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EFFECT OF CONCANAVALIN A ON Ca^{2+}BINDING, Ca^{2+} UPTAKE AND THE Ca^{2+}ATPase OF LYMPHOCYTE PLASMA MEMBRANES

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Summary: Lymphocyte plasma membranes bind $^{45}Ca^{2+}$ with three affinity sites: K_{A1} = 4.0 . 10^6 M^{-1}, K_{A2} = 8.5 . 10^4 M^{-1} and K_{A3} = 4.2 . 10^2 M^{-1}, and Ca^{2+} binding capacities are 0.10, 1.2 and 85 nmoles Ca^{2+}/mg protein. In the presence of 15 µg/ml ConA the Ca^{2+} binding constants were K_{A1} = 4.6 . 10^6 M^{-1}, K_{A2} = 4.4 . 10^4 M^{-1} and K_{A3} = 4.2 . 10^2 M^{-1}. The Ca^{2+} binding capacity was increased by ConA, to 0.13, 2.4 and 91 nmoles/mg protein. The Ca^{2+} ATPase activity of lymphocyte membranes was increased by ConA from 1 to 2 µmol P/protein x h. The $^{45}Ca^{2+}$ uptake was stimulated by ConA and PHA to about 16 %.

Introduction: Cellular Ca^{2+} metabolism consistes of Ca^{2+} binding to the cell membrane, intracellular uptake and retransport out of the cell by Ca^{2+} ATPase. Within various cell types Ca^{2+} is bound by mitochondria and by the sarcoplasmic or endoplasmic reticulum, and it is released and retransported into these intracellular Ca^{2+} stores (1-3). In lymphocytes omission of Ca^{2+} from the incubation medium or addition of EDTA at least during a certain period after mitogen addition inhibits lymphocyte activation. The Ca^{2+} ionophore A 23187 (in the presence of extracellular Ca^{2+}) elevates the cytosolic Ca^{2+} concentration and activates lymphocytes like a mitogen (1,3). Since the effect of mitogens on the various components of Ca^{2+} metabolism has not been defined, we have investigated the effect of ConA on Ca^{2+} binding, on Ca^{2+} transport into membrane vesicles and on Ca^{2+} ATPase of isolated lymphocyte cell membranes.

Methods:

Membrane preparation: The cells from sheep lymph nodes (usually about 200 g, (1 - 5 x 10^{11} cells) were suspended under continuos stirring in 200 ml 1 mM Tris/HCl pH 7.4 for 1 hour with addition

ConA, Concanavalin A; PHA, Phytohemagglutinin;
EDTA, Ethylendiamintetraaceticacid;
5-Nucl., 5-Nucleotidase; Succ.d.h., Succinatdehydrogenase,
Gluc-5-ph, Glucose-6-Phosphatase.

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Table 1: Characterisation of the cell membrane fraction.

Prot.[x]	RNA[x]	DNA[x]	Na^+ K^+ ATPase[x]	5'Nucl.[xx]	Succ.dh.[xx]	Gluc-6-ph[xx]
4.1	0.1	0.1	1.6	12.6	not detected	0.21

x mg/ml xx µmol/h x mg protein

of DNAse. The suspension was homogenized (Potter homogenizer) with 10 stokes and the homogenate was made 0.25 M with sucrose and centrifuged at 7 000 x g for 10 minutes. The supernatant was made 1.23 M with sucrose and the pellet discharged. For further purification a step gradient was used in a 25.2 Beckman Rotor: 8 ml 2 M sucrose, 9 ml 1.31 M sucrose, 35 ml sample in 1.23 M sucrose followed by 8 ml 0.8 M sucrose. After centrifugation for 16 hours at 200 000 x g, the plasma membrane was found at the border between 1.31/1.23 M sucrose.

The determination of ATPase activity, 5-nucleotidase, succinate dehydrogenase, glucose-6-phosphatase, protein, DNA and RNA was described earlier (4).

