Review Series to Celebrate Our 100th Volume

Calmodulin: a highly conserved and ubiquitous Ca^{2+} sensor

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Abstract: Calcium ions (Ca^{2+}) play critical roles in various biological phenomena. The free Ca^{2+} concentration in the cytoplasm of a resting cell is at the 10^{-7} M level, whereas that outside the cell is 10^{-3} M, creating a 10,000-fold gradient of Ca^{2+} concentrations across the cell membrane, separating the intracellular and extracellular solutions.[1),2)] When a cell is activated by external stimuli, the intracellular Ca^{2+} concentration increases to levels of 10^{-6}–10^{-5} M through Ca^{2+} entry from the extracellular solution via plasma membrane Ca^{2+} channels and/or Ca^{2+} release from intracellular stores. This transient increase in Ca^{2+} functions as an important signal mediated by Ca^{2+} sensors. Thus, Ca^{2+} signals are transmitted to intracellular loci such as distinct, localized targets of Ca^{2+} sensors. Among numerous Ca^{2+} sensors present in cells, calmodulin is a highly conserved and ubiquitous Ca^{2+} sensor.[3)]

Keywords: calmodulin, Ca^{2+} sensor, calmodulin targets, enzymatic regulation, cytoskeletal regulation

Daybreak of Ca^{2+} studies

The study of Ca^{2+} in biology was initiated by physiologists. S. Ringer first demonstrated the continuous beating activity of frog hearts in the presence of millimolar levels of Ca^{2+} in the blood.[4)] Subsequently, physiologists confirmed that extracellular Ca^{2+} in the millimolar order is required for the secretion of catecholamines from the adrenal medulla and some hormones from endocrine organs.[5)]

L.V. Heilbrunn and F.J. Wiercinski showed that microinjection of Ca^{2+} into frog skeletal muscle fibers induced contraction, indicating the involvement of increased intracellular Ca^{2+} in muscle activity.[6)] This finding subsequently influenced biochemists to understand the Ca^{2+}-mediated regulation of striated (skeletal and cardiac) muscle contraction. S. Ebashi, at the University of Tokyo, Japan, discovered a protein factor, troponin, that controls Ca^{2+}-dependent actin-myosin interactions.[7)] The troponin complex is composed of three subunits: troponin C, troponin I and troponin T. In striated muscle contraction, troponin C is the only Ca^{2+} sensor, which confers a micromolar level of Ca^{2+} sensitivity to actin-myosin interactions via actin-linked regulation.[8)] Before the discovery of troponin C, some Ca^{2+} binding proteins were reported, such as calbindin, parvalbmin, and S-100 protein.[9)] In those days, their biological significance remained unclear. Therefore, troponin C was the first Ca^{2+} sensor for muscle contraction at physiological concentrations of Ca^{2+}.

The bridge linking between cAMP and Ca^{2+} studies

In 1958, E.W. Sutherland discovered cAMP as an intracellular messenger.[10)] Nowadays, it has been widely accepted as a second messenger involved in many biological phenomena. When ligand-

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This paper commemorates the 100th anniversary of this journal and introduces the following papers previously published in this journal. Kakiuchi, S. and Yamazaki, R. (1970) Stimulation of the activity of cyclic 3′,5′-nucleotide phosphodiesterase by calcium ion. Proc. Jpn. Acad. **46** (4), 387–392 (https://doi.org/10.2183/pjab1945.46.387); Kakiuchi, S., Yamazaki, R. and Nakajima, H. (1970) Properties of a heat-stable phosphodiesterase activating factor isolated from brain extract: Studies on cyclic 3′,5′-nucleotide phosphodiesterase. II. Proc. Jpn. Acad. **46** (6), 587–592 (https://doi.org/10.2183/pjab1945.46.587).

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bound, the α-subunit of G-protein coupled receptor (GPCR) dissociates from a $\beta\gamma$ dimer, activating the neighboring membrane-bound adenylate cyclase, and resulting in cAMP generation. This increase in cAMP binds and activates protein kinase A (PKA) to trigger physiological processes.[11)]

Since the discovery of cAMP, studies in this field have focused on the regulation mechanism of cAMP levels by generation and degradations. S. Kakiuchi worked in Rall's lab in the US, particularly in quantifying cAMP in the brain.[12),13)] Many researchers competed in the field regarding the regulatory mechanisms of adenylate cyclase, and cAMP generation. After Kakiuchi returned to Japan as the director of the Nakamiya Mental Hospital in Osaka Prefecture, he started to study the control mechanism of cAMP by phosphodiesterase (PDE), involved in cAMP degradation, because this was a less competitive field. The discovery of troponin C, which regulates muscle contraction at micromolar Ca^{2+} concentration levels, had a strong impact on scientists. Kakiuchi was once such scientist who proceeded to examine the involvement of micromolar levels of Ca^{2+} in PDE regulation. However, conducting *in vitro* experiments at micromolar levels of Ca^{2+} proved challenging because of Ca^{2+} contamination released from glass test tubes, even after washing with distilled water. After repeated trials and failures, he meticulously followed the discovery process of troponin C by Ebashi, and recognized the importance of Ca^{2+}-EGTA buffer,[14)] which can control the free Ca^{2+} concentration from 10^{-7} M to over 10^{-5} M. Utilizing Ca^{2+}-EGTA buffer, they detected Ca^{2+}-dependent and -independent PDE activities in brain supernatants.[15)] Ca^{2+}-dependent activity was observed below 1×10^{-5} M of Ca^{2+}. From these findings, they discovered a Ca^{2+}-dependent PDE-activating factor in a gel- filtered fraction of brain supernatant.[16)] This factor was a natural, heat stable protein. They further confirmed that PDE was activated by a protein factor at physiological concentrations of Ca^{2+} ($5 \times 10^{-6} - 2 \times 10^{-5}$ M). Thus, these two papers were the first bridge to link cAMP and Ca^{2+} studies. Independently, Cheung in the US reported a protein activator of PDE in brain extract.[17)] However, his report did not provide information on the Ca^{2+} sensitivity of PDE activation. Three years later, Kakiuchi and Cheung's independent research lines converged when J.H. Wang, in Canada, homogeneously purified this protein factor as a Ca^{2+}-binding protein.[18),19)] This protein was subsequently named calmodulin.

