Ca\textsuperscript{2+}-Operated Transcriptional Networks: Molecular Mechanisms and In Vivo Models

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I. Introduction: The Encoded Calcium Signal

For the last 25 years, great effort has been devoted to understanding and characterizing the molecular mechanisms used by cells to transform a cytosolic Ca\textsuperscript{2+} signal into specific, finely controlled changes in gene expression. Several earlier reviews have addressed the variety of regulatory mechanisms that participate in Ca\textsuperscript{2+}-dependent gene expression in neurons (76, 208, 321) and other cells (27, 45, 179). Recent discoveries have added new elements to the “nuclear Ca\textsuperscript{2+} toolkit” and, just as important, new interactions have been reported between players that use Ca\textsuperscript{2+} to tune the function of different transcriptional networks. Furthermore, the spectacular rise of recently developed genetically modified animal models illustrates the universal, though selective, and crucial role of the calcium signal in the living animal. Advances in the understanding of the role of the calcium signal in regulating gene expression patterns in other organisms, mainly worms, yeast, and plants, have been recently reviewed (19, 68, 333) and are not discussed here.

Since the central protagonist in this review is the calcium signal, this introduction will comment on three major characteristics of the calcium signal that determine its final physiological role in the control of transcriptional networks: the specific temporal dynamics of the signal, the subcellular microdomains in which the signal is generated, and the nature of the calcium signal inside the nucleus.

In most cells, excitable and nonexcitable, stimulation of cell-surface receptors generates oscillations in intracellular free calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) of variable amplitude and frequency. The oscillatory nature of the cal-
calcium signal was from the beginning understood as a code to explain the different biological activities that it triggered in different cell systems. In a pioneering work, Lewis and colleagues (85) demonstrated that the oscillatory nature of the Ca\textsuperscript{2+} signal was in itself sufficient to enhance the nuclear detection of low-amplitude stimuli, and to reduce the effective Ca\textsuperscript{2+} threshold for activating transcription factors, while for high level of stimulation oscillations did not further increase the response (85). In trying to decipher the encoded information, it was soon obvious that frequency rather than amplitude was the important property of the oscillation for selective activation of one but not other transcriptional networks. Thus the Lewis and the Tsien groups showed in parallel that rapid oscillations stimulated nuclear factor of activated T cells (NFAT) and NF-κB-dependent transcription, whereas infrequent oscillations activated only NF-κB (85, 181). This effect of oscillation frequency has a physiological correlate in the differential inducibility at rapid and slow oscillations of interleukin (IL)-2 and IL-8, respectively (85), and it was clearly associated with the specific dynamics of posttranslational modifications and nuclear accumulation of these two nuclear effectors, fast and very transient for NFAT and much slower for NF-κB (reviewed in 129a).

It was long hypothesized that the site of Ca\textsuperscript{2+} entry determined the biological outcome of Ca\textsuperscript{2+} signaling (84, 121). In neurons, calcium flux through N-methyl-d-aspartate (NMDA) receptors or L-type calcium channels is a potent inducer of CREB phosphorylation and brain-derived neurotrophic factor (BDNF) expression. Ca\textsuperscript{2+} entry following glutamate bath application only slightly activates CREB and does not trigger BDNF expression (120, 279, 300). The apparent controversy was solved by Bading and co-workers (122), who showed conclusively that Ca\textsuperscript{2+} entry through extrasynaptic NMDA receptors opposes the increase in CREB phosphorylation and the induction of BDNF expression triggered by Ca\textsuperscript{2+} input through synaptic NMDA channels. Thus expression of BDNF in neurons is regulated by at least two different Ca\textsuperscript{2+} pools, each one associated with specific signaling cascades that lead to opposite biological effects. The mechanism(s) by which extrasynaptic Ca\textsuperscript{2+} shuts off CREB activation is not fully understood, but the action of a glutamate-regulated protein phosphatase 1 (PP1) able to specifically dephosphorylate Ser133CREB was shown in hippocampal neurons (268). This finding is consistent with previous reports (29, 119) and with the proposed role of PP1 in synaptic plasticity (7). How PP1 is specifically activated by extrasynaptic NMDA receptor stimulation and the cellular compartment where it dephosphorylates CREB are questions that remain to be elucidated.

In a recent follow-up report, Hardingham and co-workers (236) added further complexity to the calcium signal by showing that within the synaptic site of calcium entry, at least two completely different molecular pathways can be discriminated, conferring acute or long-lasting neuroprotection. Acute survival induced by Ca\textsuperscript{2+} entry through synaptic NMDA channels is thus not mediated by CREB-dependent transcriptional activity, but is exclusively related to the activation of the phosphatidylinositol (PI) 3-kinase/Akt pathway. Conversely, in the same study, the authors demonstrate that long-lasting activity-dependent neuroprotection requires CREB family activation and the elevation in nuclear Ca\textsuperscript{2+} or in nuclear Ca\textsuperscript{2+}/calmodulin signaling.

In nonexcitable cells, the first Ca\textsuperscript{2+} entry pathway identified was termed store-operated Ca\textsuperscript{2+} entry (SOCE), since it is stimulated in response to depletion of Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} stores in the endoplasmic reticulum (ER). SOCE is mediated via the activation of specific plasma membrane channels, termed store-operated calcium (SOC) channels, which are ubiquitously expressed in all cell types. However, and somewhat uniquely, the biophysical characteristics of the channel are different in various cell types. The first SOC channel to be identified, the calcium-release-activated calcium (CRAC) channel, mediates a highly Ca\textsuperscript{2+}-selective calcium current detected in T lymphocytes (9, 230). The molecular entity and the mechanisms that operate the activation of CRAC channels have remained elusive over the years that followed their functional identification (101, 180). Until recently, members of the mammalian TRP family of channels (named after the homologous Drosophila transient receptor potential channel) were considered to be good candidates for forming the CRAC channel (reviewed in Refs. 179, 237). However, TRP channels do not recapitulate the expected biophysical properties of CRAC currents in transfection experiments (247). Concerning the mechanisms for the activation of these currents, several theories were proposed including a direct conformational coupling with inositol trisphosphate (IP\textsubscript{3}) receptors (146, 159, 161, 195) or the involvement of a diffusive calcium influx factor (CIF) (252, 291).

Recently, several proteins that are essential for CRAC channel activity in Drosophila and in mammals have been identified, including the ER Ca\textsuperscript{2+} sensor Stim1 (186, 260) and the transmembrane protein Orai1/CRACM1, which is a pore subunit of the CRAC channel and forms the Ca\textsuperscript{2+}-selectivity filter of the channel (93, 249, 315, 334, 340). Transient overexpression of either Stim1 or Orai1 alone results in little or no increase in store-operated Ca\textsuperscript{2+} entry, while cotransfection of both proteins results in an increased store-operated Ca\textsuperscript{2+}-selective current (241). In basal conditions, the Stim1 protein is located in the lumen of the ER, where it senses Ca\textsuperscript{2+} levels. After store depletion, Stim1 relocates into preexisting and newly formed regions of junctional ER lying within 10–25 nm of the plasma membrane, while Orai1 accumulates at sites in the plasma membrane (PM) directly opposite to Stim1 (186,
There, Stim1 interacts with Orai1 (193, 210, 327). Taken together, these data suggest a model in which the elementary unit of CRAC is represented by ER-PM junctions (192). A similar coupling between the sarcoplasmic reticulum and the plasma membrane was previously described in skeletal and cardiac muscle directing excitation/contraction (299). While in the skeletal muscle there is a direct coordinated bidirectional interaction between plasma membrane L-type Ca²⁺ channels (LTCC) and the ryanodine receptor (RyR) in sarcoplasmic reticulum (221), in cardiac muscle calmodulin and Ca²⁺- and calmodulin-dependent protein kinase (CaMK) are required to functionally couple LTCC and RyR during cardiac excitation-contraction coupling (328).

The mechanism that regulates CRAC channels has been further advanced by the recent identification of Golli, a negative regulator of the CRAC activation pathway (92). Golli-deficient T cells show enhanced calcium entry after TCR stimulation, while golli−/− oligodendrocytes show altered Ca²⁺ transients that are associated with hypomethylination in the visual cortex and optic nerve (148). Myristoylation-dependent membrane association of Golli is essential for its inhibitory action after calcium influx in T cells (92).

Following the discovery of the Stim1 and Orai1 proteins, the potential role of TRP channels in calcium fluxes has been reevaluated, and an emerging role for TRP channels has been disclosed. Thus it has been shown that TRPC1 interacts with Stim1 in a store depletion-dependent manner and that Stim1 is required for the coupling between TRPC1 and type II IP₃ receptor (IP₃R) in platelets (188). Furthermore, it has also been shown that the structural features of Stim1 that are essential for Stim1-dependent activation of CRAC channels are identical to those involved in the binding and activation of TRPC1 in Jurkat T cells (137). Recent evidence showing that the formation of a TRPC1-Stim1-Orai1 ternary complex is essential for the generation of I₅SOC currents in salivary gland cells suggests a common molecular basis for CRAC in T lymphocytes and SOC in other cells (9, 230).

An additional important point, specific to exocrine cells, is the polarized/asymmetric nature of Ca²⁺ handling. Exocrine acinar cells are polarized, and it was therefore of interest to investigate whether Ca²⁺ extrusion has a uniform rate across both faces of the cell or whether there is preferential extrusion in one direction. It has been shown that in acinar cells the basal part is designed for Ca²⁺ uptake from the extracellular fluid into the ER, while the apical part is specialized for Ca²⁺ release from the ER to stimulate the formation of primary fluid and enzyme secretion into the acinar lumen (243). It is therefore favorable for the cells to restrict Ca²⁺ elevations to the apical/granular region. The signal is initiated at the apical pole (303) due to preferential localization of Ca²⁺ release channels in this part of the cell (176, 223). The major mechanism by which the signal is restricted to the apical part of the cell is sequestration of Ca²⁺ by active mitochondria that localize in a “belt” around the granular region (155, 288, 307). These mitochondria accumulate Ca²⁺ during agonist-evoked Ca²⁺ elevations (238).

