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Calcineurin as a Multifunctional Regulator

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Long recognized as an important regulatory mechanism in biosignal processes, modulation of the phosphorylation state of proteins has emerged as the most important mechanism for understanding signal transduction. In contrast to the multitude of protein kinases and the clear signal transduction pathways, relatively few protein phosphatases are known and their regulation is unclear. Among them, calcineurin, a calcium/calmodulin-dependent phosphatase (PP2B), is the best enzyme to unveil the phosphatase function, because it was shown to be the direct target for immunosuppressants CsA and FK506, which are powerful tools for understanding this function in diseases as well as in several tissues and organs. Although calcineurin has been found in the highest concentrations in brain, it has also been detected in many other mammalian tissues. Well characterized in T cell activation by analysing the transcription factor NFAT, the function of calcineurin, however, was less well understood in other tissues and organs. Since the mid-1990s, several novel functions of this phosphatase have been reported, revealing that it plays important roles as a multifunctional regulator under the direct regulation of calcium signaling.

Key words: Bcl-2, calcineurin, cardiac hypertrophy, immunosuppressant, ischemia.

Since the first detection of calcineurin (CaN; also called protein phosphatase 2B) two and a half decades ago (*1*), when it was identified as an inhibitor of the calmodulin (CaM)-dependent cyclic nucleotide phosphodiesterase, knowledge of its function and regulation has been growing rapidly. CaN is a major CaM-binding protein in brain and the only known serine/threonine protein phosphatase that is directly regulated by Ca^{2+}/CaM (*2, 3*). These properties makes it a potentially powerful mediator of intracellular signals, which are commonly involved in both physiological and pathological processes, especially in the nervous system. CaN exhibits a broad distribution and abundance in all kinds of tissues, but is highly enriched in neural tissue (*4*). It comprises more than 1% of the total protein content in brain (*5*), which also points towards its important roles in neuronal functions. CaN was identified as the target of the immunosuppressant FK506 and CsA and thus was shown to play an essential role in T cell activation (*6*). The demonstration that FK506 and CsA, when bound to their respective binding proteins FKBP12 and cyclophilin A, are specific inhibitors of calcineurin provided the tools needed to reveal its many other roles in the transduction of Ca^{2+} signals (*7*). In 1995, we encountered a new role of this enzyme, which might be an essential component in cell death signaling (*8, 9*). This attractive hypothesis is supported by the evidence that FK506 and CsA had a drastic neuroprotective effect in rat focal and forebrain ischemic models (*10, 11*). In 1999, Bad, a pro-apoptotic protein of the Bcl-2 family was demonstrated to be a direct substrate of CaN, and the apoptotic function was regulated in the balance between CaN and Akt/PKB (*9*). Another important finding was that CaN plays a critical role in the myocardial hypertrophy through the transcription factors NFATc/GATA4 (*12*). Further roles of this enzyme have been clarified in many tissues and organs as summarized in Fig. 1. In this review, we focus especially on the new aspect of CaN functions.

1. Characteristics of calcineurin

Calcineurin (CaN) is one member of the serine/threonine protein phosphatase family and the only known phosphatase activated by Ca^{2+} and CaM. Its 58–64-kDa catalytic subunit (calcineurin A; CaNA) contains specific domains with regulatory functions (*2*), including an autoinhibitory (AI) domain near the carboxy terminus, a CaM-binding domain, and a binding domain of a regulatory subunit, calcineurin B (CaNB) as shown in Fig. 2. In the presence of less than 10^{-7} M Ca^{2+}, CaNA tightly binds to CaNB, but the enzyme is inactive in this form (*3*).

Three mammalian isoforms of CaNA (α, β, and γ) have been identified, and their amino acid sequences are highly conserved between different organisms (*3, 13–15*). The isoforms CaNAα and β is ubiquitously distributed, while CaNAγ is specifically expressed in the testis, suggesting that it may share the signal transduction of spermatogenesis (*16*). The 19-kDa Ca^{2+}-binding CaNB, which is also highly conserved, was originally identified as a dumbbell-shaped protein with four Ca^{2+}-binding "EF-hands" (*17*). A

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Abbreviations: CaN, calcineurin; CaNA, calcineurin A; CaNB, calcineurin B; CaM, calmodulin; CsA, cyclosporin A; MPT, mitochondria permeability transition; cyt *c*, cytochrome *c*; FKBP, FK506-binding protein.

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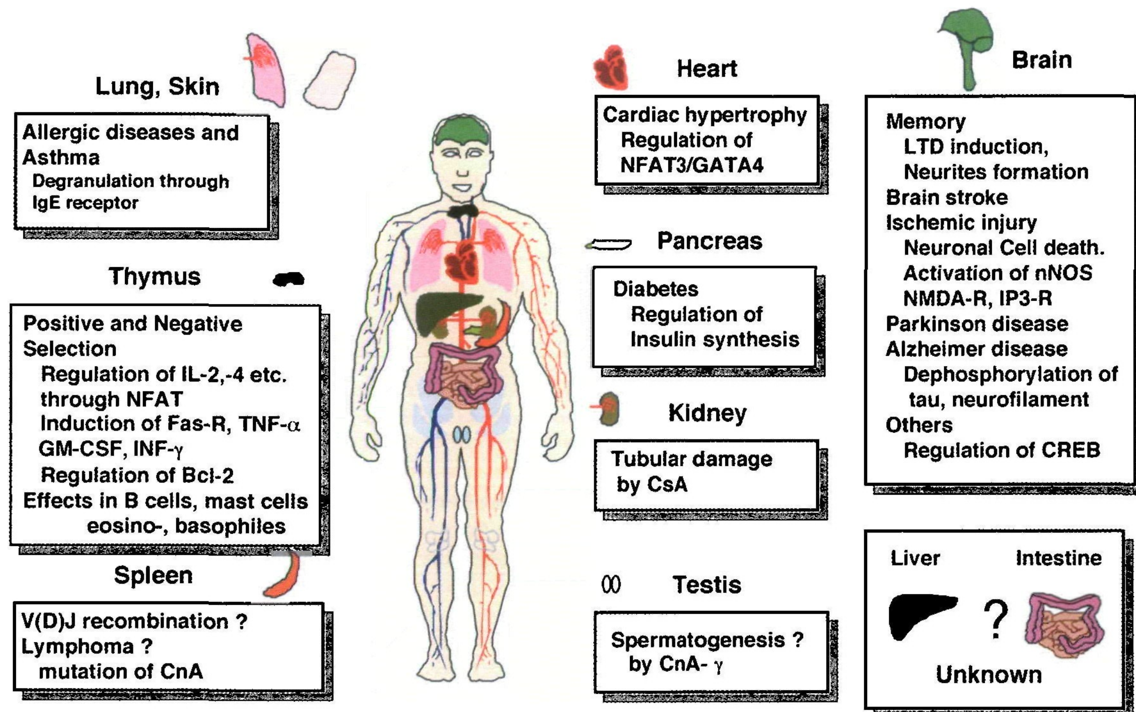

Fig. 1. **Calcineurin as a multifunctional regulator.** The function of calcineurin in organs is summarized from previous reports. Some of the direct substrates have been clarified to explain the function, but others are reported on the basis of the inhibition of the immunosuppressants CsA and FK506.

study using multidimensional NMR has demonstrated that its structure is similar to that of CaM with two adjacent Ca^{2+}-binding loops connected by a flexible helix linker (*18, 19*). There are two mammalian isoforms of calcineurin B, CaNB1 and CaNB2, which were originally found associated with CaNA. CaNB1 is ubiquitously expressed as a heterodimer with CaNAα or β, while CnB2 is expressed only in testis and binds to CaNAγ. In other species, only one form has been reported in fruit flies and the budding yeast (*20*). The CaNB subunit has an N-terminal myristoylation, which has been suggested to facilitate the interaction of CaN with lipid membranes and its localization at the plasma membrane. The role of this myristoylation, however, is still under debate. A mutational study showed that the myristoylation of CaNB was not necessary for either membrane association or function (*21*). Recently, on the other hand, the N-terminal myristoylation was demonstrated to be required for the Ca^{2+}-dependent binding of CaN to phosphatidylserine vesicles, suggesting that it may be localized at the membrane to be positioned close to its substrates (*22, 23*).

