3 significantly enhanced agonist-induced inward Cl^- currents, whereas the S36G variant did not (in this case the agonist was serotonin). The S36D variant also significantly augmented inward Cl^- currents whereas S36A and F37W did not. Whereas a statistically significant increase in Cl^- currents was only observed with RC3 and S36D, a general trend emerged where the size of the response elicited by the wild-type species and each variant was inversely related to their relative affinities for CaM: S36D> RC3>S36A>S36G>F37W = control, where S36D had the greatest biological activity and the lowest CaM affinity, and F37W had the least activity and the highest affinity. Although the affinity of recombinant S36G for CaM was not determined, this study predicts that its value would lie between S36A and F37W. Since the amount of time that RC3 or variants spend in the α-helical conformation is proportional to their affinities for CaM (Gerendasy et al., 1995a), these data suggest that CaM regulates RC3's ability to mobilize internal stores of Ca^{2+} in response to G protein-coupled metabotropic receptor stimulation by modulating the concentrations of the helical form, which is inactive, and the random coiled form, which is active. In retrospect, this is not surprising, as the primary function of CaM is to regulate target proteins in response to Ca^{2+}, although, in this case, the direction of regulation is reversed; the target's activity is inhibited by CaM when Ca^{2+} levels are low. Phosphorylation of either substrate by PKC would uncouple the regulation leading to constitutive Ca^{2+}-independent activity.

GAP-43 also stimulates the mobilization of internal Ca^{2+} stores in response to G protein-coupled receptor stimulation. Strittmatter et al. (1993) demonstrated that microinjection of GAP-43 protein into *Xenopus* oocytes caused a 10- to100-fold increase in agonist-induced, Ca^{2+}-dependent chloride channel activity. Attempts to stimulate or inhibit PKC activity have not yet been reported. Previously, this group demonstrated that that GAP-43 stimulated GTP-γ-S binding to G_0 and increased GDP release, GTP binding, and GTPase activity of both G_0 and G_{i1} (Strittmatter et al., 1990, 1991). Moreover, these workers showed that only the first 10 N-terminal amino acids of GAP-43 were required for these activities. The same N-terminal peptide 10-mer was able to modulate several phenomena in cultured cells that had previously been attributed to the whole molecule, such as neurite outgrowth and morphology (Strittmatter et al., 1994a), and filopodia formation and cell spreading (Strittmatter et al., 1994b). Thus, many activities of GAP-43 observed in vivo appear to be mediated through the interaction of its N-terminus with G_0 and consequent amplification of agonist-induced mobilization of internal Ca^{2+} stores. Since the peptide does not contain the CaM-binding domain of GAP-43, it should exhibit constitutive activity, independent of CaM or Ca^{2+}. A regulatory role for CaM has not yet been tested in vitro, but the in vivo data are consistent with this view.

A Physiological Model of RC3 Function

Regulation of CaM by RC3 and PKC

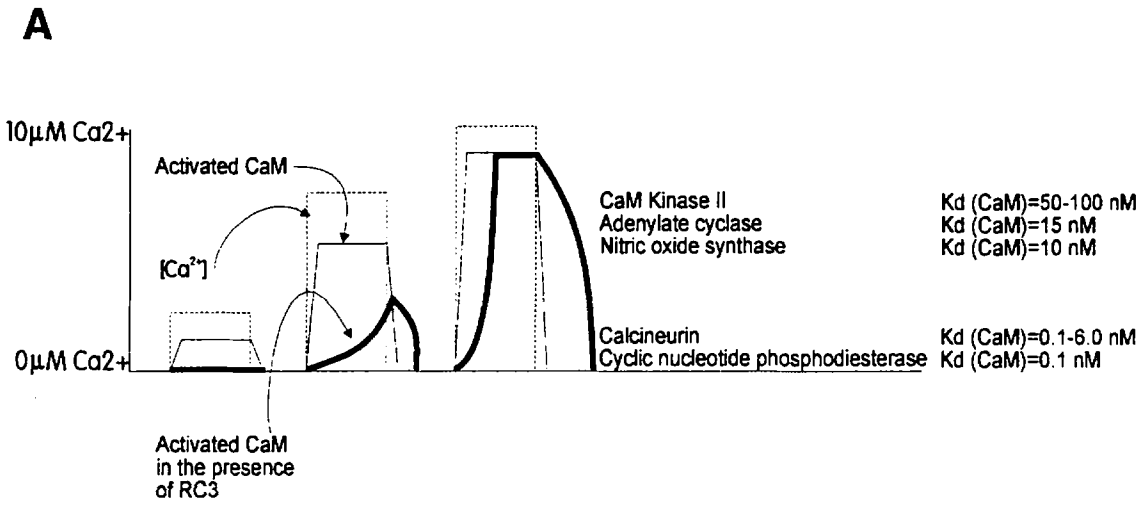
When $[Ca^{2+}]$ within a spine is very low, rate constants K_{on1}, K_{off1} and equilibrium constants

