Anal. Chem. **1997,** *69,* 3081−3085

An Optical Method for Evaluating Ion Selectivity for Calcium Signaling Pathways in the Cell

Takeaki Ozawa, Masaya Kakuta, Masao Sugawara, and Yoshio Umezawa*

Department of Chemistry, School of Science, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

Mitsuhiko Ikura

Center for Tsukuba Advanced Research Alliance and Institute of Applied Biochemistry, University of Tsukuba, Tsukuba 305, Japan, and Division of Molecular and Structural Biology, Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, 610 University Avenue, Toronto, Ontario, Canada M5G 2M9

A method for evaluating a physiologically relevant ion selectivity of Ca^{2+} signaling pathways in biological cells based on a Ca^{2+}-dependent on/off switch for cellular processes via calmodulin (CaM) chemistry is described. CaM serves as a primary ion receptor for Ca^{2+} and a given CaM-binding peptide as a target for a CaM−Ca^{2+} complex. Upon accommodating four Ca^{2+} ions in its binding sites, CaM undergoes a conformational change to form a CaM−Ca^{2+}−target peptide ternary complex. This Ca^{2+}-induced selective binding of the Ca^{2+}−CaM complex to the target peptide was monitored by a surface plasmon resonance (SPR) technique. As a target peptide, a 26-amino acid residue of M13 derived from skeletal muscle myosin light-chain kinase was used. The target peptide was covalently immobilized in the dextran matrix on top of gold, over which sample solutions containing Ca^{2+} and CaM were injected in a flow system. Ca^{2+}-dependent SPR signals were observed for Ca^{2+} concentrations from 3.2×10^{-8} to 1.1×10^{-5} M and it leveled off. The observed SPR signals were explained as due to an increase in the refractive indexes caused by a Ca^{2+} ion-switched protein/peptide interaction, i.e., Ca^{2+} ion to CaM and subsequent additional binding of the thus formed complex with immobilized M13. No SPR signals were however, induced by Mg^{2+}, K^+, and Li^+ at concentrations as high as 1.0×10^{-1} M; these results and previous spectroscopic data taken together conclude that these ions do not induce CaM/peptide interaction. Large changes in SPR signals were observed with a Sr^{2+} ion concentration over 5.1×10^{-4} M; Sr^{2+} ion behaved in this case as a strong agonist toward the Ca^{2+}-dependent on/off switch of CaM. The present system thus exhibited "physiologically more relevant" ion selectivity in that relevant metal ions could switch on the CaM/peptide or -protein interaction rather than merely be bound to CaM causing no further signal transduction. The potential use of this finding for more widely evaluating cation selectivity toward the Ca^{2+} signaling process was discussed.

The common strategy employed so far for designing biosensors is that the transducer is used to directly monitor the binding between antigen and antibody or a product of the interaction of enzymes.[1] Recently, some new types of biosensors have emerged that rely on signal transduction mechanisms by membrane receptor proteins themselves for generating the analytical signal. Typical examples are ion channel[2] or transporter proteins[3] embedded in lipid bilayer membranes. In contrast to the binding assay and related techniques, these sensors utilize the corresponding transmembrane signals, such as ion channel currents or active membrane transport for yielding, in some cases, physiologically more relevant signals compared to those with the binding assay approach: The chemical selectivity of a glutamate receptor displayed by its ion channel, for example, has been evaluated from the sum of total amount of ions passed through the glutamate receptor.[2c] The integrated channel currents were used as an amplified physiologically more relevant measure of agonist potency to activate the glutamate receptor. The chemical selectivity ratio among three agonists including glutamate ion evaluated from the integrated channel currents was found to be ∼1 order of magnitude narrower than that obtained by binding studies. Novel methods for evaluating chemical selectivities on an agonist or antagonist for receptor-type proteins are thus increasingly required in addition to sensitive and selective sensing methods of analytes.

