

Special Lectures

The role of calcineurin in immune system responses

Randall L. Kincaid, PhD *Rockville, Maryland*

In this article I offer my own perspective on regulation of the immune system, which emphasizes some of the intracellular signaling events that underlie these biologic responses. Although this article highlights work that my collaborators and I have carried out, I would like to acknowledge at the outset the important contributions of many investigators in this area whose work I will be unable to discuss in detail.

As stated elsewhere in this supplement, the immune system represents an example of remarkable complexity and involves the coordinated interaction of many cell types. On the macroscopic level, these responses are achieved through intercellular communication and the tightly controlled differentiation and expansion of specific subsets of lymphoid cells. However, these events are ultimately set in motion through the regulation of key intracellular signaling pathways, which determine the production and release of cytokines or other mediators. Early studies of lymphocyte signaling focused primarily on the initial receptor-mediated steps involving activation of tyrosine kinases and their substrates.¹ Although very informative about the effector coupling events, these studies were unable to pin-point the critical downstream steps that link stimulus recognition to the cellular response or developmental commitment. Nonetheless, the existence of these subsequent signaling events had been inferred through pharmacologic studies involving drugs that can modify a wide range of immune cell responses.

During the past 7 years, my laboratory has focused its efforts on the biologic roles of calcineurin, a protein phosphatase that is regulated by Ca^{2+} through its association with the Ca^{2+} -binding protein, calmodulin. In this respect it is unique among protein phosphatases in that its activity can

Abbreviations used

CaM:	Calmodulin
cAMP:	Cyclic adenosine monophosphate
NF-AT:	Nuclear factor for activated cells
PMA:	Phorbol, 12-myristate, 13-acetate
TNF:	Tumor necrosis factor

be directly controlled by a second messenger. This phosphatase's sensitivity to Ca^{2+} provides a means to translate effector-induced changes in second messenger concentration into altered steady-state protein phosphorylation; the latter constitutes a "common currency" of intracellular information (Fig. 1). Also, because protein phosphorylation cascades are known to be regulated via "cross-talk" arising from multiple effector-regulated events, calcineurin seems positioned to integrate early responses by influencing other phosphoprotein pathways. These factors suggested to us that by acting as a Ca^{2+} sensor, calcineurin might function as a "switching" element, enabling the amplification of primary second messenger information and the redirection of biochemical events.

Historically, our interest in calcineurin stemmed from its identification as the major calmodulin-binding protein of the central nervous system,^{2,3} in which its high expression levels suggested a role in neural function. Interestingly, this largely neuronal protein was shown to bind Ca^{2+} through its small B subunit (Fig. 2) as well as via calmodulin, leading to its being named "calcineurin."⁴ Several years later it was discovered to be a serine/threonine phosphoprotein phosphatase (type 2B),⁵ the activation of which was almost completely dependent on its high-affinity Ca^{2+} -dependent interaction with calmodulin ($K_a < 10^{-9}$ mol/L). Perhaps most significant was that it exhibited a relatively narrow substrate specificity when compared with the related protein phosphatases, PP-1 and PP-2A.⁶ These findings generated considerable excitement in the field of phosphoprotein biochemistry

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Reprint requests: Randall L. Kincaid, PhD, Veritas, Inc., 11700 Bunnell Ct., Potomac, MD 20854.

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Second Messengers and Phosphoproteins in Biological Signaling Events