Control of the activity of specific transcriptional networks by Ca²⁺ is regulated by cytosolic and/or nuclear mechanisms that decode the calcium signal specificities in terms of frequency and spatial properties. The regulation of Ca²⁺-dependent transcriptional networks does naturally demand the intervention of nuclear Ca²⁺, but existence of mechanisms that control free calcium concentration within this organelle independently of the cytoplasm remains to this day an unresolved question. A frequently proposed view is that calcium diffuses freely from the cytosol into the nucleus, and back, through nuclear pores (187). However, this view is challenged by a number of observations that support the alternative concept of independent nuclear Ca²⁺ homeostasis (81, 191 for a comprehensive review, but also a number of other specific contributions 197, 240; see Ref. 269). According to the alternative concept, the nuclear pores could be somehow gated and restrict the transient of Ca²⁺ under given physiological situations. About this, we could quote a number of observations, in diverse cell types, that have shown attenuation of cytosolic Ca²⁺ transients at the nuclear envelop, and the demonstration of single-channel currents in patch-clamped nuclear envelope containing dozens of nuclear pores. The idea of an independently regulated nuclear calcium compartment is conceptually important, as it would imply the existence of similarly independent regulatory mechanisms for the release of this calcium. More importantly, it would also allow us to consider calcium microdomains within the nucleus that would confer additional specificity to calcium effects on chromatin structure and gene expression. In a recent study that extends previous work by other laboratories (97, 103, 118), Nathanson’s group (88) identified a nucleoplasmic reticulum that is continuous with the ER and the nuclear envelope. Like the ER, the nucleoplasmic reticulum expresses IP₃Rs that mediate small calcium signals in localized subnuclear microdomains. This calcium signal then triggers the translocation of nuclear protein kinase C (PKC)-γ to the nuclear envelope in the vicinity of this domain (88). Interestingly, the mechanism can be preferentially triggered by growth factors such as hepatocyte growth factor and involves nuclear translocation of the growth factor receptor, induction of nuclear calcium signals, and the translocation of PKC-γ to the nuclear envelope without cytosolic PKC redistribution (88). It is presently not known whether increases in nuclear calcium control transcriptional networks through general mechanisms (see below) or if the specific localization of the signal in subnuclear compartments requires...
different mechanisms, such as localized changes at dinucleotide repeats in the DNA structure (83).

II. THE CALCIUM TOOLKIT FOR POSTTRANSLATIONAL MODIFICATIONS

The first known and best described set of mechanisms by which changes in the intracellular concentration of free calcium can affect gene expression is the activation of several kinases and phosphatases. Both in the cytosol and the nucleus, they introduce posttranslational modifications on appropriate molecules in the transcriptional machinery, resulting in modified expression of specific single genes or in readjustments of the activity of entire transcriptional networks.

A. Ca\(^{2+}\)/Kinase-Regulated Transcription Pathways

Several kinases are important mediators of calcium-dependent gene expression since, in one way or another, their activity is regulated by changes in calcium levels and their substrates are molecules that regulate gene expression. Among the kinases, our focus will be on two major groups, the CaMKs and the “conventional” PKC isoenzymes. A summary of the different Ca\(^{2+}\)-dependent kinases, their regulation, and specificities is portrayed in this section (Fig. 1). For a more detailed description, see monographic reviews (62, 139, 212, 239).

1. CaMKs

The CaMKs (CaMKI, CaMKII, and CaMKIV) are serine/threonine protein kinases with an NH\(_2\)-terminal catalytic domain and a COOH-terminal regulatory domain. The regulatory domain includes the Ca\(^{2+}\)/calmodulin (CaM) binding domain, which overlaps with an autoinhibitory domain such that autoinhibition is relieved upon Ca\(^{2+}\)/CaM binding. In addition, CaMKII has an association domain that permits CaMKII to form multimeric structures, whereas CaMKI and CaMKIV are monomeric enzymes. These kinases are all regulated by phosphorylation, although through different mechanisms. Whereas CaMKI and CaMKIV are phosphorylated by two Ca\(^{2+}\)/CaM-dependent protein kinase kinases (CaMKK\(_{\alpha}\) and -\(\beta\)), CaMKII binds Ca\(^{2+}\)/CaM and autophosphorylates at Thr-286. After phosphorylation, phosphoCaMKII shows a 1,000-fold increased affinity for CaM and becomes partially Ca\(^{2+}\)/CaM independent (reviewed in Ref. 134). In addition, CaMKII activity can also be regulated by proteins that have domains homologous to the CaMKII autoinhibitory domain (114), i.e., the NMDA NR2B subunit (23) or the Drosophila ether-a-go-go (Eag) potassium channel (294). In most cases, these interactions require prior autophosphorylation at Thr-286 to occur, and once in place, the enzymatic activity becomes also Ca\(^{2+}\)/CaM independent. The fact that kinase activity is preserved for some time after the initial stimulus has ended led to proposals that CaMKII might serve as a memory molecule at synapses and may underlie long-term changes in synaptic activity (reviewed in Ref. 113). The enzyme activity may terminate through the action of specific phosphatases and/or through other autophosphorylation events as is the case of CaMKII, in which autophosphorylation at Thr residues 305 and 306 blocks binding to CaM and inhibits activity of the kinase.

Four genes encode CaMKII isoenzymes. Of them, \(\alpha\) and \(\beta\)
are expressed preferentially in neuronal tissue, while \( \gamma \) and \( \delta \) are expressed in somatic cells (140). Another important distinction between CaMKs is their subcellular distribution. While at least one CaMKII isoform is predominantly a nuclear enzyme and CaMKIV is largely nuclear, CaMKI is exclusively cytosolic.

Among other substrates, CaMKs have been associated with activity-dependent direct phosphorylation of several transcription factors including CREB, CBP, and Ets-1, and to the nuclear translocation of NF-\( \kappa B \). In addition, a recent report associates CaMK's activity with the activity-dependent redistribution of SMRT, a corepressor protein related to transcriptional control through hormone receptors. Thus nuclear export of SMRT follows the nuclear extrusion of class II histone deacetylases (HDAC) and the process is blocked by the CaMKKK inhibitors N-62 and Sto-609 (205). Whether this is a direct mechanism remains to be clarified. It is conceptually important, however, since it could explain the \( Ca^{2+} \)-dependent potentiation of hormonal effects without direct intervention at the level of nuclear hormone receptors.

With such a pleiotropic role in the regulation of gene expression, it could be expected that genetic manipulation of CaMKs results in profound phenotypic changes. In the case of CaMKII\( \alpha \), which is highly expressed in the forebrain, genetic ablation results in deficient hippocampal long-term potentiation (LTP) (280), abnormal fear response and aggressive behavior (52), increased ischemia-induced neuronal damage (319), and defective plasticity in the primary visual cortex (109). Outside the brain, expression of a calcium-independent CaMKII\( \gamma \) mutant in T cells results in increased percentage of peripheral T cells with an antigen-dependent memory phenotype through a mechanism that involves NF-\( \kappa B \) activation (39). In keeping with a wider distribution of CaMKIV expression, removal of this gene results in strong phenotypic changes in the central nervous system (CNS) and in the periphery. Thus CaMKIV\( ^{-/-} \) mice are deficient in two forms of synaptic plasticity: LTP in hippocampal CA1 neurons and a late phase of long-term depression in cerebellar Purkinje neurons (129). Despite impaired LTP and CREB activation, CaMKIV/Gr-deficient mice exhibit no obvious deficits in spatial learning and memory, although emotional memory, which is associated with CREB activation in amygdala and cortex, is significantly reduced (320). Moreover, CaMKIV null mice display locomotor defects consistent with a significant reduction in the number of mature Purkinje neurons and an immature development of Purkinje cells (258). Use of CaMKIV inhibitors or expression of a kinase-dead CaMKIV mutant blocks \( Ca^{2+} \)-induced dendritic growth in cortical neurons, an effect that is strictly dependent on CREB phosphorylation (257). Outside the CNS, CaMKIV is highly expressed in the immune system and reproductive organs. Genetic deletion of the enzyme results in impaired positive selection in thymocytes and defective \( Ca^{2+} \)-dependent cytokine gene transcription in CD4\(+\) T cells (12, 251) as well as reduced male and female fertility due to defective spermatogenesis and impaired follicular development and ovulation, respectively (325, 326).

Nevertheless, the phenotype does not necessarily result from the lack of action on nuclear transcriptional effectors but could as well be related to the lack of phosphorylation of other substrates. Whereas phenotypic changes in neurons and memory CD4\(+\) T cells are clearly associated with impaired CREB phosphorylation in mutant mice, CREB-dependent transcription is perfectly normal in germ cells of CaMKIV\( ^{-/-} \) mice, suggesting that CaMKIV might use other targets in this cell type (325, 326).

2. \( Ca^{2+} \)-dependent PKC isoenzymes

To date, 11 closely related PKC isoenzymes have been described and classified into three subfamilies based on their domain structure and ability to respond to \( Ca^{2+} \) and DAG (226). The “conventional” PKC isoforms (\( \alpha, \beta I, \beta II, \) and \( \gamma \)) are regulated by DAG, which binds the C1 domain, and by \( Ca^{2+} \), which binds the C2 domain. In contrast, the “novel” PKC isoforms (\( \delta, \varepsilon, \eta, \) and \( \theta \)) are not regulated by \( Ca^{2+} \) but respond to DAG. The molecular structure of the novel PKC isoforms is similar to that of the classical isoforms except for differences in the \( Ca^{2+} \) binding domain. The third group of PKC isoforms includes the “atypical” PKC isoforms (\( \zeta, \kappa, \iota, \) and \( \mu \)), which are regulated neither by DAG nor \( Ca^{2+} \).

Elevated concentrations of intracellular \( Ca^{2+} \) activate conventional PKC isoforms, recruiting the enzyme to the inner leaflet of the plasma membrane or the nuclear envelope within minutes. This process has been associated with changes in gene expression by two different mechanisms: 1) the first involves activation of IKK and nuclear translocation of activated NF-\( \kappa B \) to activate cytokine gene expression and T-cell proliferation (123, 309). The initial finding identified PKC-\( \theta \) as responsible for the effect (183, 295). \( Ca^{2+} \)-dependent conventional PKC \( \alpha \) and \( \beta \)-isoforms were shown to activate NF-\( \kappa B \) transcription as well (267, 290). A later study identified PKC-\( \alpha \) action upstream of PKC-\( \theta \) to activate the IKK complex and NF-\( \kappa B \) in T lymphocytes following TCR activation (310).

