

- S. Brugge, *J. Biol. Chem.* **269**, 28859 (1994); C. Fujii et al., *Eur. J. Biochem.* **226**, 243 (1994); Y. Tohyama, S. Yanagi, K. Sada, H. Yamamura, *J. Biol. Chem.* **269**, 32767 (1994).
41. P. van der Geer and T. Hunter, *Annu. Rev. Cell Biol.* **10**, 251 (1994).
42. C.-H. Heldin, *Cell* **80**, 213 (1995).
43. J. V. Frangioni, A. Oda, M. Smith, E. W. Salzman, B. G. Neel, *EMBO J.* **12**, 4843 (1993).
44. C. E. Turner, *Bioessays* **16**, 47 (1994).
45. S. Tanaka et al., *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3443 (1994).
46. R. B. Birge et al., *Mol. Cell. Biol.* **13**, 4648 (1993).
47. C. Kapron-Bras, L. Fitz-Gibbon, P. Jeevaratnam, J. Wilkins, S. Dedhar, *J. Biol. Chem.* **268**, 20701 (1993).
48. C. S. Hill and R. Treisman, *Cell* **80**, 199 (1995).
49. D. Piomelli, *Curr. Opin. Cell Biol.* **5**, 274 (1993); L.-L. Lin et al., *Cell* **72**, 269 (1993); M. Durstyn, S. Durstyn, T. F. P. Molski, E. L. Becker, R. I. Sha'afi, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3142 (1994).
50. Z.-H. Qiu and C. C. Leslie, *J. Biol. Chem.* **269**, 19480 (1994).
51. J.-S. Chun and B. S. Jacobson, *Mol. Biol. Cell* **3**, 481 (1992).
52. M. P. Peppelenbosch, L. G. J. Tertoolen, W. J. Hage, S. W. De Laat, *Cell* **74**, 565 (1993).
53. J. Zhang et al., *J. Biol. Chem.* **268**, 22251 (1993).
54. H.-C. Chen and J.-L. Guan, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10148 (1994).
55. M. Wyman and A. Arcaro, *Biochem. J.* **298**, 517 (1994).
56. L. D. Chong, A. Traynor-Kaplan, G. M. Bokoch, M. Schwartz, *Cell* **79**, 507 (1994).
57. H. P. McNamee, D. E. Ingber, M. A. Schwartz, *J. Cell Biol.* **121**, 673 (1993).
58. J. A. Theriot and T. J. Mitchison, *Cell* **75**, 835 (1993).
59. A. Aderem, *Trends Biochem. Sci.* **17**, 438 (1992).
60. L. Somogyi, Z. Lasic, S. Vukicevic, H. Banfic, *Biochem. J.* **299**, 603 (1994).
61. R. A. Blake, G. L. Schieven, S. P. Watson, *FEBS Lett.* **353**, 212 (1994); J. L. Daniel, C. Dangelmaier, J. B. Smith, *Biochem. J.* **302**, 617 (1994).
62. S. T. Barry and D. R. Critchley, *J. Cell Sci.* **107**, 2033 (1994).
63. A. Woods and J. R. Couchman, *ibid.* **101**, 277 (1992).
64. D. Leavesley, M. Schwartz, M. Rosenfeld, D. Cheresh, *J. Cell Biol.* **121**, 163 (1993).
65. F. P. Lindberg et al., *J. Biol. Chem.* **269**, 1567 (1994); D. E. Clapman, *Cell* **80**, 259 (1995).
66. T. Lin et al., *J. Cell Biol.* **126**, 1585 (1994).
67. P. Huhtala, M. Humphries, J. McCarthy, Z. Werb, C. Damsky, *Mol. Biol. Cell* **5**, 64A (1994).
68. A. Hall, *ibid.* **3**, 475 (1992); A. J. Ridley and A. Hall, *Cell* **70**, 389 (1992).
69. A. J. Ridley and A. Hall, *EMBO J.* **13**, 2600 (1994).
70. J. McGlade et al., *ibid.* **12**, 3073 (1993).
71. H. Serve et al., *ibid.* **14**, 473 (1995).
72. K. Matsumoto, T. Nakamura, R. H. Kramer, *J. Biol. Chem.* **269**, 31807 (1994).
73. R. L. Klemke, M. Yebra, E. M. Bayna, D. A. Cheresh, *J. Cell Biol.* **127**, 859 (1994); P. Chen, H. Xie, M. C. Sekar, K. Gupta, A. Wells, *ibid.* **127**, 847 (1994).
74. S. Rankin and E. Rozengurt, *J. Biol. Chem.* **269**, 704 (1994); H.-C. Chen and J.-L. Guan, *ibid.*, p. 31229 (1994).
75. K. Vuori and E. Ruoslahti, *Science* **266**, 1576 (1994).
76. K. Tobe et al., *J. Biol. Chem.* **268**, 11167 (1993); K. Baltensperger et al., *Science* **260**, 1950 (1993); E. Y. Skolnik et al., *ibid.*, p. 1953.
77. I. Zachary and E. Rozengurt, *Cell* **71**, 891 (1992).
78. Y. Chen, J. Pouyssegur, S. A. Courtneidge, E. Van Obberghen-Schilling, *J. Biol. Chem.* **269**, 27372 (1994).
79. J. Sinnott-Smith, I. Zachary, A. M. Valverde, E. Rozengurt, *ibid.* **268**, 14261 (1993); S. Rankin, N. Morii, S. Narumiya, E. Rozengurt, *FEBS Lett.* **354**, 315 (1994).
80. T. Seufferlein and E. Rozengurt, *J. Biol. Chem.* **269**, 9345 (1994); N. Kumagai et al., *FEBS Lett.* **329**, 273 (1993).
81. M. Fuortes, W. Jin, C. Nathan, *J. Cell Biol.* **127**, 1477 (1994).
82. G. Berton, L. Fumagalli, C. Laudanna, C. Sorio, *ibid.* **126**, 1111 (1994).
83. M. M. Hamawy, S. E. Mergenhagen, R. P. Siraganian, *J. Biol. Chem.* **268**, 6851 (1993); M. M. Hamawy et al., *J. Immunol.* **153**, 4655 (1994).
84. A. R. de Fougerolles, X. Qin, T. A. Springer, *J. Exp. Med.* **179**, 619 (1994).
85. The authors apologize to all those whose work has been omitted from the reference list and is instead cited through reviews due to space limitations. We would like to thank R. O. Hynes, S. Shattil, and S. Thomas for careful reading of the manuscript. A. Ackroyd for her assistance in preparing the manuscript, and R. Hellmiss for generating the figures.

Calcium Signaling in Neurons: Molecular Mechanisms and Cellular Consequences

Anirvan Ghosh and Michael E. Greenberg*

Neuronal activity can lead to marked increases in the concentration of cytosolic calcium, which then functions as a second messenger that mediates a wide range of cellular responses. Calcium binds to calmodulin and stimulates the activity of a variety of enzymes, including calcium-calmodulin kinases and calcium-sensitive adenylyl cyclases. These enzymes transduce the calcium signal and effect short-term biological responses, such as the modification of synaptic proteins and long-lasting neuronal responses that require changes in gene expression. Recent studies of calcium signal-transduction mechanisms have revealed that, depending on the route of entry into a neuron, calcium differentially affects processes that are central to the development and plasticity of the nervous system, including activity-dependent cell survival, modulation of synaptic strength, and calcium-mediated cell death.

Ionic conductances in neurons have long been a focus of research in neurobiology because of their central role in the control of cell excitability and synaptic transmission. More than 40 years ago our understanding of the propagation of electrical signals in neurons was revolutionized by the work of Hodgkin, Huxley, Katz, and others

who described how voltage-sensitive sodium and potassium conductances mediate the active propagation of action potentials along the axon (1). In subsequent years, study of ionic conductances during synaptic transmission led to the current view that entry of calcium ions (Ca^{2+}) into the presynaptic terminal is the trigger for neurotransmitter release. It is now widely accepted that the propagation of electrical signals between cells is mediated by the binding of neurotransmitter molecules to their receptors, which act as ligand-gated channels that regulate the influx and efflux of ions into and from the postsynaptic cell (1).

The authors are in the Division of Neuroscience, Department of Neurology, Children's Hospital, Boston, MA 02115, USA, and Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA.

*To whom correspondence should be addressed at the Division of Neuroscience, Enders 260, Children's Hospital, 300 Longwood Avenue, Boston, MA 02115, USA.

Although the importance of ion fluxes in fast synaptic transmission and the role of intracellular calcium in modulating the biophysical properties of membranes have been known for some time (1), it has become increasingly apparent during the last decade that changes in intracellular Ca^{2+} can act much more broadly to influence events such as neuronal survival (2, 3), axon outgrowth (4), and changes in synaptic strength (5). Moreover, depending on the mode of Ca^{2+} entry and the cellular context, Ca^{2+} can mediate disparate biological effects. For example, Ca^{2+} influx through voltage-sensitive Ca^{2+} channels can lead to increased cell survival of embryonic neurons from the central and peripheral nervous systems (2). Yet Ca^{2+} influx does not always result in cell survival, and Ca^{2+} influx via the N-methyl D-aspartate (NMDA) subtype of glutamate receptors in postnatal neurons mediates excitotoxic cell death (3). Although it is not clear how Ca^{2+} could cause such dramatically different outcomes, an intriguing possibility is that the mode of Ca^{2+} entry may be a critical determinant of cell survival (6).

