J. Membrane Biol. 21, 113–124 (1975)

Calcium-Binding of Synaptosomes Isolated from Rat Brain Cortex

II. Inhibitory Effects of Magnesium Ions and Some Other Cations

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Received 12 August 1974; revised 6 December 1974

Summary. As in our previous report (Kamino, Uyesaka & Inouye, *J. Membrane Biol.* **17**:13, 1974), the absorbance changes of murexide caused by Ca^{2+} and followed up by a dual wavelength spectrophotometer were applied to measure synaptosomal Ca^{2+}-binding in the presence of cations such as Rb^+, Mn^{2+} or La^{3+}. All the cations tested showed a significant inhibition of synaptosomal Ca^{2+}-binding except Li^+. The inhibitory effects could be divided into the following three categories: (1) noncompetitive, co-operative K^+-type, which includes alkali metal ions. The potency of inhibition is $K^+ > Rb^+ > Cs^+ > Li^+$, $Na^+ = 0$; (2) competitive Mn^{2+}-type which includes many divalent cations. The inhibitory potency was found to be in the following order: $Mn^{2+} \gg Sr^{2+} > Cd^{2+}$, $Ba^{2+} > Mg^{2+}$; (3) nonspecific, noncompetitive La^{3+}-type: among the cations tested, La^{3+} and Ce^{3+} were found to markedly reduce the Ca-binding capacity of synaptosomal particles, resulting in a noncompetitive inhibition, at least in the range of Ca^{2+} concentration used.

Our previous study (Kamino, Uyesaka & Inouye, 1974) demonstrated that the mode of Ca-binding with synaptosomal particles was analogous to that of interaction of Ca^{2+} with synaptic membranes of end plate put forward by del Castillo and Katz (1952) and further studied by Jenkinson (1957) and Dodge and Rahamimoff (1967) as well as Cook and Quastel (1973) and Cook, Okamoto and Quastel (1973), while K^+-induced inhibition of synaptosomal Ca-binding appeared similar to the inhibitory effect of K^+ on miniature end-plate potential (m.e.p.p.) reported by Cook *et al.* (1973). These findings strongly suggest a similarity in the mechanism of interaction of K^+ and Ca^{2+} between the synaptosomal membrane and the synaptic membrane of end plate, thereby providing direct evidence for well-known antagonism between both ions. Jenkinson (1957) and Dodge and Rahamimoff (1967) demonstrated that Mg^{2+} inhibited the interaction of

Ca^{2+} with the synaptic membrane competitively, while effects of some ions such as Mn^{2+} or La^{3+} on the Ca-binding of presynaptic membranes have been reported hitherto (Kajimoto & Pirpekar, 1972; Meiri & Rahamimoff, 1972). The present article is concerned with the effects on synaptosomal Ca-binding of some ions including Mg^{2+}, Mn^{2+} and La^{3+}.

Materials and Methods

Essentially the same methods as those employed in previous works were used: synaptosomes of rat brain cortex were prepared by density gradient centrifugation (Kamino, Inouye & Inouye, 1973) and synaptosomal protein was assayed by the method of Lowry, Rosebrough, Farr and Randall (1951), while Ca^{2+} concentration in synaptosomal suspensions was measured by the difference spectrophotometric method with murexide (Ohnishi & Ebashi, 1963; Mela & Chance, 1968), in which the difference in absorbance at 507 nm and at 542 nm ($\Delta A_{507-542}$) was employed as described in our previous paper (Kamino *et al.*, 1974).

In the presence of Cd^{2+} or an alkali earth metal ion such as Mg^{2+}, the relationship between $\Delta A_{507-542}$ and Ca^{2+} concentration continued to remain linear but did show a deviation from that in the absence of a divalent cation because of its binding with a part of murexide. The greater the affinity to murexide, the greater was the deviation. In a limited concentration range of these ions, for instance less than 50 μM for Cd^{2+}, however, the difference spectrophotometric method with murexide was practically applicable; constructing the control calibration curve in the presence of a fixed amount of a divalent cation to be tested, we could follow changes in free Ca^{2+} concentration by titration with successive addition of a small amount of $CaCl_2$ just as in our previous work. Though Hg^{2+} also showed a slight affinity to murexide, the concentration used in this study was so small that special procedures were not required.