Studies on the crystal structure of the CaN-FKBP12-FK506 complex identified the interface between CaNB and CaNA as the binding site of the FK506-FKBP complex (*24, 25*), as shown in Fig. 2. This result sheds light on some of the fundamental properties of these proteins, namely, the molecular mechanisms of catalysis and substrate recognition, which have been proposed on the basis of cross-linking and biochemical experiments (*26–30*). It is of interest that the interaction of CaN with the residues of FK506-FKBP12 and CsA-cyclophilin A depend on the structure of these complexes. The isomerase activity of cyclophilin A and FKBP was shown not to be necessary for the interaction and CaN inhibition (*31–33*). The two structurally different drug complexes, FK506-FKBP12 and CsA-cyclophilin A, bind competitively to the same site on CaN, but the interaction sites are not identical (*30, 34*). A mutagenic study using *Saccharomyces cerevisiae* addressed the cause of this difference (*34*). Three mutations that confer dominant CsA resistance are single amino acid substitutions (T350K, T350R, Y377F) in the CaNA catalytic subunit. One mutation that confers dominant FK506 resistance alters a single residue (W430C). *In vitro* and *in vivo*, the CsA-resistant CaN mutants bind FKBP12-FK506 but have reduced affinity for cyclophilin A–CsA. The FK506 resistance mutation (W388C) blocks binding by FKBP12-FK506, but not by cyclophilin A–CsA. These studies identify cyclophilin A–CsA and FKBP12-FK506 binding targets as distinct, highly conserved regions of CaNA that overlap the binding domain for the CaNB regulatory subunit (*34*).

2. Tissue distribution and intracellular localization

CaN is widely distributed in various mammalian tissues, but its concentration in brain is 10–20 times that found in other tissues (*3, 35*). In brain, 50–70% of CaN is bound to membrane or cytoskeleton elements, and the enzyme is found in most cells, although its concentration varies greatly from one area to another (*5*). In T lymphocytes, CaN has been found intranuclearly (*36*), colocalized with one of its substrates, NFAT (Nuclear Factor of Activated T cell), which is not able to translocate into the nucleus unless it is dephosphorylated. CaN has also been found in nuclei from different species (*37*). Several research groups have reported an almost exclusively neuronal distribution of CaN in

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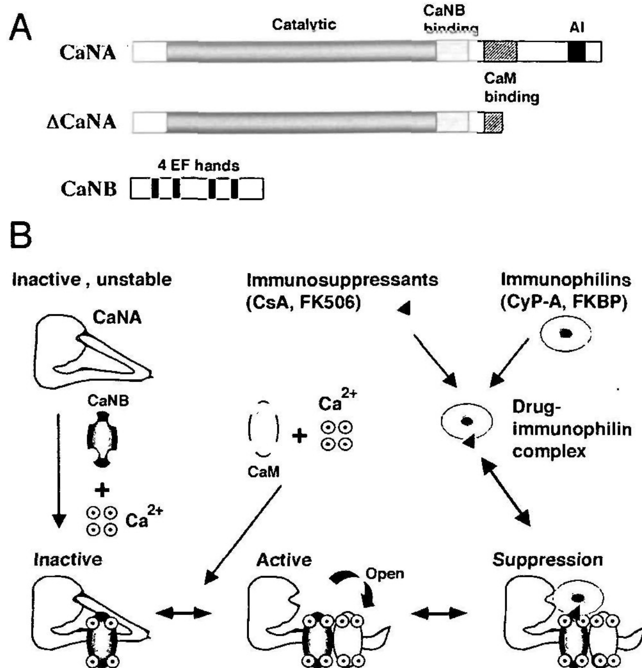

Fig. 2. **Structure and regulation of calcineurin.** A: Calcineurin A subunit (CaNA) has specific domains: a catalytic domain (catalytic), a calcineurin B (CaNB)-binding domain, a calmodulin (CaM)-binding domain, and an autoinhibitory domain (AI). A mutant (ΔCaNA) of CaNA deleted from the carboxy-terminal AI to part of the CaM-binding domain is Ca^{2+}/CaM-independent and constitutively active. A regulatory subunit CaNB, which has 4 EF-hands that bind to Ca^{2+}, has a structure similar to CaM. B: In the regulation of CaN, an increase of intracellular Ca^{2+} triggers the binding of CaM to a CaNA/CaNB heterodimer and dissociates the carboxy-terminal AI domain from the catalytic groove. This structural change activates CaNA and is reversible. On the other hand, the drug-immunophilin complexes such as CsA-cyclophilin A and FK506-FKBP bind to a CaNA/CaNB heterodimer through the latch domain of CaNB and inhibit CaN activity competitively by covering the catalytic groove.

the brain of different species (*3, 38*). The highest levels of CaN are found in the hippocampus (interestingly, in especially high concentration in CA1, in neurons selectively vulnerable to cerebral ischemia) and the striatum (*39*). In most brain regions, CaN is colocalized with FKBP, the FK506-binding protein, while cyclophilin is found in some areas that appear to lack expression of CaN (*38*). The α and β isoforms of CaNA have different spatial distribution in rat brain, Aα being the more abundant, but both show similar subcellular localization (*15*). A later study, however, suggests that the intracellular distribution of the two CaNA isoforms in the brain is different, with the α-form being located in the nuclei and the β-form in the cytoplasm (*37*). Intracellularly, CaN is located in the soma and dendrites, in close association with post-synaptic densities (PSDs), plasma membrane, dendritic microtubules and somal organelles (*3*). Developmentally, CaN is reported to be undetectable in rat brain at a very young age. The expression then increases to a peak at around 5 weeks of age, after which it remains at a high level throughout adulthood (*40*). CaN levels in rat brain are reported to increase in parallel with synaptogenesis (*41*).

3. Regulation of CaN activity

The Ca^{2+} dependence of the phosphatase activity of CaN is controlled by two structurally similar but functionally different Ca^{2+}-binding proteins, CaM and a regulatory subunit CaNB (*3*). The increase of intracellular calcium activates CaN through Ca^{2+}/CaM binding to the CaNA subunit. This binding reversibly changes the structure of CaNA, causing the AI sequence to dissociate from the catalytic groove (Fig. 2). The basal activity is stimulated more than 20-fold by the addition of an equimolar amount of CaM and is strictly the result of an increased V_{max}. The highly cooperative Ca^{2+} dependence of the CaM stimulation of CaN allows the enzyme to respond to narrow Ca^{2+} thresholds following cell stimulation. In crude tissue extracts, the CaN activity is almost completely dependent on CaM but not on added metals (*42*). Crude CaN is protected against inactivation and instability by superoxide dismutase (*43*), and this protection has also been observed in yeast cells and in hippocampal neurons after prolonged Ca^{2+} stimulation (*43, 44*). In a mutagenic study, deletion of both the AI domain and a C-terminal part of the CaM-binding domain produced a constitutively active form of CaNA (ΔCaNA in Fig. 2), which does not depend on an intracellular Ca^{2+} increase (*45–47*). The single amino acid mutation of a dominant negative mutant was also found to suppress endogenous CaN. Three mutants with substitutions of the histidine residues of CaNA to glutamate (H101Q, H160Q, and H290Q) effectively suppressed NFAT translocation when they were overexpressed in cultured cells (*36, 48, 49*).