Diverse functions of calmodulin

The early steps in the calmodulin field focused on its primary sequence and structure, then explored its functions, particularly in searching for its targets. Calmodulin was first analyzed by amino acid sequencing. It is a small protein (approximately ~150 amino acids) and its amino acid sequence is highly conserved among species, homologous to that of troponin C.[20),21)] Calmodulin has significant homology in and around the Ca^{2+}-binding sites, suggesting that Ca^{2+}-binding proteins evolved from a smaller ancestral precursor (a single Ca^{2+}-binding site) through gene duplication.[22)] Calmodulin is widely distributed in various tissues of animals and plants, whereas troponin C expression is restricted to striated muscles. The calmodulin concentration in mammalian cells is estimated to be around 2–10 μM,[23)] comparable with the intracellular Ca^{2+} concentration in activated cells. The affinity of calmodulin for Ca^{2+} (Kd = 5×10^{-7} to -5×10^{-6} M)[24)] falls within the intracellular Ca^{2+} concentration range.

Y.S. Babu *et al.* solved the crystal structure of Ca^{2+}/calmodulin,[25)] and R.H. Kretsinger *et al.*[26)] described it as well. Similar to troponin C, Ca^{2+}/calmodulin exhibits a dumbbell-like shape. The N- and C-terminal globular domains are connected by an interconnecting α-helix of approximately seven turns. Each globular domain contains two Ca^{2+}-binding sites in a helix-loop-helix (EF-hand) structure, indicating that calmodulin and troponin C each possess four Ca^{2+}-binding sites per molecule, respectively.[27)] Conformational changes in calmodulin upon Ca^{2+}-binding were further studied using solution techniques, such as nuclear magnetic resonance. By saturating the Ca^{2+}-binding sites with Ca^{2+}, calmodulin becomes more elongated, suggesting that this elongation upon Ca^{2+} binding might be required for the exposure of hydrophobic side chains to the surface and engaging in hydrophobic protein-protein interactions with target proteins.[28)] Upon Ca^{2+} binding, calmodulin undergoes a conformational change that actives it ready to bind and influence the enzymatic activity of target proteins.

More than 300 calmodulin-binding proteins (calmodulin targets) have been identified to date (representative examples are summarized in Fig. 1). Among these, calmodulin-dependent enzymes are major targets. Adenylate cycle[29)] and PDE[15),16)]

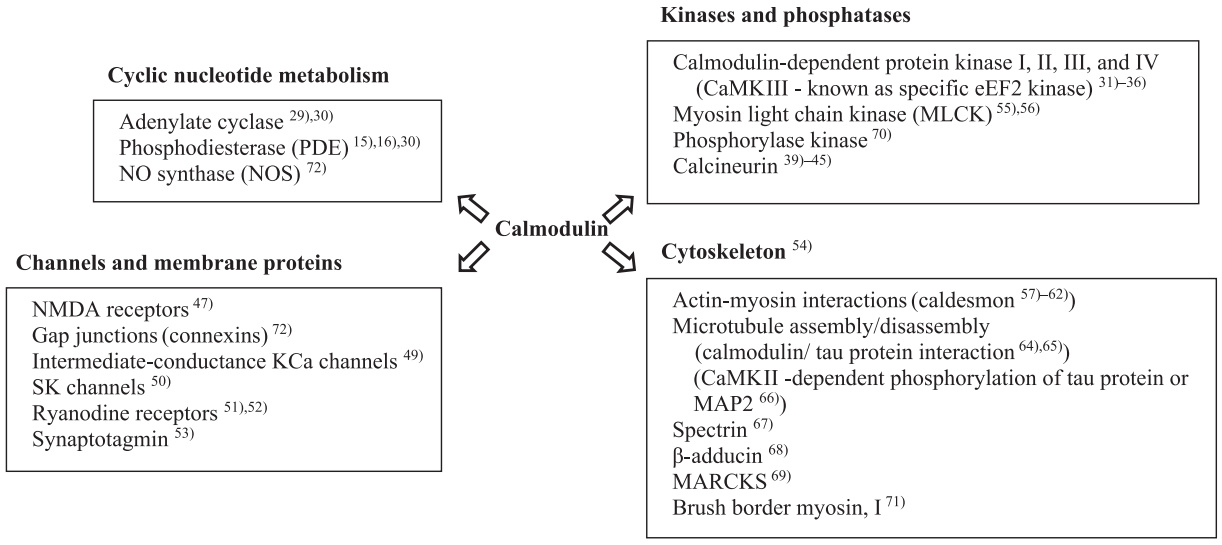

Fig. 1. Diverse arrays of calmodulin targets. Details are described in the text.

act in opposite directions on calmodulin-dependent enzymes involved in cAMP generation and degradation. Calmodulin regulates these discrepant functions through different intracellular localizations and Ca^{2+} sensitivities. Low Ca^{2+} levels (high-affinity Ca^{2+} binding) through the cell membrane preferentially bind to calmodulin on the cytoplasmic side of membrane-linked adenylate cyclase, resulting in increased cAMP levels, which triggers signal transduction. After cAMP levels reach a maximum, higher Ca^{2+} concentrations (low-affinity Ca^{2+} binding) activate calmodulin-dependent PDE, which rapidly degrades cAMP.[30)]

Ca^{2+}/calmodulin-dependent protein kinase activity was originally detected in brain synaptosomal membranes.[31)] Gel filtration chromatography was used to separate four groups of Ca^{2+}/calmodulin-dependent protein kinases; CaMKI (calmodulin-dependent protein kinase I), CaMKII, CaMKIII (known as specific eEF2 kinase), and CaMKIV.[32),33)] Among these, CaMKI, CaMKII and CaMKIV phosphorylate a broad range of substrates, indicating they are multifunctional CaMKs.[34)] CaMKII accounts for 1–2% of the total protein in the forebrain. CaMKII is highly enriched in neurons, especially in the post-synapse, and plays a critical role in neurotransmission, neuronal plasticity and neuronal network formation.[35),36)] In the heart, CaMKII acts as a molecular nexus that is linked to heart failure. Expression of some isoforms of CaMKII and autonomous CaMKII activity are increased in patients with advanced and end-stage heart failure.[37),38)] Therefore, the specific inhibition of CaMKII-mediated pathological signaling may be an effective therapeutic strategy for treating heart failure.[38)] Thus, the main functions of CaMKs are retained for their benefits for human health, and in some cases, CaMKII may be linked to pathological conditions.