The second mechanism of \( Ca^{2+} \)/PKC-mediated gene expression relates to increased nuclear activity of histone acetyltransferases (HAT). This mechanism has been described in epidermal cells where calcium induces epidermal cell differentiation and expression of several keratinocyte differentiation markers such as involucrin, filaggrin, loricrin, and transglutaminase (82). The process involves a dramatic increase in nuclear HAT activity and the specific acetylation of a subset of nuclear proteins. Calcium-induced HAT activity in epidermal cells is associated with...
CBP and P/CAF transcriptional coactivators, but only overexpression of P/CAF mimics the activation of the involucrin promoter and the specific nuclear acetylation. The effect of calcium on HAT activity is almost completely blocked by specific PKC inhibitors at concentrations that block only Ca\(^{2+}\)/DAG-dependent "conventional" PKC isoenzymes, suggesting that "conventional" PKC isoforms may phosphorylate P/CAF to direct epidermal cell differentiation (154). Calcium-induced involucrin expression nevertheless seems to require a calcium-dependent tyrosine phosphorylation of PKC-δ (78).

Genetic ablation of the different "conventional" PKCs rendered a variety of phenotypes, suggesting diversified hierarchy in different biological functions. Thus PKC-α\(^{-/-}\) mice showed cardiac hypercontractility, which protects against heart failure induced by pressure overload and rescues cardiomyopathy associated with overexpression of protein phosphatase 1 (35). B cells from PKC-β-deficient mice develop an immunodeficient phenotype that is related to their inability to activate IKK, degrade IκB, and upregulate NF-κB-mediated induction of the prosurvival protein Bcl-xL (267, 290). Finally, mice deficient in PKC-γ have a modified LTP of synaptic transmission in the hippocampus (1), and Purkinje cells show aberrant innervation from climbing fibers, while other aspects of the cerebellum including the morphology and excitatory synaptic transmission appear normal (152).

**B. Ca\(^{2+}\)/Phosphatase-Regulated Transcription Pathways**

As important as phosphorylation events, Ca\(^{2+}\)-dependent dephosphorylation of specific residues in key proteins within a given transcriptional network also induces drastic changes in the activity of the network. Interestingly, activity-dependent phosphorylation and dephosphorylation are in some occasions coupled events that act on a particular protein or even on a particular phospho residue. In those cases, the specificities of the calcium signal to activate the two events alternatively require a fine tuning that so far is not well understood. Two major phosphatases, calcineurin, also termed protein phosphatase 2B (PP2B), and PP1, will be discussed in detail in this section. A summary of the different transcriptional networks regulated by these Ca\(^{2+}\)-dependent phosphatases is shown in Figure 2.

1. **Calcineurin-regulated gene expression**

Calcineurin is a Ca\(^{2+}\)/calmodulin-activated serine/threonine phosphatase, with a catalytic A subunit that binds calmodulin and a regulatory B subunit that binds calcium. Calcineurin conveys cytosolic Ca\(^{2+}\) signals to the nucleus, regulating the activity of at least three important transcription pathways involving NFAT, TORC, and myocyte enhancer factor-2 (MEF-2) proteins (for reviews, see Refs. 27, 30, 64, 255; see also sect. III) that control responses in the immune, nervous, and cardiovascular systems. Calcineurin may also interfere with gene expression through regulation of the intracellular concentration of free calcium by dephosphorylating phospholamban (219), the endogenous regulator of the SERCA2a calcium pump, ryanodine receptors (53), as well as the IP\(_3\)R1 (43).

Three genes encode the catalytic subunit of calcineurin in mammalian cells: CnA\(_\alpha\), CnA\(_\beta\), and CnA\(_\gamma\). Genetic ablation of CnA\(_\alpha\) or CnA\(_\beta\) results in specific alterations of T-cell function, suggesting that the absence of one gene is not fully compensated by the others (37,

![Figure 2](http://physrev.physiology.org/)

**FIG. 2.** Molecular mechanisms of Ca\(^{2+}\)-dependent transcription: activity-dependent phosphatases. Representation of the main transcriptional networks regulated by Ca\(^{2+}\)/CaM-dependent protein phosphatases calcineurin and protein phosphatase 1 (PP1). For details about how these phosphatases regulate Ca\(^{2+}\)-dependent transcription, see section III in the text.
In support of distinct roles for the three proteins, lack of CnAα results in deficient T-cell response, while absence of CnBβ has profound effects on T-cell development, with fewer CD4/CD8 single positive cells and defective proliferative response. Furthermore, CnBβ-deficient mice show impaired cardiac hypertrophic response following pressure overload or isoproterenol infusion (38). The molecular basis of the functional specificity difference between α- and β-isoforms is not fully understood, although it could be related to differential expression or distinct ability to interact with endogenous inhibitors. Thus the specific regulation of the CnBα promoter by NFAT/GATA4 complexes was recently described (229), and several classes of endogenous inhibitors of calcineurin activity have been identified (see below).

Compared with other mechanisms of Ca\(^{2+}\)-dependent gene regulation, calcineurin has a particularly high affinity for Ca\(^{2+}\)/calmodulin and is at least one order of magnitude more sensitive to Ca\(^{2+}\)/calmodulin than is CaMKII. As a consequence, calcineurin responds to sustained, low-amplitude calcium transients, whereas CaMK-dependent gene expression is used preferentially in response to transient, high-amplitude calcium spikes (84, 304). It was shown, however, that adequate calcium levels for calcineurin effects on gene expression may require calreticulin-mediated supply from the endoplasmic reticulum; embryonic lethality in calreticulin deficient mice is thus reversed by cardiac-specific overexpression of calcineurin (194). Furthermore, overexpression of activated calcineurin is associated with an intermediate phase of LTP in CA1 neurons of the hippocampus (324) and with defective long-term memory, evident in both a spatial task and a visual recognition task (199). Conversely, transient inhibition of calcineurin facilitates LTP, enhancing learning and strengthening short- and long-term memory in several hippocampal-dependent spatial and nonspatial tasks (199). Taken together, these data are consistent with the idea that endogenous calcineurin restrains LTP and memory. On the other hand, calcineurin B mutant mice have defects in axonal outgrowth but die at E10.0 due to failure to correctly pattern the developing vascular system (110).

Calcineurin activity is regulated by its interaction with several endogenous proteins. The first inhibitor identified, AKAP79 (A-kinase anchoring protein-79), serves as a scaffold protein that anchors calcineurin to the membrane together with protein kinases A or C (160). Other inhibitors, such as Cabin1/Cain, A238L, and RCS (regulator of calmodulin signaling), function by blocking calcineurin binding to physiological substrates (170, 216, 250, 293), or by mimicking the interaction of calcineurin with its regulatory subunit calcineurin B as is the case of the CHF/p22 protein (184). More recently, a family of regulators of calcineurin (RCAN)-1 to -3 has been described (70, 157). RCAN-1, also known as calcipressin-1 (Csp-1), MCIP-1, and Adapt78, is the protein product of the RCAN-1 gene previously known as DSCR1 because it is localized in the Down's syndrome critical region (90, 98, 261). RCAN-1 is highly expressed in the CNS, immune system, heart, and skeletal muscle and was shown to interact physically and functionally with CnB, inhibiting both NFAT and MEF-2 signaling pathways (reviewed in Ref. 263). It has been proposed that RCAN-1 expression is regulated through a calcineurin-dependent mechanism that involves a cluster of NFAT binding sites in the promoter (reviewed in Ref. 263), indicating that RCAN-1 could function as a feedback inhibitor of calcineurin. Furthermore, it was demonstrated that glycogen synthase kinase-3 (GSK-3)-mediated phosphorylation of RCAN-1 reverses its effect on calcineurin signaling (127) and that RCAN-1 is repressed by the basic helix loop helix (bHLH) protein Hes-1 after Notch1 activation (198). On the other hand, RCAN-2, also known as Csp-2, MCIP-2, and ZAKI-4, is not regulated by calcium, but its expression in striated muscle is absolutely dependent on thyroid hormone levels (332).

Work with RCAN-1\(^{-/-}\) or RCAN transgenic mice indicates that RCAN-mediated inhibition of calcineurin activity may represent a superimposed control of the calcium signal by finely tuning the calcineurin response to the calcium signal. Thus a high-threshold gene, such as Fas ligand (87), is kept silent under moderate TCR stimulation (265), and cardiac hypertrophy is blocked after calcineurin stimulation without repression of the induction of B-type natriuretic peptide (BNP), a low-threshold calcineurin target gene in the heart (128). RCAN-1\(^{-/-}\) mice show premature cell death of Th1 lymphocytes during TCR-mediated proliferation due to aberrant expression of Fas ligand and enhanced responses in several models of cardiac hypertrophy (reviewed in Ref. 313). Conversely, targeted overexpression of RCAN-1, Cain/Cabin-1, or AKAP-79 in myocytes inhibits cardiac hypertrophy and preserves the systolic function (72, 128, 262).

Calsarcins, a family of calcineurin-interacting proteins expressed specifically in striated muscle, also inhibit calcineurin (96). Calsarcin-1-deficient mice show an excess of slow muscle fibers and develop exaggerated hypertrophy in response to mechanical stress, which is associated with marked activation of a cardiac fetal gene program (95). The fact that calsarcin-1-deficient mice show induction of the fetal gene program but not cardiac hypertrophy in response to mechanical stress, which is associated with marked activation of a cardiac fetal gene program (95). The fact that calsarcin-1-deficient mice show exacerbation of hypertrophy in basal conditions and do not respond to other hypertrophic stimuli such as chronic isoproterenol administration or exercise, supports the concept that the calcineurin response to a given calcium signal is tuned to different thresholds by the specific action of calcineurin inhibitors that modulate the physiological or pathological response (265).

Recently, an endogenous inhibitor of calcineurin, named Carabin, functions as a true feedback inhibitor.
since its expression is upregulated after TCR stimulation and calcium entry (235). Carabin induction is sensitive to inhibitors of calcineurin, indicating that Carabin constitutes part of a negative regulatory loop for the intracellular TCR signaling pathway. Knockdown of Carabin by short interfering RNA led to a significant enhancement of IL-2 production by antigen-specific T cells in vitro and in vivo (235).

The most significant and best-characterized mechanisms by which calcineurin influences transcriptional networks involves dephosphorylation of NFAT and TORC and the activation of MEF-2 proteins (30, 65, 201, 274, 336). While in the first case the effect of calcineurin is a direct enzymatic action on NFAT and TORC proteins, in the second case, calcineurin regulates the activity of MEF-2 transcription factors by three different mechanisms that are discussed in detail in section II B.

