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Ca^{2+} BINDING SITES IN PLASMA MEMBRANES OF RAT LIVER AND HEPATOMA CELLS, AND EFFECT OF CONCANAVALIN A ON THE Ca^{2+} BINDING SITES AND CELLULAR UPTAKE OF Ca^{2+}

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Summary

1. Plasma membranes isolated from rat livers and ascites hepatoma cells (AH-130, AH-7974) were assayed for specific Ca^{2+} binding sites using $^{45}Ca^{2+}$ and a Millipore filtration technique. The presence of higher (K_d = 1.4—1.5 · 10^{-5} M) and lower (K_d = 0.9—1.0 · 10^{-4} M) affinity sites in both liver and hepatoma membranes was observed. The hepatoma plasma membranes, however, showed 1.4—2.1-fold as many Ca^{2+} binding sites (higher and lower affinity sites) as the liver plasma membranes on the basis of protein.

2. Concanavalin A stimulated the specific Ca^{2+} binding to liver and hepatoma plasma membranes, showing a maximal stimulation (3—5-fold) at 100 μg/ml. Succinyl concanavalin A was less effective, whereas wheat germ agglutinin and *ricinus* lectin were ineffective.

3. Concanavalin A stimulated the Ca^{2+} uptake by AH-7974 cells. The concanavalin A-mediated stimulation of Ca^{2+} uptake showed lectin-concentrations and Ca^{2+}-concentration dependencies similar to those in the concanavalin A-mediated stimulation of Ca^{2+} binding.

Introduction

In spite of the potential importance of Ca^{2+} binding sites in plasma membranes regarding the Ca^{2+}-mediated regulation of plasma membrane functions, for instance, cyclic AMP phosphodiesterase [1], ($Na^+ + K^+$)-ATPase [2,3],

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

adenylate cyclase [4,5] and so on (Ca^{2+} binding sites in plasma membranes have been reported on liver cells [6], adipocytes [7] and some other cells), the Ca^{2+} binding parameters so far reported are not consistent with each other. Furthermore, no comparison has yet been reported on the Ca^{2+} binding sites in plasma membranes between the normal and transformed cells.

Some lectins such as phytohemagglutinin [8] and concanavalin A [9] induce the blastic transformation and mitosis in lymphocytes accompanied by the stimulation of Ca^{2+} influx although the lectin-concentration dependency in the lectin-mediated stimulation of mitosis may not necessarily coincide with that in the lectin-mediated stimulation of Ca^{2+} influx [10]. Recently, it has been reported that concanavalin A modulates the activities of certain enzymes in plasma membranes of various cells, for instance, Mg^{2+}-ATPase [11,12], Ca^{2+}-ATPase [13], ($Na^+ + K^+$)-ATPase [13—15], 5′-nucleotidase [15—20], cyclic AMP phosphodiesterase [21] as well as adenylate cyclase [14]. Some of these enzymes seem to be related to the modulation of Ca^{2+} transport [22] and cell proliferation [23,24].

The results of these earlier studies suggest intimate interactions or relationships among Ca^{2+}-dependent regulatory proteins, concanavalin A receptors and plasma membrane enzymes. In the present paper, investigations have been carried out firstly to characterize the Ca^{2+} binding sites in plasma membranes of liver and hepatoma cells, secondly to clarify the concanavalin A effect on the Ca^{2+} binding sites in plasma membranes of these cells, and thirdly to clarify the concanavalin A effect on the cellular uptake of Ca^{2+}.

Materials and Methods

Livers of male Wistar rats (130—190 g body weight) were perfused in situ with the 10 mM sodium citrate-saline solution (pH 7.5) prior to homogenization. Transplantable ascites hepatoma cells (AH-130 and AH-7974) were inoculated into the peritoneal cavity of male Donryu rats (120—140 g body weight) 1 week prior to harvesting the ascites hepatoma cells. The hepatoma cells were washed several times with the sodium citrate-saline solution to eliminate the blood cells prior to homogenization.

Plasma membranes were prepared from liver and hepatoma cells according to the method of Ray [25], and washed successively with 0.1 M Tris-HCl buffer (pH 7.5), 1 mM EDTA (pH 7.0) and 0.1 M Tris-HCl buffer (pH 7.5) each time by centrifuging at 10 000 rev./min for 20 min. The final pellet was resuspended in the same Tris buffer (0.2—1.0 mg protein/ml), and immediately used for the Ca^{2+} binding assays. Protein was assayed by the method of Lowry et al. [26] with bovine serum albumin as a standard. Purity of plasma membranes (liver) was examined by assaying the activities of 5′-nucleotidase [27], succinate dehydrogenase [28] and glucose-6-phosphatase [29].

Ca^{2+} binding to plasma membranes was assayed according to the technique of Shlatz and Marinetti [6] using $^{45}Ca^{2+}$. Plasma membranes equivalent to 20—100 μg protein suspended in 0.1 ml of the 0.1 M Tris buffer (pH 7.5) were mixed with 0.8 ml of the Tris buffer and 0.1 ml of $^{45}Ca^{2+}$ (0.1—0.5 μCi) solution of various concentrations. The whole mixture (1 ml) containing plasma membranes (20—100 μg protein) and $^{45}Ca^{2+}$ (0.4 μM—5 mM) was incubated at

37°C for 10 min with gentle shaking except when otherwise specified. After incubation, the mixture was filtered through a Millipore disk (25 mm diameter; 0.45 μm pore size), which was then quickly washed twice with 10 ml of the Tris buffer under suction. Radioactivity trapped in the filter disk was counted in a liquid scintillation spectrometer using 2,5-diphenyloxazole-1,4-bis[2-(5-phenyloxazolyl)]-benzene in toluene as a scintillation fluid. The Millipore filter disk used in the present study had been coated with 1% polylysine or 0.05% methylglycolchitosan to minimize the filter blanks (nonspecific binding of $^{45}Ca^{2+}$ to the filter in the absence of plasma membranes). To examine the effects of lectins on $^{45}Ca^{2+}$ binding to plasma membranes, 0.1 ml of a plasma membrane suspension was usually added to a 0.9-ml mixture of $^{45}Ca^{2+}$ and lectin prepared in advance instead of adding $^{45}Ca^{2+}$ and lectin solutions separately to a plasma membrane suspension. The filter blank correction was done at each $^{45}Ca^{2+}$ concentration or lectin concentration.