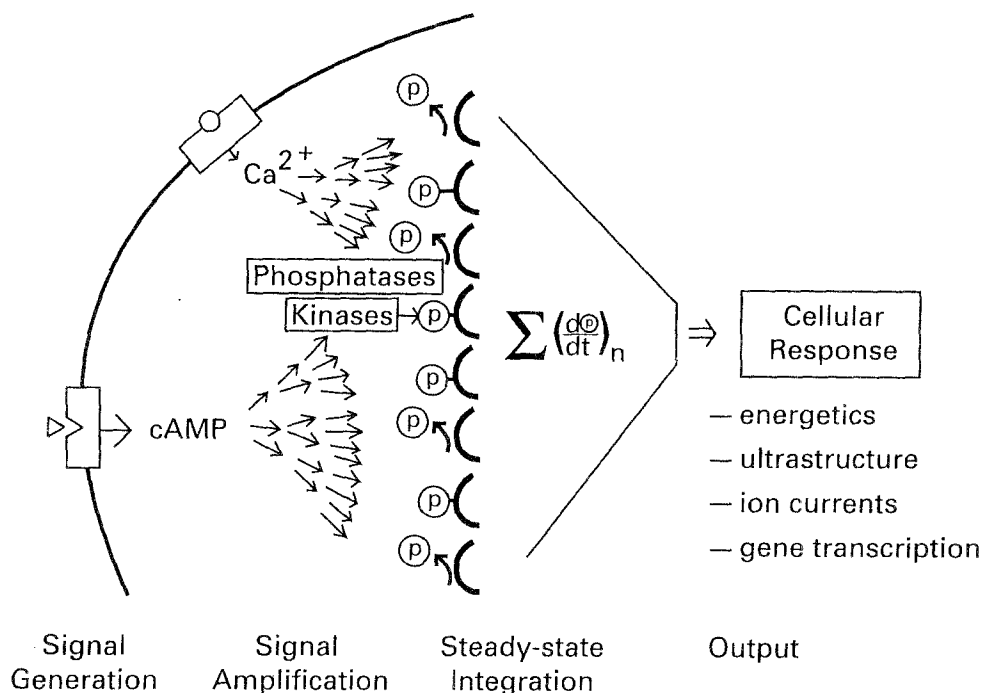


FIG. 1. Control of cellular response through second messenger-mediated regulation of phosphoprotein metabolism. Scheme for intracellular signaling is depicted, showing phosphoprotein content as the primary reservoir of information.

because it implied that calcineurin might act on a select group of biologic substrates.

Indeed, studies characterizing the mechanism of epinephrine-regulated glycogen breakdown in skeletal muscle showed that PP-2B (calcineurin) initiates the reversal of this cyclic adenosine monophosphate (cAMP) cascade by acting on a pivotal phosphoprotein, Inhibitor-1.⁷ By dephosphorylating this key substrate, an inhibitor of the broad specificity serine/threonine protein phosphatase PP-1, calcineurin activation unleashes a phosphatase cascade to rapidly amplify the Ca^{2+} signal and reverse the actions of cAMP. This illustrates an important physiologic example of how calcineurin may "switch off" a (cAMP-mediated) biochemical response to an effector by influencing a critical component common to other signaling pathways.

It has been known for some time that an elevation of intracellular Ca^{2+} is one of the earliest steps in the activation of lymphocytes.⁸ We wondered if, as in other cells, these might be calmodulin-mediated actions. In collaborative studies with Sitkovsky to determine a potential target for cal-

modulin in such early events, we established that calcineurin was the predominant calmodulin-binding protein in all populations of lymphohematopoietic cell types that we examined.⁹ Interestingly, we found that its amount varied between T and B lymphocytes and that an apparently different form of this protein was expressed in thymocytes. Although these findings did not prove that it influenced a critical lymphocyte function, we noted that as a protein phosphatase, it might easily influence cellular signaling responses in a selective way. On this basis we suggested that calcineurin, via regulation of phosphoproteins, might play a key role in Ca^{2+} -dependent lymphocyte activation.

A second and much larger current of research, which involves the mechanism of action of immunosuppressive drugs, also implicated Ca^{2+} -sensitive pathways in lymphocyte activation. It was known that a variety of immune cell responses, including lymphokine gene transcription, apoptosis, and degranulation, require Ca^{2+} and that these same pathways are blocked by the immunosuppressants, cyclosporine and FK-506 but not by a