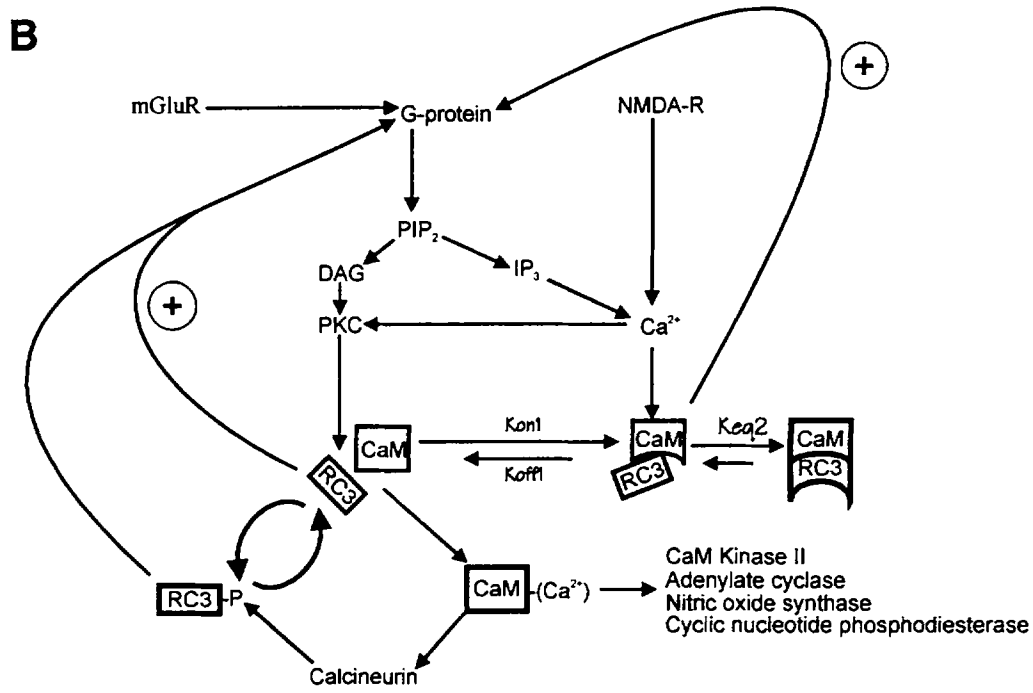
K_{eq2} ensure that little free CaM exists (Eq. 1). A sudden rise in Ca^{2+} alters these equilibria, causing the immediate dissociation of RC3 and CaM via K_{off2}, and consequent activation of Ca^{2+}/CaM-dependent proteins such as CaMKII and CaM-dependent adenylate cyclase. Reassociation depends on the re-establishment of K_{on1} and K_{eq2}, which are very small when [Ca^{2+}] is high. K_{off1} is very slow unless the complex is "shocked" with Ca^{2+}. Thus, the rate of dissociation (K_{off}) is regulated by the size of a Ca^{2+} flux and baseline Ca^{2+} levels prior to its induction. These variables determine whether CaM undergoes a sufficiently dramatic conformational change to cause rapid dissociation of the RC3-CaM complex or whether the rate of disassociation is limited to K_{off1}. In short, RC3 may serve as a biochemical "capacitor," in that it either releases Ca^{2+}/CaM gradually or in a rapid pulse, depending on the size and rapidity of a Ca^{2+} flux (Gerendasy et al., 1994a).