Ca^{2+} also appears to be a central mediator of adaptive responses (plasticity) in the nervous system. In recent years the function of Ca^{2+} in synaptic plasticity has been most extensively examined in a cellular model of plasticity called long-term potentiation (LTP) (5). LTP and long-term depression (LTD) are examples of synaptic transmission-dependent changes in synaptic efficacy, and the mechanisms that underlie this kind of synaptic plasticity are thought to represent the molecular substrates of learn-

ing and memory. The induction of LTP and LTD in hippocampal slices requires the activation of NMDA receptors (NMDAr) and the subsequent influx of Ca^{2+} into the postsynaptic cell (5).

As suggested by these examples, intracellular Ca^{2+} is likely to be a central mediator of development and plasticity in the nervous system, and consequently there has been widespread interest in understanding the mechanisms by which changes in intracellular Ca^{2+} can lead to diverse, long-lasting biochemical and cellular changes. In this review we consider the possibility that in addition to changes in the level of intracellular Ca^{2+} , the route of Ca^{2+} entry and its intracellular localization give rise to the activation of specific biochemical signaling pathways that mediate particular biological responses.

Modes of Calcium Entry

The concentration of intracellular free Ca^{2+} in most neurons is about 100 nM. This low resting concentration is maintained by the action of membrane-associated Ca^{2+} adenosine triphosphatases (Ca^{2+} pumps), which must act against a fairly steep concentration gradient to pump Ca^{2+} into the two major sinks—the extracellular space and the internal Ca^{2+} stores of the endoplasmic reticulum (ER), where the Ca^{2+} concentration is typically about 2 mM (7).

There are two major mechanisms by which Ca^{2+} enters the cytosol from these Ca^{2+} sinks. The first is by influx of extracellular Ca^{2+} through various Ca^{2+} channels embedded within the plasma membrane. The second is by release of Ca^{2+} from the internal stores. The entry of Ca^{2+} into the cytosol can be regulated by multiple mechanisms, allowing for exquisite spatial and temporal control of Ca^{2+} by signals such as neurotransmitters and growth factors that act at the cell surface.

Calcium influx from the extracellular space. In most neurons of the central nervous system (CNS) there are at least two major classes of Ca^{2+} channels: the voltage-sensitive Ca^{2+} channels (VSCCs) and the NMDAr. In addition, subsets of central neurons express other Ca^{2+} -permeable channels including the neuronal acetylcholine receptor (nAChR), the type 3 serotonin receptor (5HT₃R), and the Ca^{2+} -permeable AMPA and kainate glutamate receptors. The opening of VSCCs is regulated by the local membrane potential. On the basis of electrophysiological criteria as well as sensitivity to pharmacological agents, VSCCs can be classified into a number of subtypes (8). Inhibitors of specific Ca^{2+} channels have been used to determine the contribution of the various VSCC subtypes to the total Ca^{2+} current, and in CNS

neurons most of the voltage-sensitive Ca^{2+} current appears to be carried by the L-, N- and P-type Ca^{2+} channels (8). These Ca^{2+} channel subtypes have distinct functions within neurons. For example, N- and P-channels have been implicated in the control of neurotransmitter release (9), whereas L-channels are thought to be involved in modulating the release of certain neurotransmitters and in regulating Ca^{2+} -dependent signaling events in postsynaptic neurons (10).

Because the various subtypes of Ca^{2+} channels have distinct gating characteristics and kinetics of inactivation, the influx of Ca^{2+} through different VSCCs is likely to be acutely sensitive to the strength and duration of synaptic stimulation. The activation of specific VSCCs may also depend on the subcellular localization of the channels because VSCC subtypes are differentially distributed within individual neurons. For example, in hippocampal pyramidal neurons the L-channels are concentrated at the base of the apical dendrites (11), whereas the N-channels are more broadly distributed (12). Such differences in Ca^{2+} channel localization could allow for increases in intracellular Ca^{2+} in specific subcellular compartments in response to synaptic stimulation. As discussed in the next section, Ca^{2+} imaging experiments support this prediction.

Although much of our understanding of Ca^{2+} channels comes from the study of VSCCs, in recent years the NMDAr has been intensively studied because of its involvement in events such as synaptic plasticity and cell death. A striking feature of the NMDAr is that, in contrast to most other ion channels, it is a ligand- and voltage-gated channel (13). Opening of the channel requires the binding of the neurotransmitter glutamate as well as a depolarization of the membrane. The voltage dependence of the channel arises from the fact that at resting potential the channel is blocked by extracellular magnesium ions (Mg^{2+}). Depolarization of the membrane allows the Mg^{2+} -block to be relieved, and in the open state the NMDAr allows the entry of both Ca^{2+} and sodium (Na^+) ions. Since the induction of LTP is completely blocked by the inhibition of NMDAr as well as by chelating intracellular Ca^{2+} , the prevailing hypothesis is that Ca^{2+} influx through the NMDAr leads to the potentiation of active synapses (5).

Like Na^+ and potassium ion (K^+) channels, Ca^{2+} channels are multisubunit heteromers (13). In VSCCs, the $\alpha 1$ subunit serves as both the pore and the voltage sensor, although the other subunits also significantly affect the Ca^{2+} current amplitude (14). The cloning of various glutamate receptor subunits has revealed that a single residue in the M2 transmembrane segment plays a critical

role in determining Ca^{2+} permeability. All NMDAr subunits bear an asparagine residue at position 16 of the M2 region, and substitution of an arginine at this position dramatically reduces Ca^{2+} permeability (15). Ca^{2+} permeability through AMPA receptors is controlled by RNA editing at this same position (16).

Both VSCCs and NMDAr subunits have intracellular loops that include potential phosphorylation sites for a number of kinases. There is accumulating evidence that NMDAr function can be modulated by channel phosphorylation. For example, activators of protein kinase C (PKC) potentiate NMDAr function in central neurons, and recently it has been shown that the NR1 subunit of the NMDAr is directly phosphorylated by PKC in neuronal cell cultures (17). Recent evidence suggests that voltage-sensitive ion channels and NMDAr can also be regulated by tyrosine phosphorylation (18). This is of interest not only from the perspective of channel modulation, but also because tyrosine phosphorylation may allow these channel proteins to influence intracellular signaling through direct intermolecular interactions. A central mechanism in the propagation of intracellular signals in response to receptor tyrosine kinase activation is the interaction of phosphorylated tyrosine residues on the receptor with SH2 domain-containing signaling molecules (19). The identification of SH2 domain-containing proteins that interact with phosphorylated channel proteins could dramatically alter current views of ion channel signaling.

Calcium release from internal calcium stores. Although a major route of Ca^{2+} entry into the cytosol is through plasma membrane Ca^{2+} channels, release from internal Ca^{2+} stores also contributes significantly to the concentration of intracellular free Ca^{2+} (20). There appear to be at least two pools of internal Ca^{2+} stores in the ER, and the release of Ca^{2+} from these two pools is mediated by distinct mechanisms. The first mechanism is by the binding of inositol triphosphate (IP_3) to its receptor on the ER membrane (20). IP_3 is a central regulator of intracellular free Ca^{2+} that is generated by exposure of cells to a variety of stimuli, including growth factors and neurotransmitters that act through guanosine triphosphate (GTP)-binding protein (G protein)-linked receptors.

Another mechanism by which Ca^{2+} is released from the internal stores is by Ca^{2+} -mediated Ca^{2+} release (20). This mechanism is regulated by the activation of the ryanodine receptor (RyanR, named after its specific agonist ryanodine), which has been extensively studied in muscle cells where it plays a critical role in excitation-contraction coupling. Although RyanRs regulate intra-

cellular Ca^{2+} in both skeletal and cardiac muscle, the activation of RyanRs in these two tissues differs in an important aspect. Whereas depolarization of skeletal muscle directly activates RyanRs (by voltage-sensitive activation), in cardiac muscle Ca^{2+} influx through dihydropyridine-sensitive channels is the trigger for RyanR activation (20). RyanRs expressed in brain appear to be more closely related to cardiac muscle RyanRs, suggesting that its activation may also be mediated by Ca^{2+} influx through dihydropyridine-sensitive Ca^{2+} channels. Cardiac (and presumably neuronal) RyanR activation has a biphasic Ca^{2+} response. The RyanR-activated channel opens at Ca^{2+} concentrations of 100 nM to 1 μM , but is inhibited at Ca^{2+} concentrations above 10 μM (20). Therefore, the release of Ca^{2+} from RyanR-activated internal stores may be acutely sensitive to the magnitude of Ca^{2+} influx after synaptic stimulation.

The IP_3 receptor and RyanR have been extensively studied because of their role in the generation of intracellular Ca^{2+} waves and oscillations (20, 21). In terms of neuronal signaling an intriguing possibility is that the generation of such intracellular Ca^{2+} waves could be influenced by patterns of synaptic input. By such a mechanism information about the stimulus strength or frequency could be translated into Ca^{2+} -dependent biochemical changes within the postsynaptic cell.

There have been two important recent advances in our understanding of RyanR activation. The first is that a cellular target of the immunosuppressant FK506, called FKBP12, appears to be an integral part of the RyanR complex and can influence both the stability of the channels and their probability of being in the open state (22). The second observation is that a newly identified nucleotide, cyclic ADP-ribose (cADPR), appears to be an important effector of Ca^{2+} -dependent Ca^{2+} release. cADPR directly activates the cardiac (and presumably the neuronal) but not the skeletal RyanR. Moreover, it appears that accessory proteins, such as calmodulin, can modulate the action of cADPR on RyanR activation (23).