Calcineurin is a heterodimer comprising a 60 kDa calmodulin-binding catalytic subunit that interacts with a myristylated subunit of 19 kDa.[39),40)] Calcineurin exhibits Ca^{2+}/calmodulin-dependent protein phosphatase activity. At the post-synapse, multimeric CaMKII interacts with calcineurin, which is involved in synaptic plasticity.[41)] Calcineurin dephosphorylates the nuclear factor of activated T-cell (NFAT) family of transcription factors, causing the nuclear translocation of NFAT and activating gene expression. In T-cells, nuclear-translocated NFAT activates an adaptive immune response.[42)] The calcineurin inhibitors, cyclosporine A and FK506, are clinically used as immunosuppressants for organ transplantation to prevent organ rejection and autoimmune disorders (atopic dermatitis and psoriasis).[43)–45)] However, side effects, such as hypertension, diabetes and hypomagnesemia, are associated with decreased calcineurin function in nonimmune tissues of unknown etiology.[46)]

Calmodulin binds to channels and membrane proteins and regulates their function. Calmodulin binding to the NR1 subunit of NMDA receptors in neurons causes a marked reduction in their channel opening probability, suggesting activity-

dependent feedback inhibition and Ca^{2+}-dependent inactivation of NMDA receptors.[47] Gap junctions involved in cell-cell communication are composed of connexin family proteins. Calmodulin binds to connexins, resulting in the negative regulation of gap junction channels.[48] Ca^{2+}/calmodulin interacts with the intermediate-conductance KCa channel (IKCa1), which is necessary for the channel to open.[49] Ca^{2+} gating of small-conduction Ca^{2+}-activated K^+ channels (SK channels) is mediated by Ca^{2+}/calmodulin.[50] Ryanodine receptors are regulated by calmodulin in both the Ca^{2+}-free ($< 5 \times 10^{-7}$ M) and Ca^{2+}-bound ($> 10^{-6}$ M) calmodulin states. Ca^{2+}-free calmodulin enhances affinity of the ryanodine receptor for Ca^{2+}, whereas Ca^{2+}-bound calmodulin (Ca^{2+}/calmodulin) inhibits the channel.[51),52] Calmodulin also interacts with synaptotagmin, which is involved in exocytosis.[53]

Calmodulin binds to cytoskeletal proteins and regulates cytoskeletal reorganization.[54] As described previously, troponin C is a critical sensor of the Ca^{2+}-dependent regulation of striated actin-myosin interactions via actin-linked regulation.[8] Despite much effort, troponin complexes (C, I and T) have not yet been detected in smooth muscle or non-muscle tissues and cells. It had been reported that Ca^{2+}/calmodulin-dependent myosin light chain kinase phosphorylates the myosin light chain and activates the actin-activated myosin ATPase activity (via myosin-linked regulation) in smooth muscle and non-muscle cells.[55),56] Smooth muscle is a contractile tissue containing abundant contractile proteins, including myosin, actin, tropomyosin and calmodulin. Kakiuchi transferred to Osaka University, where he and K. Sobue began to search for calmodulin targets in smooth muscle and non-muscle tissues[54] and purified its major target protein, caldesmon, from smooth muscle.[57] This protein alternately binds to calmodulin or F-actin in a Ca^{2+}-dependent manner. At low Ca^{2+} concentration (-10^{-7} M), caldesmon binds to F-actin, but not to calmodulin,[57] and it inhibits actin-myosin interactions. When the Ca^{2+} concentration rises above over 10^{-6} M, calmodulin binds to caldesmon[57] and overcomes this caldesmon-induced inhibition.[58] Further analysis revealed that smooth muscle caldesmon and its non- muscle isoform inhibit tropomyosin-enhanced actin-myosin interaction, whereas Ca^{2+}/calmodulin overcomes this inhibition.[59),60] In these conditions, myosin phosphorylation is a prerequisite for actin-myosin interactions. Ca^{2+}/calmodulin and caldesmon control actin-myosin interactions in smooth muscle and non-muscle contractions via actin-linked regulation.[61),62] Thus, smooth and non-muscle contractions may operate via by actin- and myosin-linked dual regulation.[62]

Microtubules are one of the major cytoskeletal proteins in the brain. Marcum *et al.* reported the regulation of microtubule assembly *in vitro* by Ca^{2+}/calmodulin.[63] We identified the tau protein, a microtubule-associated protein, as a Ca^{2+}-dependent calmodulin-binding protein, and it was demonstrated that tau protein-induced microtubule assembly is regulated by Ca^{2+}/calmodulin,[64),65] conferring Ca^{2+}-sensitivity to microtubule assembly. Afterwards, Miyamoto *et al.* reported that CaMKII-dependent phosphorylation of the tau protein or MAP2 also regulates microtubule assembly.[66]

In addition to the aforementioned cytoskeletal proteins, calmodulin is also involved in cytoskeleton-mediated events such as motility, morphogenesis, and development including spectrin,[67] β-adducin,[68] and myristoylated alanine-rich C kinase substrates (MARCKS).[69]

