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CALMODULIN IN NEUROTRANSMITTER AND HORMONE ACTION

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INTRODUCTION

Calmodulin (CaM) is a ubiquitous, multifunctional Ca^{2+}-binding protein with a molecular weight of approximately 17 kd that mediates Ca^{2+}-signaling in many organisms, cell types, and tissues. Since CaM was discovered as a Ca^{2+}-binding activator of cyclic nucleotide-dependent phosphodiesterase (1, 2) and adenylyl cyclase (3, 4), it was initially considered to be a Ca^{2+}-dependent modulator of cyclic nucleotide metabolism. The detection of an increasing number of CaM-binding proteins (CaM-BP) and CaM-dependent enzymes in conjunction with the development and use of CaM inhibitors have greatly expanded the known functions of CaM. CaM acts as a Ca^{2+}-dependent regulator of cyclic nucleotide metabolism, Ca^{2+}-transport, protein phosphorylation-dephosphorylation cascades, ion transport, cytoskeletal function, and cell proliferation (5–7). CaM activates isozymes of enzymes such as adenylyl cyclase, cyclic nucleotide phosphodiesterase, Ca^{2+}, Mg^{2+} ATPase, calcineurin, nitric oxide synthetase, and several protein kinases. CaM also binds to a number of other, predominantly cytoskeletal, proteins, including microtubule-associated protein-2 (MAP-2), fodrin (spectrin), MARCKS (myristoylated alanine-rich C kinase substrate), neuromodulin, neurogranin, caldesmon, adducin, tau, and tubulin. Studies on the crystal structure show that CaM is a symmetrical dumbbell-shaped molecule with two Ca^{2+}-binding domains on each end connected by an α-helical structure (8). The Kds for the binding of the four Ca^{2+} molecules to CaM are in the μM range, with

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two higher and two lower affinity sites (9, 10). Ca^{2+} binding to the low affinity sites is enhanced by two orders of magnitude in the presence of a CaM-binding enzyme (11). Ca^{2+}-dependent binding to many proteins or enzymes would occur primarily in response to cell activation as opposed to resting Ca^{2+} levels, although CaM can bind to several proteins in the absence of Ca^{2+}. Thus, hormones and neurotransmitters that elevate intracellular Ca^{2+} ($[Ca^{2+}]i$) can activate CaM-dependent processes that will contribute to the physiological response.

In general, hormones and neurotransmitters generate CaM-mediated responses in three ways, which are distinguished temporally and mechanistically. First, a rapid activation of CaM-dependent enzymes or binding of CaM to certain CaM-BPs is elicited in response to initial receptor-mediated increases in $[Ca^{2+}]i$. Second, persistent hormone or neurotransmitter stimulation can redistribute cellular CaM from membrane fractions to cytosol, or vice versa, in a process lasting from minutes to hours. This redistribution could affect the balance of CaM activity in a cell, significantly altering the CaM response and Ca^{2+} sensitivity in a select subcellular location. Third, on a long-term basis, such as hours to days, hormone or neurotransitter stimulation can lead to changes in total cellular CaM content, suggesting a long-term need for increased sensitivity of a Ca^{2+}/CaM-dependent process and/or a compensatory response to persistent changes in Ca^{2+}. Thus, CaM can participate in and be modulated by immediate and long-term responses to hormones and neurotransmitters. This review examines these three modes of activation and regulation of CaM activity by hormones and neurotransmitters, emphasizing the manner in which the CaM activity contributes to the physiological response to these agents.

RAPID ACTIVATION OF CALMODULIN-DEPENDENT ENZYMES IN RESPONSE TO HORMONES AND NEUROTRANSMITTERS

Although it has often been postulated that activation of Ca^{2+}-mobilizing receptors would activate various CaM-dependent enzymes or stimulate CaM binding to other CaM-BPs, this result has been demonstrated convincingly in vivo for relatively few proteins. Direct activation of several CaM-dependent protein kinases and the CaM-dependent phosphatase, calcineurin, has been demonstrated after activation of cells with Ca^{2+}-mobilizing hormones or neurotransmitters. The response of the kinases or phosphatase to a stimulus can be readily examined since specific substrates or specific sites on substrates for phosphorylation/dephosphorylation can often be identified. In addition, CaM can integrate receptor-induced Ca^{2+} mobilization and receptor-induced

cAMP actions through its activation of adenylyl cyclase and cyclic nucleotide phosphodiesterase.

Activation of CaM-dependent Phosphorylation-Dephosphorylation in Response to Hormone Stimulation

Neurotransmitter- and hormone-mediated activation of CaM-dependent protein kinases can be determined by analysis of a substrate specific for that kinase, a specific phosphorylated residue on a substrate or, in the case of CaM-Kinase II, by an analysis of the autophosphorylation activation characteristics of the kinase itself. Myosin light chain kinase from smooth muscle is a major target of Ca^{2+}/CaM and has a highly restricted substrate specificity. The enzyme phosphorylates a regulatory 20-kd myosin light chain that results in actin-activated myosin MgATPase activity and activation of contractile elements. No other physiological protein substrates or Ca^{2+}-binding proteins that activate the enzyme have been identified; CaM-dependent activation of the enzyme has thus been readily determined through myosin light chain phosphorylation following Ca^{2+}-mobilizing signals (12). Phosphorylation of myosin light chain in response to insulin in vivo was detected in IM-9 human B-lymphoblasts. CaM inhibitors blocked both the myosin light chain phosphorylation and insulin-receptor capping, suggesting that an actomyosin cytoskeletal contraction, regulated by CaM and myosin light chain kinase, is involved in insulin-receptor capping (13). Phosphorylase *b* kinase is a high molecular weight (M_r of 1.3 million) tetrameric (α, β, γ, δ) CaM-activated enzyme that also has a restricted substrate specificity. Phosphorylase is the primary in vivo target of the enzyme. The δ subunit is identical to CaM and is tightly bound to the enzyme in the presence or absence of Ca^{2+}. A second molecule of CaM can also activate the enzyme in a Ca^{2+}-dependent manner (14). The enzyme is activated in response to cyclic AMP-generating or Ca^{2+}-mobilizing hormones in skeletal muscle and liver (14, 15). Activation of the enzyme by the Ca^{2+}-binding protein, troponin C, has been demonstrated in skeletal muscle so that Ca^{2+}- dependent activation by CaM cannot be automatically assumed in that tissue (14).