We postulate that RC3 buffers the biochemical consequences of small and intermediate Ca^{2+} fluxes in the dendritic spine by regulating CaM availability (Fig. 3A). CaM assumes a more compact conformation after binding to Ca^{2+}, which is further stabilized by interactions with target proteins. The latter results in a significant increase in the affinity of CaM for Ca^{2+}. Thus, stabilization of the relaxed conformation of Ca^{2+}-free CaM by RC3 or GAP-43 decreases its affinity for Ca^{2+}. The effects of small Ca^{2+} fluxes, possibly resulting from synaptic noise, would be dampened completely by the unphosphorylated form of RC3. If rate constant K_{off1} and equilibrium constant K_{eq2} (Eq. 1 and Fig. 3B) only permit slow release of CaM from its complex with RC3 in the face of moderately sized Ca^{2+} fluxes, maximum concentrations of free CaM would not be attained prior to the ebb of the flux. In the event of a large Ca^{2+} flux, dissociation of CaM from RC3 would occur rapidly leading to maximum CaM availability. These properties also apply to GAP-43 in the presynaptic neuron. Postsynaptically, a rapid influx of Ca^{2+} through the NMDA receptor and/or voltage-gated Ca^{2+} channel might be required to discharge this capacitor, whereas a slow change in Ca^{2+} levels caused by IP_3 would result in an even slower release of CaM from the RC3·CaM complex. Steady-state levels of activated CaM would not be attained under these conditions.

The stability and duration of LTP and whether LTP is induced rather than LTD appears to depend on such factors as the size and duration of pre- and/or postsynaptic Ca^{2+} fluxes (Malenka, 1994). Such variables could determine which Ca^{2+}-dependent enzymes are stimulated and ultimately, which second messenger cascades are activated. Various Ca^{2+}/CaM-dependent enzymes are listed along with their dissociation constant for CaM (Fig. 3A). These enzymes have all been proposed to be involved in LTP and LTD (Chavez-Noriega and Stevens, 1994; Malenka, 1994; Schuman and Madison, 1994). Generally those that are thought to be positive effectors, favoring LTP, such as CaM kinase II and adenylate cyclase have large dissociation constants relative to their negatively acting counterparts, calcineurin and cyclic nucleotide phosphodiesterase, which would favor LTD. Thus, the buffering effect of RC3 favors the initiation of cascades leading to LTD by keeping free CaM levels at optimal levels for negative effectors

Fig. 3. *(opposite page)* **(A)** Hypothesis that places RC3 in the postsynaptic second messenger cascade. In its role as a capacitor RC3 mediates a nonlinear, postsynaptic response to incoming stimuli. The concentration of activated CaM in the presence or absence of RC3 in response to different-sized Ca^{2+} fluxes is illustrated. By regulating [Ca^{2+}/CaM], RC3 dictates which CaM-dependent enzymes are stimulated. Positive and negative modulators of LTP are listed along with their affinities for Ca^{2+}/CaM. **(B)** Two processes are proposed to result in the disassociation of the RC3-calmodulin complex (RC3:CaM) and subsequent phosphorylation of RC3 by protein kinase C (PKC). Phosphorylation of phosphatidylinositol 4-monophosphate (PIP) results in phosphatidylinositol 4,5-bisphosphate (PIP_2), which is hydrolyzed following stimulation of the metabotropic glutamate receptor into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). The mobilization of internal stores of Ca^{2+} by IP_3 along with the possible influx of extracellular Ca^{2+} through the *N*-methyl-D-aspartate





(NMDA) receptor may cause the disassociation of RC3 and calmodulin (CaM). The final concentrations of free CaM and RC3 are dependent on the Ca^{2+}-sensitive rate constants K_{on1}, K_{off1}, and K_{eq2}, which are, in turn, influenced by the size and rapidity of the Ca^{2+} flux. Released from CaM-mediated inhibition, RC3 can amplify the Ca^{2-}-mobilizing effects of metabotropic glutamate receptor stimulation (indicated by ⊕). PKC, which is activated by DAG in the presence of Ca^{2+}, phosphorylates numerous substrates, including RC3. Other Ca^{2+}-dependent enzymes are also activated, most of which require CaM, such as Ca^{2+}/CaM-dependent kinase II. Phosphorylated RC3, (RC3-P), uncoupled from CaM-dependent inhibition constitutively amplifies the effects of metabotropic glutamate receptor stimulation. As Ca^{2+}/CaM concentrations increase, calcineurin is activated. Calcineurin either dephosphorylates RC3 directly or dephosphorylates inhibitor 1 causing activation of protein phosphatase 1 which, in turn dephosphorylates RC3.

and suboptimal levels for positive effectors. When Ca^{2+} fluxes exceed a threshold, concentrations of free CaM can rise unimpeded. This state could also be achieved, even for small Ca^{2+} fluxes, through the phosphorylation of RC3 or GAP-43 by PKC.