Certain enzymes contain calmodulin, as a subunit of the enzyme complex. Phosphorylase kinase is a multimeric enzyme $(\alpha\beta\gamma\delta)_4$ subunit complex, which demonstrates Ca^{2+}-dependent activation. The δ subunit is tightly associated with the enzyme, and identified as calmodulin.[70] Brush border myosin-I (BBMI) is another example of a Ca^{2+}-independent calmodulin-target association. BBMI consists of a single myosin heavy chain and three or four strongly associated calmodulin molecules (light chains) without Ca^{2+}.[71] However, the significance of calmodulin as light chains in BBMI remain unclear. Nitric oxide synthase (NOS) generates NO, which activates guanylate cyclase, generating cGMP. Three isoforms of NO synthase are present, neuronal NO synthase (nNOS), endothelial NO synthase (eNOS) and inducible NO synthase (iNOS). Constitutive NOS isoforms (nNOS and eNOS) require Ca^{2+}/calmodulin, whereas iNOS shows no requirement for Ca^{2+}/calmodulin which has calmodulin as a tightly bound subunit.[72]

Current methods are starting to recognize unexpected arrays of calmodulin targets. Solving the structure of the calmodulin-interacting surfaces to reveal novel target recognition sites may be necessary to uncover the further functions of calmodulin and its targets in health and disease.

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Profile

Kenji Sobue was born in Nagoya, Japan, in 1947, graduated from Iwate Medical University, Medical School, and obtained his M.D. in 1973. He entered the Department of Neuroscience at the School of Medicine, Osaka University, as a doctoral fellow, and received his Ph.D. in 1977. He started his academic career as assistant professor in 1977, and then associate professor in 1981, in the same Department at the Graduate School of, Medicine, Osaka University, In 1988, he was appointed as professor of the Graduate School of Medicine, Osaka University. He moved to Iwate Medical University as Vice President in 2011, President in 2016, and CEO in 2023. He received the Young Investigator Award of the Japan Biochemical Society in 1984 and Nakaakira Tsukahara Prize in Japan Neuroscience Society in 1990. His interests focus on the molecular organization of the cytoskeleton and the isolation and regulation of actin-linked proteins, such as caldesmon, spectrin, and other proteins, and also in the cloning of postsynaptic proteins and investigation of synaptic dynamics. Based on these studies, he expanded his interest to neuronal network formation involved in the actin cytoskeleton and their abnormalities in psychiatric disorders.

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90. *Stimulation of the Activity of Cyclic 3′,5′-Nucleotide Phosphodiesterase by Calcium Ion*

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(Comm. by Yushi UCHIMURA, M. J. A., April 13, 1970)

It has been shown recently that concentrations of 3′,5′-AMP in mammalian brain tissues increased dramatically in responding to various stimuli, i.e. decapitation,[1] electrical stimulation applied *in vitro*,[2] and certain neurohormones added *in vitro*.[1),3)] It is generally accepted that adenyl cyclases were responsible for regulating the level of 3′,5′-AMP in the tissues.

Recently, Cheung[4)] has proposed ATP and PP controls on brain cyclic 3′,5′-nucleotide phosphodiesterase, the enzyme which decomposes 3′,5′-AMP, suggesting a possibility of this enzyme to contribute to such regulation mechanism under certain conditions.

Results presented in this communication demonstrate the implication of the physiological concentrations of calcium ion regulating activity of cyclic 3′,5′-nucleotide phosphodiesterase from rat brains.

Materials and methods. *Materials purchased are* Crotalus atrox from Sigma Chemical Co., glycoletherdiamine-N,N,N′,N′-tetra-acetic acid (GEDTA) from Dojindo, Co.

Enzyme preparation. Brains from male rats of Sprague Dowley strain were homogenized in 3 volumes of 1 mM tris-HCl (pH 7.5). The homogenate was centrifuged on Hitachi 40 A rotor for 60 min. at 40000 rpm. The precipitate was washed once by the centrifugation as above and the supernatant was combined.

A portion of the combined supernatant was dialyzed overnight against 100 volumes of 1 mM tris-HCl (pH 7.5) and referred to the "supernatant" enzyme. Another portion was subjected to GEDTA treatment and referred to the "GEDTA-washed" enzyme. The procedure was carried out by repeating the cyclic process; condensing enzyme by suction through a collodion bag and diluting it with the washing medium. 0.4 mM GEDTA in 10 mM Tris-HCl (pH 7.5) was used as such the medium, and followed by 10 mM tris-HCl alone to remove excess GEDTA.

All the procedures were done at 4°C. Enzymes were kept at 4°C for a week or more, or at −80°C for several months with no appreciable loss of the activity.

Assay for enzymatic activity. The phosphodiesterase was as-

sayed according to the method of Butcher and Sutherland[5] with a slight but important modification. The procedures consist of two separate stages of incubation at 30°C. The snake venom 5′-nucleotidase, Crotalus atrox, used in the second stage incubation, required for Mg^{++} or Mn^{++} and was inhibited by EDTA or GEDTA, and therefore, the concentration of free Mg ion was adjusted to more than 3 mM prior to starting the second stage incubation. It was confirmed that the excess amount of the snake venom used throughout the studies did not release any detectable amount of P from 3′,5′-AMP during the incubation either in the presence or in the absence of Ca^{++}. Enzyme protein was assayed by procedures similar to those Lowry *et al.*[6]

Free Ca ion concentrations in media. The concentration of free Ca ion in the absence of added $CaCl_2$, i.e. the contamination level of free Ca was roughly 10^{-6} M~10^{-5} M, and therefore, those in 1 mM GEDTA should be in the order of 10^{-9} m~10^{-8} M. Estimated concentration of free Ca ion in the mixture of GEDTA, 1 mM plus $CaCl_2$, 0.33 mM (Ca buffer) was 1×10^{-6} M. These values were calculated by adopting 5×10^{5} M^{-1} as the apparent binding constant of GEDTA according to Ogawa,[7] which is considerably lower than the formerly used.[8]

Results. Since it was discovered by Sutherland and Rall[9] the requirement of the phosphodiesterase for Mg ion has been well demonstrated. It was also reported that even greater enzymatic activity was attained by Mn ion[5),10] and partial activity by Co ion.[5]

In accordance with these reports, we observed that the "GEDTA-washed" enzyme, which retained very little activity in the absence of added cations, restored good activity by the additions of Mg, Mn or Co ions (Fig. 1). 3 mM of Mg^{++} or 0.3 mM of Mn^{++} expressed the full enzymatic activity and higher concentrations of these cations were slightly inhibitory.