As the standard suspending medium, 170 mM NaCl solution containing 15 mM Tris−HCl buffer (pH 7.3) was employed. The metal ions tested in this study were Rb^{+}, Cs^{+}, Mg^{2+}, Sr^{2+}, Ba^{2+}, Cd^{2+}, Hg^{2+}, Mn^{2+}, La^{3+} and Ce^{3+}. When studying the effect of these cations, a part of NaCl was replaced with the chloride salt of the above in order to maintain constant the total osmolarity and pH of the suspending medium.

Prior to the titration of synaptosomal suspensions, synaptosomes were treated with 170 mM KCl as described previously, to elevate their avidity for Ca^{2+}, and K^{+} was washed out twice with 170 mM NaCl.

The synaptosomal binding of Ca^{2+} is expressed, as reported previously, in the following equations:

$$M + n\,Ca^{2+} \rightleftharpoons Ca_n M \tag{1}$$

and

$$\log\{\bar{X}/(1-\bar{X})\} = n \log x + \log K \tag{2}$$

where M denotes a binding site of synaptosomal membrane, K the association constant of synaptosome-Ca^{2+} complex, Ca_nM, while x and $\bar{X}$ stand for free Ca^{2+} concentration in a medium and fractional saturation of the binding sites with Ca^{2+}, respectively. From differences in $\Delta A_{507-542}$ between synaptosomal suspensions and its control (in the absence of synaptosomes), the value of Ca_nM at any x can be evaluated in terms of Ca^{2+} and so its maximum value, so-called Ca-binding capacity S, as well as $\bar{X}$ can be estimated. Applying Eq. (2), therefore, Hill plots were constructed, the value of K and n being graphically estimated. Thus, the effects of cations on K, n and S were examined.

Results

Effects of Divalent Metal Cations

The synaptosomal Ca-binding in the presence of Mg^{2+} is illustrated in Fig. 1. At first glance, the results presented appear to be similar to those with K^+ (*cf.* Fig. 4 in our previous report, Kamino *et al.*, 1974); the binding capacity S remains unaltered in the presence of the added ion, whereas the higher the concentration of the ion, the more inhibited the binding. The Hill plots clearly demonstrated, however, that it is not the case: in contrast to K^+-induced inhibition, the value of n, the co-operativity coefficient, was hardly affected ($n = 3.4$); only the value of apparent association constant of Ca-membrane complex, K', which corresponds to K in Eq. (2) in the absence of Mg^{2+}, decreases as Mg^{2+} concentration in the suspending media increases (Fig. 2*A*). With the same procedure as that applied to Mg^{2+}, it was demonstrated that other divalent cations also showed an inhibition of Ca-binding quite similar to Mg^{2+}. Some of the results are included in Fig. 2, a fact strongly suggesting the competitive nature of their inhibitory effect on synaptosomal Ca-binding.

When these divalent cations bind with the so-called "Ca-binding site" of synaptosomal membrane just in the same manner as Ca^{2+}, i.e. according to Eq. (2), these ions result in a competitive inhibition of Ca-binding and

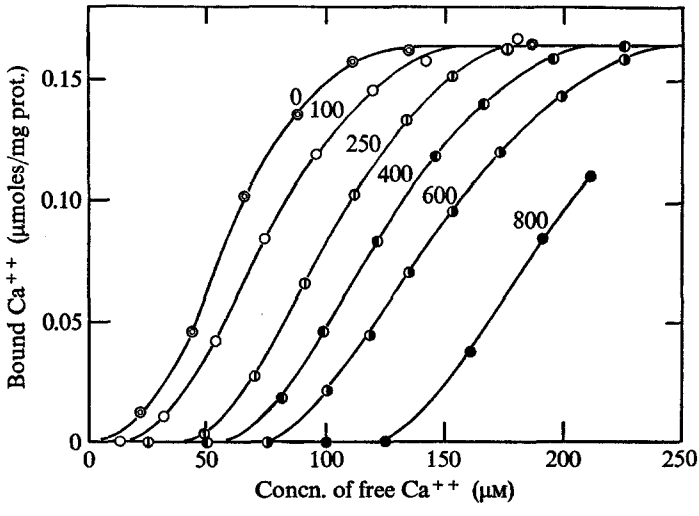

Fig. 1. Relationship between synaptosome-bound Ca^{2+} and the free Ca^{2+} concentration in suspending media (170 mM, NaCl) in the presence and absence of Mg^{2+}. These solutions contained Tris-Cl buffer (pH 7.3, 15 mM). Added $MgCl_2$ concentration: 0 (◎), 100 (○), 250 (◫), 400 (◐), 600 (◑) and 800 (●) in μM

