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Decreased cytosolic free calcium concentration of aged human lymphocytes in resting state

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Abstract – Cytosolic free calcium concentration was measured in lymphocytes from individuals over the age of 80, using quin2 and fura-2 calcium indicators. The average intracellular free calcium concentration of the samples was 62 nM, which value is roughly half the adult (age between 35 and 55) level (116 nM). It is supposed that the decline in immune function of aged individuals is connected to the decrease in free calcium concentration in their lymphocytes. We also discuss the consequences and the adaptive character of this decrease.

Key words: cytosolic free calcium; human lymphocytes; immunosenescence; calcium indicators; aging mechanism; cellular aging.

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Immunosenescence is one of the most thoroughly studied and most firmly established processes in gerontology. The decline of immune function in ageing is an important factor of the pathogenesis of various disorders characteristic of old age (1). The decrease in the proliferation ability of lymphocytes plays a major role in this process. The possible reasons of the decrease of lymphocyte proliferation can be summarised in the diminished protein synthesis, enzyme induction and availability of various co-factors of enzyme reactions (2). However, the attenuation or defects in signal transduction of lymphocytes can also contribute to the decrease of proliferation.

The intracellular calcium level is a well known regulator of various responses and the overall metabolism of living cells. Though the physiological role of calcium was discovered almost 100 years ago, the importance of intracellular calcium concentration was first emphasized by Hodkin & Keynes in 1957 (3). To our present knowledge calcium ions influence the biochemical changes of living cells in two ways. Firstly, as a "second messenger" calcium transfers the extracellular stimuli to the intracellular effector mechanisms. Calcium ions are an important part of this action as calcium ions serve as co-factors of Ca^{2+}-calmodulin-dependent protein kinase and protein kinase C. Secondly, Ca^{2+} is a charge-carrier. The calcium fluxes through the Ca^{2+}-channels of the plasma membrane alter the membrane potential and thus regulate the potential-dependent intracellular biochemical machinery (4–6).

The cytosolic free calcium concentration of aged human lymphocytes has hitherto been unknown (6). However, such evidence as a reduction in T lymphocyte cell reactivity (7, 8), decrease in transmembrane Ca^{2+} flux (9) and decline in production of interleukin-2 and its receptors (10, 11) suggests that pronounced changes should occur in the intracellular calcium concentration during aging.

In the present report we examine the intracellular calcium concentration of aged human lymphocytes (from individuals over the age of 80) using the new fluorescent techniques developed by Tsien (12, 13).

Material and methods

Material

Fura-2*, fura-2/AM, quin2/AM, A23187 and TPEN were from Calbiochem; chelex 100, DTPA, Hepes and trypan blue were obtained from Sigma Chemicals; digitonin was a Fisher Scientific product. EGTA and Triton X-100 were from Serva; DMSO was a Fluka product. $CaCl_2$ and $MgCl_2$

* *Abbreviations:* BSS: balanced salt solution; $[Ca^{2+}]_i$: free cytoplasmic calcium concentration; chelex 100: divalent cation chelating resin; DMSO: dimethyl sulfoxide; DTPA: diethylene-triamine-pentaacetic acid; EBSS: Earle's balanced salt solution; EGTA: ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; fura-2 and fura-2/AM: 1-(2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy)-2(2′-amino-5′-methylphenoxy)-ethane-N,N,N′,N′-tetraacetic acid and its pentaacetoxymethyl ester, respectively; Hepes: 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid; PBS: phosphate buffered saline; quin2 and quin2/AM: 2-((2-bis(carboxymethyl)amino-5-methylphenoxy)-methyl)-6-methoxy-8-(bis(carboxymethyl)amino)quinoline and its tetra-acetoxymethyl ester, respectively; TPEN: N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylene-diamine; Tris: tris(hydroxymethyl)-aminomethane.

were high purity products of Merck and BDH, respectively. [^{3}H]-uridine (742 MBq/mmol) was purchased from the Institute for Research, Production and Application of Radioisotopes (Prague, Czechoslovakia). All other reagents used were from Reanal, Hungary and of analytical purity.

