A Spatial-Temporal Model of Cell Activation

DANIEL L. ALKON AND HOWARD RASMUSSEN

A spatial-temporal model of calcium messenger function is proposed to account for sustained cellular responses to sustained stimuli, as well as for the persistent enhancement of cell responsiveness after removal of a stimulus, that is, cellular memory. According to this model, spatial separation of calcium function contributes to temporal separation of distinct phases of the cellular response. At different cellular sites, within successive temporal domains, the calcium messenger is generated by different mechanisms and has distinct molecular targets. In particular, prolonged cell activation is brought about by the interaction of calcium with another spatially confined messenger, diacylglycerol, to cause the association of protein kinase C with the plasma membrane. Activity of the membrane-associated protein kinase C is controlled by the rate of calcium cycling across the plasma membrane. In some instances, a single stimulus induces both protein kinase C activation and calcium cycling and thus causes prolonged activation; but in others, a close temporal association of distinct stimuli brings about cell activation via interaction of these intracellular messengers. Persistent enhancement of cell responsiveness after removal of stimuli is suggested to be due to the continued association, or anchoring, of protein kinase C to the membrane.

THE SECOND MESSENGER MODEL OF CELL ACTIVATION HAS become an accepted tenet of cell biology (*1*). Implicit in this model is the notion that a second messenger conveys information from the cell surface to the entire intracellular domain. In addition, the second messenger is thought to act in a single temporal domain, that is, the magnitude and duration of the cellular response is a direct function of the strength and duration of the message. However, when considering the second messenger function of calcium, these features often do not hold. Only during a brief response or the initial phase of a sustained response do changes in cellular response appear to mirror changes in Ca^{2+} concentration within the cytosol (*2*).

Using Ca^{2+} as our primary example, we will propose here a different model of intracellular messenger functions—a spatial-temporal model. This model, which involves Ca^{2+}, the inositol lipid system, and protein kinase C (PKC), can account for sustained cellular responses during sustained application of diverse physiologic stimuli that mediate endocrine secretion, neurotransmission, or cell proliferation. The model also can explain enhanced responsiveness that outlasts the initiating stimuli. Such persistent responsiveness can be viewed as cellular "memory," whereby a single messenger "recalls" the previous temporal and spatial association of multiple second messengers.

D. L. Alkon is head of the Section on Neural Systems and codirector of the Laboratory of Cellular and Molecular Neurobiology, National Institute of Neurologic and Communicative Disorders and Stroke, Bethesda, MD 20892. H. Rasmussen is a professor of medicine and cell biology at the Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510.

Spatial Domains of Ca^{2+} Signals

Calcium can, and often does, operate within a restricted spatial domain. Such domains include the cytosol of the sarcomere (*3*), a submembrane cytosolic domain immediately adjacent to the inner surface of the plasma membrane (*2, 4*), the mitochondrial matrix compartment (*5*), and a submembrane cytosolic domain immediately adjacent to the cytoplasmic face of the endoplasmic reticulum (*6*).

Two major sites of Ca^{2+} signal generation are the plasma membrane and the endoplasmic (or sarcoplasmic) reticulum. At either site, transduction can be transient or sustained and can be independent of or linked to the inositol phospholipid system. A major distinction between the two sites is in the magnitude of the signal generated. In the case of the plasma membrane, many agonists cause a two- to fourfold sustained increase in Ca^{2+} influx, which is usually associated with a compensatory increase in Ca^{2+} efflux rate so that any net entry of Ca^{2+} into the bulk cytosol is limited both in magnitude (1 to 5 μmol per liter of cell water) and duration (*2*). In contrast, when the Ca^{2+} pool in the endoplasmic (sarcoplasmic) reticulum is released, there is a net shift of a large amount of Ca^{2+} (20 to 200 μmol per liter of cell water) in a very brief time into the bulk cytosol (*7*).

Sarcoplasmic reticulum. Release of Ca^{2+} from the sarcoplasmic reticulum (SR) occurs via electronic or ionic coupling (or both) of events at the plasma membrane to those at the SR membrane at the T system in skeletal and cardiac muscle (*8*). In addition, there are many cell types in which an agonist-induced rise in inositol 1,4,5-trisphosphate (Ins 1,4,5P_3) concentration leads to the efflux of Ca^{2+} from a specialized compartment of the endoplasmic reticulum (*9*). Cells ranging from those in the blowfly salivary gland and mammalian smooth muscle to a variety of hormonally responsive cells behave in this manner (*10*).

Plasma membrane. Many mechanisms underlie generation of Ca^{2+} signals at the plasma membrane (*11*). These depend on a variety of Ca^{2+} channels as well as numerous regulatory mechanisms. There are voltage-dependent, receptor-operated, and second messenger–regulated Ca^{2+} channels. Within each category there are several types of channels. Furthermore, many voltage-dependent and receptor-operated channels are also regulated by second messengers (*11*). Transient or sustained increases of Ca^{2+} flux through such channels are linked to transient or sustained cellular responses. Thus, temporal domains of intracellular messenger function must, to some degree, depend on the temporal domains of membrane Ca^{2+} channel function.

Transient Cellular Responses

In neurosecretion and skeletal muscle contraction, Ca^{2+} functions as a classic second messenger. Transient stimuli elicit transient responses, release of neurotransmitter, or contraction of muscle. In neurosecretion, Ca^{2+} is elevated within the presynaptic terminal and acts largely in a subcellular domain adjacent to the inner surface of the presynaptic membrane (*12*). In the contraction of skeletal muscle, Ca^{2+} acts mainly at the cytosolic domain of the sarcomere, particularly that domain close to the Z disc (*8*). In each case, the magnitude and duration of the response is a direct function of a change in Ca^{2+} concentration within the relevant cellular domain.