The mechanism of CaNB activation has not yet been elucidated. To understand the CaNB function, we have to address two questions: why CaNA is inactive as a monomer, while other protein phosphatases with structures similar to the catalytic subunit are active as monomers; and why a constitutively active mutant of CaNA that lacks the CaM-binding site and the AI domain has 10 times higher affinity for Ca^{2+} than the native enzyme (*50*). Results indicate that Ca^{2+} binding to CaNB plays a structural rather than a regulatory role (*50*). Functional analysis using mutants of the latch domain of CaNB, which is a binding site for the drug-immunophilin complex, supports this hypothesis, since some mutants effectively suppressed CaN activity. This also indicates that the conformational folding of CaNA by CaNB is essential for allosterically activating CaNA (*29*).

4. Target substrates

CaN was originally thought to have a relatively narrow substrate specificity. Within a decade, however, many new substrates were found, although the *in vivo* relevance of some of them remains unclear. Many of the substrates are involved in various signal transduction pathways and diseases (*3, 51, 52*). They include potentially physiological substrates with clearly determined kinetic characteristics, such as NO synthase, dynamin (a GTPase involved in endocytosis), the transcription factor Elk-1, the heat shock protein hsp25, tau factor, and the NMDA receptor (*53–58*). The phosphorylation-independent tight binding of substrates, such as the transcription factor NF-ATp/1 and anti-apoptotic protein Bcl-2, may allow CaN to dephosphorylate proteins whose intracellular concentration is very low (*59–61*). However, NFAT4, another member of the NFAT family, is also a good substrate for CaN, and it binds to an N-termi-

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nal domain of CaN in an activity-dependent manner with a low affinity (*36*). This indicates that the substrate specificity of CaN is not due to a specific sequence but rather is determined by structural features (*62*). CaN also dephosphorylates phosphotyrosine, but the K_{cat} is two orders of magni-tude lower than that for phosphoserine (*63*). The synthetic peptide corresponding to residues 81–99 of the RII subunit of cAMP-dependent protein kinase is most commonly used to measure CaN phosphatase activity in cell and crude tissue extracts (*62*). This RII peptide is a poor substrate for other protein phosphatases 1, 2A, and 2C, and is usually used to quantitate the Ca^{2+}/CaM-dependent, metal-independent, okadaic acid-insensitive CaN activity (*42, 43, 64*). Difficulties are sometimes encountered in measuring CaN activity in crude extracts, because the inactive endogenous CaN is reactivated in the assay buffer including free Ca^{2+} and CaM. To solve this problem, we modified the assay using the 100-kDa pore-size filtration of crude extract to remove the free Ca^{2+} and CaM. The measurement of the CaN activity of activated CaN complex including CaNA (60 kDa), CaNB (19 kDa), and CaM (19 kDa), whose molecular mass is around 100 kDa, is performed in assay buffer without Ca^{2+} and CaM (*65*). The N-terminal half of NFAT4 is also a good substrate for the CaN activity assay, because this transcription factor is tightly regulated by CaN but not by other phosphatases (*36*). Inactivation of crude CaN by the superoxide anion and its protection and reactivation by ascorbate strongly suggest that reduced iron is required for activity (*43*).

5. Endogenous inhibitors

The drug-binding domains of CaN are conserved from yeast to man. This raises the question of what are the natural ligands interacting with these domains. Since the finding of CaN, many researchers have struggled in vain to find small endogenous analogs of FK506 and CsA. The disruption of the FKBP12-mediated anchoring of CaN to the ryanodine and IP_3 receptors by FK506 suggests that these receptors may involve a specific amino acid sequence structurally similar to such analogs (*66, 67*). The FKBP-mediated targeting of CaN to the receptors may ensure rapid modulation of Ca^{2+} release from internal stores by inducing structural change of the receptors through protein dephosphorylation (*68, 69*). Since 1995, several endogenous CaN inhibitors have been reported. The first important endogenous inhibitor of CaN is AKAP79 (A-kinase anchoring protein), which binds both PKA (protein kinase A) and CaN in post-synaptic densities, possibly to allow these enzymes to act on the same substrates (*70*), or to regulate their activity or substrate specificity (*5*). AKAP79 requires neither FK506 nor Ca^{2+} to bind CaN, and the binding sites of CaN for AKAP79 overlap with the drug-binding site. The exact role of this protein is still unknown, but it may be partly responsible for the intracellular distribution of CaN through the action mentioned above. Secondly, in 1996, CHP (Calcineurin Homologous Protein) was identified in the expression library as a binding protein to the Na^{+}-H^{+} exchanger NHE1 (*71*). This protein is homologous to CaNB and inhibits CaN activity by direct binding (*72*). Thirdly, two proteins, Cabin1/cain and CBP1/calcipressin were identified using the yeast two-hybrid system. Cabin1/cain interacts with and inhibits CaN in a phosphorylation-dependent manner in mammalian cells through a binding site on CaN, which is distinct from that of the drug-immunophilin complex (*73, 74*). CBP1/calcipressin was also identified as a CaNA-binding protein and inhibits CaN activity (*75, 76*). The conserved peptide (FLISPPxSPP) of the calcipressin family was identified as a phosphorylation and binding site. Recent studies show that calcipressin functions as a feedback inhibitor of CaN signaling, because the expression is induced in a CaN activity–dependent manner. Most interestingly, this protein is encoded on the human chromosome 21 and is the first gene in the Down's syndrome candidate region (DSCR) interval (*77*), suggesting that calcipressin plays an important role in the pathophysiology of Down's syndrome (*75, 76*). Calcipressin and another homologue, Zaki-4 (*78*), of the *DSCR1*-like gene family were termed MCIP1 and MCIP2, respectively (*79*). The last CaN inhibitor, A238L, is not an endogenous protein but is encoded by a gene of the *African swine fever virus* (ASFV) (*80*). A238L was first identified as a potent immunosuppressor that inhibited a NFκB transcription factor and the CaN activity. Further study demonstrated that A238L directly binds to CaNA and competitively inhibits its activity by binding to a site near the catalytic domain and/or its PxIxITxC/S motif, which is also the binding site for NFAT (*80*).

6. Overview of calcineurin functions in brain

Identifying the sites of action of CaN in striatal and hippocampal neurons, which are particularly rich in CaN, continues to be a major challenge. The dephosphorylation of DARPP-32 by CaN in striatal neurons was the first evidence of a protein phosphatase cascade involving CaN that is responsible for the opposite effects of glutamate and cAMP on neuronal excitability (*81*). In hippocampal neurons, the activation of CaN not only results in inhibition of the release of the neurotransmitters, glutamate and aminobutyric acid (*82*), but is also involved in the desensitization of the postsynaptic NMDA receptor–coupled Ca^{2+} channels (*83*). The complex regulation of the function of the NMDA receptors may be the basis for the proposed role of CaN in long-term potentiation (LTP) and depression (LTD) and long-term memory (*84, 85*). CaN-mediated activation of nitric oxide synthase (NOS) has also been invoked to explain glutamate neurotoxicity (*86*).