2. PP1-regulated gene expression

The calcium/calcineurin-dependent type 1 serine/threonine protein phosphatase (PP1) holoenzyme consists of a highly conserved catalytic subunit and one or more regulatory subunits. Evidence accumulated in recent years indicates that PP1 activity is modulated largely by its interaction with ~70 different auxiliary proteins that target the enzyme to distinct subcellular compartments, conferring substrate specificity (21, 138). A subset of these interacting proteins are inhibitory proteins that block the catalytic activity of PP1. These include PP1 inhibitors 1 and 2, DARPP-32 (220, 331), as well as PNUTS and NIPP-1, two inhibitory subunits of PP1 that direct the nuclear targeting and retention of the enzyme, regulating PP1 nuclear activity (8, 178). Binding to PP1 and inhibition of its activity are regulated by PKA phosphorylation in both cases (28, 156), while calcineurin dephosphorylates inhibitor-1 blocking its activity which results in PP1 activation (89). PNUTS also binds to chromatin and enhances in vitro chromosome decondensation in a PP1-dependent manner (171). Dephosphorylation of inhibitor 1 by other non-calcium-dependent phosphatases like PP2A should result in calcium-independent PP1 activation (89).

A major role of PP1 activity in the nucleus is to dephosphorylate the transcription factor CREB (44) and terminate CREB-dependent gene expression. The mechanism involves the specific interaction of PP1 with HDAC1, which in turn recruits the phosphatase to the proximity of phosphoCREB, allowing its dephosphorylation (44). Conditional overexpression of PP1 protein inhibitor-1 in mice prolongs CREB phosphorylation and improves performance in an object recognition task (102). Strikingly, the improvement was observed only when mice were exposed to a protocol of distributed training with brief intertrial intervals, suggesting that the interference with the decay of CREB phosphorylation specifically mimicked the improved acquisition associated with long intertrial protocols (102). Furthermore, conditional inhibition of PP1 activity during training also improved the information retention for up to 6 wk after intensive training in the water maze test, supporting an active role of PP1 in memory decline. Overexpression of PP1 protein inhibitor-1 also resulted in extended autophosphorylation of CaMKII at Thr-286, a finding previously observed in vitro after local application of thioprophosphorylated inhibitor-1 and associated with improved LTP at the Schaffer collateral-CA1 synapse (33).

III. CALCIUM-DEPENDENT TRANSCRIPTIONAL NETWORKS

A number of nuclear effectors whose action is regulated by changes in intracellular Ca$^{2+}$ have been described over the years. The growing list includes proteins, which are targets of calcium-dependent posttranslational modifications as well as others, whose nuclear activity or presence is regulated by the interaction with Ca$^{2+}$ or calcium binding proteins. This section reviews recent developments on the diversity of molecular mechanisms governing these calcium-sensitive transcriptional networks.

A. The CREB/CBP/TORC Pathway

It has long been accepted that Ca$^{2+}$- and cAMP-dependent pathways that control gene expression share many common players and points of cross-talk. Indeed, the accepted view involves several coincident steps, including the phosphorylation at Ser-133 of DNA-bound CREB dimers and recruitment of the coactivator paralogs CBP/p300 to trigger transcription of CRE-containing target genes (reviewed in Ref. 204). Recent studies have added new players and provided new steps into the process, some of which are strictly dependent on changes in intracellular Ca$^{2+}$ levels.

Binding of CREB as a dimer to the canonical CRE site (TGAGCTCA) has been considered the default start of the process. Work in Goodman’s laboratory using chromatin immunoprecipitation and in vivo genomic footprinting assays showed, however, that occupancy of CRE sites is regulated in a cell-specific manner and that the ability of CREB dimers to bind to a particular CRE represents an important component of gene regulation (48). In addition, core promoter configuration, including the presence of a TATA box motif and the vicinity to, and methylation state of consensus cAMP response elements near the promoter, are key factors in deciding the proportion of CREB target genes that will respond to cAMP in a given cell type (61, 342). Thus, before CREB is phosphorylated in response to...
a given stimulus in a particular cell type, genetic and epigenetic factors determine whether that cell will respond to the stimulus with an upregulation of CRE transcription just by allowing or preventing the CREB-controlled machinery to approach the specific promoter.

Evidence has accumulated showing that Ser-133 CREB phosphorylation can occur through several kinases including PKA, PKC, mitogen-activated protein kinases (MAPKs; ERK and p38), CaMK, and CaMKK. As mentioned above, Ca²⁺/calcineurin-dependent phosphorylation of PP1 terminates CREB activation and stops CRE-dependent transcription (44). Both processes have been reviewed extensively (77, 108, 204).

CBP/p300 recruitment to Ser-133 phosphoCREB is favored by signaling-dependent modifications of CBP by several kinases, including Ca²⁺- and CaMKIV-dependent phosphorylation of CBP at Ser-301, e.g., following NMDA receptor activation in cultured hippocampal neurons (135, 143). Nevertheless, serine to alanine mutation of residue 301 attenuates but does not block CREB/CBP-dependent transactivation, indicating that additional CaMKIV phosphorylation sites, particularly in the COOH terminal, or other kinases, including CaMKII, may as well participate in activity-dependent CBP induction. The Ca²⁺-dependent phosphorylation of CBP is thus an additional check-point controlling CREB/CBP-dependent transcription, although the mechanism by which phosphorylation of CBP affects its transactivating properties is not known. An intriguing possibility is that Ca²⁺-dependent CBP phosphorylation modifies its HAT activity, as previously shown for the P/CAF protein (154). In support of this mechanism, impaired chromatin acetylation in heterozygous CBP⁺₋/- mice or after inducible expression in the forebrain of a mutant form of CBP that lacks HAT activity, CBP(HAT⁻/⁻), is related to long-term memory impairment that is reversed by pharmacological inhibition of histone deacetylases (5, 166). Overexpression of constitutively active CREB or treatment with rolipram, a phosphodiesterase-4 inhibitor that enhances CREB activation, partially rescues this phenotype (5, 34).

Nonetheless, CREB phosphorylation alone is not a reliable predictor of target gene activation, and additional CREB regulatory partners are required for recruitment of the transcriptional apparatus to the promoter. It has been shown that calcium signals destabilize the CREB-CBP complex through secondary phosphorylation events on CREB (164) and that the DNA binding/dimerization domain (bZIP) in CREB mediates transcriptional response to both calcium influx and cAMP (278). In this case, the bZIP appears to contribute significantly to induction of CREB activity in response to membrane depolarizing signals, implicating this domain in CREB association with a calcium-regulated coactivator. Indeed, search for proteins able to interact with CREB and to induce CREB activity identified a conserved family of coactivators, designated TORCs, for transducers of regulated CREB activity, that enhances CRE-dependent transcription via a phosphorylation-independent interaction with the bZIP DNA binding/dimerization domain of CREB. TORC recruitment does not appear to modulate CREB DNA binding activity, but rather enhances the interaction between the glutamine-rich transactivation domain of CREB and the TAFII130 protein, a component of the TFIIID complex, following TORC recruitment to the promoter (61, 145).

The three TORC family members share an NH₂-terminal coiled-coil domain that associates as a tetramer with the bZIP domain of CREB. Contrary to CBP, expression levels of TORC proteins are very low in the cell. In basal unstimulated conditions, TORC proteins are localized mainly in the cytosol, which makes it difficult to understand their crucial role as limiting factors for CREB transcription. Later studies from the two laboratories involved in the identification of TORC proteins defined a “signaling module” that mediates calcium- and cAMP-dependent, phosphorylation-independent activation of the CREB transcription factor. The pathway involves the calcium-regulated phosphatase calcineurin and the Ser/Thr kinase SIK2, both of which associate with TORC2 in pancreatic islet cells. Under resting conditions, TORC2 is sequestered in the cytoplasm via a phosphorylation-dependent interaction with 14-3-3 proteins. Following glucose and gut hormone stimulation, calcium influx increases calcineurin activity triggering TORC2 dephosphorylation, its release from the interaction with 14-3-3 proteins, and its nuclear translocation. Concomitant stimulation of the cAMP pathway after glucose and hormone action inhibits SIK2 kinase activity, reducing TORC2 phosphorylation. Through this mechanism, the concerted action of a phosphatase/kinase module connects two signaling pathways in response to nutrient and hormonal cues (30, 274). In a recent report, Montminy’s group shows that hormonal- and energy-sensing pathways converge on the coactivator TORC2 to modulate glucose output. Under fasting conditions or after glucagon administration, TORC2 thus translocates to the nucleus and enhances CREB-dependent transcription. Conversely, signals that activate the energy-sensing kinase AMPK inhibit hepatic gluconeogenesis by promoting TORC2 phosphorylation and nuclear export. These results suggest that fasting hyperglycemia associated with elevated gluconeogenesis in type 2 diabetes could potentially be treated with compounds that enhance TORC2 phosphorylation (162). A key role for TORC proteins in muscle cells has been described to induce the expression of PGC-1α, a master regulator of mitochondrial biogenesis and energy metabolism (329).

Finally, also in line with the phosphorylation-independent regulation of CREB activity, a Ca²⁺-dependent interaction between transcriptional repressor DREAM and CREB or phosphoCREB regulates the accessibility of
CBP to the KID domain in CREB, and so compromises CRE transcription (174). This mechanism, which is described in detail later in this review, constitutes together with the Ca\(^{2+}\)-dependent modulation by TORC proteins a new possibility to modulate CREB activity independently of its phosphorylation state.

**B. The MEF-2 Crossroad of Ca\(^{2+}\) Regulation**

The MEF-2 family of myogenic transcription factors (MEF-2A to D) is an important determinant in muscle differentiation and T-cell activation through the transcriptional control of specific target genes (31, 336). It was found that MEF-2A and MEF-2C are highly expressed also in postmitotic neurons of the cerebellum and the cerebral cortex, respectively, and that MEF-2 expression is essential for the survival of these neurons (200). Transcriptional actions of MEF-2 proteins are regulated by a variety of mechanisms. In resting conditions, MEF-2 proteins are bound to DNA as part of a repressor complex that includes histone deacetylases and calmodulin binding proteins among others (189). HDAC4 and -5 interact with the so-called MEF-2 domain, located next to the MADS box in the NH\(_2\) terminal of MEF-2 proteins (189, 215, 318), resulting in the repression of MEF-2 transcriptional activation, e.g., of the c-jun promoter (318). MEF-2B and -D are normally sequestered by cabin1 (190) or the MEF-2 interacting transcriptional repressor (MITR) (285) in multiprotein complexes together with HDAC1. Cabin1 recruits chromatin-modifying enzymes, both histone deacetylases and a histone methyltransferase to repress MEF2 transcriptional activity (149, 162). As a result, transcription of target genes like nur77 is also repressed (337), and myoblast differentiation is blocked (190). HDAC4, cabin1, and MITR are Ca\(^{2+}\)/CaM binding proteins that bind calmodulin with the same domain responsible for binding of MEF-2 proteins (337, 338). Thus activity-dependent increase in nuclear Ca\(^{2+}\)/CaM complexes after stimulation releases MEF-2 proteins from the repressor complex (337, 338). This Ca\(^{2+}\)-dependent mechanism is partially counteracted in the case of MEF-2D by increased cAMP levels (26). In addition, Ca\(^{2+}\)-dependent phosphorylation of the MEF-2 domain by CaMKI and CaMKIV disrupts HDAC-mediated repression and releases MEF-2 (189, 190). Both CaMKI and CaMKIV can phosphorylate HDAC4 and -5, creating a phosphodomain that binds to 14-3-3 proteins and mediates shuttling of HDACs from the nucleus to the cytosol as well as releases repression from MEF-2 (205, 206). Noteworthy, a negative-feedback loop between MEF-2 proteins and HDAC9 has been recently disclosed by showing that HDAC9 is a direct transcriptional target of MEF-2 in myocytes, where HDAC9 associates with MEF2 proteins to suppress their transcriptional activity (117).