Calcium ions play an important role as a messenger in cellular signal transduction pathways. In order to detect the Ca^{2+} signal in cells, nature has developed a number of proteins to selectively or specifically recognize Ca^{2+}. Among many Ca^{2+} sensor proteins, calmodulin (CaM; 148 amino acid residueds 16.7 kDa) is a ubiquitous Ca^{2+} binding protein which serves as a multifunctional Ca^{2+} sensor in a variety of cellular processes such as energy and biosynthetic metabolism, cell motility, exocytosis, cytoskeletal assembly, and intracellular modulation of both cAMP and Ca^{2+} concentrations.[4] It has four Ca^{2+} binding sites within two helix−loop−helix (EF-hand) motifs, organized in two structurally similar

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S0003-2700(96)01314-5 CCC: $14.00

globular domains.[5] One of the EF-hands has high-affinity sites with binding constants for Ca^{2+} of 5×10^6 M^{-1}, and the other has low-affinity sites with binding constants of 5×10^5 M^{-1}.[6] Also a high degree of selectivity for Ca^{2+} over Mg^{2+} (>10^3 difference in stability constants) has been explored.[7] The inherent selectivity of CaM becomes a definite advantage for developing new types of Ca^{2+} ion sensors. Recently, Bachas and co-workers reported a fiber-optic sensor with a fluorescein-labeled calmodulin (F-CaM) entrapped in a dialysis membrane at the fiber tip.[8] F-CaM exhibited Ca^{2+} ion-dependent changes in its fluorescence intensity, which was found to be a measure of Ca^{2+} concentrations in sample solutions.

After the selective recognition by CaM of Ca^{2+} ion, how do CaM and other Ca^{2+} binding proteins transfer the Ca^{2+} signal to different cellular processes? In many cases, they interact with target proteins when they bind Ca^{2+}, while their Ca^{2+}-free forms do not bind the targets: Ca^{2+}-induced conformational transitions enable CaM and other Ca^{2+} binding proteins to interact (bind) with the target proteins and lead to the activation and regulation of the latter. The mechanism by which CaM recognizes the target protein has been defined by crystallography, NMR, and other spectroscopic studies combining with site-directed mutagenesis of CaM; the CaM−Ca^{2+} complex can bind to the CaM binding domains of all its target proteins with high affinity, despite their lack of amino acid sequence homology.[4b,9] The only common feature is that they are hydrophobic basic peptides that have a propensity to form an α-helix. If the molecular mechanisms of such a fine tuning of protein conformation are introduced into a sensing system, which is responsible for a Ca^{2+}-dependent on/off switch for cellular processes, then physiologically more relevant ion selectivity, i.e., agonists or antagonists toward Ca^{2+} signaling pathway via CaM, may be evaluated.

In this paper, a new optical method for evaluating such ion selectivity for the Ca^{2+} signaling pathway is described based on the above described on/off switching mechanism of the CaM-mediated Ca^{2+} signaling, which is monitored by surface plasmon resonance (SPR). The principle is schematically shown in Figure 1. CaM serves as a primary receptor for Ca^{2+} ion and M13 as a target peptide. The selective binding of CaM to M13 in the presence of Ca^{2+} or other possible interfering ions is monitored by the SPR technique. M13, a synthetic peptide of 26 amino acid residues, comprising a CaM binding domain (residues 577−602; its dissociation constant with CaM is 1 nM) of skeletal muscle myosin light-chain kinase (MLCK)[10] is immobilized covalently on the dextran matrix attached to the gold surface. Upon binding four Ca^{2+} ions, CaM in the sample solution undergoes conformational changes to form a CaM−Ca^{2+} complex. The complex thus formed is then bound to the immobilized target peptide M13, and

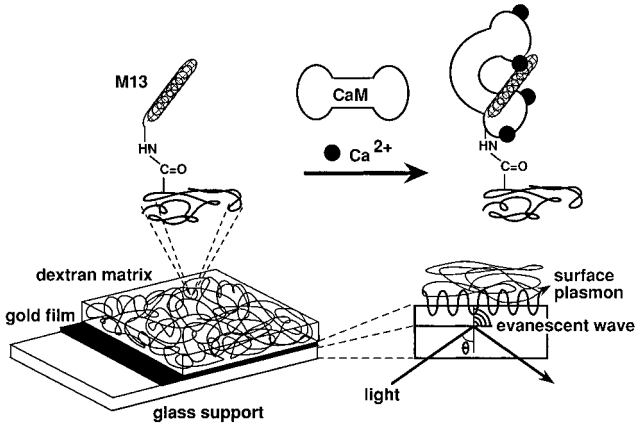

Figure 1. Principle of the physiologically relevant Ca^{2+} ion sensor.

forms a CaM−Ca^{2+}−M13 ternary complex in the dextran matrix, which is measured by SPR, a technique that sensitively responds to changes in the refractive index close to a gold surface.[11] The amount of the ternary complex thus formed is expected to be a selective and sensitive measure of the extent of the Ca^{2+} signaling, representing the physiologically more relevant selectivity of metal ions including Ca^{2+}, Mg^{2+}, Sr^{2+}, and others.

