# Early changes of 'leak flux' and the cation content of lymphocytes by concanavalin A[1]

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Summary:

The leak fluxes of $Na^+$, $K^+$, $Mg^{++}$ and $Ca^{++}$ in mouse thymocytes are increased by Concavaline A (Con A), within minutes after mitogen addition. The intracellular $Mg^{++}$ and $K^+$ concentrations were decreased and the $Na^+$ and $Ca^{++}$ contents were increased by Con A in mouse thymocytes and spleen cells.

## Introduction:

Changes in the active $Na^+$ and $K^+$ transport (1-3) and $Ca^{++}$ uptake (4,5) are events in lymphocyte transformation, measurable within the first minute after mitogen addition (3,5). It is not yet clear whether or not these permeability changes precede, and eventually may trigger, biochemical events leading to blast cell transformation. Change in the uptake of $^{42}K$ or $^{45}Ca$ does not necessarily reflect a change in total cell $K^+$ or $Ca^{++}$ content, since the $K^+$ leak influx is increased also (7). It is therefore of special interest to measure quantitatively the intracellular content of the cations shortly after mitogen addition to the lymphocytes, especially because a decrease in $K^+$ of human lymphocytes after mitogen addition has been reported earlier (6).

Since the determination of intracellular cation concentration by atomic absorption spectrophotometry has a variation coefficient of about 10 %, it is impossible to monitor the early changes. A method therefore was developed to measure the intracellular cations during the steady state of the tracer with the Phthalate-oil method (7).

[1] Throughout, please read concanavalin A for Concavaline A.

## Material and Methods:

### Preparation of Lymphocytes

Thymocytes and spleen cells were obtained from $CF_1$ mice. The thymocytes were washed in Eagle's Medium at 4° C (800 x g, 10 min) followed by further centrifugation on a Ficoll-Ronpacon gradient. (9:55 g Ficoll, 20 ml Ronpacon, 130 ml $H_2O$) at 1000 x g for 45 min). Spleen lymphocytes were separated from erythrocytes and granulocytes on a continuous gradient of 40 % Ficoll in Eagle's Medium and the Ficoll-Ronpacon mixture by centrifugation for 1 h at 1000 x g. The preparations contained more than 95 % lymphocytes. Viability was tested by incubation of 3 drops of lymphocyte suspension with one drop of trypan blue (10 g/l in 0,15 mM NaCl) at 37° C for 15 min. Cell counts were done using a haemocytometer.

### Efflux studies:

Cells were preloaded with $^{42}K$ or with $^{28}Mg$ by adding the labelled ion to Eagle's Medium plus 10 % foetal calf serum to give 12 µCi/ml $^{42}K$ or 16 µCi/ml $^{28}Mg$. After incubation for 6 hours, during which full equilibrium between extra- and intracellular $K^+$ and $Mg^{++}$ took place, the lymphocytes were washed 3 times with ice cold Eagle's Medium and than further incubated in the same medium at 37° C as a suspension of 1 x $10^7$ lymphocytes/ml. To measure the effluxes, 1 ml of cell suspension was sampled at various time intervals and processed as described earlier for the $K^+$ influx experiments (7).

### Influx studies:

Unidirectional $Na^+$ and $Ca^{++}$ influxes were measured as described earlier for $K^+$ influx (7).

### Determination of cell ion contents with radiolabelled cations:

Preloading with $^{42}K$ and $^{28}Mg$ was the same as described for the efflux experiments. For $Ca^{++}$ measurements it was necessary to preincubate the cells for 30 h in Eagle' Medium plus 10 % foetal calf serum with a $Ca^{++}$-concentration of 1 mM and 5 µCi/ml $^{45}Ca$. For $Na^+$ determination preincubation was for 4 h, the $Na^+$-concentration 80 mM (iso-osmolarity achieved with choline choloride).

After the incubation, Con A was added to one half of the cell suspension. At various times samples were removed from both tubes in quadruplicates and injected into test tubes with the n-butyl-phthalate oil phase (7). $^{42}K$, $^{28}Mg$ and $^{22}Na$ were counted in a Berthold Autogamma Counter. For $^{45}Ca$ counting and protein determination (9) the pellets were extracted with either to remove residual phthalate, the cells solubilized with 0,1 N NaOH and 0,1 ml was transfered into counting vials containing Bray's scintillation fluid and counted in a beta-counter. The variationcoefficient of this method was always below 3 %.

