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Metal ion selectivity for formation of the calmodulin–metal–target peptide ternary complex studied by surface plasmon resonance spectroscopy

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Abstract

Ion selectivities for Ca^{2+} signaling pathways of 33 metal ions were examined based on the Ca^{2+}-dependent on/off switching mechanism of calmodulin (CaM): Ca^{2+} ion-induced selective binding of CaM–Ca^{2+} ion complex to the target peptide was observed as an increase in surface plasmon resonance (SPR) signals. As the target peptide, M13 of 26-amino-acid residues derived from skeletal muscle myosin light-chain kinase was immobilized in the dextran matrix, over which sample solutions containing CaM and each metal ion were injected in a flow system. Large changes in SPR signals were also observed for Sr^{2+}, Ba^{2+}, Cd^{2+}, Pb^{2+}, Y^{3+} and trivalent lanthanide ions, thereby indicating that not only Ca^{2+} but also these metal ions induce the formation of CaM–M13–metal ion ternary complex. No SPR signal was, however, induced by Mg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} and all monovalent metal ions examined. The latter silent SPR signal indicates that these ions, even if they bind to CaM, are incapable of forming the CaM–M13–metal ion ternary complex. Comparing the obtained SPR results with ionic radii of those metal ions, it was found that all cations examined with ionic radii close to or greater than that of Ca^{2+} induced the formation of the CaM–metal–M13 ternary complex, whereas those with smaller ionic radii were not effective, or much less so. Since these results are so consistent with earlier systematic data for the effects of various metal ions on the conformational changes of CaM, it is concluded that the present SPR analysis may be used for a simple screening and evaluating method for physiologically relevant metal ion selectivity for the Ca^{2+} signaling via CaM based on CaM/peptide interactions.

Keywords: Calmodulin; Myosin light-chain kinase; Surface plasmon resonance; Calcium; Metal ion

1. Introduction

Calcium ions act as intracellular messengers that control the functions of cells in many living systems [1–3]. In the resting cell, the concentration of Ca^{2+} is less than 10^{-7} M and during activation it rises to approximately 10^{-6} M. 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+} receptor proteins, calmodulin (CaM; 148 amino acid residues, 16.7 kDa) belongs to a ubiquitous Ca^{2+} binding protein that translates this modest rise in intracellular Ca^{2+} into a physiological response such as energy and biosynthetic metabolism, exocytosis, cytoskeletal assembly and intracellular modulation of both cAMP and

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Ca^{2+} concentrations in all eukaryotic cells. CaM performs these roles by primarily accommodating four Ca^{2+} ions in its binding sites and its subsequent large conformational changes in CaM. Upon this conformational change of CaM, it allows the CaM–Ca^{2+} complex to further bind the CaM binding site on the target enzymes such as myosin light-chain kinase (MLCK) [4,5]. This sequential mechanism by which CaM–Ca^{2+} recognizes the target proteins via its conformational changes has been defined by multidimensional NMR spectroscopy and X-ray crystallography [6–12].

The change in kinase activity of MLCK based on the above described mechanism of Ca^{2+} signaling was demonstrated by measuring the amount of phosphate transferred from [γ-^{32}P]ATP into myosin light chain [13–15]. The extent of thus-measured MLCK activity was found increased with increasing concentrations of Ca^{2+} from 1.0×10^{-6} to 1.0×10^{-5} M and then leveled off. Similar results were also observed by Cd^{2+}, Pb^{2+} or Sr^{2+} at 1.0×10^{-6} to 1.0×10^{-5} M with otherwise identical systems. No phosphorylation of the light chain was, however, observed for Zn^{2+}, Al^{3+} and Hg^{2+} at their concentrations up to 1.0×10^{-3} M. Besides the effects of those metal ions on the MLCK activity, effects of metal ions on phosphodiesterase (PDE) activity, which is also one of the CaM targets, have been studied by measuring the amount of [^{3}H]adenosine produced by hydrolysis of [^{3}H]cAMP: the increase in PDE activities was induced not only by Ca^{2+} but also by Zn^{2+}, Mn^{2+}, Cd^{2+}, Hg^{2+}, Sr^{2+}, Pb^{2+}, Tb^{3+}, Sm^{3+} and La^{3+}; but no such effect was shown by Be^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+} and Ba^{2+} [16]. The metal ions activating MLCK or PDE such as Cd^{2+}, Hg^{2+}, and Pb^{2+} are known to represent serious environmental pollutants, and accumulate in various human tissues, causing neurological, muscular and retinal disorders [17–19]. These metal ions, then, raise a possibility that some of their toxicity in biological systems may be mediated through their activation of CaM. To understand the mechanism of the toxicity in the biological systems, simple methods for screening metal ions or agonists for intracellular Ca^{2+} signaling are increasingly required in addition to bioassay methods of analytes. Until now, the radiochemical analyses using radiolabeled substrates become the most powerful and widespread techniques currently used in the evaluation of the effects of agonists like those metal ions on the enzyme activities such as MLCK or PDE.