EXPERIMENTAL SECTION

Materials. Bovine brain CaM was extracted as previously described.[12] The purity of CaM was assessed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE): A major band of CaM was detected at 17 kDa and a very minor impurity was assessed near 17 kDa, which was used without further purification. Chemically synthesized HPLC-purified M13 peptide, which consists of the amino acid sequence of KRRWKKNFIAVSAANRFKKISSSGAL (expressed by one-letter abbreviations), was purchased from Queen's University Peptide Facility (Kingston, Canada) and was used without further purification. *O*,*O*′-Bis(2-aminoethyl)-ethylene glycol-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) were obtained from Dojindo Laboratories (Kumamoto, Japan). *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Other salts and solvents used were all of the highest purity available. All aqueous solutions were prepared with Milli-Q grade water (Millipore reagent water system, Bedford, MA).

Apparatus. All SPR measurements were performed on a BIAcore system of Pharmacia Biosensor Co. (Uppsala, Sweden). A gold film called Sensor Chip CM5 (a carboxymethylated dextran attached to a gold-coated glass surface) was purchased from Pharmacia. Adjustment of the pH of the buffer solutions was made by a glass electrode pH meter Model HM-18E (TOA Electronics Co., Tokyo, Japan).

Immobilization of M13 on the Carboxymethylated Dextran Matrix. Immobilization of M13 on a dextran matrix attached

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to a gold thin film was performed via primary amine groups by the amine coupling method.[13] Briefly, after equilibration of the dextran matrix with 10 mM HEPES buffer solution (pH 7.5) at a constant flow rate of 5 μL/min, the carboxymethylated dextran on the surface of the gold film was first activated with 35 μL of a NHS/EDC mixture (0.05 M NHS, 0.2 M EDC in water). Successively, a 30 μL solution containing 0.1 mg/mL M13 and 5 mM maleate (pH 6.0) was injected over the activated dextran at a flow rate of 2 μL/min, followed by deactivation of residual NHS esters with 1 M ethanolamine (pH 8.5) at a flow rate of 5 μL/min.

The immobilization process was monitored in situ by SPR. The change in the observed resonance units (RU) was 1722 ± 265 for three trials, which corresponded to 1.7 ± 0.3 ng/mm^2 M13. Since the molecular weight of CaM is 6 times higher than that of the M13 peptides, more than 10 000 RU changes in SPR signals are expected to occur if CaM−Ca^{2+} complexes bind all of the M13 molecules immobilized on the dextran matrix with a 1:1 stoichiometry.[10a] The changes in SPR signals of the order of 10 000 RU are well over the noise level (less than 1 RU) of observed SPR signals in the present study. The amount of M13 thus immobilized on the dextran matrix was, therefore, assumed to be enough for the following quantitative evaluation of CaM−Ca^{2+}−M13 complex.

Measurement of SPR Signals. The M13-immobilized dextran matrix on the gold surface was equilibrated with a running buffer (0.5 mM EGTA, 150 mM NaCl, 10 mM HEPES, pH 7.5). The operating temperature for all SPR measurements was 25.0 ± 0.1 °C. The Ca^{2+} solutions were prepared, consisting of 5 μM CaM, 0.5 mM EGTA, 150 mM NaCl, 10 mM HEPES (pH 7.5), and each concentration of Ca^{2+}. Since the change in the refractive index on the gold surface was greatly affected by a small change in ionic strengths (*I*s) of the adjacent buffer solutions, 150 mM of NaCl was added to all the sample solutions to eliminate such an ionic strength effect. The concentration of free Ca^{2+} in the presence of a given concentration of EGTA was determined by using the conditional formation constant of the Ca^{2+}−EGTA complex at a given pH, calculated from the formation constant of the Ca^{2+}−EGTA chelate (log K = 11.0, I = 0.1, 25 °C) and four acid dissociation constants of EGTA (pK_1−pK_4 = 2.08, 2.73, 8.93, and 9.54, I = 0.1, 25 °C);[14] the values of formation and dissociation constants used here were those with I = 0.1 and were not corrected for the experimental ionic strength value of 0.15 employed in the present study. The samples were loaded into an autosampler of the instrument and injected automatically into the flow cell, followed by washing the dextran matrix with a running buffer for ~3 min to remove CaM. This procedure was repeated at each concentration of free Ca^{2+}.

