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CONCANAVALIN A BINDING AND Ca^{2+} FLUXES IN RAT SPLEEN CELLS

C. HENRIK J. WOLFF and KARL E.O. ÅKERMAN *

Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10 A, SF-00170 Helsinki 17 (Finland)

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Addition of the mitogenic lectin concanavalin A to rat spleen cells results in a small increase in the steady-state Ca^{2+} content of the cells. $^{45}Ca^{2+}$ fluxes were measured under conditions where artifacts due to Ca^{2+} binding to concanavalin A could be excluded. Both $^{45}Ca^{2+}$ influx into and efflux from these cells are significantly activated by the lectin. If $^{45}Ca^{2+}$ is added 30 min after concanavalin A the rate of influx is further enhanced. The increase in $^{45}Ca^{2+}$ influx correlates well with binding of concanavalin A to the cells. At low concentrations (optimal mitogenic) of the lectin (1 and 3 μg/ml) no significant increase in $^{45}Ca^{2+}$ influx occurs but an increase in $^{45}Ca^{2+}$ efflux is still observed. The results suggest that concanavalin A binding to the cell surface causes an increase in Ca^{2+} influx into the cells and that activation of Ca^{2+} efflux occurs as a response to an increase in the cytosolic Ca^{2+} activity. Thus, Ca^{2+} may well play a role in triggering lymphocyte activation.

Introduction

Several lines of evidence implicate a role for Ca^{2+} as an intracellular messenger in mitogenic T-lymphocyte activation. It has been established that extracellular Ca^{2+} is required [1–3] for mitogenic stimulation induced by various lectins such as concanavalin A and phytohaemagglutin. The divalent cation ionophore A23187 is mitogenic [4,5] and an increase in the cytosolic Ca^{2+} activity is observed upon addition of concanavalin A or phytohaemagglutin to lymphocyte suspensions [6]. The mechanism by which the cytosolic Ca^{2+} rises as a response to mitogen addition is unclear. Some workers have observed an increase in Ca^{2+} uptake, which terminates within a few minutes [7,8], while others have reported that there is a prolonged increase in Ca^{2+} influx [9]. Also, in the case of the correlation of the mitogenic response to Ca^{2+} influx, variable observations have been made. Thus, a good correlation has been observed by some workers [8] while others [9] have claimed that Ca^{2+} influx correlates with the inhibition of DNA synthesis at high concentrations of lectins. A further complication may arise from the fact that the most commonly used lectin, concanavalin A, binds Ca^{2+} irreversibly [10,11], i.e., the bound cation cannot be removed totally from the lectin by dialysis against 1 mM EDTA [10] for several hours and $^{45}Ca^{2+}$ bound by concanavalin A is not exchanged upon addition of 40 mM $^{40}Ca^{2+}$ [11].

The aim of the present study was to re-examine Ca^{2+} fluxes in lymphocytes by measuring steady-state net Ca^{2+} uptake and steady-sate $^{45}Ca^{2+}$ exchange fluxes taking possible Ca^{2+} binding to concanavalin A and phytohaemagglutin into account.

* Present address: Department of Pathology, University of Helsinki, Helsinki, Finland.

Abbreviations: Con A, concanavalin A; PHA, phytohaemagglutinin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*-tetraacetate; Tes, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulphonate.

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Methods and Materials

Isolation of cells

The spleens of Wistar rats were cut open and the cells squeezed out with a pair of forceps into a basal medium consisting of 137 mM NaCl, 5.4 mM KCl, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 20 mM Tes (pH 7.4) at room temperature. The suspension of cells was subsequently filtered through two layers of cheese cloth or cotton-wool and centrifuged at $400 \times g$ for 10 min. The cells were then lightly suspended into 0.83% (w/v) NH_4Cl, 10 mM Tes (pH 7.4) (10 ml/spleen), and incubated for 8 min after which they were centrifuged at $500 \times g$ for 5 min and washed twice in the basal medium. The cells were finally suspended into the basal medium and stored at room temperature before use. The viability of the cells used in the experiments described below was always over 90% as measured by trypan blue exclusion.