In the present study, metal ion selectivity is evaluated by the SPR method for the Ca^{2+} signaling pathway based on on/off switching mechanism of the CaM-mediated Ca^{2+} signaling. A preliminary study of this approach was presented earlier [20]. The principle is as follows: 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 of a CaM binding domain (residues 577–602) of skeletal muscle myosin light-chain kinase (MLCK), is immobilized covalently on the dextran matrix attached to the gold surface. M13 is known to bind to the CaM–Ca^{2+} complex with a dissociation constant of 1 nM. Upon binding four Ca^{2+} ions in its binding sites of CaM, it undergoes a conformational change in the sample solution. The CaM–Ca^{2+} complex thus formed is then bound to the immobilized target peptide M13, and forms a CaM–Ca^{2+}–M13 ternary complex in the dextran matrix, which is measured by SPR, a technique that responds to changes in the refractive index close to a gold surface. The amount of the ternary complex thus formed is a selective and sensitive measure of the extent of the Ca^{2+} signaling. The obtained SPR results were compared with ionic radii of each metal ion, and all of the metal ions having ionic radii comparable to or higher than Ca^{2+} were thereby found to induce the formation of the ternary complex. Comparing the obtained SPR results and earlier systematic studies on the effect of various cations on the conformational changes of CaM, the validity and general applicability of the present SPR method for the screening and evaluation of metal ion selectivity in Ca^{2+}–CaM signaling system will be discussed.

2. Materials and methods

2.1. Materials

Chemically synthesized HPLC-purified M13 peptide, which consists of the amino acid sequence of KRRWKKNFIAVSAANRFKKISSSGAL (express-

ed by one-letter abbreviations), was kindly provided by Dr. M. Ikura (University of Toronto, Ontario, Canada). *O,O′*-bis(2-Aminoethyl)ethyleneglycol-*N*,*N*,*N′*,*N′*-tetraacetic acid (EGTA), 2-morpholinoethanesulfonic acid monohydrate (MES) 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) and dysprosium chloride hexahydrate were obtained from Nacalai Tesque (Kyoto, Japan). Erbium(III) chloride hexahydrate, thulium(III) chloride hexahydrate and lutetium(III) chloride hexahydrate were obtained from Aldrich Chem. Co. (Milwaukee, WI). Terbium chloride hexahydrate, praseodymium chloride heptahydrate and *N*-hydroxysuccinimide (NHS) were obtained from Kanto Chem. Co. (Tokyo, Japan). Other salts and solvents used were obtained from Wako Pure Chem. Ind. (Osaka, Japan). All of the salts were of the highest purity available. All aqueous solutions were prepared with Milli-Q grade water (Millipore reagent water system, Bedford, MA).

2.2. Extraction of CaM

Bovine brain CaM was extracted as previously described [21]. To remove salts and impurity from the extracted CaM, the following purification steps were added. Lyophilized CaM was dissolved in a small volume of Milli-Q water, and applied to reverse phase HPLC performed on a 801-SC system (Japan Spectroscopic, Tokyo, Japan), equipped with a Kaseisorb LC ODS-300-5 column (diameter 4.6×250 mm) and UV detector (UV-970, Japan Spectroscopic), using a linear gradient of 0% to 80% acetonitrile in 0.05% trifluoroacetic acid, at a constant flow rate of 10.0 ml/min over 60 min. The eluents were monitored by UV absorbance at 220 nm. Fifty ml of an eluent containing CaM was collected and lyophilized again. The purity of CaM was assessed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE): a single band of CaM was detected at a molecular mass of 17 000.

2.3. Apparatus

All SPR measurements were performed on a BIAcore 2000 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, Tokyo, Japan).

2.4. Immobilization of M13 on the carboxymethylated dextran matrix

Immobilization of M13 on the dextran matrix attached to a gold thin film was performed via primary amine groups by the amine coupling method [22]. 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.

2.5. Measurement of SPR signals

The M13-immobilized dextran matrix was equilibrated with a running buffer (0.5 mM EGTA, 150 mM NaCl, 10 mM Hepes (pH 7.0) or MES (pH 6.0)). The operating temperature for all SPR measurements was 25.0±0.1°C. Metal ion solutions were prepared, consisting of 5.0 μM CaM, 150 mM NaCl, 10 mM Hepes (pH 7.0) or MES (pH 6.0), and a given concentration of metal ions. The concentration of free Ca^{2+} in the presence of 0.50 mM 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 complex ($\log K = 11.0$, $I = 0.1$, 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) [23]; 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.