Media for cells

Cells were stored in 199 EBSS medium or Eagle's basal medium. Prior loading of fluorescent indicator medium was changed to modified Hank's BSS medium containing 143 mM NaCl, 1 mM Na_2SO_4, 5 mM KCl, 1 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose and 10 mM Hepes (pH 7.45) at 37°C (measuring medium, 14). Usually the measuring medium was treated with chelex 100 resin overnight at 4°C to remove accidental traces of heavy metals (15).

Blood samples and preparation of lymphocytes

Peripheral blood was taken from 30 volunteers over the age of 80. The average age was 95 years. The group examined was representative in all respects of this very old population of Hungary. They were immunologically uncompromised and had normal amounts of T and B lymphocytes. Only 25% took medication. The control, "adult" group consisted of 6 volunteers aged between 35 and 55 years. We did not increase the number of control experiments since our present results, showing the "adult" intracellular calcium concentration, are in good agreement with our earlier findings and literature data (16–19). Lymphocytes were separated using the method of Böyum (20). Peripheral T and B lymphocytes were not separated.

Loading of fura-2/AM and quin2/AM

For comparison, usually one portion of cells was loaded with fura-2/AM and another with quin2/AM. Cells were incubated with quin2/AM and fura-2/AM at final concentrations of 30 μM and 3 μM, respectively at a cell density of 5×10^7 cells/ml in Hepes-buffered modified Hank's BSS for 20 min at 37°C. In quin2/AM, after 20 min, the cell suspension was diluted 10-fold and the incubation continued for a further 40 min. After incubation, cells were centrifuged at $400 \times g$ for 10 min at room temperature, gently suspended in standard medium, setting the final cell density to 5×10^6 cells/ml, and kept at room temperature.

Fluorescence measurements

Immediately before measurement the cells were centrifuged and resuspended again in the same medium and final volume to remove the extracellular dye. The amount of extracellular indicator was routinely checked by the methods of Hesketh et al. (21) and Pollock et al. (22), adding $MnCl_2$ at a final concentration of 10 μM to a separated 2 ml aliquot of the cell suspension. Fluorescence was recorded with Perkin-Elmer MPF-448 and Jobin Yvon Spectrofluo JY3 spectrofluorimeters. The sample compartment was thermostatted at 37°C. In quin2 experiments excitation was 339 nm, emission 492 nm, using 4 and 10 nm slits; the settings for fura-2 were 340/380 and 510 nm, respectively.

Calibration of the fluorescence of calcium indicators and calculation of the intracellular calcium concentration were performed by the method of Tsien et al. (14) for quin2 and by Grynkiewicz et al. (13) and by Pollock et al. (22) for fura-2. Particular care was exercised to avoid the possible pitfalls of the measurement (especially the inappropriate correction for autofluorescence and heavy metal traces) as previously described in more detail (15).

Scanlon et al. (23) have reported a calcium-insensitive product of fura-2/AM hydrolysis. This compound may be the reason for the partial hydrolysis of the pentacetoxymethyl ester. Since it is reasonable that the overall activity of intracellular esterases decreases during aging this phenomenon may disturb our measurements. To check the validity of our data we plotted the $[Ca^{2+}]_i$ values of our measurements as the function of intracellular fura-2 concentration (Fig. 1). The lack of any correlation between the two values shows that this calcium-insensitive form of fura-2 was not present in our samples in a significant concentration. (The increase of the intracellular concentration of fura-2 should cause an increase in the amount of this insensitive form and hence an artificial increase in $[Ca^{2+}]_i$.)