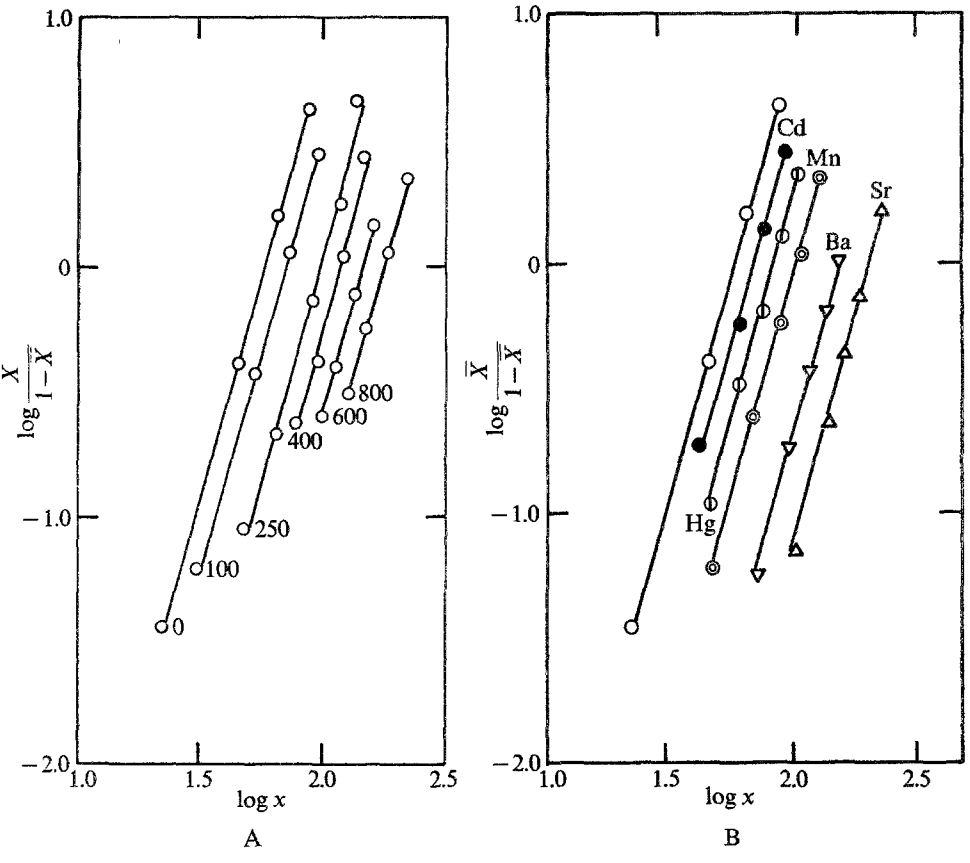

Fig. 2. Hill plots of Ca-binding by synaptosomes in the presence of various concentrations of Mg^{2+} (*A*) and of other divalent cations (*B*). • Cd^{2+} (25 μM), ⊙ Hg^{2+} (40 μM), ◎ Mn^{2+} (2.5 μM), ▽ Ba^{2+} (200 μM), △ Sr^{2+} (200 μM), ○ control. *Ordinate:* logarithm of $\bar{X}/(1-\bar{X})$, where $\bar{X}$ is fractional saturation of bound Ca^{2+}. *Abscissa*: logarithm of free Ca^{2+} concentration (x) in μM

the apparent association constant of Ca_nM complex is given by

$$K'_s = K/(1+K_D[D^{2+}]^n) \tag{3}$$

where D represents a divalent cation tested and K_D is the association constant of the complex D_nM. It follows that

$$\frac{[Ca^{2+}]'_{1/2}/[Ca^{2+}]_{1/2}}{[D^{2+}]} = K_D^{1/n} \cdot \left(1+\frac{1}{K_D[D^{2+}]^n}\right)^{1/n} \tag{4}$$

where $[Ca^{2+}]_{1/2}$ stands for Ca^{2+} concentration (x) at $\bar{X}=1/2$ (i.e. $K^{-1/n}$) and the prime over it denotes $[Ca^{2+}]'_{1/2}$ in the presence of D^{2+} (i.e. $-K'^{-1/n}$).