In vivo activation of the multifunctional CaM-dependent protein kinase, CaM-Kinase II, has been demonstrated in response to activation of Ca^{2+}-mobilizing receptors. Originally discovered in brain as a Ca^{2+}-dependent protein kinase (16, 17), CaM-Kinase II was subsequently found to have a wide tissue distribution. CaM-Kinase II from most tissues consists of α (50-kd) and β or β' (62-kd) subunits in a holoenzyme of 6–12 subunits in variable ratios (18). Its substrates include enzymes such as tyrosine hydroxylase, tryptophan hydroxylase, and pyruvate kinase, cytoskeletal proteins such as synapsin 1, MAP-2, tau, and tubulin, and transcription factors such as CREB (cAMP response element binding protein) and

C\EBPβ, a member of the bZip family of transcription factors (19–22). CaM-Kinase II initially requires Ca^{2+}/CaM for its activity but, as Ca^{2+} levels increase, it phosphorylates its own subunits as well as its substrates (18). Autophosphorylation markedly increases the affinity of the enzyme for CaM and effectively "traps" CaM within the enzyme, allowing it to maintain 85% of its initial activity while at basal levels of Ca^{2+} (23). This permits prolonged enzyme activation after stimulated Ca^{2+} levels have decreased or new lower plateau levels of Ca^{2+} are attained. Stimulus-induced activation of CaM-Kinase II can be detected either as the phosphorylation of a specific site on a substrate (e.g. the tail region of synapsin I (24)) or by measuring the Ca^{2+}\CaM-dependent and CaM-independent enzyme activity after in vivo activation using a specific peptide substrate (25). Activation of CaM-Kinase II was demonstrated in hepatocytes in response to vasopressin by analysis of tryptic phosphopeptide maps of specific substrates (26). Direct activation of the CaM kinase has been demonstrated in response to the Ca^{2+}-mobilizing hormones bradykinin in PC12 cells (25) and thyrotropin releasing hormone in the rat pituitary cell line GH_3 (27), both by analysis of specific substrates and by measurement of autonomous enzyme activity. The primary effect of the hormones on CaM-Kinase II activity is transient, peaking within 10 sec, and correlates with the spike of Ca^{2+} released from intracellular stores (25). The ability of CaM-Kinase II to maintain activation in the absence of continued Ca^{2+} stimulus makes the enzyme a particularly attractive candidate to play a role in sensitization or memory processes, such as long-term potentiation (28). CaM-dependent protein kinase III is a 140-kd protein that specifically phosphorylates elongation factor-2 (eEF-2) in a variety of tissues (29). eEF-2 is a monomeric 100-kd protein that catalyzes the ribosomal translocation reaction. Rapid (5–10s) in vivo activation of this enzyme has been demonstrated in fibroblasts in response to mitogens (30). The time course of phosphorylation of eEF-2 closely paralleled the mitogen-stimulated Ca^{2+} transient.

Receptor stimulation can also lead to activation of a CaM-activated phosphatase. Protein phosphatase 2B, or calcineurin, has two subunits of 61 and 19 kd, is broadly distributed but has a narrow substrate specificity (5). Stimulation of N-methyl D-aspartate receptors in rat striatal slices leads to dephosphorylation of DARPP-32 (31), a potent inhibitor of protein phosphatase-1 localized primarily to dopaminoceptive cells (32). DARPP-32 is phosphorylated in response to dopamine and cAMP (*d*opamine and *c*AMP-*r*egulated *p*hospho*p*rotein) and is dephosphorylated on a specific threonine site by calcineurin (31, 32). This represents a distinct level of interaction between Ca^{2+} and cAMP whereby a Ca^{2+}-generated signal inhibits a cAMP signal. Phosphorylation and activation of DARPP-32 in response to cAMP

inhibits protein phosphatase activity. Dephosphorylation and inactivation of DARPP-32 by calcineurin would subsequently activate protein phosphatase 1, counteracting the cAMP effect. Calcineurin can similarly be activated to terminate Ca^{2+}-mediated signals. Nerve growth factor (NGF) increased phosphorylation of neuromodulin (GAP-43, F1, B50, p57), a growth-associated CaM-BP, in intact isolated growth cones at a site selective for phosphorylation by protein kinase C (PKC) (33). Dephosphorylation of neuromodulin in the intact growth cones was blocked by EGTA but not okadaic acid, suggesting that calcineurin activation was responsible for the dephosphorylation. Neuromodulin has been shown to be a substrate for calcineurin in vitro (34).

Effect of CaM on Neurotransmitter- and Hormone-stimulated cAMP Metabolism

CaM-dependent adenylyl cyclase has been widely studied, in part due to its abundance in brain and the possibility that it could modulate neurotransmitter-mediated adenylyl cyclase and thus synaptic activity. There is ample evidence in vitro, but little in vivo, that CaM can modulate neurotransmitter and hormone stimulation of adenylyl cyclase activity. There are presently five identified and cloned isozymes of adenylyl cyclase; types I and III are Ca^{2+} and CaM sensitive (35, 36). Type I (115 kd) is predominantly localized in brain and was originally purified as a CaM-sensitive adenylyl cyclase (37, 38). Although CaM directly activates the catalytic subunit and does not require GTP or the GTP-binding protein, Gs, for its activation, many studies suggest that CaM plays a role in neurotransmitter-sensitive adenylyl cyclase activity. In the central nervous system, CaM synergistically activates adenylyl cyclase with dopamine in rat and bovine striatum (39–41) and bovine retina (42) and norepinephrine in bovine cerebellum (43) and rat cerebral cortex (41, 44). The synergistic or potentiating effect is not at the level of the receptor, since CaM activation is also synergistic with activators of Gs and the catalytic subunit such as guanyl nucleotides (40, 45, 46), forskolin (43, 44) and cholera toxin (47). Using partially purified Type I adenylyl cyclase, purified Gs and CaM, Harrison et al (48) showed that the synergistic reaction occurred at the level of the catalytic subunit and that each activator, CaM and Gs, directly activated the catalytic subunit. It was demonstrated, using a photoaffinity-labeled CaM, that CaM does not bind to Gs and that activated Gs does not increase the binding of CaM to adenylyl cyclase (48).