2.6. Evaluation of SPR signals

Concentrations of metal ions had to be increased to a fairly high level like 1.0×10^{-1} M for Mg^{2+}, Ba^{2+} and alkali metal ions, because the effect of those metal ions on the observed SPR signals for the present method was found to be relatively small. In such an experimental condition, we found the influence of added metal ions on the SPR signals that was CaM-irrelevant, when the concentration of each metal ion exceeded 10^{-5}–10^{-4} M. These CaM-irrelevant and non-ion-selective SPR signals originated from changes in bulk refractive indexes caused by the high concentration of metal ion solutions. In order to offset these background signals, the SPR signal measured for each sample solution in the presence of M13 was corrected for the one in the absence of M13 under otherwise identical conditions.

The measurement for each sample metal ion was repeated at least three times, including the preparation process for M13 immobilization. Even though the same sample solution flowed over the dextran matrix, changes in the SPR signals measured on each sensor chip were different from those on the other chips. In order for the SPR signals to be comparable from one sensor chip to those for another, the observed SPR signals for each sample metal ion were normalized according to the equation $R = 1000\times R_{ob}/R_{max}$, where R_{ob} is the observed SPR signal for each concentration of metal ion and R_{max} is the SPR signal for 1.0 mM Ca^{2+}, where four EF-hand sites of CaM are fully occupied with Ca^{2+}: The Ca^{2+}-induced SPR signals are always expressed as 1000 (arbitrary unit); the change in SPR signals induced by each metal cation is expressed by the value relative to the one of Ca^{2+} and thus being made directly comparable. Hereafter the normalized SPR signals, R, thus obtained are called simply SPR signals.

3. Results

3.1. Response to Ca^{2+}

Upon immobilizing M13 in the dextran matrix and conditioning the sensing membrane in a buffer solution containing 0.5 mM EGTA, Ca^{2+} sample solutions containing 5.0 μM of CaM (pH 7.0) were injected. A typical time profile of the SPR signals is shown in Fig. 1 with Ca^{2+} ion concentrations from 1.3×10^{-8} to 4.0×10^{-4} M, where injection of the Ca^{2+} sample solutions induced an immediate increase in the SPR signal until it soon leveled off. Upon running the EGTA buffer solution after the measurement of each concentration of Ca^{2+} solutions, a final baseline returned to the initial baseline of zero RU without hysteresis. In order to determine Ca^{2+}-relevant SPR signals, the Ca^{2+}-dependent signals were evaluated as the difference in SPR signals between those for the running EGTA buffer solution and for the Ca^{2+} sample solution.

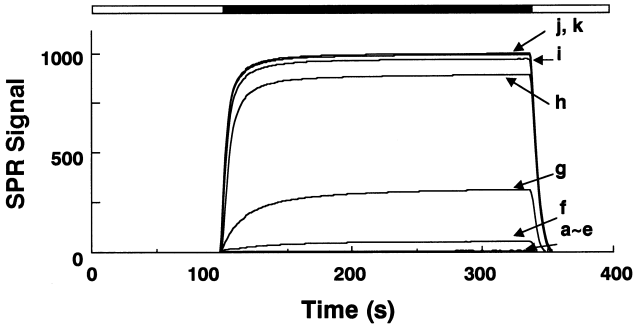

Fig. 1. Changes in SPR signals by injecting various concentrations of Ca^{2+} ion (horizontal closed bar) and a running buffer without Ca^{2+} (horizontal open bar). The surface-immobilized M13 was initially equilibrated for 100 s with a running buffer (150 mM NaCl, 0.5 mM EGTA, 10 mM Hepes buffer, pH 7.0) at a constant flow of 5.0 μl/min. Calcium ion solutions ranging from 1.3×10^{-8} to 4.0×10^{-4} M each containing 5.0 μM CaM were injected (horizontal closed bar): (a) 1.3×10^{-8}, (b) 3.4×10^{-8}, (c) 4.6×10^{-8}, (d) 5.2×10^{-8}, (e) 1.4×10^{-7}, (f) 3.1×10^{-7}, (g) 8.3×10^{-7}, (h) 1.0×10^{-5}, (i) 1.0×10^{-4}, (j) 3.0×10^{-4}, and (k) 4.0×10^{-4} M. After the end of injection, the surface-immobilized M13 was regenerated with injecting the running buffer.

