Calcium Messenger System: 
An Integrated View

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I. Introduction .......................................................... 938
II. Calcium Receptor Protein ............................................. 940
   A. Reaction sequences of calmodulin in response elements .............. 940
   B. Hysteresis in calcium-regulated enzymes ........................... 942
   C. Types of reactions controlled by calmodulin ......................... 942
   D. Amplitude versus sensitivity modulation ............................ 943
   E. C-kinase .......................................................... 944
III. Cellular Calcium Metabolism .......................................... 945
   A. Introduction ...................................................... 945
   B. Role of plasma membrane ......................................... 946
   C. Role of intracellular membranes .................................. 947
   D. Cytosolic free calcium and its measurement ........................ 948
   E. Mitochondrial calcium exchange: process and function ............... 950
IV. Calcium Metabolism in Activated Cell: A Model ........................ 956
V. Initial Biochemical Events in Calcium Messenger System ............... 960
VI. Gain Control in Calcium Messenger System ............................ 962
   A. Role of hysteresis ................................................. 962
   B. Role of C-kinase ................................................... 962
VII. Calcium Cycling Across Plasma Membrane During Sustained 
    Cellular Response ................................................ 967
VIII. cAMP and Calcium Messenger System .................................. 970
    A. General relationships ............................................ 970
    B. cAMP and branches of calcium messenger system ................... 972
    C. cAMP-mediated gain control in calcium messenger system .......... 974
IX. Conclusions .......................................................... 976

I. INTRODUCTION

What began a quarter century ago as two separate threads in the fabric of 
our knowledge of cellular control mechanisms (193, 212, 233) has merged 
today into a single dominant pattern: the synarchic regulation of cell function 
by the two messengers, calcium ion and cyclic AMP. It has become evident 
that these messengers are involved in coupling stimulus to response in a 
wide variety of differentiated cell types when these cells are called on to 
perform their specific function. In regulating cellular function, Ca\(^{2+}\) and 
cAMP nearly always function together. Their interactions are plastic rather 
than stereotyped in character.

Although the molecular and cellular mechanisms involved in the initia-
tion, propagation, reception, and termination of the cAMP message have
been known for nearly 20 years (233), these aspects of the messenger function of Ca$^{2+}$ have been elucidated largely in the past decade. It has become evident that the Ca$^{2+}$ messenger system is considerably more complex than the cAMP messenger system, that the means for translating changes in [Ca$^{2+}$] into cellular responses are more diverse than those by which cAMP acts, and that the metabolism of Ca$^{2+}$ by the cell is more complex than is the metabolism of cAMP.

The purpose of this review is to develop a model, based on this decade of experimentation, of how the Ca$^{2+}$ messenger system operates. A particular emphasis is placed on how the Ca$^{2+}$ messenger system functions in cells that display a sustained response to the sustained presence of an agonist. Also discussed are the constraints such cells face when employing Ca$^{2+}$ as a messenger. A general model of how this cellular control device operates is based on evidence drawn from a variety of specific cell types and their particular responses to extracellular messengers. This is done in the firm belief that the basic aspects of cellular Ca$^{2+}$ metabolism and the Ca$^{2+}$ messenger system are the same in all types of mammalian cells; yet each specific cell type has its own particular specialization of these components. Hence, as our knowledge of the details of cellular Ca$^{2+}$ metabolism and of the behavior and properties of the Ca$^{2+}$ messenger system in specific cells expands, it will surely be true that the quantitative aspects of these processes will differ from cell type to cell type.

The model presented is one in which the flow of information from cell surface to cell interior in the calcium messenger system proceeds by two operationally distinct branches. The first is a calmodulin (CaM) branch that is activated by a transient rise in the [Ca$^{2+}$] in the cell cytosol ([Ca$^{2+}$]C) and is responsible for the initial phase of cell response. The second is a C-kinase branch that is activated by both the rise in [Ca$^{2+}$C] and an increase in the diacylglycerol (DG) content of the plasma membrane and is responsible for the sustained phase of cell response.

To develop this general model, there are five principal features of cell Ca$^{2+}$ and its function that need to be comprehended: 1) the properties of intracellular Ca$^{2+}$ receptor proteins; 2) the various features of cellular Ca$^{2+}$ metabolism, and how these are interrelated with the properties of the Ca$^{2+}$ receptor proteins; 3) how events are initiated in the Ca$^{2+}$ messenger system; 4) the way in which information flows through two separate branches of the Ca$^{2+}$ messenger system; and 5) the relationship of the cAMP messenger system to the Ca$^{2+}$ messenger system.

Before discussing these features it is worth summarizing the four major attributes of messenger Ca$^{2+}$. First, Ca$^{2+}$ is a nearly universal messenger in animal cells. Second, it is a minatory messenger in that excess cellular Ca$^{2+}$ leads to cellular dysfunction and death. Third, it is a mercurial messenger in that the rise in its concentration in the cell cytosol is transient even in those cells displaying a sustained response. Fourth, it is a synarchic messenger in that it nearly always regulates cell function in concert with another intracellular messenger, cAMP.
II. CALCIUM RECEPTOR PROTEIN

By definition Ca$^{2+}$ receptor proteins are proteins that bind Ca$^{2+}$ with high affinity ($K_d$ $10^{-8}$-$10^{-6}$ M) and thus undergo a conformational change, which leads either to a change in function or a change in ability to interact with other proteins to change their functions. There are two classes of Ca$^{2+}$ receptor proteins: 1) true Ca$^{2+}$ receptor proteins (Table 1), such as CaM, which have no intrinsic enzymatic activity but which, when they bind Ca$^{2+}$, undergo a conformational change that alters their association with other proteins, response elements (REs), and thereby causes a change in activity of these REs (1, 2, 29, 37, 53-55, 57-61, 69, 70, 106, 109-111, 120, 125, 137, 155, 156, 161, 201, 202, 216, 217, 223, 252, 254-256, 265); and 2) Ca$^{2+}$-regulated enzymes, such as the phospholipid-dependent Ca$^{2+}$-activated protein kinase (C-kinase), which bind Ca$^{2+}$ directly and have no known specific Ca$^{2+}$-binding subunit (45, 108, 160, 166, 222, 234, 236, 238).

A. Reaction Sequences of Calmodulin in Response Elements

Calmodulin is the most universally distributed protein of the first type (53, 161, 256). The manner in which it participates in the activation of REs remains a matter of some controversy. It is generally accepted that for CaM to activate an enzyme, at least three of the four Ca$^{2+}$-binding sites on CaM must be occupied (27, 58-60, 106, 120, 125, 137, 196, 216, 217, 255). The unresolved issue is the exact reaction sequence by which this occurs. The two types of sequences proposed are illustrated in Figure 1.

Scheme 1 was developed by Huang et al. (120, 255). Its main features are 1) an ordered sequence of reactions; 2) cooperative binding of the first

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<tr>
<th>TABLE 1. Calcium receptor proteins</th>
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<td>I. True receptor proteins</td>
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<tr>
<td>A. Soluble homologous class (cytosol)</td>
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<tr>
<td>1. Calmodulin—all cells</td>
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<tr>
<td>2. Troponin C—skeletal and cardiac muscle</td>
</tr>
<tr>
<td>3. Parvalbumin—skeletal muscle</td>
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<tr>
<td>4. Myosin light chain</td>
</tr>
<tr>
<td>B. Membrane bound</td>
</tr>
<tr>
<td>1. Calmodulin</td>
</tr>
<tr>
<td>II. Calcium-activated enzymes without specific calcium receptor subunit</td>
</tr>
<tr>
<td>A. Bound to mitochondrial membrane</td>
</tr>
<tr>
<td>1. Glyceraldehyde phosphate dehydrogenase</td>
</tr>
<tr>
<td>2. Mitochondrial substrate transport protein</td>
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<tr>
<td>B. Mitochondrial matrix</td>
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<tr>
<td>1. Pyruvate dehydrogenase</td>
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<tr>
<td>2. $\alpha$-Ketoglutarate dehydrogenase</td>
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<td>C. Cytosol</td>
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<td>1. Calcium-activated, phospholipid-dependent protein kinase</td>
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FIG. 1. Two different reaction schemes for association of Ca$^{2+}$, calmodulin (CaM), and a response element (RE). Number over each arrow is the $K_d$ of that particular association reaction. Sequence of heavy lines depicts presumed major sequence of association reactions in each scheme. Scheme 1 is from work of Huang et al. (120) on activation of cyclic nucleotide phosphodiesterase. Scheme 2 is from work by Cox et al. (58) on activation of red cell Ca$^{2+}$ pump and on activation of phosphorylase kinase by added CaM. Scheme 1: first step is association of first Ca$^{2+}$ with CaM. This leads to conformational change in CaM such that second Ca$^{2+}$ binds to protein with a slightly higher affinity (lower $K_d$). After binding of second Ca$^{2+}$, the CaM species Ca$_2$-CaM undergoes dramatic change in its affinity for RE (70-fold increase in affinity for this element), hence next step is association of Ca$_2$-CaM with RE to form the complex Ca$_2$-CaM-RE. In this complex, RE is still in its low-activity state. However, binding of RE causes dramatic (150-fold) increase in affinity of Ca$_3$ for the third and fourth Ca$^{2+}$-binding sites on CaM. Binding of one or both of these Ca ions to the Ca$_3$-CaM-RE converts RE from its low-activity to its high-activity state. Scheme 2: first 3 steps are bindings of first, second, and third Ca$^{2+}$ to CaM, with each site having slightly lower affinity (higher $K_d$) for Ca$^{2+}$. The Ca$_3$-CaM form of CaM has a millionfold greater affinity for RE than either CaM, Ca$_1$-CaM, or Ca$_2$-CaM. Consequently, Ca$_3$-CaM-RE (activated form of the enzyme) forms from association of Ca$_3$-CaM with RE. Little Ca$_4$-CaM-RE forms, because of the low affinity of the fourth site for Ca$^{2+}$.

two Ca$^{2+}$ to CaM; 3) a marked change in the affinity of CaM for the RE when CaM exists in the form of Ca$_2$-CaM; 4) as a consequence of the second feature, the formation of the complex Ca$_2$-CaM-RE, in which the RE is still in a low-activity state; and 5) a marked change in the affinity of the last two Ca$^{2+}$-binding sites on CaM as a consequence of the association of Ca$_2$-CaM with RE. This change in Ca$^{2+}$-binding affinity leads to the formation of Ca$_3$-CaM-RE and Ca$_4$-CaM-RE, the high-activity states of the RE.
Scheme 2 was developed by Cox and co-workers (58). Its main features are 1) no cooperative binding of Ca$^{2+}$ to CaM; 2) a very marked change in the affinity of CaM for the RE only when CaM exists in the form of Ca$^{3+}$-CaM; 3) as a consequence of the second feature, the formation of Ca$^{3+}$-CaM-RE, in which RE is in its high-activity state; and 4) the fact that formation of Ca$^{3+}$-CaM-RE does not enhance the Ca$^{2+}$ affinity of the fourth site on CaM.

Note that Huang et al. (120, 255) developed their reaction scheme from the study of the associations of Ca$^{2+}$ and CaM with the enzyme cyclic nucleotide phosphodiesterase, whereas Cox et al. (58) developed their scheme from an analysis of the association of Ca$^{2+}$ and CaM with either [Ca$^{2+}$]-H$^+$-ATPase in the plasma membrane of the red cell or the enzyme phosphorylase b kinase (an enzyme already possessing CaM as a subunit). Hence these two schemes may actually represent the range of possible reaction sequences by which these association reactions can follow. In spite of the interpretation of Cox et al. (58), there is considerable data showing the cooperative binding of Ca$^{2+}$ to either CaM (60, 106) or troponin C (87, 102). In particular, Forsen et al. (87) have recently provided new data, using $^{45}$Ca NMR spectroscopy, that demonstrate the cooperative binding of Ca$^{2+}$ to the two high-affinity sites of CaM. These data argue against the model of Cox et al. (58). Also, it is difficult to account for positive sensitivity modulation (see sect. IID) of CaM-dependent enzymes by the scheme of Cox et al. (58), because under certain conditions some CaM-dependent enzymes are activated by [Ca$^{2+}$] in the range of 0.05 μM or less, values at which essentially no Ca$^{3+}$-CaM is formed. On the other hand, particularly in the case of the membrane-bound Ca$^{2+}$ pump, the scheme of Cox et al. may well apply and provide the basis for the hysteretic behavior of this enzyme (216, 217; sect. VII).

B. Hysteresis in Calcium-Regulated Enzymes

A feature of possible physiological importance is that these reaction sequences appear to display hysteresis (196, 201, 216, 217), i.e., once activated, the system will stay activated at a lower [Ca$^{2+}$] than that needed to activate it initially. Such hysteretic behavior has been found for both a CaM-regulated enzyme (the plasma membrane Ca$^{2+}$ pump; 216, 217) and a troponin C regulated process (skeletal muscle contraction; 201). Troponin C is a structural homologue of CaM with a restricted function—that of serving as a Ca$^{2+}$ receptor protein in the regulation of the contractile response of skeletal and cardiac muscle (48, 69, 102, 257). In skeletal muscle the strength of contraction is a function of pCa (negative log of [Ca$^{2+}$]); yet it is greater, if contraction is initiated at a high free Ca$^{2+}$ and [Ca$^{2+}$] is lowered to an intermediate value, than if contraction is initiated at a low free Ca$^{2+}$ and [Ca$^{2+}$] is raised to the same intermediate value (211) (i.e., the system displays hysteresis).

C. Types of Reactions Controlled by Calmodulin

When the pathway of information flow through the CaM branch of the Ca$^{2+}$ messenger system is considered, the rise in Ca$^{n+}$-CaM can lead either to
the direct activation of an RE via association or to the indirect activation of an RE via a CaM-dependent phosphorylation (1, 2, 15, 27, 37, 93, 101, 110). This contrasts with the way information flows in the cAMP messenger system, in which cAMP receptor proteins only influence intracellular events by controlling protein phosphorylation (55). Two important examples in which CaM regulates the function of an enzyme by a direct association are the cyclic nucleotide phosphodiesterase (54, 106, 120, 255) and the plasma membrane Ca\textsuperscript{2+} pump (29, 59, 145, 216, 217, 249-251).