Results:

Effect of Con A on leak fluxes of $Na^+$, $K^+$, $Mg^{++}$ and $Ca^{++}$

The intracellular cation concentrations in mouse thymocytes suspended in Eagle's Medium were $K^+$ = 103.6, $Na^+$ = 31.9, $Mg^{++}$ = 10.3, $Ca^{++}$ = 0.9 mM/L (Variation $\pm$ 5 %). It is general accepted that $K^+$ and $Mg^{++}$ leak from inside to outside of the cells, whereas $Ca^{++}$ and $Na^+$ leak from the outside to the inside (10-13). Mouse thymocytes lose their intracellular $K^+$ and $Mg^{++}$ very rapidly into the medium (Fig. 1). Con A greatly increases the amount of the lost cation. As soon as 5 minutes after addition of the mitogen, differences to the control cells were measured (Fig. 1). Thus, Con A has a strong effect on the passive permeability of $Mg^{++}$ and $K^+$.

In contrast to $K^+$ and $Mg^{++}$, the intracellular concentration of $Na^+$ and $K^+$ is lower than in the extracellular fluid. $Na^+$ and $K^+$ leak inwards and are actively transported out of the cells. Fig. 2 shows that the $Na^+$ influx is increased very rapidly after Con A addition. Nearly twice as much $Na^{22}$ is found intracellularly in the mitogen-treated cells compared to the controls. Using $^{45}Ca$ we found an increased uptake of $Ca^{++}$ into the thymocytes as already described by others (4,5,14).

In earlier studies it was found that mitogens activate the active $K^+$ influx and $Na^+$ outflux (7). For $Mg^{++}$ and $Ca^{++}$ a similar effect was seen. (Data not shown here). Since the active and the passive cation fluxes are increased, the question is, whether or not: firstly the enhanced loss is compensated by an intracellular uptake (intracellular concentration remaining constant); secondly an over-