Fig. 2A, replotted from Fig. 1, shows the SPR signals as a function of Ca^{2+} concentration. The magnitude of the SPR signal sharply increased with an increase in Ca^{2+} concentrations from 3.0×10^{-6} to 1.0×10^{-5} M. The median effective value, ED_{50}, defined as the metal ion concentration yielding a half-maximum SPR signal (500 a.u.), was 1.6×10^{-6} M in this particular case. When pH of the Ca^{2+} sample solution was changed from 7.0 to 6.0 or from 7.0 to 7.5, the Ca^{2+}-dependent SPR signals were shifted in parallel to higher or lower Ca^{2+} concentration side, respectively. The ED_{50} value for

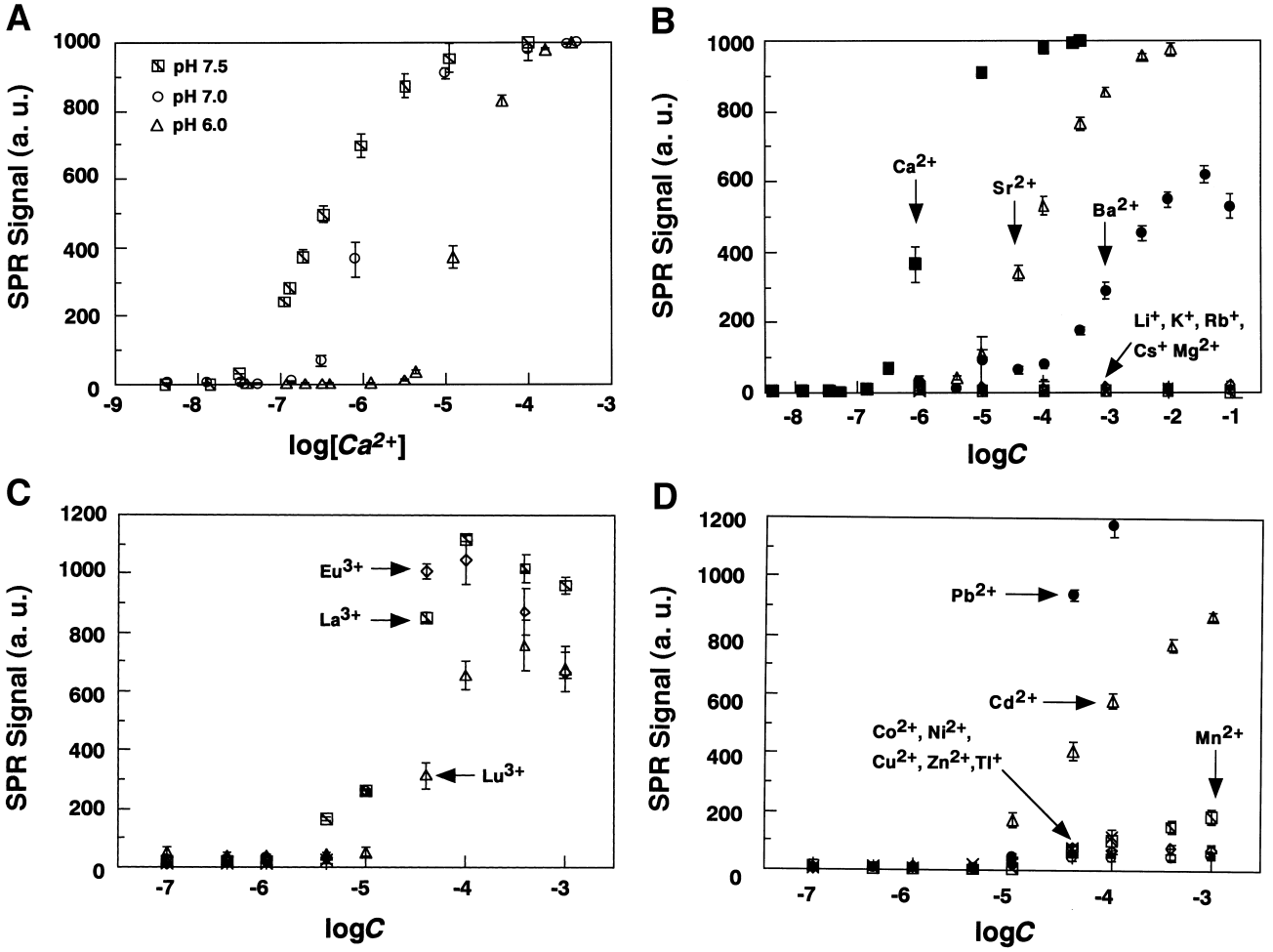

Fig. 2. Dependence of the SPR signals on the concentration of metal ions. The surface-immobilized M13 was initially equilibrated with a running buffer (150 mM NaCl, 500 μM EGTA, 10 mM Hepes/NaOH (pH 7.5 and 7.0) or 10 mM MES/NaOH (pH 6.0)) at a constant flow of 5.0 μl/min. Metal ion solutions, each containing 5.0 μM CaM, were injected successively and SPR signal changes were observed 4 min after the injection of metal ion solutions. (A) Dependence of the SPR signals on the concentrations of free Ca^{2+} ions at pH 7.5 (square with slash), pH 7.0 (open circle) and pH 6.0 (open triangle). The Ca^{2+} concentrations of each pH buffer solution were calculated based on the respective conditional stability constants for the 1:1 metal ion–EGTA complex. (B) Dependence of the SPR signals on the concentrations of Mg^{2+}, alkali and alkaline earth metal ions. The pH of a Hepes buffer solution containing 150 mM NaCl and each concentration of the metal ion was adjusted at 7.0. (C) Dependence of the SPR signals on the concentrations of La^{3+}, Eu^{3+} and Lu^{3+}. The pH of a MES buffer solution containing 150 mM NaCl and each concentration of the metal ion was adjusted at 6.0. (D) Dependence of the SPR signals on the concentrations of Cd^{2+}, Pb^{2+} and transition metal ions. The pH of a MES buffer solution containing 150 mM NaCl and each concentration of the metal ion was adjusted at 6.0.

Ca^{2+} at pH 7.5 was 3.3×10^{-7} M and the one at pH 6.0 was 1.6×10^{-5} M. These results demonstrate that the amount of the CaM–Ca^{2+}–M13 ternary complex formed in the dextran matrix increases with increasing pH values of the sample solutions.

3.2. Response to alkali and alkaline earth metal ions

The results of the SPR signals obtained upon injection of sample solutions (pH 7.0) together with a given concentration of alkali or alkaline earth metal ions are shown in Fig. 2B. A large increase in the SPR signal was observed with Sr^{2+} ion at its concentration from 3.0×10^{-6} to 1.0×10^{-2} M. Also, Ba^{2+} ion induced an increase in the SPR signals from 1.0×10^{-5} to 4.0×10^{-2} M. Its maximum response of the SPR signal is, however, nearly half of Ca^{2+} ion. The responses for Sr^{2+} and Ba^{2+} indicate that