To measure the cellular uptake of Ca^{2+}, AH-7974 cells ($2-5 \cdot 10^5$ cells) were suspended in 1 ml of the 10 mM Hepes/saline/11 mM glucose solution or the Hepes/saline-solution containing $^{45}Ca^{2+}$ (0.5 μCi) at 10^{-3}, 10^{-5} or $5 \cdot 10^{-7}$ M and concanavalin A at 0—200 μg/ml. The mixture was incubated at 37 or 20°C for scheduled intervals of time (2—30 min). The cells were then filtered through a polycation-coated Millipore disk, and washed with the 10 mM phosphate buffered saline solution (pH 7.5). Radioactivity trapped in the filter disk was counted and corrected for the filter blank as described above.

To measure the binding of concanavalin A to plasma membranes, 0.5 ml of a plasma membrane suspension was added to a 4.5-ml mixture of [^{3}H]concanavalin A and Ca^{2+} in the 0.1 M Tris buffer (pH 7.5). The final concentrations of [^{3}H]-concanavalin A and Ca^{2+} were 100 μg/ml (0.25—0.025 μCi/ml) and 10^{-5} (or 10^{-4}) M, respectively. The whole mixture was incubated at 37 or 0°C for various intervals of time (usually 10 min). After incubation, plasma membranes were spun down, and twice washed with 5 ml of the Tris buffer. Radioactivities of the supernatant and washings were counted in a liquid scintillation spectrometer, using 2,5-diphenyloxazole-1,4-bis[2-(5-phenyloxazolyl)]benzene/toluene/Nonion as a scintillation fluid. Radioactivity of plasma membrane pellet was also counted using 2,5-diphenyloxazole/toluene/Cellosolve as a scintillation fluid after oxidation of the plasma membranes with perchloric acid and hydrogen peroxide.

Binding of [^{3}H]concanavalin A to AH-7974 cells was measured as follows. 0.5 ml of a AH-7974 cell suspension ($5.6 \cdot 10^6$ cells/ml) in the Hepes/saline solution (pH 7.5) was added to 4.5 ml of a mixture of Ca^{2+} and [^{3}H]concanavalin A (0.25 μCi) in the same buffer (the final concentrations of [^{3}H]concanavalin A and Ca^{2+} were 100 μg/ml and 10^{-5} M, respectively), and the whole mixture was incubated at 37°C for 10 min. The cells were then spun down and washed twice with 5 ml of cooled 10 mM phosphate-buffered saline (pH 7.5), and the cellular pellet, the supernatant and washings were assayed for radioactivity as described in the [^{3}H]concanavalin A binding to plasma membranes.

Binding of $^{45}Ca^{2+}$ to concanavalin A was measured as follows. 1 ml of a mixture of [^{3}H]concanavalin A (0.25 μCi, 100 μg/ml) and $^{45}Ca^{2+}$ (0.2 μCi, 10^{-5} M) was incubated at 37°C (or 0°C) for 10 min (or 120 min). After incubation, the mixture was filtered through a Millipore disk and ^{3}H and $^{45}Ca^{2+}$ radio-

activities trapped in the disk were measured in a liquid scintillation spectrometer using 2,5-diphenyloxazole-1,4-bis[2-(5-phenyloxazolyl)]benzene in toluene as a scintillation fluid. The amount of $^{45}Ca^{2+}$ bound to [^{3}H]concanavalin A was corrected for the nonspecific binding of $^{45}Ca^{2+}$ to the disk in the absence of [^{3}H]concanavalin A.

The reagents used in the present study were as follows. $^{45}CaCl_2$ (0.52 and 0.58 Ci/mol) was from New England Nuclear, Boston, U.S.A. and $CaCl_2 \cdot 2H_2O$ (reagent grade) was from Wako Pure Chem. Ind., Tokyo, Japan. The concentrations of $CaCl_2$ solutions were checked by atomic absorption spectrophotometry. DL-Isoproterenol hydrochloride, concanavalin A, insulin and α-methyl-D-mannoside were from Sigma Chem. Co., St. Louis,U.S.A. Methylglycol-chitosan hydrochloride, 2,4-dinitrophenol and colchicine were from Wako Pure Chem. Ind. 3′,5′-Cyclic adenosine monophosphate was from Seikagaku Kogyo Co., Tokyo. Poly-L-lysine hydrochloride and L-epinephrine were from Tokyo Chem. Ind., Tokyo, Japan. Aldosterone and RCA_{60} (*Ricinus communis* lectin) were from E. Merck, Darmstadt, and Boehringer, Mannheim, Germany, respectively. Wheat germ agglutinin and succinyl concanavalin A were from E.Y. Laboratories, San Mateo, California, U.S.A. Hydrocortisone was from Nakarai Chemicals, Kyoto, Japan, cytochalasin B from Aldrich Chem. Co. Inc., Milwaukee, U.S.A. Concanavalin A used in the present study contained 0.085% Ca^{2+} (2.1 mol Ca^{2+} per mol concanavalin A) and electrophoretically pure (single band) (Sigma Chem. specification). [^{3}H]Concanavalin A ([*Acetyl*-^{3}H]-*N*-acetylated) (17.166 Ci/mmol) was purchased from the New England Nuclear and is supposed to give 100% activity in the agglutination assay (New England Nuclear specification).

Results

1. Kinetics of $^{45}Ca^{2+}$ binding to plasma membranes and plasma membrane concentration dependence of $^{45}Ca^{2+}$ binding

Preliminary investigations have revealed that the binding of $^{45}Ca^{2+}$ (10^{-6} M) to plasma membranes (liver and hepatomas) reaches an equilibrium at 10 min of incubation at 37°C, whereas an almost similar level of binding equilibrium is attained after 2 h of incubation at 0°C (data not shown). The binding of $^{45}Ca^{2+}$ (10^{-5} M) to plasma membranes (liver) (37°C, 10 min) was proportional to the amounts of plasma membranes added to the incubation mixture up to a concentration of 100 μg protein per ml (data not shown). Therefore, in the present study $^{45}Ca^{2+}$ binding to plasma membranes was assayed after 10 min of incubation at 37°C using plasma membranes at concentrations from 20 to 100 μg protein per ml except when otherwise specified.