The synergistic interaction between CaM and hormonal activation of adenylyl cyclase has been shown in peripheral tissues as well. CaM potentiated the activation of adenylyl cyclase by thyrotropin in the human thyroid gland

(49), by vasopressin in the pig kidney cell line, LLC-PK_1, (50) and in cultured renal papillary collecting tubule cells (51), by ACTH in Y-1 mouse adrenal tumor cells (52) and by α-melanocyte stimulating hormone in mouse B16 melanoma cells (53). CaM has also potentiates activation of adenylyl cyclase by prothoracicotropic hormone in the prothoracic glands of the tobacco hornworm, *Manduca sexta* (54). Activation of adenylyl cyclase by CaM has also been reported in kidney (55), heart, aortic smooth muscle, pituitary tumor cells, platelet, adrenal medulla, enterocytes, and pancreatic islets (see 56). The presence of CaM-sensitive adenylyl cyclase in nonneuronal tissues is controversial. Alternate explanations for Ca^{2+} and CaM stimulation include an action of Ca^{2+}-dependent proteases, CaM-mediated protection from Ca^{2+} inhibition or protein kinase C stimulation (57). While these criticisms are valid and must be considered, recent studies have provided some clarification on this issue. Using the clones for CaM-sensitive adenylyl cyclases, Xia et al (58) have found that the message for Type I adenylyl cyclase was selectively localized in nervous tissue, in brain, retina, and adrenal medulla. By contrast, Type III CaM-sensitive adenylyl cyclase, which was originally considered selective for olfactory tissue (36), was localized in brain and some peripheral tissues, including heart atrium, aorta, lung, and adrenal cortex (59). No message was found in some tissues, e.g. kidney, where CaM-sensitive adenylyl cyclase activity has been reported, so the above cautions must still be considered. mRNA for Type I adenylyl cyclase is highly concentrated in the neocortex and cells of the hippocampal formation that are implicated in learning and memory (60). A role for this enzyme in learning and memory is also suggested by the fact that the Drosophila learning mutant, *rutabaga,* is deficient in CaM-sensitive adenylyl cyclase (61) and by biochemical studies of stimulus convergence during classical conditioning in *Aplysia* (46). Adenylyl cyclase in mechanosensory neurons of *Aplysia* was synergistically activated by both Ca^{2+}/CaM and serotonin only when the Ca^{2+} pulse temporally overlapped the addition of facilitatory transmitter, suggesting that the synergistic response could play a role in classical conditioning in this system.

The Type I CaM-sensitive adenylyl cyclase is required for neurotransmitter inhibition in certain areas of brain. CaM was required for inhibition of adenylyl cyclase by opiates and adenosine in rat hippocampus, cerebral cortex, and cerebellum (62) and for adenosine P-site inhibition in cerebral cortex (63). On the contrary, Ca^{2+}\CaM had no effect on inhibition of adenylyl cyclase by opiates in the striatum (62). In reconstitution studies using purified porcine atrial muscarinic acetylcholine receptor, purified Gi and partially purified Gs-activated Type I adenylyl cyclase, adenylyl cyclase activated by Ca^{2+}\CaM was more sensitive to inhibition by carbachol than unstimulated or GTPγS-

activated adenylyl cyclase. Gi_α inhibited adenylyl cyclase only when the enzyme was activated by Ca^{2+}\CaM (64).

It is conceivable that a Ca^{2+}-mobilizing hormone or neurotransmitter would lead to CaM enhancement of an adenylyl cyclase-coupled receptor activity but this has only recently been demonstrated in vivo. The free Ca^{2+} required for CaM activation of Type I adenylyl cyclase measures 500 nM when fura-2 is used in the assay to measure free Ca^{2+} (48), indicating that a Ca^{2+}-mobilizing stimulus would be required for CaM to activate the enzyme. A CaM inhibitor and CaM antibodies inhibited the ACTH-stimulated increase in adenylyl cyclase in Y-1 adrenal tumor cells but the effect of CaM was attributed to a CaM-mediated potentiation of Gs-ACTH receptor coupling rather than a direct effect on the cyclase (52). In general, CaM-stimulated adenylyl cyclase has not been demonstrated in cultured cells, which would present a useful system in which to test receptor-potentiating effects of CaM and the interaction of Ca^{2+}-mobilizing hormones with adenylyl cyclase- coupled receptors. Although activation of Ca^{2+}-mobilizing muscarinic receptors leads to increases in cAMP in A9 L cells (65) and SH-SY5Y cells (66), CaM-sensitive adenylyl cyclase has not been convincingly demonstrated in those systems (see (67)). Activation of CaM-dependent adenylyl cyclase in response to phospholipase C-coupled muscarinic receptors, however, has recently been demonstrated in human embryonic kidney 293 cells transfected with DNA for Type I adenylyl cyclase. Muscarinic receptor stimulation did not increase cAMP accumulation in nontransfected control cells (68). mRNA for Type III adenylyl cyclase has been found in PC12 pheochromocytoma cells (59) and a very low level of enzyme activation by CaM was also reported (69), suggesting that these cells may be a useful system in which to investigate in vivo synergistic interactions among Gs-coupled receptors, Type III CaM-dependent adenylyl cyclase and phospholipase C-coupled receptors.