There is another class of REs that is not activated by Ca\textsuperscript{2+} receptors such as CaM but is activated by the direct binding of Ca\textsuperscript{2+} to the RE (108, 160, 191, 234). An example is the mitochondrial glyceraldehyde phosphate dehydrogenase, which is activated by changes in the Ca\textsuperscript{2+} of the cell cytosol without the participation of CaM (108). The enzymes of this class are arbitrarily classified as components of the CaM branch of the Ca\textsuperscript{2+} messenger system.

**D. Amplitude Versus Sensitivity Modulation**

An increase in the amplitude of the Ca\textsuperscript{2+} message occurs when a hormone or other extracellular messenger interacts with its plasma membrane receptor and initiates the flow of information through the CaM branch of the Ca\textsuperscript{2+} messenger system. This increase in the [Ca\textsuperscript{2+}]\textsubscript{i} leads to the association of Ca\textsuperscript{2+} with binding sites on either CaM or directly on Ca\textsuperscript{2+}-activated enzymes. The binding of Ca\textsuperscript{2+} to these sites leads to further events that result in the activation of a variety of REs. The consequence of these activations is thought to be the observed physiological responses of the cell. The hallmark of amplitude modulation is that the response is directly related to an increase in the amplitude of the Ca\textsuperscript{2+} message. There is a correlation between the increase in [Ca\textsuperscript{2+}]\textsubscript{i} and the magnitude of the initial cellular response.

In contrast, a change in the function of a Ca\textsuperscript{2+}-regulated enzyme can be induced by a change in the sensitivity of a Ca\textsuperscript{2+}-dependent reaction to activation by Ca\textsuperscript{2+} (190, 196). Sensitivity modulation can be achieved in at least two ways: by an increase or decrease in [CaM] or by a change in the structure (or concentration) of the RE such that its association with CaM-Ca\textsubscript{n} is increased. The classic example of the latter is phosphorylase \(b\) kinase (37, 55). This enzyme is in turn a CaM-dependent, Ca\textsuperscript{2+}-activated protein kinase and a substrate for the cAMP-dependent protein kinase (55). It can exist in two stable forms: nonphosphorylated and phosphorylated. In its nonphosphorylated form it displays half-maximal activity (\(K_a\)) at a [Ca\textsuperscript{2+}] of \(\sim 3.0\) \(\mu\)M, and in its phosphorylated form its \(K_a\) is 0.5 \(\mu\)M. This is an example of positive sensitivity modulation, in which a change in the structure of an RE (phosphorylation) has the effect of increasing the sensitivity of the system to activation by Ca\textsuperscript{2+}. Examples of negative sensitivity modulation are also known. Myosin light-chain kinase is one (1, 27, 57). It too is a CaM-dependent protein kinase that is a substrate for the cAMP-dependent protein kinase.
In its nonphosphorylated form it displays a $K_a$ of 0.8 $\mu$M, and in its phosphorylated form its $K_a$ is 8 $\mu$M. (For a fuller discussion of amplitude and sensitivity modulation see refs. 191, 196.)

E. C-Kinase

Covalent changes in RE structure such as phosphorylation are not the only means by which sensitivity modulation is achieved. The phospholipid-dependent C-kinase is one example in which another type of sensitivity modulation is observed (127, 128, 136, 166, 179, 234–236, 238). This protein kinase is not activated either by a cAMP receptor protein or by an interaction with CaM. It is directly activated by $Ca^{2+}$. When existing free in the cytosol, its sensitivity to activation by $Ca^{2+}$ is low (Fig. 2). However, in the presence of a DG containing at least one polyunsaturated fatty acid either in position 1 or position 2 and in the presence of a mixture of phospholipids containing at least phosphatidylserine, C-kinase is extremely sensitive to activation by $Ca^{2+}$ (Fig. 3). This complex lipid mixture alters enzyme conformation in such a way that the sensitivity to activation by $Ca^{2+}$ is increased as much as 1,000-fold, and the $V_{\text{max}}$ of the enzymatic reaction is increased 4- to 10-fold. Note that the tumor-promoting phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) can substitute for DG as a positive sensitivity modulator of this enzyme (45, 146, 177).

It seems that in most cells, except brain, kidney, and liver, the C-kinase is almost exclusively free in the cytosol when the cell is in its nonactivated state, when enzyme is not associated with diacylglycerol (DG), its $V_{\text{max}}$ is low, and it is quite insensitive to activation by $Ca^{2+}$ (—). In activated state, when associated with DG containing at least 1 polyunsaturated fatty acid and a mixture of phospholipids including phosphatidylserine, its $V_{\text{max}}$ is high, and it is extremely sensitive to activation by $Ca^{2+}$ (—). If these studies on isolated enzyme are extrapolated to situation in cell, presumably in nonactivated cell most C-kinase exists free in cytosol in its low-activity, low-sensitivity state (—), and in activated cell it is bound to plasma membrane, where it exists in its high-activity, high-sensitivity state (—). [Modified from Takai et al. (235).]
FIG. 3. Schematic representation of pools of Ca$^{2+}$ in an idealized mammalian cell. CaP, plasma membrane pool; CaZ, endoplasmic reticulum pool; CaX, mitochondrial nonionic pool; Ca-Y, uncharacterized nonionic pool in cytosol; Ca$^+$, free Ca$^{2+}$ in cytosol (center) or mitochondria (right). Shaded areas: left represents plasma membrane; right represents inner mitochondrial membrane. Arrows denote pathways of Ca$^{2+}$ flow. $\Theta$, Active transport step. Sizes of 2 major intracellular pools are based on data obtained in hepatocytes [Joseph and Williamson (126)]. Sizes of CaZ and CaX are calculated to be ~50 μmol/kg cell H2O. Note, however, that the respective size of these 2 pools varies from one cell type to another, depending on extent of smooth endoplasmic reticulum and density of mitochondria in particular cell type.

state (135, 166, 238). One consequence of a hormone-receptor interaction in the Ca$^{2+}$ messenger system is an increase in the DG content of the plasma membrane (10, 136, 236). As a result the C-kinase binds (or is activated if already bound) to the inner surface of the plasma membrane, associates with the proper mix of phospholipids, and becomes a fully activated Ca$^{2+}$-sensitive protein kinase (142, 143, 146, 179). It is quite possible that the simultaneous initial rise in [Ca$^{2+}$] also serves, with DG, to increase the rate of association of this enzyme with the plasma membrane (140). It has not yet been possible to characterize these complex association reactions between Ca$^{2+}$, C-kinase, DG, and membrane phospholipids in detail, but both a rise in [DG] and in [Ca$^{2+}$] will probably be found to affect the rate of association of this enzyme with the plasma membrane and hence will affect its activation.

III. CELLULAR CALCIUM METABOLISM

A. Introduction

Having considered the characteristics of Ca$^{2+}$-regulated enzymes in isolation, it is necessary to consider the intracellular milieu in which they operate (3, 6–9, 30–32, 34, 35, 38, 43, 44, 46–48, 64, 69, 83, 85, 126, 131, 151, 159, 160, 170, 175, 183, 185, 186, 189–192, 194–196, 198, 199, 204, 208, 221, 230, 231, 243, 260), particularly the features of cellular Ca$^{2+}$ metabolism that place constraints on the function of these proteins in situ. The important facts about cells and Ca$^{2+}$ (Fig. 3) are that 1) Ca$^{2+}$ is a cellular toxin (32, 44, 77,
2) the $[\text{Ca}^{2+}]_i$ is extremely low, $\sim 0.1 \mu\text{M}$ (7-9, 17, 26, 31, 38, 50, 64, 98, 167, 182, 183, 201, 203, 204, 227, 239, 241-244), relative to the extracellular $[\text{Ca}^{2+}]$, which is quite high, $\sim 1000 \mu\text{M}$ (a 10,000-fold $[\text{Ca}^{2+}]$ gradient exists across the plasma membrane); 3) there is in most cells a pool of activator $\text{Ca}^{2+}$ located in the plasma membrane and/or the endoplasmic reticulum (28, 30, 31, 35, 37, 41, 46, 47, 62-64, 80, 89, 94, 98, 124, 126, 141, 162, 167, 168, 184, 197, 217, 221, 227, 229-231, 237, 259); 4) there is a relatively large pool of nonionic but exchangeable Ca within the mitochondrial matrix space in exchange with a small pool of free $\text{Ca}^{2+}$ within this space; and 5) even under basal conditions, the free $[\text{Ca}^{2+}]$ in cytosol and in the mitochondrial matrix space are similar, and these two pools are in rapid exchange with one another.

**B. Role of Plasma Membrane**

The remarkable fact is that it costs the cell $< 1\%$ of its basal expenditure of energy to maintain this gradient. This efficiency is achieved by the fact that the plasma membrane is relatively impermeable to $\text{Ca}^{2+}$; $\text{Ca}^{2+}$ enters the nonactivated cell at a rate of $4 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} \text{cell H}_2\text{O}$ (31) and is pumped back out of the cell by an ATP-dependent $\text{Ca}^{2+}$ pump that is actually a $\text{Ca}^{2+}$-$\text{H}^+$-ATPase (100, 178, 226). Consequently, to maintain $\text{Ca}^{2+}$ homeostasis, the hydrolysis of 4 $\mu\text{mol} \cdot \text{ATP} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} \text{cell H}_2\text{O}$ is required (based on a $\text{Ca}^{2+}$:ATP ratio of 1 for the plasma membrane $\text{Ca}^{2+}$ pump). The typical skeletal muscle cell has a basal rate of ATP turnover of 10,000 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} \text{cell H}_2\text{O}$, and some $10-20\%$ of this is utilized by the $\text{Na}^+$-$\text{K}^+$-ATPase to maintain cellular $\text{Na}^+$ and $\text{K}^+$ homeostasis, compared with $<1\%$ to maintain $\text{Ca}^{2+}$ homeostasis (22). However, this estimated energy requirement for maintaining cellular $\text{Ca}^{2+}$ homeostasis fails to take into account the continued cycling of $\text{Ca}^{2+}$ across both the inner mitochondrial membrane and the membrane of the endoplasmic reticulum. There has been no direct measurement of these fluxes, but from the kinetics of $\text{Ca}^{2+}$ exchange in intracellular pools, it can be estimated that the total cost to the cell of maintaining $\text{Ca}^{2+}$ homeostasis is no more than 20 $\mu\text{mol} \cdot \text{ATP} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} \text{cell H}_2\text{O}$ or 0.2% of the basal energy metabolism of the cell.

Not only does the plasma membrane play the predominant role in maintaining cellular $\text{Ca}^{2+}$ homeostasis, but it has a vital function in stimulus-response coupling when the cell employs the $\text{Ca}^{2+}$ messenger system. It subserves this function in two ways. First, $\text{Ca}^{2+}$ is bound both to the inner surface of the membrane itself and considerably more is bound to the glycocalyx (a poorly defined structure on the external surface of the membrane) (34, 35, 47, 83, 150, 237). Second, on activation of a cell by a particular extracellular messenger, specific $\text{Ca}^{2+}$ channels may open either in a transient or sustained fashion to allow $\text{Ca}^{2+}$ to enter the cell down its steep concentration gradient (8, 9, 28, 31, 35, 46, 48, 66, 83, 86, 88, 94, 115, 116, 130, 141, 147, 151, 154, 159,
There may be as many as three types of such channels (200). The two major ones are the potential-operated (or voltage-dependent) channels (POC) and the receptor-operated channels (ROC). In addition, some Ca\(^{2+}\) enters via the Na\(^{+}\) channel. From our point of view in this discussion, the single most important point about either the POC or ROC is that when these are opened by agents that induce a sustained cellular response, they remain open as long as the agent is present and response continues (31, 141, 221).

The least understood aspect of plasma membrane function and cell Ca\(^{2+}\) is the function of membrane-bound Ca\(^{2+}\) (31). For example, in heart cells, Fleckenstein (83) concludes that the Ca\(^{2+}\) bound to the glycocalyx is an important source of the Ca\(^{2+}\) that enters the cell during each systole. Similarly, Loutzenhisier and van Breeman (149) found that when the prostaglandin H\(_2\) analogue U-44069 activates \(^{45}\)Ca influx into arterial smooth muscle, this influx exhibits two phases: an initial rapid but transient phase and a slower sustained phase. They conclude that the initial phase represents the entry of extracellularly bound Ca\(^{2+}\) into the cell. A similar conclusion was reached by Tan and Tashjian (237) in their studies of the thyrotropin-releasing hormone (TRH)-induced activation of pituitary cells in culture. In contrast, Schulz (221) has shown that when pancreatic exocrine cells are induced to secrete enzymes by natural agonists, there is a rapid net efflux of Ca\(^{2+}\) from the cell, which she attributes to the loss of Ca\(^{2+}\) from an internal (possibly plasma membrane) pool. In studies in adrenal glomerulosa cells and \(\beta\)-cells of pancreatic islets, activation of secretion in these cells by angiotensin II (ANG II) or glucose, respectively, causes the release of Ca\(^{2+}\) from a dantrolene-sensitive intracellular pool (89, 112, 124, 141, 151, 259). This presumably means that the Ca\(^{2+}\) comes from the endoplasmic reticulum, because in skeletal muscle the major action of dantrolene is to inhibit Ca\(^{2+}\) release from the sarcoplasmic reticulum (62, 188, 247).

C. Role of Intracellular Membranes

In addition to events occurring at the plasma membrane, events at two other membranes determine the [Ca\(^{2+}\)]\(_{i}\): the membrane of the endoplasmic reticulum and the inner mitochondrial membrane (see Fig. 3). Both have pump-leak systems oriented so that there is an active extrusion of Ca\(^{2+}\) from and a passive leak of Ca\(^{2+}\) back into the cytosol. Each of these two membranes has a unique role. The mitochondrial membrane pump-leak system serves two functions: at low [Ca\(^{2+}\)]\(_{i}\) it is responsible for stabilizing the [Ca\(^{2+}\)]\(_{i}\), and at high [Ca\(^{2+}\)]\(_{i}\), the mitochondria serve as a reservoir for the storage of Ca\(^{2+}\) during periods of excessive cellular Ca\(^{2+}\) accumulation. The endoplasmic reticulum serves as a source of Ca\(^{2+}\) for the initial phase of cell activation in many cells (11, 28, 31, 46, 63, 69, 85, 98, 124, 141, 197, 207, 208, 290, 247, 260).