Not only can Ca^{2+} enter the cytosol from the extracellular environment or internal Ca^{2+} stores, but in each case multiple receptors can potentially participate in regulating the entry of Ca^{2+} into the cell, and the intracellular Ca^{2+} signal can be significantly affected by Ca^{2+} -binding proteins that can act as local Ca^{2+} buffers (7). What is the biological importance of having multiple mechanisms by which intracellular Ca^{2+} can be regulated? One possibility is that specific patterns of synaptic stimulation may lead to localized increases in intracellular Ca^{2+} . This could result, for example, from the differential activation of

plasma membrane Ca^{2+} channels or from the activation of different intracellular Ca^{2+} stores. Such localized increases in intracellular Ca^{2+} could then allow for a large number of cellular responses to be mediated by the activation of specific Ca^{2+} -sensitive biochemical pathways.

Imaging of Intracellular Calcium Dynamics

The existence of multiple routes of Ca^{2+} entry into the cytosol could allow for localized increases in intracellular Ca^{2+} in response to different kinds of stimuli. Indeed, recent Ca^{2+} -imaging experiments support the hypothesis that the mode of Ca^{2+} entry is an important determinant of the spatial pattern of Ca^{2+} increase in neurons. These imaging experiments indicate that synaptic stimulation can lead to highly localized increases in intracellular Ca^{2+} . Consequently the spatially restricted activation of Ca^{2+} -sensitive second messenger molecules may allow for the activation of distinct biochemical signaling pathways.

Direct imaging of intracellular Ca^{2+} with Ca^{2+} -sensitive dyes (24) has provided important insights into the regulation of intracellular Ca^{2+} in neurons and its biological consequences. For instance, excitotoxic stimulation of cultured CNS neurons is associated with a biphasic increase in cytosolic Ca^{2+} (3, 25). There is an initial transient increase in intracellular Ca^{2+} followed by a variable period (typically on the order of hours) during which Ca^{2+} is maintained at resting levels. Eventually there is a loss of Ca^{2+} homeostasis that leads to cell death. The source of Ca^{2+} entry appears to be an important determinant of eventual loss of Ca^{2+} homeostasis and cell death. When NMDAr or VSCCs on spinal cord neurons in culture are activated so that they lead to equivalent initial increases in intracellular Ca^{2+} , NMDAr activation results in significantly more cell death (6). Therefore the biochemical pathways that eventually lead to cell death appear to be preferentially activated by NMDAr stimulation.

Although many of the initial Ca^{2+} imaging experiments were done on cells in culture, the development of confocal fluorescence microscopy has allowed for the imaging of Ca^{2+} in single neurons in brain slices in response to synaptic stimulation. It has been of particular interest to determine how synaptic activity affects intracellular Ca^{2+} in hippocampal CA1 neurons where the relation between patterns of synaptic activity and synaptic plasticity has been extensively explored. Transsynaptic stimulation of pyramidal neurons from the CA1 region of the hippocampus at frequencies up to 20 Hz produces an increase in the intracellular Ca^{2+} concentration from less than

100 nM to over 1 μM in the proximal apical and basal dendrites (Fig. 1A) (26). Moderate, and somewhat more sustained, Ca^{2+} increases are also seen in the soma. The overall pattern of Ca^{2+} increase appears to be independent of the location of afferent fibers being stimulated and can be mimicked by direct depolarization of the postsynaptic cell by current injections. This increase in intracellular Ca^{2+} levels is mediated largely by the activation of VSCCs. At stimulation frequencies higher than 20 Hz, which reliably lead to the induction of LTP, an additional component of the Ca^{2+} increase can be seen. At these higher frequencies, there is a marked increase in intracellular free Ca^{2+} in dendritic regions that receive direct synaptic input (Fig. 1A). This localized increase in Ca^{2+} is mediated by the activation of NMDAr and may therefore be directly involved in the induction of LTP (26). High-resolution imaging experiments indicate that synaptic stimulation of hippocampal neurons can produce Ca^{2+} increases in individual dendritic spines (27). These observations support the possibility that highly localized changes in postsynaptic Ca^{2+} levels may allow for the synapse specificity of LTP.

Whereas the experiments on hippocampal neurons show how the frequency of stimulation can influence the pattern of intracellular Ca^{2+} increase, experiments on Purkinje neurons provide evidence that accumulation of intracellular Ca^{2+} may be a mechanism by which input from a number of synapses could be locally integrated (28). Recent experiments indicate that stimulation of parallel fiber inputs onto terminal spiny dendrites of Purkinje neurons leads to an increase in postsynaptic Ca^{2+} within a restricted region of the terminal dendrites and associated spines (Fig. 1B) (28). What is surprising is that the recruitment of more parallel fibers to the presynaptic input by increasing stimulus strength gives rise to an increase in the localized intracellular Ca^{2+} concentration without notably changing the spatial distribution of the Ca^{2+} increase within the dendrite. This increase in Ca^{2+} can be detected even in response to subthreshold stimuli (which do not result in the generation of action potentials) and suggests a mechanism by which subthreshold synaptic inputs could be integrated in a localized region of the cell and possibly lead to modulation of synaptic efficacy in a restricted cellular compartment (28). This observation is significant because Ca^{2+} increases in Purkinje cell dendrites are required for LTD of parallel fiber inputs and for activity-dependent changes in inhibitory inputs (29).

The above experiments are just a few examples from an emerging body of evidence suggesting that local Ca^{2+} increases

may reflect temporal and spatial integration of inputs in restricted regions of the cell (30). These Ca^{2+} -imaging experiments complement long-standing electrophysiological studies which indicate that neuronal activity can give rise to increases in postsynaptic Ca^{2+} through the activation of various Ca^{2+} -permeable channels. Further elucidation of the mechanisms that control increases in intracellular Ca^{2+} in response to various stimuli should be facilitated by the development of high-resolution Ca^{2+} -imaging technology and will likely provide important new insights.

Activation of Calcium-Sensitive Second Messengers

As Ca^{2+} enters the cytosol, it encounters a number of proteins that regulate its biochemical effects. Central among them is calmodulin, a small Ca^{2+} -binding protein, which can act as an intracellular Ca^{2+} sensor (7, 31). Ca^{2+} -calmodulin (CaM) binds to a number of enzymes and modulates their activity. These include CaM-dependent protein kinases (CaM kinases), protein phosphatases, and adenylate cyclases. These enzymes can either act locally at the synapse by inducing the modifications of pre-existing synaptic proteins or can mediate more general cellular responses by activating molecules involved in the regulation of gene expression.

Calcium-calmodulin-dependent protein kinases. Of the five CaM kinases that have been identified, CaM kinases II and IV have been best characterized. Both have broad substrate specificities and, therefore, may regulate a number of Ca^{2+} -dependent biochemical events. Whereas CaM kinase II has been extensively studied as a potential mediator of Ca^{2+} -dependent synaptic changes, CaM kinase IV and certain isoforms of CaM kinase II may be specifically involved in mediating transcriptional activation of gene expression in response to changes in intracellular Ca^{2+} . Our focus in this section will be on CaM kinase II and its mediation of biochemical changes in response to increases in synaptic Ca^{2+} .

A number of studies on the localization, activation, and perturbation of CaM kinase II present a compelling case that CaM kinase II is directly involved in the regulation of synaptic plasticity (31). CaM kinase II is enriched in the cortex and hippocampus and is concentrated both pre- and postsynaptically. CaM kinase II isolated from brain contains α and β subunits in a ratio of about 4:1 (31). Unlike protein kinase A (PKA) and PKC, in which there are separate catalytic and regulatory subunits, each subunit of CaM kinase II has catalytic activity. In the resting state, an autoinhibitory domain of the kinase keeps the catalytic site

Fig. 1. Temporal and spatial integration of synaptic inputs by localized increases in intracellular calcium. (A) Schaffer collateral stimulation of hippocampal CA1 neuron. Low-frequency stimulation of hippocampal neurons leads to increases in intracellular Ca^{2+} levels in the proximal basal and apical dendrites, as well as in the soma (green stippling) via the activation of VSCCs. At higher frequencies of stimulation, an additional component of Ca^{2+} increase mediated by the NMDAr can be seen that is restricted to the active synaptic regions (red stippling). (B) Parallel fiber stimulation of Purkinje cell dendrites. At low stimulus strength parallel fiber activation results in a localized increase in intracellular Ca^{2+} in Purkinje cell dendrites (red stippling). Recruiting more parallel fibers to the presynaptic volley by increasing stimulus strength results in a greater increase in postsynaptic Ca^{2+} without affecting the subcellular distribution of the Ca^{2+} increase. [Adapted from (26, 28)]

inaccessible (32). This inhibition can be relieved by the binding of CaM to the autoinhibitory domain. The kinase thus activated can not only phosphorylate substrates at serine residues but also undergoes autophosphorylation at a number of sites.

The autophosphorylation of CaM kinase II causes the enzyme to switch to a Ca^{2+} -independent form, that is, the kinase maintains catalytic activity even after intracellular Ca^{2+} concentrations have returned to basal levels (33). This is the property of CaM kinase II that initially led to the proposal that it could function as a "memory molecule" and might contribute to long-term modulation of synaptic function. Site-directed mutagenesis experiments indicate that of the multiple autophosphorylation sites, phosphorylation at Thr^{286} is both necessary and sufficient for the generation of Ca^{2+} -independent activity (34). Autophosphorylation of Thr^{286} also markedly increases the affinity of the kinase for calmodulin. After Thr^{286} phosphorylation, the time constant for dissociation of calmodulin increases to about 10 s from about 0.2 s prior to autophosphorylation, and this trapping of calmodulin can allow CaM kinase II to remain in an active state even as intracellular Ca^{2+} levels decline (35).