2. Binding of $^{45}Ca^{2+}$ to plasma membranes of liver and hepatoma (AH-130 and AH-7974) cells as a function of $^{45}Ca^{2+}$ concentration — $^{45}Ca^{2+}$ binding parameters

Amounts of $^{45}Ca^{2+}$ bound to plasma membranes (nmol/mg protein) plotted against $^{45}Ca^{2+}$ unbound ($^{45}Ca^{2+}$ binding curves) and the corresponding Scatchard plots for $^{45}Ca^{2+}$ concentration ranges from $4 \cdot 10^{-7}$ to $1—5 \cdot 10^{-4}$ M are shown in Fig. 1a. The $^{45}Ca^{2+}$ binding curves and the corresponding

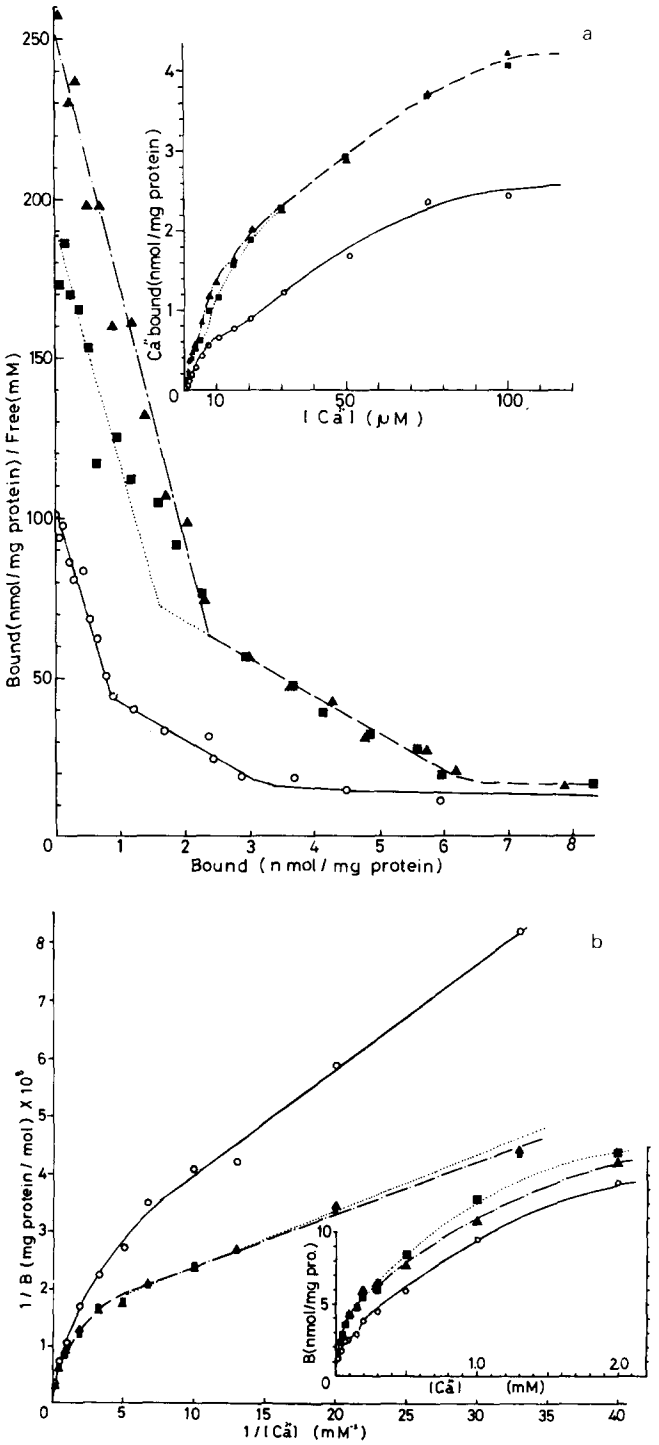

Fig. 1. Binding of $^{45}Ca^{2+}$ to plasma membranes of liver and hepatoma cells at various $^{45}Ca^{2+}$ concentrations. The incubation mixture contained plasma membranes of liver (○: 61 μg protein), AH-130 (■: 83 μg protein) or AH-7974 (▲: 64 μg protein) and 0.2—0.5 μCi $^{45}Ca^{2+}$ (0.4 μM—5 mM) in 1 ml. (a) $^{45}Ca^{2+}$ binding to these plasma membranes at $^{45}Ca^{2+}$ concentrations from 0.4 to 100 μM (upper inset) and the Scatchard plots of the $^{45}Ca^{2+}$ bindings. (b) $^{45}Ca^{2+}$ binding at $^{45}Ca^{2+}$ concentrations from 30 μM to 2.0 mM (lower inset) and the Lineweaver-Burk plots of the bindings.

Lineweaver-Burk plots of $^{45}Ca^{2+}$ binding, for $^{45}Ca^{2+}$ concentration ranges from $3 \cdot 10^{-5}$ M to $2–5 \cdot 10^{-3}$ M, are shown in Fig. 1b.

There appears to exist at least 2 specific binding sites with different affinities in both liver and hepatoma plasma membranes. Whether or not there may be a third specific binding site with a smaller affinity for Ca^{2+} is not clear from the Scatchard plots (Fig. 1a). However, the fact that the Lineweaver-Burk plots (Fig. 1b) at higher $^{45}Ca^{2+}$ concentrations show convex curves approaching the zero point, suggests that the binding at $^{45}Ca^{2+}$ concentrations higher than $2 \cdot 10^{-4}$ may be of a nonspecific type with a negative cooperativity.

From the Scatchard plots of $^{45}Ca^{2+}$ binding the $^{45}Ca^{2+}$ binding parameters (N = number of binding sites, K_d = dissociation constant) were calculated. The results indicated that the hepatoma plasma membranes contain Ca^{2+} binding sites of both the higher ($K_d = 1.4–1.5 \cdot 10^{-5}$ M) and lower ($K_d = 0.9–1.0 \cdot 10^{-4}$ M) affinities similar to the liver plasma membranes. The numbers of specific binding sites (nmol/mg protein) with higher affinity were 1.6 ± 0.1 (n = 4) (liver), 2.6 ± 0.3 (n = 2) (AH-130) and 3.4 ± 0.2 (n = 2) (AH-7974), whereas the numbers of specific binding sites (nmol/mg protein) with lower affinity were 5.6 ± 0.4 (n = 2) (liver), 7.8 (n = 1) (AH-130) and 7.7 ± 0.2 (n = 2) (AH-7974) (n = numbers of experiments), indicating that the numbers of these specific binding sites are 1.4–2.1-fold larger in the hepatoma plasma membranes as compared to the liver plasma membranes.