Conditions of incubation during isotope experiments

The cells were diluted into the basal medium to a final concentration of 10^7 cells/ml. 2 μCi/ml ^3H_2O, 0.8 mM $CaCl_2$ and 5 mM glucose were added and incubation was carried out at 35°C in a shaking water bath. $^{45}Ca^{2+}$ or [^{3}H]acetyl-concanavalin A was added as indicated in the figure legends. At certain time intervals, samples (250 μl) were taken from the suspension and layered onto the top of a mixture of dinoylphthalate and butylphthalate (30:60, v/v) containing 250 μl of 4 mM EGTA in the basal medium at the top in order to remove surface-bound Ca^{2+} [12,13]. Thereafter, the cells were immediately spun through the oil in a Janetzki bench centrifuge. The supernatant on the top of the oil was removed by suction. 0.5 ml H_2O was pipetted onto the top of the oil to wash the walls of the tubes, subsequently the H_2O and oil were sucked off and the pellet was homogenized in 10% (v/v) perchloric acid. After extraction for at least 1 h the acid was neutralised with 0.5 M Tris base and the total content of the tubes transferred into scintillation vials and counted in an LKB/Wallace Minibeta (1211) apparatus using gate settings distinguishing $^{45}Ca^{2+}$ from ^{3}H.

Materials

Radioisotopes were obtained from the Radiochemical Centre (Amersham, Bucks, U.K.). Concanavalin A and phytohaemagglutin were from Sigma Chemical Co. (St. Louis, U.S.A.) Ionophore A23187 was from Calbiochem-Behringer Corp. (La Jolla, U.S.A.). All the other reagents used were of the highest grade available.

Results

Effect of concanavalin AS on $^{45}Ca^{2+}$ uptake by rat spleen cells

After isolation the cells were preincubated for 1 h at 35°C in order to achieve real steady-state conditions (cf. Ref. 14). ^3H_2O was included in all incubations as a relative measure of cell number [8] in the pellets, since the cells often form sediments of small aggregates and under some conditions there was a reduction in the number of cells during the incubation due to attachment to the walls of the flasks. In control experiments there was a good correlation between the protein content and amount of ^3H_2O in the pellets (not shown). After

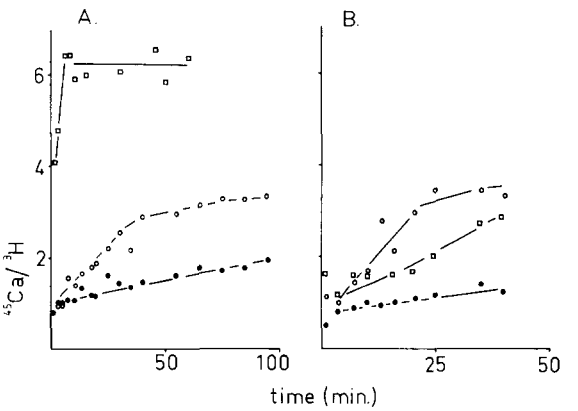

Fig. 1. Effect of concanavalin A on $^{45}Ca^{2+}$ uptake by rat spleen cells. 10^7 cells/ml were preincubated for 1 h in the basal medium containing in addition 2 μCi/ml ^3H_2O, 0.8 mM $CaCl_2$ and 5 mM glucose. (A) At time = 0, additions were made of 1 μCi/ml $^{45}Ca^{2+}$ (●), a mixture of $^{45}Ca^{2+}$ and 25 μg/ml concanavalin A (□) or concanavalin A pretreated with 10 mM $CaCl_2$ together with $^{45}Ca^{2+}$ (○) giving a final Ca^{2+} concentration of 1 mM in the incubation under all conditions. (B) At time = 0, addition of $^{45}Ca^{2+}$ (●) or 25 μg/ml concanavalin A pretreated with 10 mM $CaCl_2$ and $^{45}Ca^{2+}$ added after 5 min (□) or 30 min (○). Incubation otherwise as in A. Results are expressed as nmol Ca^{2+}/μl pellet H_2O.

the preincubation period $^{45}Ca^{2+}$ was added with or without concanavalin A (Fig. 1). Under the control conditions there is an apparent fast initial phase followed by a slower phase of $^{45}Ca^{2+}$ influx. If $^{45}Ca^{2+}$ is allowed to come into contact with concanavalin A prior to addition of the lectin to the lymphocyte suspension, there is a large apparent increase in Ca^{2+} uptake upon association of the lectin with the cells (Fig. 1A). Since concanavalin A binds Ca^{2+} with an apparent K_d of the order of 0.3 mM [15], the lectin was preincubated with 10 mM Ca^{2+} prior to its addition together with $^{45}Ca^{2+}$ to the cells. In this case concanavalin A induces a slow increase in $^{45}Ca^{2+}$ influx into the cells. The largest difference in $^{45}Ca^{2+}$ content as compared to untreated cells was obtained after 40–60 min of incubation. Addition of phytohaemagglutin gives virtually identical results under the above conditions (not shown).