both ions can be a substitute for Ca^{2+} ion to induce the formation of CaM–metal–M13 complex at their examined concentration ranges. The ED_{50} values for Sr^{2+} and Ba^{2+} ions were 8.5×10^{-5} and 3.9×10^{-2} M, respectively. In contrast, no change in the SPR signals was observed for Li^{+}, K^{+}, Rb^{+}, Cs^{+} and Mg^{2+} ions even at their concentrations up to 1.0×10^{-1} M, indicating no formation of the ternary complex with these ions.

3.3. Response to rare earth metal ions and other metal ions

Since trivalent rare earth cations and some divalent transition metal ions such as Cr^{2+} and Cu^{2+} tend to precipitate in solutions at neutral pH by hydrolysis, it needs to keep their solution pH lower. The affinity of CaM for metal ions is known to gradually decrease with decreasing pH from 8.0 to 5.0 and sharply decrease with its pH lower than 5.0 [24]. All SPR measurements for various fixed concentrations of rare earth and transition metal ions were therefore performed at pH 6.0, where even Mn^{2+} ion, which is the most insoluble in solutions among transition and rare earth metal ions examined, is dissolved in solutions of its concentration up to $\sim10^{-4}$ M.

The results of the SPR signals for La^{3+}, Eu^{3+} and Lu^{3+} are shown in Fig. 2C. Large increases in the SPR signals were observed for all rare earth metal ions with concentrations from 1.0×10^{-5} to 1.0×10^{-4} M. Upon further increasing their concentration above 1.0×10^{-4} M, the SPR signals leveled off and then decreased. The initial increases in the SPR signals with increasing those metal ion concentrations indicate that all trivalent rare earth metal ions examined induce the conformational change of CaM to form the CaM–metal–M13 ternary complex. The decreases in the SPR signals following its maximum may be due to an allosteric feedback regulation of CaM by the metal ions. It has been demonstrated by Tb^{3+} fluorescence measurements that increasing the concentration of Tb^{3+} ion greater than 5.0×10^{-5} M inhibited activities of CaM-dependent phosphodiesterase, which was explained to be due to aggregation of CaM caused by increased Tb^{3+} ion concentration [25]. Although the target of CaM in this case is different from MLCK used in the present study, the decrease in the present SPR signal for Tb^{3+} ion may likewise originate from the aggregation of CaM. The decreases in the SPR signals for the other lanthanide ions may also cause the aggregation of CaM as the Tb^{3+} case. ED_{50} values of all rare earth metal ions, estimated from the SPR signals for their concentrations from 1.0×10^{-6} to 1.0×10^{-4} M, are listed in Table 1.

