Biochemical Pharmacology, Vol. 30, No. 12, pp. 1395–1405, 1981.
Printed in Great Britain.

0006-2952/81/121395-11 $02.00/0

COMMENTARY

PROPERTIES AND FUNCTIONS OF CALMODULIN

CHARLES O. BROSTROM* and DONALD J. WOLFF

Department of Pharmacology, College of Medicine and Dentistry of New Jersey, Rutgers Medical School, Piscataway, NJ 08854, U.S.A.

Calcium ion is widely recognized as a major regulator of intracellular metabolism throughout the animal kingdom. Stimuli effecting increases in the intracellular concentrations of free Ca^{2+} are well established to increase the activities of many enzyme systems and specialized cellular processes, including muscle contraction, cell motility, and exocytotic secretion from endocrine and exocrine tissue [1, 2]. The cation has particularly important functions in intercellular communication systems and is frequently classified as a second messenger in the mediation of responses to various humoral substances.

The mechanisms by which Ca^{2+}-dependent signals are translated into biological responses are currently being subjected to intense scrutiny by many workers. Such mechanisms clearly require intracellular receptors for Ca^{2+} that rapidly and reversibly bind the cation in the range of free concentrations occurring upon physiologic stimulation of resting cells. The range of free Ca^{2+} concentrations within cells is believed to vary from a resting value of approximately 10^{-7} M for most cells to maximal concentrations of about 10^{-5} M in highly stimulated cells. Through the efforts of many research workers over the past two decades, a number of Ca^{2+}-binding proteins have been characterized that have properties which would be expected for putative intracellular Ca^{2+} receptors. These proteins include the variant forms of troponin C from cardiac and skeletal muscle, parvalbumin, a vitamin D inducible protein, calsequestrin, and calmodulin (also frequently termed "Ca^{2+}-dependent regulator" or "CDR"). Calsequestrin appears to be exclusively involved in the accumulation of Ca^{2+} by elements of the endoplasmic reticulum. With the exception of calmodulin, the remaining proteins are of limited tissue distribution and appear to be concerned with tissue specific processes.

Calmodulin has a broad, if not ubiquitous, distribution in the tissues of eukaryotes. The protein has been prepared, frequently to homogeneity [3–10], from many chordate sources. It has also been found in a variety of invertebrate species and tissues [11], in the slime mold, *Physarum polycephalum* [12], in a fungus, *Blastocladiella emersonii* [13], in protozoan sources [12], and in various higher plants [14] but not in bacteria [15]. The protein varies in concentration from source to source. Mammalian brain [7] and testis [8] and the electroplax of *Electrophorus electricus* [6] are particularly rich sources. Subcellular fractionation studies have localized the protein predominantly in the soluble fraction, although some is associated with the particulate fractions as well. Binding of calmodulin to particulate fractions has been reported to require Ca^{2+} and appears to occur at specific sites [16, 17]. Binding is saturable, reversible, and temperature- and trypsin-sensitive. By indirect immunofluorescence techniques, calmodulin appears to be generally distributed throughout the cytoplasm of interphase proliferating cells in tissue culture [18, 19]. During mitosis, much of the protein appears to associate with the mitotic apparatus. Another study conducted with tissue slices of rat liver reports that calmodulin is associated with cytoplasm, nucleus, plasma membrane, and glycogen particles [20]. Related distributions were found in slices of skeletal muscle and adrenal.

In contrast to other Ca^{2+}-binding proteins, calmodulin possesses the exciting property of conferring reversible activation upon a number of enzymes (Table 1). The ability of the protein to serve as a multifunctional Ca^{2+}-dependent regulator, by virtue of physical interactions with a group of otherwise very different enzymes, may be unique among regulatory proteins. It is this ability that has generated the current widespread interest in the biochemistry of calmodulin and provides the basis of an interesting pharmacology. As will be discussed later, the ability of calmodulin to activate these enzymes is blocked by various neuroleptics, such as the phenothiazines. While the remainder of this commentary summarizes the properties and functions of calmodulin, the reader is also referred to other recent reviews on the topic for additional information and alternate viewpoints [21–26].

Physical and chemical properties of calmodulin

Calmodulin is resistant to denaturation by heating

Table 1. Calmodulin activated enzymes

Cyclic nucleotide phosphodiesterase
Adenylate cyclase
Myosin light chain kinase
Phosphorylase *b* kinase
Glycogen synthase kinase
NAD^+ kinase
$(Ca^{2+} + Mg^{2+})$ ATPase

* Address correspondence to: Charles O. Brostrom, Ph.D., Department of Pharmacology, Rutgers Medical School, P.O. Box 101, Piscataway, NJ 08854, U.S.A.

(99°), urea (8 M), detergents, high salt concentration, and acidic pH. Identical tryptic peptide maps have been reported for calmodulin from cow, pig, rabbit, rat, and chicken brain [27]. The protein has been sequenced completely from bovine brain [28] and partially from bovine uterus [9], rat testis [29], and the coelenterate *Renilla reniformis* [30], and was found to have 148 amino acid residues. Approximately ten minor changes in the sequences of these calmodulins have been found, almost all of which involve differences in amidation states or conservative amino acid substitutions. The protein from animal sources has one histidine, no tryptophan or cysteine, and one trimethyllysine residue. The predominance of acidic to basic amino acid residues is responsible for the low isoelectric point (3.9). The high ratio of phenylalanine to tyrosine provides the low molar extinction coefficient ($\varepsilon_{276}^{1\%} = 1.8$). Calmodulin has a molecular mass of approximately 16,800, calculated from its amino acid sequence, and appears to exist as a monomeric species. This molecular mass lies about midway in a series of reported values determined by the techniques of sedimentation equilibrium and sodium dodecylsulfate (SDS)-analytical gel electrophoresis.

Calmodulin exhibits a high degree of α-helical conformation, and there is general agreement that the protein binds four Ca^{2+}. The four Ca^{2+} binding sites appear to correspond to four domains with considerable degrees of homology in amino acid sequence. Starting at the amino terminus of the protein, the degree of homology is highest between the first and third domains and between the second and fourth domains. These findings have been interpreted to reflect the occurrence of genetic redundancy in the codon for calmodulin in ancestral cells in the distant past [28].

Only poor agreement exists in the literature regarding the respective affinities for Ca^{2+} of the four binding sites of calmodulin. Most often the experimental data have been interpreted as defining two types of binding sites, with (depending on the report) one class of sites binding two or three Ca^{2+} with K_d values ranging from 0.2 to 4 μM and the other class binding one to three Ca^{2+} with K_d values ranging from 1 to 800 μM [3, 5, 7, 31–33]. One group, using a Ca^{2+}–EGTA* buffer system, has reported that four equivalent sites exist with a K_d of 2.5 μM [8]. The exact bases for these discrepancies in binding data are unclear but may relate, in part, to conditions and procedures employed in this determination. With the exception of the earliest report [3], all of these studies employed equilibrium dialysis. In most of these studies Ca^{2+} binding was determined by measuring the differences between $^{45}Ca^{2+}$ inside and outside the compartment containing calmodulin and subsequently calculating the amount of bound Ca^{2+} from the specific radioactivity of the Ca^{2+} added to the system. Such values may have been distorted by the presence of contaminating Ca^{2+} or other divalent cations in the system competitively binding to the protein.

Two approaches have been used to overcome these constraints. Wolff *et al.* [33], using reagents freed of contaminating divalent cations by chromatography on Chelex 100, directly determined Ca^{2+} concentrations after equilibrium dialysis by atomic absorption spectrophotometry. This technique allowed the determination of total Ca^{2+} involved in the system such that any Ca^{2+} contamination was taken into account. Determinations of binding of Ca^{2+} in the absence of other cations provided three binding sites with a K_d of 0.2 μM and one site with K_d of 1 μM. Both Mg^{2+} and Mn^{2+} were competitive inhibitors of Ca^{2+} binding. At 1 mM Mg^{2+} only three sites were saturated with Ca^{2+}, all with an apparent K_d of 3 μM. These experiments were conducted at low ionic strength. Dedman *et al.* [8], using a Ca^{2+}–EGTA buffer system, obtained different results. Using this buffer at a high concentration of total Ca^{2+} minimizes the effects of Ca^{2+} contaminants and provides a larger molar excess of Ca^{2+} over the potential number of binding sites in the system while maintaining low free Ca^{2+} concentrations. Free Ca^{2+} values which must be calculated for these buffers depend on an accurate assessment of the association constant of EGTA for Ca^{2+}, a value that varies as a squared function of the pH range near neutrality. By this technique four equivalent binding sites of K_d 2.5 μM were found. This K_d was not affected by the addition of Mg^{2+}. The variance between these results and those of Wolff *et al.* [33] was not explicable in terms of source [21] but may have related to the observation that calmodulin binds EGTA [34].