3. Effects of various reagents on $^{45}Ca^{2+}$ binding to plasma membranes

Taking into consideration that Ca^{2+} might be an intermediate in some hormonal actions [30,31], the effects of various hormones such as norepinephrine (10^{-7} M), epinephrine (10^{-7} M, 10^{-8} M), isoproterenol (10^{-7} M), insulin (10^{-7} M), aldosterone (10^{-7} M, 10^{-9} M) and hydrocortisone (10^{-7} M, 10^{-9} M) on the $^{45}Ca^{2+}$ (10^{-5} M) binding to liver plasma membranes were first investigated. Although the data are not presented, these hormones did not show any significant effects on the $^{45}Ca^{2+}$ binding in contrast to the stimulatory effect of epinephrine, glucagon and hydrocortisone and the inhibitory effect of insulin reported by Shlatz and Marinetti [31]. Similarly, 3',5'-cyclic AMP ($2 \cdot 10^{-4}$–10^{-9} M), 2,4-dinitrophenol ($5 \cdot 10^{-5}$ M), colchicine ($5 \cdot 10^{-4}$ M) and cytochalasin B (10 and 100 μg/ml) did not show significant effects either (data not shown).

Binding of $^{45}Ca^{2+}$ (10^{-5} M) to plasma membranes was scarcely affected by low concentration (1 mM) NaCl or KCl, but was inhibited (60%) by high concentrations (150 mM) of NaCl. Mg^{2+} and Sr^{2+} (1 mM) slightly (less than 25%) inhibited, but Ca^{2+} (1 mM) markedly (80%) inhibited $^{45}Ca^{2+}$ binding, suggesting that the $^{45}Ca^{2+}$ binding sites are rather specific for Ca^{2+}.

Next, the effects of various lectins such as concanavalin A, succinyl concanavalin A, wheat germ agglutinin and *Ricinus communis* lectin (RCA_{60}) on the $^{45}Ca^{2+}$ (10^{-5}, 10^{-4} and $5 \cdot 10^{-3}$ M) binding to liver plasma membranes were investigated. The results (data not shown) indicated that concanavalin A (100 μg/ml) markedly stimulated the $^{45}Ca^{2+}$ binding to plasma membranes,the stimulation being larger as $^{45}Ca^{2+}$ concentration was lower. The stimulatory effect of succinyl concanavalin A (100 μg/ml) was much smaller than that of concanavalin A, whereas the other lectins (10–150 μg/ml) did not affect the

$^{45}Ca^{2+}$ binding to liver plasma membranes. It should be noted that the concanavalin A-mediated stimulation of $^{45}Ca^{2+}$ binding was observed similarly in the presence of saline (150 mM NaCl), although the net $^{45}Ca^{2+}$ binding to liver plasma membranes was reduced by saline as described earlier.

4. Kinetics of $^{45}Ca^{2+}$ binding to plasma membranes in the presence of concanavalin A, and concanavalin A concentration and temperature dependence of concanavalin A-mediated stimulation of $^{45}Ca^{2+}$ binding

Kinetics of $^{45}Ca^{2+}$ (10^{-5} M) binding to liver plasma membranes was compared under 3 different conditions; (a) plasma membrane suspension was mixed with $^{45}Ca^{2+}$ solution, (b) plasma membrane suspension was added to a mixture of $^{45}Ca^{2+}$ and concanavalin A already prepared, and (c) plasma membrane suspension, $^{45}Ca^{2+}$ solution and concanavalin A solution were mixed immediately prior to incubation at 37°C. The final concentrations of plasma membranes, $^{45}Ca^{2+}$ and concanavalin A were 26 μg protein/ml, 10^{-5} M, and 100 μg/ml, respectively.

The kinetics of $^{45}Ca^{2+}$ binding (data not shown) indicated that the binding of $^{45}Ca^{2+}$ in (a) and (b) reaches a plateau (equilibrium) level after 10 min of incubation at 37°C although the final binding level in (b) was much higher than that in (a). On the other hand, the $^{45}Ca^{2+}$ binding in (c) took a much longer time (about 1 h or so) before reaching a plateau level, which was, nevertheless, quite close to the final binding level in (b). Therefore in the experiments examining the concanavalin A effect on Ca^{2+} binding, plasma membranes were added to a mixture of $^{45}Ca^{2+}$ and concanavalin A prepared in advance, and the whole mixture was incubated at 37°C for 10 min except when otherwise specified.

Fig. 2 shows the concanavalin A concentration dependence in the $^{45}Ca^{2+}$

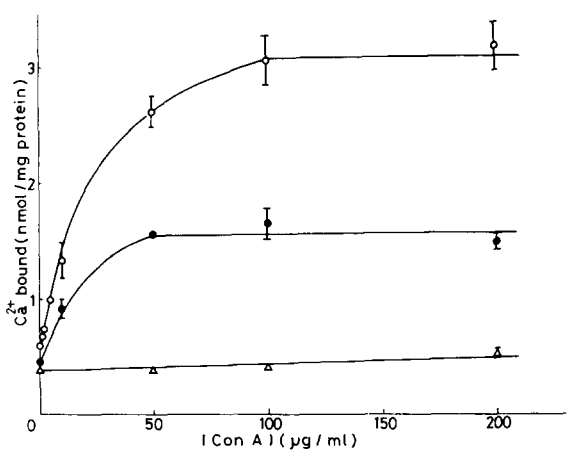

Fig. 2. Concentration dependence of concanavalin A effect on $^{45}Ca^{2+}$ binding at 37 and 0°C, and disappearance of the concanavalin A effect in the presence of α-methyl-D-mannoside. The incubation mixture (1 ml) contained liver plasma membranes (40—70 μg protein), 0.2 μCi $^{45}Ca^{2+}$ (10^{-5} M), concanavalin A (the concentrations are indicated in the abscissa) and α-methyl-D-mannoside (0.1 M). $^{45}Ca^{2+}$ binding to plasma membranes was assayed at the end of 10 min incubation at 37°C in the absence of α-methyl-D-mannoside (○) or in the presence of α-methyl-D-mannoside (△), and at the end of 120 min incubation at 0°C in the absence of α-methyl-D-mannoside (●).

binding to liver plasma membranes as measured at 37°C (10 min) and 0°C (2 h) as well as the effect of α-methyl-D-mannoside on the concanavalin A-mediated stimulation of $^{45}Ca^{2+}$ binding. It seems clear that the stimulation of $^{45}Ca^{2+}$ binding by concanavalin A becomes maximal at 100—200 μg/ml (0.9—1.8 μM as tetramers) at both 37 and 0°C although the stimulation was larger at 37°C than at 0°C. Addition of α-methyl-D-mannoside completely abolished the concanavalin A effect.