Responses for Mg^{2+}, K^+, Li^+, and Sr^{2+} of the present sensing system were evaluated by a separate solution method similar to the ones used in ion-selective electrode methodology.[15] For doing so, the concentration of interfering ions had to be increased to an extreme level like ~10^{-1} M, because the effect of interfering ions was in many cases found to be very small. In such an experimental condition, we found CaM-independent SPR signals with the concentration of analyte ions (primary or interfering ions) exceeding 1.0 × 10^{-2} M. This unexpected CaM-independent and non-ion-selective SPR signals may have originated from a large bulk refractive index change caused by the high concentration of analyte ion solutions. In order to offset these unwanted signals, the SPR signal measured in each sample solution without CaM was subtracted from the one with CaM under otherwise identical conditions.

The observed changes in the refractive index on the gold surface were defined as the resonance units. All SPR signals were measured at a constant flow rate of 5 μL/min. The precision of the observed SPR signals was evaluated upon five times repetitive injections of 1.0 × 10^{-4} M free Ca^{2+} solution: The SPR signal with thus obtained standard deviation was 3286 ± 10 RU.

RESULTS AND DISCUSSION

The sensitivity of SPR depends on the molecular weight of proteins and has a lower limit of detection of 10 pg/mm^2.[16] However, small molecules or ions are difficult to detect because they contribute only small changes in the mass, giving also only minimum refractive index changes.[17] The reason why M13 was immobilized in the present system rather than CaM was that we expected much larger SPR signals by doing so than the other way, because the molecular weight of CaM is 6 times higher than that of M13. The present approach, relying on the formation of a large molecular complex, a CaM−Ca^{2+}−M13 ternary complex, enables one to measure small ions and molecules like Ca^{2+} by the SPR method.

(i) Response for Ca^{2+}. When M13 was immobilized on the dextran matrix and a Ca^{2+} ion sample solution containing CaM was injected, binding of the CaM−Ca^{2+} complex to the immobilized M13 was revealed as a response of the SPR signal. A typical time profile of the SPR signals with increasing concentrations of Ca^{2+} ion from 4.1 × 10^{-9} to 1.1 × 10^{-5} M is shown in Figure 2. Upon conditioning the sensing membrane in a buffer solution containing 0.5 mM EGTA, an initial baseline (marked by bar L in Figure 2) for the SPR signal was 13 207 RU. With injection of the Ca^{2+} sample solutions, the SPR signals increased and leveled off within 3 min. At low Ca^{2+} concentrations, this SPR signals did not return to the baseline upon running the buffer solution containing 0.5 mM EGTA. Upon conditioning with the buffer solution containing EGTA after the measurement for 1.1 × 10^{-5} M Ca^{2+} solution, a final baseline (bar R in Figure 2) for the SPR signal was 13 897 RU, which is higher than the initial baseline (13 207 RU).

The small difference of the SPR signals (690 RU) between the initial and final baselines may originate from Ca^{2+}-independent irreversible adsorption of a small amount of CaM on the dextran matrix and/or on the immobilized M13. Klevit et al. have reported by the NMR method that, in the absence of free Ca^{2+}, there is an ionic interaction between CaM and M13 if the concentration of CaM and M13 is high (500 μM), and adding high concentrations of NaCl results in their dissociation.[18] In the present system, the sample solutions contained 150 mM of NaCl and the concentration of CaM was only 5 μM. Therefore, the ionic interaction between