In contrast, activation of CaM-dependent cyclic nucleotide phosphodiesterase activity by CaM has been demonstrated in response to Ca^{2+}-mobilizing signals in vivo. At present, at least seven different isozymes of CaM-dependent phosphodiesterase, ranging from 59 to 75 kd, have been identified in a variety of tissues (70). Exposure of intact coronary artery strips to histamine or 50 mM KCl increased $[Ca^{2+}]i$, induced contraction, and increased the EGTA-sensitive phosphodiesterase activity (71). Activation of phospholipase C-coupled muscarinic receptors in dog thyroid slices (72) and 1321N1 cells (73) results in an inhibition of cAMP accumulation, which can be accounted for by a Ca^{2+} stimulation of CaM-dependent cyclic nucleotide phosphodiesterase activity. The phosphodiesterase was identified by comparing relative capacities of phosphodiesterase inhibitors to block the muscarinic-

mediated effect in vivo and CaM-stimulated phosphodiesterase activity in vitro (73).

ALTERATION OF CALMODULIN LOCALIZATION BY HORMONES AND NEUROTRANSMITTERS

A number of hormones and neurotransmitters alter the subcellular localization of CaM in a cell within minutes after the stimulus. The relative subcellular distribution of CaM varies from tissue to tissue (74). Although some tissues such as brain have a high CaM content (1–10 μM (74)), the large number and high concentration of many CaM-BPs in some cells make it unlikely that CaM is free; this has been shown experimentally (75). Further, CaM may vary in accordance with the concentration of its targets (6, 9–11). Binding of CaM to many CaM-BPs is predominantly Ca^{2+}-dependent, but Ca^{2+}-independent binding does occur (76–78). The fact that hormones and neurotransmitters can modify the distribution of CaM in cells suggests that an alteration in CaM binding and activity contributes to the physiological response to the stimuli.

Neurotransmitter-stimulated translocation of CaM was originally demonstrated in rat brain striatal membranes in response to cAMP and dopamine (DA) (79, 80). DA and D-1 receptor agonists stimulate a release of CaM from rat striatal membranes to cytosol within 15 to 90 min and can be mimicked by amphetamine and apomorphine treatment both in vivo and in striatal slices in vitro (Table 1). The redistribution process can be altered by modifying synaptic dopaminergic activity; amphetamine-induced translocation of CaM in vivo is accentuated in striata of rats that are behaviorally sensitized after repeated administration of amphetamine (81, 82) or haloperidol (M. E. Gnegy, unpublished results). The synaptic localization and the specific CaM-BPs affected are as yet unknown, although DA- or amphetamine-stimulated CaM translocation leads to a reduction of DA-activated adenylyl cyclase activity and an activation of a cytosolic EGTA-sensitive phosphodiesterase (80, 81, 83, 84). Other psychoactive compounds such as morphine, enkephalin and cocaine elicit a relatively rapid redistribution of CaM in neural tissue (see Table 1). As shown in Table 1, hormone-mediated CaM translocation has also been demonstrated in peripheral tissue. In dog cardiac sympathetic ganglia, for instance, a 30 min stimulation of preganglionic nerves or treatment with angiotensin increased cytosolic CaM in proportion to the heart rate increase (85). Steroid hormones alter the cellular localization of CaM but with a longer time frame. Calcitriol stimulates a translocation of CaM from cytosol to brush border membranes in intestine (86) and to myofibrils in skeletal muscle (87; see below). Stimuli leading to cell growth can induce a translocation of CaM into the nuclei. Estrogen treatment of immature chicks

enriched the proportion of CaM in liver nuclear matrix (88). $Alpha_1$ receptor-mediated release of Ca^{2+} from the endoplasmic reticulum during proliferative activation of rat liver elicited a transient peak of cytosolic CaM that subsequently translocated into the nuclear matrix by 12 to 16 hr after hepatectomy (89).

In most cases the molecular mechanism of the stimulus-induced CaM translocation is not known. The DA- and amphetamine-mediated translocation of CaM in rat striatum can be mimicked by cAMP and agents that increase cAMP (79, 80). Although it is assumed that cAMP is activating protein kinase A (PKA), the phosphorylated substrate has not yet been identified. PKA could phosphorylate CaM-BPs and decrease their affinity for CaM, as has been shown for phosphodiesterase (90) or myosin light chain kinase (91). Cyclic AMP could also affect the cellular Ca^{2+} flux by phosphorylating a Ca^{2+} channel or directly enhancing IP_3-induced Ca^{2+} release (92). Certainly, the likely candidate to effect a change in the subcellular localization of CaM is Ca^{2+}. Ca^{2+}-mobilizing hormones and neurotransmitters could alter the localization of CaM by increasing a Ca^{2+} flux in the cell and altering CaM binding to intracellular CaM-BPs. The Ca^{2+} concentration requirement for binding of CaM to a CaM-BP varies among the CaM-BPs (9) such that a Ca^{2+} pulse could dissociate CaM from binding to one protein and increase its binding to another. A sequential activation of adenylyl cyclase and phosphodiesterase by CaM in response to Ca^{2+} influx has been suggested since the Ca^{2+} requirements for activation of adenylyl cyclase by CaM may be lower than those for phosphodiesterase (6, 56).

CaM translocation in response to a Ca^{2+}-mobilizing hormone has been demonstrated in the human neuroblastoma cell line, SK-N-SH (93). In these cells, activation of m_3 muscarinic receptors leads to an immediate inositol trisphosphate (IP_3)-mediated peak influx of Ca^{2+}, followed by a plateau phase due to the influx of extracellular Ca^{2+} (94). Muscarinic stimulation led to a four- to fivefold increase in CaM in the cytosol, with an accompanying decrease in membrane-bound CaM. The redistribution was maximal by 15 min and was maintained for 2 hr before returning toward normal. Cytosolic levels of CaM remained elevated for at least 4 hr (94) but returned to normal levels within 2 hr in the presence of cycloheximide (L. A. Mangels, unpublished results). Both the Ca^{2+} ionophore ionomycin and a phorbol ester were required to equal the magnitude of carbachol-mediated translocation, suggesting that both IP_3 production and protein kinase C (PKC) activation are required for the translocation, although the phorbol ester had the greater effect. In addition, the PKC inhibitor, H-7, almost completely blocked the carbachol-mediated translocation. It therefore appears that muscarinic receptor activation resulting in a change in cellular Ca^{2+} flux and PKC-stimulated phosphorylation directs CaM localization in SK-N-SH cells. A similar result was