Physiological stimuli lead to the activation of CaM kinase II and to the generation of the Ca^{2+} -independent form of the enzyme (36). Particularly noteworthy are changes in CaM kinase II activity that accompany the induction of LTP. Stimuli that give rise to LTP in hippocampal slices

also lead to significant increases in CaM kinase II enzymatic activity (36). Immunostaining of cultured hippocampal neurons with a phospho-specific antibody that recognizes the Thr^{286} -phosphorylated form of CaM kinase II indicates that activation of NMDAr, which is required for LTP induction, leads to autophosphorylation of CaM kinase II at Thr^{286} (36).

Perturbation experiments also support a role for CaM kinase II in the induction of LTP. For example, microinjection of a peptide that selectively inhibits CaM kinase II activity by binding to its catalytic site prevents the induction of LTP in the CA1 region of the hippocampus (37), and a targeted gene disruption of the CaM kinase II α -subunit produces a deficiency in the induction of LTP (37). The CaM kinase II null mutant mice also have a deficit in certain spatial learning tasks. Taken together, these observations support a role for CaM kinase II in learning and memory.

Because CaM kinase II is thought to mediate certain neuronal adaptive responses, there is considerable interest in identifying the substrates of this enzyme. Most putative targets of CaM kinase II have been identified by *in vitro* kinase assays. In several instances physiological stimuli that lead to increased intracellular Ca^{2+} levels result in the phosphorylation of these substrates at sites that are also phosphorylated by CaM kinases II *in vitro*. Substrates defined in this way include MAP2, synapsin I, and the non-NMDA glutamate receptor subunit, GluR1 (31, 38). Because MAP2 is

a microtubule-associated protein, its phosphorylation may be involved in Ca^{2+} -induced structural changes at the synapse. Both synapsin I (a synaptic vesicle-associated protein) and GluR1 are involved in synaptic transmission, and their phosphorylation by CaM kinase II may modulate synaptic efficacy. Indeed, phosphorylation of GluR1 by CaM kinase II increases channel conductance (38). Such a mechanism may be involved in the change in postsynaptic response following the induction of LTP (39). These observations suggest that CaM kinase II may regulate neuronal adaptive responses, at least in part by regulating the function of various synaptic proteins.

Calcium-calmodulin-dependent adenylate cyclases. Although CaM kinases are important transducers of Ca^{2+} signals, changes in intracellular Ca^{2+} can also influence biochemical events by activating other second messenger systems. One example is the ability of Ca^{2+} to influence cyclic AMP (cAMP) levels by the activation of CaM-dependent adenylate cyclases. Ca^{2+} regulation of cAMP concentration may be quite important because CaM-sensitive adenylate cyclase is required for certain forms of learning. For example, the *Drosophila* learning mutant *rutabaga* lacks CaM-sensitive adenylate cyclase activity (40), and a Ca^{2+} -sensitive adenylate cyclase is involved in classical conditioning of the *Aplysia* gill withdrawal reflex (41). Also, inhibitors of PKA prevent the induction of LTP in hippocampal slices, suggesting that the cAMP pathway contributes to synaptic plasticity (42). Finally, mice carrying a targeted mutation of the type I adenylate cyclase are deficient in certain tests of spatial learning (43).

Several CaM-sensitive adenylate cyclases have been identified in the brain. Of particular interest is the type I Ca^{2+} -sensitive adenylate cyclase (I-AC), which is highly expressed in the hippocampus and neocortex, regions associated with activity-dependent plasticity (44). Although it remains to be shown that CaM-sensitive adenylate cyclase is activated in neurons in response to synaptic stimulation *in vivo*, preliminary reports are consistent with such a possibility. For example, in I-AC-transfected human kidney 293 cells, Ca^{2+} ionophores cause a marked increase in cAMP concentrations (45). Moreover, adenylate cyclase activity can be synergistically activated by Ca^{2+} and G_s -coupled receptors (46). This observation suggests that Ca^{2+} may serve to amplify neurotransmitter-induced increases in cAMP. By such a mechanism Ca^{2+} influx could act as a switch to permit biochemical modifications that require a certain threshold of cAMP levels. It will be of interest to determine if Ca^{2+} -sensitive adenylate cyclases are activated in response to NMDAr

stimulation and if such a mechanism is involved in Ca^{2+} -dependent adaptive responses in neurons.

Propagation of Calcium Signals to the Nucleus

Ca^{2+} signals are also propagated to the nucleus, where they elicit profound changes in gene expression that are critical for proper neuronal function. Although the induction of LTP is a rapid event that most likely involves modifications of existing synaptic proteins, the long-term maintenance of LTP depends on new protein synthesis and transcription (47). Likewise, a requirement for new gene expression has been demonstrated for certain forms of associative learning in the marine mollusc *Aplysia* (48). It is also likely that Ca^{2+} regulation of neuronal survival is mediated by the activation of specific genes (2). In each of these cases, the Ca^{2+} signal must be transmitted to the nucleus, and recent observations suggest that there are at least two major mechanisms by which this may be achieved. One possibility is that changes in intracellular Ca^{2+} could directly lead to the activation of Ca^{2+} -sensitive nuclear enzymes. Alternatively, the activation of Ca^{2+} -sensitive cytosolic enzymes (such as a CaM kinase) could result in the activation of target signaling molecules that translocate to the nucleus to influence gene expression. This would be analogous to the mechanism by which growth factor signals are propagated to the nucleus (19).

Calcium activation of CaM kinases within the nucleus. Ca^{2+} signals can be propagated to the nucleus by the direct interaction of Ca^{2+} with effector molecules localized within the nucleus. Both the activation of NMDAr and VSCCs can produce increases in the nuclear Ca^{2+} concentration (25). Moreover, certain CaM kinase II isoforms and CaM kinase IV have been localized to the nucleus where they may be directly activated when nuclear Ca^{2+} interacts with calmodulin (49). As there is evidence that nuclear CaM kinases phosphorylate specific transcription factors that regulate expression of Ca^{2+} -responsive genes, it seems likely that this is one mechanism for propagating Ca^{2+} signals to the nucleus. However, cytosolic CaM kinases may also propagate Ca^{2+} signals to the nucleus via translocation. Consistent with this possibility is the recent observation that CaM kinase IV activation is not only dependent on CaM binding, but also requires phosphorylation by a newly characterized protein kinase (49). It remains to be determined if the activator protein functions within the nucleus or if it phosphorylates CaM kinase IV in the cytoplasm, and the active CaM kinase IV is then translocated to the nucleus.

Calcium activation of the Ras signaling pathway. It has recently been shown that the Ras signaling pathway is activated by increases in intracellular Ca^{2+} (50). This pathway has been extensively studied in the context of signaling by receptor tyrosine kinases (19). Once activated, Ras appears to associate with and activate the serine-threonine kinase Raf, followed by the activation of a cascade of phosphorylation events that result in the sequential activation of the kinase MEK-1, MAP kinases, and ribosomal S6 kinases (Rsk). The Ras signaling pathway is believed to transmit extracellular signals across the plasma membrane and cytoplasm to the nucleus where it effects changes in gene expression (19).

Ca^{2+} influx through L-VSCCs triggers Ras activation within 30 s of stimulation and precedes the phosphorylation and activation of the dual-specificity kinase MEK-1 and its direct target MAP kinase (50). The expression of a dominant interfering form of Ras in PC12 cells prevents the activation of MAP kinase, indicating that Ca^{2+} activation of MAP kinase requires Ras function. Because activation of both L-VSCCs and NMDAr leads to MAP kinase activation in primary cultures of hippocampal and cortical neurons, a similar Ras-dependent pathway may be activated in response to NMDAr stimulation, although this remains to be demonstrated. It will be important to determine the mechanism by which Ca^{2+} stimulates Ras. In growth factor-stimulated cells, Ras activation is triggered by a GTP/GDP exchange factor, SOS, that upon growth factor receptor tyrosine kinase activation interacts indirectly with the activated growth factor receptor via the adaptor protein Grb-2. This interaction is believed to then localize SOS to the plasma membrane where it activates Ras. It is possible that Ca^{2+} activates Ras by somehow activating a growth factor receptor or by a pathway that might involve an unknown Ca^{2+} -sensitive GDP/GTP exchange factor.

Ca^{2+} activation of the Ras-dependent MAP kinase signaling pathway could have a number of important consequences in neurons. MAP kinase is present in dendrites and could therefore mediate biochemical events in response to local changes in dendritic Ca^{2+} . MAP kinase substrates include the cytoskeletal proteins Tau and MAP2, and phosphorylation of these molecules may mediate the effects of Ca^{2+} on neuronal morphology. However, as is the case of cells exposed to growth factors, it seems likely that a major function of Ca^{2+} -activated MAP kinase is to propagate the Ca^{2+} signal to the nucleus. By analogy to growth factors, Ca^{2+} activation of MAP kinase might influence gene transcription by phosphorylating and activating transcription factors such as c-Myc and Elk-1 (51). Thus,

the MAP kinase signaling pathway could play a role in mediating Ca^{2+} -induced adaptive responses that require new gene expression.

Calcium Regulation of Gene Expression

Once Ca^{2+} signals are propagated to the nucleus, they influence gene transcription by activating or inhibiting the function of various transcription factors (52). A number of genes have been identified that are regulated by changes in intracellular Ca^{2+} . These genes can be divided into two classes on the basis of their kinetics of activation and their function in the cell. The rapidly induced genes, termed immediate-early genes (IEGs), do not require the synthesis of new proteins for their transcription and often encode transcription factors (such as c-Fos and c-Jun) that regulate subsequent waves of gene expression. Many IEGs are induced not only in response to changes in the intracellular Ca^{2+} level but also by exposure to a variety of extracellular stimuli that include growth factors and neurotrophins. In contrast to IEGs, the delayed response genes (DRGs) are induced slowly and typically require new protein synthesis for their expression. The proteins encoded by the DRGs usually act within the cell to influence cell physiology more directly. It is generally thought that transcription of the DRGs is regulated at least in part by factors encoded by the IEGs.