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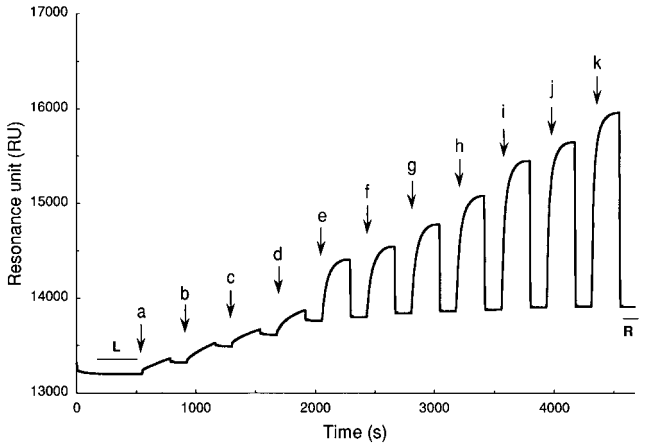

Figure 2. Changes in SPR signals by injecting various concentrations of Ca^{2+} ion (arrows a−k) and running buffer without Ca^{2+}. The surface-immobilized M13 was initially equilibrated during the time L with a running buffer (150 mM NaCl, 0.5 mM EGTA, 10 mM HEPES buffer, pH 7.5) at a constant flow of 5 μL/min. Calcium ion solutions ranging from 4.1×10^{-9} to 1.1×10^{-5} M each containing 5 μM CaM were injected successively at each arrow: (a) 4.1×10^{-9}, (b) 8.3×10^{-9}, (c) 1.4×10^{-8}, (d) 3.2×10^{-8}, (e) 1.1×10^{-7}, (f) 1.3×10^{-7}, (g) 1.9×10^{-7}, (h) 3.3×10^{-7}, (i) 9.6×10^{-7}, (j) 3.3×10^{-6}, and (k) 1.1×10^{-5} M. After the end of each injection, the surface-immobilized M13 was regenerated with the running buffer during a period R.

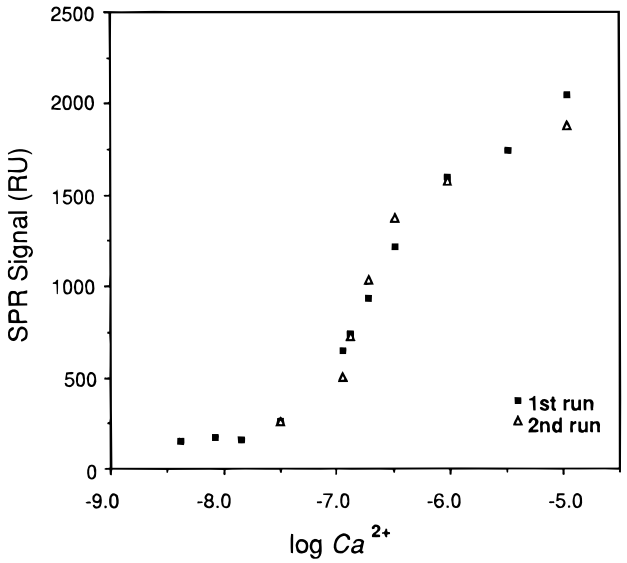

Figure 3. Dependence of the observed SPR signals on the concentrations of free Ca^{2+} ion. The data sets from just two separate runs are shown to demonstrate the reproducibility of the method.

CaM and immobilized M13 was initially expected to be negligible but might be remaining to a certain extent. In order to determine Ca^{2+}-dependent SPR signals, the measured SPR signal was corrected for the irreversible adsorption of CaM: We evaluated each Ca^{2+}-dependent SPR signal as the difference in SPR signals between those for Ca^{2+} sample solutions and for the following EGTA running buffer solution (see Figure 2). Hereafter the difference of SPR signals thus obtained is called simply the SPR signal.

Figure 3, which is replotted from Figure 2, shows the dependence of SPR signals thus defined as a function of Ca^{2+} concentrations. The magnitude of the SPR signal sharply increased with an increase in Ca^{2+} concentrations from 3.2×10^{-8} to 9.6×10^{-7} M, a moderate increase followed up to 1.1×10^{-5} M