Sustained Cellular Responses to Sustained Stimuli

Implicit in the classic second messenger model is the concept that, during a sustained cellular response, there is a sustained increase in second messenger concentration. However, with the introduction of new methods for measuring changes in the intracellular Ca^{2+} concentration, aequorin (*13*) or fura 2 (*14*), it has become evident that the first messenger leads to only a transient increase in intracellular Ca^{2+} even though a sustained cellular response is often induced (*2, 15*). Even though intracellular Ca^{2+} concentration, as measured by these methods, remains low during the sustained phase of the response, there is, nonetheless, an increased rate of Ca^{2+} influx (or actually Ca^{2+} cycling) across the plasma membrane (*2, 16*). This cycling is essential for the sustained response. Yet in many cell types that display a sustained response, simply increasing Ca^{2+} cycling across the plasma membrane is insufficient to induce a response (*16*). There is need for both an increase in Ca^{2+} cycling and the presence of a plasma membrane–associated, Ca^{2+}-activated response element. Two Ca^{2+}-activated response elements that function in this way are adenylate cyclase (*17*) and PKC (*18*).

Ca^{2+} and cAMP as synarchic messengers. Although we will focus on sustained cellular responses mediated by the inositol lipid–linked pathway, sustained Ca^{2+}-linked responses also occur in situations in which this pathway is not activated. In particular, adenosine 3′,5′-monophosphate (cAMP) and Ca^{2+} can interact as synarchic messengers to mediate sustained cellular responses (*19*). Commonly this interaction involves a sustained increase in Ca^{2+} cycling across the plasma membrane associated with a sustained activation of adenylate cyclase (*2*). There are several different pathways by which these two events can be linked; a primary increase in Ca^{2+} influx can result in the calmodulin-dependent activation of adenylate cyclase (*17*); the simultaneous coupling of a hormone to two distinct receptor types can bring about the opening of a receptor-operated Ca^{2+} channel via one receptor subtype and the activation of adenylate cyclase via another (*16*); or the primary event can be an activation of adenylate cyclase leading to the cAMP-dependent opening of specific Ca^{2+} channels (*16*).

In these cases, a sustained cellular response depends on the sustained increase in both Ca^{2+} influx and cAMP production; neither messenger alone is sufficient. It is not yet clear how these two messengers interact. One possibility is that exemplified by the glycogenolytic cascade within which the cAMP-dependent phosphorylation of phosphorylase b kinase, a Ca^{2+}-calmodulin–regulated enzyme, leads to a modulation of its sensitivity to activation by Ca^{2+}. The phosphorylated form of phosphorylase b kinase is activated by a tenfold lower Ca^{2+} concentration then is the nonphosphorylated form (*19*). Sensitivity modulation of other Ca^{2+}-calmodulin–regulated kinases is likely to be a common regulatory mechanism (*20*). Additionally, cAMP may bring about the inhibition of phosphoprotein phosphatases that catalyze the dephosphorylation of the phosphoprotein products of Ca^{2+}-dependent kinases (*21*).

Ca^{2+} and protein kinase C. A variety of endocrine, autonomic, and neural systems display sustained responses to the sustained presence of extracellular messengers acting via the inositol lipid–PKC pathways (*2, 7, 10, 22*). For example, studies in adrenal glomerulosa cells

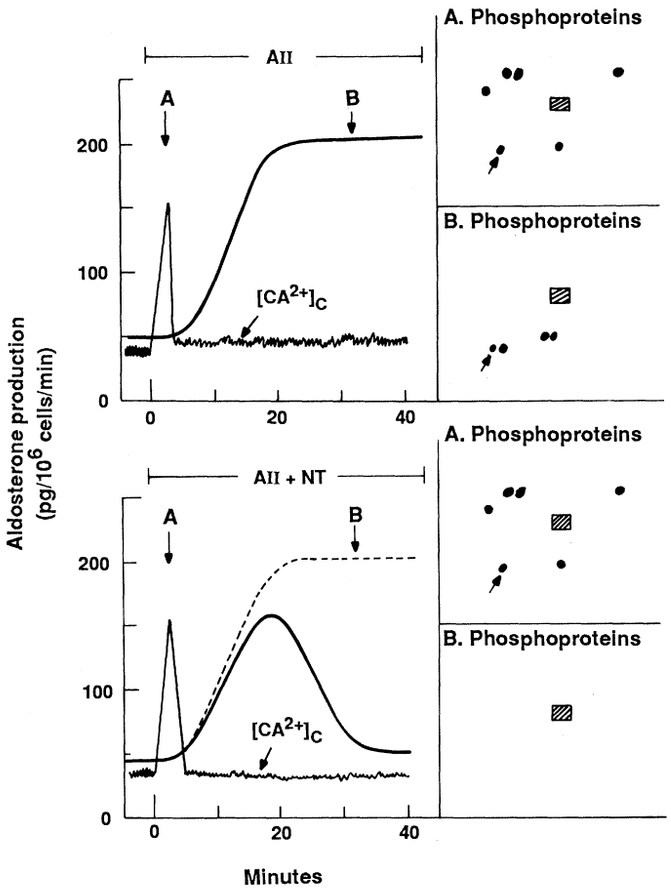

Fig. 1. Temporal events in cell activation and the crucial role of plasma membrane Ca^{2+} flux in the sustained phase of the response of adrenal glomerulosa cells to (**upper left**) $10^{-9}M$ angiotensin II (AII) in control cells and cells exposed continuously to (**lower left**) $10^{-6}M$ of the Ca^{2+} channel antagonist, nitrendipine (NT). On the left are shown the time course of change in aldosterone production rate (——) and in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_c$) (▬▬). A and B represent the time points (1 and 30 minutes, respectively) at which protein extracts were prepared from cells preincubated with ^{32}P for analysis of protein phosphorylation patterns by autoradiography of two-dimensional polyacrylamide gels. Schematic representation of the resulting autoradiographs are shown for control cell (**upper right, A and B**) and for nitrendipine-treated cells (**lower right, A and B**). The black dots represent the individual low molecular weight (25 to 64 kD) proteins that show an increase in phosphorylation in response to angiotensin II treatment. The hatched square in each figure signifies the position of a reference protein with unchanged phosphorylation. Note that in the control cells (upper figures) angiotensin II increases the intracellular Ca^{2+} concentration only transiently but induces a monotonic rise in aldosterone secretory rate to a sustained plateau and that these changes are associated with different patterns of phosphoproteins at 1 minute (the peak of the Ca^{2+} transient) and at 30 minutes (during the sustained phase of the response). Only one protein is common to both patterns (arrow). In the presence of a concentration of nitrendipine that inhibits the angiotensin II–induced increase in plasma membrane Ca^{2+} influx rate but does not inhibit the basal rate of Ca^{2+} influx (not shown), angiotensin II induces the same initial Ca^{2+} transient (because of a release of intracellular Ca^{2+}) and the same initial pattern of change in protein phosphorylation. These are associated with the same initial rise in aldosterone secretory rate. However, this increase in secretory rate is not sustained and by 30 minutes is close to its basal value, at which time none of the four late-phase proteins are phosphorylated.