Calcium regulation of immediate-early genes. Much of our understanding of Ca^{2+} signaling within the nucleus comes from experiments on the transcriptional regulation of IEGs (53). The view emerging from these experiments is that the transcription factor CREB and related molecules are critical mediators of Ca^{2+} -dependent gene expression. CREB was initially identified as a factor that binds to the sequence element 5'-TGACGTCA in the somatostatin gene promoter, and it mediates the transactivation of this gene in response to changes in cAMP levels (54). Subsequently the somatostatin cAMP response element (CRE) has been identified in the regulatory regions of many different genes whose transcription is activated by increases in cAMP. The role of CREB in mediating Ca^{2+} responses first became apparent from analysis of the c-Fos promoter (55). The Ca^{2+} response element (CaRE) in this gene is similar to the CRE and indeed both the c-Fos CaRE and the consensus CRE can confer a Ca^{2+} response when placed in the regulatory region of a heterologous gene (55).

CREB is a member of the bZIP family of transcription factors that includes ATF-1, CREM, and c-Jun, and is distantly related to C/EBP β (56, 57). Several of these tran-

scription factors are capable of mediating a Ca^{2+} response in transient transfection experiments. CREB and these related factors can form homo- or heterodimers through their leucine zipper domains, and the regulation of dimer formation may provide a mechanism by which transactivation via CaREs is regulated. Although CREB homo- and heterodimers bind CaREs with high affinity, binding to the promoter is not sufficient for CREB-dependent transcription, and in fact a CREB-containing complex appears to be bound to the CaRE even before the activation of Ca^{2+} signaling pathways. Influx of Ca^{2+} must therefore give rise to some modification of the protein complex bound to the CaRE to allow for inducible transactivation.

The activation of CREB in response to increased levels of cAMP or Ca^{2+} appears to be regulated by the inducible phosphorylation of a specific amino acid, Ser¹³³, which acts as a critical positive regulatory site (57). Mutation of this site prevents CREB-dependent transcription in response to increases in the level of intracellular cAMP or Ca^{2+} . Because the Ser¹³³ mutation does not influence CREB stability, dimer formation, or binding to DNA, it is thought that phosphorylation at this site promotes the assembly of the polymerase II transcription complex at the TATA box and leads to the initiation of transcription. Recently two CREB-binding proteins, CBP and p300, have been characterized that may mediate the interaction of Ser¹³³-phosphorylated CREB with the polymerase II transcription machinery (58). For example, CBP interacts specifically with CREB only after it is phosphorylated at Ser¹³³, and in cotransfection experiments CBP enhances CREB/CRE-dependent transcription. However, it has not yet been demonstrated that CBP function is critical for Ca^{2+} -dependent CREB-mediated transcription.

Phosphorylation of CREB at Ser¹³³ may involve the direct entry of Ca^{2+} into the nucleus where it could bind to calmodulin and activate CaM kinases. Consistent with this possibility, both CaM kinase IV and certain CaM kinase II isoforms have been localized to the nucleus, and these enzymes can catalyze the phosphorylation of CREB at Ser¹³³ *in vitro* (55). However, CaM kinases II and IV may not be expressed in all cells in which Ca^{2+} influx is known to trigger CREB Ser¹³³ phosphorylation, so that other Ca^{2+} signaling pathways that lead to CREB phosphorylation must be activated in certain cells. In this regard it is relevant that activation of VSCCs or the nerve growth factor (NGF) receptor in PC12 cells results in the activation of another CREB kinase that appears to be unrelated to CaM kinases (59). The NGF-activated CREB kinase appears to be com-

posed of a single polypeptide of 105 kD that is activated by a Ras-dependent mechanism. It will be of interest to determine whether activation of CREB kinase in response to VSCC activation is also mediated by the activation of the Ras-dependent kinase cascade.

Recent experiments suggest that phosphorylation of CREB at sites in addition to Ser¹³³ may also affect CREB's ability to activate transcription. Once it is phosphorylated at Ser¹³³ CREB becomes a substrate for glycogen synthetase kinase 3 (GSK3), which catalyzes the phosphorylation of CREB at Ser¹²⁹ (60). Mutating Ser¹²⁹ to an alanine impairs CREB-dependent transcription in cells exposed to activators of adenylyl cyclase. This suggests that the phosphorylation of Ser¹²⁹ may contribute to CREB activation under certain circumstances.

The phosphorylation of CREB at another site, Ser¹⁴², appears to inhibit CREB's transcriptional-activating potential. The inhibitory effect of Ser¹⁴² phosphorylation was first revealed in experiments comparing the effects of CaM kinase IV and CaM kinase II in mediating CREB-dependent transactivation of a CRE-driven reporter gene. CaM kinase II, which phosphorylates both Ser¹³³ and Ser¹⁴², is much less effective at inducing CREB-dependent transcription than CaM kinase IV, which only phosphorylates Ser¹³³ (61). These findings suggest that the phosphorylation of CREB at Ser¹⁴² inhibits CREB's ability to activate transcription even when Ser¹³³ is fully phosphorylated. Nevertheless, while phosphorylation of CREB at Ser¹⁴² appears to be an important regulatory event under these circumstances, the phosphorylation of endogenous CREB at this site has yet to be documented.

The analysis of CREB-dependent transcription in neurons has provided further evidence that Ca^{2+} influx through VSCCs and NMDAr leads to the activation of distinct signaling pathways. The analysis of NMDAr-mediated signaling has been facilitated by the development of methods that allow the transfection of primary neuronal cultures (62). In hippocampal neurons, as in PC12 cells, the activation of VSCCs effectively stimulates transcription of a CaRE-containing reporter. However, in hippocampal neurons NMDAr activation fails to trigger significant levels of CaRE-dependent transcription, suggesting that the route of Ca^{2+} entry may determine the specific transcription complexes that are activated (62). Ca^{2+} influx through the NMDAr does lead to the induction of c-Fos and other IEGs. NMDAr-mediated c-Fos activation requires the presence of a second Ca^{2+} -responsive element, the serum response element (SRE), which binds the transcription factors serum response factor (SRF) and Elk-1 (62) (Fig. 2).

The failure of NMDA receptors to mediate CREB-dependent transcription is not due to a lack of CREB Ser¹³³ phosphorylation because both VSCC and NMDAr activation lead to phosphorylation of CREB at this site (62). The recent evidence that CREB function can be regulated by phosphorylation at other sites suggests an interesting mechanism by which Ca²⁺ influx through the NMDAr and VSCC could result in differential activation of CREB-mediated transcription. If NMDAr activation either leads to phosphorylation of CREB at the inhibitory site (Ser¹⁴²) or fails to induce CREB phosphorylation at the second positive regulatory site (Ser¹²⁹), it could explain why CREB is incapable of mediating an NMDA response even though CREB is effectively phosphorylated at Ser¹³³ under these conditions.

Another mechanism by which the specificity of the CREB-mediated transcriptional response could be regulated is by differential dimerization. CREB can form heterodimers with a number of other bZIP transcription factors such as CREM, ATF-1, and C/EBP β (56, 57). C/EBP β is phosphorylated by CaM kinase II on a residue within its leucine zipper, and phosphorylation at this site potentiates the transcription-activating potential of C/EBP β (63). The location of the phosphorylation site

within the C/EBP β leucine zipper raises the possibility that phosphorylation at this site might alter C/EBP β homo- or heterodimerization. The formation of different heterodimers after leucine zipper phosphorylation could in turn influence C/EBP β -mediated transcription. If heterodimers between different CREB family members have distinct sequence-binding specificities or differ in their ability to activate or inhibit transcription via a particular regulatory sequence, one could generate a wide spectrum of nuclear responses by the activation of distinct Ca²⁺ signaling pathways.

Calcium regulation of "effector" genes. Within neurons there are multiple Ca²⁺ signaling pathways that are differentially activated by specific classes of Ca²⁺ channels. In turn, activation of these distinct Ca²⁺ signaling pathways can regulate the formation of transcription complexes and influence gene expression. How does the activation of these various Ca²⁺ signaling pathways ultimately produce specific biological outcomes?

As a way of understanding how Ca²⁺ influences particular biologic responses, there has been considerable interest in identifying Ca²⁺-inducible genes that may directly influence physiological responses. One of the first examples in which the differential regulation of gene expression by

Ca²⁺ has been linked to distinct biological outcomes is the regulation of cell survival in response to Ca²⁺ channel activation (64). The survival of embryonic cortical neurons in culture is differentially regulated by the activation of VSCCs and NMDArs. Whereas VSCC activation promotes the survival of these neurons, NMDAr activation is ineffective. Recent experiments indicate that BDNF can act as an endogenously produced neurotrophic factor for these cells, and the expression of BDNF is differentially regulated by Ca²⁺ channel activation (64). The activation of NMDAr leads to only a weak and transient increase in BDNF expression, but the induction of BDNF in response to VSCC activation is much more robust and sustained. Consistent with the interpretation that VSCC-dependent increase in cell survival is mediated by an up-regulation of a trophic factor produced by these cells, neutralizing antibodies to BDNF completely inhibit the VSCC-mediated increase in cell survival (64). These experiments not only suggest a mechanism by which VSCC activation can influence cell survival, they provide important evidence in support of the view that differential regulation of gene expression by distinct Ca²⁺ channels can mediate diverse biological effects.