An increase in influx is still observed if $^{45}Ca^{2+}$ is added 5 min after concanvalin A and the rate of influx is even enhanced after 30 min incubation in the presence of the lectin (Fig. 1B). Note that under all the above conditions, there is a change in the total Ca^{2+} activity of the incubation from 0.8 to 1 mM (Fig. 1). Under control conditions this does not, however, affect the distribution of $^{45}Ca^{2+}$.

In order to find out whether the $^{45}Ca^{2+}$ association with the cells under the above conditions represents a real influx into the cells, EGTA, to chelate Ca^{2+}, in combination with the bivalent cation ionophore A23187 [16], was added after the influx was completed. Fig. 1A demonstrates that there is a release of the $^{45}Ca^{2+}$, only under conditions where concanavalin A has been preincubated with 10 mM Ca^{2+}, to the same extent as the control, while most of the $^{45}Ca^{2+}$ remains associated with the cells under conditions where $^{45}Ca^{2+}$ has been allowed to come into previous contact with the lectin. This suggests that the apparent influx of $^{45}Ca^{2+}$ is due to binding of the isoptope to concanavalin A. To examine further this point, α-methylglucoside was added in order to remove the bound lectin from the cells [11]). In this case (Fig. 2B), the $^{45}Ca^{2+}$ is lost from the cells only under conditions where concanavalin A has had previous contact with the isotope. If α-methylglucoside was added prior to concanavalin A, no

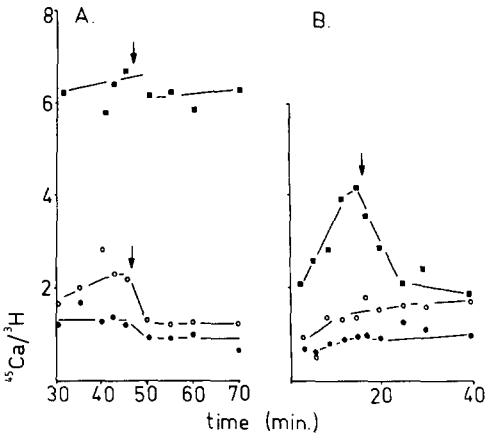

Fig. 2. Effect of ionophore A23187 in combination with EGTA and of α-methylglucoside on $^{45}Ca^{2+}$ association with rat spleen cells. Conditions as in Fig. 1. (A) Addition of 1 μCi/ml $^{45}Ca^{2+}$ (●), a mixture of $^{45}Ca^{2+}$ and 25 μg/ml concanavalin A (■) or concanavalin A pretreated with 10 mM $CaCl_2$ together with $^{45}Ca^{2+}$ (○) at time = 0. At the time denoted by the arrow, 5 μM A23187 was added together with 2 mM EGTA (●, ○, ■). (B) Conditions as in A (●, ■) except that $^{45}Ca^{2+}$ was added 30 s after the concanavalin A/10 mM Ca^{2+} mixture (○). At the time denoted by the arrow, 0.2 M α-methylglucoside was added (●, ○, ■).

effect of the lectin on $^{45}Ca^{2+}$ uptake was observed under any of the above conditions (not shown).

When cells are preincubated in the presence of Ca^{2+} together with $^{45}Ca^{2+}$ for 1 h prior to concanavalin A addition, in order to reach a steady state, the lectin causes a very modest increase in $^{45}Ca^{2+}$ uptake (Fig. 3). Although the change is small it has been reproduced several times.

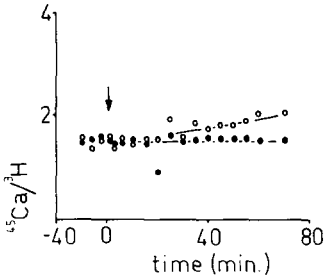

Fig. 3. Effect of concanavalin A on the steady-state Ca^{2+} content of rat spleen cells. Conditions were as in Fig. 1 except that 1 μCi/ml $^{45}Ca^{2+}$ was present throughout the preincubation period (●, ○). Addition of 25 μg/ml concanavalin A as indicated by arrow (○).

Correlation between Ca^{2+} influx and concanavalin A binding to cells

An increase in Ca^{2+} influx is observed above 3 μg/ml concanavalin A (Fig. 4). With different concanavalin A concentrations the increase in $^{45}Ca^{2+}$ influx correlates well with the concanavalin A binding to the cells as measured using [^{3}H]acetylconcanavalin A, except at very low concentrations of the lectin. The apparent K_d for concanavalin A binding to cells (about 10 μg/ml) found in this study corresponds fairly well with previous reports on binding of the lectin to lymphocytes [17].