and tracheal smooth muscle have provided evidence for a temporal sequence of events underlying the action of the agonists, angiotensin II and carbacholamine, respectively (*23*). In these cases and in the cases of other peptide and amine hormones (*2, 7, 10, 22*), there is a temporal sequence of linked events in inositol lipid metabolism, Ca^{2+} metabolism, and protein kinase activities that mediate a sustained cellular response. One can distinguish at least two distinct phases of response during which Ca^{2+} serves as a messenger, but in which its site of action and molecular targets differ. During the initial phase, Ca^{2+} acts as a messenger in the bulk cytosol on calmodulin-regulated protein kinases leading to the phosphorylation of one specific subset of cellular proteins (*2, 23*). During the sustained phase, Ca^{2+} acts as a messenger in a submembrane domain of the plasma membrane (see below) on PKC to induce the phosphorylation of a second subset of cellular proteins (Fig. 1).

The complex temporal relationships between inositol lipid metabolism, inositol phosphate turnover, Ca^{2+} metabolism, and protein phosphorylation underlying such sustained responses are only partially understood. When a hormone (for example, angiotensin II) interacts with its receptor, a specific phospholipase C is activated resulting in the hydrolysis of phosphatidyl 4,5-bisphosphate (PIP_2) to yield Ins 1,4,5P_3 and diacylgylcerol (DG). As a consequence, the DG content of the plasma membrane increases and the concentration of Ins 1,4,5P_3 in the cytosol rises. However, the rise in Ins 1,4,5P_3 lasts only 1 minute or less because Ins 1,4,5P_3 is metabolized either to inositol tetrakisphosphate (Ins 1,3,4,5P_4) by a Ca^{2+}-dependent kinase (*24*) or to Ins 1,4P_2 by a specific phosphatase (*24*). Nonetheless, Ins 1,4,5P_3 production continues so that during the sustained phase of the response, the content of Ins 1,3,4,5P_4 remains elevated, as does that of its metabolite, inositol 1,3,4-trisphosphate (Ins 1,3,4P_3) (*24*).

There is also a temporal sequence of changes in cellular Ca^{2+} metabolism (*2*). Initially a transient rise in intracellular Ca^{2+} concentration is associated with a net efflux of Ca^{2+} from the cell. These two events are a reflection of an Ins 1,4,5P_3–induced mobilization of Ca^{2+} from an intracellular pool contained in a specific component of the endoplasmic retriculum, which has been located in smooth muscle by electron probe analysis just beneath the plasma membrane (*25*). When this Ca^{2+} is mobilized, much of it is pumped out of the cell as a result of the Ca^{2+}-calmodulin–dependent activation of the Ca^{2+} pump in the plasma membrane (*26*). The Ca^{2+} transient is accompanied and followed by a sustained increase in the rate of Ca^{2+} influx across the plasma membrane without a net increase in cellular Ca^{2+} content (*2, 16*). Hence, there is a compensatory increase in Ca^{2+} efflux across the plasma membrane such that during the sustained phase of the response, a sustained increase in Ca^{2+} cycling across the plasma membrane occurs with neither net accumulation of Ca^{2+} by the cell nor a significant increase in the Ca^{2+} concentration in the cell cytosol.

Three aspects of this Ca^{2+} cycling are noteworthy: (i) The first is the mechanism by which it is induced. It is not yet clear how a hormone of this class increases plasma membrane Ca^{2+} influx. Either Ins 1,3,4,5P_4 or Ins 1,4,5P_3 (or both) might act as intracellular regulators of plasma membrane Ca^{2+} channel activity (*27*), but it remains possible that the change in Ca^{2+} flux is a more direct consequence of hormone-receptor interaction. (ii) The second important aspect of Ca^{2+} cycling is the nature of its autoregulatory mechanisms. At least two major mechanisms operate to bring about a compensatory increase in Ca^{2+} efflux rate after a hormonally induced increase in Ca^{2+} influx. The first is the direct Ca^{2+}-calmodulin–dependent allosteric activation of the plasma membrane Ca^{2+} pump (*26*), and the second results from the PKC-dependent phosphorylation of the Ca^{2+} pump (*28*). (iii) Finally, Ca^{2+} cycling serves as a messenger. The initial Ca^{2+} transient in the cytosol initiates cellular responses by activating calmodulin-dependent protein kinases that catalyze the phosphorylation of a specific subset of cellular proteins (Fig. 1) involved in the initiation of the response (*29*). However, because the rise in Ca^{2+} concentration is transient, the activation of these enzymes is also transient, albeit the phosphorylation events outlast the rise in Ca^{2+} concentration. The Ca^{2+} transient also acts in concert with the rise in the DG content of the plasma membrane to cause the translocation of the Ca^{2+}-insensitive form of PKC from the cell cytosol to the plasma membrane (*2, 18, 30*). The plasma membrane–associated form of this enzyme is Ca^{2+}-sensitive, that is, the sensitivity of the enzyme changes because of its association with the membrane lipids. PKC may either "read" the rate of Ca^{2+} flux across the membrane directly or, more likely, respond to an elevation of Ca^{2+} concentration at or near the endoplasmic face of the plasma membrane. In either case, the sustained increase in Ca^{2+} cycling across the plasma membrane regulates the activity of the plasma membrane–associated PKC, and thus the extent of phosphorylation of a second subset of cellular proteins (Fig. 1) responsible for mediating the sustained phase of the cellular response (*23, 29*). This linking of plasma membrane–associated PKC to the sustained phase of cellular response departs from the classical second messenger concept. Now, Ca^{2+} acts only at the plasma membrane rather than throughout the cytosolic domain.