The effect of these cations were stimulated when Ca ion was included in the system, as are the cases in Fig. 2, where $CaCl_2$ was added in all the reaction tubes to final 5×10^{-4} M while other experimental conditions essentially identical to those in Fig. 1. In addition to its stimulatory effect, inclusion of Ca ion changed shapes of dose response curves of the cations so that the inhibitions due to excess Mg^{++} or Mn^{++} were relived.

With the optimum concentration of Mg^{++} (3 mM) present in media, activities of the "GEDTA-washed" enzyme were tested with various additions of other components. The results presented in Table I demonstrate clearly that minute amounts of Ca ion stimulated enzymatic activity markedly. It should be noted that the stimulatory

effect of Ca ion required Mg^{++} or Mn^{++} (Co^{++}) as a basic cation in medium and Ca ion alone revealed very poor enzymatic activity as shown in Table I (Exp. 3) or in Fig. 2 (shaded bar).

Results in Fig. 3 show the stimulatory effect of Ca ion as a function of its concentration. The stimulation was observed with Ca ion below 1×10^{-5}M, within the concentrations where regulatory roles of Ca ion in physiological processes could be seen. Enzymatic activities in the presence of Ca ion lower than 1×10^{-6}M was not analyzed directly. However, the results in Fig. 4 involve such cases of 10^{-9}M $\sim10^{-8}$M and will be mentioned later.

Experiments using chelating agents on the "supernatant" enzyme differentiated two types of inhibitions, one caused by EDTA and the other by GEDTA (Fig. 4). Parallel relationship was found in degrees of the inhibition and concentrations of EDTA in media. The enzyme was completely inactivated as EDTA level increased over 3mM. The essential nature of this inhibition seems to be due to depriving of free Mg ion in the media. These observations coupled with those shown in Table I (Exp. 3) confirmed the absolute requirement for Mg^{++} for basic activity of the phosphodiesterase.

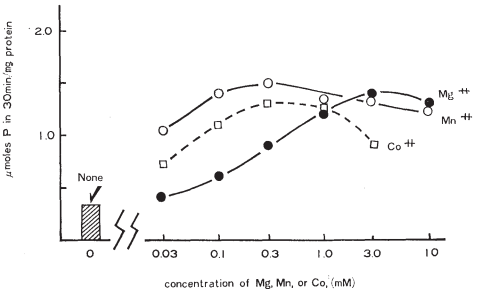

Fig. 1. The effect of divalent cations on the "GEDTA-washed" enzyme. Reaction mixtures contained $MgCl_2$ (——●——). $MnCl_2$ (——○——), or $CoCl_2$ (- - -□- - -) as the only divalent cation. Other experimental conditions were essentially the same as those in Table I.

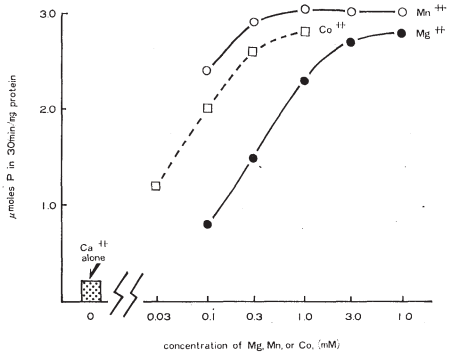

Fig. 2. The effect of divalent cations on the "GEDTA-washed" enzyme in the presence of 5×10^{-4}M Ca ion.
Reaction mixtures contained a fixed concentration of $CaCl_2$ (final 5×10^{-4}M) plus one of the following cations; $MgCl_2$ (——●——), $MnCl_2$ (——○——), or $CoCl_2$ (- - -□- - -). Other experimental conditions were essentially the same as those in Table I.

The inhibition caused by GEDTA was clearly seen with its concentration as low as 1×10^{-4} M and could not be explained by possible changes in free Mg^{++} concentration in the media. GEDTA is known

Table I. Activity of the "GEDTA-washed" enzyme under various conditions

Basic system	Further addition	Activity†)
Experiment 1	none*)	1.2
	GEDTA, 1 μmol**)	1.0
complete system	GEDTA, 1 μmol+$CaCl_2$, 0.33 μmols***)	1.0
	$CaCl_2$, 0.03 μmols	1.9
	$CaCl_2$, 1.0 μmol	2.0
Experiment 2	GEDTA, 1 μmol**)	1.0
	GEDTA, 1 μmol+$CaCl_2$, 0.33 μmols***)	1.0
complete system	$CaCl_2$, 0.03 μmols	1.3
—dithiothreitol	$CaCl_2$, 1.0 μmol	1.4
Experiment 3	GEDTA, 1.0 μmol+$CaCl_2$, 0.33 μmols***)	<0.1
complete system	$CaCl_2$, 0.03 μmols	0.4
—$MgCl_2$	$CaCl_2$, 1.0 μmols	0.4
Experiment 4	GEDTA, 1.0 μmol+$CaCl_2$, 0.33 μmols***)	0.0
complete system	$CaCl_2$, 0.03 μmols	0.0
—3′, 5′-AMP		
Experiment 5	GEDTA, 1.0 μmol+$CaCl_2$, 0.33 μmols***)	0.0
complete system	$CaCl_2$, 0.03 μmols	0.0
—enzyme		

†) μmols P in 30 min per mg protein.
As is described in the text, estimated concentrations of free Ca^{++} are as follows.
*): 10^{-6}M~10^{-5}M, **): 10^{-9}M~10^{-8}M, ***): 1×10^{-6}M.
Complete system consisted of, in one ml of the reaction mixture, 40 μmols of Tris-maleate (pH 6.9), 3 μmols of $MgCl_2$, 1 μmol of dithiothreitol, 1.2 μmols of 3′, 5′—AMP, and the "GEDTA-washed" enzyme protein.

to have a selective affinity for Ca ion compared with Mg ion; the latter is practically not affected by GEDTA under the experimental conditions. The observations agree well with the idea that minute amounts of Ca ion are involved in the phosphodiesterase system. Comparison of Fig. 3 and Fig. 4 indicates that the degrees of the inhibition caused by GEDTA on the "supernatant" enzyme is comparable to those of the activation by Ca ion on the "GEDTA-washed" enzyme.