Table 1 Stimulus-induced calmodulin translocation

Agent[a]	Tissue/Cell	Subcellular distribution[b]	Time[c]	Reference
Dopamine	Rat striatal slices	M→C	60–90 min	80
Apomorphine	Rat striatum	M→C	60 min	138
Amphetamine 1.5 mg/kg	Rat striatum	M→C	40 min	84, 138
Morphine	Rat striatum	M→C	30 min	139
Leu-enkephalin	NG108-15	M→C	15 min	106
Cocaine	Rat striatum	C→M	30 min	140
Amphetamine 5 mg/kg	Rat striatum	C→M	40 min	138
Angiotensin II	Canine sympathetic ganglia	M→C	10 min	85
Gonadotropin releasing hormone	Rat pituitary	C→M	35 min	104
Carbachol	SK-N-SH	M→C	15–30 min	93
Bradykinin	PC12 cells	M→C	1 min	94a
Calcitriol	Chick intestine	C→brush border membranes	4–24 hr	86
	Chick myoblast cultures	C→myofibrils	4–12 hr	87
	Chick soleus muscle, in vitro	C→myofibrils	5–30 min	87
Estrogen	Immature chick liver	C→nuclear matrix	4 hr	88
Norepinephrine	Proliferating rat liver	C→nuclear matrix	12–24 hr	89

[a] Drug is given to whole animal or cell unless otherwise noted

[b] M represents a membrane preparation, generally a 100,000 × g pellet. C represents a cytosol preparation, generally a 100,000 × g preparation

[c] In in vivo brain studies, time CaM was measured in fractions after administration of drug to animal. In other studies, time of maximal effect.

demonstrated in PC12 cells where either a bradykinin-stimulated calcium flux or a PKC activator elicited a rapid increase in soluble CaM and activation of CaM-Kinase II (94a). While cAMP was a potent stimulus for CaM translocation in rat striatum, it was less effective in SK-N-SH cells. Ca^{2+} and PKC-stimulation increased cytosolic CaM by four- to fivefold in SK-N-SH cells, while dibutyryl cAMP and cAMP-producing transmitters increased cytosolic CaM by only 50% (67). It is therefore likely that the mechanism of neurotransmitters and hormones in redistributing CaM is cell-specific and depends upon the types and localization of CaM-BPs as well as changes in Ca^{2+} flux within the cell.

The PKC-phosphorylated substrate responsible for CaM release in SK-N-SH cells is not yet known but there are several possible candidates. Many CaM-binding proteins are PKC substrates, including neuromodulin (76), neurogranin (77), and MARCKS (95). The PKC-phosphorylated site on each protein is either near to or within the CaM-binding site such that PKC phosphorylation and CaM binding are mutually exclusive. CaM binding to MARCKS is Ca^{2+}-dependent but CaM binds neuromodulin and neurogranin with greater affinity in the absence of Ca^{2+}. The dual requirement for ionomycin and TPA to mimic carbachol in translocating CaM in SK-N-SH cells suggests that neuromodulin or neurogranin are the most likely PKC substrates involved in CaM translocation; both Ca^{2+} and PKC-mediated phosphorylation dissociate CaM from neuromodulin and neurogranin. Neuromodulin has been detected in SK-N-SH cells (93). Unlike MARCKS, which has a widespread tissue and cellular distribution, neuromodulin and neurogranin are neurospecific (34, 77). Neuromodulin is primarily localized in axons and terminals while neurogranin is concentrated in perikarya and dendrites. Neuromodulin has been implicated in the regulation of neurite outgrowth, adult neuronal plasticity, neurotransmitter release, and polyphosphoinositide metabolism (34). The high concentration of neuromodulin in brain (1 μM) and CaM-binding properties of neuromodulin have led to the assertion that neuromodulin sequesters CaM along the cytoplasmic surface of neuronal membrane domains until a Ca^{2+} and PKC signal causes it to dissociate (76). It is likely that neurogranin serves the same function postsynaptically.

Potential targets for CaM following muscarinic receptor-induced CaM redistribution in SK-N-SH cells have been examined through the introduction of a photoreactive CaM derivative (calmodulin-diazopyruvamide, CaM-DAP) into the cells using a scrape-loading technique (96). CaM-DAP was fully characterized and shown to exhibit Ca^{2+}-binding and enzyme activation properties identical to native CaM (97). Carbachol stimulated an increase in photoaffinity labeling of CaM-BPs with relative molecular masses of 55, 105, and 163 kd, after correcting for CaM-DAP binding. The time course of

labeling was distinct for the proteins. Carbachol-stimulated binding of CaM-DAP to the 55- and 105-kd proteins was maximal by 15–30 min, following the time course of CaM translocation, while maximal labeling of the 163-kd protein displayed a slower time course, with increases maintained for 2–4 hr. Using immunoblotting techniques, it was found that carbachol stimulated the binding of CaM-DAP to calcineurin (55 kd) and adducin (102 kd), a cytoskeletal protein, in a time-dependent manner. On the other hand, CaM-DAP bound equally well to phosphodiesterase in the presence or absence of carbachol, indicating that CaM can bind to phosphodiesterase at low $[Ca^{2+}]i$ or that the resting level of Ca^{2+} in the scrape-loaded cells is slightly elevated. The affinity of CaM for the phosphodiesterase is very high (23, 98), suggesting that a small change in either Ca^{2+} or CaM could result in activation of the enzyme. Thus, carbachol-stimulated CaM translocation in SK-N-SH cells may affect the activity of CaM-dependent enzymes and may alter aspects of cytoskeletal function. Indeed, one function of receptor-mediated CaM translocation could be to increase the Ca^{2+} sensitivity in a select localization of the cell. Increased CaM binding to an enzyme reduces the $[Ca^{2+}]$ required for maximal activation (9, 11).