If this simple picture is an accurate one, it follows that the capacity of
different cell types to cope with Ca\(^{2+}\) overload is a function of their complement of mitochondria and that the capacity of different cell types to use internal Ca\(^{2+}\) as an initiator of cell response depends on the size of the Ca\(^{2+}\) pool contained in their smooth endoplasmic reticulum. Given that the extent of each of these intracellular membranes differs among cell types, it is evident that the respective sizes of these intracellular Ca\(^{2+}\) pools vary from cell type to cell type. A most interesting example of the variation in the size of the endoplasmic reticulum pool is seen in different types of rabbit vascular smooth muscle cells (47). Stimulation of these cells in medium-sized vessels by nor-epinephrine (NE) leads to both a release of Ca\(^{2+}\) from an intracellular pool and an uptake of Ca\(^{2+}\) from the extracellular pool. In contrast, in the smooth muscle cells of small resistance vessels there is practically no release of Ca\(^{2+}\) from an intracellular pool. Nearly all the Ca\(^{2+}\) for stimulus-response coupling comes from the extracellular pool.

As a way of discussing the functions of these pools, it is useful to present a model of an idealized mammalian cell in which the size of the two pools is approximately equal, as found in liver cells, where the total cellular Ca\(^{2+}\) is 100–150 \(\mu\text{mol/kg cell H}_2\text{O}\) (160; see Fig. 3). The pool of activator Ca\(^{2+}\) is contained in the endoplasmic reticulum and/or plasma membrane. It is released on activation of the cell by an appropriate extracellular messenger. Usually there is a four- to sixfold increase in the rate of Ca\(^{2+}\) influx across the plasma membrane via either a Ca\(^{2+}\) ROC and/or POC in the plasma membrane at approximately the same time that the release of intracellular Ca\(^{2+}\) occurs. However, the release process results in a single bolus of Ca\(^{2+}\) entering the cytosolic space, but the increase in Ca\(^{2+}\) influx is sustained as long as hormone or agonist is present. These combined changes lead to a rise in the \([\text{Ca}^{2+}]_c\) from values of 0.1–0.2 \(\mu\text{M}\) to 0.6–2 \(\mu\text{M}\).

Note that a large bolus of Ca\(^{2+}\), in the range of 46–66 \(\mu\text{mol}\), is needed for a small rise in \([\text{Ca}^{2+}]_c\) (Table 2). There are two reasons for this. First, as soon as the \([\text{Ca}^{2+}]_c\) rises, the plasma membrane Ca\(^{2+}\) pump is activated by Ca\(^{2+}\)-CaM (29, 59, 145, 216, 217, 249–251), and so Ca\(^{2+}\) efflux from the cell increases. Second, a considerable amount of the released Ca\(^{2+}\) is taken up into a nonionic intramitochondrial Ca pool (see CaX in Fig. 3). Not only is the plasma Ca\(^{2+}\) pump activated by a rise in \([\text{Ca}^{2+}]_c\), but it remains activated when the \([\text{Ca}^{2+}]_c\) falls (34, 216, 217). It seems possible that in the activated cell, when the rates of both plasma membrane Ca\(^{2+}\) influx and efflux via the pump are high, the free \([\text{Ca}^{2+}]_c\) immediately beneath the cytoplasmic face of the plasma membrane is increased even though the \([\text{Ca}^{2+}]_c\) in the bulk cytosol is near its original basal value.

D. Cytosolic Free Calcium and Its Measurement

The single most important and dynamic pool of Ca\(^{2+}\) in the cell is that in the cytosol. Free \([\text{Ca}^{2+}]_c\) is in relatively rapid exchange with Ca\(^{2+}\) in the
TABLE 2. Calcium required to change cytosolic free calcium on activation of hypothetical cell

<table>
<thead>
<tr>
<th>Condition</th>
<th>( \text{Ca}^{2+}, \mu\text{mol/kg Cell H}_2\text{O} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exchangeable cell ( \text{Ca}^{2+} )</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>110</td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td>45</td>
</tr>
<tr>
<td>Mitochondrial matrix</td>
<td>45</td>
</tr>
<tr>
<td>Change in free ( \text{Ca}^{2+} ) needed to raise ( [\text{Ca}^{2+}]_c )</td>
<td></td>
</tr>
<tr>
<td>Increase in ( [\text{Ca}^{2+}]_c )</td>
<td>0.9</td>
</tr>
<tr>
<td>Assume ( [\text{CaM}] )</td>
<td>5</td>
</tr>
<tr>
<td>Assume when ( [\text{Ca}^{2+}]_c = 1.0 \mu\text{M} ), ( \sim 5% ) of ( \text{CaM} ) is ( \text{Ca}_2^-\text{CaM} ) and remainder is ( \text{Ca}_2^-\text{CaM} ) and ( \text{Ca}_2^-\text{CaM} ); ( \text{Ca}^{2+} ) required to saturate all these sites</td>
<td>5</td>
</tr>
<tr>
<td>( \text{Ca}^{2+} ) required to fill mitochondrial matrix pool when ( [\text{Ca}^{2+}]_c = 1.0 \mu\text{M} )</td>
<td>100-150</td>
</tr>
<tr>
<td>This pool will only fill partially, however, so assume ( \text{Ca}^{2+} ) required</td>
<td>40-60</td>
</tr>
<tr>
<td>Total required for activation</td>
<td>46-66</td>
</tr>
<tr>
<td>Source of ( \text{Ca}^{2+} ) for activation</td>
<td></td>
</tr>
<tr>
<td>Increased plasma membrane influx in 1 min</td>
<td>12-15</td>
</tr>
<tr>
<td>Release of ( \text{Ca}^{2+} ) from endoplasmic reticulum</td>
<td>5-45</td>
</tr>
</tbody>
</table>

Estimates of amount of \( \text{Ca}^{2+} \) necessary to raise cytosolic free \( \text{Ca}^{2+} \) concentrations (\( [\text{Ca}^{2+}]_c \)) from 0.1 to 1.0 \( \mu\text{M} \) in cell that in nonactivated state has pool of 50 \( \mu\text{mol/kg Cell H}_2\text{O} \) in endoplasmic reticulum and 50 \( \mu\text{mol} \) in mitochondrial matrix.

extracellular pool and the mitochondrial and plasma membrane–endoplasmic reticulum intracellular pools. It is the change in \( [\text{Ca}^{2+}]_c \) that is thought to act as the primary messenger in the \( \text{Ca}^{2+} \) messenger system. Hence, accurate measurement of the \( [\text{Ca}^{2+}]_c \) is of primary importance in defining the properties of the \( \text{Ca}^{2+} \) messenger system. Until recently such measurements were confined to large cells, because the only methods for getting either photoproteins, such as aequorin (25), or organic \( \text{Ca}^{2+} \) indicators, such as arsenazo III (214), into the cytosolic space was by microinjection techniques (6, 7). These techniques led the way to an analysis of the changes in \( [\text{Ca}^{2+}]_c \) resulting from cell activation. The first major use of aequorin for this purpose was made by Ashley and Ridgway (6, 7) in barnacle muscle cells. As had been predicted, they showed that when these cells were stimulated to contract, there was a transient rise in the \( [\text{Ca}^{2+}]_c \) from a value in the 0.1 \( \mu\text{M} \) range to a value in the 1–2 \( \mu\text{M} \) range, which preceded the contractile response.

Recently two more general methods have been developed for measuring \( [\text{Ca}^{2+}]_c \) in small cells. The first is that developed by Tsien and associates (241–243) employing a fluorescent analogue of ethylene glycol-bis(\( \beta \)-aminoethyl-ether)-\( N,N' \)-tetraacetic acid (EGTA) named quin 2. By preparing this compound as a complex ester, it is possible to make it sufficiently lipophilic so that it crosses the plasma membrane of the intact cell. Once within the cytosolic space the ester form is acted on by nonspecific esterases, and the acid \( \text{Ca}^{2+} \)-binding form of the molecule is generated. It then acts as a flu-
orescent indicator of intracellular free Ca\textsuperscript{2+}. The major disadvantage in the use of the quin 2 compound is that high concentrations (~0.5 mM) are required to obtain a measurable Ca\textsuperscript{2+} signal. At these concentrations the compound acts as a Ca\textsuperscript{2+} buffer. Hence any transient increase in [Ca\textsuperscript{2+}], measured after cell activation is likely to be a minimal value, underestimating to some extent the peak of the transient, whereas any steady-state value measured during the sustained phase of cell activation is likely to be a maximal value, overestimating the true value.

The second method, developed first by Borle and Snowdowne (33) and Morgan and Morgan (167), is that of making the plasma membrane of the cell reversibly permeable to aequorin by incubation in a hypotonic medium containing a Ca\textsuperscript{2+} chelator (Mg\textsuperscript{2+} and ATP) with a subsequent incubation in an isotonic medium containing Ca\textsuperscript{2+}. Sufficient aequorin can be taken up by the cells so that a measurable Ca\textsuperscript{2+} signal can be produced on cell activation. The advantage of this method over the quin 2 method is that the molar amount of cytosolic aequorin is low, contributing little Ca\textsuperscript{2+}-buffering capacity. The disadvantage is that one alters the permeability of the plasma membrane sufficiently that certain cytosolic constituents will leak out during the process of aequorin uptake. It is not yet clear whether the same “permeabilization technique” will work equally well in diverse cell types nor whether the functional properties of the cells so treated are identical to those of untreated cells.

The data derived from the use of these methods are discussed in section IV.

E. Mitochondrial Calcium Exchange: Process and Function

The experimental approaches to the study of the function of mitochondria in cellular Ca\textsuperscript{2+} homeostasis have progressed from an analysis of how isolated mitochondria “buffer” [Ca\textsuperscript{2+}] to how they behave when they are coincubated with microsomes and to how they function in situ in intact cells whose plasma membranes have been permeabilized. From these studies, two opposing views have developed about the function of the mitochondrial Ca\textsuperscript{2+} pool. One view is that this pool serves the combined function of stabilizing the [Ca\textsuperscript{2+}], and of serving as a sink for Ca\textsuperscript{2+} in times of Ca\textsuperscript{2+} excess. The other is that this pool serves as a source of activator Ca\textsuperscript{2+}. To understand these opposing points of view it is necessary to understand the processes of mitochondrial Ca\textsuperscript{2+} transport as well as the factors determining the intramitochondrial distribution of Ca\textsuperscript{2+} between its free and complexed forms.

1. Calcium stabilizing and storage function of mitochondria

The first experiments were done by Nicholls (175) with isolated mitochondria. They were designed to answer a simple question: can mitochondria
buffer the extramitochondrial or cytosolic \([\text{Ca}^{2+}]\)? He showed that if isolated mitochondria were incubated in media containing micromolar amounts of \(\text{Ca}^{2+}\), the extramitochondrial \([\text{Ca}^{2+}]\) would become stable at a value in the range 0.8 µM. The addition of small aliquots of extra \(\text{Ca}^{2+}\) would lead to a transient rise in the free \([\text{Ca}^{2+}]\). The mitochondria would rapidly accumulate this \(\text{Ca}^{2+}\), and the medium \([\text{Ca}^{2+}]\) would return to its original value. Conversely, if aliquots of EGTA were added to lower the free \([\text{Ca}^{2+}]\), sufficient \(\text{Ca}^{2+}\) would be released by the mitochondria to restore the free \([\text{Ca}^{2+}]\) to its original value. Brinley et al. (36) took this type of analysis one step further, employing mitochondria in the axoplasm of squid axons (a normal cytosolic milieu), and showed a similar capacity of these organelles to buffer or stabilize the extramitochondrial \([\text{Ca}^{2+}]\). Factors such as pH, \([\text{Mg}^{2+}]\), and the total \(\text{Ca}^{2+}\) content of the isolated mitochondria were shown to influence the set point around which the free extramitochondrial \([\text{Ca}^{2+}]\) was fixed.

In a further elaboration of this approach, Becker et al. (12) analyzed the ability of both isolated endoplasmic reticulum and isolated mitochondria from liver to fix the \([\text{Ca}^{2+}]\) in the medium and then determined what occurred when both organelles were present simultaneously. They found both isolated organelles could fix the free \([\text{Ca}^{2+}]\), but the endoplasmic reticular \(\text{Ca}^{2+}\) transport system fixed it at a lower value (0.2 vs. 0.5 µM) than did the mitochondria. When the two organelles were incubated together with small \(\text{Ca}^{2+}\) loads, the free \([\text{Ca}^{2+}]\) was fixed at the level determined predominantly by the activity of the endoplasmic reticulum. However, they also showed that the capacity of the endoplasmic reticulum to accumulate \(\text{Ca}^{2+}\) was quite limited compared with the much greater capacity of the mitochondria, so that with successive \(\text{Ca}^{2+}\) loads the mitochondria became the major \(\text{Ca}^{2+}\) pool.

Murphy and co-workers (170) then studied the ability of permeabilized liver cells to fix the free \([\text{Ca}^{2+}]\) using the so-called null-point method, in which a \(\text{Ca}^{2+}\)-sensitive dye is placed in media containing successively higher concentrations of free \(\text{Ca}^{2+}\). Aliquots of the permeabilized cells are then placed in these different media, and the media in which no change in the absorbance of the \(\text{Ca}^{2+}\)-sensitive dye is seen is taken to indicate the free \([\text{Ca}^{2+}]\) in the cytosol of the permeabilized cells. These investigators found that unstimulated cells have a free \([\text{Ca}^{2+}]\) of 0.25 µM, and those treated with a hormone like phenylephrine have a value closer to 0.6 µM.