Activation of nonrepressed MEF-2 proteins is also a Ca\(^{2+}\)-dependent process, for which two major mechanisms have been proposed. First, as shown in cerebellar neurons by Greenberg’s group, calcium influx through voltage-sensitive calcium channels triggers activation of MKK6-p38 MAPK resulting in the phosphorylation of Ser-387 located in the transactivation domain of MEF-2C (202). Second, the Ca\(^{2+}\)/CaM-dependent phosphatase calcineurin activates MEF-2 through a posttranscriptional mechanism that either increases its DNA binding activity in cerebellar granules (201) or triggers interaction of MEF-2 and NFAT proteins and recruitment of CBP to these complexes in T lymphocytes (32, 336). Ca\(^{2+}\)/CaM-dependent signaling thus prevents formation of the MEF-2/HDAC complexes, induces nuclear export of class II HDACs, activates MEF-2-dependent transcription, and as a result stimulates cytokine expression, myogenesis, and neuronal survival. An integrated view of different Ca\(^{2+}\)-dependent mechanisms regulating MEF-2 transcriptional activity is shown in Figure 3.

Abrogation of MEF-2C by homologous recombination in mice leads to early embryonic lethality at E9.5 due to cardiac defects (182). Specifically, mutant mice lacking the MEF-2C gene show a severely hypoplastic right ventricular chamber and outflow tract, suggesting that these cardiogenic regulatory factors may act in a common pathway for development of the anterior heart field and its derivatives. It was recently shown that expression of the BOP nucleoprotein in the developing heart depends on the direct MEF-2C binding to a MEF-2-responsive element in the Bop promoter. Consistent with this, mice deficient in the BOP transcriptional activity reproduce the MEF-2C\(^{-/-}\) phenotype. MEF-2C is thus necessary and sufficient to recapitulate endogenous BOP expression in the anterior heart field and its cardiac derivatives during mouse development. The Bop promoter also directs transcription in the skeletal muscle lineage, but only cardiac expression is dependent on MEF-2C. These findings identify Bop as an essential downstream effector gene of MEF-2C in the developing heart and reveal a transcriptional cascade involved in development of the anterior heart field and its derivatives (246).

Although MEF-2C is the only family member shown to have a role in early heart formation, MEF-2A is the predominant mef2 gene product expressed in postnatal cardiac muscle. In accordance with this, most mice lacking MEF-2A die suddenly within the first week of life and exhibit pronounced dilation of the right ventricle, myofibrillar fragmentation, mitochondrial disorganization, and activation of a fetal cardiac gene program (224). The few MEF-2A\(^{-/-}\) mice that survive to adulthood show a deficiency in cardiac mitochondria and susceptibility to sudden death. These MEF-2A-mutant mouse phenotypes reveal an essential role for MEF-2A in maintenance of mitochondrial content and cytoarchitectural integrity in
postnatal cardiac myocytes and show that other MEF-2 isoforms cannot fully support these activities. Surprisingly, MEF-2 transcriptional activity, revealed by the expression of a MEF-2-dependent reporter transgene, is enhanced in the hearts of MEF-2A-mutant mice, reflecting the transcriptional activation of residual MEF-2D. The MEF-2A-mutant mouse phenotype clearly differs from that of MEF-2C mutants, which die at E9.5 from severe cardiovascular abnormalities. In light of the overlapping expression patterns and similar DNA-binding activities of the four vertebrate Mef2 gene products, it is likely that these proteins have distinct as well as partially redundant functions.

The generation of conditional MEF-2CloxP/loxP mice recently showed that myocardial-specific removal of MEF-2C results in viable offspring, demonstrating that whereas MEF-2C is required for early development of the heart, it is not necessary for the formation of the heart after looping morphogenesis (316). As MEF-2C is also highly expressed in postmitotic neurons, smooth muscle cells, and skeletal muscle precursors, MEF-2CloxP/loxP mice could serve as a useful tool for dissecting the role of MEF-2C in these various cell types.

The NFAT family of transcription factors, NFATc1 to -c4 and NFAT5, also known as TonEBP, are proteins evolutionarily related to the Rel/NF-kB family. Dephosphorylation of NFAT proteins by calcineurin exposes their nuclear localization signal and allows nuclear translocation. Once in the nucleus, NFAT proteins bind DNA, alone or in complexes with other nucleoproteins, e.g., the Fos-Jun heterodimers, to activate transcription of specific sets of genes depending on the cell type (reviewed in Refs. 130, 255). The process is terminated by casein kinase I or the sequential action of PKA and GSK-3, constitutive NFAT kinases that rephosphorylate NFAT, exporting the protein back to the cytosol (24, 111). In addition, MAPKs p38 and JNK are inducible NFAT kinases, which may couple the termination of the calcineurin cascade with different signaling pathways, simultaneously conferring an additional specificity since p38 and JNK show distinct affinities for the different NFAT family members (57, 333). Encoded in the Down's syndrome critical region, the dual-specificity tyrosine-phosphorylation regulated kinases (DYRK) 1A and 2 are physiological negative regulators of NFAT activation and have been implicated in the regulation of NFAT phosphorylation (15, 116). DYRK1A, which is localized in the nucleus, acts as a priming kinase that enables additional phosphorylation of NFAT by CK1 and GSK-3 leading to NFAT inactivation (15, 116). DYRK2, which is localized in the cytoplasm, functions as a “maintenance” kinase that sustains the phosphorylation state of cytoplasmic NFAT in resting cells (116).

Selective activation and action of export kinases therefore explain how individual NFAT proteins might be differentially regulated in a single cell type in response to calcium stimulation. The process as a whole is extremely rapid, giving NFAT proteins the remarkable ability to sense dynamic changes in intracellular Ca$^{2+}$ levels and frequencies of Ca$^{2+}$ oscillations (84). Ca$^{2+}$-dependent regulation of the calcineurin/NFAT transcriptional network encompasses both activation and repression of several target genes. Gene profiling shows that activation follows...
the coordinated action of NFAT:AP-1 complexes, whereas NFAT in the absence of AP-1 recruits corepressors and silences the expression of genes (196). Indeed, it was more recently suggested that repression of CDK4 by NFAT involves the recruitment of histone deacetylases to a site just 3′ of the transcription start site of the CDK4 gene (18).

Genetic ablation of single NFAT genes generally results in mild phenotypes, indicating a certain degree of redundancy. There are, nevertheless, notable exceptions: 1) mice with a deletion in the NFATc1 gene show impaired formation of cardiac valves and septa, suggesting a role for calcineurin/NFATc1 in the cardiac endothelium during cardiac morphogenesis (71, 253) acting together with GATA5 to control the expression of endothelin-1 (225), and to direct the differentiation of the Th2 response (330, 335); and 2) deletion of NFATc2 alone moderately increases cytokine production by Th2 and mast cells (284, 312), whereas for instance, deletion of NFATc3 does not alter cytokine expression (234). In most cases, however, prominent phenotypes are observed only when two or more NFAT proteins are lacking. Deletion of both NFATc1 and -c2 is thus required for important loss of cytokine production in T cells (242); deletion of both NFATc2 and NFATc3 results in preferential Th2 cytokine production (254). Deletion of both NFATc3 and NFATc4 isoforms results in serious defects in sensory axon projections, which are mimicked by calcineurin inhibition during embryonic development; these mice die in utero due to failure of normal vascular patterning. Death is the result of derepression of the vascular endothelial growth factor (VEGF) gene, and due to the growth of vesicles into the nervous system and somites (110, 151). Finally, deletion of three members, NFATc2, -c3, and -c4, is required to observe striking defects in axonal outgrowth in the central and peripheral nervous systems. Cultured primary neurons, sensory or commissural, from these triple knockout mice are unable to respond to neurotrophins or netrin-1 with efficient axonal outgrowth. These data indicate that the ability of embryonic axons to respond to growth factors with rapid outgrowth requires activation of a calcineurin/NFAT pathway, while survival effects are independent (112). These results are in line with the aberrant sensory behavior described in Caenorhabditis elegans bearing a loss-of-function mutation in TAX-6, a calcineurin catalytic subunit (168). Also indicative of functional redundancy, overexpression of constitutively active versions of NFATc1 and NFATc2, in which a large fraction of the phosphorylated serines have been mutated to alanines to mimic the dephosphorylated form, elicit a similar increase in expression of most cytokine genes (218). Finally, overexpression of a constitutive active form of NFATc4 results in cardiac hypertrophy and eventual heart failure, a phenotype observed also after expression of activated calcineurin (217).