Sustained neuronal responses to sustained stimuli. Although typical unitary neuronal responses such as action potentials or synaptic potentials last only a fraction of a second, the information processing in neural systems depends on responses that persist as long as the stimulus persists. Sustained neuronal and muscular responses can be elicited by sustained sensory stimuli, neurochemical agonists, or

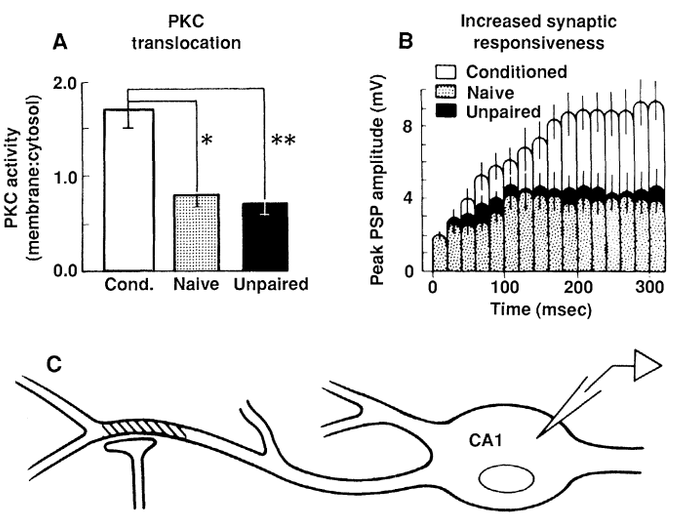

Fig. 2. Enhanced synaptic responsiveness parallels protein kinase C (PKC) translocation after classical conditioning of the eye blink reflex in rabbits. (**A**) The proportion of PKC associated with the plasma membrane is greatest in regions surrounding and including the CA1 pyramidal cells microdissected from classically conditioned rabbits compared to controls (naïve and unpaired groups). Values from three groups are significantly different ($P < 0.01$) by one-way analysis of variance. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.025$) by t test comparions of each control group with conditioned group. (**B**) Summation of postsynaptic potentials (PSP) is greatest in responses of CA1 pyramidal cells from conditioned rabbits when compared to cells from naïve rabbits or to cells from rabbits given unpaired stimuli. Synaptic potentials were elicited by electrical stimulation (50 Hz) of presynaptic fibers (Schaeffer collaterals). (**C**) A model CA1 pyramidal cell showing position of recording microelectrode in cell soma. Hatched area represents postsynaptic regions in proximal dendrites where specific binding of radioactive phorbol ester was localized by autoradiographic analysis. The data are from (*50, 51*).

changes of membrane potential. Just as for the endocrine examples already described, interaction of second messengers is often necessary for sustained responses.

Interaction of Ca^{2+} and cAMP occurs in, for example, cardiac muscle cells, *Aplysia* neurons (*31*), and *Hermissenda* photoreceptors (*32*). In the presence of epinephrine, elevation of cAMP regulates Ca^{2+} channels activated by depolarization of cardiac muscle cells (*33*). In the presence of serotonin (5-HT), cAMP elevation also controls voltage-dependent Ca^{2+} channels of *Aplysia* central neurons (*34*). Indirect control of voltage-dependent Ca^{2+} influx can also be effected by the cAMP-dependent reduction of K^+ currents, as illustrated by *Aplysia* sensory cell soma responses to 5-HT (*32*). In these cases, the change of ionic flux, whether Ca^{2+}, K^+, or both, is sustained as long as neurotransmitter is present, but reverses upon removal of the agonist. K^+ currents (and thus indirectly Ca^{2+} currents) of *Hermissenda* sensory cells, the type B photoreceptors, are also reduced by cAMP (*35*).

Interaction of the calmodulin and PKC branches of the phosphoinositol pathway has also been implicated in the generation of sustained neuronal responses. Regulation of the *Hermissenda* type B K^+ currents involves both the Ca^{2+}-calmodulin–dependent kinase and the PKC pathways (*36*). Depolarization of the type B cell activates a sustained voltage-dependent Ca^{2+} influx, which is accompanied and followed by sustained reduction of two K^+ currents, I_A and $I_{Ca^{2+}K^+}$. This reduction is enhanced by injection of either Ca^{2+}-calmodulin type II kinase or PKC, or prior exposure to phorbol ester. Such exposure brings about the translocation of the PKC from the cytosolic to the membrane compartment of the cell. Similarly, application of phorbol ester to hippocampal CA1 neurons produces a reduction of a Ca^{2+}-dependent K^+ current (*37*). Phorbol esters also increase Ca^{2+} currents of *Aplysia* bag cell neurons (*38*). Likewise, K^+ currents are controlled by both Ca^{2+} and DG in mammalian neuroblastoma-glioma hybrid cells (*39*).

Coupling of Events in Different Domains

As illustrated by the actions of angiotensin II on adrenal cells, the initial Ca^{2+} transient is linked to both the initial and late phosphorylation events and thereby partly determines the magnitude of both phases of the responses (*2, 23*). The initial phosphorylation events (about 30 minutes) outlast the Ca^{2+} transient (less than 2 minutes). How this coupling of temporal domains is achieved is not yet clear. It may involve simple hysteresis of the Ca^{2+}-calmodulin–dependent activation of the kinase (*20*), a sustained inhibition of a phosphoprotein phosphatase, or the autophosphorylation of the kinase so that its activity is no longer dependent on Ca^{2+} and calmodulin (as is the case with the type II calmodulin-dependent protein kinase) (*40*).