Several studies have reported *in vitro* dephosphorylation of tau by CaN (*57*). Tau is a microtubule-associated protein, which, in its less phosphorylated form, stabilizes the cytoskeleton by linking together its critical components such as neurofilaments and microtubuli. The action of CaN on structural proteins such as tau (other substrates of CaN are GAP-43, MAP-2, and tubulin) is of physiological importance not only for structural reasons in the neuron but also for neurite formation (*87*). An *in vivo* study using CaNAα knock-out mice (*88*) has confirmed the ability of CaN to dephosphorylate tau by showing increased tau phosphorylation in these animals in comparison to the wild type. Tau protein has been shown to be involved in Alzheimer's disease (AD). Hyperphosphorylated tau has less affinity for microtubuli and aggregates to form a large part of the neurofibrillary tangles characteristically found in the brains of AD patients. An imbalance between "tau kinases" and "phosphatases" has therefore been proposed as a pathophysiological mechanism in AD. One recent report found decreased CaN activity in human AD brains compared to age-matched controls, supporting this hypothesis of an

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important role for CaN in the pathogenesis of AD (*89*).

Several studies have clarified the involvement of CaN in the process of the formation of long-term memory. This requires new protein synthesis (*90*), which means that the intracellular signals during this memory development must reach the nucleus and alter the gene expression. Ca^{2+} was first described as an important regulator of gene expression in 1985 (*91*). A well-known example of Ca^{2+}-activated transcription involves the transcription factor CREB, cyclic AMP response element binding protein, which is of importance in memory formation (*92*). When phosphorylated by PKA, CREB can translocate into the nucleus and interact with the cAMP response element in the promoter region of several genes, increasing their level of expression. CREB activity is also regulated by CaN through the dephosphorylation of inhibitor-1 and the subsequent activation of protein phosphatase-1, which dephosphorylates CREB and prevents its nuclear translocation (*93*). This mechanism thus demonstrates that CaN is an important factor influencing the process of memory formation. CaN has been found to increase in rat hippocampus during aging (*94*), a process which also results in impaired Ca^{2+} homeostasis intracellularly. This study showed that the increased CaN correlated with memory deficits, further supporting the involvement of CaN in memory. Also, NMDA receptors are involved in synaptic plasticity and, like CREB, they are influenced by CaN activity. CaN causes a decreased rate of Ca^{2+} entry through NMDA receptors both by decreasing the opening time of the channel and by blocking desensitisation (*5*). A CaN overexpression study in mice showed impaired long-term memory in these animals (*85*), while another report demonstrated enhanced learning and memory capacity when CaN was reversibly inhibited in mice (*95*). The balance between phosphatases and kinases seems to be of importance for the choice between LTD and LTP, the alternative mechanisms in memory formation. The explanation for this could be the different affinities for Ca^{2+} of the important enzymes in this respect. CaN has a higher affinity for Ca^{2+} than CaMKIIα, a kinase involved in LTP, and might therefore be activated earlier during Ca^{2+} influx or after a low frequency of stimulation, when the Ca^{2+} levels have not yet reached a high enough concentration to activate CaMKIIα, leading to LTD instead of LTP (*96*). CaN also participates in the regulation of the function of the IP_3- and ryanodine-receptors (*67*), modulating their ability to release Ca^{2+}. These receptors also have an important function in skeletal muscle by regulating the amount of Ca^{2+} released from the sarcoplasmic reticulum.

A new role for CaN has also been proposed in apoptosis (*8*) and in the redistribution of the integrins required for the migration of neutrophils on vibronectin in response to Ca^{2+} transients (*97, 98*). The inhibition of the CaN-mediated regulation of Na^+,K^+-ATPase by immunosuppressive drugs in the kidney (*99*) may be responsible for their nephrotoxicity, whereas in cerebellar neurons, the CaN activation of the Na^+,K^+-ATPase is required to prevent neurotoxicity because of excessive Na^+ entry induced by glutamate binding to NMDA receptors (*100*). Regulation of two intracellular Ca^{2+} channels (the ryanodine receptor involved in excitation–contraction coupling in skeletal muscle and other excitable cells and the IP_3 receptor involved in Ca^{2+} release by hormones and neurotransmitters) can potentially affect all cellular processes under Ca^{2+} control. The pathway of CaN in cell death has been closed up recently, especially in ischemic brain damage and neuronal degeneration. Evidence that immunosuppressants CsA and FK506 have a potent neuroprotective effect in animal models mimicking human ischemic and degenerative diseases has focused attention more closely on this pathway (*10, 11*). An overview of the new role of calcineurin in ischemic diseases with an accompanying explanation of the recent background follows.

7. Calcineurin in ischemic brain damage

Brain ischemia leads to specific damage that is accompanied by a cascade of biochemical events. The influx of calcium into cells, a key event among several factors in the pathogenesis of ischemic brain damage, reflects the opening of calcium channels in membranes, secondary to the presynaptic release of glutamate and membrane depolarization. Calcium is a ubiquitous intracellular messenger and an activator of biochemical cascades of potentially adverse nature. The traditional view is that cell death caused by ischemia has the structural and biochemical characteristics of necrosis, *i.e.*, a type of cell death that is characterized by the swelling of cells and intracellular organelles, by membrane rupture, and by an inflammatory response. However, it has been realized that ischemic cell death may take on the characteristics of apoptosis, particularly in global/forebrain ischemia of a short duration, or in the periphery of a focal ischemic lesion (*101–104*). This section discusses ischemia in the context of recent ideas with a special focus on the role of mitochondrial dysfunction and calcium responsive enzyme, CaN.

7-1. Types of ischemia. The sequence set in motion by ischemia triggers reactions that may lead to brain damage (*105*). This is of two major types. In one, typically caused by brief periods of global or focal ischemia, damage occurs as selective neuronal necrosis, affecting neuronal populations which are particularly vulnerable to ischemic and some other insults (*106–108*). Such damage is typically delayed and has so far been called "delayed neuronal cell death," because the typical pathological findings of damage appeared 2–3 days after ischemic reperfusion in specific regions such as the CA1 sector of the hippocampus and cerebral cortex for models of forebrain ischemia, and the penumbral region for those of middle cerebral artery (MCA) occlusion. In general, damage is inversely proportional to the length of the preceding ischemia (*109*). The second type of ischemic brain damage is pan-necrosis ("infarction"), which involves not only neurons but also glial cells and vascular elements. It typically occurs after a focal ischemia of relatively long duration, *e.g.*, that caused by middle cerebral artery (MCA) occlusion [for data in primates and rats, see reviews (*110, 111*)]. However, infarction is also observed in forebrain/global ischemia of long duration, particularly if the ischemia is incurred in hyperglycemic subjects (*112, 113*). A third and relatively minor type of tissue damage is trans-synaptic neuronal necrosis, *e.g.*, that which occurs as delayed neuronal damage following MCA occlusion (*114, 115*). The affected areas (*e.g.*, the substantia nigra and certain thalamic nuclei) are not part of the ischemic territories, but they are synaptically connected with those that are (*116*).

7-2. Bioenergetic status of ischemia. When released

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in ischemic events, glutamate stimulates both ionotropic (NMDA, AMPA receptor) and metabotropic receptors, which first activate ion channels that provide major entry pathways for calcium. Since Ca^{2+} is an important second messenger in signal transduction, a cascade of potentially harmful reactions is triggered, encompassing enhanced lipolysis and proteolysis, as well as changes in protein phosphorylation and gene expression (*117–119*). At least two events qualify as putative mechanisms of brain damage. The first is an alteration of the signal transduction pathway, which starts with the stimulation of receptors for glutamate and growth factors, and continues with the generation of second messengers, which modulate downstream signaling by influencing cytosolic transcription factors, some of which are kinases and phosphatases, and which regulate gene expression and protein synthesis (*120, 121*). The second is one in which the resumption of oxygen supply leads to grossly enhanced production of ROS and, thereby, to free radical-mediated damage (*122–124*). Finally, the stimulation of NO synthase (NOS), a Ca^{2+}-CaM dependent enzyme, leads to the production of ROS. Clearly, the reactions outlined provide a direct link between the loss of Ca^{2+} homeostasis and the production of free radicals. In brain damage triggered by a nonphysiologic Ca^{2+} transient, the mitochondria occupy a central role. This is not only because mitochondrial dysfunction underlies the bioenergetic failure that triggers the initial loss of cell Ca^{2+} homeostasis, but also because the mitochondria absorb a major fraction of the cell Ca^{2+} load once they become re-energized during recovery from an insult, or during reperfusion (*125–127*). Another important factor to induce brain damage that is activated by intracellular Ca^{2+} increase is CaN, which is the only protein phosphatase directly stimulated by Ca^{2+}.