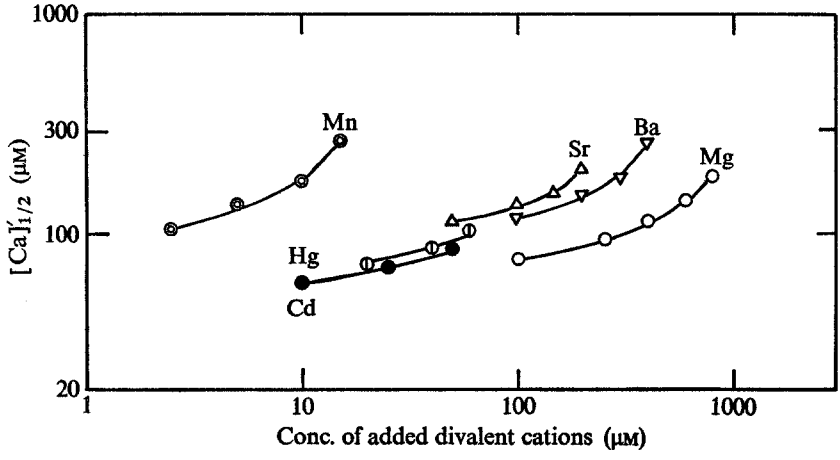

Fig. 3. Correlation of external Ca^{2+}-concentration at the half saturation of synaptosomal Ca^{2+} binding ($[Ca]_{1/2}$) with added divalent cation concentration. Each mark: the same as in Fig. 2. *Ordinate:* $[Ca]'_{1/2}$ in log scale. *Abscissa:* added divalent cation concentration (μM) in log scale

For large $[D^{2+}]$, the amount of D^{2+} bound with the synaptosomes is quite small compared with that of added D^{2+}. Using $[D^{2+}]$ expressed as the latter, therefore, it is expected that $\log[Ca^{2+}]'_{1/2}$ *vs.* $\log[D^{2+}]$ plots for respective divalent cations run in parallel and are linear with a slope of 45° for large values of $[D^{2+}]$, while $K_D^{1/n}$ could be estimated from the ratio in the left-hand side of Eq. (4) for such a high value of $[D^{2+}]$. As seen in Fig. 3, the results obtained on $[Ca^{2+}]'_{1/2}$ *vs.* $[D^{2+}]$ relation appear consistent with Eq. (4) except for Cd^{2+} and Hg^{2+}; because of marked interaction of these ions with murexide, observations for higher concentration were impossible, while Hg was not expected to be fully ionized under our experimental conditions. But their effects on $[Ca^{2+}]'_{1/2}$ are very similar to that of Sr^{2+} and Ba^{2+}, so that these divalent cations appear to interact with the sites in a manner similar to that of Sr^{2+}. The value of the ratio $R=(K_D/K)^{1/n}$, are presented in Table 1, which shows that the affinity of the synaptosomal Ca-binding sites to Ca^{2+} is about 4 times as high as that to Mg^{2+}. The value of R for Sr^{2+} and Ba^{2+} also is of the same order of magnitude as that obtained on the end plate (Dodge & Rahamimoff, 1967). The value of R for Mn^{2+} is about 20 and such a high affinity to the Ca-binding sites of this ion is also consistent with the results obtained on the end plate (Meiri & Rahamimoff, 1972). Thus, it might be said that the divalent cations tested competitively inhibit the binding with Ca^{2+} of synaptosomal membranes and the order of decreasing affinity of Ca-binding sites to these ions is given as $Mn^{2+} \gg Ca^{2+} > Sr^{2+} > (Hg^{2+} > Cd^{2+}) = Ba^{2+} > Mg^{2+}$.

Table 1. The value of $R(=(K_D/K)^{1/n})$ for various divalent cations tested

Divalent cations	$(K_D/K)^{1/n}$
Mg^{2+}	0.23 ± 0.06
Ba^{2+}	0.67 ± 0.11
Sr^{2+}	0.98 ± 0.05
Hg^{2+}	(>0.98) [a]
Cd^{2+}	(>0.67)
Mn^{2+}	19.1 ± 2.3

[a] Hg was not expected to be fully ionized under this experimental condition.