Table 1
Median effective values (ED_{50}) for each lanthanide ion

Metal ion	Log[ED_{50}]
Y^{3+}	−4.26
La^{3+}	−4.76
Ce^{3+}	−4.78
Pr^{3+}	−4.82
Nd^{3+}	−4.77
Sm^{3+}	−4.73
Eu^{3+}	−4.81
Gd^{3+}	−4.79
Tb^{3+}	−4.70
Dy^{3+}	−4.69
Ho^{3+}	−4.66
Er^{3+}	−4.56
Tm^{3+}	−4.57
Yb^{3+}	−4.47
Lu^{3+}	−4.18

Responses of SPR signals for Cd^{2+}, Pb^{2+}, Tl^{+} and other typical transition metal ions are shown in Fig. 2D. The SPR signals for Cd^{2+} increased with its concentration from 1.0×10^{-5} M to 1.0×10^{-3} M. In the case of Pb^{2+}, the SPR signals also increased from its concentration of 4.0×10^{-6} M. The maximum response of the SPR signal for Pb^{2+} was higher than 1000 a.u. and did not reach its plateau. The ED_{50} values of the SPR signals for Cd^{2+} and Pb^{2+} were 7.1×10^{-6} M and 2.6×10^{-6} M, respectively, both being nearly equal to that for Ca^{2+} (pH 6.0). For Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} and Tl^{+} ions, none of these ions exhibited an increase in SPR signals at their concentrations up to 1.0×10^{-3} M. A moderate increase in the SPR signal was observed with Mn^{2+} ion, although its maximum was below 200 a.u.

3.4. Comparison of the ED_{50} values with ionic radius for each metal ion

Median effective values (ED_{50}) for each metal ion and its ionic radius are listed in Table 2. The metal

ions examined were classified into the following two categories: Group I ions, monovalent metal ions such as Li^+, Na^+, K^+, Rb^+ Cs^+ and Tl^+, were silent in inducing the SPR signals, indicating no formation of CaM–metal–M13 ternary complex. Group II includes di- and trivalent cations, among which Ca^{2+}, Sr^{2+}, Ba^{2+}, Cd^{2+}, Pb^{2+}, Y^{3+} and all lanthanide ions examined were found to induce the conformational change for CaM to bind M13.

Metal ions such as Cd^{2+}, Eu^{3+} and Tb^{3+} have been used as isomorphous analogues of Ca^{2+} for studying structures of EF-hand sites of CaM and magnitude of the binding constants for each metal ion. All of these metal ions have been chosen because of similarity in ionic radii between Ca^{2+} and those metal ions [3,26]. It is therefore of great interest to investigate what relations exist between ionic radii of each metal ion and the ability of forming CaM–M13–metal ion ternary complexes. In order to examine each metal ion concentration requisite for the formation of the ternary complex of equal amount to the Ca^{2+} case, Ca^{2+} equivalent factor (CEQ) was here defined as,

$$\mathrm{CEQ} = \frac{\mathrm{ED}_{50}^{\mathrm{Metal}}}{\mathrm{ED}_{50}^{\mathrm{Ca}}}$$

Table 2
Comparison between the median effective values (ED_{50}) and ionic radii for each metal ion

Metal ion	Log[ED_{50}]	CEQ	Ionic radii (nm)
Ca^{2+}	−5.81 (pH 7)	1.00	0.100
	−4.80 (pH 6)	1.00	
Mg^{2+}	–	–	0.072
Cr^{2+}	–	–	0.080
Mn^{2+}	–	–	0.083
Co^{2+}	–	–	0.074
Ni^{2+}	–	–	0.069
Cu^{2+}	–	–	0.073
Zn^{2+}	–	–	0.075
Sr^{2+}	−4.07	1.51×10^{-2}	0.113
Ba^{2+}	−1.41	3.23×10^{-5}	0.142
Cd^{2+}	−4.15	2.20×10^{-1}	0.095
Pb^{2+}	−4.59	6.17×10^{-1}	0.118
Y^{3+}	−4.26	2.88×10^{-1}	0.090
Ln^{3+} [a]	−4.81	2.40×10^{-1}	0.086
	∼−4.18	∼1.03	∼0.106

[a] Ln means all the lanthanide ions in the present study.