Of even greater interest is the observations that the enzyme still retains about a half the original activity even in the presence of excess amount of GEDTA (1~4 mM), where the estimated concentrations of free Ca ion should be in the order of 10^{-9} M~10^{-8} M. The results would suggest a possible occurrence of two separate enzymatic activities in the preparation, the calcium dependent and the calcium independent.

The effect of dithiothreitol seems to enhance the calcium dependent activity (Exp. 2 in Table I). The effect of Ca ion could be replaced with strontium or barium ions in some extent.

Comments. It is now widely accepted and well documented[11])

that the calcium ion is the final activator of the contractile system under physiological conditions. Ebashi and Endo[11] reported that the maximum tension was attained at pCa less that 4.9 and threshold pCa was around 5.9 by using skinned fibres. These values are close to those in Fig. 3, where the maximum stimulation of the phosphodiesterase was observed at pCa near 4.5 and threshold pCa was observed around 5.5 (with 3mM Mg present). It was observed both in the contractile system[11] and on the phosphodiesterase (Fig. 3) that the increase in free Mg ion concentration up to 8~10 mM decreased the Ca sensitivities.

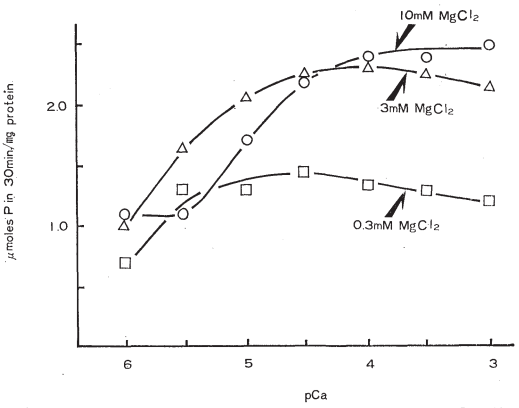

Fig. 3. Activity of the "GEDTA-washed" enzyme as a function of concentrations of Ca ion.
$CaCl_2$ were added into reaction mixtures to produce the indicated concentrations, except 1×10^{-6}M which was achieved by the addition of GEDTA, 1.0 μmol plus $CaCl_2$, 0.33 μmol. Other experimental conditions were essentially the same as those in Table I.

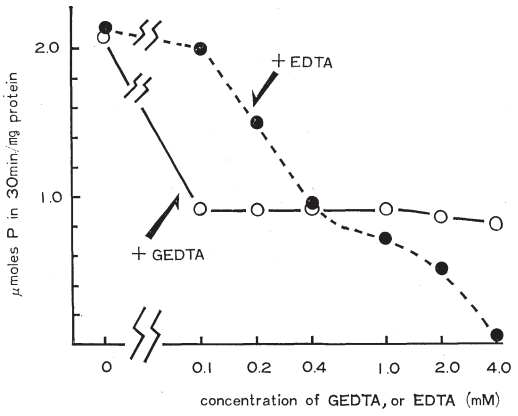

Fig. 4. The effect of chelating agents on the "supernatant" enzyme.
One ml of reaction mixtures contained 40 μmols of Tris-maleate (pH 6.9), 5 μmols of dithiothreitol, 3 μmols of $MgSO_4$, 2 μmols of 3′,5′-AMP, the "supernatant" enzyme, and indicated amounts of EDTA (---●---) or GEDTA (——○——).

Taking the above comparisons into consideration, it would be highly probable that the calcium ion is controling the phosphodiesterase activity under physiological status and thus affecting 3′,5′-AMP level in the tissues *in vivo*. It is of interest that Namm *et al.*,[12] by using isolated and perfused rat hearts, have shown the changes in the tissue level of 3′,5′-AMP as the concentration of Ca in the medium changed. They have proposed a hypothesis that Ca ion either inhibits adenyl cyclase activity or stimulates the phosphodiesterase activity.

This communication provides the first direct evidence of the calcium ion acting upon the phosphodiesterase, and supplies another

clue to the probable interrelationship between the 3′,5′-AMP system and the calcium ion.[13)–16)]

We would like to acknowledge the continuing encouragement of Dr. H. Nakajima, Nakamiya Mental Hospital. Our thanks are also due to Drs. H. Wada and I. Sano, Osaka University, Medical School and Dr. M. Kurokawa, Institute of Brain Research, University of Tokyo for many helpful suggestions. The work was supported in part by Research Grant from Pharmacophsychiatry Research Foundation.

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137. *Properties of a Heat-stable Phosphodiesterase Activating Factor isolated from Brain Extract*

Studies on Cyclic 3′,5′-Nucleotide Phosphodiesterase. II

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(Comm. by Yushi UCHIMURA, M. J. A., June 12, 1970)

In the previous paper,[1] we demonstrated the occurrence of two separate phosphodiesterase activities*) in crude extract from rat brain for 3′,5′-AMP break down; namely the calcium dependent enzyme activity and the calcium independent enzyme activity. The former is controlled by physiological concentrations of calcium ions (5×10^{-6}M~2×10^{-5}M), and differs from the latter in PH dependency and other enzymatic characters.[2]

During the course of the investigation, we found that a heat-stable and nondialyzable factor, present in crude brain homogenate, was participating in phosphodiesterase, particularly in the calcium dependent enzyme activity, and we isolated this phosphodiesterase activating factor (PAF) from rat brain homogenate.[3] This present communication describes on some properties of PAF, including its effect on the calcium dependent phosphodiesterase activity.