Effects of Alkali Metal Ions

In a previous paper (Kamino *et al.*, 1973) we reported that the synaptosomal particles swell in RbCl or CsCl solutions though to a lesser degree than in KCl, but never in LiCl as in NaCl, while such a synaptosomal swelling was closely related to the amount of membrane-bound Ca. We examined, therefore, the effect on synaptosomal Ca-binding of these alkali metal ions. Not surprisingly, it was found that in 170 mM LiCl solution the binding of synaptosomes with Ca^{2+} was hardly affected, but Rb^+ and Cs^+ inhibited it in the same way as K^+-induced inhibition; the binding capacity remains unaltered but the co-operative coefficient n increases as the external concentration of both these ions increases. Here, some examples of Hill plots for these ions are presented in Fig. 4. The results with these alkali metal ions will be reported in detail in relation to the synaptosomal swelling. The values of n at 170 mM of these ions are as follows: $(n)_K \fallingdotseq 7.8$, $(n)_{Rb} \fallingdotseq 5.0$, and $(n)_{Cs} \fallingdotseq 4.5$. Of course, $(n)_{Li} \fallingdotseq (n)_{Na} \fallingdotseq 3.4$ (*cf.* Fig. 2 as well as our previous paper, 1974). It may be concluded, therefore, that inhibition of synaptosomal Ca-binding by the alkali metal ions is, as a general rule, noncompetitive and of a co-operative nature because of increase in n and the order of its increasing magnitude of n for the ions tested is as follows:

$$0 = \mathrm{Na},\ \mathrm{Li} < \mathrm{Cs} < \mathrm{Rb} < \mathrm{K},$$

a series which resembles quite well that of their potency in inducing synaptosomal swelling.

Effects of La^{3+}

The Ca-binding curves of synaptosomes in the presence of La^{3+} are presented in Fig. 5. It is obvious from the figure that La^{3+} markedly reduced the binding capacity (S). A trivalent ion, Ce^{3+}, was found to show

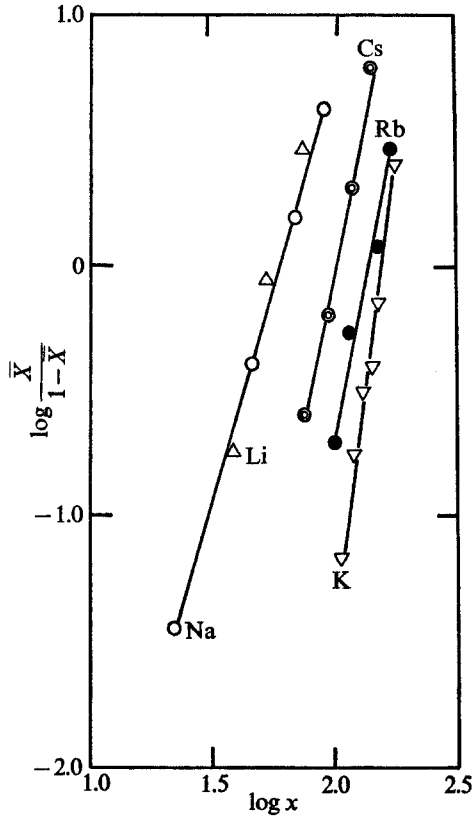

Fig. 4. Hill plots of Ca-binding by synaptosomes in the KCl (170 mM), RbCl (170 mM) CsCl (170 mM), LiCl (170 mM), NaCl (170 mM) solution. *Ordinate:* logarithm of $\bar{X}/(1-\bar{X})$, where $\bar{X}$ is fractional saturation of bound Ca^{2+}. *Abscissa:* logarithm of free Ca^{2+} concentration (x) in μM. ○ in NaCl, △ in LiCl, ◎ in CsCl, ● in RbCl, ▽ in KCl

an inhibition quite similar to that with La^{3+}. When the binding capacity at a given concentration (C) of La^{3+} or Ce^{3+}, S_c is applied, instead of S in the absence of La^{3+}, so-called normal capacity, S_o, to calculate fractional saturation of synaptosomes with Ca^{2+}, ($\bar{X}_c$), the $\bar{X}_c$ *vs.* x relations at any concentration were found to coincide (Fig. 6). Such a result indicates that the mode of Ca-binding is not so seriously affected by La^{3+}, the inhibitory effect being only a decrease in S, a fact in sharp contrast with K^+-induced inhibition which has a co-operative character in synaptosomal binding with Ca^{2+}.