In addition, the magnitude of the sustained cellular response correlates directly with the magnitude of the initial Ca^{2+} transient. This temporal coupling appears to be due to the fact that PKC translocation to the plasma membrane depends on a synergistic interaction between the rise in plasma membrane DG content (*23*) and the initial Ca^{2+} transient (see above).

The linking of plasma membrane–associated PKC to the sustained phase of cellular response departs from the classical second messenger concept in another way. Information is not conveyed from cell surface to cell interior by the second messenger. Rather, it is conveyed in the form of one or more products of either PKC activity or metabolism. In effect, a new plasma membrane–linked transducing system forms as part of the temporal sequence of cell activation. In some instances, the molecular targets of this plasma membrane–associated PKC are ion channels or enzymes in or on the plasma membrane (*36–38, 41*) but in other cases functional changes in other subcellular domains occur. For example, there are changes in steroidogenesis in the mitochondria, gene activation in the nucleus, glycogenolysis in glycogen particles, cell shape due to changes in intermediate filaments, and protein synthesis in the endoplasmic reticulum. Hence, it is of interest how changes in the activity of plasma membrane–associated PKC cause changes in such intracellular events.

Protein kinase C may convey information to the cell interior by (i) causing global changes in intracellular ionic concentrations via a mechanism such as activation of the plasma membrane Na^+/H^+ exchanger (*42*); (ii) the cycling of membrane components containing activated PKC from plasma membrane to intracellular membranes; (iii) the Ca^{2+}-dependent proteolytic conversion of plasma membrane–associated PKC to a smaller cytosolic kinase, protein kinase M, which no longer requires either Ca^{2+} or lipid for its activity (*43*); (iv) causing an integrated change in the structure of the cytoskeleton or intracellular matrix (*44*); or (v) one or more protein kinase cascades, as exemplified by the appearance of a cytosolic S6 kinase in response to a change in PKC activity (*45*). There is some evidence for several of these mechanisms, and, in particular, for the protein kinase cascades (*46*).

Sustained Cellular Responses That Outlast Stimuli

In either endocrine or neural cells, responses may persist after a brief intense stimulus. In such cases the persistent response is the same response as that that occurred during the stimulus but is of lesser amplitude (*47*). There is, however, another way in which a sustained response can persist after an input stimulus. In this case there is an altered state of cellular responsiveness that is only evident upon presentation of a subsequent stimulus. Information has been stored or "remembered" by the cell and can be recalled by subsequent stimulus presentation. Such "memory" can last for hours or even days and can provide a mechanism for cellular or behavioral (or both) modifiability or "plasticity."

One type of altered cellular responsiveness after exposure to a hormone is exemplified by the up and down regulation of cell surface receptor number—up regulation (the increase in receptor number) leads to increased responsiveness and down regulation to decreased responsiveness. In contrast, prolonged association of the Ca^{2+}-sensitive form of PKC with the plasma membrane is an example of modified responsiveness that occurs independent of and subsequent to receptor activation. Activation of cAMP-regulated pathways, on the other hand, has not yet been shown to alter long-term changes in cellular responsiveness.

Sustained enhancement of neuronal responsiveness. In several neuronal systems, memory (as defined in classical psychological terms) has been correlated with a persistent increase in neuronal responsiveness and, in some cases, a persistent association of PKC with the plasma membrane. These include classical conditioning of the mammalian eye blink response, classical conditioning of *Hermissenda* light response, and long-term potentiation.

Classical conditioning of the rabbit nictitating membrane causes enhanced impulse activity of CA1 hippocampal pyramidal cells in response to conditioned stimulus presentations in vivo (*48*). Increased excitability and evidence of persistent reduction of a Ca^{2+}-dependent K^+ current, measured in vitro, are correlated with conditioning measured in vivo (*49*). In hippocampal slices taken from conditioned (and control) animals at least 24 hours after training, intracellular recordings from the CA1 pyramidal cells reveal persistent reduction of the impulse afterhyperpolarization (AHP), even after synaptic interactions are effectively eliminated.

This AHP reduction, due to reduction of a specific K^+ current ($I_{Ca^{2+}K^+}$), causes the CA1 cell to respond more to depolarizing signals. Concomitant with this AHP reduction is a conditioning-specific enhancement of synaptic potential summation (Fig. 2) in response to electrical stimulation of presynaptic fibers (Schaeffer collaterals) (*50*). This increased CAl responsiveness is accompanied by (*51*) and can be explained by persistent PKC translocation (Fig. 2). Thus 24 hours after conditioning, membrane-associated PKC is increased and cytosolic PKC is decreased in the microdissected CA1 region of the hippocampus (Fig. 2). A similar translocation, induced in vitro by phorbol ester, reproduces the electrophysiologic differences (AHP reduction, enhanced synaptic potential summation). Furthermore, there is persistent (at least 1 hour) translocation of PKC in hippocampal slices exposed to elevation of extracellular K^+ combined with exposure to the glutamate agonist, *N*-methyl-D-aspartate (NMDA) (*51*). Thus, sustained elevation of Ca^{2+} influx together with PKC translocation appear to be responsible for persistent enhancement of neuronal excitability. In this case, the interaction of second messengers, such as Ca^{2+} and DG, could mediate the temporal association of distinct sensory inputs that become related during classical conditioning. Other forms of associative learning may also be mediated by such temporal associations.

Classical conditioning of the mollusk *Hermissenda* also results in a persistent increase of neuronal excitability (*52*). For at least 2 to 3 days after acquisition of the classically conditioned response, the type B photoreceptors show increased excitability, which has been causally implicated in the retention of the learned behavior (*52*). Conditioning-specific reductions of two K^+ currents (I_A and $I_{Ca^{2+}K^+}$) underlie the persistent increase of neuronal excitability (*53*). One current ($I_{Ca^{2+}K^+}$) is the same as or similar to the current that is modified in the CA1 pyramidal neurons in conditioned rabbits. Activation of voltage-dependent Ca^{2+} influx, together with activation of either Ca^{2+}-calmodulin kinase or PKC (*36*), simulate the conditioning-specific reduction of *Hermissenda* K^+ currents and thus cause persistent enhancement of neuronal responsiveness. Microgel analysis reveals that a 20-kD protein, a substrate of PKC and Ca^{2+}-calmodulin–dependent kinase in the *Hermissenda* eye (in which the photoreceptors are located), shows increased phosphorylation for at least 2 to 3 hours after classical conditioning (*54*). Depolarizing conditions that simulate the effects of conditioning as *Hermissenda* neurons similarly regulate the phosphorylation of the 20-kD protein (and possibly others) (*55*). Thus, a long-lasting translocation of PKC into the membrane compartment of the *Hermissenda* type B cell could account for the observed phosphorylation differences as well as the conditioning-specific changes in K^+ currents.