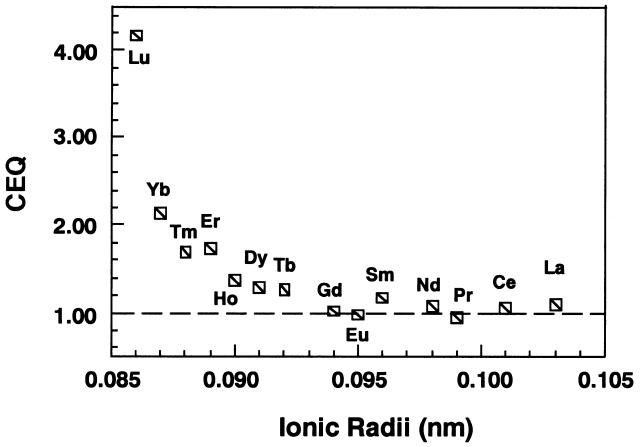

Fig. 3. The variation of CEQ values with the ionic radii of lanthanide metal ions. The CEQ value for Ca^{2+} in a pH 6.0 buffer solution is unity (= 1) by definition and is marked (dashed line) for reference.

where pH of the sample solutions for ED_{50}^{Metal} is the same as that for ED_{50}^{Ca}. This CEQ value is an empirical factor; if the CEQ value for a given metal ion is equal to 1.0, the concentration of that metal ion providing the same amount of CaM–M13–metal ion complex is the same to that of Ca^{2+}. It should be noted that the CEQ value shows normalized ED_{50} in the direction of the *x*-axis, whereas the SPR signals are normalized in the *y*-axis (see Section 2). The results are listed in the third column (CEQ) in Table 2 and the variation of CEQ values with the ionic radii for lanthanide ions is shown in Fig. 3. The CEQ values from Lu^{3+} to Gd^{3+} decreased with the ionic radii and leveled off from Gd^{3+} to La^{3+}. The CEQ values from Gd^{3+} to La^{3+} are almost equal to that of Ca^{2+}, while the ionic radii of these lanthanide ions are close to that of Ca^{2+}. These results confirm that the metal ion having ionic radii close to that of Ca^{2+} induces the formation of the CaM–M13–metal ion ternary complex.

The usefulness of introducing CEQ values is due to that the surface concentration of modified M13 is not necessarily the same between different surface preparations, causing formation of scattered amount of M13–CaM–metal ion complexes. The amount of the ternary complex for each metal ion is therefore measured relative to that for Ca^{2+} to normalize this unwanted change in the M13–CaM–metal ion complexes. This set of measurements was achieved for each metal ion by using a respective single surface

preparation. It was assumed that chemistry of surface modification is uniquely controlled in the immobilization process of M13. In other words, the amine coupling with the carboxyl groups in the dextran matrix is always achieved at the same places using any of five lysine residues or the N-terminals of M13. The validity of this assumption was supported by the fact that the obtained CEQ values measured with different surface preparations remained constant for a given metal ion. It is also noted that the immobilization with lysine side chains would reduce the affinity of CaM for the peptide, compared to the one in the bulk solution.

4. Discussion

4.1. Ion selectivity for the formation of CaM–metal–M13 complexes

According to the present SPR results, among 33 metal ions, only limited numbers of ions exhibited the formation of CaM–metal–M13 ternary complexes. In earlier works, the effects of metal ions on conformational changes of CaM in the absence of M13 have been studied by fluorescence, NMR, ESR and electrophoretic mobility shift assay (EMSA) measurements. Ohki et al. identified Ca^{2+}- and Mg^{2+}-induced structural changes using two-dimensional NMR by monitoring chemical shift changes of individual amino acid residues of CaM during the Ca^{2+} and Mg^{2+} ion titrations [27]. In contrast to the large structural changes induced by Ca^{2+}, Mg^{2+} ion was found to cause only localized conformational changes within the four Ca^{2+}-binding loops of CaM. Recently, Malmendal et al. gave more detailed information on the structural changes of the N-terminal domain accommodating Mg^{2+}, through ^{1}H and ^{15}N chemical shifts and line widths in NMR spectra [28]. N-Terminal loop I has slower binding kinetics compared with C-terminal loop II and the Mg^{2+}-saturated loop II undergoes small conformational exchange on a 100-μs time scale, which is similar to that of Mg^{2+} exchange in loop II. Also, the structural change of CaM upon binding respective metal ions has been evaluated by ESR spectral changes with a specific probe such as spin-labeled tyrosine, and/or methionine [29,30]. Ca^{2+}, Cd^{2+} and lanthanide ions induced increases in the peak heights of ESR spectra, whereas no such effect was induced during titration of Mg^{2+} ion. Cheung et al. have reported systematic studies on conformational changes of CaM by various metal cations by measuring enhancement of tyrosine fluorescence intensity at 307 nm upon each cation binding to CaM [16]: increases in the fluorescence were induced by Ca^{2+}, Sr^{2+}, Cd^{2+}, Hg^{2+}, La^{3+}, Tb^{3+} and Sm^{3+}; Mn^{2+} and Zn^{2+} also induced a slight increase of the fluorescence; but no effect of Be^{2+}, Mg^{2+}, Ni^{2+} and Ba^{2+} was shown on the fluorescence intensity. Comparing ionic radii of each metal ion with its corresponding effect on conformational changes of CaM, Cheung et al. have concluded that all cations with ionic radii close to that of Ca^{2+} induced the conformational changes of CaM, whereas those with different ionic radii were not effective, or much less so. The same results of the effects of ionic radii on the conformational changes of CaM have been confirmed by the EMSA method [16]. In all of these studies, how each metal ion induced the conformational changes of CaM was a primary concern rather than what metal ion induced the interaction of the formed metal–CaM complexes with target proteins or peptides.