The difference, $S_o - S_c$, may be regarded as representing the sites capable of binding Ca^{2+}, but firmly occupied by La^{3+} at the concentration C. Applying Langmuir's adsorption isotherm, the formation of LaM complex

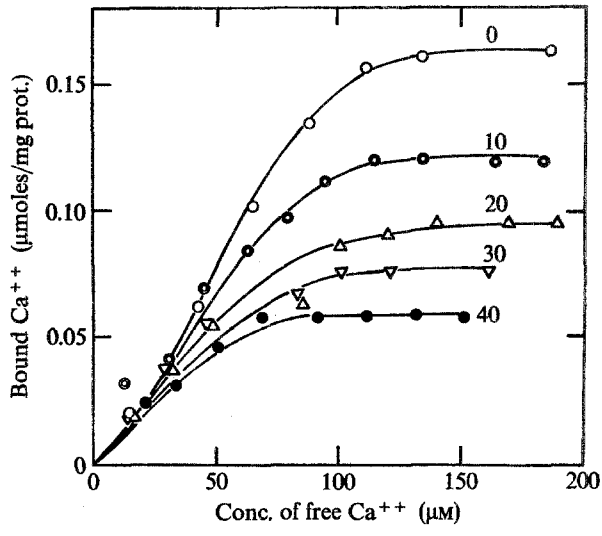

Fig. 5. Relationship between synaptosome-bound Ca^{2+} (μM/mg protein) and the free Ca^{2+} concentration (μM) in suspending media in the presence of La^{3+}. La^{3+} concentration: ○ 0 μM, ◎ 10 μM, △ 20 μM, ▽ 30 μM, ● 40 μM. Suspending media 170 mM NaCl containing Tris-Cl buffer (pH 7.3, 15 mM)

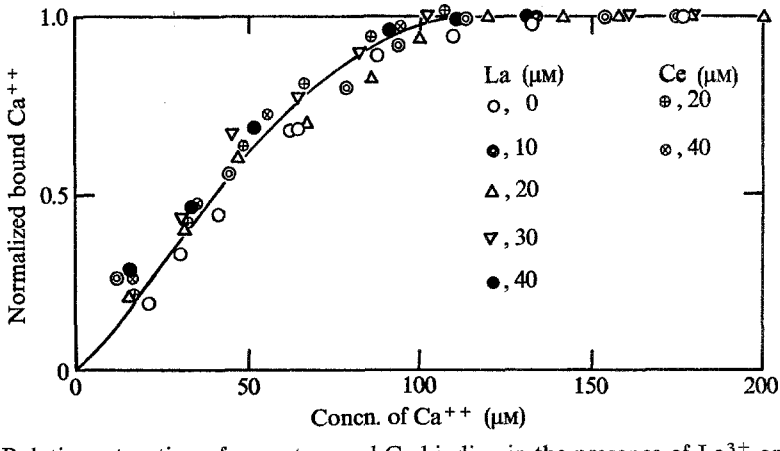

Fig. 6. Relative saturation of synaptosomal Ca-binding in the presence of La^{3+} or Ce^{3+}. *Ordinates:* degree of saturation normalized to Ca-binding capacity in the presence of La^{3+}, $(S_c)^{La}_{max}$ or Ce^{3+}, $(S_c)^{Ce}_{max}$. *Abscissae:* free Ca^{2+} concentration (μM). Each mark for La^{3+} the same as in Fig. 5, representing La^{3+} concentrations. Concentration of Ce^{3+}: 20 μM ⊕, 40 μM ⊗