Ca^{2+} binding: To the binding buffer (150 mM KCl, 5 mM $MgCl_2$, 50 mM Tris/HCl, pH 6.8) 0.1 µCi $^{45}Ca^{2+}$ (spec.activity 22.2 µCi/mg Ca^{2+}, NEN chemicals) was added. 1 µCi $^{45}Ca^{2+}$ was added for Ca^{2+} concentrations above 1 x 10^{-5} M, and 2 µCi $^{45}Ca^{2+}$ werem added for concentrations exceeding 1 x 10^{-3} M. The protein concentration of plasma membrane was 1 mg per sample. After 30 minutes incubation at 37°C 1 ml of sample was layered over 2 ml of 0.5 M sucrose and centrifuged at 30 000 x g for 30 minutes (Spinco), the pellet cut out and solubillized in 0.1 M NaOH. The radioactitivy was measured in a scintillation counter (LS 100 Beckman). To define the trapped space, 10 mM EDTA was added.

Ca^{2+} uptake: For Ca^{2+} uptake the same buffer and temperature (37°C) were used as in the binding experimentes, and 5 mM ATP and 5 mM oxalate were added. The reaction was started by addition of $^{45}Ca^{2+}$ and ConA. After 30 minutes the plasma membranes were centrifuged through 0.5 M sucrose. The pellet was counted for $^{45}Ca^{2+}$.

Results: The plasma membrane fraction used is only slightly contaminated with DNA and RNA (Tab.1). There is some contamination with endoplasmic reticulum as indicated by the low glucose-6-phosphatese activity. Succinate dehydrogenase activity was not measurable, which excluded significant contaminations by mitochondria. This agrees with the electron microscopic pictures (Fig.1) showing some ribosomes but no mitochondria. The membrane preparation consists of vesicles, many of which are open. Increasing the calcium concentration in the medium increases the amount of Ca^{2+} bound to the plasma membrane. The correlation between membrane-bound Ca^{2+} and Ca^{2+} concentration in the medium was analysed by Scatchard plots (Fig.2). There are three binding