Evidence from a number of systems suggests that CaM translocation may be integrally involved with a cytoskeletal alteration in the response to the transmitter. This altered response is suggested in SK-N-SH cells by the carbachol-stimulated binding of CaM to the cytoskeletal protein, adducin. Binding of CaM to adducin inhibits the ability of adducin to promote actin-spectrin associations (99). Since homogenization could disrupt many cytoskeletal elements, depending upon the contents of the homogenization buffer, translocation of CaM into the cytosol could represent binding of CaM to cytoskeletal elements that have been destabilized in response to stimuli or dissociation from membrane-bound elements. Many CaM-BPs are cytoskeletal proteins (5), and many of these are substrates for PKC. Neuromodulin is localized in the membrane skeleton in growth cones and neuronal cultures (34). MARCKS is a filamentous actin crosslinking protein whose activity is regulated by PKC-mediated phosphorylation and CaM-binding (100). MARCKS is postulated to represent a crossbridge between the actin cytoskeleton and the substrate-adherent plasma membrane during chemotaxis. Physiological activities such as neurosecretion and growth-factor-dependent mitogenesis are accompanied by cytoskeletal rearrangement and the PKC-dependent MARCKS phosphorylation. PKC activation can lead to the translocation of MARCKS from membrane to cytosol (101), but it is not known whether CaM is similarly redistributed under those conditions. In the intestine, calcitriol induces a redistribution of CaM to a membrane fraction, where it binds to brush border myosin I (102), a 110-kd protein linking plasma membrane and actin filament bundle in microvilli (78). Hormonal stimulation

can lead to increases in CaM and cytoskeletal CaM-BPs in the cell nuclei as well as in the cytosol. CaM translocation into the nucleus following liver cell proliferation (89) was followed by an increase in nuclear content of two CaM-BPs believed to be α-spectrin and myosin light chain kinase; this increase suggested that the translocated nuclear CaM could activate a nuclear contractile system during proliferative activation (103). The translocation of CaM could be involved in secretion or vesicle movement, processes known to involve the cytoskeleton. The GnRH-stimulated redistribution of CaM from cytosol to plasma membranes in pituitary gonadotrophs could be involved in GnRH-directed changes in distribution of secretory granules and clathrin-coated vesicles (104, 105). In SV40-transformed 3T3 cells, the virally induced increase in CaM correlated with an attenuation of microtubule assembly; CaM increases the sensitivity of microtubule disassembly to Ca^{2+} (105). Therefore, the redistribution of CaM could alter cytoskeletal structure, vesicular, ion or protein transport, and Ca^{2+} sensitivity in the cell resulting in changes in secretion, cell shape, motility, or cell proliferation.

NEUROTRANSMITTER- AND HORMONE-MEDIATED LONG-TERM CHANGES IN CALMODULIN CONTENT

Long-term or repeated treatment with hormones, neurotransmitters, neurotransmitter antagonists, or psychoactive drugs has led to increased CaM content in a variety of tissues. Increases in CaM in rat brain have been reported after repeated treatment of rats with antipsychotic drugs, amphetamine, morphine, and cocaine (Table 2) in areas appropriate to the neurotransmitter and receptor population affected by the drug (82, 106, 107). The percent change in CaM after most treatments is relatively modest (25–50%), but a small change in CaM could have profound effects on Ca^{2+} signal transduction events. Increases in the level of CaM may affect the Ca^{2+} set point for activation of intracellular processes. It is not yet known whether the increases in CaM are generalized within a certain area or a cell or are concentrated in select cells. Since many CaM-BPs, such as CaM-dependent phosphodiesterase (108), are localized in select brain cells, the physiological response to the increase will depend upon the cell in which CaM is increased and the area, such as a dendritic spine, in which it is increased.

The role of the elevated brain CaM after repeated treatment with psychotropic drugs is difficult to assess but may be related to drug-induced plasticity. Repeated treatment with amphetamine, morphine, cocaine, and antipsychotic drugs results in a behaviorally manifested sensitization to dopaminergic drugs (109, 110). A sensitization-inducing regimen of amphetamine led to an increase in CaM in rat striatum and limbic forebrain that exhibited characteristics (persistence and withdrawal-dependent increases)

Table 2 Long-term increases in calmodulin content induced by persistent treatment with neurotransmitters, drugs, or hormones

Agent	Dose	Tissue/Cell	Subcellular fraction[a]	Time[b]	Reference
Haloperidol	0.5 mg/kg, 20 d	Rat striatum	M	14 d	141
	0.5 mg/kg, 10 d		M	2–9 d	142
	1 mg/kg, 24 d		M	4 d	84
	1 mg/kg, 21 d		M	2 d	107
Amphetamine	2.5 mg/kg, 10 d	Rat striatum	C	10 d	143
	1.25 mg/kg, 21 d		C	2 d	107
	5 mg/kg, 21 d		M	2 d	107
	escalating dose, 4 wk		C & M	2d–4 wk	82, 143
	escalating dose, 4 wk	Rat limbic forebrain	M	2d–4 wk	82
Cocaine	30 mg/kg, 21 d	Rat striatum	M	30 min	140
Morphine	20–30 mg/kg, 5d	Rat midbrain, thalamus, cerebral cortex, striatum	M	24 hr	144
Melatonin	10^{-9}M	N1E-115 cells & MDCK cells	H	3–6 d	145
Thyrotropin	0.1 mU TSH/ml	Cultured porcine thyroid cells	H	3–7 d	124
PGE_2	50 μmol/l	Cultured porcine thyroid cells	H	3–7 d	124
Estrogen	40 μg/kg s.c.	Rabbit myometrium	C & M	6 d	118
Progesterone	800 μg/kg s.c.	Rabbit myometrium	C & M	10 d	118
Calcitriol	10^{-10}M	Chick embryo myoblasts	H	12 hr	136
NGF	50 ng/ml	PC12 cells	H	2–16 d	122

[a] The subcellular fraction in which the increase in CaM was measured, membrane, cytosol or homogenate.