D. The DREAM/KChIP Family of Ca2+-Sensitive Transcriptional Repressors

DREAM (downstream responsive element antagonist modulator), also termed KChIP-3 (potassium channel interacting protein-3) or calsenilin, is a multifunctional protein of the DREAM/KChIP subfamily of calcium sensors (KChIP-1 to -4) (11, 42, 46). Depending on the cell type and the physiological conditions, DREAM shows multiple subcellular localizations, in the nucleus, cytosol, or cell membrane (Fig. 4). DREAM is widely expressed in the brain, in particular in sensory neurons where it has a predominantly nuclear localization (Fig. 4). All four members of the family are structurally and functionally related (185), and they are coexpressed in many different brain areas (Fig. 5), as well as in other tissues including the

**Fig. 4.** Subcellular distribution of DREAM/KChIP3. A: immunocytochemistry with specific antibodies for DREAM showing the preferential nuclear localization of endogenous DREAM protein in freshly isolated mouse sensory neurons. Note absence of staining in the nucleolar compartment and weak signal in cytoplasm. B: confocal image showing the preferential membrane localization of the DREAM-EGFP signal after overexpression together with the interactive protein Kv4.2 potassium channel. [Adapted from Ruiz-Gomez et al. (264a).]
immune system, thyroid gland, and reproductive organs \((11, 42, 46, 271)\). All four DREAM/KChIP family members share the unique property to bind as homo- or heterotetramers to a specific site in the DNA, the DRE site, and repress transcription in a \(Ca^{2+}\)-dependent manner \((46, 66, 185, 232)\). Mutation within any of the three functional calcium binding EF-hand motifs in DREAM results in mutant proteins, EFmDREAMs, that retain the ability to bind the DRE sequence and to repress transcription, but are insensitive to calcium and remain bound to the DRE site during activity-dependent stimulation, blocking \(Ca^{2+}\)-dependent derepression \((46)\). Since DNA binding and repressor function is dependent on DREAM/KChIP heterotetramers, EFmDREAM proteins function as dominant active mutants of endogenous KChIP proteins during basal or \(Ca^{2+}\)-induced cell activation \((107, 271)\). A scheme depicting the mechanism of \(Ca^{2+}\)-insensitive repression by EFmDREAM is shown in Figure 6. In addition, derepression of DRE-dependent transcription is regulated by PKA or PI3-kinase activation \((173, 270)\). cAMP-dependent derepression is associated with specific protein-protein interactions between DREAM and \(\alpha\) or \(\epsilon\)-CREM, two nuclear effectors of the transcriptional actions of cAMP \((173)\). This interaction requires two leucine-charged resi-

**FIG. 5.** Differential expression of DREAM/KChIP mRNA in different brain areas. Analysis was performed by quantitative real-time PCR using specific TaqMan probes and primers for each of the four KChIPs, 1-4. Coexpression of two or more KChIPs is observed in most areas analyzed.

**FIG. 6.** DREAM-mediated transcriptional repression. \(A\): basal repression of DREAM target genes upon binding of DREAM/KChIP heterotetramers to the DRE site. \(B\): stimulated derepression of DREAM-mediated repression by different stimuli including \(Ca^{2+}\) \((i)\), DREAM phosphorylation \((ii)\), or the interaction of DREAM with other nucleoproteins such as \(\alpha\)-CREM \((iii)\). Once DREAM is detached from the DRE site, transcription of a given target gene will proceed driven by the specific combination of enhancers (E) and silencers (S) present in the promoter region. \(C\): blockage of \(Ca^{2+}\)-mediated derepression by the presence of EFmDREAM within the DREAM/KChIP heterotetramer bound to the DRE site.
due rich domains (LCDs) present in DREAM and α/ε-CREM (173). The LCD motif was first described in nuclear coactivators (NCoA-1, p/CIP) and corepressors (NCoR, SMRT) and has been implicated in protein-protein interactions with nuclear hormone receptors and CBP (136, 172, 308). The presence of functional LCDs within the DREAM protein may thus allow DREAM to interact with several other nucleoproteins to regulate transcriptional events not directly related to the presence of DRE sites. Indeed, we have reported that a LCD-dependent DREAM/CREB interaction displaces CREB from CRE sites and prevents CBP recruitment to phosphoCREB, which results in a reduction of CRE-dependent transcription without direct binding of DREAM to the CRE site (174). The DREAM-CREB interaction is Ca\(^{2+}\) dependent, and Ca\(^{2+}\)-insensitive DREAM mutants (EFmDREAM) also act as dominant repressors of Ca\(^{2+}\)-dependent CREB-mediated transcription (174). In addition, LCD- and Ca\(^{2+}\)-independent protein-protein interactions between DREAM and several other nucleoproteins have been reported (259, 275).

DREAM was identified through its binding to the DRE in the proximal promoter of the prodynorphin gene (46, 47). Consistent with a role for DREAM in the regulation of the prodynorphin gene, DREAM\(^{-/-}\) mice show upregulation of prodynorphin expression in spinal cord, which results in a hypoalgesic phenotype (56). Curiously, genetic ablation of the functionally related neuronal calcium sensor-1 gene in C. elegans results in worms with defects in learning and memory for isothermal sensitivity (105). Nevertheless, except for the modified noxious sensitivity and an anomalous response to chronic cannabinoid administration (56), no other phenotypic changes have been described in DREAM\(^{-/-}\) mice, supporting the idea that functional redundancy between members of the family compensates for the genetic ablation of DREAM/KChIP-3. To overcome this problem, an EFmDREAM dominant active mutant was used in conventional transgenesis to block activity-dependent DREAM-KChIP-mediated derepression and to address the analysis of the physiological significance of this group of proteins. In two recent reports, overexpression of EFmDREAM in T cells or in neurons has dramatic effects on the response to TCR engagement or to extracellular depolarizing conditions, respectively (107, 271). In T lymphocytes, endogenous DREAM expression is downregulated in response to TCR stimulation, and overexpression of EFmDREAM results in reduced proliferative response after polyclonal TCR activation or following an antigen-specific response (271). The phenotype could be associated with decreased expression of several Th1 and Th2-specific cytokine genes including IL-2, IL-4, and interferon (IFN)-γ, suggesting DREAM as a new target for the development of immunosuppressant drugs (271). Overexpression of EFmDREAM in the cerebellum of transgenic mice significantly reduces mRNA and protein levels of the sodium/calcium exchanger-3 (NCX3) without modifying the expression of NCX1 and NCX2. Reduced NCX3 protein levels in cerebellar granules results in increased levels of free cytosolic Ca\(^{2+}\), making transgenic neurons more vulnerable to increased Ca\(^{2+}\) influx following partial opening of voltage-gated plasma membrane Ca\(^{2+}\) channels induced by increasing K\(^{+}\) in the culture medium. Lentiviral-mediated overexpression of the NCX3 exchanger in cerebellar transgenic granules restores normal Ca\(^{2+}\) homeostasis, indicating that the phenotypic change in these transgenic neurons is related exclusively to the downregulation of NCX3 by DREAM (107). DREAM/KChIPs are the only EF-hand calcium sensors so far known to bind specifically to DNA and directly regulate transcription in a Ca\(^{2+}\)-dependent manner. Recently, however, a novel DNA binding protein called Freud-1 (5’ repressor element under dual repression binding protein-1) was reported to bind to the FRE site in the proximal promoter of the serotonin 1A receptor gene and to mediate its Ca\(^{2+}\)-dependent repression (233). Freud-1 is evolutionarily conserved and has two DM-14 basic repeats, a predicted helix-loop-helix DNA binding domain, and a C2 region, calcium/phospholipids binding domain, similar to those found in conventional PKC isoenzymes. An intact C2 domain is required for Freud-1 to mediate derepression, whereas treatment with inhibitors of CaM or CaMKs reversed Ca\(^{2+}\)-mediated Freud-1 inhibition, suggesting an additional regulatory mechanism that needs further investigation. Freud-1 thus represents a novel calcium-regulated repressor that, at least in transient transfection experiments, negatively regulates basal 5-HT\(_{1A}\) receptor expression.

E. The NF-κB/IκB Couple

NF-κB is expressed in nearly all cell types and is implicated in the regulation of numerous genes mediating immune, inflammatory, and stress responses (for reviews, see Refs. 104, 147). NF-κB is a heterodimer of p65 (RelA) with p50 or p52. This heterodimer is anchored by a group of proteins termed IκB, which retain NF-κB in the cytosol by masking its nuclear localization signal (25). Following site-specific IκB hyperphosphorylation at S32 and S36 by two IκB kinases, IKKα and IKKβ (79, 211), the inhibitor molecule becomes susceptible to site-specific ubiquitination and subsequent degradation by the proteasome complex (36, 273). This releases NF-κB, allowing nuclear translocation. In addition, more recent studies in T lymphocytes and neurons identified CaMKII as an activator of NF-κB. In response to T-cell receptor (TCR) activation, nuclear translocation of NF-κB can be blocked by inhibitors of CaM or CaMK, or mimicked by overexpression of a constitutively active form of CaMKII (141). In basal conditions in neurons, the p65:p50 NF-κB form is selec-
F. Novel Mechanisms Associated With Ca$^{2+}$-Dependent Gene Expression

Search for new Ca$^{2+}$-sensitive transcriptional effectors using a transactivator trap strategy led to the identification of CREST (for calcium responsive transactivator) from a cerebral cortex cDNA library (4). Overexpression of CREST as a Gal4 fusion protein drives expression of an UAS reporter in nondifferentiated embryonic rat cortical neurons in culture to respond to calcium depolarization or bath application of glutamate, two well-characterized stimuli that increase intracellular calcium concentration and trigger a variety of cellular responses including dendrite outgrowth and neuronal death (122).

CREST does not bind directly to DNA; therefore, it must associate with other nucleoproteins to activate transcription. Indeed, CREST has been shown to interact with CBP through its last nine COOH-terminal amino acids, a mechanism that could explain CREST-mediated dendritic growth, a process associated with CREB phosphorylation after Ca$^{2+}$/cAMP neuronal stimulation (reviewed in Ref. 256). Interestingly, CREST$^{-/-}$ mice show a reduction in dendritic growth and branching that is specific for the basal rather than apical dendrites in cortical pyramidal neurons (4). Given the pleiotropic effects of the CREB/CBP/TOCR transcriptional network, future studies should identify other CREST interactors in the nucleus responsible for this specific action on dendritic maturation. In addition, CREST homodimerization has been proposed to be necessary to block an inhibitory domain located next to a nuclear localization signal in the COOH-terminal 150 amino acids of the protein (248).

A number of interesting questions about CREST can be raised: 1) which are the final gene targets of CREST that mediate the effect on dendrite growth; 2) 80% of CREST$^{-/-}$ mice die before adulthood within the second or third week after birth, indicating that CREST may be relevant for additional biological effects; and 3) how calcium signals actually activate CREST. This is an important issue, since no calcium binding motifs have been observed in CREST and not even its interaction with CBP has been shown to be induced upon neuronal depolarization (4).

Cleavage of the COOH-terminal end of voltage-gated calcium channels (VGCCs) is a well-known phe-

nomena, and the cleaved fragments have been implicated in regulating channel activity by reducing Ca$^{2+}$ influx (142). Recently, however, the COOH-terminal intracellular fragment of the Ca$_{1.2}$ subunit of the L-type VGCC, named, calcium channel-associated transcriptional regulator (CCAT), was shown to localize in the nuclei of GABAergic inhibitory interneurons in the cortex and hippocampus and to regulate transcription of selective target genes (106, 222).