A variety of data supports the conclusion that Mg^{2+} and other divalent cations compete for the Ca^{2+} binding sites on calmodulin. Included are: (a) direct binding studies indicating that 4 moles of Mg^{2+} or Mn^{2+} bind per mole of calmodulin and are displaced by Ca^{2+} [33], (b) shifts in dichroic spectra [33, 35] and nuclear magnetic resonance signals [36] occurring with the addition of Mg^{2+}, and (c) an increase in the apparent K_m for Ca^{2+} of various calmodulin-dependent enzymes occurring as a function of increasing Mg^{2+} concentration. These enzymes include forms of cyclic nucleotide phosphodiesterase [37], adenylate cyclase [38], ATPase [39], myosin light chain kinase [40], and phosphorylase kinase [41].

Mechanism of enzyme activation by calmodulin

With the exception of phosphorylase *b* kinase which has a nondissociable endogenous calmodulin component [42], all enzymes known to be activated by direct interaction with calmodulin (Table 1) dissociate from the protein on anion exchange columns equilibrated with Ca^{2+} chelators. Regulation of these enzymes by calmodulin (C) is generally believed to proceed by two sequential, fully reversible mass action expressions:

$$n\mathrm{Ca}^{2+} + \mathrm{C} \rightleftharpoons \left(\mathrm{Ca}^{2+}\right)_n \mathrm{C} \qquad (1)$$

$$x\left(\mathrm{Ca}^{2+}\right)_n \mathrm{C} + \mathrm{Enzyme}_{(\mathrm{inactive})} \rightleftharpoons [(\mathrm{Ca}^{2+})_n\mathrm{C}]_x \cdot \mathrm{Enzyme}_{(\mathrm{active})}. \qquad (2)$$

* EGTA = ethyleneglycolbis (aminoethylether) tetraacetate.

In this model, Ca^{2+} first interacts with calmodulin to form a complex capable of activating the enzyme (Eqn. 1). This complex then associates with the inactivated form of the enzyme forming an activated ternary complex (Eqn. 2); X stands for an unknown number of $(Ca^{2+})_n$ · calmodulin complexes.

Saturation of calmodulin with Ca^{2+} (Eqn. 1) results in the occurrence of conformational changes in the protein involving increases in α-helical content as reflected by increases in ellipticity, an enhanced tyrosine fluorescence, and a negative tyrosine difference spectrum [8, 32, 33, 35, 43]. It is important to note, however, that saturation of the protein with Mg^{2+} (1 mM) elicits similar but somewhat less extensive changes without providing a complex capable of enzyme activation. For example, Wolff *et al.* [33] found that preparations of calmodulin that were initially free of divalent cations increased in α-helical content from 28 to 42 per cent with added Ca^{2+} and from 28 to 39 per cent with Mg^{2+}. Lesser changes in responses to Mg^{2+} were found by Walsh *et al.* [35] at higher ionic strength with EGTA-containing buffers. At present the number (n) of Ca^{2+} which must bind to convert calmodulin to an activating form is in question. At 1 mM Mg^{2+} an activating calmodulin complex for the cyclic nucleotide phosphodiesterase forms with n equal to 3 [33]. An n equal to 4 is reported for the myosin light chain kinase based on kinetic rather than binding data [40]. It is difficult to distinguish whether complexes with $n < 3$ form activating species since the three remaining sites appear to bind Ca^{2+} with equivalent affinities. No information is available regarding whether Ca^{2+} binds sequentially or randomly to the various domains.

The activation of the enzyme by the Ca^{2+}–calmodulin complex (Eqn. 2) appears to result from a conformational change induced in the enzyme as the active ternary complex forms [44–46]. Various data indicate that calmodulin without Ca^{2+} does not bind to the inactive enzyme [47]. Dissociation of the ternary complex presumably could either occur as a direct reversal of equation 2 or as a consequence of a dissociation of Ca^{2+} from the ternary complex followed by reversion of calmodulin to an inactive conformation and dissociation from the enzyme. Data are not available to distinguish among these possibilities, and it is not known whether the ternary complex has a higher affinity for Ca^{2+} than does free calmodulin.

Several interesting inferences can be drawn from the above model. Clearly, if one increases the calmodulin concentration in equation (1) without changing the Ca^{2+} concentration, more Ca^{2+}–calmodulin complex will form. Therefore, the apparent K_m of the enzyme for Ca^{2+} would be determined, in part, by the molar ratios of calmodulin to enzyme molecules. Only at ratios less than unity would the apparent K_m of the enzyme for Ca^{2+} be equal to the dissociation constant of Ca^{2+} from calmodulin. Ca^{2+} sensitivity as a function of calmodulin concentration is highlighted in Fig. 1 (panel A) for the Ca^{2+}-dependent phosphodiesterase. A second factor that would affect Ca^{2+} sensitivity would be the presence of cations competing for the Ca^{2+} binding sites on calmodulin (Eqn 1). The effect of Mg^{2+} concentration on the sensitivity of the phosphodiesterase is also illustrated in Fig. 1 (panel B). Similar effects have been found for the myosin light chain kinase [40]. A third determinant of Ca^{2+} sensitivity would be the relative affinity of the inactive enzyme for the Ca^{2+}–calmodulin complex. Factors acting to increase the affinity of an enzyme for the complex would provide apparent increases in sensitivity of the enzyme to Ca^{2+}. Presumably enzymes with high relative affinities for the Ca^{2+}–calmodulin complex would be preferentially activated over those with poor affinity in cells exposed to mild stimuli. A range of affinities for calmodulin does apparently exist among the proteins of Table 1. For example, phosphorylase *b* kinase contains a complement of calmodulin which has not proven separable without denaturation of the enzyme [42]. Myosin light chain kinase has dissociable calmodulin but is activated by 1 mole calmodulin per mole enzyme [25]. Cyclic nucleotide phosphodiesterase requires high molar ratios of CDR to enzyme for full activation to occur [37].

Regulatory roles of calmodulin

As indicated in Table 1, a considerable number of enzymes are reported to be subject to Ca^{2+}-dependent regulation by calmodulin, and there is a high probability that others remain to be identified. All of these enzymes appear to function at rate-limiting points in various cellular processes, with calmodulin coupling intracellular free Ca^{2+} flux to enzyme activation. Processes affected include cyclic nucleotide metabolism, glycogen metabolism, Ca^{2+} transport systems, and certain cell motility and contractile functions. While some of these processes are

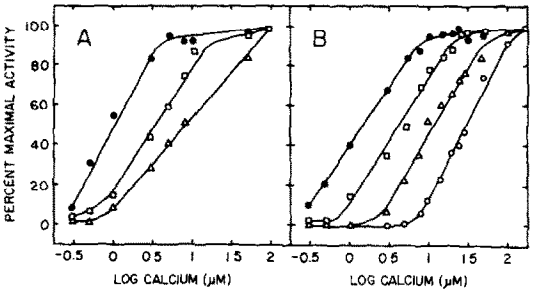

Fig. 1. (A) Effect of calmodulin on Ca^{2+} sensitivity of porcine brain cyclic nucleotide phosphodiesterase. Ca^{2+} concentration dependence of phosphodiesterase activity was determined, as described previously [33], in incubations containing fixed concentrations of calmodulin-dependent phosphodiesterase, 25 μM cyclic GMP, 1 mM $MgSO_4$, and either 10 (△), 100 (□) or 1000 (●) ng calmodulin. Maximal activities were, respectively, 395, 1060 and 1050 nmoles cyclic GMP hydrolyzed · min^{-1} · ml enzyme. (B) Effect of Mg^{2+} on Ca^{2+} sensitivity of calmodulin-dependent phosphodiesterase. Ca^{2+} concentration dependence of calmodulin-dependent phosphodiesterase was determined in incubations containing 33 ng calmodulin and fixed concentrations of calmodulin-dependent phosphodiesterase at 0.3 (●), 1 (□), 3 (△) and 10 (○) mM $MgSO_4$. Maximal activities were, respectively, 685, 840, 1075 and 998 nmoles cyclic GMP hydrolyzed · min^{-1} · ml enzyme. Cations were of spectrograde quality. Reagents were freed of divalent cations by Chelex-100 pretreatment.

found throughout many cell types, calmodulin regulation is not necessarily found in all tissues which possess a given process. For example, the calmodulin-dependent forms of adenylate cyclase and phosphodiesterase have limited tissue distribution with the phosphodiesterase apparently being the more widely distributed of the two [21]. For extensive details regarding the major properties of the various proteins listed in Table 1, the reader is referred to a number of recent reviews [21–26]. The discussion to follow in this section will be limited to several issues of current interest in which new data have been reported.