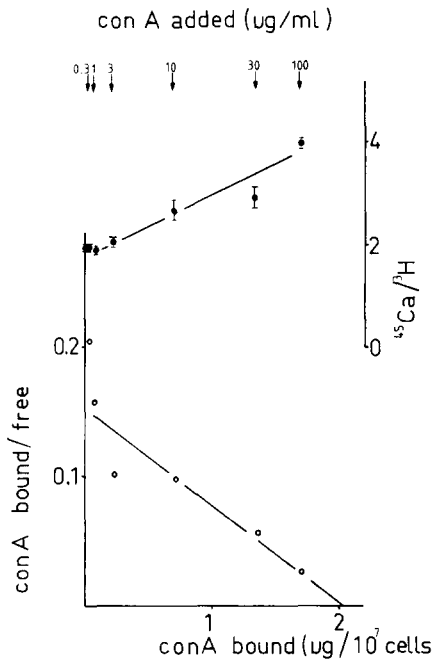

Fig. 4. $^{45}Ca^{2+}$ influx and concanavalin A binding at increasing concentrations of concanavalin A (con A). Incubation conditions were as in Fig. 1. After the preincubation period, $^{45}Ca^{2+}$ was added with or without varying concentrations of concanavalin A (indicated at the top of the figure) pretreated with 10 mM $CaCl_2$ as in Fig. 1A and the incubation was continued for 30 min before samples were taken. Each point is the average of four separate determinations ± S.D. In a separate experiment, [^{3}H]acetyl concanavalin A was added with varying concentrations of concanavalin A (indicated at the top of the figure) under conditions otherwise similar to those above and the incubation was continued for 30 min before samples were taken and spun through oil. Results were calculated assuming a similar binding constant of [^{3}H]acetylconcanavalin A and concanavalin A. Abscissa: concanavalin A bound (μg/10^7 cells) per concanavalin A free (μg/ml). Ordinate: concanavalin A bound (μg/10^7 cells). Each point is the average of three determinations.

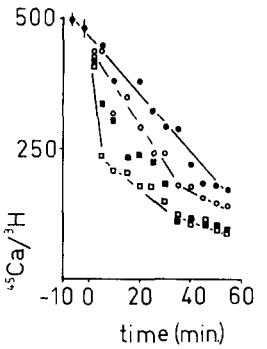

Fig. 5. Effect of concanavalin A on $^{45}Ca^{2+}$ efflux from rat spleen cells. The cells were preincubated in the presence of 20 μCi/ml $^{45}Ca^{2+}$ in the basal medium (endogenous Ca^{2+} content 8 μM) for 30 min at room temperature. Thereafter, they weer washed twice by centrifugation at 500 × g for 5 min and the top of the pellet washed twice before suspending the cells into the basal medium containing 2 μCi/ml ^3H_2O, 1 mM $CaCl_2$ and 5 mM glucose at 35°C (●) at time = −10 min. At time = 0, additions were made of 1 μg/ml (○), 3 μg/ml (■) or 30 μg/ml (□) concanavalin A. Samples were taken and spun without EGTA pretreatment.

Effect of concanavalin A on $^{45}Ca^{2+}$ efflux

Incubation of lymphocytes at room temperature results in net Ca^{2+} uptake into cells [18]. If cells are preincubated at room temperature in the presence of $^{45}Ca^{2+}$, repeatedly washed and suspended at 35°C in a medium containing Ca^{2+}, a linear $^{45}Ca^{2+}$ efflux occurs (Fig. 5). If concanavalin A is added a considerable increase in the rate of efflux is observed even at very low concanavalin A concentrations (1 μg/ml).

Discussion

The results of the present study demonstrate that $^{45}Ca^{2+}$ binding to concanavalin A and phytohaemagglutin may easily cause an apparent artefactual increase in $^{45}Ca^{2+}$ influx into cells. The strong binding of Ca^{2+} to concanavalin A may, however, be used as a simple qualitative assay for the binding of the lectins to cells. In Ca^{2+} flux studies with cells it is of importance to ensure that the active concanavalin A-Ca^{2+} complex [10] has

been formed before addition of the lectin to cells. This may well partially explain differences observed in various laboratories concerning lectin-induced Ca^{2+} fluxes [7–9,11].