could be expressed as

$$\frac{C}{\bar{m}_c B} = \frac{C}{m_o} + \frac{k}{m_o} \tag{5}$$

or

$$k\frac{B}{C} = \frac{m_o}{\bar{m}_c} - B \tag{5'}$$

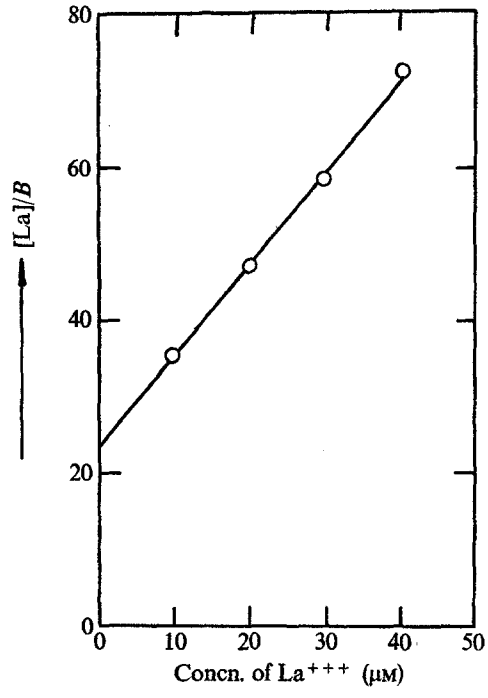

Fig. 7. Langmuir plot of apparent La-binding. *Ordinate:* [La]/*B* (*see text*). *Abscissa:* La^{3+}

where $B=(S_o-S_c)$, i.e. fractional saturation of Ca-binding sites with La^{3+}, k a constant, while $\bar{m}_c$ and m_o denote the average number of the bound ions per site at a given concentration and the maximum value of $\bar{m}_c$. As is well-known, Klotz (1946) already showed that Eqs. (5) or (5′) were practically applicable to the binding of proteins with ions, providing an elegant explanation for their correspondence to the mass action law. Then k is regarded as the overall dissociation constant of LaM complex. We attempted, therefore, to apply Eq. (5) or (5′), the latter being known as the Scatchard plot. As shown in Fig. 7, a linear correlation of the slope $\simeq 1$ was obtained between C/B and C, a finding which suggests that $\bar{m}_c/m_o$ is practically 1 in spite of $\bar{m}_c$ being dependent on C. This does not indicate, however, that $\bar{m}_c=m_o=1$ in every case. The intercept on the ordinate gives k/m_o because of $\bar{m}_c$ as $C=0$ being 1. The value of k/m_o is 24.6 ± 1.3. Application of the Scatchard plot (B/C *vs.* B) gives the same result, in which the intercept on the abscissa is 1 because of $m_o/\bar{m}_c$ for $C\to\infty$ being 1.

It may thus be concluded that the binding of synaptosomal Ca-binding sites with La^{3+} and Ce^{3+} is too firm to dissociate La^{3+} with the raised external Ca^{2+} concentrations used, resulting in an apparent irreversible loss

of Ca-binding capacity; however, the sites remaining unoccupied by La^{3+} or Ce^{3+} were practically unaffected. Thus, the inhibition by La^{3+} and Ce^{3+} of synaptosomal Ca-binding is noncompetitive and practically irreversible.

Discussion

The results presented above clearly demonstrate that the inhibitory effect on synaptosomal Ca-binding of the cations tested can be classified into three types:

(1) *Competitive type* which is typically observed with the effect of Mg^{2+}: a finding that Mg^{2+} occupied the same sites on synaptosomal membranes in competition with Ca^{2+} was also reported in the paper by Madeira and Antunes-Madeira (1973). These workers used the membrane fractions prepared from synaptosomes by subfractionation with a density gradient centrifugation, and applied Scatchard plots to analyze the mode of binding of Ca^{2+} and Mg^{2+}. Because of differences in the methods of preparation and analysis, actual comparisons are not feasible. Applying the Hill's plot, we found that Hill's coefficient (n) of synaptosomal Ca-binding was about 3.4 so that it would be quite natural that the Scatchard plot would not be linear. These workers explained such a nonlinearity of Scatchard plot of synaptosomal Ca-binding as the presence of two sorts of binding sites. But their results appear, as a general trend, consistent with ours; the sum of the binding capacity for Ca^{2+} (and so Mg^{2+}) of both sites as well as the average of their Ca^{2+} concentration for half-maximal saturation are nearly in the same order of magnitude as those obtained in the present study.