Calmodulin-dependent adenylate cyclase (for review see Ref. 21)

Increasing numbers of tissues have been reported to possess adenylate cyclase activities that are activated by Ca^{2+} [21, 48–53]. It seems likely that many, if not all, such activities will prove to require calmodulin. The interrelationships between the Ca^{2+}-dependent adenylate cyclase and the more generally distributed Ca^{2+}-independent form(s) remain to be clarified. Such properties as the apparent K_m for ATP [54], inhibition by high free Ca^{2+} concentrations [38], and thermal stabilities with Ca^{2+} chelators are similar for the Ca^{2+}-dependent form of adenylate cyclase from brain and the form(s) that are not activated by Ca^{2+}. In common with other forms, the Ca^{2+}-dependent enzyme is reported to be activated by cholera toxin [55], GTP and 5′-guanylyl imidodiphosphate (GMPPNP) [56, 57], F– [38], and Mn^{2+} [58].

Westcott *et al.* [59] have recently reported that the calmodulin-dependent adenylate cyclase from brain is largely deactivated by calmodulin-Sepharose affinity chromatography conducted with 1% Lubrol PX. Deactivation involved the removal of a heat-labile, trypsin-digestible, and *N*-ethyl maleimide-sensitive factor(s), and was accompanied by loss of enzyme activation by calmodulin, NaF, and GMPPNP [57]. Activation of the enzyme by calmodulin was restored by incubation of the enzyme at lower detergent concentrations with GMPPNP and a detergent-dispersed fraction from cerebral cortex. The dependence of the reconstitution on GMPPNP may have related to the well-established requirement of various adenylate cyclase activities for a specific GTP binding protein [60]. Since the reconstitution resulted in a calmodulin-dependent enzyme rather than a Ca^{2+}-independent form, interconversion of forms apparently did not occur.

In an early study, washed particulate preparations of C-6 glioma cells were found by Brostrom *et al.* [61] to possess a norepinephrine-stimulated adenylate cyclase with a biphasic response to Ca^{2+}. Low Ca^{2+} concentrations ($\simeq 0.1\ \mu M$) in conjunction with calmodulin activated the enzyme approximately 40 per cent, whereas high Ca^{2+} concentrations were inhibitory. Increasing calmodulin concentrations reduced both the apparent K_{act} and K_i for Ca^{2+}. The significance of the effect of calmodulin on Ca^{2+}-dependent inhibition is unclear, but it has been reported to occur for CHO cells [62] and brain adenylate cyclase [63] preparations. More recently, cyclic AMP accumulation by intact C-6 cells has been examined following a 5-fold depletion of intracellular Ca^{2+} stores with EGTA washes [64]. The ability of the cells to accumulate cyclic AMP in response to β-adrenergic agonists was reduced 60–70 per cent by Ca^{2+} depletion without effects on the binding of agonists. Rates of accumulation of cyclic AMP were restored by the addition of Ca^{2+} to the extracellular media in a manner blocked by verapamil, an inhibitor of plasmalemmal Ca^{2+} influx. Trifluoperazine at micromolar concentrations blocked the Ca^{2+}-dependent component of cyclic AMP accumulation without affecting the underlying Ca^{2+}-independent rate of accumulation. Most recently, the rate of activation of adenylate cyclase in intact C-6 cells and the concomitant accumulation of cyclic AMP in response to cholera toxin have been found to be greatly reduced by Ca^{2+} depletion (M. Brostrom, unpublished results). Similar responses were found for other cell lines in culture including chinese hamster ovary (CHO) cells, pituitary (GH_3) cells, neuroblastoma (Neuro 2A) cells, and neuroblastoma-glioma (NG108-15) hybrid cells. Calmodulin has not been proven, as yet, to be involved in these effects of Ca^{2+} on cyclic AMP accumulations in intact cells.

The adenylate cyclase of *Bordetella pertussis* has been recently reported by Wolff *et al.* [15] to be activated 100- to 1000-fold by calmodulin. Activation was prevented by EGTA but, once activated, the enzyme was only slightly inhibited by the chelator. Since calmodulin is apparently not present in this prokaryote, this activation is presumably actifactual. The broader significance of this result, however, may be that calmodulin became an integral Ca^{2+} metalloprotein component of the enzyme. If so, forms of mammalian adenylate cyclase that are not activated significantly by Ca^{2+} or inhibited by EGTA may, by analogy with this prokaryotic enzyme, have calmodulin as an intrinsic metalloprotein component. It is also possible that activation of the prokaryotic enzyme by calmodulin was indirect, with calmodulin activating a primary enzyme which then activated the adenylate cyclase.

Calmodulin-dependent cyclic nucleotide phosphodiesterase (for reviews see Refs. 21, 22 *and* 25)

Only one of a number of forms of cyclic nucleotide phosphodiesterase is known to require calmodulin for activity. Although structural and functional relationships may exist among these forms, they have not been clarified. The form of phosphodiesterase activated by calmodulin has a higher apparent K_m for cAMP ($\simeq$ 50–200 μM) than for cGMP ($\simeq$ 2–20 μM), but the question of whether it functions physiologically as a cGMP or cAMP phosphodiesterase, or both, has not been resolved. Since it was the first enzyme shown to be activated by calmodulin and is easily assayed, it has been studied extensively by many workers.

Recent work has focussed on the purification and physical characterization of this phosphodiesterase [65–68]. Ho *et al.* [65], in early work, reported purification of the enzyme from bovine heart extracts of 4000- to 5000-fold to an estimated 80 per cent of homogeneity. This preparation had a specific activity of 120 μmoles cAMP hydrolyzed $\cdot$ min^{-1} $\cdot$ mg protein (120 units). The preparation was unstable and of low

yield and was not subjected to a detailed characterization. Subsequent preparations of the phosphodiesterase have depended heavily on affinity chromatography using either calmodulin–Sepharose or blue dextran–Sepharose. Klee *et al.* [66] purified the enzyme 3000-fold in 2 per cent yield from bovine brain and described it as being homogeneous and activated 8- to 10-fold by calmodulin with a specific activity of 80–100 units. The enzyme hydrolyzed both cAMP and cGMP with respective apparent K_m values of 100 and 2.5 to 9 μM in the presence of Ca^{2+} and calmodulin. The enzyme was described as having three subunits of M_r–61,000, 59,000, and 15,000 respectively. LaPorte *et al.* [67] purified the enzyme approximately 14,000-fold from bovine heart in 10 per cent yield with a specific activity of 275 units. The enzyme was activated 10-fold by calmodulin and Ca^{2+}. This preparation ran as a single band on SDS-polyacrylamide gel electrophoresis with an apparent M_r of 57,000. Through dimethyl suberimidate cross-linking studies the enzyme was determined to be a dimer of these units, capable of binding two moles of calmodulin, to form an overall tetramer. The variance in these results with those of Klee *et al.* [66] was interpreted to arise from a contamination of the Klee preparation with a co-purifying calmodulin-binding protein with subunits of M_r 61,000 and 15,000. Morrill *et al.* [68] purified the enzyme 2000-fold from bovine brain to apparent homogeneity and a specific activity of 170 units. The enzyme was described as existing as a dimer of two identical subunits of 59,000 M_r. The enzyme was activated 2-fold by calmodulin in conjunction with Ca^{2+} and exhibited apparent K_m values of 9 ± 1 μM for cGMP and 150 ± 50 μM for cAMP. These results support the subunit studies of LaPorte *et al.* [67] and are consistent with the kinetic studies of Klee *et al.* [66]. The poor degree of activation by calmodulin may have arisen from proteolytic activation during preparation of the enzymes.

The purification of the phosphodiesterase to homogeneity should allow potential physical relationships of the Ca^{2+}-dependent phosphodiesterase subunits with other phosphodiesterase enzymes to be determined by such techniques as immunologic cross-reaction studies. It should also prove possible to characterize more fully the interactions of the enzyme with Ca^{2+} and calmodulin, perhaps, gaining insight into how calmodulin promotes activation of the enzyme and how the ternary complex (Ca^{2+}-calmodulin–enzyme) dissociates.