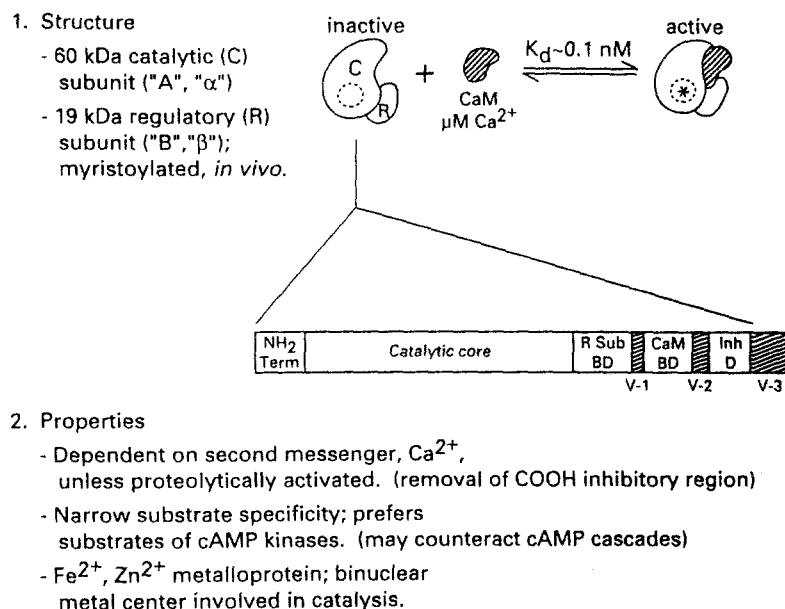


FIG. 2. Structure and properties of calcineurin, a Ca^{2+} - and calmodulin-dependent protein phosphatase. Expanded projection in middle of figure outlines the general domain structure for catalytic subunit. (Modified from Kincaid RL, O'Keefe SJ. Calcineurin and immunosuppression: a calmodulin-dependent protein phosphatase acts as the "gatekeeper" to interleukin-2 gene transcription. *Adv Prot Phosphatases* 1993;7:543-83, with permission.)

related drug, rapamycin.¹⁰ The extensive amount of data correlating immunosuppressant action and Ca^{2+} provided an important insight into the general response mechanism by which lymphocyte commitment is achieved and suggested that a unique Ca^{2+} -sensitive switching component, shared by these biologic responses, is targeted by such pharmacologic agents.

Considerable progress had been made in identifying the cellular receptors ("immunophilins") for the immunosuppressants. These cyclosporine-binding proteins (cyclophilins) and FK-506 binding proteins (FKBPs) appeared unrelated structurally but both had peptidyl-prolyl isomerase activity.^{11, 12} However, inhibition of this isomerase activity did not correlate with immunosuppressive activity, which suggested that immunophilins were not the final physiologic targets of drug action. These paradoxes were resolved in a hallmark collaborative study between the Schreiber and Weissman laboratories in which affinity chromatography with immobilized immunosuppressant-binding proteins yielded three polypeptides that bound to these matrixes only in the presence of both drug and Ca^{2+} ; these peptides corresponded to calmodulin and the A and B subunits of calcineurin.¹³ Furthermore, the drug-bound immunophilins inhibited

phosphatase activity, which supports the notion that cyclosporine and FK-506, when complexed to their binding proteins, block the actions of this Ca^{2+} - and calmodulin-dependent enzyme to exert their immunosuppressive and/or toxic effects.

In collaborative studies with O'Keefe et al., we examined the biologic role of calcineurin in the pathways required for T-cell expansion and set forth several requirements that we felt were essential to prove such an involvement. These were as follows: (1) that expression of a constitutively active form of calcineurin should substitute for Ca^{2+} in activation of the IL-2 gene; (2) that overexpression of calcineurin *in vivo* should decrease the pharmacologic sensitivity of T cells to immunosuppressants, and (3) that a "knock-out" of calcineurin function should block the activation of the IL-2 gene. To examine the first two requirements, we used a co-transfection strategy in which the calcineurin A and B subunits, either alone or separately, were introduced into the human lymphoma cell line, Jurkat, along with a chloramphenicol acetyl transferase reporter gene for the human IL-2 promoter.