The results presented in Figs. 2 and 3 demonstrate that Sr^{2+} and Ba^{2+} also show this type of inhibition. As seen in Table 1, relative affinity for the synaptosomal membranes of the alkali earth metal ions referred to Ca^{2+} appears similar to, or at least not so different from, that for the synaptic membrane of end plate studied with e.p.p. (Jenkinson, 1957; Dodge & Rahamimoff, 1967) or for barnacle muscle fiber membrane with Ca^{2+} spike (Hagiwara & Takahashi, 1967).

Other divalent cations (Mn^{2+}, Hg^{2+} and Cd^{2+}) were also found to show inhibition of competitive type. Meiri and Rahamimoff (1972) suggested that powerful inhibitory effects on e.p.p. of Mn^{2+} were due to its binding in competition with Ca^{2+}. On the other hand, the Ca^{2+} spike of barnacle muscle fiber membrane was competitively inhibited by Mn^{2+} (Hagiwara & Takahashi, 1967).

(2) *Noncompetitive-specific (co-operative) type* which is seen in the inhibitory effect of the alkali metal ions: the present study demonstrated that

Rb^+ and Cs^+ also exhibit a specific co-operative inhibition just as K^+ did (Kamino *et al.*, 1974), the potency of their inhibition being in the order of $K^+ > Rb^+ > Cs^+$. Such a finding is consistent with our observation on synaptosomal swelling (Kamino *et al.*, 1973) and provides an indirect support for a view that synaptosomal swelling is related to some changes in the state of synaptosomal membrane and/or Ca-binding sites on the membrane (Kamino *et al.*, 1974). Many types of tissues swell in high K^+ media (nervous tissues, Lipton, 1973; nerve fibers, Hill, 1950). It seems not so improbable, therefore, that the alkali metal ions, especially K^+, interact with other cell membranes through such a specific inhibition of Ca-binding (or Ca^{2+} influx) as found on synaptosomes. Wolff, Huebner and Siegel (1972) have isolated a pure phosphoprotein from pig brain which binds Ca^{2+}, and in which Mg^{2+}, Mn^{2+}, Sr^{2+} and Ba^{2+} compete with Ca^{2+} for the same binding site, but in which potassium ions inhibit noncompetitively the Ca-binding. Their results are consistent with our observations on synaptosomal membrane.

(3) *Nonspecific, noncompetitive type* which was observed only with trivalent ions (La^{3+} and Ce^{3+}) among the ions tested: A powerful inhibition by La^{3+} of binding with Ca^{2+} as described on the synaptosomes was already reported on cardiac sarcoplasmic reticulum (Krasnow, 1972), while the Ca influx of barnacle muscle fibers was inhibited by La^{3+} far more intensely than by Mn^{2+} (Hagiwara & Takahashi, 1967).

Since La^{3+} and Ce^{3+} are trivalent, it is hardly expected that these ions occupy Ca-binding sites in competition with Ca^{2+} by one-to-one kinetics, resulting in a simple competitive type of inhibition as observed on divalent cations. As seen in Fig. 7, La^{3+} binding with the Ca-binding sites shows simple Langmuir-type kinetics with respect to the external La^{3+} concentration and so it possibly binds sites other than the Ca sites. Indeed, nonspecific binding (or deposition) of La^{3+} with cardiac muscle membrane was electron-microscopically observed (Langer & Frank, 1972). Thus, the mode of binding of La^{3+} and Ce^{3+} with Ca-binding sites appears to be a part of its interaction with cellular membrane and the nonspecificity of its nature.

In contrast, La^{3+} inhibition of Ca^{2+} spike in barnacle muscle fibers was reported to show one-to-one kinetics that is a competitive inhibition (Hagiwara & Takahashi, 1967). At present, it is difficult to explain how to give rise to such a difference between synaptosomes and muscle cells showing Ca^{2+} spike.

The results obtained in the present study strongly suggest that the nature of interaction of the cations studied here with Ca^{2+} in the synapto-

somal membrane is quite similar to that observed on other biological membranes or on a Ca-binding protein isolated by Wolff *et al.* (1972).

The authors wish to thank Miss Mary Ohara, Kyoto University for assistance with the manuscript. This work was supported in part by Grant No. 748052 from the Ministry of Education of Japan, and a grant from the Interdisciplinary Research Institute of Environmental Sciences.

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