7-3. Mitochondria as a center of cell death. The concept has recently emerged of a mitochondrial permeability transition (MPT) pore as a major pathogenetic factor in brain ischemia and of MPT pore assembly that can lead to mitochondrial dysfunction and cell death. The mitochondrial permeability transition (MPT) pore is reported to be a voltage-sensitive and Ca^{2+}-activated channel in the inner mitochondrial membrane that is indiscriminately permeable to solutes with a molecular mass < 1,500 Da (*128–130*). An MPT pore consists of three components: a voltage-dependent anion channel (VDAC), an adenosine nucleotide translocator (ANT), and cyclophilin D, which is an isoform of a cyclophilin family member and is expressed specifically in mitochondrial matrix (*131*). The role of an MPT pore in the heart and liver in reperfusion damage has been discussed by several authors (*131, 132*). The importance of an MPT pore in ischemia is demonstrated by coupling both the accompanying depolarization and the mitochondrial release of an apoptosis-inducing factor (AIF) and of cytochrome *c* (cyt *c*), which can induce cell death by activating caspase-9/caspase-3 (Fig. 3). These proteases have been called the

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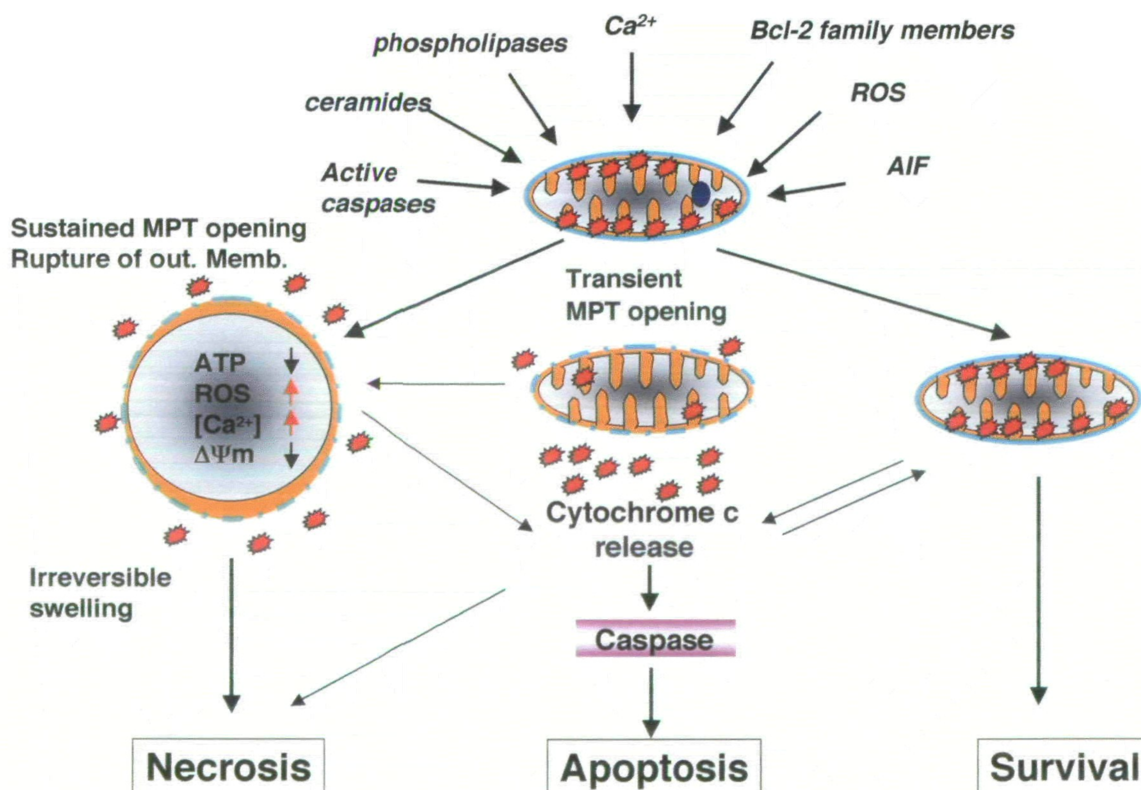

Fig. 3. **Mitochondrial function in ischemia.** Ischemia leads to energy failure and cell depolarization, and it also induces a massive release of glutamate. Glutamate stimulates both ionotropic (NMDA, AMPA receptor) and metabotropic receptors. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) increase and signal transduction pathway occur. Ca^{2+} also activates Ca^{2+}-dependent enzymes, especially CaN. $[Ca^{2+}]_i$ increase leads to the Ca^{2+} overload, and mitochondria request Ca^{2+} due to the energy-dependent manner. At a certain point, it reaches the assembly of the Mitochondrial Permeability Transition (MPT) pore. Pore opening accompanies the release of mitochondrial protein and proteases such as cytochrome *c* and AIF. Finally, the process leads to cell death.

executioners of cell death, since they activate a series of enzymes, causing a breakdown of DNA, fodrin, and poly (ADP-ribose) polymerase (PARP) (*133–136*). The assembly of an MPT pore probably also contributes to reperfusion damage in the brain. Indirect evidence supporting this notion was afforded by the finding that the immunosuppressant CsA, when given in such a way that the low permeability to CsA across the BBB was bypassed, dramatically decreased the neuronal necrosis in the CA1 sector and other areas in the brain (*11, 137*) and aborted post-ischemic seizures in hyperglycemic animals (*138*). CsA proved effective when given 30 min after the start of reperfusion (*11, 137*) but not when given after 2 h (personal communication from Dr. Friberg), suggesting that a CsA-sensitive MPT is not likely to operate many hours or days after the ischemia, *i.e.*, at the time when the delayed damage becomes manifest.

7-4. The role of calcineurin in ischemic brain damage. Calcineurin is highly localized in vulnerable regions of the brain such as in CA1 pyramidal cells and has been suggested to be crucial in the vulnerable neurons, especially after ischemia. Some reports suggested the alteration of CaN after transient forebrain ischemia (*139–141*). In particular, a decrease of CaN immunoreactivity of CA1 pyramidal cells was reported 1.5 days after ischemia, and a gradual loss during the time course of the neuronal death was also suggested (*139*). On the other hand, transient MCA occlusion in rat produced small lesions in the dorsolateral striatum, and CaN immunoreactivity was shown to be preserved until morphological degeneration appeared 8 h after 30 min of ischemia (*142*). These results suggest that the immunoreactivity of CaN tends to be preserved until a remarkable degeneration of neurons has occurred. One can then envisage a scenario where Ca^{2+} enters cells, combines with CaM, and activates CaN, which acts on its substrates, including NFAT, NOS (nitric oxide synthase), and the NMDA receptor (*143*).