Calmodulin-dependent protein phosphorylation (for reviews see Refs. 21, 25, 26, and 69)

A rapid expansion has occurred during the past several years in our understanding of regulatory mechanisms involving Ca^{2+}-dependent phosphorylation of specific proteins in various cell types. The existence of a calmodulin-dependent myosin light chain kinase has been reported in smooth [70], skeletal [71, 72], and cardiac muscle [73] cells as well as in non-muscle cells [74, 75]. When activated by calmodulin and Ca^{2+}, this protein kinase phosphorylates the regulatory (P) light chain (M_r 20,000) of myosin [76]. In smooth muscle this phosphorylation event has been proposed by Sobieszek and Small [77] to permit myosin to interact with actin, allowing the initiation of contraction. Subsequent dephosphorylation of the light chain is a Ca^{2+}-independent event. This model of contraction has achieved wide, but not universal, support. Ebashi and co-workers [78–80] reported that Ca^{2+}-dependent superprecipitation of chicken gizzard actomyosin (believed to reflect actin-myosin interaction) and its subsequent re-solubilization by chelators occurs with a time dependence markedly discrepant from light chain phosphorylation and dephosphorylation. These workers reported that chicken gizzard and bovine aortic smooth muscle possess a Ca^{2+}-binding protein, termed leiotonin C (M_r 18,000), which differs from both troponin C and calmodulin [80, 81]. Leiotonin C is reported to be a dissociable component of a leiotonin–protein complex that in the presence of Ca^{2+}, interacts with aortic actin and allows superprecipitation with myosin to occur. Although calmodulin could functionally interchange with leiotonin C, it was not the native component of the complex. Further study will be required to resolve the respective contributions of calmodulin-dependent myosin light chain kinase and leiotonin in the control of actin–myosin interaction in smooth muscle.

Myosin light chain kinase has been purified from turkey gizzard and serves as a substrate for the catalytic subunit of cyclic AMP-dependent protein kinase [82]. The myosin light chain kinase is phosphorylated at a single site with a resultant 2-fold decrease in its rate of catalysis of myosin light chain phosphorylation. It is conceivable that phosphorylation of myosin light chain kinase in response to elevated cyclic AMP concentrations in smooth muscle cells may relate to the mechanism by which cAMP induces smooth muscle relaxation.

Skeletal muscle myosin light chain kinase (M_r 77,000–80,000) was originally purified by Pires and Perry [83]. These workers reported that the enzyme was stimulated by micromolar concentrations of Ca^{2+} to phosphorylate a partially purified light chain fraction in incubations without added calmodulin. The enzyme transferred phosphate from ATP, but not from GTP, ITP, CTP or UTP, and was inactive in phosphorylating casein, mixed calf thymus histones, phosphorylase *b*, or troponin. More recently, Nairn and Perry [72] have reported that this preparation of enzyme requires calmodulin for expression of activity when purified regulatory light chain is used as substrate. The previous lack of calmodulin dependence was attributed to calmodulin contamination of the light chain fraction. Recently, myosin light chain kinase has also been purified from rabbit skeletal muscle and bovine cardiac muscles by protocols involving calmodulin affinity chromatography [84]. The physical and kinetic properties of the myosin light chain kinase purified from these diverse sources are shown in Table 2. The most pronounced difference among these enzymes involves their maximum rates of light chain phosphorylation, with the enzyme from heart exhibiting a 100- to 1000-fold lower specific activity. Walsh *et al.* [73] have speculated that this low activity may be due to proteolytic modification during isolation.

The function of myosin light chain phosphoryl-

Table 2. Properties of various, purified myosin light chain kinases

Source	M_r	K_m for light chain (μM)	K_m for ATP (μM)	Specific activity (μmoles·min^{-1}·mg^{-1})	Ref.
Human platelets	105,000	18	121	3.1	75
Bovine heart	85,000	21	175	0.03	73
Rabbit skeletal muscle	80,000[a] 80,000[c]	40–50[a] 24[c]	200–400[b] 280[a]	15–30[a] 4.3[c]	72[a], 83[b] 84[c]

ation *in vivo* appears to vary from one tissue to another. Hidaka *et al.* [85, 86] have proposed an essential involvement of calmodulin in the process of smooth muscle contraction. This proposal was based upon pharmocological studies conducted with isolated rabbit aortic strips induced to contract by such agents as KCl, $CaCl_2$, norepinephrine, histamine, and prostaglandin F2α. *N*-(6-Aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7) and various psychotropic drugs, such as chlorpromazine, were found to relax the contracted strips. These relaxing drugs are known to bind to calmodulin and to inhibit its biological activity. The order of potency of the drugs in inhibiting contractility paralleled their potencies as inhibitors of the calmodulin-dependent form of cyclic nucleotide phosphodiesterase. The slow time course of the contraction–relaxation cycle in smooth muscle would be compatible with enzymatic mediation through a phosphorylation–dephosphorylation mechanism. Similar considerations are not applicable to skeletal muscle systems where the contraction–relaxation cycle is rapid and where Ca^{2+}-induced contraction is effected by an allosteric interaction on the thin filament effected by troponin C. The role of light chain phosphorylation in skeletal muscle is, therefore, unclear. Manning and Stull [87] have examined light chain phosphorylation in relation to isometric tension development in rat extensor digitorum longus muscle. Following a 1-sec tetanic contraction, light chain phosphate increased from 0.1 to 0.75 mole/mole light chain. However, while relaxation occurred rapidly, light chain phosphate content declined slowly and correlated to post-tetanic potentiation of peak twitch tension. These workers suggested that light chain phosphorylation is not obligatory for contraction in skeletal muscle but may play a role in post-tetanic potentiation. Comparable studies with intact tissue are as yet unreported for heart and non-muscle cells, and the functional role of light chain phosphorylation in these systems remains to be determined.

Phosphorylation b *kinase*

Phosphorylase *b* kinase (M_r 1.28×10^6) from skeletal muscle is a Ca^{2+}-dependent enzyme [88] that comprised four types of subunits (α, β, γ and δ).

The δ subunit has been identified as calmodulin [42]. Calmodulin appears to associate at two sites of very different affinity on the enzyme [89–91]. Binding at one site occurs in stoichiometric amounts with the other subunits and is nearly irreversible. Dissociation does not occur readily with either Ca^{2+} chelators or 8 M urea in the presence of Ca^{2+}, but it is reported to occur with chelators plus 8 M urea [89] or with enzyme denaturation [42]. Binding at the second site confers additional activity upon the enzyme as measured with such substrates as phosphorylase *b*, glycogen synthase, and phosphorylase kinase (autophosphorylation) [91]. Variable degrees of enzyme activation ranging from 2- to 6-fold have been observed at both pH 6.8 and 8.2 [89–91]. These reports, however, are not in accord regarding the pH at which the larger fold activation occurs. This activation is prevented by other calmodulin-binding proteins. Calmodulin binding at the lower affinity site is reversible with EGTA on calmodulin–Sepharose affinity columns. Phosphorylase kinase preparations, exposed to limited proteolysis by trypsin, lose the ability to be activated by exogenous calmodulin [90, 91]. Trypsin-treated phosphorylase kinase, however, retains its Ca^{2+} dependence, but at a much increased affinity [89]. This cation requirement is apparently expressed through the endogenous calmodulin component integrated into the enzyme at the high affinity binding site. Phenothiazine antipsychotic agents have been reported by Walsh *et al.* [91] to inhibit both the basal activity of phosphorylase kinase and the increment in activity occurring upon addition of calmodulin. Shenolikar *et al.* [89] report that only the increment was inhibited.

Troponin C and the complete troponin complex (TNC–TNI-TNT), but not the troponin I-troponin C complex, are also reported to substitute for calmodulin at the weaker binding site in the activation of phosphorylase kinase [89, 92]. A 200-fold higher concentration of either troponin C or the complex as compared to calmodulin was required for half-maximal activation. Arguments based on considerations of the relative concentrations of calmodulin and troponin in skeletal muscle have been advanced by Cohen *et al.* [92] to suggest that troponin C, rather than calmodulin, may be the more important physiological regulator at the second binding site of phosphorylase kinase.

Glycogen synthase kinase

After phosphorylase kinase from skeletal muscle was found to activate glycogen synthase [93–95], the earlier reports of a calmodulin-dependent glycogen synthase kinase from that tissue [96, 97] were placed in perspective. Most recently, however, Payne and Soderling [98] reported the discovery of a glycogen

synthase kinase from liver that is highly calmodulin dependent and not identical with phosphorylase kinase. This enzyme appeared to be specific for glycogen synthase in that it did not phosphorylate muscle phosphorylase, myosin light chain, casein, or histone.