Similar experiments have recently been carried out by Streb and Schulz (230) in isolated permeabilized pancreatic acinar cells via a \(\text{Ca}^{2+}\) electrode to measure free \([\text{Ca}^{2+}]\). Their results were quite similar to those obtained by Murphy et al. (170). The permeabilized cells were able to maintain a free \([\text{Ca}^{2+}]\) of 0.42 µM when aliquots of \(\text{Ca}^{2+}\) were added to the medium. Both mitochondrial and ATP-dependent nonmitochondrial uptake of \(\text{Ca}^{2+}\) occurred. If mitochondrial uptake was blocked, the time necessary to achieve a steady-state value was reduced, but the final value was the same. However, if nonmitochondrial uptake was blocked, the time necessary to achieve a steady state was prolonged, and the final value of \([\text{Ca}^{2+}]\) was increased to 0.66 µM.
There are some differences in the absolute values of free [Ca$^{2+}$] determined by different investigators, particularly in regard to the mitochondrial set point. However, a variety of technical factors can account for these differences. Note that during the preparation of isolated cells, these cells accumulate considerable Ca$^{2+}$, which is stored in the mitochondria. As techniques have been refined and an awareness of this problem has become manifest, the estimates of the set point at which mitochondria fix the free Ca$^{2+}$ and the estimates of the in situ content of total mitochondrial Ca$^{2+}$ have become lower. For example, Joseph and Williamson (126) have recently shown that when liver cells contain low amounts of total Ca$^{2+}$ (<0.9 nmol/mg dry wt), approximately half is in the endoplasmic reticulum and half is in the mitochondria. Furthermore the endoplasmic reticulum is the major pool from which Ca$^{2+}$ was mobilized by vasopressin or phenylephrine action. As total cell Ca$^{2+}$ increased, the capacity of the endoplasmic reticulum to store Ca$^{2+}$ was exceeded. The mitochondria contained progressively greater amounts of total Ca$^{2+}$ (126). Under these conditions the hormones mobilized Ca$^{2+}$ from the mitochondrial pool without an apparent effect on the pool in the endoplasmic reticulum. However, it is quite possible that under the conditions employed, an initial mobilization of Ca$^{2+}$ from this latter pool could not be observed. The most important conclusion from these studies from the point of cell physiology is that the mitochondria are the organelles with the capacity to store relatively large amounts of Ca$^{2+}$.

Before considering this point further, there is an additional aspect of mitochondrial Ca$^{2+}$ metabolism that needs to be understood. It is now clear that there are separate influx and efflux pathways for Ca$^{2+}$ across the membrane (3, 43, 175, 176, 185). The capacity of the influx pathway is considerably greater than the efflux pathway. The influx pathway operates as a Ca$^{2+}$ uniporter driven by the large membrane potential (150 mV, inside negative), and the efflux pathway operates as a Ca$^{2+}$:2H$^+$ or Ca$^{2+}$:2Na$^+$ antiporter. Given this information, initial speculations were that the [Ca$^{2+}$] gradient across the mitochondrial membrane would be quite large. Even with minimal estimates it was realized early that if all the Ca$^{2+}$ in the mitochondrial matrix space were ionized, the concentration would be several millimoles per liter. Clearly this was not the case. Carafoli and Lehninger (44) showed that massive loading of Ca$^{2+}$ by mitochondria required the presence of both ATP and phosphate (P$_i$) even with a substrate present. They concluded that under these conditions much of the Ca$^{2+}$ existed in some form of Ca$^{2+}$-P$_i$-ATP complex. This work did not address the form of Ca$^{2+}$ in freshly isolated mitochondria that were not massively loaded. An experiment carried out by Rasmussen et al. (192) provided an insight into this problem [as did an experiment by Chappell and Crofts (49)]. This experiment is reproduced in the upper panel of Figure 4. When isolated liver mitochondria are incubated in an acetate medium and then a small aliquot of Ca$^{2+}$ is added, the Ca$^{2+}$ is accumulated in an energy-dependent manner. Simultaneously the mitochondria swell and accumulate acetate, i.e., the acetate serves as the counter ion, and Ca$^{2+}$ acetate,
**FIG. 4.** Determinants of \([\text{Ca}^{2+}]_m\) in mitochondrial matrix space ([\(\text{Ca}^{2+}]_m\)). A: experiment showing effect of phosphate addition to mitochondria that had accumulated \(\text{Ca}^{2+}\) while incubated in an acetate-buffered medium [modified from Rasmussen et al. (192)]. B: schematic representation of interplay between primary activity of proton pump coupled to electron transport (ET) and fluxes of \(\text{Ca}^{2+}, \text{H}^+, \text{OH}^-, \text{and the HPO}_4^{2-}\) across mitochondrial membrane as well as representation of formation of nonionic complexes of \(\text{Ca}[\text{Ca}_4(\text{PO}_4)_4]\) within mitochondrial matrix space. Because of high pH and high \([\text{HPO}_4^{2-}]_m\), \([\text{Ca}^{2+}]_m\) (free \(\text{Ca}^{2+}\)) is quite low, but total \(\text{Ca}^{2+}\), mostly as \(\text{Ca}_4(\text{PO}_4)_4(\text{ATP})\), is relatively quite high.

being a soluble salt, increases the osmotic activity within the mitochondrial matrix space. Addition of \(\text{HPO}_4^{2-}\) at this point leads to a shrinkage of the mitochondria (an \(\text{HPO}_4^{2-}:2\text{Ac}^-\) exchange has taken place), and the rate of respiration actually falls because the intramitochondrial free \([\text{Ca}^{2+}]_m\) has been reduced. Hence there is less cycling of \(\text{Ca}^{2+}\) across the membrane. Under
physiological circumstances the major cytosolic anion is phosphate. Hence when Ca\(^{2+}\) is taken up by mitochondria, HPO\(_4^{2-}\) is taken up by HPO\(_4^{2-}\) :2OH\(^-\) exchange and Ca\(^{2+}\) phosphates are formed. According to the Mitchell mechanism of mitochondrial energy transduction, the primary event is the development of an electrochemical proton gradient across the membrane, so that not only a membrane potential but also a pH gradient (pH\(_m\) > pH\(_e\)) exists. This is normally ~1 pH unit (176). In an alkaline environment the solubility of Ca\(^{2+}\) phosphates are extremely low. This means that the major determinants of the free [Ca\(^{2+}\)] within the mitochondrial matrix space, [Ca\(^{2+}\)]\(_m\), are the extra- and intramitochondrial [HPO\(_4^{2-}\)]\(_s\), the intramitochondrial pH, and the K\(_m\) and V\(_{\text{max}}\) of the efflux pathway (Fig. 4, lower panel).

A point of considerable uncertainty is the concentration of free Ca\(^{2+}\) in the mitochondrial matrix space. When this was measured by Hansford and Castro (107) via a variation of the null-point method, the value was found to be quite similar to that of the free [Ca\(^{2+}\)]\(_s\); i.e., there is practically no Ca\(^{2+}\) gradient across the mitochondrial membrane in the normal resting cell. Yet studies of the kinetics of cellular Ca\(^{2+}\) exchange clearly show that the cytosolic Ca\(^{2+}\) is in rapid exchange with the mitochondrial Ca\(^{2+}\) (30, 31). The other point emphasized by Hansford and Castro (107) is that <1% of the total mitochondrial Ca\(^{2+}\) is free. To double the free [Ca\(^{2+}\)]\(_m\), for example, it is necessary to approximately double the total Ca\(^{2+}\) content within the mitochondrial matrix space.

With this picture of the metabolism of Ca\(^{2+}\) by mitochondria in mind, it is now possible to consider the role of these organelles in cellular Ca\(^{2+}\) homeostasis. Comprehension of this role of the mitochondria can be most easily achieved by considering the rate of Ca\(^{2+}\) uptake into mitochondria as a function of the [Ca\(^{2+}\)]\(_s\). The rate of mitochondrial Ca\(^{2+}\) uptake (or the rate of Ca\(^{2+}\) efflux from cytosol) increases slowly when the [Ca\(^{2+}\)]\(_s\) rises from 0.1 to ~0.5 μM. Then above ~0.6 μM the rate of uptake increases dramatically as the [Ca\(^{2+}\)]\(_s\) rises. When the [Ca\(^{2+}\)]\(_s\) is <0.6 μM, the mitochondrial pools are in equilibrium exchange with the cytosolic pool; above this value, however, the [Ca\(^{2+}\)]\(_m\) rises higher than [Ca\(^{2+}\)]\(_s\), because the efflux pathway for Ca\(^{2+}\) out of the mitochondria is saturated (175). Hence when the [Ca\(^{2+}\)]\(_s\) rises above 0.6 μM, the rate of mitochondrial Ca\(^{2+}\) influx exceeds efflux and a net accumulation of Ca\(^{2+}\) by the mitochondria occurs; i.e., a state of disequilibrium exists.

These two components of the mitochondrial Ca\(^{2+}\) influx profile define two related mitochondrial functions. When the [Ca\(^{2+}\)]\(_s\) is between 0.1 and ~0.5 μM, the free [Ca\(^{2+}\)]\(_m\) has a very similar value to that of the [Ca\(^{2+}\)]\(_s\) (see Fig. 3), and the plasma membrane is largely responsible for maintaining cellular Ca\(^{2+}\) homeostasis. The [Ca\(^{2+}\)]\(_m\) is in rapid exchange with a large nonionic Ca pool in the mitochondrial matrix space: [Ca\(^{2+}\)]\(_m\) = 0.15 μM and total Ca = 50 μmol/kg cell H\(_2\)O. This arrangement means that the [Ca\(^{2+}\)]\(_m\) pool is coupled to the nonionic Ca pool in the mitochondria. When the [Ca\(^{2+}\)]\(_m\)
rises, $[Ca^{2+}]_m$ rises and more Ca$^{2+}$ is deposited into the nonionic mitochondrial pool. In fact a stable increase in $[Ca^{2+}]_m$ is possible only when this nonionic pool again has a steady-state relationship with $[Ca^{2+}]_m$. If, for example, one assumes that initially the $[Ca^{2+}]_m$ is 0.1 μM and the total Ca is 50 μmol, then to bring about a stable increase in $[Ca^{2+}]_m$ from 0.1 to 0.2 μM requires that total Ca increases from 50 to ~100 μmol/kg cell H$_2$O. Thus at low $[Ca^{2+}]_m$ the nonionic mitochondrial Ca pool acts to stabilize the $[Ca^{2+}]_m$. This stabilizing property of the mitochondrial Ca pool explains why such a large bolus of Ca$^{2+}$ is necessary to bring about such a relatively small change in $[Ca^{2+}]_m$ during the initial phase of cell activation (see Table 1). It also means that minor changes in the rate of Ca$^{2+}$ influx into the cell have little effect on $[Ca^{2+}]_m$; i.e., there is a built-in device to maintain the stability of the $[Ca^{2+}]_m$. This stabilization is required if, as noted in section II A, very small changes in $[Ca^{2+}]_m$ bring about large changes in the activity of CaM-regulated or other Ca$^{2+}$-regulated cellular REs.

Because the rate of mitochondrial uptake is a much steeper function of $[Ca^{2+}]_m$ (at $[Ca^{2+}]_m$ above ~0.6 μM) and because the mitochondrial efflux pathway is saturated, the second function of the mitochondria in cellular Ca$^{2+}$ homeostasis is defined as a sink for Ca$^{2+}$ during Ca$^{2+}$ overload.

From the point of view of the function of the Ca$^{2+}$ messenger system, these features of mitochondrial Ca$^{2+}$ metabolism define the range over which a change in $[Ca^{2+}]_m$ can be employed as messenger. For example, if, as might be expected from the $[Ca^{2+}]_m$-activation profile of an enzyme such as phosphorylase b kinase, the $[Ca^{2+}]_m$ rises from 0.1 to 1.0 μM when a cell is activated and the mitochondria function in situ as they do in vitro, then it can be calculated that the capacity of the mitochondria to store Ca$^{2+}$ would be exceeded within a few hours if $[Ca^{2+}]_m$ is maintained at 1.0 μM throughout this time period (190, 191). Conversely, if the $[Ca^{2+}]_m$ in the activated cell is maintained at values <0.5 μM, the cell could function in its activated state for hundreds of hours without running the risk of Ca$^{2+}$ overload. A very small difference in $[Ca^{2+}]_m$ in this critical range of 0.5–1.0 μM makes an extremely large difference in whether cellular Ca$^{2+}$ homeostasis is maintained.

2. Mitochondria as source of activator calcium

A different proposal as to the function of the mitochondrial Ca pool has been made by investigators studying the function of the Ca$^{2+}$ messenger system in the activation of hepatic glucose production by phenylephrine, vasopressin, and/or ANG II (23, 39, 50, 71, 85, 91, 126, 138, 170, 199, 231, 260). A considerable amount of data has been presented in support of the concept that when any one of these hormones acts, there is an increase in $[Ca^{2+}]_m$, associated with a net efflux of Ca$^{2+}$ from the cell. Furthermore, considerable data show that a major source of this released Ca$^{2+}$ is the mitochondrial pool. Hence it has been proposed that when one of these hormones acts, an
unknown second messenger is generated that acts to mobilize the mitochondrial Ca pool, and this pool provides the Ca$^{2+}$ responsible for the activation of glycogenolysis.

This model is radically different from the one developed in our discussion. A number of considerations lead us to question this hypothesis. It is our point of view for several reasons that the Ca$^{2+}$ messenger system in the hepatocyte functions as it does in smooth muscle cells, adrenal glomerulosa cells, or pancreatic acinar cells.

First, many of the studies on which the alternative model is based suffer from three technical problems. 1) Efflux of Ca$^{2+}$ from hepatocytes was measured when these cells were incubated in a medium containing a very low [Ca$^{2+}$], $\sim 50 \mu$M. 2) The Ca$^{2+}$ content of mitochondria was measured by methods that take 30 min to obtain an isolated mitochondrial fraction, and the assumption is made that these organelles neither gain nor lose Ca$^{2+}$ during this period. 3) Many of the studies with isolated cells employed cells that contained a higher than physiologically normal amount of total (and mitochondrial) Ca$^{2+}$.

Second, it has been reported that when an indicator of the [Ca$^{2+}$], such as quin 2 is employed, glucagon as well as phenylephrine addition leads to a rise in the [Ca$^{2+}$], (50). However, the glucagon-induced rise in [Ca$^{2+}$] is delayed and is of less magnitude, compared with that seen after phenylephrine. Our interpretation of these data is that when glucagon acts, the initial event is the cAMP-dependent activation of glycogenolysis. The rapid phosphorolysis of glycogen leads to a fall in the [HPO$_4^{2-}$], and this in turn leads to a release of [Ca$^{2+}$] from the mitochondria (see Fig. 4). Hence, rather than being a cause of the glycogenolysis, the release of mitochondrial Ca$^{2+}$ is a consequence of this metabolic event. The higher the original content of Ca$^{2+}$ in the cell and in its mitochondria, the greater the subsequent release of mitochondrial Ca$^{2+}$ induced by this mechanism.

Third, several groups have reported that when hepatocytes containing physiologically relevant amounts of total Ca$^{2+}$ are employed, phenylephrine or vasopressin cause the mobilization of Ca$^{2+}$ from the plasma membrane-endoplasmic reticular pool of Ca$^{2+}$ (39, 126, 239). In a recent study, Shears and Kirk (224) have confirmed these data and have shown, using a newly developed technique for the rapid fractionation of cells, that the Ca$^{2+}$ content of the mitochondria actually increases rather than decreases 30 s after cell activation by vasopressin.