Measurement of [^{3}H]-uridine uptake

Lymphocytes were incubated for 2 h at a cell density of 1×10^7 cells/ml at 37°C with A23187 at final concentrations indicated. Control samples were incubated with the solvent DMSO at a final concentration of 0.1% (v/v). After 2 h 2 μCi (74 MBq) [^{3}H]-uridine was added to the 1 ml samples and the samples incubated for an additional 30 min. The samples were then cooled to 0°C and 1 ml 1 M perchloric acid was added to each. After 15 min incubation samples were centrifuged and washed three times with 2 ml 0.5 M perchloric acid. Finally, the samples were incubated with 0.5 ml of 0.5 M perchloric acid at 90°C for 30 min and their radioactivity determined from 2×0.2 ml aliquots in a scintillation cocktail containing 5.9 g/l diphenyloxazole, 160 mg/l 1,4-di(2-5-phenyl)-oxazoyl)

benzene, 30% (v/v) Triton X-100 and 70% (v/v) toluene. All data points were measured in triplicate.

Results

The intracellular calcium concentration of adult human peripheral blood lymphocytes has been measured by many laboratories (16–19). The average of their $[Ca^{2+}]_i$ values is 122 nM. This value is in good agreement with our results, which show a $[Ca^{2+}]_i$ of 116 ± 6 nM (Table 1).

The results in Table 1 indicate a dramatic decrease in the intracellular free calcium concentration of lymphocytes from individuals over the age of 80. The average $[Ca^{2+}]_i$ is 62 ± 3 nM, which is roughly half the adult level. The difference between the $[Ca^{2+}]_i$ in the two groups has a level of significance $(p) < 0.001$ (Table 1A). The SEM is $< 5\%$. This value is remarkably low if we compare the usual SD in the data of samples from the elderly. Our results are in agreement with those of Peterson et al. (24) who reported a similar $[Ca^{2+}]_i$ decline in aged fibroblasts.

Since there are no available data in the literature on the intracellular calcium concentration of aged lymphocytes we increased the validity of our measurements by parallel determination of $[Ca^{2+}]_i$ with quin2 and fura-2. Quin2 was used to compare with previous data on adult $[Ca^{2+}]_i$ levels in the literature. However, fura-2 has a number of advantages over quin2, e.g. it gives up to 30-fold brighter fluorescence and can therefore be used at a much lower final concentration, thus allowing the examination of many more physiological conditions. Fura-2 also has greater selectivity for Ca^{2+} than other divalent cations and has a much lower sensitivity to the more abundant intracellular heavy metal ion, Zn^{2+}, than quin2 (13, and P. Csermely, unpublished data).

Table 1B shows that no significant difference can be observed when comparing the $[Ca^{2+}]_i$ values measured by quin2 or fura-2. Since the two chelators have differing sensitivity to intracellular heavy metals, the lack of any differences in $[Ca^{2+}]_i$ measured by them suggests that our $[Ca^{2+}]_i$ data were not disturbed by changes in the intracellular concentration of heavy metals during aging. This is in agreement with the data in Fig. 1: if heavy metals had any influence, the $[Ca^{2+}]_i$ should decline at low intracellular concentrations of fura-2 (15, 21). The fact that the addition of the intracellular heavy metal chelator, TPEN, did not change quin2 or fura-2 fluorescence (data not shown) also suggests that heavy metals did not significantly disturb our $[Ca^{2+}]_i$ values.

The data in Table 1C. show no significant changes in $[Ca^{2+}]_i$ values in male and female volunteers from the group over 80 years.

The decrease in the free Ca^{2+} concentration of cytosol has several consequences. It is known, for example, that the activation of T lymphocytes causes changes in the potential of the plasma mem-

Table 1. Cytosolic free calcium concentration in lymphocytes[a]

	No. samples	Mean (nM)	± SD	± SEM
A. Comparision of adult (between 35 and 55 years) and old (> 80 years) data				
Adult	6[b]	116	15	6
Old	30	62	13	3
		$p < 0.001$		
B. Comparision of fura-2 and quin2 measurements of individuals > 80 years				
Fura-2	24[c]	61	14	3
Quin2	6[c]	65	16	6
C. Comparision of female and male data > 80 years				
Female	16	61	15	4
Male	14	63	12	3

[a] Isolation of peripheral blood lymphocytes and measurement of their intracellular calcium concentration was performed as described in Material and methods. The level of significance (p) was determined using Student's t test.