A coupling seems to exist between CaN and the Bcl-2 family of proteins. Thus, Bcl-2 has been found to form a tight complex with CaN, resulting in the targeting of CaN to Bcl-2 sites on cytoplasmic membranes (*61*). When CaN is bound to Bcl-2, it is unable to promote the nuclear translocation of NFAT, and Bcl-2 blocks the cell death resulting from CaN overexpression. Interestingly, the pro-apoptotic Bax interferes with interactions between CaN and Bcl-2 (Fig. 4). Both toxic and protective actions of this phosphatase have been reported in different models of ischemia. The direct target of the toxic actions of CaN found in BHK (*8*) and HEK293 cells is the pro-apoptotic Bcl-2 family member Bad (*9*), which causes its release from its cytosolic anchor, 14-3-3 protein, and enables it to bind to the anti-apoptotic Bcl-X_L protein. This results in loss of the anti-apoptotic function of Bcl-X_L (Fig. 5). Glutamate-induced Ca^{2+} influx caused CaN activation in hippocampal neurons, triggering mitochondrial targeting of Bad and apoptosis. Both could be suppressed by a dominant-inhibitory mutant of CaN, or by pharmacological inhibition of CaN (*9*). The opposite effect of calcineurin on a Bcl-2 family member has not yet been fully clarified, but one possible explanation is that the expression of Bcl-2 is lower than that of Bad in vulnerable region of brain. Under a physiological concentration of Ca^{2+} in normal neuronal functions, CaN is important as a regulator of synaptic transmission. On the other hand, under pathological conditions, such as ischemia, a massive increase of Ca^{2+} concentration may induce the full activation of CaN. This may lead to a toxic action against neu-

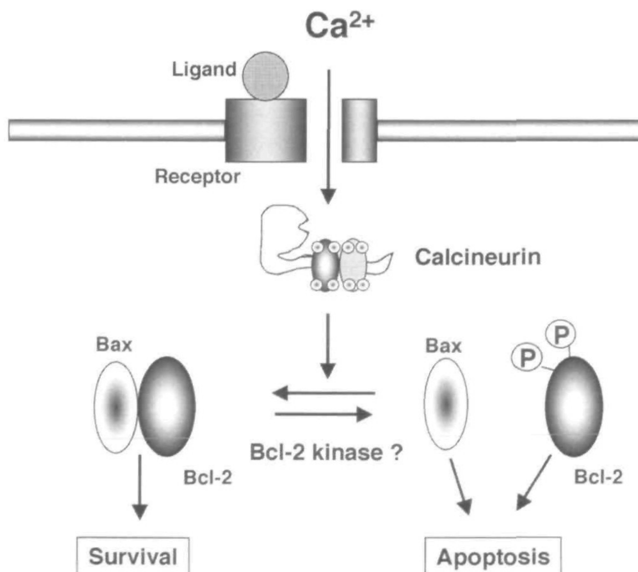

Fig. 4. **Calcineurin acts as an anti-apoptotic protein through dephosphorylating Bcl-2.** Calcineurin was found to bind directly to Bcl-2, an anti-apoptotic protein. This binding might be necessary to maintain the anti-apoptotic ability of Bcl-2. In several tumor cells expressing large excess of endogenous Bcl-2, Bcl-2 was phosphorylated after treatment with anti-cancer drugs such as Taxol, and its anti-apoptotic activity was decreased or lost (*180*). These findings suggest that Bcl-2 might be regulated by the balance between calcineurin and Bcl-2 kinases.

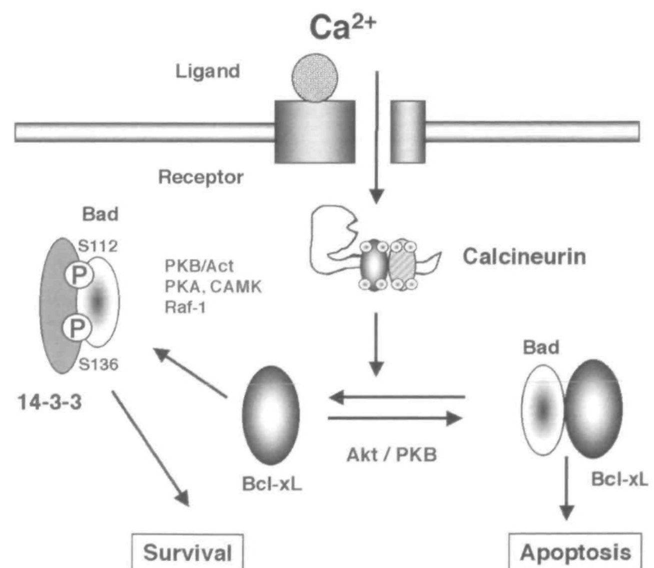

Fig. 5. **Calcineurin acts as a death inducer through dephosphorylating Bad.** In contrast to the relation between calcineurin and Bcl-2, the dephosphorylation of a pro-apoptotic protein, Bad, by CaN triggers the binding of Bad to Bcl-xL to induce apoptosis. The reason for opposite effect of calcineurin in the regulation of cell death is not clear, but might be due to the different expression of a Bcl-2 family member in tissues.

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ronal cells (Fig. 6).

7-5. Immunosuppressants FK506 and CsA as anti-ischemic drugs. The anti-ischemic effect of CsA in focal ischemia was first suggested by Shiga and Butcher *et al.* (*144*), who occluded the MCA in rats for 90 min using an intraluminal filament technique. Later, Sharkey and Butcher (*10*) found a robust effect of FK506 in MCA occlusion induced by endothelin injection. Subsequent results have given information that is pertinent to the following questions. First, is there a difference between the anti-ischemic effects of CsA and FK506? Second, what is the therapeutic window of opportunity for CsA and FK506? Third, if the two immunosuppressants differ in the mechanisms of their anti-ischemic effects, what is the molecular explanation?

In a later study, FK506 failed to reduce NO production *in vivo* during brain ischemia (*145*), indicating a neuroprotective mechanism of this compound is rather than reducing NO. The CaN inhibitor cyclosporin A (CsA) provides more potent protection against brain damage in certain ischemia models (*137*). In the experiments of Friberg *et al.* (*146*), the CsA analogue MeVal-CsA, which showed a specific inhibition of isomerase activity without suppressing CaN activity, had effects similar to that of CsA. Since this analogue blocks the MPT pore *in vitro*, it was suggested that CsA and its analogues work by preventing the opening of an MPT pore, a probable candidate mechanism by which the neuroprotection of CsA is provided through its target molecule cyclophilin D. However, recent results obtained in rats, subjected to 90–120 min of permanent MCA occlusion, demonstrate that CsA and FK506 are equally efficacious when given 1 to 3 h after the start of recirculation, infarct volume being reduced to 35–40% of the control (*147*). In these experiments, the low rate of penetration of CsA across the BBB was circumvented by an increase in the systematically administered dose (*146*), or by direct intracarotid infusion (*147*). However, when CsA was infused 5 min after the start of recirculation, following 2 h of transient MCA occlusion, the size of the infarct was reduced to 5–10% of the control (*147*). Since this additional effect was not observed when FK506 was given at the same time point, the results suggested that a CsA-sensitive MPT pore remains to be directly demonstrated the critical role in view of the *in vitro* results discussed above. At the present time, therefore, experiments on both forebrain and focal ischemia suggest that calcineurin pathway underlies the