IV. CALCIUM METABOLISM IN ACTIVATED CELL: A MODEL

As previously mentioned (sect. IIID), with the aid of several new techniques (6–9, 25, 26, 33, 214, 241, 242) it has become possible to directly measure the [Ca$^{2+}$] in resting and activated cells. Resting values of [Ca$^{2+}$] range from 0.05 to 0.35 $\mu$M (5–9, 17, 26, 38, 50, 64, 81, 98, 167, 182, 183, 201, 204, 217, 243, 244). It is not clear whether this sevenfold range of values actually exists
in different cell types or whether this is a reflection, in part at least, of the limitations of these new techniques to measure the "true" [Ca\(^{2+}\)] in situ. This range may also reflect the fact that isolation of cells by several techniques leads to an increased permeability of their plasma membranes to Ca\(^{2+}\) and hence to a higher total Ca\(^{2+}\) content and a higher [Ca\(^{2+}\)]\(_{i}\), than actually exists in situ. Clearly, however, the ratio of the concentrations of CaM to REs is a critical determinant of the sensitivity of these elements to activation by changes in [Ca\(^{2+}\)]. Hence it may in fact be necessary that the [Ca\(^{2+}\)]\(_{i}\) is higher in cells with lower CaM contents or, because the Ca\(^{2+}\) pump of the plasma membrane is a CaM-dependent RE, the lower [CaM] may in fact determine the higher [Ca\(^{2+}\)]\(_{i}\) by defining the set point around which the Ca\(^{2+}\) pump operates. From the point of view of cellular Ca\(^{2+}\) homeostasis, this inter-relationship between [Ca\(^{2+}\)]\(_{i}\), [CaM], and the activity of the plasma membrane Ca\(^{2+}\) pump provides an elegant autoregulatory system. The only potential disadvantage to a particular cell type of possessing less CaM and hence operating at a higher basal [Ca\(^{2+}\)]\(_{i}\) is that this cell is likely to be at a greater risk of cellular Ca\(^{2+}\) intoxication.

It is believed that a rise in [Ca\(^{2+}\)]\(_{i}\) occurs in many cells in response to an appropriate extracellular messenger. Direct measurement has verified this belief. Rises in [Ca\(^{2+}\)]\(_{i}\) to the range of 0.65-1.5 \(\mu\)M have been observed. The most interesting and somewhat unexpected finding is that even in those cells displaying a sustained response, the rise in [Ca\(^{2+}\)]\(_{i}\) is transient (Fig. 5). For example, as reported by Snowdowne and Borle (227) with TRH-mediated secretion in isolated pituitary cells or by Morgan and Morgan (167) with ANG II-mediated contraction in intact smooth muscle, there is only a transient rise in [Ca\(^{2+}\)]. It appears from these data that on activation of cells of this type, the [Ca\(^{2+}\)]\(_{i}\) rises from ~0.1-0.3 \(\mu\)M to values no greater than 0.6-1.5 \(\mu\)M and then falls within a few minutes to values \(\ll\)0.5 \(\mu\)M and often to values indistinguishable from the basal ones.

Investigators have also considered the converse question: whether a rise

![FIG. 5. Change in light output from intracellular aequorin (a measure of intracellular free Ca\(^{2+}\)) in GH\(_{3}\) pituitary cells in response to thyrotropin-releasing hormone (TRH) (solid line below trace). Note that addition of 1 \(\mu\)M TRH caused transient increase in light output, lasting only 40 s, after which the value fell to control level. Under similar conditions, prolactin secretion is sustained for at least 15–30 min. Cells were loaded with aequorin by hypsometric shock (36) and then placed into the curette of an aequorin photometer. Cells were mixed with Cytodex 1 beads to entrap them in place so they could be perfused with physiological media. Using Allen-Blinks calibration method, estimated basal free Ca\(^{2+}\) was 0.3 \(\mu\)M, and it rose to peak of 1.8 \(\mu\)M after TRH. Similar results have been reported from use of another Ca\(^{2+}\) indicator, quin 2 (98). [Courtesy of Dr. Snowdowne. Figure is representative of other data from studies of GH\(_{3}\) cells by Snowdowne and Borle (227).]
FIG. 6. Schematic representation of changes in cellular Ca\textsuperscript{2+} metabolism and Ca\textsuperscript{2+} fluxes after sustained activation of hypothetical cell by sustained presence of hormone (shaded bar). Top panel depicts change in [Ca\textsuperscript{2+}] in cytosol ([Ca\textsubscript{c}]; see Fig. 5). Shaded profile (upper panel) represents composite of data from several recent studies. All show sharp rise in [Ca\textsuperscript{2+}] in range of 0.9-1.5 μM followed by fall to a value either very close to original basal value or in some cases as much as 30-40% of peak value. It is not clear whether this range of values reported during sustained phase of response reflects true difference in behavior of different cell types or results from technical artifacts in these difficult measurements. Center panel is plot of rates of Ca\textsuperscript{2+} influx and efflux across the plasma membrane derived from kinetic analysis of Borle and Uchikawa (24). Key points are that once cell is activated, rate of Ca\textsuperscript{2+} influx remains high during
in $[Ca^{2+}]_i$ alone is sufficient to bring about sustained activation of specific cells. Studies of the effect of the divalent ionophore in several different systems have shown that ionophore addition often leads to an initial $Ca^{2+}$-dependent response, but this response is not sustained and may be less than the maximal response induced by the natural agonist in spite of a sustained elevation of $[Ca^{2+}]_i$ (128, 183). Likewise data from studies of the regulation of smooth muscle contraction have led to the conclusion that the rise in $[Ca^{2+}]_i$ and the associated $CaM$-dependent phosphorylation of myosin light chain are not sufficient to account for the sustained contractile response (4, 52, 167).

From the available data it is possible to present a tentative scheme of the sequence of changes in $Ca^{2+}$ metabolism that take place in a cell displaying a sustained response to the sustained presence of a hormone (or other extracellular messenger) (Fig. 6). When hormone interacts with its receptor, there are two more-or-less simultaneous events: 1) a release of $Ca^{2+}$ from the plasma membrane and/or endoplasmic reticulum and 2) an increase in the rate of $Ca^{2+}$ influx into the cell. These two changes lead to a rapid rise in the $[Ca^{2+}]_i$. The rise in $[Ca^{2+}]_i$ is short-lived because much of the released $Ca^{2+}$ is taken up into the nonionic $Ca$ pool in the mitochondrial matrix and because, as $[Ca^{2+}]_i$ rises, the plasma membrane $Ca^{2+}$ pump is activated by $Ca^{2+}$-$CaM$. Furthermore the plasma membrane-endoplasmic reticular pool(s) of $Ca^{2+}$ is nonrenewable; once it has lost its bolus of $Ca^{2+}$, it does not regain it as long as the cell remains activated (221). As a consequence of these three features, the $[Ca^{2+}]_i$ falls to a steady-state value of no more than 0.5 $\mu$M (newer evidence suggests this value is in the range of 0.25–0.3 $\mu$M; see Fig. 5). In this state, plasma membrane $Ca^{2+}$ influx remains high, the $[Ca^{2+}]_i$ in the subcellular domain just beneath the plasma membrane may be increased (see sect. VII), and plasma membrane efflux remains high but does not quite balance influx, resulting in a very slow net accumulation of $Ca^{2+}$ by the cell (34, 221). This accumulated $Ca^{2+}$ is stored largely in the mitochondrial matrix pool.

One of the most interesting questions that remains largely unexplored is what happens to cellular $Ca^{2+}$ metabolism when, after a long period of stimulation, the extracellular messenger concentration falls and cellular response is terminated. As shown by Schulz (221), the activator (nonmitochondrial) pool of $Ca^{2+}$, which is rapidly depleted with addition of extracellular messenger, rapidly refills when extracellular messenger is removed. At this point the cell contains considerably more total $Ca^{2+}$ than it did before messenger addition, but it is no longer in an activated state. Much of this extra $Ca^{2+}$ is presumably in the mitochondria. It is slowly lost from this pool into sustained phase of response. After time delay, related presumably to $Ca^{2+}$-dependent activation of plasma membrane $Ca^{2+}$ pump, rate of $Ca^{2+}$ efflux also rises to 80% or more of influx rate. Thus in maximally activated cell there is net gain in total $Ca^{2+}$ (34, 221). Lower panel represents hypothetical changes in free calcium ($[Ca]_c$: ———) and total $Ca^{2+}$ (-----) in mitochondria as result of above processes.
the cytosol and then into the extracellular pool, without flooding the cytosol, so that reactivation of the cell is not seen. The details of this process of controlling the disposal of mitochondrial Ca^{2+} during the postactivated phase of cellular activity remains to be examined.

V. INITIAL BIOCHEMICAL EVENTS IN CALCIUM MESSENGER SYSTEM

Having provided this scheme, it is now possible to consider how information flows from cell surface to cell interior. One of the least-defined features of the Ca^{2+} messenger system is the initial sequence of events after a hormone interacts with its receptor. Nevertheless, within the past few years new methods and new insights have begun to provide a coherent picture of the most probable consequences of hormone-receptor interaction. Hence we present current views of these events based on a variety of data (10, 13, 14, 18, 19, 20, 67, 72–74, 78, 84, 113, 117–119, 121–123, 127, 133, 136, 148, 158, 163–165, 169, 179, 187, 205, 208, 210, 219, 222, 228, 229, 235, 248), but considerably more information is needed to present a complete description of these processes.

When a hormone interacts with its receptor, it is thought that a specific phospholipase C is activated that catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate in the plasma membrane (Fig. 7). There is only a small pool of this phosphoinositide, but more can be rapidly formed from

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

**FIG. 7.** Schematic illustration of turnover of membrane phosphoinositides and cell activation. Interaction of occupied hormone receptor (R) with a specific phospholipase C (PLC), by a mechanism not yet known, leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIn_{4,5}P_{2}). This leads to production of diacylglycerol (DG) rich in arachidonic acid or other polyunsaturated fatty acids and to inositol triphosphate (InP_{3}). The latter compound may serve as mediator of Ca^{2+} release from intracellular source, but it is also rapidly dephosphorylated to inositol bisphosphate (InP_{2}), inositol monophosphate (InP_{1}) and eventually free inositol (In), which may recombine with phosphatidic acid (PA_{1}) to reform phosphatidylinositol (PIn). Generated DG serves as activator of C-kinase (not shown) and as substrate for a DG lipase, which causes the release of monoglyceride (MG) and of arachidonic acid (AA), which in turn serves as substrate for synthesis of leukotrienes (LT), prostaglandins (PG), and thromboxanes (TX), many of which may serve either an autocrine or paracrine function in controlling cellular response.
precursor pools of phosphatidylinositol 4-phosphate and phosphatidylinositol (PI). The hydrolysis of the bisphosphate leads to the production of DG (which is rich in arachidonic acid) and of inositol 1,4,5-trisphosphate (Ins 1,4,5P$_3$) (18, 67, 187). It is also possible that the initial event is the simultaneous hydrolysis of both phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, leading to the generation of DG from both pools and the production of inositol 1,4-diphosphate as well as Ins 1,4,5P$_3$.

There is general agreement that the DG serves two functions. It is a sensitivity modulator of the Ca$^{2+}$-activated phospholipid-dependent protein kinase, C-kinase. Its interaction with this enzyme causes the enzyme to bind to the plasma membrane, resulting in a further positive sensitivity modulation of the enzyme by the phospholipids in the membrane so that the enzyme becomes highly sensitive to activation by Ca$^{2+}$ (179; see Fig. 2). The DG also serves as a source for arachidonic acid, the substrate for leukotriene, prostaglandin, prostacyclin, and thromboxane synthesis (13, 179, 210, 211). However, it is not yet clear whether this DG is the sole source of the arachidonic acid released on activation of the cell (84, 113, 121, 123, 133, 144, 206, 222, 245). The activation of phospholipase A$_2$ may cause the hydrolysis of other lipids, which could serve as an additional source of arachidonic acid. Whether phospholipase A$_2$ activation is a direct consequence of hormone-receptor interaction or of some event that follows phospholipase C activation also remains to be determined (144, 169).

The second product of phospholipase C activity is Ins 1,4,5P$_3$. Streb et al. (229) have recently shown that Ins 1,4,5P$_3$ induces the release of Ca$^{2+}$ from an intracellular store (presumably the plasma membrane–endoplasmic reticulum pool) in permeabilized acinar cells from the pancreas. It is possible that Ins 1,4,5P$_3$ is a general second messenger for the release of activator (or trigger) Ca$^{2+}$ in many cells, but this also remains to be determined. Other candidates for this function are several leukotrienes or other products of the lipoxygenase or epoxygenase pathways of arachidonic acid metabolism (46, 113, 117, 133, 157, 163, 164, 171–173, 187, 208, 210, 222, 228, 253, 263, 264).

The other event that occurs is an increased rate of Ca$^{2+}$ influx across the plasma membrane via either a POC or an ROC channel. Considering only the ROC, the mechanism of its opening is not yet clear. It is possible that the hydrolysis of the phosphatidylinositol 4,5-bisphosphate leads directly to a change in membrane structure and thereby to a change in Ca$^{2+}$ permeability. It is equally possible that Ins 1,4,5P$_3$ serves to change plasma membrane permeability or that one or more of the products of arachidonic acid metabolism are involved (Fig. 7). At various times a prostaglandin, a leukotriene, or phosphatidic acid has been proposed as a mediator of this process in different cell types. No clear answer is available. On the other hand, several recent lines of evidence suggest that the various products of arachidonic acid metabolism serve as autocrine and/or paracrine agents that temporally and/or geographically extend or restrict many of these tissue responses.
In spite of these uncertainties, two common events apparently occur when the Ca\(^{2+}\) messenger system is activated in a cell displaying a sustained response: a transient rise in the \([\text{Ca}^{2+}]_c\) and a prolonged increase in the DG content of the cell (10).