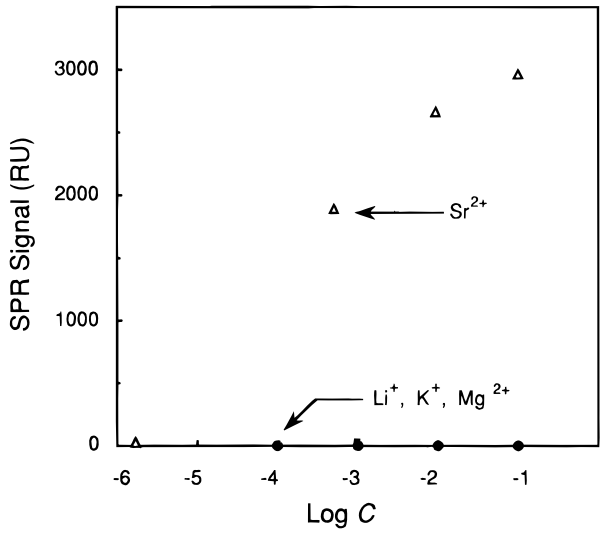

Figure 4. Dependence of SPR signals (ΔRU) on the concentration of Mg^{2+}, Sr^{2+}, Li^{+}, and K^{+} ions, respectively, each of which was dissolved in 5 μM CaM, 150 mM NaCl, 0.5 mM EGTA, and 10 mM HEPES buffer (pH 7.5). The concentration of each free metal ion was calculated based on the respective conditional stability constant for the 1:1 metal ion−EGTA complex.

free Ca^{2+}. Below 3.2×10^{-8} M Ca^{2+} concentration, small SPR signals were still observed. Upon injection of a CaM-containing sample solution free of Ca^{2+} ions, similar changes in the SPR signal (~40 RU) were also observed (data not shown). From these observation, we found that this small SPR signal was a Ca^{2+}-independent signal, which originated from bulk refractive index changes between the sample solutions and the running buffer.

A change in SPR signals was ~2000 RU when the concentration of Ca^{2+} increased from 3.2×10^{-8} to 1.1×10^{-5} M. This change was only 20% of 10 000 RU of the calculated values (vide supra), showing that roughly 20% of the M13 immobilized on the dextran matrix formed the ternary complex and the rest of M13 molecules did not form the complex. This may probably be because immobilization of M13 on the dextran matrix was not highly controllable; in particular, part of five lysine residues or the N terminal of M13 could be immobilized on the dextran matrix, which possibly hindered sterically the formation of the ternary complex or affected the specific conformational changes of the M13 peptide by an increase in α-helix content.[18]

The detection limit of the free Ca^{2+} concentration with the present sensing system was 1.6×10^{-8} M, defined as the concentration that gives a signal of 3 times the standard deviation of the background output. The detection limit for the present Ca^{2+} sensing was comparable to that of a fluorescein-labeled CaM sensor (5×10^{-8} M)[8] but inferior to those of optical ion sensors based on Ca^{2+} ionophores ETH1001 or ETH129,[19] which exhibited a detection limit of lower than 10^{-9} M free Ca^{2+}.

The working range of the calibration curve for the present method extends from 3.2×10^{-8} to 1.1×10^{-5} M free Ca^{2+}, which is 1 order of magnitude wider on the higer concentration side than that of the fluorescein-labeled CaM sensor.[8]

(ii) Response for Mg^{2+}. The results obtained according to the Experimental Section (vide supra) are shown in Figure 4. No changes in the SPR signals were observed even at Mg^{2+} ion concentrations up to 1.0×10^{-1} M.

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The binding sites for Mg^{2+} ions in CaM are known to be the same as those for Ca^{2+}.[20] The dissociation constants between CaM and Mg^{2+} ions, obtained by competitive binding reaction of Mg^{2+} to the Ca^{2+} binding sites of CaM with the flow dialysis method, range from 10^{-4} to 10^{-3} M at each binding site in CaM ($K_{d1}-K_{d4} = 0.70 \times 10^{-4}$, 2.7×10^{-4}, 1.6×10^{-4}, and 0.90×10^{-4} M, respectively).[6] Therefore, at 1.0×10^{-1} M Mg^{2+}, all four binding sites of CaM are occupied by Mg^{2+}. Our results indicate that the $Mg^{2+}-$CaM complex, even if formed, is incapable of binding to M13, thereby inducing no SPR signal.