Regulation of RC3 by CaM and PKC

Our model (Gerendasy et al., 1995a, b) places RC3 in the dendritic spine in the context of PKC and CaM, the two proteins with which it is believed to interact (Fig. 3B). A cascade is initiated by an influx of Ca^{2+} through the NMDA receptor and/or voltage dependent Ca^{2+} channel, which is essential for the induction of Hebbian LTP in the dentate gyrus, commissural-CA3 pathway, and the CA1 region of the hippocampus (Brown et al., 1990; Madison et al., 1991) and is required for phosphorylation of RC3 in hippocampal slice preparations. The NMDA receptor is expressed in many of the same regions of the forebrain as RC3 (Insel et al., 1990; Nakanishi, 1992). Although the NMDA receptor may play a role in physiological phenomena relevant to RC3, it is not essential since any event leading to increased $[Ca^{2+}]$ within a dendritic spine would cause RC3 to relax its grip on CaM and stimulate its phosphorylation by PKC. A transient increase in Ca^{2+} is also sufficient for the induction of LTP (Madison et al., 1991; Lynch et al., 1983; Malenka et al., 1988). We further hypothesize that stimulation of increased G protein GDP release and GTP binding by RC3, and consequent increased IP_3-mediated mobilization of internal Ca^{2+}, occurs only when the allosteric inhibition of RC3 stimulatory activity by CaM is removed by a Ca^{2+} flux through the NMDA receptor and/or voltage-gated Ca^{2+} channel just prior to, or coincidentally with, the activation the metabotropic glutamate receptor. Under such circumstances the effect of G protein stimulation is amplified, resulting in more production of IP_3 and DAG, causing increased mobilization of internal Ca^{2+} and greater activation of PKC. The latter can phosphorylate RC3, thereby uncoupling its G-protein-stimulatory activity from Ca^{2+}-sensitive allosteric regulation and, at the same time, eliminating its buffering effect on CaM. This may explain why PKC activation within the postsynaptic neuron and simultaneous stimulation of the NMDA and metabotropic glutamate receptors are required for the induction of LTP (Malinow et al., 1989; Madison et al., 1991; Silva et al., 1992). Bortolotto et al. (1994) observed a conditioning effect when the NMDA and metabotropic glutamate receptor were stimulated simultaneously that eliminated the need for further stimulation of the metabotropic glutamate receptor. According to our model, the phosphorylation of RC3 could be responsible for this effect.

As phospho-RC3 levels increase, so will concentrations of Ca^{2+} and free CaM. Together these stimulate calcineurin activity causing the dephosphorylation of RC3, either directly or through the activation of protein phosphatase 1. This, in turn, leads to sequestration of CaM, a decrease in Ca^{2+} levels, and a consequent decrease in calcineurin activity. Thus, the equilibrium concentration of phospho-RC3 would be determined by the local concentrations of RC3, CaM, and calcineurin. This model is homeostatic in that it provides a mechanism to maintain steady-state levels of phospho-RC3. Di Luca et al. (1996) used electrospray mass spectroscopy coupled to liquid chromatography to determine the ratio of phosphorylated to unphosphorylated RC3 in rat cortical and hippocampal extracts. They found that phosphorylated RC3 exceeded unphosphorylated by a factor of 2.4. Based on the work of Ramakers et al. (1995), a high frequency tetanus, sufficient to induce LTP, takes approx 1 h to increase the phosphorylation state of RC3 and an additional hour for the system to return to baseline. A low frequency tetanus, sufficient to induce of LTD, takes approx 10 min to decrease the phosphorylation state of RC3 and an additional 20 min to return to baseline.

Since RC3 and CaM dissociate with nonlinear kinetics, the Ca^{2+}-mobilizing activity of RC3, which is regulated by CaM, will also exhibit nonlinear characteristics. Any nonlin-

ear changes in the Ca^{2+}-mobilizing activity of RC3 will be mirrored by a change in CaM availability because RC3 and CaM regulate each other. We propose the term calpacitin for proteins with such functions. RC3/neurogranin is, therefore a postsynaptic calpacitin, GAP-43/neuromodulin is a presynaptic calpacitin, while PEP-19 is a cerebellar calpacitin.