[b] Number of "adult" samples was not increased in these experiments since the average intracellular calcium concentration correlates well with our earlier data and the values given in the literature.

[c] Number of experiments with fura-2 was greater since this indicator has much better properties (see text) than quin2. A few experiments with quin2 were also included for comparision with the majority of data in the literature.

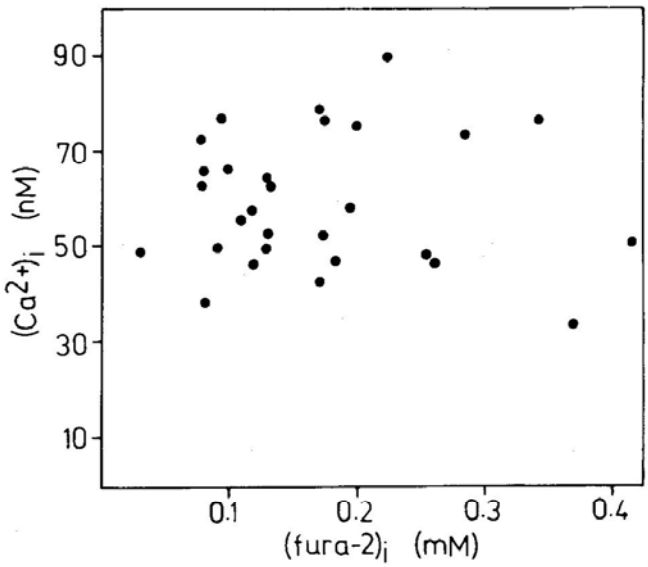

Fig. 1. Correlation of the intracellular concentration of Ca^{2+} and fura-2. Data points are from individual experiments of the experimental series in Table 1. Determination of intracellular calcium concentration ($[Ca^{2+}]_i$) was performed as described in Material and methods. The intracellular concentration of fura-2 ($[fura\text{-}2]_i$) was determined comparing the total fura-2 fluorescence to a calibration curve of free fura-2 fluorescence. For the calculation of $[fura\text{-}2]_i$ the intracellular volume of peripheral blood lymphocytes was taken as 240 fl (44).

brane (25, 26). The concentration gradient of calcium ions across the lymphocyte plasma membrane is 10^4, since the Ca^{2+} concentration in serum is in the range of 1.05–1.3 mM and the cytosolic free calcium concentration in the resting state in adults is roughly 120 nM. This enormous concentration difference generates a substantial diffusion force and membrane potential (Nernst potential) in lymphocytes.

Fig. 2 shows this Ca^{2+}-derived potential as the function of intracellular calcium concentration at 5° and 37°C. The Ca^{2+}-derived membrane potential (E_{Ca}) of older persons is higher (see "old" arrow in the abscissa) than the "adult" value. This increase in the Ca^{2+}-derived component should only be regarded as a tendency, as we did not take into account the possible changes in the relative permeability of Ca^{2+} (steady-state potential, Goldman-Hodgkin-Katz potential), the electrogenic Ca^{2+}-pumps, and other intracellular mechanisms of Ca_i regulation.

In older persons depolarization during late lymphocyte activation is made more difficult by the increase in Ca^{2+}-derived component of membrane potential. Thus, depolarization may also link the decrease of $[Ca^{2+}]_i$ in aged lymphocytes to the overall decrease in lymphocyte activation. However, these consequences of the decrease in $[Ca^{2+}]_i$ must be regarded with caution, since changes in intracellular concentrations of other main ions (K^+, Na^+, H^+, Mg^{2+}, Cl^-, etc.) may compensate for the increase of the Ca^{2+}-derived component in the overall membrane potential.