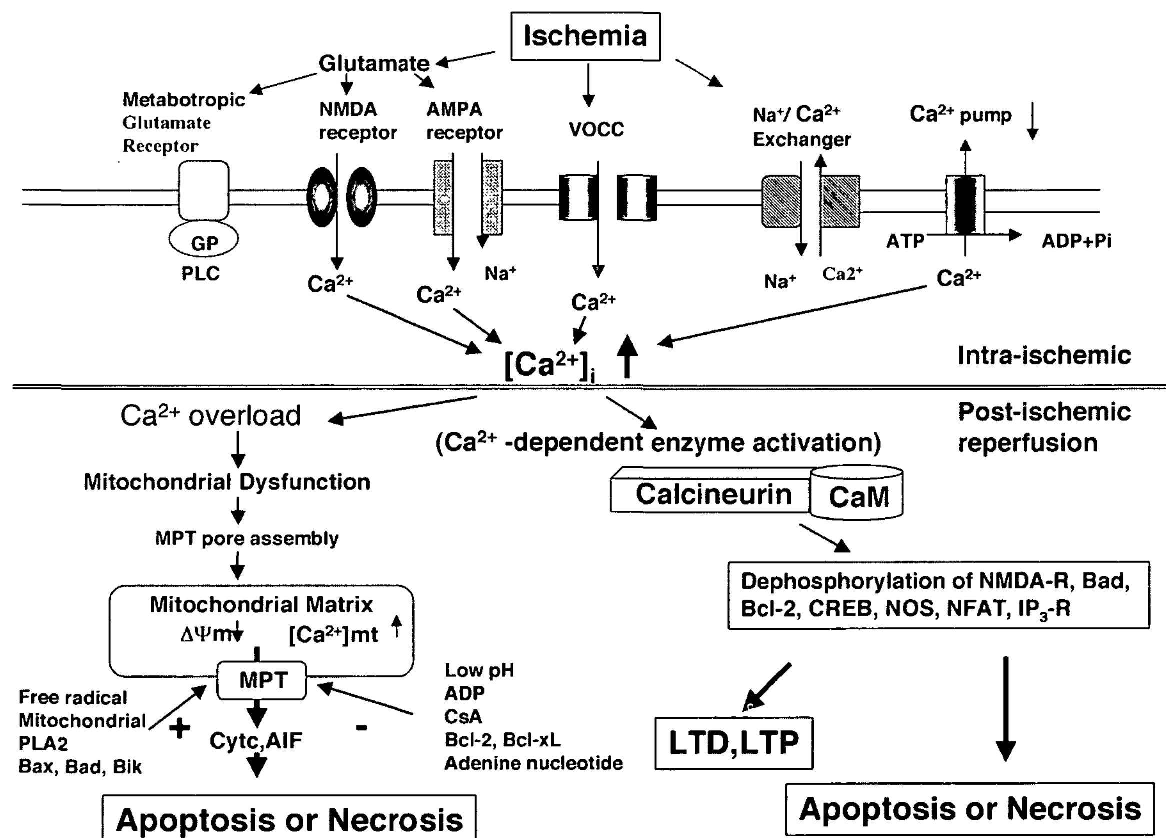

Fig. 6. **Overview of signaling pathways in ischemia.** This schema is cited from Siesjö *et al.* (*105*) with permission. It indicates how mitochondria can trigger necrotic or apoptotic cell death. It also suggests that the "cell death trigger" is a decrease in $\Delta\psi m$, which is promoted by mitochondrial Ca^{2+} accumulation or oxidative stress and counteracted by cyclosporin A (CsA) and the anti-apoptotic members of the Bcl-2 gene family (such as Bax, Bik, and Bad). Sustained mitochondrial permeability transition (MPT) is apt to cause large amplitude mitochondrial swelling with outer membrane rupture, leading to a massive release of mitochondrial proteins (and glutathione). The immediate result is collapse of the ΔH^+, uncoupling of phosphorylation, a spurt of production of reactive oxygen species (ROS) and cessation of ATP production, while the ultimate result is necrosis. Spurred on by the release of cytochrome *c* and other apoptogenic factors, this triggers cell death by a series of events that involve Apaf-1 and the activation of caspases, including caspase-3, the "executioner" of cell death.

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shared effects of CsA and FK506. CsA has a more potent neuroprotective effect than FK506, probably through inhibiting cyclophilin D in mitochondria as well as suppressing CaNA activity.

8. CaN in muscle tissues

Recent advances in molecular technology, including the creation of transgenic and knockout animals, have revealed unexpected functions of several gene products. These technologies have shed light on novel functions of calcineurin in the cardiovascular system and skeletal muscle from the perspectives of diseases or development of these organs. The aim of this section is to point out some of the functions of CaN in muscular tissues to show the many different mechanisms this phosphatase has been found to be involved in.

Tissues showing a moderately high level of CaN expression are skeletal and cardiac muscle. The functions of these tissues are highly regulated by the intracellular concentration of Ca^{2+}, and it is therefore not surprising that the only serine/threonine protein phosphatase that is known to be regulated by Ca^{2+} and CaM has been found to be involved in many cellular mechanisms, both physiologically and pathologically, in the cell types making up these tissues. How CaN can detect a specific Ca^{2+} signal to turn on specific mechanisms, when the intracellular Ca^{2+} level in muscle cells is subject to very frequent changes during the normal contraction/relaxation cycle? This has been explained by the fact that, for its activation, CaN requires a relatively low increase in intracellular Ca^{2+} of longer duration (*148*). This feature is typical of muscle cells of the slow type due to greater tonic motor neuron activity (*149*). A situation of sustained, increased intracellular Ca^{2+} also seems to be the case in the pathogenesis of cardiac hypertrophy (*150*). Several excellent reviews and thorough research reports have been published regarding the involvement of CaN in muscle cell functions and pathology, and studies are presently being performed to further elucidate the details. Some contradictory results have been obtained, especially in *in vivo* studies, possibly due to experimental differences, but there is no argument that CaN is also an important enzyme in these tissues.

To some degree, CaN in muscular tissue is working through the same substrates as in other tissues, although the isoforms might differ. In skeletal muscle, two isoforms of the transcription factor NFAT are especially abundant (*151*): NFAT2 and NFAT4. In cardiac muscle, NFAT3 is the predominantly expressed NFAT family member (*152*). The transcription factor MEF2 (Myocyte Enhancer binding Factor 2), which acts in concert with NFAT, is also expressed in skeletal muscle and is in some situations involved in the function of CaN (*153*). Another functional partner of NFAT is the transcription factor GATA: in skeletal muscle in the combination NFAT2/GATA2, and in cardiac muscle NFAT3/GATA4 (*149*). The type of endogenous CaN inhibitors and modulating proteins also differ between tissues. In skeletal muscle, the CaN-inhibiting proteins MCIP1 (Myocyte enriched CaN Interacting Protein) and MCIP2 are highly expressed (*149*). These proteins are the products of the genes *DSCR1* (Down Syndrome Candidate Region 1) and *ZAKI-4* respectively (*79*). The latter was renamed *DSCR1L1* (DSCR1-Like protein 1) when its similarities with *DSCR1* were discovered (*154*). *DSCR1* is also highly expressed in cardiac tissue (*77*). The human *DSCR1* gene is located on chromosome 21 and its overexpression in Down's syndrome has been suggested to be partly responsible for some of the clinical features of this syndrome (*77*). The gene is expressed in mice during the critical period of the development of the heart and during neurogenesis (*155*), suggesting its involvement in the pathogenesis of the specific cardiac and neurological changes seen in Down's syndrome patients.