The method used in the present study, in contrast, was based on the direct observation by SPR of the interaction of CaM–metal ion complexes with the target peptide, M13. Tables 1 and 2 summarize the classification of metal ion selectivity into two groups as to whether or not given metal ions can induce conformational change in CaM, thereby forming the respective metal ion–CaM–M13 ternary complex [26]. All monovalent metal ions examined were found to be silent in SPR signals indicating that no formation of CaM–metal–M13 ternary complex induced. Metal ions of their ionic valences of +2 or +3, Sr^{2+}, Ba^{2+}, Cd^{2+}, Pb^{2+}, Y^{3+} and lanthanide ions, of which ionic radii are nearly equal to or higher than Ca^{2+}, turned out to induce the CaM–M13 interaction. No such effect was however observed with di- and trivalent metal ions of which ionic radii are equal to or less than 0.83 Å. This SPR result on the relation between the formation of the ternary complex and ionic radii of each metal ion was consistent with the conclusion drawn by the fluorescence and EMSA methods. The only difference is that in the present SPR method, Ba^{2+} produced the active

CaM conformation, whereas Cheung et al. found that Ba^{2+} failed to induce the characteristic change in tyrosine fluorescence or mobility shift on gel electrophoresis. This may, however, originate from the difference in the examined concentration range of Ba^{2+}. Cheung et al. studied the effect of Ba^{2+} up to 500 μM, in which concentration range Ba^{2+} failed to induce the conformational changes of CaM. But in the present study, higher concentrations of Ba^{2+} were examined, in which the SPR signal leveled off at nearly half of Ca^{2+} and then it decreased; this decrease may be due to that Ba^{2+} induced the aggregation of CaM, which inhibited the conformational changes of CaM and/or binding to M13 as discussed in the case of rare earth metal ions. Otherwise, considering the fact that CaM possesses many carboxyl groups and some of them interact with the basic amino acid residues in M13 [7,9], Ba^{2+} may bind to the carboxyl groups in CaM via charge–charge interaction and thereby interfere with the binding of CaM to M13.

Recently, the effects of divalent and trivalent metal ions on the M13-binding abilities of CaM were further investigated by fluorescence changes of a single tryptophan residue in M13, which caused a large blue shift and an increase in the intensity of the emission spectrum [31]. The ions such as Ca^{2+}, Sr^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, La^{3+} and Lu^{3+} were shown to induce the blue shifts, whereas no spectral change was obtained for Mg^{2+}. This result is in good agreement with the one obtained in the present study except for the case of Zn^{2+}; our SPR data showed that Zn^{2+} did not induce the formation of Zn^{2+}–CaM–M13 ternary complex, whereas a blue shift occurred in the fluorescence spectra. The exact reason is unclear why the SPR result for Zn^{2+} in the present study is markedly different from that obtained by the fluorescence method. However, we concluded that no formation of the ternary complex took place in view of the following earlier information, (i) the ionic radius of Zn^{2+} is much smaller than that of Ca^{2+} [26], (ii) Zn^{2+} binds to 'auxiliary' cation-binding sites that are different from those reserved for four Ca^{2+} [32], and (iii) Zn^{2+} does not induce the large conformational changes of CaM [16].

In conclusion, the present SPR method provided metal ion selectivity for Ca^{2+} signaling pathways based on selective binding of CaM with Ca^{2+} and resulting binding of the Ca^{2+}–CaM complex with the target peptide, M13. Metal ions of their ionic valences of +2 or +3, Cd^{2+}, Pb^{2+}, Sr^{2+}, Ba^{2+}, Y^{3+} and lanthanide ions, of which ionic radii are nearly equal to or higher than Ca^{2+}, induced the formation of the CaM–metal–M13 ternary complex. This results for metal ion selectivity were consistent with those obtained from earlier fluorescence measurements based on the conformational changes of CaM itself, supporting the validity of the SPR method for evaluating metal ion selectivity for the Ca^{2+} signaling as observed in its agonist-like nature for those cations described above. The present approach will provide a means of evaluating toxicological effects of not only metal ions but also organic compounds on the Ca^{2+} signaling pathway through CaM. However, after thus screening and evaluating each metal ion or agonist by the present method, actual toxicological studies need to be substantiated with some in vivo systems, e.g., by measuring the level of biomarkers such as concentration or activity changes of specific target enzymes when animals are exposed to the ions or organic compounds.

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