Compatibility with the BCM Theory of Neuroplasticity

Bienenstock, Cooper, and Munro (BCM) (1982) formulated a theory of developmental neuroplasticity to explain activity-dependent synaptic changes within the visual cortex of the kitten (Fig. 4): persistent synaptic activity that falls below a certain modification threshold, 0m, leads to weakening of a given synapse (LTD); while the converse leads to synaptic strengthening (LTP). They additionally theorized that 0m changes as a function of time-averaged postsynaptic activity such that a lack of persistent activity increases the ease with which LTP can be induced and continuous activity biases the synapse toward LTD. Mayford et al. (1995) demonstrated that θm is shifted towards the right, favoring LTD, in transgenic mice that express a pseudo-phosphorylated (Thr286Asp) sequence variant of the α subunit of CaMKII, providing a glimpse of the molecular processes underlying the BCM theory. Autophosphorylated CaMKII binds CaM extremely tightly in the presence of Ca^{2+} and releases it slowly in its absence (Meyer et al., 1992), suggesting that this form prevents excessive potentiation by restricting CaM availability and, consequently, shifting θm toward LTD. Placed in the context of the BCM theory, our hypothesis suggests that RC3 and CaMKII, and the degree to which they are phosphorylated, are both major, although opposing, determinants of θm (Fig. 4). As RC3 becomes phosphorylated it increases the Ca^{2+}-mobilizing capacity of the metabotropic glutamate receptor and makes CaM more accessible to CaMKII and other CaM-dependent enzymes, driving the system towards LTP. As CaMKII becomes autophosphorylated and RC3 is dephosphorylated, LTD becomes favored again. Our model predicts that the ratios of phosphorylated to unphosphorylated forms of RC3 and CaMKII determine the LTP-LTD threshold.

LTD induction is more pronounced in young rats (Dudek and Bear, 1993). Mayford et al. (1995) showed that this was also true in mice and reasoned that it might be caused by increased levels of phosphorylated CaMKII in younger rodents. They found greater Ca^{2+}-independent CaMKII activity in hippocampal extracts derived from younger mice, suggesting that the ratio of phosphorylated to unphosphorylated CaMKII decreases with age. Our model predicts that the ratio of phosphorylated to unphosphorylated RC3 will be smaller in young animals and increase with age. Thus, in young rodents, higher concentrations of unphosphorylated RC3 will displace θm to the right (Fig. 4), thereby increasing the ease with which LTD can be induced. In older rodents, greater amounts of phosphorylated RC3 will shift θm to the left, favoring LTP. Since LTD is easier to induce after induction of LTP, our model also predicts that the steady-state ratio will decrease after induction of LTP. Since RC3 is phosphorylated in the first hour after LTP induction, a decrease in the phosphorylation ratio would not occur until the second hour. Likewise, the induction of LTD should cause an increase in the ratio after the initial decrease.

A Possible Role in the Regulation Metabolism

Immunohistochemical electron microscopy reveals that RC3 associates with a subset of mitochondria in the cell body (Neuner-Jehle et al., 1996). The observation is intriguing because mitochondria are notorious Ca^{2+} sinks that have a large influence on ambient Ca^{2+} concentrations and are probably, themselves, affected

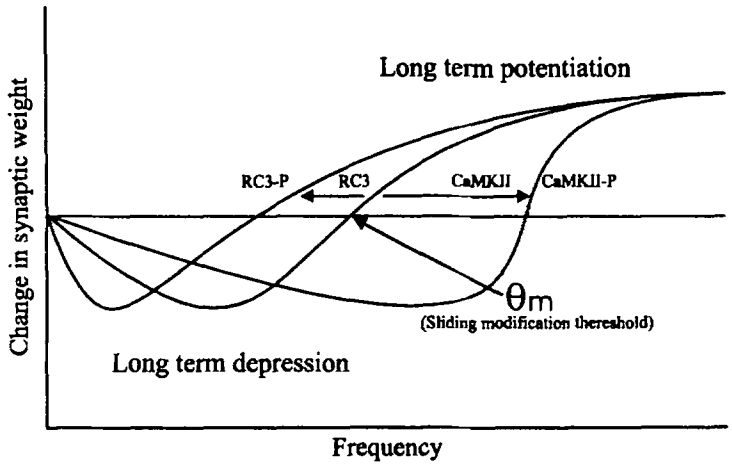


Fig. 4. RC3 and the BCM theory. The cellular and anatomical localization of RC3, its high abundance and its CaM-binding properties suggest that RC3 may play an opposing role to the α subunit of CaM/kinase II (Mayford et al., 1995) as a determinant of the postsynaptic sliding modification threshold, proposed by Bienenstock, Cooper, and Munro (1982). Thus, the phosphorylation of RC3 and CaM/kinase II may be principle mechanisms by which postsynaptic machinery keeps track of prior activity.