Genes encoding various growth factors and growth factor receptors have been shown to be regulated by neuronal activity in vivo and by Ca²⁺-dependent mechanisms in vitro (65). For example, the expression of BDNF and NGF is induced in hippocampal slices by stimuli that lead to the induction of LTP. Because these factors are known to regulate cell morphology, their induction during LTP suggests that they may be involved in mediating morphological changes at the synapse that might accompany long-term changes in synaptic strength. These genes are also induced in the hippocampus after limbic seizures. Given the trophic effects of these molecules on various CNS neurons, it is possible that they act as neuroprotective factors in response to an excitotoxic assault.

A number of genes that encode components of the synapse have also been shown to be regulated by neuronal activity (66). For example, the activation of VSCCs leads to the induction of tyrosine hydroxylase and the neuropeptide vasoactive intestinal peptide (VIP) in peripheral ganglia. Several synaptic vesicle-associated proteins are also induced by the activation of Ca²⁺ signaling pathways. The activation of these genes in response to synaptic stimulation may be directly involved in mediating long-lasting changes at the synapse. We now know of a number of neurotrophic molecules and synaptic components that are regulated by Ca²⁺ influx into neurons. The function of

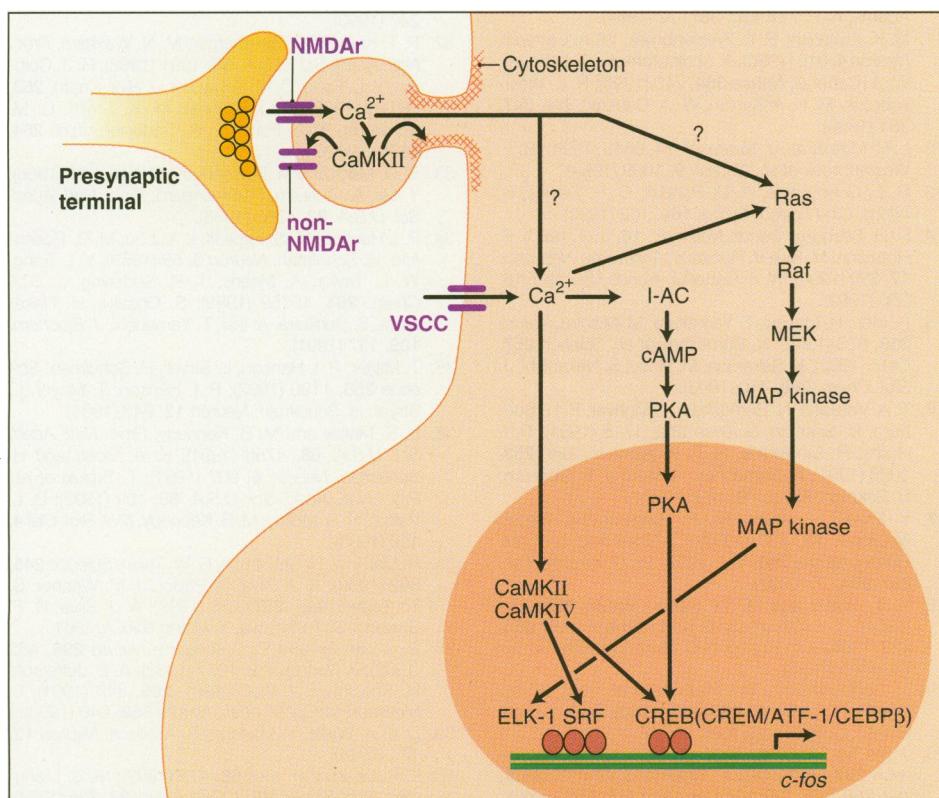


Fig. 2. A diagrammatic representation of various Ca²⁺ signaling pathways that are activated in response to Ca²⁺ influx via VSCCs and the NMDAr in neurons. Different routes of Ca²⁺ entry can lead to the activation of distinct Ca²⁺ signaling pathways which can then mediate specific Ca²⁺-dependent biological responses.

these molecules makes it likely that they are directly involved in coupling the changes in intracellular Ca^{2+} levels to long-term biological responses.

Perspective

There has been substantial progress in our understanding of how changes in intracellular Ca^{2+} effect long-term physiological changes in neurons. This has been possible because of advances on a number of fronts. The elucidation of the primary structure of various Ca^{2+} channels, the development of sophisticated Ca^{2+} -imaging systems, the identification of a number of Ca^{2+} -sensitive enzymes, and the ability to transfet reporter genes into cultured neurons and examine their regulation have all contributed greatly to our understanding of Ca^{2+} signaling.

A central feature of Ca^{2+} signaling is that there are multiple routes by which Ca^{2+} can enter the cytosol (Fig. 2). Whereas VSCCs and the NMDAr are the major plasma membrane channels through which Ca^{2+} can enter the cell from the extracellular space, release of Ca^{2+} from internal Ca^{2+} stores can also significantly influence intracellular Ca^{2+} concentrations. It appears that Ca^{2+} influx through specific plasma membrane Ca^{2+} channels or via release from specific internal stores can result in highly localized increases in intracellular Ca^{2+} . In addition to the spatial pattern of Ca^{2+} increase, temporal characteristics such as the duration of increase or the frequency of Ca^{2+} oscillations are likely to be important determinants of the activation of specific Ca^{2+} -sensitive enzymes by distinct routes of Ca^{2+} entry. Another mechanism by which the route of Ca^{2+} entry might give rise to the activation of specific signaling pathways may involve the association of intracellular signaling molecules with Ca^{2+} channel proteins. Thus, multiple modes of Ca^{2+} entry may lead to the activation of distinct intracellular signaling pathways.

The activation of Ca^{2+} -sensitive enzymes can mediate long-term adaptive responses by two major mechanisms. The posttranslational modification of preexisting proteins in local regions of Ca^{2+} increase (such as the postsynaptic spine) are likely to be involved in the induction of synapse-specific changes such as those that accompany LTP and LTD. Other cellular responses such as Ca^{2+} -dependent cell survival appear to require the propagation of Ca^{2+} signals to the nucleus and changes in the pattern of gene expression. A number of Ca^{2+} -regulated genes have been identified that can directly influence cell physiology. These include genes that encode neurotrophic factors and their receptors, neurotransmitters and neuropeptides, and synaptic vesicle proteins. It will be

of great interest to find out how specific Ca^{2+} signaling pathways lead to the activation of particular effector genes and ultimately to specific biological responses.