Importantly, the nuclear pool of CCAT is rapidly extruded in response to calcium entry following neuronal membrane depolarization (106); CCAT-regulated gene expression is sensitive to calcium stimulation. Whether CCAT is found in the nuclei of other cell types that express L-type channels and regulates gene expression in nonneuronal cells is not known.

Transcriptional regulation by CCAT does not seem to involve direct interaction with DNA but rather involves interaction with other nucleoproteins, such as p54(nrb)/NonO (nuclear RNA- and DNA-binding protein of 54 kDa, also known as Non-POU domain-containing octamer-binding protein) (264).

Since other Ca$_{2+}$ subunits have been reported to be cleaved in neurons (126, 167, 322) and one of them, the Ca$_{2.1}$ subunit, generates a COOH-terminal fragment that has been localized to Purkinje cell nuclei (163), additional effectors to be described may share with CCAT this novel Ca$^{2+}$-dependent mechanism to control gene expression.

IV. CALCIUM-DEPENDENT PROTEIN-PROTEIN INTERACTIONS THAT MODULATE GENE EXPRESSION

Like DREAM, other calcium sensors, mainly CaM but also S-100 and calreticulin, are multifunctional proteins able to interact with various nuclear targets after Ca$^{2+}$ binding to regulate the activity of transcription factors or enzymes that influence chromatin structure to eventually change gene expression. From the viewpoint of control of transcriptional networks by nuclear Ca$^{2+}$, two types of interactions will be discussed because of their significance: 1) the interaction between Ca$^{2+}$-bound calcium sensors and transcription factors of the bHLH (63) and the nuclear receptors superfamilies (40, 74) and Sox proteins (14) and 2) the Ca$^{2+}$-mediated changes in chromatin structure.

A. Ca$^{2+}$ Sensors in the Nucleus

CaM, a small acidic protein, is the prototypic calcium sensor (reviewed in Ref. 129a). CaM contains four EF-hand Ca$^{2+}$-binding motifs, distributed in pairs embedded within two separate globular regions at the NH$_{2}$ and COOH termini. A flexible central helix completes the
characteristic dumbbell-shaped structure (289). S-100 proteins are also EF-hand proteins able to interact in a Ca\(^{2+}\)-dependent manner with many targets, some of which are common with CaM. Of particular importance here is the property of both CaM and S-100 proteins to bind to DNA in a sequence-specific manner through their HMG box and by interacting with specific partner proteins (for a recent review, see Ref. 133). Interaction of Ca\(^{2+}\)-bound CaM with the HMG domain of Sox9, an early embryonically expressed member of the family that regulates chondrogenesis, testis formation, and neural crest development (133), blocks its nuclear import and consequent transcriptional activity (14). Missense mutations at the Sox9 HMG box affect CaM interaction and result in campomelic dysplasia/autosomal sex reversal (CD/SRA), a severe bone malformation syndrome in which most XY individuals show male-to-female sex reversal (14). Ca\(^{2+}\) regulation of Sox9 activity is thus crucial for its biological effects. Since the HMG box is common to all members of the Sox family, it can be predicted that calcium plays a major role during organ development by selectively regulating the activity of specific Sox proteins.

B. Ca\(^{2+}\)-Dependent Changes in Chromatin Structure

Ca\(^{2+}\)-dependent enzymes not only modify the activity of specific transcriptional networks but also act on chromatin-associated proteins, histones (H) and high mobility group (HMG) proteins, to evoke selective modifications in the nucleosomal environment that encompass specific genes to either expose or mask their regulatory sites to transcriptional regulators. An example is the correlation between c-fos induction and the increased sensitivity to DNaseI of the c-fos gene (91), which is related to phosphorylation of histone H3 and HMG proteins (153).

Histones are essential components for the arrangement of the nucleosomes, and posttranslational modifications, including acetylation, phosphorylation, methylation, and ADP-ribosylation, have profound effects on chromatin structure and on gene expression. The HMG proteins are non-histone chromosomal proteins of low molecular mass, fundamental for stabilizing the enhancerosome at promoter regions and mediating transcriptional elongation (80). Because of their critical role in transcription, they are also called architectural transcription factors, although they do not make sequence-specific contact with the DNA.

Of the posttranslational modifications, phosphorylation and acetylation/deacetylation are the best characterized in terms of their association with activity-dependent changes in chromatin structure. Early work from Schulman’s laboratory (323) reported the specific phosphorylation of histone H3, but not other histones, and of HMG protein-17 in isolated nuclei from untreated cells in response to an increase in external Ca\(^{2+}\) in vitro. The precise mechanism by which phosphorylation of these nucleosomal proteins facilitates transcription is not known; however, it was elegantly demonstrated that phosphorylation of HMG盒 proteins facilitates transcription in vivo (225).
loration follows the hyperacetylation and decreases methylation on the same NH2-terminal histone tails (58, 266). On this basis, phosphorylation was proposed to stabilize an open conformation, perhaps blocking access of transcriptional corepressors associated with HDACs and DNA methyltransferases. An important issue has been to identify the kinase(s) responsible for histone phosphorylation leading to transcriptional activation. \(\text{Ca}^{2+}/\text{CaM}\)-dependent phosphorylation of histone H3 was reported first, suggesting the involvement of a nuclear CaMK (317). More recently, the mitogen- and stress-activated kinases, MSK1 and MSK2, two effector kinases downstream of ERK and p38, were demonstrated to be responsible for the H3 and HMG protein 14 phosphorylation associated with immediate early gene activation (283, 302) and cytokine gene expression during inflammation (266). Notably, MSK kinases are also responsible for the direct activation of NF-\(\kappa\)B p65 subunit and CREB (73, 314). Several \(\text{Ca}^{2+}\)-dependent kinases thus phosphorylate histone H3 and specific transcription factors to coordinate their activation transcriptional networks that participate in the immediate early or the inflammatory responses. The system is reset by the calcineurin-dependent nuclear protein phosphatase 1, which has been implicated in H3 dephosphorylation (49).

Phosphorylation of histones and HMG proteins has been studied mainly in vitro and in cultured cells. Nonetheless, two recent reports suggest a role in vivo, showing that a light pulse during the subjective nighttime stimulates MSK1 phosphorylation of H3 (41, 67) in hypothalamic neurons of the suprachiasmatic nucleus, a major central circadian clock. The effect of light is specific, and the kinetics of H3 phosphorylation parallel the early induction of c-fos and m\(\text{Per1}\) (the mouse ortholog of the Drosophila gene period) in the same neuron (67). Moreover, systemic blockage of GABA\(_{\text{A}}\) receptors with baclofen prevents light-induced H3 phosphorylation and c-fos and m\(\text{Per1}\) gene expression (67). These results correlate well with circadian variation in phosphoCREB in suprachiasmatic neurons and light-induced upregulation of CRE-dependent transcription during the subjective nighttime (227). The architectural transcription factor HMG-I(Y) regulates expression of the rhodopsin gene in terminally differentiated retinal photoreceptors (50). The mechanism involves the interaction of HMG-I(Y) with the Crx homeoprotein, which participates in the circadian regulation of rhodopsin gene expression (50). In addition, phosphorylation of HMG-I(Y) by PKC also regulates its activity as reported in tumoral cell lines (20).

Acetylation/deacetylation, linked predominantly to activation and repression, respectively, are to date probably the most extensively studied posttranslational histone modifications. It is commonly accepted that acetylation partially neutralizes the positive charge of histones, reducing their affinity for DNA, thereby creating an open structure that permits the access of other proteins to the DNA. The reverse effect is achieved after histone deacetylation. Activity-dependent changes in histone acetyltransferase activity have been presented earlier in this review. Therefore, we now present recent studies focused on activity-dependent mechanisms that modify the activity of histone deacetylases. This includes protein-protein interactions, changes in the subcellular localization and posttranslational modifications, as well as regulation of their availability either by controlling HDAC expression or proteolytic processing.

Mammalian HDACs are classified into three different groups (I, II, and III) according to their sequence homology to yeast HDACs: reduced potassium dependency 3 (Rpd3), histone deacetylase 1 (Hda1), and silent information regulator 2 (Sir2), respectively. In general, HDACs do not make direct contact with DNA and depend on other nucleoproteins for recruitment to specific locations in the genome. HDACs are normally bound to scaffold proteins like Sin3 and are included in large multiprotein complexes (Sin3, NuRD/NRD/Mi2, and the CoREST complexes) that are multifunctional (for a review, see Ref. 280a). The main mechanism that regulates HDAC activity is the protein-protein interaction within the multiprotein complexes. An example of this is the increased enzymatic activity of HDAC3 after the interaction with the silencer complex SMRT/N-CoR (silencing mediator of retinoid and thyroid receptor/nuclear receptor corepressor) (6, 125).

The activation mechanism involves the displacement of an inhibitor protein called TCP-1 ring complex (Trichome) that is bound to and blocks HDAC3 enzymatic activity (115). Another major mechanism for the regulation of HDAC activity is through phosphorylation. For class I HDACs, phosphorylation by casein kinase II and/or PKA results in increased activity, except for HDAC8, for which it is reduced after PKA phosphorylation (175, 245, 311). In the case of class II HDACs, phosphorylation regulates their subcellular localization and their ability to interact with other proteins. \(\text{Ca}^{2+}\)-dependent phosphorylation of HDAC4 and -5 by CaMKIV thus results in unbinding from MEF-2 proteins and in a multistep \(\text{Ca}^{2+}\)-regulated process that involves the interaction with 14-3-3 proteins, producing important changes in gene expression that result in drastic phenotypic modifications (337, 338). It has been demonstrated that spontaneous electrical activity is sufficient for nuclear export of HDAC4, while stimulation of calcium flux through synaptic NMDA receptors or L-type calcium channels induces the nuclear export of HDAC5. Nuclear import of HDAC4 but not -5 is mediated by sumoylation (158). These differences in the activation thresholds for HDAC4 and HDAC5 nuclear export and the distinct import processes could provide a mechanism for input-specific gene expression (51). Dephosphorylation of HDACs is also a \(\text{Ca}^{2+}\)-sensitive process, since most class I and II HDACs form complexes that include PP1 (44,
biologically active protein (305). Each of the first five promoters of both IFN-
and IFN-
In Th1 cells, STAT1 and STAT4 act downstream of IL-12 transcription factors to determine the Th1/Th2 lineage. STAT elicits the lineage-specific expression of transcription factors T-bet and GATA3 for Th1 and Th2 differentiation, respectively. At the same time, NFAT cooperates functionally with STAT proteins at cytokine regulatory regions, initiating long-range changes in Dnase I hypersensitivity and histone modification throughout the regulatory regions of the IFN- and IL-4 genes (3, 13, 296). The appearance of new Dnase I-hypersensitive sites in the IFN- and IL-4 genes during Th1 and Th2 cell differentiation is blocked by CsA, demonstrating the Ca\textsuperscript{2+}-dependent involvement of NFAT proteins in the remodeling of cytokine genes (2). Although NFAT proteins bind to the promoters of both IFN- and IL-4 during the early stages of naive T-cell activation, after initiation of Th1 cell differentiation, the locus of IL-4 is silenced resulting in the specific binding of NFAT to the IFN- promoter. Similarly, during Th2 cell differentiation and after silencing of the IFN- locus, NFAT binds to the IL-4 promoter.