While this manuscript was in preparation, a report demonstrating an activator from brain homogenate was published by Cheung.[4] In that report, he presented that the factor was a protein nature (MW of some 40,000) and stable to heat, to acidic PH's, and to 8M urea. The experimental data on those lines, however, were not shown there.

Materials and methods. *Crotalus atrox* was obtained from Sigma Chemical Co. Sepharose 6B was the product of Pharmacia Fine Chemicals, Sweden, which was washed thoroughly with 1×10^{-2}M Tris-HCl (pH 7.4) before use.

Activity of phosphodiesterase was assayed by procedures slightly modified from the original method of Butcher and Sutherland.[5] In the 1st stage incubation, enzyme protein was incubated with 3′,5′-AMP in 1 ml of reaction medium at 30°C for 30 min, and was inactivated at the end of the incubation by heating in a boiling water

*) Abbreviations used; phosphodiesterase, cyclic 3′,5′-nucleotide phosphodiesterase; GEDTA, glycoletherdiamine-N, N, N′, N′-tetraacetic acid; DTT, dithiothreitol.

bath for 3 min. 5′-AMP formed was converted to adenosine plus Pi during separate 2nd stage incubation which was carried out at 30°C for 20 min with excess amount *C. atrox*. The reaction was stopped by adding perchloric acid in final 3%, and the centrifuged supernatant of the reaction mixture was assayed for Pi.[6] Enzyme protein was assayed by procedures similar to those of Lowry *et al.*[7]

Enzyme preparation. Brains from male rats of Sprague Dowley strain were homogenized in 3 volumes of 1 mM Tris-HCl (pH 7.4). The homogenate was centrifuged on Hitachi 55TA rotor for 90 min. at 45,000 rpm. The precipitate was washed once by the centrifugation as above and the supernatant was combined. The combined sup. was dialyzed overnight against 10 mM Tris-HCl (pH 7.4) and referred to "45,000 sup". Acetic acid was added to the "45,000 sup" to make pH 5.0~5.1, and the precipitate formed was collected by centrifugation. PH of the collected precipitate, suspended in the original volume of 10 mM Tris buffer, was brought back to 7.4~7.5 with NaOH.

Insoluble material was removed by centrifugation. The supernatant was fractionated by the addition of saturated ammonium sulfate (pH 7), and the 20%~50% fraction, dissolved in minimum quantity of the eluent medium, was subjected to gel filtration column chromatography using Sepharose 6B with 10 mM Tris-HCl (pH 7.4) plus 2 mM $MgSO_4$ as the eluent solution. Enzyme activity was eluted in the fraction B (see Fig. 1). All the procedures were carried out

Table I. Effect of PAF, and of heat-treated PAF on phosphodiesterase activity

Addition	Enzyme activity	
	without DTT	with 10mM DTT
	(μmols P per mg protein)	
None	2.2	4.9
PAF, 15 μg	4.2	8.6
PAF, 15 μg, heat treated*)	3.9	8.1
PAF, 30 μg,	4.7	8.9
PAF, 30 μg, heat treated*)	4.7	8.3
PAF, 60 μg	5.3	8.9
PAF, 60 μg, heat treated*)	5.5	8.6

*) The reaction tube containing Tris-HCl, $MgSO_4$ and the indicated amount of PAF was heated for 5 min. at 80°C, chilled, and received the other components (DTT, enzyme, substrate).

The assay system contained, in one ml, 40 μ mols of Tris-HCl (pH 7.4), 4 μ mols of $MgSO_4$, 2 μ mols of 3′, 5′-AMP, and 36 μg of Fr. B of the Sepharose eluate as enzyme source.

at 4°C, and the enzyme preparations were stored at −80°C if they were not for immediate use.

Isolation of PAF. It was observed that considerable loss in the yield of the total enzyme activity took place as the purification stage advanced, while crude enyzme by itself, tested under the experimental conditions, could be tolerated for several weeks at 4°C without much loss of the activity. These facts led to a search for enzyme activating factor(s) in crude brain extract.

A fraction (protein content of 16 mg), which contained such activating factor was isolated free from the phosphodiesterase by gel filtration column chromatography of "45,000 sup" (190 mg protein), by similar procedures shown in Fig. 1. This fraction, after condensed through collodion bags, was used for the following studies as PAF source.

Properties of PAF. Effect of PAF was seen either in the absence or presence of DTT (10 mM) in the assay system (Table I). Separate experiments showed 1~10 mM DTT by itself produced supramaximal activation effect on crude enzyme, and hence, PAF worked in different way as DTT did.

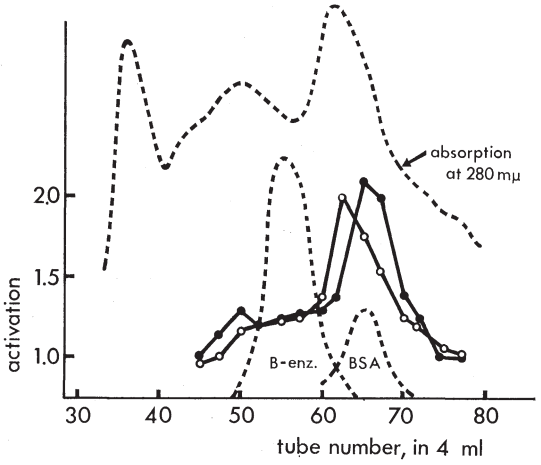

Fig. 1. Sepharose 6B Column Chromatography of "45,000 sup". Native "45,000 sup" (——●——), or heat-treated "45,000 sup" (——○——) was analyzed for phosphodiesterase activating factor (s) by gel filtration column chromatography, as described in the text. Sepharose 6B column, ϕ25 mm×93 cm, was eluted with 10 mM Tris-HCl, pH 7.5 (V_0=135 ml). The Fig. indicates that PAF was eluted after phosphodiesterase (dotted line, Fr. B) and in comparable position to bovine serum albumin (dotted line, BSA). PAF was assayed by using Fr. B as purified enzyme source and measuring the ratio of the enzyme activities with or without the addition of column fractions in the system. The ordinate shows such activation ratio.