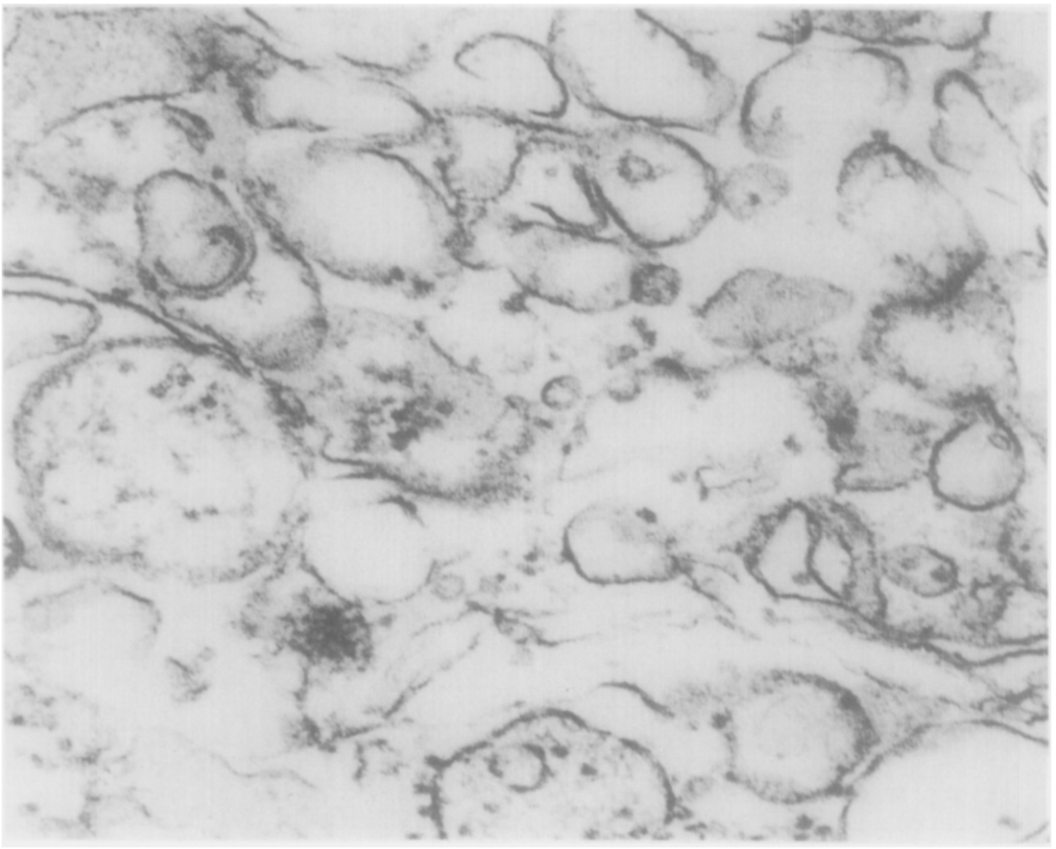

Fig. 1: Electromicroscopic picture of the membran fraction used.

sites for Ca^{2+}, with high, intermediate and low affinity. The dissociation constants and the amount of Ca^{2+} bound to each site are listed in Tab.2. In the presence of 15 µg ConA/ml, the affinity and the capacity of the high-affinity sites are increased by 15 % and 13 % respectively. The affinity of the intermediate-affinity binding site is reduced, whereas its Ca^{2+} binding capacity is doubled. The low-affinity Ca^{2+} binding sites are not affected by ConA. The increase in Ca^{2+} binding capacity by ConA and PHA is dose-dependent. At all Ca^{2+} concentrations used (5, 50, 500 µM Ca^{2+}), there is an increased Ca^{2+} binding to the plasma membrane with increasing lectin concentration. At 15 - 30 µg/ml there is an optimum and at the very high concentration of 100 µg/ml there is a decrease of calcium binding. 1 mM α-methyl mannoside inhibits the ConA effect totally (not shown).

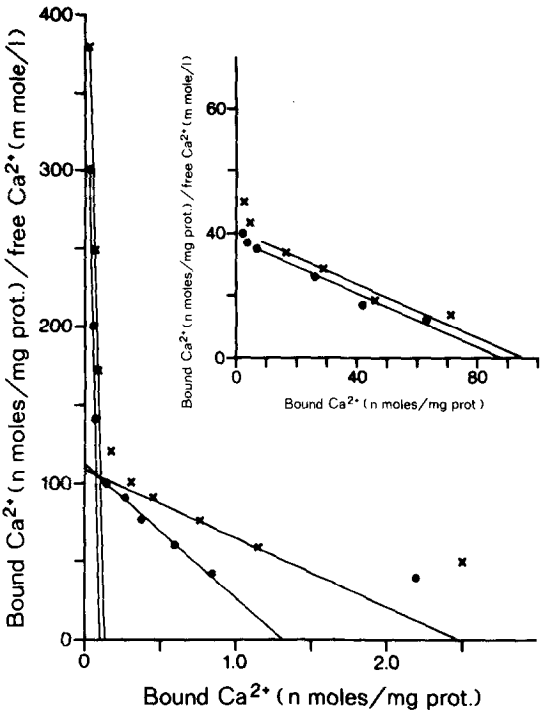

Fig. 2: Scatchard plot of Ca^{2+} binding to lymphocyte cell membranes in absence (•) and in presence of 15 µg/ml ConA (x).

There is a maximal Ca^{2+} uptake in the presence of ATP and oxalate. Without ATP or oxalate, Ca^{2+} uptake at $5 \cdot 10^{-7}$ M Ca^{2+} corresponds to high-affinity Ca^{2+} binding (Tab.2) NaN_3 was added to exclude mitochondrial Ca^{2+} uptake, and no effect of this agent was seen (Tab.3).