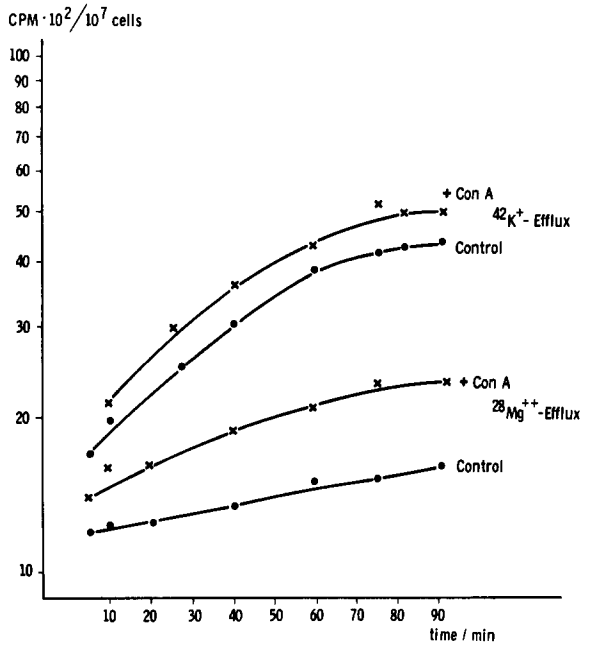


Figure 1 $^{42}K^{+}$ and $^{28}Mg^{++}$ Efflux

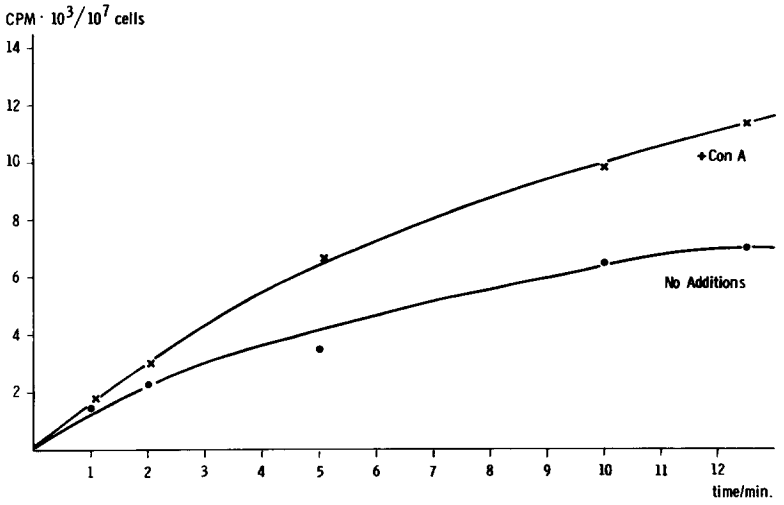


Figure 2 $^{22}Na$ influx in mouse thymocytes

shooting active transport is caused by the mitogen ($K^{+}$ and $Mg^{++}$ increased, $Ca^{++}$ and $Na^{+}$ decreased); and thirdly the leak fluxes exceed the pump fluxes ($K^{+}$ and $Mg^{++}$ decreased, $Ca^{++}$ and $Na^{+}$ increased). To decide between these possibilities the ion contents with radiolabelled cations were determined. As soon as 5 minutes after Con A

Table 1

$^{42}K$ and $^{28}Mg$ content of thymocytes and spleen cells after Con A addition

| Time min | Con A 15/ug/ml | Thymocytes $^{42}K$ CPM/ug Protein | Thymocytes $^{28}Mg$ CPM/ug Protein | Spleen Cells $^{42}K$ CPM/ug Protein | Spleen Cells $^{28}Mg$ CPM/ug Protein |
|---|---|---|---|---|---|
| 5 | - | 47,5 ± 1,3 | 28,2 ± 1,09 | 36,9 ± 0,98 | 24,7 ± 0,87 |
| | + | 45,6 ± 1,1 | 25,1 ± 1,11 | 35,2 ± 0,97 | 23,1 ± 0,91 |
| 10 | - | 47,8 ± 1,3 | 27,9 ± 1,13 | 37,0 ± 0,94 | 24,9 ± 0,88 |
| | + | 45,0 ± 1,2 | 24,2 ± 1,15 | 33,2 ± 0,95 | 22,6 ± 0,84 |
| 30 | - | 47,9 ± 1,3 | 27,6 ± 1,14 | 38,1 ± 0,97 | 24,0 ± 0,89 |
| | + | 40,8 ± 1,1 | 23,4 ± 1,15 | 31,9 ± 0,92 | 21,3 ± 0,76 |
| 60 | - | 49,6 ± 1,4 | 27,1 ± 1,13 | 37,6 ± 0,98 | 23,9 ± 0,87 |
| | + | 40,2 ± 1,2 | 22,5 ± 1,05 | 30,4 ± 0,91 | 20,0 ± 0,79 |
| 120 | - | 50,1 ± 1,4 | 26,6 ± 1,0 | 38,2 ± 1,0 | 23,0 ± 0,79 |
| | + | 39,5 ± 1,2 | 20,1 ± 0,92 | 30,1 ± 0,92 | 18,9 ± 0,81 |

**Table 2**

**$Na^{22}$-content of thymocytes and spleen cells after Con A addition**

| Time min | Con A (15 µg/ml) | Ouabain ($5 \times 10^{-3}$ M) | Thymocytes CPM/µg Protein | Spleen Cells CPM/µg Protein |
|---|---|---|---|---|
| 5 | - | - | 17,2 ± 0,21 | 12,9 ± 0,16 |
| | + | - | 17,9 ± 0,22 | 13,2 ± 0,16 |
| | - | + | 28,2 ± 0,25 | 22,4 ± 0,19 |
| 10 | - | - | 16,9 ± 0,20 | 11,7 ± 0,14 |
| | + | - | 18,0 ± 0,21 | 12,6 ± 0,17 |
| | - | + | 34,9 ± 0,38 | 27,5 ± 0,25 |
| 30 | - | - | 15,8 ± 0,20 | 11,2 ± 0,15 |
| | + | - | 18,6 ± 0,21 | 12,9 ± 0,19 |
| | - | + | 40,2 ± 0,49 | 34,1 ± 0,39 |
| 60 | - | - | 15,7 ± 0,20 | 11,3 ± 0,17 |
| | + | - | 19,0 ± 0,22 | 12,0 ± 0,18 |
| | - | + | 43,6 ± 0,53 | 37,1 ± 0,51 |
| 90 | - | - | 16,3 ± 0,20 | 11,5 ± 0,15 |
| | + | - | 19,3 ± 0,22 | 13,8 ± 0,18 |
| | - | + | 44,2 ± 0,55 | 37,4 ± 0,52 |
| 120 | - | - | 16,8 ± 0,21 | 11,9 ± 0,17 |
| | + | - | 22,1 ± 0,25 | 14,1 ± 0,19 |
| | - | + | 44,4 ± 0,57 | 38,1 ± 0,57 |

addition the decrease in the intracellular $K^+$ content was seen (Tabl. 1). Spleen cells (containing B and T cells) and thymocytes (almost pure T cell population) showed the same changes. Within two hours the decrease in $K^+$ was about 20 %. Thus, after Con A treatment the intracellular $K^+$ fell from 103.6 to 80 mM whereas in the control cells $K^+$ concentration remained constant (Tabl. 1).