Borle [14] has stressed the importance of using steady-state conditions in Ca^{2+} flux studies on intact cells. Under such conditions, we observe an increase in $^{45}Ca^{2+}$ influx into rat spleen cells upon addition of concanavalin A or phytohaemagglutin while the net Ca^{2+} gain is modest. The lack of Ca^{2+} uptake is probably due to a simultaneous increase in Ca^{2+} efflux as a response to an increase in the cytosolic Ca^{2+} concentration. Tsien et al. [6] have observed a doubling of the cytosolic Ca^{2+} activity of lymphocytes upon addition of concanavalin A or phytohaemagglutin as measured by using a permeable fluorescent Ca^{2+} indicator. In other systems, a doubling of net Ca^{2+} uptake causes a change of one to two orders of magnitude in the cytosolic Ca^{2+} activity [13]. Thus, very little net gain of Ca^{2+} in lymphocytes is expected.

Our results show a good correlation between concanavalin A binding to cells and $^{45}Ca^{2+}$ influx, although at very low (optimal mitogenic) concentrations no increase in influx while enhanced Ca^{2+} efflux is observed. It has been demonstrated previously that about 10% concanavalin A receptor occupancy is required for mitogenesis [17]. Thus, our results are in agreement with those of Hesketh et al. [9], who observed an increase in Ca^{2+} influx, which correlated with loss of DNA synthesis (48 h after lectin addition) at high concanavalin A concentrations. However, even at high concanavalin A concentrations DNA synthesis is normally activated (or even enhanced) if concanavalin A is removed by addition of α-methylmannoside after 5 h incubation [19]. Thus, Ca^{2+} may well be the initial trigger in mitogenic T-lymphocyte activation. A prolonged large increase in the cytosolic Ca^{2+} induced by mitogens would cause a strain on intracellular Ca^{2+} buffers such as mitochondria [20] and other active Ca^{2+}-transport systems like those in the plasma membrane [18]. At low concentrations of the mitogens, Ca^{2+} influx may be compensated for by an increase in efflux leading only to a small increase in the cytosolic Ca^{2+} activity throughout the 24 h required for commitment to DNA synthesis [19]. Furthermore, the possibility that Ca^{2+} is released from internal storage sites as in the case of skeletal muscle and various other tissues [14,21,22] ought to be considered.

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References

1 Milner, S.M. (1979) Cell Biol. 3, 35–43
2 Whitney, R.B. and Sutherland, R.M. (1973) Cell. Immunol. 6, 137–147
3 Allwood, G., Asherson, G.L., Davey, M.J. and Godford, P.J. (1971) Immunology 21, 509–516
4 Luckasen, J.R., White, J.G. and Kersey, J.H. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4088–5090
5 Maino, V.G., Green, N.M. and Crumpton, M.J. (1974) Nature 251, 324–327
6 Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) Nature 295, 68–71
7 Hume, D.A., Vijaykumar, E.K., Schweinberger, F., Russel, L.M. and Weideman, M.J. (1978) Biochem. J. 174, 711–716
8 Freedman, M.H., Raff, M.C. and Gomperts, B. (1975) Nature 255, 378–382
9 Hesketh, T.R., Smith, G.A., Houslay, M.D., Warren, G.B. and Metcalfe, J.C. (1977) Nature 267, 490–494
10 Sherry, A.D., Newman, A.D. and Gutz, C.G. (1975) Biochemistry 14, 2191–2192
11 Larner, A., Rebhun, L.I., Larner, J. and Oron, Y. (1980) Mol. Cell. Biochem. 32, 123–130
12 Scott, I.D., Åkerman, K.E.O. and Nicholls, D.G. (1980) Biochem. J. 192, 873–880
13 Åkerman, K.E.O. and Nicholls, D.G. (1981) Eur. J. Biochem. 115, 67–73
14 Borle, A.B. (1981) Rev. Physiol. Biochem. Pharmacol. 90, 14–153
15 Reed, P.W. and Lardy, H.A. (1972) J. Biol. Chem. 247, 6970–6977
16 Kalb, A.J. and Levitzki, A. (1968) Biochem. J. 109, 669–673
17 Andersson, J., Sjöberg, O. and Möller, G. (1972) Transplant. Rev. 11, 131–177
18 Dani, A.M., Cittadini, A., Calviello, G., Festucci, G. and Terranova, T. (1978) Mol. Cell. Biochem. 22, 139–145
19 Stenzel, K.H., Rubin, A.L. and Novogrodsky, A. (1978) Exp. Cell Res. 115, 285–292
20 Åkerman, K.E.O. and Nicholls, D.G. (1982) Rev. Physiol. Biochem. Pharmacol., in the press
21 Carafoli, E. and Crompton, M. (1978) Cur.. Top. Membranes Transp. 10, 151–216
22 Endo, M. (1977) Physiol Rev. 57, 71–108