5. $^{45}Ca^{2+}$ binding parameters for liver and hepatoma plasma membranes in the presence of concanavalin A

$^{45}Ca^{2+}$ binding to plasma membranes was investigated at various $^{45}Ca^{2+}$ concentrations (10^{-6} M through $5 \cdot 10^{-3}$ M) in the presence of 100 μg/ml concanavalin A. The $^{45}Ca^{2+}$ binding curves and the Scatchard plots of $^{45}Ca^{2+}$ binding in the presence of concanavalin A are shown in Fig. 3. The numbers of Ca^{2+} binding sites (N) and the dissociation constants (K_d) were obtained from these plots.

It seems clear that the concanavalin A-mediated stimulation of $^{45}Ca^{2+}$

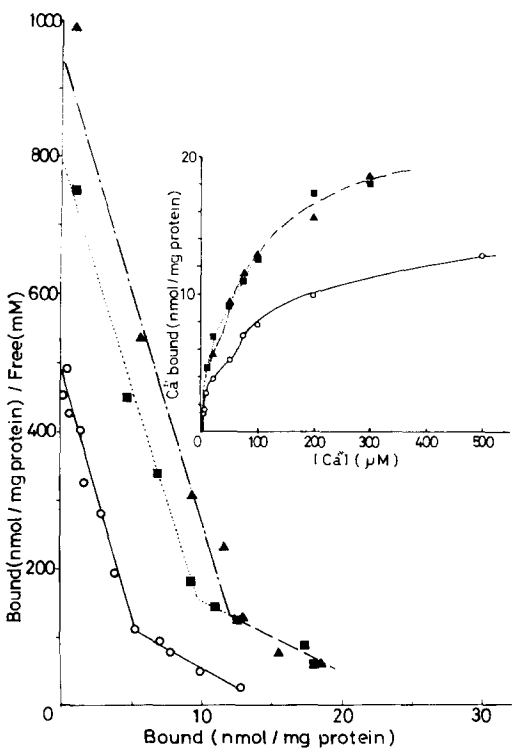

Fig. 3. Binding of $^{45}Ca^{2+}$ to plasma membranes of liver and hepatoma cells in the presence of concanavalin A. 0.2—0.5 μCi $^{45}Ca^{2+}$ (various concentrations) premixed with concanavalin A (100 μg) in Tris buffer (0.9 ml) was added to 0.1 ml of a suspension of plasma membranes of liver (○: 45 μg protein), AH-130 (■: 20 μg protein) and AH-7974 (▲: 33 μg protein) cells, and $^{45}Ca^{2+}$ binding was assayed at 10 min of incubation at 37°C. The inset shows the $^{45}Ca^{2+}$ binding curves up to $^{45}Ca^{2+}$ concentrations of $3—5 \cdot 10^{-4}$ M corresponding to the higher and lower affinity sites. The Scatchard plots of binding indicate two specific binding sites (the higher and lower affinity sites).

binding occurs not only in the liver plasma membranes but also in the hepatoma plasma membranes. Furthermore, it was found that the stimulatory effect is mainly due to an increase in the number of available binding sites of both higher and lower affinities (N higher = 7.2 ± 0.7 (n = 2) (liver), 11.6 ± 0.1 (n = 2) (AH-130) and 13.7 (n = 1) (AH-7974), and N lower = 15.2 ± 0.5 (n = 2) (liver), 24.7 (n = 1) (AH-130) and 24.2 (n = 1) (AH-7974): units are nmol/mg protein) and not to an increase in the affinity for Ca^{2+} (K_d higher = 1.3—1.5 · 10^{-5} M and K_d lower = 1.0 · 10^{-4} M).

The ratios of $^{45}Ca^{2+}$ bound to plasma membranes in the presence of concanavalin A to that in the absence of concanavalin A were plotted against $^{45}Ca^{2+}$ concentration in Fig. 4, suggesting that the higher affinity sites are more strongly (5-fold) activated than the lower affinity sites (3-fold). The $^{45}Ca^{2+}$ binding to the nonspecific sites occurring at $^{45}Ca^{2+}$ concentrations higher than 2 · 10^{-4} M does not seem to be affected by concanavalin A in accordance with earlier results. It should be noted that the concanavalin A-mediated stimulation of $^{45}Ca^{2+}$ binding to liver plasma membranes was not affected by either colchicine (0.5 mM) or cytochalasin B (10 and 100 μg/ml) (data not shown).

6. Additional features of concanavalin A-mediated stimulation of $^{45}Ca^{2+}$ binding to plasma membranes

In order to examine whether the concanavalin A-mediated stimulation of $^{45}Ca^{2+}$ binding may be reversible or not, plasma membranes (liver) were preincubated (37°C, 10 min) with concanavalin A only (Expt. I), concanavalin A and Ca^{2+} added separately (Ca^{2+}, concanavalin A) (Expt II), or concanavalin A and Ca^{2+} added as a mixture (Ca^{2+} plus concanavalin A) (Expt. III). After preincubation, the plasma membranes were spun down, resuspended in the Tris buffer and then assayed for $^{45}Ca^{2+}$ binding in the presence or the absence of concanavalin A.