Hippocampal long-term potentiation is the sustained enhancement of postsynaptic responses after high-frequency stimulation of presynaptic fibers (Schaeffer collaterals) (*56*). Long-term potentiation lasting 1 to 2 hours appears to be induced by Ca^{2+} influx through NMDA-sensitive and voltage-sensitive channels (*57, 58*) and to be maintained by PKC translocation (*59*).

In these examples of long-term change in neuronal responsiveness, the change in excitability is the same in that there is no difference in the firing pattern of the cells between control and trained animals when these cells are resting. Only in response to an input that depolarizes the CA1 neurons or the type B cells do the excitability differences appear. An enhanced cellular response (here, neuronal exictability) is "recalled" as a consequence of a molecular mechanism for memory, at least in part involving the persistence of PKC in the plasma membrane compartment.

Sustained enhancement of endocrine responsiveness. A similar process of recall of biological memory occurs in endocrine cells. When pancreatic islets are exposed to glucose (*60*) or adrenal glomerulosa cells are exposed to angiotensin II (*61*) for a period of 20 or more minutes and the agonist is then removed, the response declines rapidly and within 10 to 15 minutes has returned to its basal state. Reintroduction of agonist elicits a response that is significantly greater than the initial one. Furthermore, serial additions lead to progressively greater increases in response rate, that is, there is a cumulative memory (*60, 61*).

For either aldosterone secretion from the adrenal glomerulosa or insulin secretion from the beta cell, only an increase in Ca^{2+} influx rate is necessary to recall the enhanced secretory response (*60, 61*). This is analogous to classical conditioning, which requires presentation of paired stimuli for the induction of a memory, but subsequent presentation of a single stimulus (the conditioned stimulus) is sufficient to recall the memory. During the recall of the angiotensin-induced memory, the proteins that become phosphorylated are related to PKC activation and not to calmodulin-dependent protein kinase activation (*60*). Exposure of adrenal cells to either adrenocorticotropin or high K^+, agents that can also induce sustained aldosterone secretion, but by a mechanism not involving PKC activation, do not elicit a similar type of memory.

These results implicate a change in PKC function as the basis for memory in both neural and endocrine systems (Fig. 3). Upon activation of the cell, the PKC is converted from its Ca^{2+}-insensitive form (cytoplasmic) to its Ca^{2+}-sensitive form (membrane). Once in its Ca^{2+}-sensitive, membrane-associated form the activity of the enzyme is controlled by the rate of Ca^{2+} cycling across the plasma membrane. When agonist is removed, Ca^{2+} cycling, the membrane DG content, and the secretory response all decay rapidly (*7, 23*). However, a portion of the PKC remains in a Ca^{2+}-sensitive, membrane-associated form (*62*) that can be reactivated by any agent that increases Ca^{2+} influx rate (*61*). Cumulative memory to periodic stimulation can be accounted for by assuming that with each reexposure to agonist, there is an incremental increase in the PKC associated with and then anchored to the membrane. We postulate that there are three states of PKC—a soluble form in the nonactivated cell; a membrane-associated form in the activated cell, which rapidly leaves the membrane when Ca^{2+} and DG contents fall; and a membrane-anchored form, which remains in the membrane after DG content and Ca^{2+} flux rate have returned to their basal values. This latter state can account for increased cell responsiveness (Fig. 3). This anchoring of the PKC to the membrane may involve a covalent modification of the enzyme, perhaps autophosphorylation (*63*) or, more likely, acylation of the enzyme with palmitic or myristic acid (*64*).

Sustained responsiveness during cell proliferation. The role of Ca^{2+} in growth factor action has been clarified for BALB/c 3T3 cells (*65*). In these cells three specific mitogens are required: platelet-derived growth factor (PDGF), which renders the cells competent; epidermal growth factor (EGF), which primes these competent cells; and insulin-like growth factor II (IGF-II), which induces proliferation in the competent, primed cell.

Exposure to EGF primes the cells in two ways. It alters them so that IGF-II can induce a sustained increase in Ca^{2+} influx rate across the plasma membrane with only a transient rise in intracellular Ca^{2+} concentration, and it brings about the appearance of a Ca^{2+}-sensitive response element; these are the same two components that are seen when the PKC branch of the Ca^{2+} messenger system is activated. Hence, the primed cell is in an operational state similar to the memory-competent endocrine cell. An agent (IGF-II or BAY K 8644) that induces a sustained increase in Ca^{2+} influx rate will induce a proliferative response. The nature of the Ca^{2+}-sensitive response element is unknown; however, it could be membrane-anchored PKC or another plasma membrane–associated Ca^{2+}-sensitive transducer.

A Spatial-Temporal Model of Cell Activation

Our model of cell activation applies to the sustained phase of a cellular response as well as to sustained modification of cell responsiveness. To cause sustained responses, the flux of Ca^{2+} across the plasma membrane rather than diffusion of Ca^{2+} from the cell surface to the cell interior is critically important. As a consequence of this Ca^{2+} influx the concentration of Ca^{2+} is elevated immediately adjacent to the inner surface of the plasma membrane. Here an elevated Ca^{2+} concentration stimulates PKC that has been transformed into its membrane-associated, Ca^{2+}-sensitive form. In this model (Fig. 3), therefore, a number of new principles are incorporated: (i) Ca^{2+} acts as a messenger within a restricted (submembranous) cellular domain; (ii) Ca^{2+} interacts with another messenger, DG (or cAMP), to bring about sustained cellular responses; (iii) when Ca^{2+} interacts with other second messengers in this restricted domain, it can activate the cell for a much longer time; (iv) the molecular events underlying the initial and sustained phases of the response differ; and (v) the PKC-linked system provides a mechanism ("distal" to the receptor) by which an increase in cell responsiveness is manifest after a previous exposure to first messengers.