In control experiments we showed that the expression vector alone did not affect the reporter gene response to pharmacologic activation; that is,

both stimuli, phorbol, 12-myristate, 13-acetate (PMA) and a Ca^{2+} ionophore (ionomycin) were required to elicit promoter activity. Similar results were observed when either the wild-type catalytic (A) subunit or regulatory (B) subunit alone were co-transfected with the reporter gene. Because it was known that calcineurin could become permanently activated by cleavage of an inhibitory domain near the carboxyl terminus of the A subunit,^{14, 15} Dr. Tamura in my laboratory had constructed a series of deletion mutants to characterize this domain¹⁶ and the putative calmodulin (CaM) binding domain¹⁷ deduced by homology to those of other enzymes. Therefore when a constitutively active form of the A subunit, designated ΔCaMAI (i.e., lacking both the *CaM* binding and *AutoInhibitory* domains) was introduced into Jurkat cells, the IL-2 promoter was activated synergistically with PMA in the complete absence of Ca^{2+} ionophore.¹⁸ This data strongly supported calcineurin's role as the Ca^{2+} -sensitive element in the signaling pathway for IL-2 activation. Interestingly, although this stimulation was dramatic and was greater than 100-fold, it was much less than the activation achieved with PMA plus ionomycin in control cells. This suggested that additional Ca^{2+} -regulated events may augment calcineurin to give greater activation of the IL-2 gene.

These initial experiments involving transfection of the A subunit constructs showed only a modest (1.5- to 2-fold) shift in the dose-response profile to immunosuppressants despite biochemical evidence that the levels of the A subunit were at least 10-fold higher than those in control cells. To see if the B subunit was required for maximal change in drug sensitivity, both A and B subunits were transfected together. When this was carried out, there was a 50- to 100-fold increase in the K_i for FK-506, which indicates that the A.B holoenzyme rather than the A (or B) subunit alone interacts with the immunophilin-drug complexes.¹⁹ Transfection experiments carried out by Dr. Ueki in my group, using deletion mutants of the A subunit that interrupt the putative B subunit binding domain,²⁰ support this hypothesis, as do recent biochemical studies showing crosslinking of the immunophilin-drug complex to the B subunit component of the holoenzyme.^{21, 22} Significantly, despite the much higher levels of the calcineurin holoenzyme in co-transfected cells, there was still submaximal activation of the IL-2 promoter, which again suggests that other Ca^{2+} -regulated pathways serve to amplify the calcineurin-initiated activation events.

The last criterion to demonstrate a biologic role

for calcineurin in IL-2 promoter regulation was to prevent IL-2 gene activation by "knocking out" calcineurin; this was very difficult to accomplish in part because of the redundancy of A subunit genes in Jurkat cells. Dr. Muramatsu in my laboratory has achieved a "pseudo" knock-out by preparing a dominant-negative construction encoding the B subunit-binding region of the A subunit. Because the B subunit is required for phosphatase activity,²³ this "sequestration" approach overcomes problems associated with the multiplicity of genes for the catalytic subunit. When a fusion protein containing this domain was overexpressed in Jurkat cells, activation of the IL-2 promoter by PMA plus ionomycin was almost completely suppressed (submitted). However, with a variety of expression conditions for this dominant-negative form, maximal levels of inhibition never exceeded 85%. The inability to achieve a complete blockade may reflect incomplete removal of the B subunit tightly bound in the holoenzyme over the course of the transfection experiment, the relative instability of the dominant negative fusion protein or competition with significant amounts of "free" B subunit. Dr. Matsushita in my group, using chromatographic methods, has shown that a sizable percentage of the B subunit in these cells does not appear to be complexed with the A subunit (unpublished). The presence of excess regulatory subunit in Jurkat cells also explains the PMA-sensitive promoter activation and the small shift in drug sensitivity seen when the activated A subunit, ΔCaMAI , is transfected alone; indeed, if there were no free B subunit in these cells, such findings would be paradoxical. The potential physiologic significance of excess B subunit in lymphoid cells is presently unclear.

These experiments, in addition to those in several other studies,^{24, 25} clearly implicated calcineurin in the physiologic regulation of the IL-2 gene. However, with respect to gene activation, several important issues remained unresolved. First, was phorbol ester (i.e., activation of protein kinase C pathways) necessary, a priori, for calcineurin-dependent events? Was calcineurin important only for IL-2 gene activation, or was it likely to play a larger role in T-cell receptor-mediated cytokine regulation? Were other effectors (other than those acting through the T-cell receptor) likely to transduce their effects through calcineurin? These questions address the scope of calcineurin's role in mediating other physiologic immune responses. I believe that pharmacologic data pointing to an involvement in other events cannot be taken as proof of a role, although it is usually persuasive.