As shown in Table I, plasma membranes pretreated in Expts. I and II showed the basal $^{45}Ca^{2+}$ binding as well as the concanavalin A-mediated stimulation

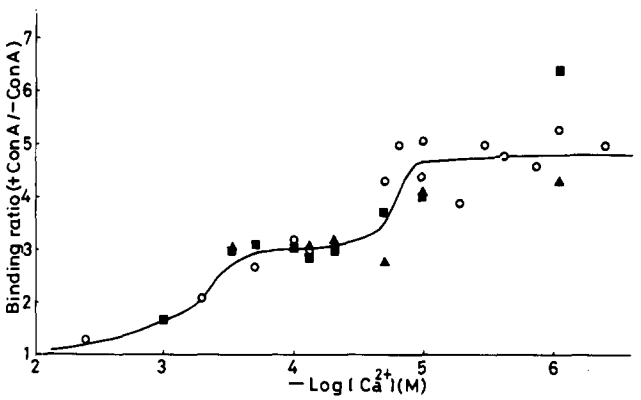

Fig. 4. Ratio of $^{45}Ca^{2+}$ binding in the presence of concanavalin A to that in the absence of concanavalin A at various $^{45}Ca^{2+}$ concentrations. Ratios of $^{45}Ca^{2+}$ bound to plasma membranes of liver (○), AH-130 (■) or AH-7974 (▲) in the presence of concanavalin A (100 μg/ml) (see Fig. 3) to that in the absence of concanavalin A (see Fig. 1) were plotted against $^{45}Ca^{2+}$ concentrations (from 0.4 μM to 5 mM.

TABLE I

REVERSIBILITY OF CONCANAVALIN A EFFECTS

Plasma membranes (liver; 250—1000 μg protein) in 1 ml Tris buffer (pH 7.5) were preincubated at 37°C for 10 min in the presence of the additives listed in the table (additives in preincubation), and then plasma membranes were spun down and resuspended in 1 ml Tris buffer. A 0.1-ml aliquot of plasma membrane suspension was added to 0.9 ml of the Tris buffer containing the additives listed in the Table (additives in binding assay mixture), and the whole mixture was incubated at 37°C for 10 min, followed by assaying $^{45}Ca^{2+}$ bound to plasma membranes. The final concentrations of $^{45}Ca^{2+}$ and concanavalin A were 10^{-5} M and 100 μg/ml, respectively.

Expt. No.	Additives in preincubation	Additives in binding assay mixture	Relative binding *
I	None	$^{45}Ca^{2+}$	1.0
	None	$^{45}Ca^{2+}$ plus concanavalin A	4.8
	Concanavalin A (100 μg/ml)	$^{45}Ca^{2+}$	0.9
	Concanavalin A (100 μg/ml)	$^{45}Ca^{2+}$ plus concanavalin A	4.3
II	Ca^{2+} (10^{-4} M)	$^{45}Ca^{2+}$	1.0
	Ca^{2+} (10^{-4} M)	$^{45}Ca^{2+}$ plus concanavalin A	5.4
	Ca^{2+} (10^{-4} M), concanavalin A (100 μg/ml)	$^{45}Ca^{2+}$	1.2
	Ca^{2+} (10^{-4} M), concanavalin A (100 μg/ml)	$^{45}Ca^{2+}$ plus concanavalin A	4.3
III	Ca^{2+} (10^{-5} M)	$^{45}Ca^{2+}$	1.0
	Ca^{2+} (10^{-5} M) plus concanavalin A (100 μg/ml)	$^{45}Ca^{2+}$	1.0
	Ca^{2+} (10^{-5} N) plus concanavalin A (100 μg/ml)	$^{45}Ca^{2+}$ plus concanavalin A	2.6

* $^{45}Ca^{2+}$ binding as compared to the binding in the first line in each experiment.

similarly to the plasma membranes preincubated without concanavalin A. However, plasma membranes pretreated in Expt. III gave the basal binding similarly to the plasma membranes preincubated without concanavalin A, but the concanavalin A-mediated stimulation was significantly smaller (2.6-fold) than that in Expts. I and II (4.3—5.4-fold). These results suggest that concanavalin A alone (Expt. I) or concanavalin A and Ca^{2+} added separately (Expt. II) does not cause long lasting alterations in the plasma membranes at least within a 10 min period of preincubation in contrast to a long lasting change in plasma membranes preincubated with concanavalin A plus Ca^{2+} (Expt. III).

Next, the replaceability of Ca^{2+} (or $^{45}Ca^{2+}$) previously bound to plasma membranes with $^{45}Ca^{2+}$ (or Ca^{2+}) added subsequently was investigated. As shown in Table II, Ca^{2+} (or $^{45}Ca^{2+}$) bound to plasma membranes in the absence of concanavalin A (1st incubation) appeared to be easily exchanged with $^{45}Ca^{2+}$ (or Ca^{2+}) added later (2nd incubation), reaching an equilibrium by the end of 10 min of the 2nd incubation (Expts. 1—3). However, Ca^{2+} (or $^{45}Ca^{2+}$) bound to plasma membranes in the presence of Ca^{2+} (or $^{45}Ca^{2+}$) plus concanavalin A does not seem to be easily exchanged with $^{45}Ca^{2+}$ (or Ca^{2+}) added later (Expts. 4 and 5).

In a parallel experiment we have observed the kinetics of Ca^{2+} replacement, finding that the replacement of Ca^{2+} bound to plasma membranes in the absence of concanavalin A is accomplished within 10 min, but the replacement of Ca^{2+} bound to plasma membranes under the condition of concanavalin A plus Ca^{2+} with $^{45}Ca^{2+}$ added later was almost negligible during the first 10 min period (see also Expt. 4 in Table II) but the replacement started to proceed later, reaching an equilibrium at 45 min of incubation (data not shown).

TABLE II

REPLACEABILITY OF Ca^{2+} (OR $^{45}Ca^{2+}$) PREBOUND TO PLASMA MEMBRANES IN THE PRESENCE OR ABSENCE OF CONCANAVALIN A WITH $^{45}Ca^{2+}$ (OR Ca^{2+}) ADDED LATER

Plasma membranes (liver) in 0.9 ml Tris buffer were preincubated at 37°C for 10 min in the presence of the additives listed in the Table (1st incubation), and then some complementary additives in 0.1 ml of the buffer (2nd incubation) were added and the whole mixture was incubated at 37°C for another 10 min period, followed by assaying $^{45}Ca^{2+}$ bound to plasma membranes. The concentrations of Ca^{2+}, $^{45}Ca^{2+}$ and concanavalin A in the final (2nd) incubation mixture were 10^{-5} M, 10^{-5} M and 100 μg/ml, respectively.