Future Directions

It is not known whether the cAMP messenger system also operates in a similar spatial-temporal context. The way cAMP has been measured has confused this issue. Three common experimental methods have contributed to the belief that upon activation of adenylate cyclase there is a rapid and sustained increase in intracellular cAMP. These are (i) the use of supraphysiologic concentrations of first messenger; (ii) the routine use of phosphodiesterase inhibitors to inhibit cAMP hydrolysis; and (iii) the measurement of total rather than intracellular cAMP even though there is a significant efflux of cAMP from the cell (*66*). When the change in the intracellular content of cAMP is measured as a function of time after exposure to a physiological concentration of agonist in the absence of a phosphodiesterase inhibitor, there is in some systems an initial 2- to 5-fold rise in cAMP content, which then falls to only 1.25- to 1.5-fold higher than basal (*67*). This subsequent fall is due to an activation of phosphodiesterase and not an inhibition of cyclase. During the sustained phase of the response there are high rates of cAMP synthesis and hydrolysis—a feature operationally similar to the increase in plasma membrane Ca^{2+} cycling seen in the Ca^{2+} messenger system. Hence, the pathways of information flow during the initial and sustained phases of cAMP-mediated responses may be more complex than presently thought.

There is another class of extracellular messengers, exemplified by insulin and peptide growth factors, that act through surface receptors that generate neither cAMP nor Ca^{2+} but possess intrinsic tyrosine kinase activity (*68*). In several of these tyrosine kinase–linked systems, one consequence of receptor activation is the phosphorylation of a specific ribosomal protein, S6, due to the appearance of a soluble (serine-specific) protein kinase, S6 kinase (*45*). Hence, in these systems, the situation may be analogous to the PKC-linked system; a plasma membrane–associated kinase activates intracellular events via one or more intermediate steps that are likely to involve protein kinase cascades.

Perhaps the most important issue raised by this spatial-temporal model of cell activation is that of additional temporal domains. For example, in the case of angiotensin II action, our model has focused on the first few hours of such a response. However, in vivo when angiotensin II concentration remains high for a longer period, there is hypertrophy of the glomerulosa cell accompanied by both an increase in the number of angiotensin II receptors on the cell surface (*69*) and an increase in the amount of the key enzyme responsible for the conversion of cholesterol to aldosterone (*70*). Additionally, if the stimulus continues for days to weeks, there is a hyperplasia of the cell of the zona glomerulosa (*70*). The spatial and temporal events underlying these later phases of the sustained response are not known.

Similarly, in neural systems, we have addressed only memory lasting for days. Even in a primitive organism such as *Hermissenda*, associative memory can persist for weeks. Furthermore, after a classically conditioned response is no longer retained, it can be relearned with a briefer period of training with paired stimuli (*52*). Such behavior may depend on gene activity or protein synthesis changes (or both) that occur as consequences of the type of second messenger activation described in our model. Support for this supposition comes from observations that PKC activation can regulate protein synthesis in fully differentiated *Hermissenda* neurons

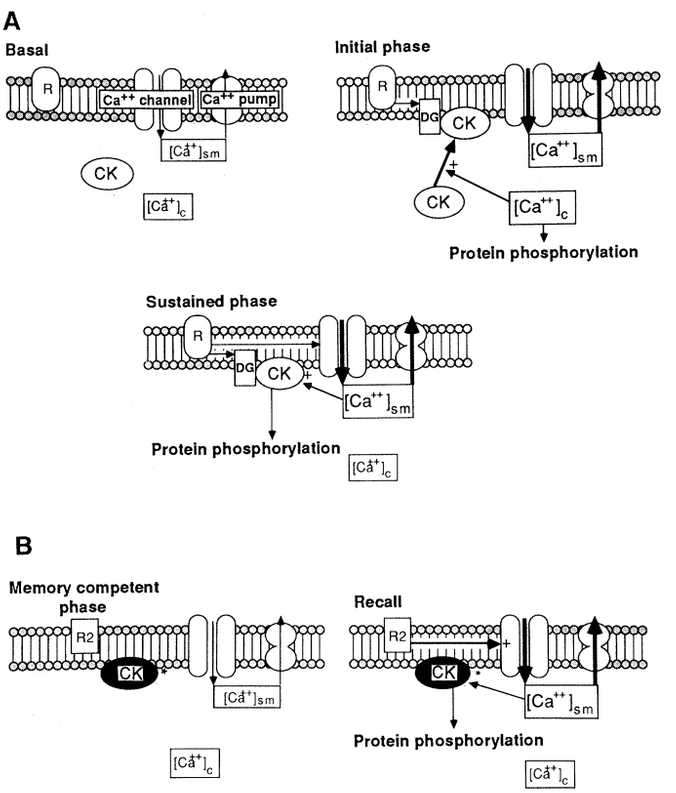

Fig. 3. The cell biology of PKC that is relevant to (A) sustained cellular response and to (B) altered cell responsiveness. The sequence of schematic models depicts the various states of PKC in the cell during and after cell activation. (**A**) In the nonactivated cell (basal), the PKC (CK) is largely in its soluble Ca^{2+}-insensitive form in the cytosol. During the initial phase of cell activation two signals, the rise in the DG content of the plasma membrane and the rise in the Ca^{2+} concentration in the cell cytosol ($[Ca^{2+}]_c$), act synergistically to cause the translocation of the enzyme to the plasma membrane. The resulting association of kinase with the membrane leads to its conversion to a Ca^{2+}-sensitive form. During the sustained phase, the activity of this Ca^{2+}-sensitive form of PKC is controlled by the Ca^{2+} concentration in a subdomain of the plasma membrane ($[Ca^{2+}]_{sm}$). The $[Ca^{2+}]_{sm}$ is determined by the rates of Ca^{2+} influx and efflux (Ca^{2+} cycling) across the plasma membrane. (**B**) During the sustained phase of the response, the plasma membrane–associated PKC undergoes a covalent modification (possibly acylation) so that upon removal of agonist, the PKC remains in a membrane-associated, Ca^{2+}-sensitive form (*) even after Ca^{2+} flux and membrane DG content return to their basal values. This state represents the memory competent phase of cell activation. Recall of this memory can be achieved simply by increasing Ca^{2+} influx rate. Reestablishing an increase in Ca^{2+} cycling across the membrane leads to a reactivation of PKC activity.