[b] For haloperidol, amphetamine, morphine and cocaine, represents the time *after* the last dose of drug given. Length of time during which drug was administered is given under Agent. For other agents, represents the length of time agent was given to tissue, animal or cell.

similar to those of behavioral sensitization (82, 109). The behavioral sensitization elicited by these drugs is conceivably a form of synaptic plasticity; drug-induced sensitization to some psychoactive drugs shares characteristics and neurochemical changes with other forms of synaptic plasticity such as long-term potentiation and sensitization in Aplysia (persistence, enhanced neurotransmitter release, enhanced Ca^{2+} activities) (28, 109, 111). In contrast to the latter models of plasticity, alterations in Ca^{2+} fluxes have not yet been identified in drug-induced sensitization. Drugs known to block receptor-mediated Ca^{2+} flux into the cell, however, block the induction of amphetamine- and morphine-induced sensitization (109). CaM could play a role in several neurochemical changes reported to result from repeated treatment with the psychoactive drugs, such as increase in stimulus-induced neurotransmitter release (109) or morphological changes in synaptic structures (112, 113), both of which involve altered cytoskeletal interactions. A role for CaM in neurotransmitter release has been demonstrated through CaM-Kinase II-induced phosphorylation of the vesicular-located protein, synapsin I. CaM-Kinase II-mediated phosphorylation of the actin-binding synapsin I is proposed to relax cytoskeletal contraints and allow more vesicles to migrate toward the active zone in the terminal (24). Morphometric analyses have demonstrated changes in density of synapses, and changes in the areas of presynaptic terminals, dendritic spines, and postsynaptic densities in response to amphetamine or antipsychotic drugs (112, 113). Synaptic remodeling is considered to be important for information storage and has been demonstrated to require calcium-dependent processes (114). Dynamic changes in cytoskeletal proteins are proposed to regulate the shape of nerve cells and synapses as well as the movement of cytosolic components within the cell, processes known to involve CaM (115, 116). Therefore, an increase in CaM and cytoskeletal CaM-BPs could be involved with morphological alterations in the synapse that contribute to sensitization. In addition, CaM, through activation of CaM-Kinase II and phosphorylation of transcription factors (20–22), could contribute to changes in transcription, which is an important mechanism in plasticity (117).

Despite the finding that CaM content in cells may be normally constitutive in response to many hormones (105), increases in CaM content can be induced by hormones in a variety of systems (see Table 2). Changes in CaM-BPs may parallel long-term increases in CaM. In rabbit myometrium, a cycloheximide-sensitive increase in myosin light chain kinase activity paralleled that of CaM after estrogen or progesterone treatment (118). Consistent increases in $[Ca^{2+}]i$ could lead to compensatory increases in CaM with subsequent induction of CaM-BPs. Homeostatic increases in CaM-BPs are not a generalized consequence to rises in $[Ca^{2+}]i$ and CaM, however. Treatment of PC12 cells with NGF, which increases $[Ca^{2+}]i$ and CaM, results in a down-regu-

lation of CaM-Kinase III over a period of hours (119) that could result in a decrease in phosphorylation of eEF-2. Since NGF causes PC12 cells to pass to a differentiated state, down-regulation of CaM-Kinase III would prevent eEF-2 phosphorylation, permitting cells to stop dividing and differentiate from the G_1 stage (29).

The increases in CaM mass induced by calcitriol and NGF have been correlated with increases in CaM mRNA. Five mRNAs for CaM have been found in rat, mouse, and human tissues that are derived from three different genes (120, 121) and all code for a CaM identical in amino acid sequence. Selective regulation of CaM mRNAs has been reported (7, 122, 123). The possibility exists for regulation of specific CaM mRNAs, especially those of genes I and II, by second messengers since different palindromic sequence elements are found in some promotor regulatory regions (120). Two near-consensus cAMP regulatory elements and a consensus AP-2 element are found in the promotor-regulatory region of rat CaM gene II. Cyclic AMP increased CaM levels in cultured porcine thyroid cells (124) and PC12 cells (123) and may be the signal for the prereplicative surge of cytosolic CaM in proliferatively activated rat liver cells (125). NGF and cAMP selectively increased the transcripts from genes I and II in PC12 cells within 3 to 6 hr (123). CaM synthesis can follow an increase in $[Ca^{2+}]i$ (6, 7). Ca^{2+}/CaM can directly alter transcription through the CaM-Kinase II-mediated phosphorylation of the transcription factors, CREB (20, 21), and C\EBPβ (22). In PC12 cells, NGF increases the $[Ca^{2+}]i$, increases the CaM content and selectively increases a 1.7-kb transcript from the CaM II gene (122). These events appear to be mediated by cAMP; NGF activates PKA in PC12 cells (119), but the occurrence of Type III Ca^{2+}\CaM-dependent adenylyl cyclase in those cells could contribute to the cAMP response (59). Robust and rapid changes in transcription of a CaM gene have been demonstrated in response to touch in the plant *Arabidopsis*. Sensory stimulation leads to 10–100-fold increases in touch (TCH) genes, one of which appears to encode *Arabidopsis* calmodulin. TCH1 and other CaM-related TCH genes are elevated within 10 to 30 min after stimulation (126). The touch stimulus results in significant increases in cytoplasmic Ca^{2+}, underscoring the need for an induction of CaM-related genes to ensure the efficacy of Ca^{2+} as a second messenger and maintenance of cellular homeostasis (126).

ROLE OF CAM IN CALCITRIOL-MEDIATED CALCIUM UPTAKE IN INTESTINE AND SKELETAL MUSCLE

Just as it can be difficult to establish that CaM plays a role in the in vivo activation of an enzyme, it is also difficult to demonstrate that CaM plays an integral role in the physiological response to a hormone. In addition to being

activated in response to Ca^{2+}- mobilizing neurotransmitters, CaM may be directly involved in the physiological responses to a wide variety of hormones, including those that elicit steroidogenesis (52, 127–129) and calcitriol (86). A CaM requirement in stimulus-induced physiological response can be examined more easily in peripheral than central systems due to the availability of isolated cells or tissues, measurable physiological responses such as steroidogenesis, ion transport or secretion, and the ability to utilize in vivo treatment with CaM inhibitors. The use of designated CaM inhibitors has proved helpful in identifying a potential role of CaM in a physiological process but their lack of specificity can lead to erroneous conclusions and they must be used with caution. CaM inhibitors such as trifluoperazine, chlorpromazine, the napthalene sulfonamide, W7, and calmidazoleum inhibit other enzymes such as PKC, calpain, and Ca^{2+}, Mg^{2+} ATPase, block adrenergic or dopaminergic receptors, or have other nonspecific membrane effects (130–132). The phenothiazines are most notable in the number of other reactions that they affect. Although there are differences in the potency in which the drugs may affect CaM and another system, often higher overlapping concentrations of the drugs are used in vivo to ensure cell permeability. It is also possible for CaM to bind so tightly to an effector protein that the inhibitors cannot block the activity (133). Stronger evidence of CaM involvement is obtained when a blockade of a hormone-mediated process in vivo by a CaM inhibitor is coupled with a demonstrated effect of CaM on hormone action in vitro.