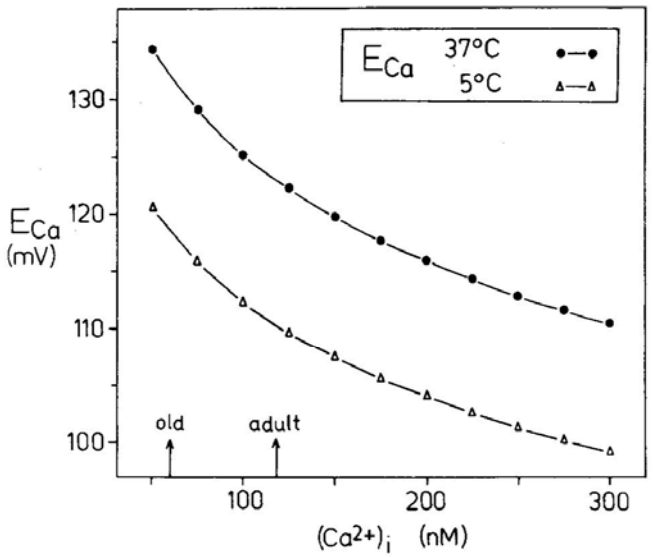

Fig. 2. Ca-derived component of the resting membrane potential (E_{Ca}) as a function of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) at 5°C and at 37°C. The Ca-derived component of the resting membrane potential was calculated using the Nernst equation

$$E_{Ca} = \frac{RT}{zF} \ln \frac{[Ca^{2+}]_0}{[Ca^{2+}]_i}$$

where R = gas constant, T = absolute temperature, z = 2 (charge of Ca^{2+}), F = Faraday constant, and $[Ca^{2+}]_0$ and $[Ca^{2+}]_i$ = the extra- and intracellular Ca^{2+} concentrations, respectively. $[Ca^{2+}]_0$ was taken to 1.2 mM in our calculations. The E_{Ca} values are only rough estimates since we did not take into account the cross effects between the membrane potential of different ions, the relative permeability of different ions, etc. (see text). Arrows ("old" and "adult") indicate the intracellular calcium concentrations characteristic of human peripheral blood lymphocytes from "old" and "adult" donors, respectively (see Table 1).

The conductivity of plasma membrane calcium channels is diminished with aging (9, 27). In panel A of Fig. 3 the logarithm of the ratio of extra- and intracellular calcium concentrations is plotted as the function of $[Ca^{2+}]_i$. The triangles represent the logarithm of Ca^{2+} concentrations if the extracellular calcium concentration is equal to the calcium concentration of human serum (1.2 mM). Since the logarithm of calcium concentrations is proportional to the diffusion force of Ca^{2+}, this diffusion force increases with aging (compare the logarithm values at vertical arrows "adult" and "old"). This increase in Ca^{2+}-derived diffusion force may compensate the decrease in the conductivity of Ca^{2+} channels and may thus be regarded as an adaptation of aging lymphocytes.

The free calcium concentration of serum is strictly regulated around 1.2 mM. Panel B of Fig. 3 shows that Ca_i of lymphocytes is greatly changed if the extracellular calcium concentration is changing. For example, if the concentration of extracellular Ca^{2+} increases above 1.5 mM $[Ca^{2+}]_i$ is elevated to the level observed after the addition of mitogens, and lymphocytes begin to proliferate (28, 29). The influence of the extracellular calcium concentration on Ca_i has also been established in other cell types, such as bovine parathyroid cells (30) and rat pituitary cells (31).

The circles of panel A of Fig. 3 represent the logarithm of the ratio of extra- and intracellular calcium concentrations where the extracellular calcium concentration is not constant, but corresponds to the given $[Ca^{2+}]_i$ value as shown in panel B. This curve contains a plateau where small changes in the logarithm of Ca^{2+} concentration (diffusion force) may induce large changes in $[Ca^{2+}]_i$. The extracellular Ca^{2+} concentration of this well-regulated region is around 1.2 mM (see horizontal arrow) which is the calcium concentration of human serum. The vertical arrows show that the $[Ca^{2+}]_i$ values of both the "adult" and "aged" group are *within* this well-regulated region.