Calmodulin-dependent protein phosphorylation in brain

Autophosphorylation of certain rat brain cytosolic proteins is stimulated by Ca^{2+} in conjunction with calmodulin [99]. At least three calmodulin-dependent protein kinase activities have been partially purified from cytosolic substrate proteins by affinity chromatography on calmodulin–Sepharose and resolved from each other by subsequent gel filtration on Sepharose 6B [100]. These activities exhibited varying degrees of specificity for phosphorylase *b* to *a* conversion, tryptophan monooxygenase activation, casein phosphorylation, and myosin light chain phosphorylation. Calmodulin-dependent activation of brain tryptophan-5-monooxygenase in medium containing MgATP had been observed previously by these workers [101]. This observation has been confirmed and extended by Kuhn *et al.* [102] who noted the suppression of the calmodulin-dependent activation of the enzyme by various antipsychotic agents. These observations suggest that calmodulin may confer Ca^{2+}-dependent regulation on serotonin biosynthesis, since tryptophan-5-monooxygenase is the rate-limiting enzyme in this anabolic pathway.

Ca^{2+}-dependent autophosphorylation of membrane bound proteins has also been observed in brain [103, 104] and other tissues [105, 106]; however, the functions of these phosphorylated proteins and the nature of the enzyme(s) catalyzing their phosphorylation are unknown.

Calmodulin and Ca^{2+} transport (for reviews see Refs. 21, 26, and 107)

The original findings that a $(Ca^{2+} + Mg^{2+})$ ATPase [108, 109] and Ca^{2+} transport in "inside out" human erythrocyte vesicles [110, 111] are each stimulated several-fold or more by calmodulin have been supported by subsequent research with the erythrocyte system [107]. Calmodulin, therefore, appears to couple intracellular free Ca^{2+} concentrations to a graded activation of an ATPase responsible for pumping Ca^{2+} from the cell. Transport of Ca^{2+} across the plasmalemma by this system would continue until intracellular free Ca^{2+} concentrations dropped below the values necessary to maintain the Ca^{2+}–calmodulin. In effect, the K_a of calmodulin for Ca^{2+} would determine the basal free Ca^{2+} that could be reached by the resting cell, barring other types of Ca^{2+} pumps.

A strong data base is lacking for generalizing this model to other cell types. Sobue *et al.* [112] have reported that synaptic plasma membranes from rat brain, but not other brain fractions, contain a $(Ca^{2+} + Mg^{2+})$ ATPase that is activated 2-fold by calmodulin. A small but reproducible stimulation of Ca^{2+} transport in canine cardiac sarcoplasmic reticulum has also been reported [113, 114]. This increment in transport has been attributed by Le Peuch *et al.* [114] to phosphorylation of phospholamban in the sarcoplasmic reticulum by a calmodulin-dependent protein kinase. Phosphorylation by the cAMP-dependent protein kinase at a different site on phospholamban also stimulated Ca^{2+} transport. Wuytack *et al.* [115] have recently compared Ca^{2+} transport and $(Ca^{2+} + Mg^{2+})$ ATPase in microsomal fractions from porcine coronary smooth muscle, canine heart, and human erythrocytes, in response to calmodulin. Despite various modifications in preparatory and assay conditions, large effects of calmodulin on either variable were found only with the erythrocyte preparations. It is presently difficult to evaluate the significance of the small stimulations ($\simeq$ 30–40 per cent) of the $(Ca^{2+} + Mg^{2+})$ ATPase and of Ca^{2+} transport found thus far in response to calmodulin in the heart and smooth muscle microsomal systems.

Calmodulin and other enzymes

Calmodulin has been reported to confer Ca^{2+}-dependent activation on an NAD kinase found in plants [116]. Calsequestrin, purified to homogeneity, has been reported to possess protein kinase activity that is activated to a small extent (1.5-fold) by calmodulin in conjunction with Ca^{2+}, using phosphorylase *b* as substrate [117]. A platelet phospholipase may be stimulated by calmodulin [118]. A Ca^{2+}-dependent protein kinase found in many tissues and studied in detail by Nishizuka and co-workers [119] has properties that may arise from non-dissociable calmodulin. Calmodulin inhibits histone phosphatase activity by substrate-directed effects [120, 121].

Microtubule assembly-disassembly (for review see Ref. 24)

Marcum *et al.* [122] have reported that, in the presence of Ca^{2+}, calmodulin inhibits the polymerization *in vitro* of brain microtubules and promotes disassembly of preformed tubules. This effect requires high (approximately 10^{-4} M) concentrations of calmodulin. Rabbit skeletal muscle troponin C was a more effective inhibitor, whereas carp muscle parvalbumin was less effective. These studies have been confirmed and extended by Nishida *et al.* [123, 124] who reported that the concentration of Ca^{2+} required for half-maximum inhibition of microtubule assembly decreased with increasing calmodulin concentration. The authors proposed that tubulin interacted selectively with a Ca^{2+}-calmodulin complex (but not with Ca^{2+}-free calmodulin) to form a non-polymerizable ternary complex. The binding of calmodulin to purified tubulin dimer free of microtubular-associated proteins was shown to occur in a Ca^{2+}–dependent manner. More recently, these workers have purified calmodulins and tubulins from porcine brain and sea urchin and have examined the effects of calmodulin on microtubule polymerization in cross recombinations [125]. Both sea urchin and brain calmodulin associated with brain tubulin, inhibiting its polymerization in a Ca^{2+}-dependent manner. These calmodulins exhibited the same mobility on SDS polyacrylamide electrophoresis and the same ability to activate brain cyclic nucletide phosphodiesterase. But neither porcine brain calmodulin nor sea urchin calmodulin inhibited sea urchin microtubule assembly. Addition of microtubular-associated proteins did not restore cal-

modulin sensitivity. While the calmodulins from the two systems were closely related or identical, the tubulin proteins were not. Thus, the interaction of calmodulin with tubulin has several features which bring into question the potential physiologic significance of calmodulin as a regulator of microtubule depolymerization. The effect of calmodulin on depolymerization is expressed at much higher calmodulin concentrations than those required for regulation of other systems. Specificity for calmodulin in preference to other Ca^{2+}-binding proteins is lacking in the polymerization process. Although microtubule polymerization–depolymerization is a universal process for eukaryotes, calmodulin does not affect depolymerization in sea urchin.

Functional interchangeability of various calciproteins

Skeletal muscle contains at least three homologous proteins with high affinity for Ca^{2+}—troponin C, calmodulin, and parvalbumin. Cardiac muscle possesses a variant form of troponin C and calmodulin, and smooth muscle has calmodulin and leiotonin C. The presence of two or more of these closely related proteins within a given cell type introduces the possibility of overlapping functionalities. The comparative ability of these proteins to confer Ca^{2+} dependence on diverse calcium-modulated enzyme systems has been examined by many investigators. It has been reported that skeletal muscle troponin, troponin C, or parvalbumin preparations can substitute for calmodulin in conferring Ca^{2+} dependence on cyclic nucleotide phosphodiesterase [126, 127], adenylate cyclase [15], myosin light chain kinase [72], phosphorylase kinase [92], or microtubule depolymerization [122].

Most commonly, calmodulin is found to be 100–1000 times more potent than troponin C, whole troponin, or parvalbumin in activating these enzymes. The brain microtubule depolymerization response is an exception in that it is more sensitive to troponin C than to calmodulin. These great differences in potencies demand extremely high degrees of resolution of troponin, troponin C, and parvalbumin from calmodulin in their purification from skeletal muscle. Such purification is complicated by the similarity of the proteins and the difficulty of detecting trace contaminations of calmodulin by conventional techniques, such as stained gel electrophoretography. Therefore, additional criteria are necessary to ensure that biological activity does not originate from trace contaminations of calmodulin in the calciprotein preparation. Recently, for example, Cohen *et al.* [92] demonstrated that whole troponin or troponin C can activate phosphorylase kinase at concentrations 100-fold higher than those required for calmodulin to produce activation. Activation did not appear to originate from calmodulin contamination of the troponin preparations since the activation was not prevented by a brain calmodulin binding protein, calcineurin [128], capable of reversing activation by authentic calmodulin.

Comparable degrees of activation by troponin C [126] and parvalbumin [127] of the brain cyclic nucleotide phosphodiesterase have been reported to occur at 600- and 1000-fold higher concentrations, respectively, than that required for calmodulin. This observation was examined further by Wolff and Brostrom [21] and LeDonne and Coffee [129] who were unable to find activation of similar phosphodiestrase preparations by parvalbumin. Stevens *et al.* [130] found no activation of phosphodiesterase with troponin C. Wolff and Brostrom [21] found activation at 2000-fold higher concentrations of troponin C than of calmodulin, but the activation was suppressed by chlorpromazine at concentrations reflecting the dissociation constant of the drug from calmodulin rather than from troponin C. The activation was, therefore, attributed to a calmodulin contaminant in the preparation of troponin C.

The ability of rabbit skeletal muscle troponin C or frog parvalbumin to substitute for brain calmodulin in activating myosin light chain kinases from cardiac, skeletal, and smooth muscle has been studied by Walsh *et al.* [131]. No biological activity was reported for these proteins at concentrations 1000-fold higher than activating concentrations of calmodulin. A similar lack of activity for troponin C and parvalbumin in activating erythrocyte ATPase has been noted [108, 109].