Collaborative studies carried out with Kubo and

Ransom have enabled us to show that calcineurin is also a player in the regulation of the IL-4 gene in cells of the Th2 subset. In these cells, which play an important modulatory function in coordinating the B- and T-cell responses to immunogens, activation of the IL-4 gene plays a central role and this process is sensitive to cyclosporine and FK-506. Because experimental models for IL-4 gene activation do not require PMA stimulation, we felt that this might also enable an assessment of the need for protein kinase C in calcineurin-regulated gene activation, as seems to be the case for IL-2. In these studies we found that transfection of Δ CaMAI into responsive cells caused IL-4 promoter activation without any other treatment,²⁶ indicating that calcineurin is sufficient to mediate the effects on gene induction in the absence of phorbol esters.

In relation to its scope of action in immune responses, collaborative work with Goldfeld and Strominger has examined the potential role of calcineurin in the induction of tumor necrosis factor (TNF)- α , a mediator produced by both B and T cells that affects many steps in the inflammatory response. These studies also used Δ CaMAI to look at the activation of the TNF- α promoter in the absence of other stimuli and found that the gene was induced on expression of this activated form of calcineurin.²⁷ Furthermore, this study demonstrated that stimulation through the IgG receptor could initiate signaling responses mediated by calcineurin: thus the involvement of calcineurin is not restricted to activation through the T-cell receptor. Collectively, these data and those on the interleukins show that calcineurin plays an obligatory role in transducing signals in several subsets of lymphoid cells whose end results vary considerably—cell proliferation, modulating sensitivity of cells to receptor activation, enhancing cell response to inflammatory stimuli, and so forth. Also, based on pharmacologic data, it seems likely that other cytokine responses, notably that of IL-5, may also be mediated by calcineurin.

A topic that is central to understanding cytokine transcriptional responses is the precise mechanism of calcineurin's action. Put more simply, if calcineurin is directly involved in gene activation, what is the primary component targeted for dephosphorylation? In truth, the unambiguous identification of principal substrates for protein phosphatases has been extraordinarily difficult because of their generally broad and overlapping specificities. However, in T cells, there is ample precedent for the involvement of a transcription factor that is

sensitive to immunosuppressants and is needed for the earliest stages of the activation response, the Nuclear Factor for Activated T cells, or NF-AT.²⁸ This factor contains a resident nuclear component, now known to consist of *jun* and *fos*,²⁹ and a pre-existing cytosolic component (NF-AT_c), whose translocation to the nucleus is required for activity.³⁰ In light of this, it was proposed early on that calcineurin may directly dephosphorylate NF-AT_c on cell activation, which leads to the induction of gene activity.³¹ This proposal has been supported both by pharmacologic evidence using electrophoretic mobility shift assays and recently by the demonstration that calcineurin can dephosphorylate NF-AT_c in vitro.³² Therefore it seems likely that calcineurin plays a role in regulating the state of NF-AT_c phosphorylation required for its functional activation. However, several points weaken this hypothetical mechanism somewhat, at least from a biochemical perspective.

Collaborative studies carried out with O'Neill et al. showed that in transfection studies with Δ CaMAI, calcineurin was capable of regulating several promoter elements within the IL-2 promoter. Activation in synergy with phorbol ester was observed with IL-2A (OAP), IL-2C (NF- κ B), and IL-2E (NF-AT), whereas inhibition was seen with IL-2B (AP-1).³³ All of these effects were sensitive to reversal by immunosuppressants, which confirmed calcineurin's likely participation. For the OAP and NF-AT elements, the activation characteristics were similar to those observed with the intact IL-2 promoter and was submaximal when compared with vector control, whereas with NF- κ B activation in the presence of PMA was not further stimulated by addition of ionomycin. This latter result is intriguing because it suggests that in contrast to NF-AT and OAP activation pathways, calcineurin alone is sufficient to account for the Ca²⁺-dependent stimulation of NF- κ B. Given the breadth of transcriptional responses that are influenced by NF- κ B (and OAP), it is tempting to consider a wider role for calcineurin in modulating transcription than just in lymphocyte activation.