(*71*) and that associative training of *Hermissenda* leads to a delayed increase of messenger RNA synthesis days after the training experience (*72*). Exploration of the cellular and molecular events underlying these later temporal domains of cell response represents the next logical stage in the analysis of cell activation.

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Research Article

A Persistent Untranslated Sequence Within Bacteriophage T4 DNA Topoisomerase Gene *60*

WAI MUN HUANG, SHI-ZHOU AO, SHERWOOD CASJENS, RICHARD ORLANDI, REGINA ZEIKUS, ROBERT WEISS, DENNIS WINGE, MEI FANG

A 50-nucleotide untranslated region is shown to be present within the coding sequence of *Escherichia coli* bacteriophage T4 gene *60*, which encodes one of the subunits for its type II DNA topoisomerase. This interruption is part of the transcribed messenger RNA and appears not to be removed before translation. Thus, the usual colinearity between messenger RNA and the encoded protein sequence apparently does not exist in this case. The interruption is bracketed by a direct repeat of five base pairs. A mechanism is proposed in which folding of the untranslated region brings together codons separated by the interruption so that the elongating ribosome may skip the 50 nucleotides during translation. The alternative possibility, that the protein is efficiently translated from a very minor and undectable form of processed messenger RNA, seems unlikely, but has not been completely ruled out.

T4 DNA TOPOISOMERASE IS A PHAGE ENCODED TYPE II ATP-dependent (ATP, adenosine triphosphate) topoisomerase that is capable of changing DNA topology. The enzyme catalyzes transient double-stranded breaks in the DNA backbone through which the DNA strand is passed, resulting in the changing of DNA linking numbers in steps of two (*1*). The T4 enzyme is a complex formed by the products of three genes in the early region of the genome; gene *39* encodes the 60-kilodalton (kD) subunit (p39), gene *52* encodes the 50-kD subunit (p52), and gene *60* encodes the 18-kD subunit (p60) (*2*). These gene products are required for normal T4 DNA replication and may be involved in the initiation event of T4 chromosomal DNA replication (*2*), although the exact roles of these proteins in phage DNA metabolism and development have yet to be clearly defined. In order to obtain large quantities of the T4 protein for structural and functional analyses, we have been cloning the genes for these three T4 subunits in uninfected *Escherichia coli* cells and studying the expression products. The sequences of p39, which is the ATP-utilization subunit, and p52, which has the cutting and joining function, have been reported (*3*).

We now describe the cloning and nucleotide sequence of the T4 gene encoding the p60 DNA topoisomerase subunit. We made the surprising discovery that T4 gene *60* is a split gene with 50 nontranslated nucleotides present within the coding region of the gene. The gene can be efficiently translated even in *Escherichia coli* cells carrying clones of gene *60*, without added phage functions. Interrupted genes whose messenger RNA (mRNA) is processed by a group I self-splicing mechanism have been found in phage T4 and its related phages (*4*). However, unlike other split genes of T4, the expression of gene *60* cannot be easily accounted for by a simple removal of the interrupting sequence via self-splicing of the primary transcript. In fact, there is no indication that the interruption is removed. The sequence of the message in the neighborhood of the interruption suggests that a highly folded structure may be generated and could form a basis for an unprecedented type of posttranscriptional mRNA handling in which the translational machinery bypasses the interruption without removing it.

Cloning and expression of T4 gene *60*. T4 gene *60* is located downstream from gene *39*, another topoisomerase subunit gene, on the circular T4 genetic map (*5*). We have shown that gene *39* is located on a 3-kb Eco RI fragment of cytosine-containing T4 DNA (*3*). Using the 3-kb Eco RI fragment and the 3′-most Hind III–Eco RI subfragment as hybridization probes, we identified a 3-kb Hind III fragment as the overlapping fragment on which gene *60* is expected to reside. The 3-kb Hind III fragment was inserted into the Hind III site of pT7-5 (*6*) under the transcriptional control of a T7 RNA promoter. The recombinant plasmid, pT60-3, is capable of rescuing five mutants in gene *60* (Fig. 1) (*7*). Subcloning portions of the 3-kb insert showed that two of the gene *60* mutants, amE416 and amE1217, were rescued by the central Eco RI fragment alone, and the remaining three mutants were rescued by the neighboring 2.4-kb Eco RI–Hind III fragment (Fig. 1). This result shows that gene *60* spans the second Eco RI site.

In order to determine whether pT60-3 carries the entire coding sequence of T4 gene *60*, we put it into a host system in which T7 RNA polymerase can be provided from a second plasmid (*6*). In this expression system, T7 RNA polymerase, expressed from a bacteriophage lambda (λ) P_L promoter, and a temperature-sensitive λ

W. M. Huang, S.-Z. Ao, S. Casjens, R. Orlandi, and M. Fang are in the Department of Cellular, Viral and Molecular Biology, University of Utah Medical Center, Salt Lake City, UT 84132. R. Zeikus and R. Weiss are in the Department of Human Genetics and Howard Hughes Medical Institute, University of Utah Medical Center, Salt Lake City, UT 84132. D. Winge is in the Department of Medicine, University of Utah Medical Center, Salt Lake City, Utah 84132. S.-Z. Ao was on leave from Shanghai Institute of Biochemistry, Academia Sinica, Shanghai, China.