Resistant to heat. Results in Table I show the effect of PAF unchanged after it was heated for 5 min. at 80°C. The possibility that heating might have produced secondary decomposed compounds which showed similar activating effect was tested as follows. Three ml. of "45,000 sup" (16 mg protein in 8 mM Tris-HCl, pH 7.4) was heated for 4 min in a boiling water bath, then centrifuged for 30 min at 18,000 rpm on Marusan. The supernatant was column chromatographed as shown in Fig. 1. Another 3 ml of native "45,000 sup" was run on the same column as a control. Results in Fig. 1 show both the chromatograms coincide in the shapes, and only the single

Table II. Effect of PAF on phosphodiesterase activity tested in the presence of high and low concentrations of calcium ions

Addition	Enzyme activity, in the presence of		[I] minus [II]
	[I] $CaCl_2$, 0.1 mM	[II] GEDTA, 1 mM $CaCl_2$, 0.33mM	
Exp. I. without DTT	(μ mols P per mg protein)		
None	1.3	0.9	0.4
Boiled extract, 34.4 μg	1.4	0.9	0.5
Boiled extract, 172 μg	1.9	0.9	1.0
Boiled extract, 860 μg	2.2	0.9	1.3
Exp. II. with DTT, 1 mM			
None	1.6	0.9	0.7
Boiled extract, 34.4 μg	1.8	0.9	0.9
Boiled extract, 172 μg	2.0	0.9	1.1
Boiled extract, 860 μg	2.4	1.0	1.4

One ml of reaction mixture contained 80 μ mols of Tris-Maleate, pH 6.9, 3 μ mols of $MgCl_2$, 2 μ mols of 3′, 5′-AMP, 25 μg of enzyme protein, and the indicated amount of boiled extract as the PAF source, Either 0.1 μ mol of $CaCl_2$ or the mixture of 1 μ mol of GEDTA plus 0.33 μ mols of $CaCl_2$ was added in the system to produce free calcium ion concentration of 1×10^{-4}M or 1×10^{-6}M. The water and reagents were carefully made free from the contamination of calcium ions.

Both the enzyme and the PAF used in this study were prepared directly from "45,000 sup", and the contaminating free calcium ions were removed as follows. "45,000 sup" was subjected to cyclic process; condensing it by suction through collodion bags and diluting it with the wash medium of 0.4 mM GEDTA in 10 mM Tris buffer, finally with 10 mM Tris buffer alone to remove excess GEDTA from the preparation.

The reaction tubes, containing the indicated amounts of the "washed 45,000 sup" as PAF source were heated for 3 min in a boiling water bath, chilled, and received the other components of the assay mixture including another addition (25 μg) of the "washed 45,000 sup" as the enzyme source. The boiled extract (PAF) designated in the table was prepared as above, and its protein contents should not be compared to those in Table I where the purified PAF was used.

peak was observed in both the cases.

It is nondialyzable, tested by using the collodion bag, made by Sartorius Membrane Co., West Germany. Fig. 1 shows that PAF was eluted from the gel filtration chromatography in the comparable position to bovine serum albumin (MW=67,500). Its effect was partially destroyed by trypsine attack (0.2 mg trypsine/mg protein, 30°C for 30 min at pH 7.5). It was not extracted from the tissue by aceton, or chloroform- methanol (2:1). It was eluted from the residue by water. It distributed both in the supernatant and in the particulate fractions of cells.

PAF paticipates in the calcium dependent phosphodiesterase activity. Results in Table II show that the effect of PAF is concerning solely with the calcium dependent phosphodiesterase activity. In this study, "45,000 sup", washed by 0.4 mM GEDTA followed by 10 mM Tris-HCl (pH 7.4), was used as both the enzyme source and the PAF source. For the latter, the preparation was heated for 3 min in a boiling water bath before using; the validity of the heated extract was already discussed in the results of Fig. 1.

As was shown in the previous paper,[1] the calcium dependent phosphodiesterase activity and the independent activity were tentatively defined that the former was requiring for calcium ions over 1×10^{-5} M for its full activity, and that the latter was distinguished from the former under reduced concentrations (below 1×10^{-6} M) of free calcium ions in the medium. It is uncertain, at present time, whether that PAF works on the enzyme which requires calcium ion for the activity, or that PAF plus calcium ion act upon the enzyme for the "activation".

We found bovine serum albumin, added 0.2 mg~1 mg/ml, had similar activating effect on the enzyme. This effect, however, is observed both on the calcium dependent and on the independent enzyme activities and would be nonspecific, enzyme stabilizing in its nature.

Comments. The discoveries[5),8)–11)] that brain is abundant in 3′,5′-AMP system and that levels of 3′,5′-AMP in brain tissues change dramatically in response to various stimuli, together with the accumulated facts[12)] that 3′, 5′-AMP system works as the messenger system in hormone actions in general facilitated the investigations on the enzymes involved in 3′,5′-AMP system in brain. Brain phosphodiesterase, cyclic 3′, 5′-nucleotide decomposing enzyme, has been studied for its probable control on 3′, 5′-AMP levels in the tissue, and ATP, PP, citrate, and a snake venom factor were reported by Cheung[13)–15)] such as factors that affected the enzyme activity. He suggested the presence of an activator(s) also in brain homogenate.[16)]

This present communication clarified several facts on the activator (PAF) isolated from rat brain, that it is stable to heat, that it is high molecular (comparable to those of bovine serum albumin; 67,500), and that it participates in the calcium dependent phosphodiesterase activity.

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