VI. GAIN CONTROL IN CALCIUM MESSENGER SYSTEM

Given these properties of the Ca\(^{2+}\) messenger system and the above model of the sequence of changes in Ca\(^{2+}\) metabolism of the activated cell, the question becomes one of determining how a cell displays a sustained response in the face of a transient rise in \([\text{Ca}^{2+}]_c\). Because during the prolonged phase of cellular response the \([\text{Ca}^{2+}]_c\) is considerably lower than that needed to achieve maximal or near-maximal activation of CaM-dependent enzymes, there must be some way in which a type of gain control is achieved in this messenger system. Several possibilities exist as to how this might be brought about: 1) the CaM-dependent REs display hysteresis (216, 217); 2) the C-kinase pathway serves a distinct function during the sustained phase of cellular response (140, 193, 266); and 3) positive sensitivity modulation of Ca\(^{2+}\)-regulated REs by changes in [cAMP] allow CaM-dependent REs to function at a lower \([\text{Ca}^{2+}]_c\) (195).

A. Role of Hysteresis

As discussed above, there is now evidence that Ca\(^{2+}\)-CaM-regulated (or troponin-regulated) REs display hysteresis (201, 216, 217). However, the magnitude of these effects is relatively small (at most the gain of 0.3 pCa units) in the cytosolic enzymes examined (201), yet the fall in pCa is greater. Moreover, work in both vascular and tracheal smooth muscle has shown that although the phosphorylation of myosin light chain increases during the early phase of cell activation, it does not remain high and within 8-10 min returns to values close to the initial ones in spite of a sustained contractile response (4, 5, 52, 99, 167, 226). In this cell type, hysteretic behavior of a CaM-regulated enzyme does not provide long-term gain control. In the case of the plasma membrane Ca\(^{2+}\) pump, however, there is a unique situation in which a sustained hysteresis appears to operate (216, 217). This type of behavior may be of crucial importance to the maintenance of a high rate of Ca\(^{2+}\) efflux across the plasma membrane of the activated cell (34), even when the \([\text{Ca}^{2+}]_c\) in the bulk cytosol has fallen back toward its basal value (227).

B. Role of C-Kinase

Exploration of the role of the C-kinase branch of the Ca\(^{2+}\) messenger system began with observations of Kaibuchi et al. (128) and Kawahara et al. (132) in blood platelets. Use was made of the fact that the divalent cation
ionophore A23187 will increase \([\text{Ca}^{2+}]_c\) without activating the C-kinase branch, and the phorbol ester TPA will activate the C-kinase branch (45) without causing a rise in \([\text{Ca}^{2+}]_c\) (15, 201, 203, 223). The effects of these two agents were compared with those of thrombin, a natural activator of the platelet-release reaction. When thrombin acts, the \([\text{Ca}^{2+}]_c\) rises and myosin light chain becomes phosphorylated as a consequence of the \(\text{Ca}^{2+}-\text{CaM}\)-dependent activation of myosin light-chain kinase. Also, a 40,000-\(M_r\) protein becomes phosphorylated as a consequence of a rise in DG content and the activation of C-kinase. Thrombin produces a maximal release reaction. When A23187 is employed, \([\text{Ca}^{2+}]_c\) rises (201) and myosin light chain becomes phosphorylated (214), but the 40,000-\(M_r\) protein does not become phosphorylated. The platelet-release reaction is submaximal. When TPA is employed, the phosphorylation of the 40,000-\(M_r\) protein occurs, the \([\text{Ca}^{2+}]_c\) does not rise nor does myosin light chain become phosphorylated, and the release reaction is submaximal. The combination of A23187 and TPA mimics all the effects of thrombin and induces a maximal release reaction. Thus, at the very least, events in both branches of this system are required to obtain an appropriate cellular response, and there appears to be a synergism between events in the two branches of the system. Likewise there is evidence in neutrophils that a rise in free \(\text{Ca}^{2+}\) alone is not sufficient to produce maximal or sustained responses (183), and conversely TPA can induce sustained responses without causing any apparent increase in free \([\text{Ca}^{2+}]_c\) (223). Thus it is probable that a similar synergism between events in the two branches of the system exists in the normal activation of these cells.

Studies in other cell types suggest that the two branches may have unique roles in the temporal integration of cellular response. We discuss data obtained from three cell types—the adrenal glomerulosa cell (140), the \(\beta\)-cell of the islets of Langerhans (266), and the vascular smooth muscle cell (H. Rasmussen, J. Forder, I. Kojima, and A. Scriabine, unpublished observations).

The function of the adrenal glomerulosa cell is to synthesize and secrete aldosterone (65, 75, 76, 88, 90, 140, 259). These processes are responsive to a number of extracellular messengers, e.g., \(K^+\), \(\text{ANG II}\), \(\text{ACTH}\), and serotonin. In most mammals the major stimulators of aldosterone secretion are thought to be \(\text{ANG II}\) and \(K^+\). A variety of data indicate that both \(\text{ANG II}\) and \(K^+\) act via the \(\text{Ca}^{2+}\) messenger system to regulate aldosterone production. This process can be studied with collagenase-isolated glomerulosa cells in a perifusion system. When \(\text{ANG II}\) acts, there is a monotonic increase in aldosterone production, starting a few minutes after hormone addition, rising to a plateau that is then sustained for several hours, as long as the \(\text{ANG II}\) is present (Fig. 8, lower left panel). Addition of A23187 instead of \(\text{ANG II}\) leads to an initial rise in aldosterone production but then to a slow decline, so that values at 30 min are one-third or less of those seen during \(\text{ANG II}\) perifusion (Fig. 8, lower middle panel). Addition of TPA leads to no initial rise in aldosterone production but to a very gradual increase, which reaches a maximal value.
FIG. 8. Temporal integration of insulin secretion from β-cells in islets of Langerhans (top) and of aldosterone secretion from adrenal glomerulosa cells (bottom) by combined activation of cells by A23187 and 12-O-tetradecanoyl-phorbol-13-acetate (TPA). With insulin secretion (266): left panel shows typical biphasic insulin secretory response induced by increase in medium glucose concentration from 2.75 to 7 mM; center panel shows individual responses to 2.5 μM A23187 (-----) and to 5 nM TPA (-----); and right panel shows response to combined addition of A23187 and TPA. With aldosterone secretion (140): left panel illustrates aldosterone secretory response induced in porcine adrenal glomerulosa cells by addition of 1 × 10^{-9} M angiotensin II; center panel shows individual responses to 1.25 μM A23187 (-----) and to 5 nM TPA (-----); right panel shows response to combined effects of A23187 and TPA.

only after 90 min or more of constant perifusion that is ~40% of that seen with ANG II (Fig. 8, lower middle panel). Combined perifusion with A23187 and TPA leads to a secretory pattern almost identical to that seen after ANG II action (Fig. 8, lower right panel). Furthermore, if the Ca²⁺ permeability of the plasma membrane is greatly increased by adding high concentrations of A23187, it is possible to clamp the intracellular free Ca²⁺ by perifusing these cells with media in which the free [Ca²⁺] is fixed by Ca²⁺-EGTA buffers. Under these circumstances the TPA-dependent increase in aldosterone secretion can be shown to be a direct function of the [Ca²⁺], in the range of 0.05–1.0 μM. Using a slightly different approach in “leaky” adrenal medullary cells, Knight and Baker (139) have shown a similar Ca²⁺ dependency for the TPA-induced stimulation of catecholamine secretion.

An interesting feature of the response of these adrenal cells to ANG II is that when ANG II acts, there is both a transient mobilization of Ca²⁺ from an intracellular pool and an increase in Ca²⁺ uptake by the cell. The ANG II-induced Ca²⁺ efflux can be blocked by the drug dantrolene, which in skeletal muscle inhibits Ca²⁺ efflux from the sarcoplasmic reticulum (I. Kojima, K. Kojima, and H. Rasmussen, unpublished observations). When dantrolene is present, maximally stimulatory concentrations of ANG II induce
only a half-maximal aldosterone secretory response. Conversely, if dantrolene is added after ANG II has induced the Ca\(^{2+}\) efflux from the intracellular pool and the maximal rate of aldosterone production has been reached, it has no inhibitory effect on the rate of aldosterone secretion. Likewise, dantrolene does not inhibit A23187-TPA-induced secretion. These data suggest that when a hormone (e.g., ANG II) activates a cell via the Ca\(^{2+}\) messenger system, the initial transient rise in the \([\text{Ca}^{2+}]_i\) is important not only in activating the CaM branch but also in determining the amount of C-kinase that becomes membrane bound and activated.

The function of the \(\beta\)-cells in the islets of Langerhans is to synthesize, store, and secrete insulin (51, 103, 104, 111, 130, 151–154, 159, 163, 164, 261, 266). Again numerous extracellular messengers regulate these processes. Of particular importance are changes in plasma glucose concentration (95, 104, 112, 151). The process of glucose-dependent insulin secretion from isolated islets can be studied in vitro via a perifusion system. When medium glucose concentration is increased, there is a biphasic pattern of insulin secretion: an initial sharp increase reaching a peak in 5–7 min, followed by a sharp fall to values <50% of the peak value, and then a slow and progressive rise to a plateau that can be maintained for hours (Fig. 8, upper left panel). The bulk of the experimental evidence supports the concept that the Ca\(^{2+}\) messenger system mediates glucose-induced insulin secretion. However, the precise mechanism has remained obscure. Calmodulin and CaM-regulated enzymes have been described (112). Also the presence of the C-kinase has been detected (238), and it has been shown that TPA stimulates insulin secretion (152, 153, 266), but its effects on both insulin secretion and islet cell Ca\(^{2+}\) metabolism differ from those of glucose. Based on these data and our observations in the adrenal glomerulosa cell, a comparison was made of the effects of A23187 and/or TPA on the time course of insulin secretion to those of glucose (266). Addition of A23187 to the perifusate induces an initial insulin secretory peak indistinguishable from that evoked by glucose, but the rate of insulin secretion then falls to nearly the basal value (Fig. 8, upper middle panel). There is no secondary or sustained phase of insulin secretion. In contrast, when TPA is added to the perifusate, there is no initial peak of insulin secretion but only a slowly progressive rise to a plateau value 60% of that seen after glucose action (Fig. 8, upper middle panel). The combination of A23187 and TPA induces an insulin secretory pattern similar to that induced by an elevation of glucose concentration (Fig. 8, upper right panel).

Another agent capable of stimulating insulin release is tolbutamide, a sulfonylurea. As shown by Henquin (115), when the extracellular glucose is 75 ng/dl or less, addition of 100–500 \(\mu\)g/ml of tolbutamide induces only an initial phase of insulin secretion, similar to that produced by A23187. Tolbutamide appears to act by increasing Ca\(^{2+}\) uptake into the islet cells (130, 147). Recently, Malaisse and co-workers (152) showed there is a synergism between the action of the sulfonylurea, gliclazide, and TPA (152). Based on
these facts, the effect of TPA and tolbutamide on the time course of insulin secretion was examined. The results were similar to those obtained when A23187 and TPA are employed: tolbutamide alone induces a first phase, TPA induces a second phase, and their simultaneous administration induces a biphasic pattern of insulin secretion similar to that induced by an increase in glucose concentration (W. Zawalich, K. Zawalich, and H. Rasmussen, unpublished observations).

The typical vascular smooth muscle cell contracts in response to either ANG II or NE (4, 28, 35, 45, 46, 52, 63, 150, 162, 213). When responding to either of these agonists, the cell displays a biphasic pattern of response: an initial or rapid phase followed by a slow tonic phase. It is well accepted that Ca$^{2+}$ is the factor that couples stimulus to response in these cells (20, 28, 149). One of its effects is that of activating the CaM-dependent, myosin light-chain kinase (1, 27, 57, 109). The resulting increase in myosin light-chain phosphorylation is thought to be responsible for the contractile response (134). However, recent work has shown that when either ANG II or NE acts, the rise in [Ca$^{2+}$], is transient and is not maintained throughout the tonic phase of the response (167), and during the tonic phase of maintained tension, the phosphorylation of the myosin light chain declines to nearly basal values (when extracellular Ca$^{2+}$ is 1.6 mM; 4, 52, 99). These data led Aksoy et al. (4, 5) to conclude that there are two Ca$^{2+}$-dependent regulatory processes involved in the control of smooth muscle contraction. Our own recent results (H. Rasmussen, J. Forder, I. Kojima, and A. Scriabine, unpublished observations) show that TPA induces a slowly progressive, Ca$^{2+}$-dependent increase in the tension developed by vascular smooth muscle (Fig. 9) and that either A23187 or brief recurrent electrical pulses enhance this response. Furthermore, the TPA-induced contraction is reversed by the addition of forskolin, an activator of adenylate cyclase (Fig. 9). Thus the proposed second Ca$^{2+}$-dependent mechanism by which the tonic phase of smooth muscle contraction

![Fig. 9. Response of vascular smooth muscle to successive additions of the phorbol ester TPA and of forskolin, an activator of adenylate cyclase. TPA (100 nM) was added at time zero to rabbit ear arterial muscle incubated in medium containing 2.5 mM Ca$^{2+}$. Starting ~50 min later, a slowly developing but progressive increase in pressure is seen, so by 87 min, pressure has risen from 7.5 to 43.5 mmHg. Addition of 25 μM forskolin at this point causes a prompt and dramatic fall in pressure back to its basal value within 2 min. (Modified from the unpublished data of H. Rasmussen, J. Forder, I. Kojima, and A. Scriabine.)](http://physrev.physiology.org/Downloaded from http://physrev.physiology.org)
is maintained appears to be the C-kinase pathway of the Ca$^{2+}$ messenger system.

These results led to the hypothesis that in many cells there are two branches of the Ca$^{2+}$ messenger system that have distinct temporal roles (Fig. 10; 195). The CaM branch, operating via amplitude modulation, is largely responsible for the initial cellular responses. The C-kinase branch, operating via sensitivity modulation, is largely responsible for the sustained cellular responses. This latter branch operates at a lower steady-state [Ca$^{2+}$], than the CaM branch, thus providing a type of gain control in this cellular control system. It is likely that this role of the C-kinase branch is a general one in cells that employ the Ca$^{2+}$ messenger system to achieve sustained responses (56, 68, 96, 97, 105, 114, 129, 218, 240).

Studies in platelets (179) and in liver cells (93) indicate that CaM-regulated protein kinases phosphorylate a different subset of cellular proteins than does C-kinase. If this is a general property of these two types of kinases, then the final REs, whose functions are changed by events in the two branches of the Ca$^{2+}$ messenger system, are different.