Basically the same changes were observed in the $Mg^{++}$ content of the cells (Tabl. 1). Con A caused a rapid loss of $Mg^{++}$ in spleen cells and in thymocytes. Within two hours the $Mg^{++}$ content of the cells was 15 % lower compared with the control.

The intracellular $Na^+$ content on the other hand was increased by Con A (Tabl. 2). The effect was seen 10 minutes after mitogen

Table 3

$Ca^{45}$-content of thymocytes and spleen cells after Con A addition

| Time min | Con A 15/ug/ml | Thymocytes CPM/ug Protein | Spleen cells CPM/ug Protein |
|---|---|---|---|
| 5 | - | 1,07 ± 0,02 | 0,85 ± 0,01 |
| | + | 1,28 ± 0,02 | 0,99 ± 0,01 |
| 30 | - | 1,12 ± 0,02 | 0,83 ± 0,01 |
| | + | 1,38 ± 0,02 | 1,11 ± 0,01 |
| 60 | - | 1,11 ± 0,02 | 0,84 ± 0,01 |
| | + | 1,41 ± 0,o2 | 1,13 ± 0,01 |
| 120 | - | 1,09 ± 0,02 | 0,85 ± 0,01 |
| | + | 1,41 ± 0,02 | 1,15 ± 0,01 |

addition. There were no significant differences between thymocytes and spleen cells. The $Na^+$-content increased about 20 %.

The $Ca^{++}$-content of the cells increased after mitogen addition. (Tabl. 3) in both thymocytes and spleen cells. After two hours the increase was similar to that of $Na^+$ content and was about 20 %.

Discussion:

The data indicate that Con A changes the intracellular content of the four tested cations in both thymocytes and spleen cells. Thus, the concentration gradient inside-to-outside is decreased. These changes are explained by the increase of leakiness of the cells. In earlier studies a decreased $K^+$ content and an almost unchanged $Na^+$ content in human lymphocytes after PHA was found (6). The decreased $K^+$ of 30 % after 12 hours incubation is in good agreement with the data shown here and makes it very likely that the mouse lymphocyte exhibit the same alteration of the intracellular $K^+$ as human lymphocytes. The flame photometry was too insensitive to monitor the $Na^+$ changes. In our hands it was impossible to discriminate between an $Na^+$ content of 32 mM in the control and 38 mM in the Con A treated cells by this method. Con A did not

induce a replacement of lost cell $K^+$ by $Na^+$ or $Mg^{++}$ by $Ca^{++}$. The total cation content of the cell decreased and this should induce a shrinkage of the cells. It is at this time difficult to predict whether or not the observed changes may trigger other biochemical events leading to blast cell transformation but it seems unlikely that $K^+$ plays an important role as it is apparently in other systems (15,16). The changes in intracellular $K^+$ and $Na^+$ however may be the driving force for the enhanced active $K^+$ and $Na^+$ pump flux (1-3,7,8), the increased Ouabain binding (8,17) and the $Na^+$-$K^+$ as well as the $Mg^{++}$ and $Ca^+$-ATPase activity (7,8,18-20).

It is an unexpected finding that the same changes were observed in the thymocytes and spleen cells (containing B cells) because the T-cells are much more stimulated by Con A than the spleen cells (measured by $H^3$-TdR-incorporation). Furthermore preliminary results with PHA, LPS and Pokeweed mitogen show a similiar effect to Con A and the same effect on thymocytes and spleen cells. Since it was however shown that the binding of the mitogen to T- or B-cells took place in the same quantity (19) this binding already alters the cell membrane and causes the described changes. It is known that the binding of Con A to the B-cells induces other early biochemical events seen in lymphocytes transformation, and that after binding of Con A to Ehrlich ascites tumor cells a rapid net $Na^+$ entry and $K^+$ loss was observed (22).

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