Changes in conformation of CaM upon binding Ca^{2+} and its subsequent binding to its target peptide have been studied by NMR,[10a] CD,[18] variation in tryptophan fluorescence,[20] and spectral differences of benzophenone derivatives of a target peptide.[21] A Ca^{2+}-specific change in the conformation of CaM was found to be the requisite for binding to its target protein or peptide. The influence of Mg^{2+} on the conformation of CaM has also been investigated by ^{1}H NMR or ^{25}Mg NMR with Mg^{2+} titration,[7] Fourier transform infrared spectroscopy (FT-IR),[22] and electron paramagnetic resonance (EPR) studies.[23] Contrary to the Ca^{2+} case, it was found that, although Mg^{2+} binds at the Ca^{2+}-binding sites of CaM (which has been confirmed by competitive binding between Ca^{2+} and Mg^{2+} for CaM),[7] it did not induce the subsequent large change in conformation and therefore did not bind to its target protein. The obtained result in the present study of silent SPR signals for Mg^{2+} up to 1.0×10^{-1} M is consistent with the conclusion drawn by the above NMR, FT-IR, and EPR studies. In view of the above considerations, it is concluded that the present sensing system is a simple new approach for evaluating "physiologically more relevant" cation selectivity for the Ca^{2+} signaling pathway.

(iii) Responses for K^+ and Li^+. In the case of K^+ and Li^+, no changes in the SPR signals were observed at concentrations up to 1.0×10^{-1} M.

Four K^+ ions occupy all the binding sites of CaM according to their binding constants ($K_{d1}-K_{d4} = 3.7 \times 10^{-3}$, 10.6×10^{-3}, 8.7×10^{-3}, and 1.5×10^{-3} M, respectively).[6a] However, the present SPR result indicates that the binding of K^+ ion to CaM does evokes neither any CaM conformational changes nor its subsequent binding to M13. Though the dissociation constant between CaM and Li^+ is not known, the present result indicates that Li^+ ion after all evokes no conformational change for CaM to bind M13.

(iv) Response for Sr^{2+}. A large SPR response was observed by Sr^{2+} indicating that, upon binding with CaM, Sr^{2+} induces the conformational change of CaM and formation of the CaM$-Sr^{2+}-$M13 ternary complex resulting at the surface.

The observed SPR result with Sr^{2+} concludes that a concentration of more than 5.1×10^{-4} M Sr^{2+} ion behaves as a strong interfering ion or agonist toward the Ca^{2+}-dependent on/off switch of CaM for intracellular processes. The result implies that Sr^{2+} ion may be toxic in the CaM signaling pathway in a living cell. The response of SPR signals for Sr^{2+} ion is consistent with Sr^{2+} ion selectivities previously reported by CaM binding enzyme activities such as guanylate cyclase[24] and cyclic adenosine 3′:5′-monophosphate phosphodiesterase.[25] With the present approach, we do not need to measure the enzyme activities, but the interaction between CaM and its target peptide is directly monitored by the SPR technique. It would therefore be useful for more widely evaluating agonist selectivity or toxicity for various other divalent cations and also for finding possible inhibitors (antagonists) specific for CaM signaling.

CONCLUSION

A simple new method was developed for evaluating ion selectivity in the Ca^{2+} signaling pathway in the cell, using a naturally occurring selective interaction of CaM with Ca^{2+} and its binding peptide M13 derived from myosin light-chain kinase. Based on the cellular Ca^{2+} signal transduction mechanism, Ca^{2+} ion-switched CaM binding to its target peptide M13 was monitored by SPR. The sequential mechanism from the Ca^{2+} recognition by CaM through the Ca^{2+}-switched binding to CaM to M13 was confirmed from the SPR signals dependent on Ca^{2+} ion concentrations, illustrating the usefulness of the present approach for evaluating a physiologically more relevant metal ion selectivity for the Ca^{2+} signaling as observed in its agonistlike nature for Sr^{2+} ions. The present approach may also provide a means of evaluating toxicological effects of metal cations or organic compounds on the Ca^{2+} signaling pathway through CaM and also as observing a protein/protein interaction between CaM binding protein and CaM whose amino acid sequences are changed by genetic engineering.

ACKNOWLEDGMENT

This work was financially supported from Grants for Scientific Research by the Ministry of Education, Science and Culture, Japan, and from the Nissan Science Foundation (Tokyo, Japan). We are grateful to Prof. Mitsuaki Yoshida, The Institute of Medical Science, The University of Tokyo, for kindly providing us with plenty of machine time for the SPR measurement at his Institute.

Received for review December 31, 1996. Accepted May 19, 1997.[⊗]

AC9613141

[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1997.

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