It appears, therefore, that calmodulin is unique among these calciproteins in being multifunctional. Troponin C and parvalbumin show weak, if any, biological activity in conferring Ca^{2+} dependence in diverse enzyme systems in accord with their restricted tissue distributions. In constrast, calmodulin has been shown to be effective in forming complexes with troponin I that can restore Ca^{2+} dependence to reconstituted actomyosin [132]. There is no evidence, however, that calmodulin is associated with thin filaments or is a native factor conferring Ca^{2+} dependence on skeletal or cardiac muscle contraction.

Interaction of drugs with calmodulin (for reviews see Ref. 21)

Phenothiazine antipsychotic agents inhibit all enzymes that require calmodulin for activity. This inhibition is reversed by increasing the concentration of calmodulin in the incubation [45, 56, 85, 133] but not by increasing the concentration of Ca^{2+}. These inhibitory effects are traceable to the original observation by Levin and Weiss [134] that calmodulin binds phenothiazine at two types of sites. One of these sites is inconsequential, in that binding occurs with only low affinity with or without the presence of Ca^{2+}. The other side binds 2 moles phenothiazine/mole calmodulin with high affinity ($K_d \simeq 1$–$10\ \mu M$) in a Ca^{2+}-dependent manner. Binding to this site was also reported with Sr^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, and Mn^{2+} but not with Mg^{2+} or Ba^{2+}. Binding was reversed by EGTA. Troponin C also was reported to bind phenothiazine in a Ca^{2+}-dependent manner, although, with less affinity. It is believed, therefore, that the phenothiazine binds to the active conformation of calmodulin and blocks the ability of the protein to interact with, and activate, calmodulin-dependent enzymes. Although this model is consistent with the experimental data base for these enzymes, phenothiazine antipsychotics have lipid solubilities and detergent properties that provide them with a wide range of effects on membranes and

membrane-associated processes [135, 136]. These effects are probably largely independent of calmodulin. Forms of adenylate cyclase not known to be activated by Ca^{2+} and calmodulin are inhibited by phenothiazines [137]. For example, the dopamine-sensitive adenylate cyclase from brain is exquisitely sensitive to inhibition by trifluoperazine ($K_i = 8 \times 10^{-9}$ M) [138]. Jackowski *et al.* [139] recently reported that pretreatment of liver plasma membranes with trifluoperazine caused marked depletion of membrane Ca^{2+} stores and inhibited the activity of an associated adenylate cyclase. Following removal of the drug by washing, the enzyme could be totally reactivated by the addition of Ca^{2+}. Calmodulin without Ca^{2+} partially restored the activity. The calmodulin-dependent phosphodiesterase can be activated by various phospholipids without Ca^{2+} or calmodulin, and this activation is reversed by phenothiazines [45].

Levin and Weiss [140] have reported that high affinity, Ca^{2+}-dependent binding to calmodulin is a general property of clinically effective antipsychotic agents, and that this binding parallels the ability of these compounds to inhibit calmodulin-dependent phosphodiesterase activity. Antianxiety and antidepressant agents bound more poorly and were less effective inhibitors. Other agents, such as LSD, amphetamine, phenobarbital, morphine, and various biogenic amines, that affect the central nervous system but are devoid of antipsychotic activity, neither bound nor inhibited. The affinity of binding to calmodulin qualitatively paralleled the potency of the agents as antipsychotics and their ability to produce extrapyramidal effects. Some lack of stereospecificity was found in this study. In a more extensive investigation of stereospecificity, Norman *et al.* [141] have found that both the clinically active and inactive isomers of a series of neuroleptics were equally effective inhibitors of the phosphodiesterase. The IC_{50} values for inhibition correlated well with the octanol:H_2O partition coefficients of the drugs. These workers argued that the observed lack of stereospecificity and the relatively high concentrations of neuroleptic required for enzyme inhibition effectively ruled out an involvement of calmodulin in the therapeutic actions of these drugs. Additional insight into this issue may be gained with the development of additional inhibitors of calmodulin function. One such agent is *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide studied extensively by Hidaka *et al.* [85, 86].

Conclusions and perspectives

A wealth of new literature continues to support the conclusion that calmodulin is a multifunctional, Ca^{2+}-dependent enzyme regulator. Its properties and distribution are in accord with its functioning as a major, if not the predominant, Ca^{2+} receptor protein in most cell types. Calmodulin acting in this capacity would couple transient increases in intracellular free Ca^{2+} resulting from extracellular stimuli to the activation of rate-limiting enzymes in diverse metabolic processes. In this capacity it would co-ordinate the processes involved in maintaining the functional status of the cell. Indeed, by activation of plasmalemmal Ca^{2+} transport systems responsible for pumping Ca^{2+} from the cell, calmodulin could presumably determine the concentration of Ca^{2+} in the resting cell and the termination of Ca^{2+}-dependent responses.

At present a number of important problems remain to be solved. It is not currently known how many additional calmodulin-regulated enzymes remain to be discovered. Indeed, additional enzymes patterned after phosphorylase kinase may exist, possessing calmodulin as a non-dissociable subunit. There is also an excellent possibility of creating artifactual enzyme activations *in vitro* from the addition of large amounts of calmodulin to enzyme incubations. It is to be hoped that additional corroborating evidence can be obtained *in vivo* for stimulatory effects *in vitro*. We presently know very little about concerted control by calmodulin of Ca^{2+}-dependent enzymes within the same cell type or the relationship between calmodulin-regulated enzymes in different cell compartments. Various reviewers [1, 2] have emphasized the many complex interrelationships between second messenger systems involving Ca^{2+}, cAMP, and possibly cGMP. These systems appear to function as discrete yet interdigitated elements of an overall biological control mechanism. Much more information will be required before the systems and their interrelationships can be fully appreciated in terms of a common theme with species and tissues variations. For example, the large group of enzymes that are phosphorylated by the various calmodulin and cyclic nucleotide-dependent protein kinases could conceivably respond catalytically with functionally independent, complementary, or antagonistic reactions. The relationships between these protein kinases could be universal or vary between selected cell populations by virtue of different cellular protein substrate components. Alternatively, the profile of protein kinase activities could vary with cell type. In systems of such complexity, the possibjlities for future pharmacological intercession would appear to be extremely promising.

Acknowledgements—The authors thank Mrs. Carolyn Clark for her care in typing this manuscript. Preparation of the manuscript was supported by U.S. Public Health Service Grants NS 11340 and NS 11252.

REFERENCES

1. H. Rasmussen, D. B. P. Goodman and A. Tenenhouse, *CRC Crit. Rev. Biochem.* **1**, 95 (1972).
2. M. J. Berridge, *Adv. Cyclic Nucleotide Res.* **6**, 1 (1975).
3. D. J. Wolff and F. L. Siegel, *J. biol. Chem.* **247**, 4180 (1972).
4. T. S. Teo, T. H. Wang and J. H. Wang, *J. biol. Chem.* **248**, 588 (1973).
5. Y. M. Lin, Y. P. Liu and W. Y. Cheung, *J. biol. Chem.* **249**, 4943 (1974).
6. S. R. Childers and F. L. Siegel, *Biochim. biophys. Acta* **405**, 99 (1975).
7. D. M. Watterson, W. G. Harrelson, P. M. Keller, F. Sharief and T. C. Vanaman, *J. biol. Chem.* **251**, 4501 (1976).
8. J. R. Dedman, J. D. Potter, R. L. Jackson, J. D. Johnson and A. R. Means, *J. biol. Chem.* **252**, 8415 (1977).
9. R. J. A. Grand and S. V. Perry, *Fedn Eur. Biochem. Soc. Lett.* **92**, 137 (1978).