Table 2: Affinity and capacity of $^{45}Ca^{2+}$ binding sites.

	high affinity	intermediate affinity	low affinity
control			
K_A (M^{-1})	$4.0 \cdot 10^6$	$8.5 \cdot 10^4$	$4.2 \cdot 10^2$
nmole Ca^{2+}/mg prot.	0.10 ± 0.01	1.2 ± 0.1	85 ± 5
ConA 15 µg/ml			
K_A (M^{-1})	$4.6 \cdot 10^6$	$4.4 \cdot 10^4$	$4.2 \cdot 10^2$
nmole Ca^{2+}/mg prot.	0.13 ± 0.01	2.4 ± 0.1	91 ± 6

Values were calculated from Fig.2

Table 3: Characterisation of Ca^{2+} transport.

ATP	$MgCl_2$	Oxalate	NaN_3	nmole Ca^{2+}/mg protein x 30 minutes
-	-	-	-	0.07
5 mM	-	-	-	0.14
5 mM	5 mM	-	1 mM	1.07
5 mM	5 mM	-	-	1.02
5 mM	5 mM	5 mM	-	2.34
5 mM	5 mM	5 mM	1 mM	2.37
-	-	5 mM	-	0.21

Calcium transport was measured at 37°C for 3 minutes. Ca^{2+} concentration was 5 x 10^{-7} M. The pH 6.8. Averages of 3 experiments, each in duplicate.

Fig.3 shows the time course of Ca^{2+} uptake. During the first 5 minutes there is a rapid $^{45}Ca^{2+}$ uptake. ConA as well as PHA increased the total amount of Ca^{2+} taken up by the plasma membranes. After 30 minutes in the presence of mitogen, the uptake is increased by about 16 % compared to the control. The ConA-induced increase in Ca^{2+} uptake was abolished by 1 mM α-methyl mannoside (not shown).

The presence of a Ca^{2+} ATPase was shown by its determination as $(Mg^{2+} + Ca^{2+})$-ATPase minus Mg^{2+} ATPase, in the absence or presence of EDTA. Its activity is doubled by 15 µg/ml ConA (Tab.4). Dornaud et al. (5) also found a stimulation of Ca^{2+} ATPase from lymphocytes by ConA.

Discussion: The plasma membrane preparation used contained about 5 % endoplasmic reticulum, as indicated by the glucose-6-

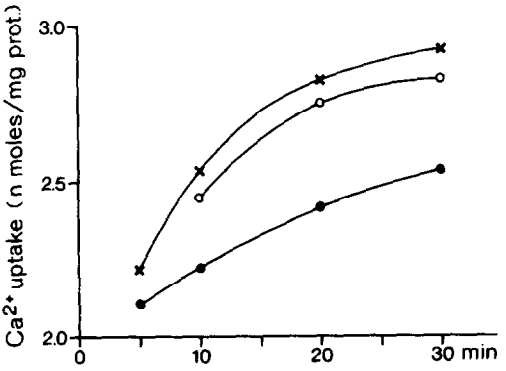

Fig. 3: Time course of Ca^{2+} uptake in the presence of 15 µg/ml ConA (x), 10 µg/ml PHA (o), control (•).
Ca^{2+} concentration was 50 µM.

Table 4: Activation of Ca^{2+} ATPase from lymphocyte plasma membranes by ConA.

EDTA (mM)	ConA (µg/ml)	total ATPase (µmol P_i/h x mg prot.)	Ca^{2+} ATPase (µmol P_i/h x mg prot.)
0.5	-	1.02	-
0.5	15	1.41	-
-	-	2.05	1.03
-	15	3.41	2.00

The ATPase activity was measured in the presence of 0.1 mM Ca^{2+}, 10^{-3}M ouabain, 2.5 mM Tris ATP and 2 mM Mg Cl_2 in 70 mM imidazole/HCl buffer pH 7.4. Mean of 3 experiments.

phosphatase activity. Since there is no ConA-binding to endoplasmic reticulum at mitogenically effective concentration of ConA (6), this contamination will not influence the results with ConA.