8-1. CaN in the cardiovascular system. CaN is important early in the development of the cardiovascular system, especially for the development of the cardiac valves and septum, as recently shown by two different groups (*156, 157*). CaN is involved in this process through its regulation of NFAT. This was shown by disrupting the NFAT gene in mice, an insult which resulted in the impaired development of the aortic and pulmonary valves and cardiac septum and early embryonal death. In normal animals, NFAT has a nuclear localization only in endocardial cells destined to participate in valve formation. The CaN inhibitor FK506 given to wild-type embryos prevents the nuclear translocation of NFAT. CaN has also been shown, by many experimental approaches, both *in vitro* using cultured neonatal cardiomyocytes and *in vivo*, to be involved in the development of cardiac hypertrophy. The evaluation of the *in vivo* effect of treatment with CaN inhibitors, however, has produced contradictory results, and further studies are needed to investigate the reasons for this. The Ca^{2+}/CaN pathway that is suggested to participate in the cardiac hypertrophic response is the induction of the nuclear translocation of NFAT3 (NFATc4) by CaN, and the subsequent interaction of NFAT3 with the transcription factor GATA4 (*12*). GATA4 was known to be involved in the induction of the expression of hypertrophic target genes in the heart and was found, by use of a yeast two-hybrid system (*12*), to interact with NFAT3. It was shown *in vitro* that GATA4 together with NFAT3 activated the transcription of *BNP* (B-type Natriuretic Peptide), which is known to be upregulated in cardiac hypertrophy (*158*). Yet another role of CaN is in the process of apoptosis in cardiomyocytes. However, results pertaining to this role point in different directions (*48, 159*), which leaves the question to be answered in the future.

8-2. CaN in skeletal muscle. CaN has been shown to be involved in several processes in skeletal muscle function: differentiation, regulation of gene expression, determination of fiber type and mechanisms leading to hypertrophy. In 2000, Friday *et al.* (*160*) reported *in vitro* data showing a role for CaN in the early stage of differentiation of skeletal muscle cells, but without the involvement of NFAT, an otherwise common and important substrate for CaN, through which CaN often exerts its effects. Several studies have investigated the role of CaN in muscle remodelling in adaptation to changed physiological demands, by hypertrophy or change of fiber type. The requirement of CaN for skeletal muscle hypertrophy was shown in an animal model of functional overload. Treatment with cyclosporin A or FK506 in the model prevented this hypertrophy (*161*). In skeletal muscle, CaN regulates induction of the expression of genes for hypertrophy through the transcription factor NFAT, and its interaction with GATA2 (*149*). Results of an *in vitro* study of cultured myogenic cells suggested a model in which CaN upregulates slow fiber-specific gene promoters

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also by the dephosphorylation of NFAT, which in combination with the transcription factor MEF2 induces the expression of genes in the "slow fiber programme" (*162*). This model was also tested in an *in vivo* experiment using the CaN inhibitor cyclosporin A, which when given to intact animals resulted in a conversion of slow fibers to fast fibers. One possible clinical implication based on these results comes from the finding that slow muscle fibers seem to be less vulnerable to dystrophy (*163*).

9. Calcineurin in the immune system

The identification of its substrates and the use of specific CaN inhibitors or CaN knockout or overexpressing animals are gradually revealing the roles of CaN in the immune system. A key to understanding the function of CaN was the discovery that NFAT was the direct substrate of calcineurin (*59*). Molecular cloning of the first NFAT1/p revealed the C-terminal transactivation and DNA-binding domain is distantly related to that of the Rel family of transcription factors (*59*). To date, five separate genes encoding NFATs have been cloned and generally denoted NFAT1/p, NFAT2/c, NFAT3, NFAT4, and NFAT5 (*164, 165*). Despite their structural and functional similarities, it is now apparent that the NFATs show tissue distribution patterns far beyond their eponymous borders. Among them, the expression of NFAT4 is restricted to $CD4^{+}$, $CD8^{+}$-double positive lymphocytes. NFAT1 is also expressed in immune cells and non-immune cells. In contrast, NFAT2/c and NFAT3 are expressed in cardiac and skeletal muscles (*164*). As described in the section above, these two NFATs have been shown to play an important role in the development and hypertrophic disorder of the heart (*12*). In agreement with the intricate expression patterns of the NFATs, a large number of cytokine genes have been shown to contain NFAT sites in their promoter region, including IL-2, IL-3, IL-4, granulocyte macrophage colony stimulating factor (GM-CSF), TNFα, interferon-γ and the Fas ligand, all of which are responsive to NFATs (*164*). The nuclear translocation of NFATs (except for NFAT5), in response to an increase of intracellular Ca^{2+} induced by the occupancy of the T cell receptor (TCR/CD28), is dependent upon their dephosphorylation by CaN (*59*). Recent studies have revealed a phosphorylation-dependent intramolecular NLS-masking mechanism that switches on and off the nuclear localization signal of NFATs (*166*). The involvement of NFAT kinases such as GSK-3, JNK1, and MKK-8A has been proposed in the mechanism of the nuclear translocation (*167, 168*). The clearest picture of the intramolecular mechanism by which NFAT4 is regulated by the balance between CaN and kinases has been demonstrated in the *in vitro* expression system (*36, 166*). Casein kinase 1α, which was identified by affinity chromatography using GST fusion protein of N-terminal NFAT4, subsequently phosphorylates a contiguous domain (Z domain) that directly masks the NLS of NFAT in a phosphorylation-dependent manner (*166*). An essential feature of NFAT signaling is the presence of a constitutive nuclear export mechanism superimposed onto a regulated import control. When the intracellular calcium concentration drops back below 150 nM, NFATs are exported from the nucleus with a $t_{1/2}$ ~ 12 min (*36*). Nuclear export is now known to be an active process involving nuclear export signal (NES) sequences recognized by NES receptors such as CRM1 (*169, 170*). The basic regulatory features underlying the remarkable sensitivity of NFAT to calcium signals and their transduction mechanisms are now apparent. These translocation signal sequences are under dual regulation by the calcium-sensitive phosphatase CaN, which can simultaneously unmask import signals while it masks export signals (*171, 172*).

As it does in T cells, CaN plays an important role in the regulation of gene expression in response to Ca^{2+} signals in yeast (*173–177*). The two major sites of action of CaN in this organism are in the pheromone response pathway (*178*) and the adaptation to high salt stress (*173*). The induction of the genes involved in these two pathways has now been shown to be regulated differentially by the Ca^{2+}-dependent and FK506-sensitive interaction of a single transcription factor (Tcn1p also named Crz1p) with CaN (*174, 175*). Other processes under CaN control include Ca^{2+} sequestration, cytokinesis, sporulation, and mating (*176, 177, 179*).

Conclusion

This report has summarized the recent findings of calcineurin functions in several organs. Within a decade, two major concepts have emerged: one is that calcineurin is involved in the signal transduction pathway of cardiac hypertrophy through the cooperative action with GATA4/NFAT2/c; the other, also unexpected, is that this enzyme plays a critical role in the pathway of cell death, especially in ischemic brain damage through a Bcl-2 family member and other unknown targets. Both findings are extremely exciting, because the specific inhibitors CsA and FK506 have a potent effect to suppress cardiac hypertrophy and ameliorate ischemic brain damage, even though these results were shown only in animal models. The common concept that these drugs are immunosuppressants that are effective only for the suppression of the immune system should be changed. In addition, recent advances of molecular technology using animals have brought mitochondria back into the limelight. These old organelles have been shown to be a center of the cell death pathway as well as of bioenergetic metabolism. The analysis of an MPT pore located in both the outer and inner membrane of mitochondria also shed light on the isomerase activity of cyclophilin D. The present review covers only some calcineurin's functions; new roles of this phosphatase and its related proteins are being reported almost daily. However, the struggle continues to determine the true substrate and how it physiologically functions. To address these questions, the immunosuppressants CsA and FK506 are powerful tools to unveil the complex phenotype. These drugs, however, are also specific inhibitors of isomerases of immunophilins such as cyclophilins and FKBPs, which have been reported to be involved in the protein folding or chaperon function. We have to be extremely careful to understand the effect of these immunosuppressants.

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