10. H. W. Jarrett and J. T. Penniston, *J. biol. Chem.* **253**, 4676 (1978).
11. D. Waisman, F. C. Stevens and J. H. Wang, *Biochem. biophys. Res. Commun.* **65**, 975 (1975).
12. J. Kuznicki, L. Kuznicki and W. Drabikowski, *Cell Biol. Int. Rep.* **3**, 17 (1979).
13. S. L. Gomes, L. Mennucci and J. C. Maia, *Fedn Eur. Biochem. Soc. Lett.* **99**, 39 (1979).
14. J. M. Anderson and M. J. Cormier, *Biochem. biophys. Res. Commun.* **84**, 595 (1978).
15. J. Wolff, G. H. Cook, A. R. Goldhammer and S. A. Berkowitz, *Proc. natn Acad. Sci. U.S.A.* **77**, 3841 (1980).
16. S. Kakiuchi, R. Yamazaki, Y. Teshima, K. Uenishi, S. Yasuda, A. Kashiba, K. Sobue, M. Oshima and T. Nakajima, *Adv. Cyclic Nucleotide Res.* **9**, 253 (1978).
17. A. Vandermeers, P. Robberecht, M. Vandermeers-Piret, J. Rathe and J. Christopher, *Biochem. biophys. Res. Commun.* **84**, 1076 (1978).
18. M. J. Welsh, J. R. Dedman, B. R. Brinkley and A. R. Means, *Proc. natn. Acad. Sci. U.S.A.* **75**, 1867 (1978).
19. B. Andersen, M. Osborn and K. Weber, *Cytobiologie* **17**, 354 (1978).
20. J. F. Harper, W. Y. Cheung, R. W. Wallace, H. L. Huang, S. N. Levine and A. L. Steiner, *Proc. natn Acad. Sci. U.S.A.* **77**, 366 (1980).
21. D. J. Wolff and C. O. Brostrom, *Adv. Cyclic Nucleotide Res.* **11**, 27 (1979).
22. J. H. Wang and D. M. Waisman, *Curr. Topics cell Regulat.* **15**, 47 (1979).
23. W. Y. Cheung, *Science* **207**, 19 (1980).
24. A. R. Means and J. R. Dedman, *Nature, Lond.* **285**, 73 (1980).
25. C. B. Klee, T. H. Crouch and P. G. Richman, *A. Rev. Biochem.* **49**, 489 (1980).
26. M. P. Walsh, C. J. LePeuch, B. Vallet, J. C. Cavadore and J. G. Demaille, *J. molec. cell. Cardiology*, **12**, 1091 (1980).
27. T. C. Vanaman, F. Sharief, J. L. Awramik, P. A. Mandel and D. M. Watterson, in *Contractile Systems in Non Muscle Systems* (Eds. S. V. Perry, A. Margreth and R. S. Adelstein), pp. 165–176. North-Holland and Elsevier, Amsterdam (1976).
28. D. M. Watterson, F. Sharief and T. C. Vanaman, *J. biol. Chem.* **255**, 962 (1980).
29. J. R. Dedman, R. L. Jackson, W. E. Schreiber and A. R. Means, *J. biol. Chem.* **253**, 343 (1978).
30. T. C. Vanaman and F. Sharief, *Fedn Proc.* **38** (3), 788 (1979).
31. T. S. Teo and J. H. Wang, *J. biol. Chem.* **248**, 5950 (1973).
32. C. B. Klee, *Biochemistry* **16**, 1017 (1977).
33. D. J. Wolff, P. G. Poirier, C. O. Brostrom and M. A. Brostrom, *J. biol. Chem.* **252**, 4108 (1977).
34. J. Haiech, B. Vallet, R. Aquaron and J. G. Demaille, *Analyt. Biochem.* **105**, 18 (1980).
35. M. Walsh, F. C. Stevens, K. Oikawa and C. M. Kay, *Can. J. Biochem.* **57**, 267 (1979).
36. K. B. Seamon, *Biochemstry* **19**, 207 (1980).
37. C. O. Brostrom and D. J. Wolff, *Archs Biochem. Biophys.* **172**, 301 (1976).
38. C. O. Brostrom, M. A. Brostrom and D. J. Wolff, *J. biol. Chem.* **252**, 5677 (1977).
39. S. Katz, B. D. Roufogalis, A. D. Landman and L. Ho, *J. supramolec. Struct.* **10**, 215 (1979).
40. D. K. Blumenthal and J. T. Stull, *Biochemistry*, **19**, 5608 (1980).
41. M. Kilimann and L. M. G. Heilmeyer, Jr., *Eur. J. Biochem.* **73**, 191 (1977).
42. P. Cohen, A. Burchell, J. G. Foulkes, P. T. W. Cohen, T. C. Vanaman and A. C. Nairn, *Fedn Eur. Biochem. Soc. Lett.* **92**, 287 (1978).
43. M. Yazawa, H. Kuwayama and K. Yagi, *J. Biochem. Tokyo* **84**, 1253 (1978).
44. J. H. Wang, T. S. Teo, H. C. Ho and F. C. Stevens, *Adv. Cyclic Nucleotide Res.* **5**, 179 (1975).
45. D. J. Wolff and C. O. Brostrom, *Archs Biochem. Biophys.* **173**, 720 (1976).
46. Y. P. Liu and W. Y. Cheung, *J. biol. Chem.* **251**, 4193 (1976).
47. C. O. Brostrom and D. J. Wolff, *Archs Biochem. Biophys.* **165**, 715 (1974).
48. P. V. Avdonin and V. A. Tkachuk, *Dok. Akda. Nauk USSR* **238**, 13 (1978).
49. T. Morishima, *Agric. biol. Chem. Tokyo* **43**, 1127 (1979).
50. S. Leoni, S. Spagnuolo and A. Panzali *Fedn Eur. Biochem. Soc. Lett.* **92**, 63 (1978).
51. I. Valverde, A. Vandermeers, R. Anjaneyulu and W. J. Malaisse, *Science* **206**, 225 (1979).
52. G. W. G. Sharp, D. E. Wiedenkeller, D. Kaelin, E. G. Siegel and C. B. Wollheim, *Diabetes* **29**, 74 (1980).
53. N. C. LaDonne and C. J. Coffee, *Fedn Proc.* **38**, 317 (1979).
54. T. J. Lynch, E. A. Tallant and W. Y. Cheung, *Archs Biochem. Biophys.* **182**, 124 (1977).
55. J. Moss and M. Vaughan, *Proc. natn. Acad. Sci. U.S.A.* **74**, 4396 (1977).
56. M. A. Brostrom, C. O. Brostrom and D. J. Wolff, *Archs biochem. Biophys.* **191**, 341 (1978).
57. W. A. Toscano, Jr., K. R. Westcott, D. C. LaPorte and D. R. Storm, *Proc. natn Acad. Sci. U.S.A.* **76**, 5582 (1979).
58. E. J. Neer, *J. biol. Chem.* **254**, 2089 (1979).
59. K. R. Westcott, D. C. LaPorte and D. R. Storm, *Proc. natn. Acad. Sci. U.S.A.* **76**, 204 (1979).
60. E. M. Ross and A. G. Gilman, *A. Rev. Biochem.* **49**, 533 (1980).
61. M. A. Brostrom, C. O. Brostrom, B. M. Breckenridge and D. J. Wolff, *J. biol. Chem.* **251**, 4744 (1976).
62. D. Evain, C. Klee and W. B. Anderson, *Proc. natn. Acad. Sci. U.S.A.* **76**, 3962 (1979).
63. M. T. Piascik, P. L. Wisler, C. L. Johnson and J. D. Potter, *J. biol. Chem.* **255**, 4176 (1980).
64. M. A. Brostrom, C. O. Brostrom and D. J. Wolff, *J. biol. Chem.* **254**, 7548 (1979).
65. H. C. Ho, E. Wirch, F. C. Stevens and J. H. Wang, *J. biol. Chem.* **252**, 43 (1977).
66. C. B. Klee, T. H. Crouch and M. H. Krinks, *Biochemistry* **18**, 722 (1979).
67. D. C. LaPorte, W. A. Toscano, Jr. and D. R. Storm, *Biochemistry* **18**, 2820 (1979).
68. M. E. Morrill, S. T. Thompson and E. Stellwagen, *J. biol. Chem.* **254**, 4371 (1979).
69. R. S. Adelstein, *A. Rev. Biochem.* **49**, 921 (1980).
70. R. Dabrowska, D. Aromatorio, J. M. F. Sherry and D. J. Hartshorne, *Biochem. biophys. Res. Commun.* **78**, 1263 (1977).
71. K. Yagi, M. Yazawa, S. Kakiuchi, M. Oshima and K. Uenishi, *J. biol. Chem.* **253**, 1338 (1978).
72. A. C. Nairn and S. V. Perry, *Biochem. J.* **179**, 89 (1979).
73. M. P. Walsh, B. Vallet, F. Autrie and J. G. Demaille, *J. biol. Chem.* **254**, 12,136 (1979).
74. M. J. Yerna, R. Dabrowska, D. J. Hartshorne and R. D. Goldman, *Proc. natn. Acad. Sci. U.S.A.* **76**, 184 (1979).
75. D. R. Hathaway and R. S. Adelstein, *Proc. natn. Acad. Sci. U.S.A.* **76**, 1653 (1979).
76. J. V. Small and A. Sobieszek, *Eur. J. Biochem.* **76**, 521 (1977).
77. A. Sobieszek and J. V. Small, *J. molec. Biol.* **112**, 559 (1977).
78. T. Mikawa, Y. Nonomura and S. Ebashi, *J. Biochem., Tokyo* **82**, 1789 (1977).