It may well be, as is widely held, that NF-AT pathways are the physiologically relevant targets of calcineurin with respect to transcriptional regulation. In fact all the responses to IL-2, IL-4, and TNF- α just described appear to require an NF-AT-like component. However, the apparent pleiotropic effects seen in the promoter element transfection experiments raise an important issue of how this phosphatase regulates the NF-AT (and other) responses. As mentioned previously, calcineurin

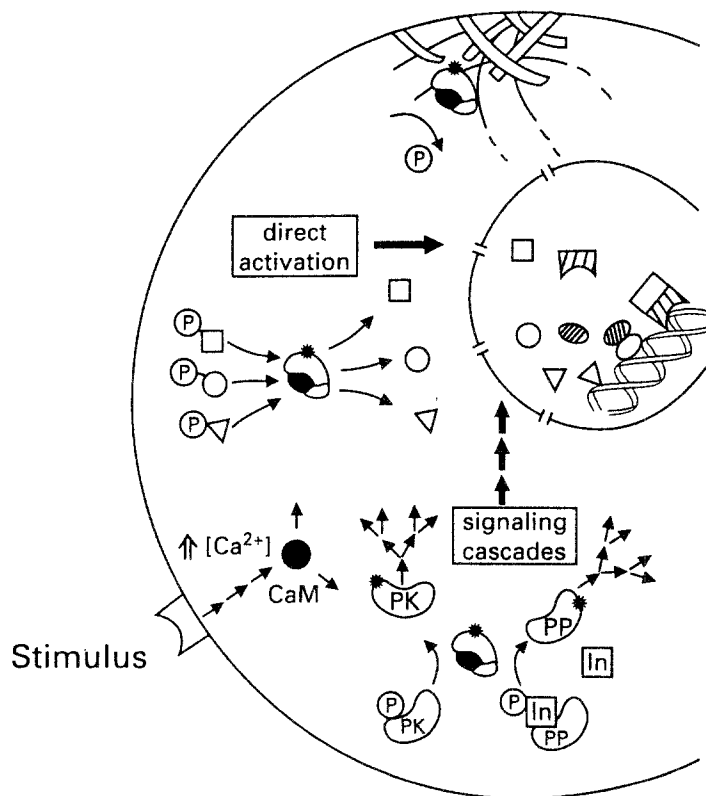


FIG. 3. Alternative models for transcriptional regulation by calcineurin. Ca^{2+} - and calmodulin (CaM)-activated form of calcineurin may directly regulate individual transcription factors (*upper portion*) or may control gene induction indirectly through dephosphorylation of a protein kinase (PK), protein phosphatase (PP), or their inhibitors. (Modified from Kincaid RL, O'Keefe SJ. Calcineurin and immunosuppression: a calmodulin-dependent protein phosphate acts as the "gate-keeper" to interleukin-2 gene transcription. *Adv Prot Phosphatases* 1993;7:543-83, with permission.)

has a relatively narrow substrate specificity. Therefore it seems perplexing that it exerts broad effects on several distinct transactivation pathways. Furthermore, collaborative studies,³⁴ as well as the findings presented here by Arai and others,^{35, 36} on the molecular heterogeneity of NF-AT_c-like components indicate that this is actually a family of factors whose structures show similarity over a rather small (*rel*-like) domain. Thus not only does calcineurin appear to act on multiple trans-activation pathways, but its effects on NF-AT pathways are actually transduced by several unique transcription components.

Based on these observations, the mechanism by which calcineurin activates gene induction could be either direct (dephosphorylation of a transcription factor itself) or indirect and potentially involves several phosphorylation cascades (Fig. 3). By analogy to its mode of regulating glycogen breakdown, calcineurin may actually dephosphorylate an as yet unknown component of a signaling pathway that activates a subset of transcription

factors. Such a possibility would provide a plausible explanation for the apparent multiplicity of promoter element targets for this protein phosphatase and, if true, suggests that calcineurin's primary substrate in these transduction events has yet to be discovered.