REFERENCES AND NOTES

- B. Hille, *Ionic Channels of Excitable Membranes* (Sinauer, Sunderland, MA, 1992).
- J. L. Franklin and E. M. Johnson Jr., *Trends Neurosci.* **13**, 501 (1992); N. C. Spitzer, *ibid.* **17**, 115 (1994).
- D. W. Choi, *Neuron* **1**, 623 (1988); D. M. Hartley, M. C. Kurth, L. Bjerknes, J. H. Weiss, D. W. Choi, *J. Neurosci.* **13**, 1993 (1993); D. W. Choi, *Trends Neurosci.* **11**, 465 (1988); *ibid.* **7**, 369 (1987).
- F. Collins, M. F. Schmidt, P. B. Guthrie, S. B. Kater, *J. Neurosci.* **11**, 2582 (1991).
- T. V. P. Bliss and G. L. Collingridge, *Nature* **361**, 31 (1993); D. V. Madison, R. C. Malenka, R. A. Nicoll, *Annu. Rev. Neurosci.* **14**, 379 (1991); R. C. Malenka, *Cell* **78**, 535 (1994); M. F. Bear and R. C. Malenka, *Curr. Opin. Neurobiol.* **4**, 389 (1994).
- M. Tymianski, M. P. Charlton, P. L. Carlen, C. H. Tator, *J. Neurosci.* **13**, 2085 (1993).
- P. L. Pederson and E. Carafoli, *Trends Biochem. Sci.* **12**, 146 (1987); D. E. Clapham, *Cell* **80**, 259 (1995).
- P. Hess, *Annu. Rev. Neurosci.* **13**, 337 (1990); R. W. Tsien, D. Lipscombe, D. V. Madison, K. R. Bley, A. P. Fox, *Trends Neurosci.* **11**, 431 (1988); R. W. Tsien, P. T. Elinor, W. A. Horne, *Trends Pharmacol. Sci.* **12**, 349 (1991); E. W. McCleskey, *Curr. Opin. Neurobiol.* **4**, 304 (1994).
- L. M. Kerr and D. Yoshikami, *Nature* **308**, 282 (1984); O. D. Uchitel et al., *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3330 (1992); T. J. Turner, M. E. Adams, K. Dunlap, *Science* **258**, 310 (1992); T. Takahashi and A. Moriyama, *Nature* **366**, 156 (1993).
- L. D. Hirning et al., *Science* **239**, 57 (1988); G. G. Holz, K. Dunlap, R. M. Bream, *J. Neurosci.* **8**, 403 (1988); G. R. Artalejo, S. Rossie, R. L. Pearlman, A. P. Fox, *Nature* **358**, 63 (1992); C. R. Artalejo, M. E. Adams, A. P. Fox, *ibid.* **367**, 72 (1994).
- M. K. Ahijanian, R. E. Westenbroek, W. A. Catterall, *Neuron* **4**, 819 (1990); A. Sculpooreanu, T. Scheuer, W. A. Catterall, *Nature* **364**, 240 (1993); R. E. Westenbroek, M. K. Ahijanian, W. A. Catterall, *ibid.* **347**, 281 (1990).
- L. R. Mills et al., *J. Neurosci.* **14**, 6815 (1994); R. E. Westenbroek et al., *Neuron* **9**, 1099 (1992).
- G. L. Collingridge, A. D. Randall, C. H. Davies, S. Alford, *Ciba Found. Symp.* **164**, 162 (1992).
- P. H. Seeburg, *Trends Neurosci.* **16**, 359 (1993); F. Hofmann, M. Biel, V. Flockerzi, *Annu. Rev. Neurosci.* **17**, 399 (1994); W. A. Catterall, *Trends Neurosci.* **16**, 500 (1993).
- H. Mori, H. Masaki, T. Yamakura, M. Mishira, *Nature* **358**, 673 (1992); N. Burnashev et al., *Science* **257**, 1415 (1992); K. Sakurada, M. Masu, S. Nakanishi, *J. Biol. Chem.* **268**, 410 (1993).
- T. A. Verdoorn, N. Burnashev, H. Monyer, P. H. Seeburg, B. Sakman, *Science* **252**, 1715 (1991); R. I. Hume, R. Dingledine, S. F. Heinemann, *ibid.* **253**, 1028 (1991); N. Burnashev, H. Monyer, P. Seeburg, B. Sakman, *Neuron* **8**, 189 (1992).
- Y. En-Avi, L. Anksztajn, P. Bregestovski, *Trends Neurosci.* **15**, 333 (1992); L. Chen and L.-Y. M. Huang, *Nature* **356**, 421 (1992); W. G. Tingley et al., *ibid.* **364**, 70 (1993).
- Y. T. Wang and M. W. Salter, *Nature* **369**, 233 (1994); G. F. Wilson and L. K. Kaczmarek, *ibid.* **366**, 433 (1993); X-Y. Huang, A. D. Morielli, E. Peralta, *Cell* **75**, 1145 (1993).
- J. Schlessinger, *Curr. Opin. Genet. Dev.* **4**, 25 (1994); T. Hunter, *Cell* **80**, 225 (1995); G. B. Cohen, R. Ren, D. Baltimore, *ibid.*, p. 237.
- J. Ma, M. Fill, C. M. Knudson, K. P. Campbell, *Science* **242**, 99 (1988); E. Niggli and W. J. Lederer, *ibid.* **250**, 565 (1990); V. Henzi and A. B. McDermott, *Neuroscience* **46**, 251 (1992); M. J. Berridge, *Nature* **361**, 315 (1993); A. I. Kaplin et al., *J. Biol. Chem.* **269**, 28972 (1994).
- Z. Xu, G. S. Kopf, R. M. Schultz, *Development* **120**, 1851 (1994); J. Amundson and D. Clapham, *Curr. Opin. Neurobiol.* **3**, 375 (1993); P. Thorn et al., *Cell* **74**, 661 (1993); S. Kume et al., *ibid.* **73**, 555 (1993); A. Galione et al., *Science* **261**, 348 (1993); V. Sorrentino and P. Volpe, *Trends Pharmacol. Sci.* **14**, 98 (1993); D. D. Friel and R. W. Tsien, *J. Neurosci.* **14**, 4007 (1994); *Neuron* **8**, 1109 (1992); D. Lipscombe et al., *ibid.* **1**, 355 (1988).
- G. P. Ahern, P. R. Junankar, A. F. Dulhunty, *FEBS Lett.* **352**, 369 (1994); A. B. Brillantes et al., *Cell* **77**, 513 (1994); A. P. Timerman et al., *J. Biol. Chem.* **268**, 22992 (1993); A. Galione et al., *Science* **261**, 348 (1993).
- H. C. Lee et al., *Nature* **370**, 307 (1994); A. Galione et al., *ibid.* **365**, 456 (1993); L. G. Meszaros, J. Bak, A. Chu, *ibid.* **364**, 76 (1993); J. Morrisette et al., *FEBS Lett.* **330**, 270 (1993); W. R. Buck, E. E. Hoffmann, T. L. Rakow, S. S. Shen, *Dev. Biol.* **163**, 1 (1994); H. C. Lee, R. Aarhus, T. F. Walseeth, *Science* **261**, 352 (1993).
- G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J. Biol. Chem.* **260**, 3440 (1985).
- R. D. Randall and S. A. Thayer, *J. Neurosci.* **12**, 1882 (1992); A. Hernandez-Cruz, F. Sala, P. R. Adams, *Science* **247**, 858 (1990); D. A. Przywara et al., *FASEB J.* **5**, 217 (1991); J. D. Kosciusko et al., *Brain Res.* **616**, 273 (1993).
- W. G. Regehr, J. A. Connor, D. W. Tank, *Nature* **341**, 533 (1989); W. G. Regehr and D. W. Tank, *ibid.* **345**, 807 (1990).
- P. B. Guthrie, M. Segal, S. B. Kater, *ibid.* **354**, 76 (1991); W. Muller and J. A. Connor, *ibid.*, p. 73.
- J. Ellers, G. J. Augustine, A. Konnerth, *ibid.* **373**, 155 (1995).
- A. Konnerth, J. Dreessen, G. J. Augustine, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7051 (1992); M. Kano, U. Rexhausen, J. Dreessen, A. Konnerth, *Nature* **356**, 601 (1992); I. Llano, N. Leresche, A. Marty, *Neuron* **6**, 565 (1991).
- R. Yuste and L. C. Katz, *Neuron* **6**, 333 (1991); R. Yuste, M. J. Gutnick, D. Saar, K. R. Delaney, D. W. Tank, *ibid.* **13**, 23 (1994); G. J. Stuart and B. Sakmann, *Nature* **367**, 69 (1994).
- P. I. Hanson and H. Schulman, *Annu. Rev. Biochem.* **61**, 559 (1992); H. Schulman, *Curr. Opin. Cell Biol.* **5**, 247 (1993).
- P. T. Kelly, R. P. Weinberger, M. N. Waxham, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4991 (1988); R. J. Colbran, Y. L. Fong, C. M. Schworer, *J. Biol. Chem.* **263**, 18145 (1988); R. J. Colbran, M. K. Smith, C. M. Schworer, Y.-L. Fong, T. R. Soderling, *ibid.* **264**, 4800 (1989).
- S. G. Miller and M. B. Kennedy, *Cell* **44**, 861 (1986); Y. Lai, A. G. Nairn, P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4253 (1986).
- P. I. Hanson, M. S. Kapiloff, L. L. Lou, M. G. Rosenfeld, H. Schulman, *Neuron* **3**, 59 (1995); Y. L. Fong, W. L. Taylor, A. Means, T. R. Soderling, *J. Biol. Chem.* **264**, 16759 (1989); S. Ohshiro, H. Nakazawa, S. Sehikara, A. Imai, T. Yamauchi, *J. Biochem.* **109**, 137 (1991).
- T. Meyer, P. I. Hanson, L. Stryer, H. Schulman, *Science* **256**, 1199 (1992); P. I. Hanson, T. Meyer, L. Stryer, H. Schulman, *Neuron* **12**, 943 (1994).
- S. S. Molloy and M. B. Kennedy, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4756 (1991); K. A. Ocorr and H. Schulman, *Neuron* **6**, 907 (1991); T. Suzuki et al., *Proc. Natl. Acad. Sci. U.S.A.* **89**, 109 (1992); B. L. Patton, S. S. Molloy, M. B. Kennedy, *Mol. Biol. Cell* **4**, 159 (1993).
- R. Malinow, H. Schulman, R. W. Tsien, *Science* **245**, 862 (1989); A. J. Silva, R. Paylor, J. M. Wehner, S. Tonegawa, *ibid.* **257**, 206 (1992); A. J. Silva, C. F. Stevens, S. Tonegawa, Y. Wang, *ibid.*, p. 201.
- E. J. Nestler and P. Greengard, *Nature* **296**, 452 (1982); *J. Neurosci.* **2**, 1011 (1982); A. B. Jefferson, H. Schulman, *J. Biol. Chem.* **266**, 346 (1991); E. McGlade-McCullough et al., *Nature* **362**, 640 (1993).
- D. J. A. Wyllie, T. Manage, R. A. Nicoll, *Neuron* **12**, 127 (1994).
- L. R. Levin et al., *Cell* **68**, 479 (1992); M. S. Livingston, P. P. Sziber, W. G. Quinn, *ibid.* **37**, 205 (1984).
- S. Schacher, V. F. Castellucci, E. R. Kandel, *Science* **240**, 1667 (1988); Y. Yovell, E. R. Kandel, Y. Dudai, T. W. Abrams, *Neurochemistry* **59**, 1736 (1992).
- D. M. Chetkovich, R. Gray, D. Johnston, J. D. Sweet, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6467 (1991).