No.	Additives in 1st incubation	Additives in 2nd incubation	Relative * binding
1	Ca^{2+}, $^{45}Ca^{2+}$	None	1.00
2	Ca^{2+}	$^{45}Ca^{2+}$	0.75 ± 0.15 (2)
3	$^{45}Ca^{2+}$	Ca^{2+}	0.85 ± 0.15 (2)
4 **	(Ca^{2+} plus concanavalin A)	$^{45}Ca^{2+}$	Nil (2)
5	($^{45}Ca^{2+}$ plus concanavalin A)	Ca^{2+}	4.05 ± 0.15 (2)
6	$^{45}Ca^{2+}$, (Ca^{2+} plus concanavalin A)	None	2.70 ± 0.20 (2)
7	Ca^{2+}, ($^{45}Ca^{2+}$ plus concanavalin A)	None	2.65 ± 0.15 (2)

* $^{45}Ca^{2+}$ binding as compared to the binding in No. 1. Average of 2 experiments.

** When $^{45}Ca^{2+}$ binding was assayed at 45 min of incubation (2nd) instead of 10 min, a relative binding similar to that in No. 6 and 7 was observed.

7. Effect of concanavalin A on $^{45}Ca^{2+}$ uptake by AH-7974 cells

Fig. 5a shows the kinetics of $^{45}Ca^{2+}$ ($5 \cdot 10^{-7}$ M) uptake by AH-7974 cells at 37 and 20°C in the Hepes/saline solution. The uptake seems to be very rapid and reaches an equilibrium within 10 min of incubation. Fig. 5b shows the

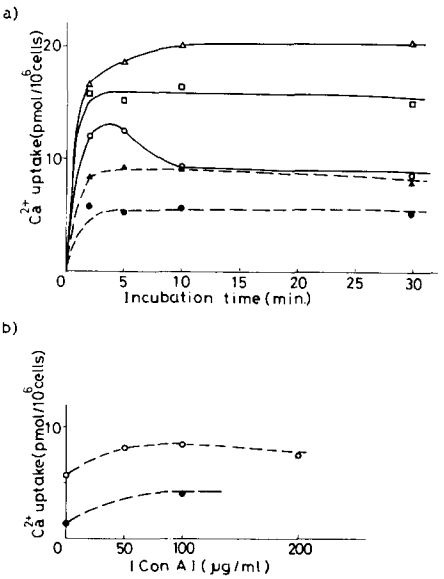

Fig. 5. Effects of concanavalin A, sodium azide and temperature on $^{45}Ca^{2+}$ uptake by AH-7974 cells. (a) Kinetics of $^{45}Ca^{2+}$ uptake. Solid lines with open symbols correspond to 37°C, and broken lines with closed symbols correspond to 20°C. Cell concentrations were $2.2 \cdot 10^5$ cells/ml (at 37°C) and $3.9 \cdot 10^5$ cells/ml (at 20°C), and concanavalin A concentrations were 0 μg/ml (○ and ●), 50 μg/ml (□) and 100 μg/ml (△ and ▲). (b) Concanavalin A concentration dependence of $^{45}Ca^{2+}$ uptake in the absence (○) and presence (●) of 50 mM NaN_3 as measured at 2 min of incubation at 20°C.

dose-dependence in the concanavalin A-mediated stimulation of $^{45}Ca^{2+}$ ($5 \cdot 10^{-7}$ M) uptake by the cells as assayed at 2 min of incubation at 20°C, showing a maximal stimulation at 100 μg concanavalin A/ml.

Addition of NaN_3 (50 mM) resulted in a marked decrease in the $^{45}Ca^{2+}$ uptake by the cells, probably reflecting the inhibition of intracellular translocation of $^{45}Ca^{2+}$ into mitochondria. It should be noted that the concanavalin A-mediated stimulation of $^{45}Ca^{2+}$ uptake was, nevertheless, observed, and actually the stimulation was more marked in the presence of azide than that in the absence of azide (Fig. 5b).

The concanavalin A-mediated stimulation of $^{45}Ca^{2+}$ uptake in the presence of 100 μg/ml concanavalin A was about 100% at $5 \cdot 10^{-7}$ M $^{45}Ca^{2+}$ (Fig. 6a) and about 40% at 10^{-5} M $^{45}Ca^{2+}$ (data not shown) as assayed at 10 min of incubation. On the other hand, no appreciable stimulation of $^{45}Ca^{2+}$ uptake by concanavalin A was observed at 10^{-3} M $^{45}Ca^{2+}$ (data not shown). Moreover, the stimulation was not observed in the Hepes/saline/glucose solution either, suggesting that glucose at a large concentration may interfere with the concanavalin A binding to the cell surface similarly to α-methyl-D-mannoside.

Discussion

In the present study we have shown that the plasma membranes of rat liver and some ascites hepatoma cells possess at least two types of specific binding sites for Ca^{2+}, higher and lower affinity sites. Ca^{2+} binding sites with much lower affinities are also present in the plasma membranes but they seemed to be of a nonspecific nature with negative cooperativity. The Ca^{2+} binding sites with K_d of the order of mM have been related to acidic phospholipids [6]. The possible presence of Ca^{2+} binding sites with much higher affinity (K_d of 10^{-6} M or so) cannot be excluded because Ca^{2+} binding analysis has not been performed under the condition of very low Ca^{2+} concentrations using an appropriate Ca^{2+} buffer system.

The present study showed that the plasma membranes of AH-130 and AH-7974 cells possess 1.4—2.1-fold as many Ca^{2+} binding sites as compared to the plasma membranes of liver cells on the protein basis. Whether such a quantitative difference (and not a qualitative difference) concerning the Ca^{2+} binding sites in plasma membranes between livers and hepatomas may be generalized or not remains to be examined by further experiments.

The Ca^{2+} binding parameters for liver plasma membranes so far reported (Refs. 3, 6 and this paper) are not completely consistent with each other. This may be due to the differences in the assay methods, the techniques adopted for plasma membrane preparation, and the purity of plasma membranes used for Ca^{2+} binding assays. In the present study Millipore filters coated with polycations were used so that the blank bindings are reduced, improving the accuracy of binding assays.