Studies on the role of CaM in calcitriol-mediated Ca^{2+} uptake in both intestine and skeletal muscle suitably illustrate issues of hormone-mediated changes in CaM and the role of CaM in the physiological response. A role for CaM in calcitriol-mediated Ca^{2+} uptake in intestine is suggested both by in vivo experiments using CaM inhibitors and by in vitro studies in purified brush border membrane vesicles (BBMV). The calcitriol-induced increase in Ca^{2+} uptake in chick duodenal BBMVs was paralleled by an robust increase in BBMV CaM content as well as an increase in duodenal Ca^{2+} transport in vivo (86). Ca^{2+} uptake was maximal at 4 hr while the CaM content increased to 24 hr. The lack of change in total CaM indicated a calcitriol-stimulated redistribution of CaM into BBMVs. The increased Ca^{2+} uptake was blocked by the active CaM antagonists, trifluoperazine and W7, but not by the less active W13 or inactive W5 and W12. The calcitriol-mediated increase in Ca^{2+} and CaM content in chick BBMVs was paralleled by a cycloheximide-insensitive increase in CaM binding to a 105-kd protein (102). CaM bound to this protein equally well in the absence and presence of Ca^{2+} and the localization of CaM and the 105-kd CaM-BP in the villus paralleled areas of maximal Ca^{2+} uptake (134). The 105-kd CaM-BP was identified as brush border myosin I (BBMI), an intestine-specific protein that exists as a helical array

of cross-bridges linking the plasma membrane and actin filament bundle in microvilli. The BBMI-CaM complex is a mechanochemical molecule containing ATPase activity that is involved in vesicle transport along actin rootlets and attachment of membranes to microvillar actin core (78). BBMI is a PKC substrate, but only when bound to membranes or vesicles through phosphatidylserine (78). Unlike neuromodulin or MARCKS, the CaM-binding site on BBMI is not phosphorylated by PKC. Instead, the site of Ca^{2+}-stimulated phosphatidylserine binding and CaM-binding site could overlap, such that BBMI binds either to phosphatidylserine or CaM. The molecular mechanism by which calcitriol effects BBMI phosphorylation or binding to CaM or phosphatidylserine in membranes has not been established. BBMI and CaM could participate in a vesicular transport of intestinal Ca^{2+}; it is postulated that Ca^{2+} is internalized in endocytic vesicles that travel to the basolateral membrane, where exocytotic release of vesicular contents occurs (135).

In skeletal muscle, as in intestine, calcitriol increases Ca^{2+} fluxes through a nongenomic as well as a genomic mechanism (87). In chick embryo skeletal muscle myoblasts and vitamin D-deficient soleus muscle, calcitriol-increased ^{45}Ca uptake was blocked by the CaM inhibitors, flufenazine and compound 48/80. In parallel with the increase in ^{45}Ca uptake, there was an increase of CaM in the microsomal, mitochondrial and myofibrillar fractions and a concomitant decrease in the cytosol (87). Calcitriol treatment of skeletal muscle myoblasts, however, increased CaM synthesis in addition to altering its distribution (136). Increases in CaM mRNA levels in the calcitriol-treated myoblasts paralleled a general calcitriol-mediated stimulation of [^{3}H]thymidine incorporation into DNA, suggesting it could be involved in the mitogenic action of the hormone. In soleus muscle calcitriol has a rapid action in increasing Ca^{2+} flux ($\leq$ 10 min) in contrast to the slower action in myoblasts ($\leq$ 24 hr). Both the calcitriol-induced Ca^{2+} uptake and the calcitriol-induced redistribution of CaM in soleus muscle are inhibited by the CaM antagonists, fluphenazine and compound 48/80. In the soleus muscle, calcitriol stimulated binding of CaM to two microsomal proteins of 28 kd and 30 kd within 1 min of exposure to the hormone (137). This binding was inhibited by treatment of microsomal membranes with a PKA inhibitor or alkaline phosphatase. This inhibition suggests that a PKA-mediated protein phosphorylation was responsible for CaM binding that could be involved in the rapid uptake of Ca^{2+} in the soleus muscle.

SUMMARY

Although CaM exists in abundance in many cells, it can be regulated by hormones and neurotransmitters on several levels in a variety of tissues and systems. Neurotransmitter action can lead to a rapid and direct activation of

CaM-dependent enzymes or binding of CaM to other CaM-BPs, while persistent stimulation results in a redistribution of CaM and CaM-BPs on a slightly longer time-scale. Long-term neurotransmitter or hormone action or changes in their activity due to drug intervention may lead to changes in cellular CaM content. Both the change in localization of CaM and the long-term increases in CaM content will result in an increase in the sensitivity of Ca^{2+}-related processes in select areas. The change in CaM content may be a homeostatic response that would signal an enhanced requirement and sensitivity for a Ca^{2+}/CaM-dependent process or a compensatory reaction titrating chronic changes in Ca^{2+} within the cell. Both CaM content and localization are highly responsive to changes in $[Ca^{2+}]i$, but other messengers such as cAMP play a distinct role in both processes. Many changes in CaM, both short and long-term, may involve rearrangements of cytoskeletal proteins, since many CaM-BPs have cytoskeletal localizations and binding of CaM to many of the proteins affects cytoskeletal protein-protein interactions. Therefore, changes in CaM distribution and content, besides altering the activities of the many CaM-dependent enzymes, could also be involved in restructuring in cytoskeletal processes, such as synaptic morphology, vesicular or protein transport, or secretion, that result from an initial neurotransmitter or hormone stimulation.

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