The main focus of immunologic research on calcineurin thus far has been on its mechanism of regulating cytokine gene induction. However, particularly in relation to allergic responses, there remain important observations on degranulation mechanism that need to be addressed. Cyclosporine and FK-506 are known to prevent the degranulation of mast cells³⁷ and neutrophils, in addition to their effects on cytotoxic T cells, suggesting that calcineurin plays an obligatory role in these events. Inasmuch as these effects do not require gene induction, the mechanism by which calcineurin may regulate them is clearly different. Historically, there is experimental evidence that calcineurin inhibition can block secretory and membrane fusion events in widely divergent species,^{38, 39} sug-

gesting that very fundamental events needed for exocytosis are controlled by this phosphatase. To date the steps involved in this process are not understood; however, I will offer some rather speculative possibilities that derive from recent collaborative studies of neuronal development.

Our collaborative work with Ferreira and Kosik has shown that calcineurin plays a pivotal role in the regulation of cytoskeletal structure in a model for neuronal growth and differentiation. Although at face value this may seem unrelated to mechanisms of immune cell degranulation, it is worthwhile reviewing those findings. By use of immunocytochemical methods, calcineurin was localized to lamellopodial domains of the developing neurons, which exhibit active microtubule and microfilament reorganization. These cells develop small neurite processes, one of which ultimately undergoes elongation to become an axon. Interestingly, on incubation with cyclosporine or a peptide inhibitor of calcineurin, neurite extension appeared normal, but axonal development was blocked⁴⁰; this suggests that this phosphatase has a role in the dynamic organization of morphology and cytoskeletal "architecture." At a biochemical level, inhibition of calcineurin was correlated with an increase in certain hyperphosphorylated forms of the tau protein, a microtubule-associated protein that appears key to stabilizing microtubule assembly. One can easily imagine that local transients in Ca^{2+} might provide information that influences the local stability of the cytoskeleton, and by inference, the susceptibility of such domains to membrane fusion events.

Unfortunately no firm data exist to implicate a specific calcineurin substrate in the process of exocytosis or vesicle release; thus, the mechanism of regulating degranulation is unclear. The observation that in neural cells calcineurin is compartmentalized in discrete cellular domains may suggest that it modulates vesicle movement and release by dephosphorylating structural components near the cytoskeletal/membrane interface. It is also important to consider the significance of such compartmentalization with respect to broader issues of substrate preference. Even phosphoproteins that are relatively poor substrates *in vitro* may become strong candidates for dephosphorylation if they have "privileged access" to this phosphatase by virtue of their colocalization. I believe that this area will likely become an important frontier of research in our understanding of calcineurin's mechanism of action in immune regulation both in relation to degranulation and transcriptional control.

In summary, the unique regulatory and biochemical properties of calcineurin place it in a pivotal position for controlling immune cell responses. Rather than serving a similar function in all cell types, it can influence a wide variety of cellular responses—proliferation, differentiation, programmed cell death, mediation of inflammatory response, regulation of vesicle transport and/or release, and morphologic response, each of which reflects the agenda for Ca^{2+} that is characteristic of a given cell type. Nonetheless, there appears to be a common philosophic role, that of a "switching" element whose effectiveness relies on a relatively restricted substrate specificity and tight regulation by Ca^{2+} . Given the universal importance of the Ca^{2+} signaling paradigm, it is probable that additional examples of lymphohematopoietic cell responses will be found in which this phosphatase plays a central role.

I am deeply indebted to my colleagues at the Department of Molecular Immunology, Merck Research Laboratories, and the Department of Inflammation Biology and Immunology, Syntex Research Laboratories, for their work on regulation of the promoters for IL-2 and IL-4. I also thank my collaborators at Harvard University with whom we worked on TNF- α regulation and control of neuronal morphology. Most important, I would like to thank my postdoctoral fellows, Taro Muramatsu and Sachio Matsushita (present address, Kurihama National Hospital) and Kazue Ueki and Jun'ichi Tamura (present address, Gunma University Medical School) for their tireless research efforts during their stay at the Section on Immunology, National Institute of Alcoholism and Alcohol Abuse, National Institutes of Health, where much of the work was carried out.

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