The purity of plasma membranes (liver) used in the present study was checked enzymically, showing the specific activities of 5′-nucleotidase, glucose-6-phosphatase and succinate dehydrogenase (μmol/mg protein per h) of 40.1 ± 12.7 ($n = 6$), 1.16 ± 0.27 ($n = 3$) and 0.20 ± 0.04 ($n = 7$), respectively (n is numbers of plasma membrane preparations). These results together with the

specific activities of mitochondria and microsomes (data not shown) may suggest a mitochondrial contamination of about 4% and a microsomal contamination of about 5%, respectively. The specific activity of 5'-nucleotidase was similar to that reported by Bermann et al. [32]. The fact that 2,4-dinitrophenol or sonication of plasma membrane preparations did not affect the $^{45}Ca^{2+}$ binding (data not shown) may also reduce the possibility for possible involvement of some vesicular transport systems in the present binding experiments. The Ca^{2+} binding parameters obtained for liver plasma membranes seem somewhat close to those for the adipocyte plasma membranes [7].

In the present study we have shown that concanavalin A gives rise to a markedly stimulated Ca^{2+} binding to plasma membranes of both liver and hepatoma cells. The concanavalin A-mediated stimulation was found due to the increase in the specific binding sites (both higher and lower affinity sites) for Ca^{2+} (Fig. 3). Such differences in the stimulatory effect between the application methods (concanavalin A and $^{45}Ca^{2+}$ added separately versus concanavalin A and $^{45}Ca^{2+}$ added as an already prepared mixture) as observed in the present study seem to be due to a conformational change in concanavalin A molecules occurring after the addition of Ca^{2+} [33]. Accordingly divalent succinyl concanavalin A was less effective than tetravalent concanavalin A.

The mechanisms of concanavalin A-mediated stimulation of Ca^{2+} binding have not yet been fully elucidated. However, the possibility that the apparent stimulation of Ca^{2+} binding might be mediated by Ca^{2+} bound to the lectin molecules which are then bound to plasma membranes seems rather unlikely mainly from the following reasons: (1) The average amount of $^{45}Ca^{2+}$ (at 10^{-5} M) bound to one concanavalin A molecule was only 0.10 mol (37°C) or 0.16 mol (0°C) (data not shown). (2) NaCl at 150 mM reduced the $^{45}Ca^{2+}$ binding but did not alter the concanavalin A effect (the binding of [^{3}H]-concanavalin A to plasma membranes as well as the binding of $^{45}Ca^{2+}$ to concanavalin A was not affected by 150 mM NaCl). (3) The concanavalin A effects were similar (Fig. 4) in both the liver and the hepatoma plasma membranes inspite of that the basal Ca^{2+} binding is smaller in the liver membranes than in the hepatoma ones and the [^{3}H]concanavalin A binding ([^{3}H]-concanavalin A at 100 μg/ml in the presence of 10^{-5} M Ca^{2+}, 37°C, 10 min) to liver and hepatoma plasma membranes was almost similar (1.3—1.5 nmol/mg protein) (data not shown). (4) As shown in Fig. 6, the binding of [^{3}H]-concanavalin A to plasma membranes at 0°C reaches the same plateau level already at 10 min of incubation as the binding of it at 37°C (see (3)) whereas the binding of $^{45}Ca^{2+}$ to plasma membranes at 0°C in the presence or the absence of concanavalin A reaches a plateau at 2 h. The possibility that some cryptic Ca^{2+} binding sites may be activated as the results of Ca^{2+}-activated concanavalin A binding to plasma membranes is now under investigations in our laboratory.

In the present study we have shown that $^{45}Ca^{2+}$ uptake by AH-7974 cells is also stimulated by concanavalin A. The concanavalin A-induced stimulation of $^{45}Ca^{2+}$ uptake showed the concanavalin A concentration and $^{45}Ca^{2+}$ concentration dependencies similar to those in the concanavalin A-mediated stimulation of Ca^{2+} binding. However, the concanavalin A-induced stimulation of $^{45}Ca^{2+}$ uptake is not merely due to the increased amount of $^{45}Ca^{2+}$ bound to the cell

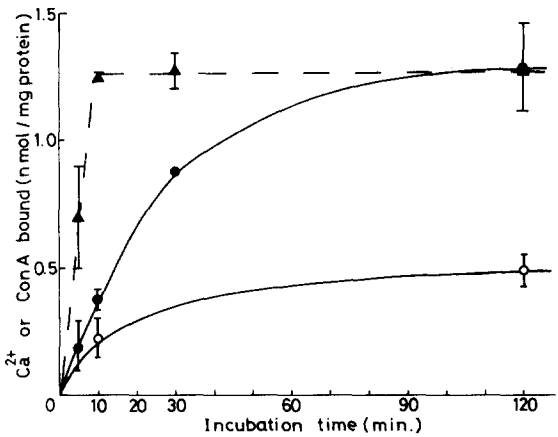

Fig. 6. Kinetics of $^{45}Ca^{2+}$ binding and [^{3}H]concanavalin A binding to plasma membranes (liver) at 0°C. Solid lines correspond to the binding of $^{45}Ca^{2+}$ (10^{-5} M) in the absence (○) and presence (●) of concanavalin A (100 μg/ml). A broken line with closed triangles corresponds to the binding of [^{3}H]-concanavalin A (100 μg/ml) in the presence of Ca^{2+} (10^{-5} M). Concentrations of plasma membranes were 34 μg protein/ml ($^{45}Ca^{2+}$ binding kinetics) and 79 μg protein/ml ([^{3}H]concanavalin A binding kinetics), respectively.

surface but it is also due to the increased influx of $^{45}Ca^{2+}$ into the cells as suggested by a marked inhibition of $^{45}Ca^{2+}$ uptake by azide. The rather quick uptake of $^{45}Ca^{2+}$ by AH-7974 cells seems to be in accord with the $^{45}Ca^{2+}$ uptake by T-cells [9].

At the moment the biochemical identities of Ca^{2+} binding sites and especially of those activated by concanavalin A are not clear. Whether they may be Ca^{2+}-dependent regulatory protein, Ca^{2+}-carrier protein, Ca^{2+}-ATPase or others remains to be investigated together with the mechanisms of activation of Ca^{2+} binding sites by concanavalin A.

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