The Ca^{2+} binding constants of the low-affinity Ca^{2+} binding site may be sialic acid, which has a $K_A = 1.21 \cdot 10^2\ M^{-1}$ (7), or phospholipids. The reported Ca^{2+} binding constants for the same phospholipids range from 10^5 to $10^1 M^{-1}$ (8-9). Ca^{2+} binding sites with higher affinity are Ca^{2+}-binding proteins. A high-affinity Ca^{2+}-binding protein is Ca^{2+} ATPase. At 22°C this enzyme binds 2 moles Ca^{2+}/mole with a $K_A = 3 \cdot 10^6\ M^{-1}$ and 1 mole Ca^{2+}/mole with a $K_A = 10^3\ M^{-1}$ (10). A small increase in ConA-induced Ca^{2+} binding may be produced by ConA itself. At 15 µg/ml ConA, $5 \cdot 10^{12}$ molecules ConA are bound per mg lymphocyte-membrane protein as measured with ^3H ConA (unpublished). Each ConA molecule binds up to 4 Ca^{2+} (11,12). Thus, a maximum of 0.003 nmoles Ca^{2+}/mg can be bound by ConA. However, at the low Ca^{2+} concentrations of high-affinity Ca^{2+} binding, only a small part of the Ca^{2+}-binding sites of the ConA molecules may be occupied. Therefore, the increase in Ca^{2+} binding is not caused by binding of Ca^{2+} to ConA. This is confirmed by the result that PHA also increases Ca^{2+} binding (Fig.3,4).

ConA produces an increase in phospholipid unsaturation (13) and membrane fluidity and an increase in amidation of amino acids (14) which changes the visco-elastic properties of cell-membrane proteins. Possibly these changes may lead to an increase in Ca^{2+}-binding capacity by a change in conformation or by alteration of the plasma membrane, exposing new Ca^{2+} binding

sites. If there is a non-exchangeable Ca^{2+} pool in lymphocyte membrane, as has been found in adipocyte membranes (15), ConA may transform some unexchangeable Ca^{2+} into an exchangeable Ca^{2+} pool without increasing the total amount of bound Ca^{2+}.

Ca^{2+} uptake by the lymphocyte-membrane preparation is rather small. It includes $^{45}Ca^{2+}$ binding, $^{45}Ca^{2+}$ exchange, $^{45}Ca^{2+}$ transport and passive diffusion into the leaky membrane vesicles. It is not possible to differentiate between these compartments. Ca^{2+} is transported by Ca^{2+} ATPase. The activity of the Ca^{2+} ATPase in membrane preparation from erythrocytes is 2 µmol/mg protein x h (16). In lymphocytes the corresponding value is 1 µmol/mg protein x h (Tab.4) and thus in the same range. The ratio Ca^{2+} transport/Ca^{2+}ATPase in erythrocyte ghosts seems to be 1 (16) ; in muscle microsomes ratios up to 2 have been determined (2). However, in the lymphocyte membrane prepration, Ca^{2+} transport is much smaller than Ca^{2+} ATPase activity. The reasons may be that the lymphocyte-membrane vesicles are in part open or leaky and that there is a mixed sidedness of the vesicles, with right-side-out vesicles not actively accumulating Ca^{2+}. Probably for the same reasons the ConA-induced increase in Ca^{2+} ATPase avtivity is higher than the ConA-induced increase in Ca^{2+} uptake. ConA was also bound to cell membranes of other cells (12,13,17). Moreover, it increased $^{45}Ca^{2+}$ binding to liver plasma membranes (17) and stimulated $^{45}Ca^{2+}$ uptake in hepatoma cells (17). Therefore, the reported effects of ConA and PHA on Ca^{2+} metabolism of lymphocytes are possibly not specific. Very recent experiments with rabbit thymocytes revealed that ConA induced release of mitochondrial Ca^{2+} and increased cytosolic Ca^{2+} concentration (18).

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