43. Z. Wu *et al.*, *ibid.* **92**, 220 (1995).
 44. Z. Xia, C. D. R. Refsdal, K. M. Merchant, D. M. Dorsa, D. R. Storm, *Neuron* **6**, 431 (1991).
 45. E. J. Choi, S. T. Wong, T. J. Hinds, D. R. Storm, *J. Biol. Chem.* **267**, 12440 (1992).
 46. S. Impey, G. A. Wayman, Z. Wu, D. R. Storm, *Mol. Cell. Biol.* **14**, 8272 (1994); G. A. Wayman *et al.*, *J. Biol. Chem.* **269**, 25400 (1994).
 47. U. Frey, Y.-Y. Huang, E. R. Kandel, *Science* **260**, 1661 (1993); P. V. Nguyen, T. Abel, E. R. Kandel, *ibid.* **265**, 1104 (1994); A. J. Cole, D. W. Saffen, J. M. Baraban, P. F. Worley, *Nature* **340**, 474 (1989); P. K. Stanton and J. M. Savrey, *J. Neurosci.* **4**, 3080 (1984); U. Frey *et al.*, *Brain Res.* **452**, 57 (1988).
 48. P. Goelet, V. F. Castellucci, S. Schacher, E. R. Kandel, *Nature* **322**, 419 (1986).
 49. R. P. Matthews *et al.*, *Mol. Cell. Biol.* **14**, 6107 (1994); H. Tokumitsu *et al.*, *J. Biol. Chem.* **269**, 28640 (1994); M. E. Selbert *et al.*, personal communication.
 50. L. B. Rosen, D. D. Ginty, M. J. Weber, M. E. Greenberg, *Neuron* **12**, 1207 (1994); H. Bading and M. E. Greenberg, *Science* **253**, 912 (1991).
 51. R. J. Davis, *J. Biol. Chem.* **268**, 14553 (1993).
 52. M. Sheng and M. E. Greenberg, *Neuron* **4**, 477 (1990); D. D. Ginty, H. Bading, M. E. Greenberg, *Curr. Opin. Neurobiol.* **2**, 312 (1992); A. Ghosh, D. D. Ginty, H. Bading, M. E. Greenberg, *J. Neurobiol.* **25**, 294 (1994).
 53. T. H. Murphy, P. F. Worley, J. M. Baraban, *Neuron* **7**, 625 (1991); J. K. Yoon and L. F. Lau, *Mol. Cell. Biol.* **14**, 7731 (1994); T. G. Hazel, D. Nathans, L. R. Lau, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8444 (1988); Y. K. Yoon and L. F. Lau, *J. Biol. Chem.* **268**, 9148 (1993); M. E. Greenberg, E. B. Ziff, L. A. Greene, *Science* **234**, 80 (1986); J. I. Morgan and T. Curran, *Nature* **322**, 552 (1986).
 54. M. R. Montminy, K. A. Sevarino, J. A. Wagner, G. Mandel, R. H. Goodman, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6682 (1986); K. K. Yamamoto, G. A. Gonzalez, W. H. Biggs, M. R. Montminy, *Nature* **334**, 494 (1988); M. R. Montminy and L. M. Bleijskij, *ibid.* **328**, 175 (1987).
 55. M. Sheng, G. McFadden, M. E. Greenberg, *Neuron* **4**, 571 (1990); M. Sheng, M. A. Thompson, M. E. Greenberg, *Science* **252**, 1427 (1991); P. K. Dash, K. A. Karl, M. A. Colicos, R. Prywes, E. R. Kandel, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5061 (1991).
 56. N. S. Foulkes, E. Borrelli, P. Sassone-Corsi, *Cell* **64**, 739 (1991); J. L. Meinhardt *et al.*, *Mol. Cell Biochem.* **127**, 179 (1993); T. E. Mayer and J. F. Habener, *Endocrine Rev.* **14**, 269 (1993).
 57. G. A. Gonzalez and M. R. Montminy, *Cell* **59**, 675 (1989).
 58. R. P. S. Kwok *et al.*, *Nature* **370**, 223 (1994); J. Arias *et al.*, *ibid.*, p. 226; J. C. Chirivita *et al.*, *ibid.* **365**, 855 (1993); Z. Arani, D. Newsome, E. Oldread, D. M. Livingston, R. Eckner, *ibid.* **374**, 81 (1995); J. R. Lundblad *et al.*, *ibid.*, p. 85.
 59. D. D. Ginty, A. Bonni, M. E. Greenberg, *Cell* **77**, 713 (1994).
 60. C. J. Fiol *et al.*, *J. Biol. Chem.* **269**, 32187 (1994).
 61. F. H. Cruzalegui and A. R. Means, *ibid.* **268**, 26171 (1993); H. Enslen *et al.*, *ibid.* **269**, 15520 (1994); H. Enslen and T. R. Soderling, *ibid.*, p. 20872; P. Sun, H. Enslen, P. S. Myung, R. A. Maurer, *Genes Dev.* **8**, 2527 (1994); S. E. Tan, R. J. Wenthold, T. R. Soderling, *J. Neurosci.* **14**, 1123 (1994); M. A. Selber *et al.*, in preparation; R. P. Matthews *et al.*, *Mol. Cell Biol.* **14**, 6107 (1994).
 62. H. Bading, D. D. Ginty, M. E. Greenberg, *Science* **260**, 181 (1993); D. D. Ginty *et al.*, *ibid.*, p. 238.
 63. M. Wegner, Z. Cao, M. G. Rosenfeld, *ibid.* **256**, 370 (1992).
 64. A. Ghosh, J. Carnahan, M. E. Greenberg, *ibid.* **263**, 1618 (1994).
 65. B. Lu, M. Yokoyama, C. F. Dreyfus, I. B. Black, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6289 (1991); F. Zafra, D. Lindholm, E. Castren, J. Hartikka, H. Thoenen, *J. Neurosci.* **12**, 4793 (1992); S. J. Birren, J. M. Verdi, D. J. Anderson, *Science* **257**, 395 (1992); C. M. Gall and P. J. Isackson, *ibid.* **245**, 758 (1989); P. J. Isackson, M. M. Huntsman, K. D. Murray, C. M. Gall, *Neuron* **6**, 937 (1991); F. Zafra *et al.*, *EMBO J.* **9**, 3545 (1990); F. Zafra *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10037 (1991).
 66. R. E. Zigmond, M. A. Schwartzchild, A. R. Rittenhouse, *Annu. Rev. Neurosci.* **12**, 415 (1989); Y. Sun, M. S. Rao, S. C. Landis, R. E. Zigmond, *J. Neurosci.* **12**, 3717 (1992); M. L. Tsauer, M. Sheng, D. H. Lowenstein, Y. N. Jan, L. Y. Jan, *Neuron* **8**, 1055 (1992); M. Kokai *et al.*, *Brain Res.* **23**, 323 (1994).
 67. We apologize to the scientists whose work we were unable to discuss due to space constraints. We thank R. Segal, W. Regehr, M. Sheng, L. Rosen, and H. Dudek for comments and L. Ghosh for help in the preparation of this manuscript. Support for this review was provided by NIH grant NS28829 (M.E.G.) and a postdoctoral fellowship from The Medical Foundation (A.G.).

Localization of Protein Kinases by Anchoring Proteins: A Theme in Signal Transduction

Daria Mochly-Rosen

A fundamental question in signal transduction is how stimulation of a specific protein kinase leads to phosphorylation of particular protein substrates throughout the cell. Recent studies indicate that specific anchoring proteins located at various sites in the cell compartmentalize the kinases to their sites of action. Inhibitors of the interactions between kinases and their anchoring proteins inhibit the functions mediated by the kinases. These data indicate that the location of these anchoring proteins provides some of the specificity of the responses mediated by each kinase and suggest that inhibitors of the interaction between the kinases and their anchoring proteins may be useful as therapeutic agents.

Stimulation of many signaling cascades results in activation of protein kinases, which in turn phosphorylate their respective substrates, leading to diverse physiological responses. These diverse effects result, at least in part, from compartmentalization of a number of signaling molecules including protein kinases. Components in tyrosine kinase-mediated signaling pathways are compartmentalized (1) as are several serine and threonine protein phosphatases and some protein kinases (2). This review summarizes data indicating that: (i) cell stimulation causes translocation of several serine and threonine protein kinases to new sites,

presumably to alter their access to their substrates; (ii) compartmentalization of these kinases results from their binding to specific anchoring proteins at their respective sites; and (iii) compartmentalization is required for the physiological functions of these kinases.

Adenosine 3',5'-Monophosphate (cAMP)-Dependent Protein Kinase

The cAMP-dependent protein kinase (PKA) is composed of two regulatory and two catalytic subunits. There are several gene products for each of these subunits and multiple PKA isoforms. Both the catalytic subunit and the type II PKA holoenzyme can be differentially compartmentalized at

specific subcellular locations both before and after cell stimulation. Type II PKA is found in the cell particulate fraction, often anchored through the regulatory domain (RII) near its protein substrates. For example, this enzyme associates with microtubules (3) near some of its known substrates, the microtubule-associated proteins (4). Binding of cAMP to the holoenzyme releases the catalytic subunits, enabling them to phosphorylate their substrates. Therefore, localization of the type II kinase near its substrates may ensure rapid phosphorylation of specific substrates in response to increases in the intracellular concentration of cAMP ($[cAMP]$) (5). The dissociated catalytic subunits may translocate to new subcellular sites to phosphorylate other substrates. In Madin Darby bovine kidney cells, for example, the holoenzyme is localized on the Golgi complex (6). When $[cAMP]$ increases, the distribution of the regulatory subunit remains unchanged, whereas the catalytic subunit is first found in the cytoplasm and then in the nucleus (6). Translocation of the catalytic subunit into the nucleus in response to increases in $[cAMP]$ has also been demonstrated by microinjection of fluorescently tagged recombinant regulatory and catalytic subunits (7). Before stimulation, both subunits are found in the cytosol (Fig. 1). When $[cAMP]$ increases after cells are treated with forskolin, some of the catalytic subunit begins to dissociate from the regulatory subunit and diffuses into the nucleus. Translocation of the catalytic subunit is transient (6); 1 hour after removal of forskolin, the catalytic subunit returns to the

The author is in the Department of Molecular Pharmacology, School of Medicine, Stanford University, Stanford, CA 94305-5332, USA.