VII. CALCIUM CYCLING ACROSS PLASMA MEMBRANE DURING SUSTAINED CELLULAR RESPONSE

A puzzling feature of the activated cell, when employing the Ca$^{2+}$ messenger system as a means of achieving a sustained cellular response, is that both membrane Ca$^{2+}$ influx and efflux remain high even though the increase in [Ca$^{2+}$] is only transient. Recent experimental data provide insights into how this Ca$^{2+}$ cycling is achieved and its possible function.

Considering the data obtained by Morgan and Morgan (167), concerning the rise in [Ca$^{2+}$], when vascular smooth muscle cells are activated by appropriate agonists, along with those obtained by Askoy et al. (4, 5), concerning the degree of myosin light-chain phosphorylation as a function of time after agonist administration to similar muscles, a consistent picture emerges. When the agonist acts, there is a transient rise in [Ca$^{2+}$], which leads to the activation of myosin light-chain kinase and the phosphorylation of myosin light chain. This leads to the initial phase of contraction. The [Ca$^{2+}$], then falls to values similar to or slightly above the basal values. Shortly after this, myosin light-chain phosphorylation declines, in large part presumably because myosin light-chain kinase is no longer markedly activated. Hence if myosin light-chain kinase displays hysteresis, it is not sufficiently prolonged to maintain the enzyme in its fully activated state when [Ca$^{2+}$] falls and remains low for periods $>10$ min.

In contrast to the situation with myosin light-chain kinase, the activation of the Ca$^{2+}$ pump in the plasma membrane by CaM displays a different response. First, in those cells in which hormone or extracellular messenger leads to an increase in Ca$^{2+}$ influx and a sustained response, there is a con-
FIG. 10. Schematic representation of pathways of information flow in Ca\(^{2+}\) messenger system. When hormone (H) interacts with its receptor (R) on the plasma membrane (shaded area), a phospholipase C (PLC) is activated, leading to hydrolysis of phosphoinositol 4,5-bisphosphate (4,5P\(_{2}\)). Either as a consequence of or simultaneously with this activation there is increased [Ca\(^{2+}\)] in cytosol caused by both release from intracellular pool (plasma membrane-endoplasmic reticulum) and increased entry of Ca\(^{2+}\) into cell. Rise in cytosolic [Ca\(^{2+}\)] ([Ca\(^{2+}\)_c]) activates response elements (REs) in calmodulin (CaM) branch of system (left), including calmodulin (CM)-dependent protein kinase(s) (PK), leading to phosphorylation of subset of cellular proteins (Pr). These events lead to initial cellular response (left). However, because the plasma membrane Ca\(^{2+}\) pump is activated and much of released Ca\(^{2+}\) is taken up by mitochondria, [Ca\(^{2+}\)_c] falls after a few minutes to steady-state values of \(<0.5 \mu M\), so flow of information through CaM branch is incapable of sustaining cellular response. Activation of PLC also causes rise in diacylglycerol (DG) content of plasma membrane. This event along with initial rise in [Ca\(^{2+}\)]_c brings about activation of C-kinase (C-K) branch of system (left). C-kinase catalyzes phosphorylation of different set of cellular proteins (Pr_b) responsible for sustained phase of cellular response (right). When flow of information through the 2 branches is temporally integrated (lower right center), integrated cellular response is observed. C-kinase branch is mechanism by which type of gain control is achieved in Ca\(^{2+}\) messenger system. Also shown is intracellular activator pool of Ca\(^{2+}\) (Ca-Z), which contributes to initial rise in [Ca\(^{2+}\)]_c and may be released as consequence of generation of inositol 1,3,4-triphosphate (In-P\(_3\)), a second product of PLC action.

continued high rate of Ca\(^{2+}\) influx, four- to fivefold greater than the basal value (34, 221). Yet the rate of net Ca\(^{2+}\) accumulation by the cell is considerably less than predicted, because not only is the rate of Ca\(^{2+}\) influx high but the rate of efflux via the plasma membrane Ca\(^{2+}\) pump is also increased (34).
Furthermore, work by Scharff and co-workers (216, 217) in intact red cells has shown that if these cells are exposed to low concentrations of A23187, there is an immediate sharp rise in cellular Ca\textsuperscript{2+} content followed very shortly by a fall to a steady-state value only slightly above the basal value. To examine this phenomenon, they have employed this experimental system and a simple two-step model of the CaM activation of the pump, nCa\textsuperscript{2+} + CaM $\rightleftharpoons$ Ca\textsubscript{n}-CaM; Ca\textsubscript{n}-CaM + E\textsubscript{l} $\rightleftharpoons$ Ca\textsubscript{n}-CaM-E\textsubscript{l}, where E\textsubscript{l} and E\textsubscript{h} represent, respectively, the low-$V_{\text{max}}$, high-$K_{m,\text{Ca}}$ form of the pump, and E\textsubscript{h} represents the high-$V_{\text{max}}$, low-$K_{m,\text{Ca}}$ form of the pump. They conclude from their analysis that once the [Ca\textsuperscript{2+}]\textsubscript{i} rises, the enzyme is shifted from its E\textsubscript{l} to its E\textsubscript{h} form but then remains there when the [Ca\textsuperscript{2+}]\textsubscript{i} falls back to near its basal value. This conclusion provides the most direct and logical explanation of why in a hormonally activated cell the rate of Ca\textsuperscript{2+} efflux across the plasma membrane remains high during its sustained phase of activation even though the [Ca\textsuperscript{2+}]\textsubscript{i} is similar to or just above its basal value. The value of this type of autoregulatory behavior is obvious. It minimizes the possibility of excessive Ca\textsuperscript{2+} accumulation by the cell. The unanswered question is why does one Ca-CaM-regulated enzyme, the plasma membrane Ca\textsuperscript{2+}-H\textsuperscript{+}-ATPase, behave so differently from another, the cytosolic myosin light-chain kinase, particularly because the kinetics of activation of the two isolated enzymes are so similar (58). Before attempting to answer this question, there are two other experimental findings to consider.

These findings come from two different studies exploring the same question: the Ca\textsuperscript{2+} dependency of C-kinase activity in situ. The results of the studies by Knight and Baker (139), examining this phenomenon in "leaky" adrenal medulla cells, and those of Kojima et al. (140), examining it in adrenal glomerulosa cells using a Ca\textsuperscript{2+}-clamp method, clearly show that the effect of TPA on C-kinase activity is dependent on the [Ca\textsuperscript{2+}]\textsubscript{i}. However, the [Ca\textsuperscript{2+}]\textsubscript{i} necessary to obtain maximal or near-maximal effects is on the order of 1 $\mu$M. On the other hand the above model of how the Ca\textsuperscript{2+} messenger system operates argues that a [Ca\textsuperscript{2+}]\textsubscript{i} in the range of 0.15–0.25 $\mu$M must be sufficient to keep this enzyme activated in the intact cell.

A hypothesis that can make sense of these disparate observations is that in the activated cell, in which a high rate of influx of Ca\textsuperscript{2+} across the plasma membrane is coupled with a high rate of Ca\textsuperscript{2+} efflux across this same membrane; the local [Ca\textsuperscript{2+}] at the endoplasmic face of the plasma membrane is higher than the [Ca\textsuperscript{2+}]\textsubscript{i} of the bulk cytosol (Fig. 11). This would mean 1) the Ca\textsuperscript{2+} pump would remain activated by Ca\textsubscript{n}-CaM, and 2) the C-kinase bound to this surface of the plasma membrane would remain highly active, although the [Ca\textsuperscript{2+}]\textsubscript{i} in the bulk cytosol is not sufficient to maintain it in that state. This hypothesis would explain why the C-kinase is membrane bound: to place it in a unique subcellular domain of high [Ca\textsuperscript{2+}] and high DG content.

There are many implications of this hypothesis, but we only discuss one: the role of Ca\textsuperscript{2+} in sustained smooth muscle contraction. If, as appears to be the case, the [Ca\textsuperscript{2+}]\textsubscript{i} is barely elevated during the sustained phase of
smooth muscle contraction (167) and the activated C-kinase has only one location (the plasma membrane), then during the sustained phase of smooth muscle contraction, changes in \([\text{Ca}^{2+}]_c\) probably are not directly involved in regulating the interactions of actin with myosin, but rather changes in the \([\text{Ca}^{2+}]_c\) at the cytoplasmic face of the plasma membrane will alter the state of phosphorylation of one or more cytosolic proteins, which may then interact with the contractile proteins.

VIII. cAMP AND CALCIUM MESSENGER SYSTEM

A. General Relationships

The pervasive and multiple relationships between the \(\text{Ca}^{2+}\) and cAMP messenger systems have been discussed previously in considerable detail (16, 190, 194). We present only a summary of the major features of these relationships. The major conclusion drawn previously was that these two intracellular messengers nearly always function in concert to couple stimulus to response in differentiated animal cells responding to stimuli that evoke their specialized work function. However, the patterns of interaction displayed by these two synarchic messengers varies among cell types.

At least five patterns of synarchic regulation are recognizable: 1) co-
ordinate control, in which a hormone (or other extracellular messenger) interacts with two separate receptors with similar affinities for the hormone, and consequently both the \([\text{Ca}^{2+}]_k\) and \([\text{CAMP}]_k\) rise; 2) hierarchical control, in which one messenger activates the \(\text{Ca}^{2+}\) messenger system, and either a higher concentration of this first messenger or another first messenger leads to an activation of the \(\text{CAMP}\) system (the rise in \([\text{CAMP}]_k\) in such a system enhances the response to the initial first messenger); 3) redundant control, in which the two messenger systems are activated by a distinctly different first messenger, and activation of either induces cellular response; 4) antagonistic control, in which a rise in \([\text{Ca}^{2+}]_k\) initiates a cellular response, and a rise in \([\text{CAMP}]_k\) inhibits the \(\text{Ca}^{2+}\)-induced response; and 5) sequential control, in which an increase in \([\text{Ca}^{2+}]_k\) leads to a rise in \([\text{CAMP}]_k\), or vice versa.

It is possible to consider the interactions between \(\text{CAMP}\) and \(\text{Ca}^{2+}\) in another fashion: how does \(\text{Ca}^{2+}\) affect the basic components of the \(\text{CAMP}\) messenger system? Conversely, how does \(\text{CAMP}\) influence events in the \(\text{Ca}^{2+}\) messenger system? Finally, how do these two messengers interact in the control of RE function?

As shown in Table 3, \(\text{Ca}^{2+}\) acting via \(\text{CaM}\) may activate phosphodiesterase and may either activate or inhibit adenylate cyclase in different cells \((155, 246)\) or in the same cell at different \([\text{Ca}^{2+}]_k\). Thus a rise in \([\text{Ca}^{2+}]_k\) may cause either a rise or a fall in \([\text{CAMP}]_k\). Likewise, changes in \([\text{CAMP}]_k\) act at multiple sites within the \(\text{Ca}^{2+}\) messenger system. A rise in \([\text{CAMP}]_k\) can increase the plasma membrane influx of \(\text{Ca}^{2+}\) via voltage-dependent \(\text{Ca}^{2+}\) channels \((42, 116, 200, 202)\). It may also increase efflux across the plasma membrane by direct effect on the \(\text{Ca}^{2+}\) pump or indirectly by an activation of the \(\text{Na}^{+}\) pump. In many cells a rise in \([\text{CAMP}]_k\) stimulates the uptake of \(\text{Ca}^{2+}\) by the endoplasmic reticulum. It is also clear that a rise in \([\text{CAMP}]_k\) may either enhance

### Table 3. Interaction between \(\text{CAMP}\) and \(\text{Ca}^{2+}\) messenger systems

<table>
<thead>
<tr>
<th>I. (\text{Ca}^{2+}) and (\text{CAMP}) system</th>
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<tbody>
<tr>
<td>A. Activates phosphodiesterase</td>
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<tr>
<td>B. Activates adenylate cyclase</td>
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<tr>
<td>C. Inhibits adenylate cyclase</td>
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<tr>
<th>II. (\text{CAMP}) and (\text{Ca}^{2+}) system</th>
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<tr>
<td>A. Increases plasma membrane influx via potential-dependent channels</td>
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<tr>
<td>B. Increases plasma membrane efflux</td>
</tr>
<tr>
<td>1. (\text{Ca}^{2+}) pump</td>
</tr>
<tr>
<td>2. (\text{Na}^{+})-(\text{K}^{+}) pump</td>
</tr>
<tr>
<td>C. Increases (\text{Ca}^{2+}) uptake into endoplasmic reticulum</td>
</tr>
<tr>
<td>D. May regulate phosphatidylinositol turnover</td>
</tr>
<tr>
<td>E. Enhances or suppresses events in either (\text{CaM}) or (\text{C-kinase}) branch of (\text{Ca}^{2+}) messenger system by positive or negative sensitivity modulation</td>
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<tr>
<th>III. (\text{CAMP-Ca}^{2+}) interactions</th>
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<tbody>
<tr>
<td>A. Control the phosphorylation of sequential proteins in a cascade</td>
</tr>
<tr>
<td>B. Both may catalyze phosphorylation of same protein via separate protein kinases</td>
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or suppress responses in either the CaM or the C-kinase branch of the Ca\(^{2+}\) messenger system. These effects are mediated, at least in part, by the sensitivity modulation of Ca\(^{2+}\)-activated proteins.

The two messenger systems interact in another manner in controlling the phosphorylation state of proteins. In some cases a cAMP-dependent protein kinase controls the phosphorylation of one protein in an enzyme cascade, and a Ca\(^{2+}\)-dependent protein kinase controls the phosphorylation of a subsequent enzyme in the cascade. In other instances both Ca\(^{2+}\) and cAMP-dependent protein kinases catalyze the phosphorylation state of the same substrate protein. Even though the protein is the same, the phosphorylation site is different. Yet in the known cases of this duality of kinase-dependent phosphorylations, the change in (substrate) enzyme function is qualitatively the same, i.e., either an increase or decrease in function.

**B. cAMP and Branches of Calcium Messenger System**

Three tissues in which the effects of cAMP on the function of both branches of the Ca\(^{2+}\) messenger system have been partially defined are the \(\beta\)-cell of the islets of Langerhans, the blood platelet, and smooth muscle.