79. M. Hirata, T. Mikawa, Y. Nonomura and S. Ebashi, *J. Biochem., Tokyo* **82**, 1793 (1977).
80. M. Hirata, T. Mikawa, Y. Nonomura and S. Ebashi, *J. Biochem., Tokyo* **87**, 369 (1980).
81. T. Mikawa, Y. Nonomura, M. Hirata, S. Ebashi and S. Kakiuchi, *J. Biochem., Tokyo* **84**, 1633 (1978).
82. R. S. Adelstein, M. A. Conti, D. K. Hathaway and C. B. Klee, *J. biol. Chem.* **253**, 8347 (1978).
83. E. M. V. Pires and S. V. Perry, *Biochem. J.* **167**, 137 (1977).
84. M. Yazawa and K. Yagi, *J. Biochem., Tokyo* **84**, 1259 (1978).
85. H. Hidaka, T. Yamaki, T. Tatsuka and M. Asano, *Molec. Pharmac.* **15**, 49 (1979).
86. H. T. Hidaka, Y. M. Naka, T. Tanaka, H. Hayashi and R. Kobayashi, *Molec. Pharmac.* **17**, 66 (1980).
87. D. R. Manning and J. T. Stull, *Biochem. biophys. Res. Commun.* **90**, 164 (1979).
88. C. O. Brostrom, F. L. Hunkeler and E. G. Krebs, *J. biol. Chem.* **246**, 1969 (1971).
89. S. Shenolikar, P. T. W. Cohen, P. Cohen, A. C. Nairn and S. V. Perry, *Eur. J. Biochem.* **100**, 329 (1979).
90. A. A. DePaoli-Roach, J. B. Gibbs and P. J. Roach, *Fedn Eur. Biochem. Soc. Lett.* **105**, 321 (1979).
91. K. X. Walsh, D. M. Millikin, K. K. Schlender and E. M. Reimann, *J. biol. Chem.* **255**, 5036 (1980).
92. P. Cohen, C. Picton and C. B. Klee, *Fedn Eur. Biochem. Soc. Lett.* **104**, 25 (1979).
93. P. J. Roach, A. A. DePaoli-Roach and J. Larner, *J. Cyclic Nucleotide Res.* **4**, 245 (1978).
94. N. Embi, D. B. Rylatt and P. Cohen, *Eur. J. Biochem.* **100**, 339 (1979).
95. T. R. Soderling, V. S. Sheorain and L. H. Ericsson, *Fedn Eur. Biochem. Soc. Lett.* **106**, 181 (1979).
96. A. K. Srivastava, D. M. Waisman, C. O. Brostrom and T. R. Soderling, *J. biol. Chem.* **254**, 583 (1979).
97. D. B. Rylatt, N. Embi and P. Cohen, *Fedn Eur. Biochem. Soc. Lett.* **98**, 76 (1979).
98. M. E. Payne and T. R. Soderling, *J. biol. Chem.* **255**, 8054 (1980).
99. T. Yamauchi and H. Fujisawa, *Biochem. biophys. Res. Commun.* **90**, 164 (1979).
100. Y. Yamauchi and H. Fujisawa, *Fedn Eur. Biochem. Soc. Lett.* **116**, 141 (1980).
101. T. Yamauchi and H. Fujisawa, *Biochem. biophys. Res. Commun.* **90**, 28 (1979).
102. D. M. Kuhn, J. P. O'Callaghan, J. Juskevich and W. Lovenberg, *Proc. natn. Acad. Sci. U.S.A.* **77**, 4688 (1980).
103. R. J. DeLorenzo and S. D. Freedman, *Biochem. biophys. Res. Commun.* **80**, 183 (1978).
104. H. Schulman and P. Greengard, *Nature, Lond.* **271**, 478 (1978).
105. H. Schulman and P. Greengard, *Proc. natn. Acad. Sci. U.S.A.* **75**, 5432 (1978).
106. M. Landt and J. M. McDonald, *Biochem. biophys. Res. Commun.* **93**, 881 (1980).
107. B. D. Roufogalis, *Can. J. Physiol. Pharmac.* **57**, 1331 (1979).
108. R. M. Gopinath and F. F. Vincenzi, *Biochem. biophys. Res. Commun.* **77**, 1203 (1977).
109. H. W. Jarrett and J. T. Penniston, *Biochem. biophys. Res. Commun.* **77**, 1210 (1977).
110. D. J. MacIntyre and W. J. Green, *Biochim. biophys. Acta* **510**, 373 (1978).
111. F. L. Larsen and F. F. Vincenzi, *Science* **204**, 306 (1979).
112. K. Sobue, S. Ichida, H. Yoshida, R. Yamazaki and S. Kakiuchi, *Fedn Eur. Biochem. Soc. Lett.* **99**, 199 (1979).
113. S. Katz and M. A. Remtulla, *Biochem. biophys. Res. Commun.* **83**, 1878 (1978).
114. C. J. Le Peuch, J. Haiech and J. G. Demaille, *Biochemistry* **18**, 5150 (1979).
115. F. Wuytach, G. De Schutter and R. Casteelo, *Biochem. J.* **190**, 827 (1980).
116. J. M. Anderson and M. J. Cormier, *Biochem. biophys. Res. Commun.* **84**, 595 (1978).
117. M. Varsanye and L. M. G. Heilmeyer, Jr., *Fedn Eur. Biochem. Soc. Lett* **103**, 85 (1979).
118. P. Y. K. Wong and W. Y. Cheung, *Biochem. biophys. Res. Commun.* **90**, 473 (1979).
119. T. Mori, Y. Takai, R. Minakuchi, B. Yu and Y. Nishizuka, *J. biol. Chem.* **255**, 8378 (1980).
120. R. L. Khandelwal, M. C. Kotello and J. H. Wang, *Archs Biochem. Biophys.* **203**, 244 (1980).
121. D. J. Wolff, J. M. Ross, P. N. Thompson, M. A. Brostrom and C. O. Brostrom, *J. biol. Chem.*, in press.
122. J. M. Marcum, J. R. Dedman, B. R. Brinkley and A. R. Means, *Proc. natn. Acad. Sci. U.S.A.* **75**, 3771 (1978).
123. E. Nishida, H. Kumagai, I. Ohtuki and H. Sakai, *J. Biochem., Tokyo* **85**, 1257 (1979).
124. H. Kumagai and E. Nishida, *J. Biochem., Tokyo* **85**, 1267 (1979).
125. E. Nishida and H. Kumagai, *J. Biochem., Tokyo* **87**, 143 (1980).
126. J. R. Dedman, J. D. Potter and A. R. Means, *J. biol. Chem.* **252**, 2437 (1977).
127. J. D. Potter, J. R. Dedman and A. R. Means, *J. biol. Chem.* **252**, 5609 (1977).
128. J. H. Wang and R. Desai, *J. biol. Chem.* **252**, 4175 (1977).
129. N. C. LeDonne and C. J. Coffee, *J. biol. Chem.* **254**, 4317 (1979).
130. F. C. Stevens, M. Walsh, H. C. Ho, T. S. Teo and J. H. Wang, *J. biol. Chem.* **251**, 4495 (1976).
131. M. P. Walsh, B. Vallet, J. C. Cavadore and J. G. Demaille, *J. biol. Chem.* **255**, 335 (1980).
132. G. W. Amphlett, T. C. Vanaman and S. V. Perry, *Fedn Eur. Biochem. Soc. Lett.* **72**, 163 (1976).
133. B. U. Raess and F. F. Vincenzi, *Molec. Pharmac.* **18**, 253 (1980).
134. R. M. Levin and B. Weiss, *Molec. Pharmac.* **13**, 690 (1977).
135. P. Seeman, *Pharmac. Rev.* **24**, 583 (1972).
136. P. Seeman, A. Staiman and M. Chau-Wong, *J. Pharmac. exp. Ther.* **190**, 123 (1974).
137. J. Wolff and A. B. Jones, *Proc. natn. Acad. Sci. U.S.A.* **65**, 454 (1970).
138. Y. C. Clement-Cormier, J. W. Kebabian, G. L. Petzold and P. Greengard, *Proc. natn. Acad. Sci. U.S.A.* **71**, 1113 (1974).
139. M. M. Jackowski, R. A. Johnson and J. N. Exton, *Biochim. biophys. Acta* **630**, 497 (1980).
140. R. M. Levin and B. Weiss, *J. Pharmac. exp. Ther.* **208**, 454 (1979).
141. J. A. Norman, A. H. Drummond and P. Moser, *Molec. Pharmac.* **16**, 1089 (1979).