V. EXEMPLIFYING THE COMPLEXITY OF CALCIUM-DEPENDENT GENE EXPRESSION: THE BRAIN-DERIVED NEUROTROPHIC FACTOR COMPENDIUM

The mouse BDNF gene consists of five 5' noncoding exons and one 3' exon that includes the entire ORF of the biologically active protein (305). Each of the first five exons has a 5' flanking region that is transcribed and a splice donor site at the 3' end. Exon VI contains the only splice acceptor site and two polyadenylation signals. By alternative usage of the five promoters followed by splicing with exon VI and alternative usage of the two polyadenylation signals, transcription of the BDNF gene in mice results in 10 different BDNF transcripts. In the rat, the third exon is missing; thus exon III corresponds to mouse exon IV, and so on. Nonetheless, the overall organization and regulation of the promoter regions associated with each exon is basically conserved (305).

Early induction of BDNF transcription in the rat brain following neuronal activation occurs through the differential usage of BDNF promoters I and III or IV (99, 213, 305), while in cultured rat cortical neurons, activation of voltage-sensitive calcium channels by increased extracellular K\textsuperscript{+} concentrations results in the preferential upregulation of BDNF\textsubscript{III} transcripts (279, 300). Several regulatory sites have been associated with the activity-dependent expression of rat BDNF transcripts: binding sites for CREB and USF on promoter I (297), RE-1/NRSE sites recognized by members of the REST/NRSF family on promoter II (306, 344, 345), C/EBPs and Sp1 sites that regulate expression from promoter IV (298), and an estrogen-responsive element near exon V (282). In exon III, the initial studies (279, 300) identified two main sites: 1) a hemi-palindromic CRE site that binds CREB and is located between 40 and 30 bp upstream of the exon III mRNA start site, and 2) a Ca\textsuperscript{2+}-responsive sequence (CRS-1) located between 72 and 47 bp upstream from the start site. Because the nature of the nuclear factor(s) binding to the CRS-1 site was unknown, activity-dependent regulation of BDNF was initially explained as a kinase-dependent process with Ca\textsuperscript{2+}-mediated CaMKIV-dependent CREB phosphorylation. Several recent discoveries have modified this simplified notion.

Analysis of the rat CRS-1 by Greenberg's laboratory has identified three independent Ca\textsuperscript{2+}-responsive regulatory sites: the CaRE1, CaRE2, and the CaRE3/CRE site located 3' from the CRS-1 site. While the CaRE3/CRE site corresponds to the originally identified calcium responsive element and mediates transactivation of BDNF by the activated CREB/CBP pathway, the other two sites interact with proteins newly characterized as Ca\textsuperscript{2+}-responsive regulators of BDNF transcription. The CaRE1 sequence is located at the 5' end of the previously defined CRS-1 site and binds a new protein termed CaRF, calcium responsive factor, identified using a one-hybrid approach in yeast (301). Ability to bind to the CaRE1 sequence resides in the NH\textsubscript{2} terminal of CaRF, while the domain(s) responsible for its transactivating properties is located at the COOH terminal. The CaRE2 sequence includes an E-box (CANNTG) that binds to the upstream stimulatory factors 1 and 2 (USF1/2) (52). USF1 and USF2 are ubiquitously expressed bHLH proteins that bind to E-boxes as homo-
or heterodimers to activate transcription of target genes (272, 281). In addition, work in our laboratory characterized the binding of transcriptional repressor DREAM to two DRE sites located within the CRS-1 sequence (209). This indicates that DREAM may participate directly in the basal as well as in the Ca\(^{2+}\)-dependent regulation of BDNF\(_{III}\) transcripts. Neither the nuclear translocation of CaRF and USF nor their binding to the CaRE1/CaRE2 sequences, respectively, is affected by changes in the Ca\(^{2+}\) concentration. This indicates that the stimulus-dependent transactivating response of CaRF or USF proteins may be mediated by so far unknown posttranscriptional modifications of these factors or by undisclosed CaRF- and USF-interacting proteins that presumably are responsible to sense and to respond to the Ca\(^{2+}\) signal (55, 301). Taken together, these results indicate that a CREB-independent, Ca\(^{2+}\)-dependent regulation of BDNF occurs but may require additional factors. Whether BDNF is a bona fide target gene for CaRF, DREAM, and USF proteins nonetheless remains to be firmly established in vivo using appropriate genetically modified mouse models.

Recent studies added a further degree of complexity to the regulation of BDNF expression by showing that DNA methylation and possibly chromatin remodeling control activity-dependent expression of the BDNF gene. Methylation of several CpG islands at the 5’ flanking region of BDNF exon III is dramatically reduced following depolarization of primary cultured cortical neurons (202). This finding correlates with the activity-dependent reduction in the binding of methyl-CpG binding protein 2 (MeCP2) to BDNF exon III promoter and the increase in BDNF expression in the brains of DNA methyltransferase I\(^{-/-}\) (Mmnt) and MeCP2\(^{-/-}\) mice (54, 202). Silencing of BDNF upon MeCP2 binding involves HDAC1 recruitment through the coressor mSin3A and/or the histone methyltransferase SUV39H1 that dimethylates histone H3 on K9, a hallmark of inactive chromatin structure (94, 202). The mechanism controlling demethylation of CpG islands after K\(^{+}\)-induced depolarization is presently unknown, although Ca\(^{2+}\)-dependent phosphorylation of MeCP2 regulates its unbinding from methylated DNA (54). Whether demethylation follows or precedes MeCP2 release from BDNF CpG islands and the signal(s) responsible for resetting basal transcription activity at BDNF promoter III after activity-dependent induction are not known.

BDNF is an important neurotrophic factor for neuronal connectivity and brain function (reviewed in Ref. 273a), and heterozygous BDNF deficient mice show important deficits in learning and memory (165). It is therefore not surprising that three devastating neurological disorders have been associated with mutations in proteins that affect or participate in the activity-dependent transactivation of the BDNF gene. These are the Rett syndrome, the Rubinstein-Taybi syndrome, and Huntington’s disease. In all three cases, a casual relationship with BDNF has been shown in transgenic models that reproduce partial aspects of the disease, although a formal demonstration of modified BDNF levels in the brains of these patients has not yet been provided.

The Rett syndrome, an X-linked neurological disorder, is characterized by arrested neurological development and cognitive decline and is produced in most cases by mutations in the MeCP2 protein (10, 276), the methyl CpG binding protein that controls BDNF\(_{III}\) transcription (54, 202). How these mutations relate to changes in BDNF expression in Rett syndrome-derived neurons is not known. The Rubinstein-Taybi syndrome is a rare congenital disorder characterized by severe mental retardation as well as retarded and abnormal skeletal growth. The disease is caused by heterozygous mutations of the CBP gene that affect the COOH-terminal domain of the protein (244). Interventions that increase CREB activity overcome memory deficits in CBP\(^{-/-}\) or heterozygous CBP\(^{-/-}\) mice (5, 166), although changes in BDNF expression in these animal models have not been reported.

Huntington’s disease, a dominantly inherited neurodegenerative disorder, is characterized by chorea, cognitive abnormalities, and psychiatric disturbances and is caused by a polyglutamine expansion in the huntingtin protein (reviewed in Ref. 123a). Both gain- and loss-of-
function of the mutated protein have been associated with mechanisms that affect BDNF expression. The gain-of-function mechanism involves the interaction of mutated huntingtin with CBP and other nucleoproteins such as p53 and TBP that impairs cAMP-dependent transcription (150, 273, 286, 292). The loss-of-function mechanism relates to the ability of wild-type huntingtin, absent in the polyglutamine extended mutated version, to interact with the neuron-specific repressor REST/NRSF, preventing its nuclear localization and repressive effect on the transcription of the BDNF promoter II (344, 345).

A summary of the mechanisms operating on the expression of BDNF exon III is shown in Figure 7. Further studies are needed to understand the global and exon-specific mechanisms by which Ca\textsuperscript{2+} regulates BDNF gene expression in different neuronal populations. It will be particularly important to assess whether the multiple mechanisms regulating activity-dependent regulation of BDNF promoter III operate at the same time in a given neuron, and the Ca\textsuperscript{2+} level requirements that are needed to activate each of these mechanisms and hence the type of stimulus that will successfully trigger one specific pathway.

VI. SUMMARY AND FUTURE DIRECTIONS

In addition to the well-known calcium-activated kinase- and phosphatase-dependent mechanisms that control gene expression, recent years have witnessed an eruption of new calcium-sensitive proteins and calcium-dependent pathways. This has added further complexity to, and raised new questions about, the mechanisms that impose spatial and temporal restrictions on the synchronically induced transcriptional activity at a given genomic locus to control its expression profile. Also, it will be very important to define the hierarchy of calcium-operated pathways that simultaneously operate on the expression of a given gene.

New experimental work has provided insights into the nature and specificities of the calcium signal itself, although it is still unclear how the different pools of free intracellular calcium differentially activate or repress a given transcriptional network. Many still unknown self-regulatory mechanisms probably participate in the fine-tuning of the transcriptional response in absolute terms, as well as of the temporal characteristics of the response.

Finally, recent publications highlight the existence of a nuclear pool of phosphoinositides and the emerging role of nuclear inositol phosphates in gene expression (228, 277, 287). New evidence has argued in favor of a similarly regulated nuclear calcium pool (88), although further studies should establish indisputably that such a pool is truly nuclear and independent from the ER pool. Mechanisms that might regulate the release of Ca\textsuperscript{2+} from such a nuclear pool as well as mechanisms that control the passage of calcium ions through the nuclear pore should also be provided. Whether the nuclear pools of phosphoinositides and calcium maintain a certain level of cross-talk to affect chromatin remodeling and to influence transcriptional networks more directly are open questions looking for an answer.

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