In the case of the \(\beta\)-cell there is substantial evidence that a rise in [cAMP] leads to an enhanced response to a standard change in medium glucose concentration (112, 151). Likewise the recent data of Zawalich et al. (266) show that a forskolin-induced rise in [cAMP] enhances (222) and a fall in [cAMP] inhibits the response of the islet to combined A23187-TPA. Thus changes in [cAMP] influence events in both the C-kinase and CaM branches of the Ca\(^{2+}\) messenger system. It is not yet clear whether these effects of cAMP can be explained solely by a CAMP-dependent increase in [Ca\(^{2+}\)], or whether a rise in [cAMP] alters the activity of some component of the C-kinase pathway, as it is known to do in the CaM pathway (190).

The effects of forskolin on insulin secretion are equally striking when a combination of tolbutamide and TPA are used to evoke secretion (W. Zawalich, K. Zawalich, and H. Rasmussen, unpublished observations). Addition of forskolin to a perifusate containing TPA alone, causes a significant (synergistic) increase in the TPA-induced rate of insulin secretion without changing the pattern of this response; i.e., there is no first phase of secretion in response to TPA in either the presence or absence of forskolin. The simultaneous perfusion of islets with forskolin and tolbutamide produces only an additive response. The simultaneous perfusion of 200 nM TPA, 10 \(\mu\)M forskolin, and 200 \(\mu\)M tolbutamide, in the absence of added glucose, leads to a biphasic pattern of insulin secretion similar both qualitatively and quantitatively to that evoked by a physiologically relevant rise (from 2.75 to 10 mM) in glucose concentration. Hence a combination of these three "messengers" mimics the response to the natural agonist glucose. The logical conclusion based on evidence to date (W. Zawalich, K. Zawalich, and H. Rasmussen, unpublished observations), albeit incomplete, is that when the
glucose concentration rises only slightly, both [Ca\(^{2+}\)], and plasma membrane DG content in the β-cell increase, leading to the activation of both branches of the Ca\(^{2+}\) messenger system with little or no change in [cAMP]. However, when the glucose concentration rises to higher values, 10–12 mM or greater, in addition to increases in [Ca\(^{2+}\)] and DG, there is an activation of adenylate cyclase, possibly by Ca\(^{2+}\)-CaM (246). This rise in [cAMP] acts as a positive-feedback regulator of Ca\(^{2+}\) entry into the cell (116). Consequently, there is an increase in rate of insulin production during both phases of the secretory response. Other factors can independently activate or inhibit adenylate cyclase activity and thereby determine the magnitude of the insulin secretory response to a given rise in glucose concentration.

These data imply that in islet cells, the basic messenger system regulating insulin secretion is the Ca\(^{2+}\) messenger system; the function of the cAMP messenger system is that of determining the set point around which the Ca\(^{2+}\) messenger system operates. It is not yet clear whether this is achieved solely by cAMP-dependent increases in either the initial Ca\(^{2+}\) transient in the cytosol and/or in the steady-state level of [Ca\(^{2+}\)], during the sustained phase of secretion or whether cAMP may also increase Ca\(^{2+}\) flux through the POCs in the plasma membrane (42, 116, 258), increase the [Ca\(^{2+}\)]\(_{i}\) at the cytoplasmic face of the plasma membrane, and thereby determine the activity of C-kinase (see Fig. 11). Changes in [cAMP] may also exert effects via the sensitivity modulation of one or more Ca\(^{2+}\)-sensitive REs.

It is of interest that forskolin potentiates the effect of carbachol, cholecystokinin-octapeptide, or A23187 on the secretion of amylase from the acinar cells of the exocrine pancreas without influencing the carbachol-induced mobilization of intracellular Ca\(^{2+}\). From these data, Heisler (114) suggests that one action of cAMP in this tissue is that of increasing carbachol-induced Ca\(^{2+}\) entry during the sustained phase of secretion. However, such an effect would not account for the potentiation of A23187-induced secretion. It is likely that cAMP also acts at a more distal site in the Ca\(^{2+}\) messenger system.

Platelet activation represents the opposite relationship. A rise in [cAMP] leads to an inhibition of thrombin-induced platelet activation (81). This result means that a rise in [cAMP] leads to an inhibition of events in both the CaM and C-kinase branches of the Ca\(^{2+}\) messenger system. In this case a rise in [cAMP] clearly induces a fall in [Ca\(^{2+}\)] (81). This effect alone may be sufficient to inhibit the flow of information in both pathways, but the results do not rule out the possibility that a rise in [cAMP] brings about the negative sensitivity modulation of an element in the C-kinase pathway, as it does in the CaM pathway. Furthermore, Nishizuki (179) has interpreted his data to mean that a rise in [cAMP] leads to an inhibition of DG production by blocking the hydrolysis of polyphosphoinositides. Alternatively, cAMP could act by increasing DG removal via an enhancement of the rate of PI synthesis.

The situation in smooth muscle is similar to that in platelets. A rise in cAMP causes a relaxation of many types of smooth muscle, such as vascular smooth muscle (28). Several mechanisms have been proposed to account for
the effects of cAMP: 1) it exerts negative sensitivity modulation on myosin light-chain kinase; 2) it stimulates the uptake of Ca^{2+} by intracellular (endoplasmic reticular) membranes; and/or 3) it stimulates the efflux of Ca^{2+} from the cell directly by enhancing the activity of the Ca^{2+} pump or indirectly by enhancing Na^{+}:Ca^{2+} exchange via the activation of the Na^{+}-K^{+}-ATPase (28,190). The first mechanism appears relatively unimportant in an already contracted muscle, because during the sustained phase of contraction little myosin light chain is phosphorylated (4,5). The second mechanism may play an important role in many types of smooth muscle cells but must be relatively unimportant in the smooth muscle cells in resistance vessels (47). The third mechanism is clearly important but is difficult to study. The major issue in smooth muscle cells, as in platelets, is whether cAMP-induced relaxation is mediated solely by a cAMP-induced fall in [Ca^{2+}]. The question is posed dramatically by the results shown in Figure 9. Addition of forskolin to a rabbit ear artery partially contracted by TPA leads to a prompt and rapid fall in tension (H. Rasmussen, J. Forder, I. Kojima, A. Scriabine, unpublished observations). Whether the effects of cAMP on cellular Ca^{2+} metabolism are sufficient to account for the rapidity of this effect remains to be determined. It is quite possible that cAMP has other effects on the events in the C-kinase branch of the Ca^{2+} messenger system: either by altering the behavior of the kinase itself, by altering the activity of the phosphoprotein phosphatase known to act on the phosphorylated substrates of C-kinase (209), or by influencing the expression of the function of one or more of these phosphoprotein products of C-kinase.

C. cAMP-Mediated Gain Control in Calcium Messenger System

The effects of cAMP on the events in the Ca^{2+} messenger system are so common as to suggest that a major function of changes in [cAMP] is that of influencing events in the Ca^{2+} messenger system by altering the amplitude of the Ca^{2+} message and/or the sensitivity of one or more REs to activation by Ca^{2+}. In doing so, one of the important functions of the cAMP messenger system may be to provide an alternate means of achieving gain control in the Ca^{2+} messenger system. This is particularly the case in those cell types in which Ca^{2+} and cAMP serve as either coordinate or sequential messengers (190). In this circumstance it is possible that a rise in [Ca^{2+}] is largely responsible for initiating response, and a CAMP-dependent increase in the sensitivity of Ca^{2+}-regulated REs to activation by Ca^{2+} is responsible for maintaining response (Fig. 12), even when [Ca^{2+}] falls from its initial peak value to one closer to the basal value.

A specific example of this type of gain control may operate in the adrenal glomerulosa cell when it is activated to secrete aldosterone by an increase in the extracellular [K^{+}] (I. Kojima, K. Kojima, and H. Rasmussen, unpublished observations). As noted in section viB, K^{+} and ANG II increase aldosterone secretion in a Ca^{2+}-dependent manner. However, there are several
FIG. 12. Representation of alternative means of achieving gain control in Ca^{2+} messenger system by cAMP-dependent positive sensitivity modulation of response elements (REs) in Ca^{2+} messenger system. This can occur in cellular systems in which Ca^{2+} and cAMP serve as synarcheic messengers in either a coordinate, sequential, or hierarchical pattern. Either a coordinate or hierarchical pattern is illustrated. When one hormone ([H]_H) acts in coordinate fashion, it binds to 2 types of plasma membrane receptors (R_1 and R_2), which are coupled either to adenylate cyclase (AC) or to the Ca^{2+} channel. Consequently both [cAMP] and [Ca^{2+}] rise in cell cytosol. Rise in Ca^{2+} causes the activation of nonphosphorylated form of RE, which regulates a cellular response (A → B). However, rise in [Ca^{2+}] is transient, and so response would be transient, except that cAMP activates its protein kinase (PK), which catalyzes phosphorylation of RE (RE → RE-P). The phosphorylated form (RE-P) is more sensitive to activation by Ca^{2+}, and so response A → B continues at high rate even though [Ca^{2+}] is considerably lower than its initial peak value. Alternatively, same type of gain control can be achieved if one hormone ([H]_H) acts only on Ca^{2+} messenger system and another ([H]_H) acts only on the cyclase system.

differences between ANG II and K^+ action: 1) K^+ has only a small effect on Ca^{2+} efflux, which is not blocked by dantrolene; 2) dantrolene has no inhibitory effect on K^+-induced secretion but does cause a significant inhibition of ANG II induced aldosterone secretion; 3) ANG II but not K^+ induces the rapid breakdown of phosphatidylinositol 4,5-bisphosphate (78); 4) K^+ but not ANG II (92) causes an increase in the cAMP content of glomerulosa cells (259); and 5) K^+ causes a depolarization of the plasma membrane. These data imply that when K^+ acts, 1) it brings about the opening of a voltage-dependent Ca^{2+} channel in the plasma membrane, and the resulting entry of Ca^{2+} into the cell is the only source of activator Ca^{2+}; 2) it does not activate the PI cycle (78), and therefore does not lead to an increase in the DG content of the plasma membrane nor activate C-kinase; and 3) by some means it activates adenylate cyclase. Hence it is possible that the K^+-induced increase in cAMP provides a type of gain control during K^+-activated aldosterone secretion as depicted in Figure 12. Indirect support for this possibility is provided by
recent observations concerning the effects of forskolin on aldosterone secretion (I. Kojima, K. Kojima, and H. Rasmussen, unpublished observations). If adrenal glomerulosa cells are perifused with low doses of forskolin, a very slight but sustained increase in aldosterone secretion is observed. Conversely, when cells are perifused with A23187, a transitory increase in aldosterone production is seen. When cells are perifused simultaneously with A23187 and forskolin, there is a synergism in their actions, and a marked and sustained rate of aldosterone secretion is seen. Hence perifusion with either A23187-TPA or A23187-forskolin induces a similar sustained rate of secretion. These results support the concept that gain control in the Ca\textsuperscript{2+} messenger system can be achieved by at least two different mechanisms (see Figs. 10 and 12) and that both mechanisms may operate in the same tissue.

IX. CONCLUSIONS

The current model of the organization and operation of the Ca\textsuperscript{2+} messenger system has been developed over the past several years. Various aspects of it have been discussed in recent chapters and reviews (190, 191, 194–196). During the development of our studies, some of the concepts have been refined and some of the quantitative aspects of Ca\textsuperscript{2+} pool sizes, the [Ca\textsuperscript{2+}]\textsubscript{i}, and the nature of the association reactions between Ca\textsuperscript{2+}, CaM, and their REs have been refined or modified.

The model presented emphasizes recent data concerning the functions of the two branches of the Ca\textsuperscript{2+} messenger system, how events are initiated in each, and how their roles are integrated to achieve an integrated temporal response. In choosing this emphasis, other important features of cellular Ca\textsuperscript{2+} metabolism and the Ca\textsuperscript{2+} messenger system have been only briefly mentioned. For example, in our discussion of the cell components involved in the maintenance of cellular Ca\textsuperscript{2+} metabolism, the Na\textsuperscript{+}:Ca\textsuperscript{2+} exchange process across the plasma membrane has received scant attention (8, 24, 31, 198). This process is particularly prominent in excitable cells such as heart and nerve, which display a different type of cellular response than discussed in this review (195). On the other hand, Na\textsuperscript{+}:Ca\textsuperscript{2+} exchange may also play a significant role in Ca\textsuperscript{2+} exchange across the plasma membrane of smooth muscle cells (28). This process of Na\textsuperscript{+}:Ca\textsuperscript{2+} exchange provides an alternative by which Ca\textsuperscript{2+} is driven out of the cell across the plasma membrane against a large [Ca\textsuperscript{2+}] gradient. The exact function of this pathway, its quantitative importance in different cell types, and the factors that may regulate its expression are not yet clear. Hence this pathway has not been considered in detail. This aspect of how cell Ca\textsuperscript{2+} homeostasis is maintained and how the Ca\textsuperscript{2+} messenger system functions is yet to be defined.

The model of cell activation presented in this review is based largely on results obtained in endocrine cells, in which the time constants of response are minutes and hours. However, other cells clearly respond much more
rapidly either to natural agonists or to phorbol esters. For example, neutrophils display an immediate response, in terms of the respiratory burst, to TPA. One explanation of the immediacy of this response is that the C-kinase in these cells is already bound to the plasma membrane. In this location it may not function primarily as a means of gain control, as in the endocrine cells discussed here, but as a simultaneous and independent branch (along with the CaM branch) by which information flows from cell surface to cell interior. If this is the case, it follows that the model of the relationship between the two branches of the Ca\textsuperscript{2+} messenger system, which has been developed in our review, is not the only way in which these two branches can be functionally related.

By highlighting the very elegant and elaborate means by which the cell employs Ca\textsuperscript{2+} as a messenger and yet maintains Ca\textsuperscript{2+} homeostasis, and in so doing limits the magnitude and duration of any change in [Ca\textsuperscript{2+}], we underscore a major reality of cellular existence: all mammalian cells exist in a hostile, Ca\textsuperscript{2+}-rich environment and are faced with the continued threat of cellular Ca\textsuperscript{2+} intoxication (83, 174, 181, 215, 239, 262). Only by understanding this minatory aspect of cellular Ca\textsuperscript{2+} can the magnificence of the evolutionary adaptations allowing Ca\textsuperscript{2+} to serve as an intracellular messenger be fully appreciated. This review is an attempt to convey some appreciation for both the beauty and simple utility of the product: the Ca\textsuperscript{2+} messenger system as it appears to operate in highly specialized mammalian cells.

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980

HOWARD RASMUSSEN AND PAULA Q. BARKETT

Volume 64


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