by local Ca^{2+} fluxes (for a review, *see* Gunter et al., 1994). Mitochondria rapidly absorb Ca^{2+} through a uniporter. The influx is driven by an electrochemical gradient and is energetically "downhill." Calcium efflux is slower and energetically "uphill." Most of the evidence, to date, suggests that mitochondria do not regulate cytosolic Ca^{2+} concentrations, although they may protect the cytosol against damage from persistently high levels. Rather, mitochondrial Ca^{2+} uptake likely serves to regulate metabolism.

There are several Ca^{2+}-sensitive dehydrogenases in the tricarboxcylic acid cycle (Hansford, 1994; Gunter et al., 1994). Hajnoczky et al. (1995) demonstrated that high frequency IP_3-mediated Ca^{2+} oscillations in the cytosol of hepatocytes was efficiently transmitted into the mitochondria, causing a sustained activation of mitochondrial metabolism. Rizzuto et al. (1994) observed fast, transient uptake of Ca^{2+} by mitochondria in several different cell types when, and only when, the cells were stimulated by agonists coupled to the IP_3 receptor. Other methods of raising cytoplasmic $[Ca^{2+}]$ such as blocking intracellular Ca^{2+} ATPases did not have this effect. The resultant increase in mitochondrial Ca^{2+} levels was sufficient to activate Ca^{2+}-sensitive dehydrogenases, although it was not directly tested in the study. In these studies only 30% of the mitochondria in a given cell responded to cytoplasmic microdomains of high Ca^{2+}.

Neuner-Jehle et al. (1996) reported that RC3 associates with the membranes of some, but not all mitochondria. Assuming that a similar heterogeneity exists in the mitochondria of neurons, RC3 may associate with those mitochondria that are able to respond to changes in external Ca^{2+}. Numerous axons form glutamatergic synapses with the cell bodies of cortical and hippocampal pyramidal cells and there is no reason to assume that RC3 does not amplify IP_3-mediated Ca^{2+} fluxes in the somata as well as the dendrites. We suggest that, in addition to playing a local role in LTP and dendritic spine development, RC3 also regulates neuronal metabolism by increasing the mobilization of internal Ca^{2+} stores in the vicinity of those mitochondria that are receptive to changes in cytoplasmic Ca^{2+}. In so doing, it would couple energy production with the synthetic events that accompany long-term structural changes in dendrites, dendritic spines and synapses.

Summary

Collectively, the work reviewed here suggests that RC3 and GAP-43 perform similar functions on opposite sides of the synapse. We propose that they buffer CaM levels and amplify the release of internal Ca^{2+} stores in response to mGluR receptor stimulation. RC3 and CaM dissociate slowly or rapidly, depending on the size of a Ca^{2+} flux and since CaM regulates RC3-mediated mobilization of internal Ca^{2+}, our model predicts that changes in CaM availability and amplification of agonist-induced mobilization of internal Ca^{2+} stores both exhibit nonlinear kinetics in the presence of RC3 or GAP-43. The system, therefore, displays the nonlinear properties of a capacitor. According to our model, the capacitance of the system, which is regulated by PKC-mediated phosphorylation, determines the LTP/LTD threshold. Furthermore, we suggest that RC3 regulates metabolism in a manner that is closely coupled to the synthetic requirements of RC3-mediated remodeling of synaptic spines that accompanies memory, learning, and LTP. Based on functional similarities and sequence homology, we propose that RC3 and GAP-43 constitute two members of a protein family that we have called the calpacitins. Since, RC3 and GAP-43 contain sequence homologies with PEP-19, and also appear to share several biochemical properties with this protein, we suggest that PEP-19 is also a member of this family, although it differs in that appears not to be regulated by PKC phosphorylation and not to be cell-compartment specific.

Note Added in Proof

Recently Juan Bernal and coworkers demonstrated that RC3 expression was regulated by thyroid hormone at the level of transcription in immortalized hypothalamic cell line GT1-7 (personal communication). Increased transcription of RC3 mRNA was induced by thyroid hormone after 6 h in the absence of protein synthesis, suggesting a direct transcriptional effect through nuclear receptors. Neither retinoic acid nor dexamethasone influenced RC3 expression.

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