How do the changes in $[Ca^{2+}]_i$ influence lymphocytes? Our previous experiments showed that in older persons, where the immune function is diminished, the $[Ca^{2+}]_i$ of peripheral lymphocytes is decreased. The experiments in Fig. 4 show that the

increase in $[Ca^{2+}]_i$ has a biphasic effect on lymphocytes. The activation of lymphocytes was monitored with the uptake of [^{3}H]-uridine after 2 h of stimulation which sensitively indicates the increase in RNA synthesis. The different $[Ca^{2+}]_i$ levels were set by increasing concentrations of the Ca-ionophore A23187. Lymphocytes are not activated if $[Ca^{2+}]_i$ is below a certain level ($\approx$200 nM). If $[Ca^{2+}]_i$ increases above approximately 2–10 μM the uptake of [^{3}H]-uridine is inhibited, and at higher intracellular calcium concentrations calcium has a marked cytotoxic effect. If $[Ca^{2+}]_i$ is in the range of 100 μM a number of Ca^{2+}-dependent proteases, lipases, DN-ases and RN-ases are activated causing the deterioration and, consequently, the death of the cell (32).

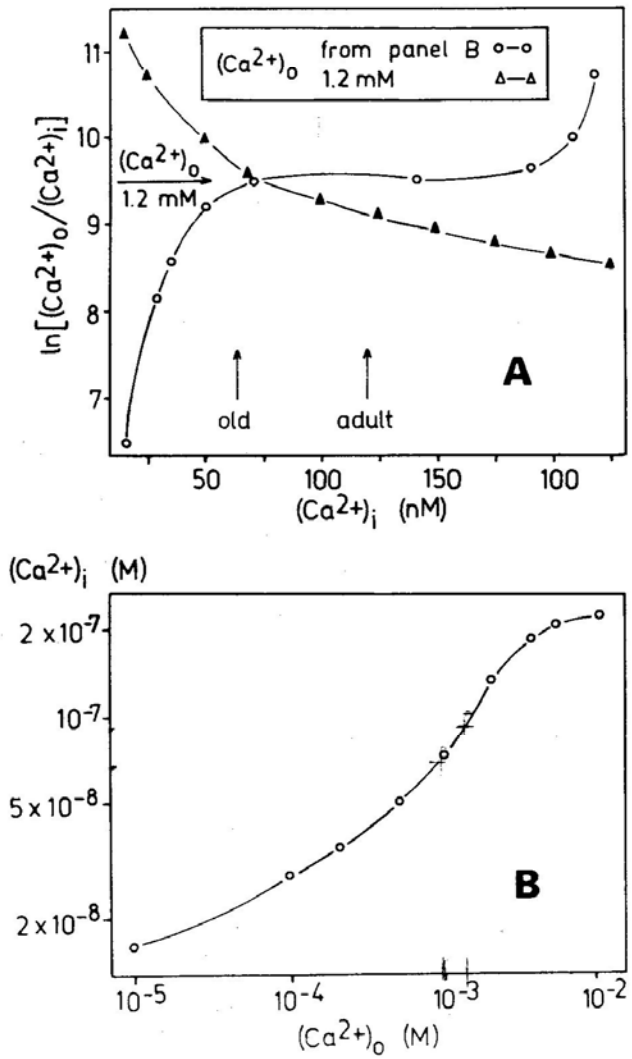

Fig. 3. Dependence of the intracellular calcium concentration ($[Ca^{2+}]_i$) on the calcium concentration of the medium ($[Ca^{2+}]_0$) (A) and the logarithm of $[Ca^{2+}]_0/[Ca^{2+}]_i$ as the function of $[Ca^{2+}]_i$ in lymphocytes (B).
Panel A: The logarithm of $[Ca^{2+}]_0/[Ca^{2+}]_i$ as the function of $[Ca^{2+}]_i$. ▲---▲: $[Ca^{2+}]_0 = 1.2$ mM; o---o: $[Ca^{2+}]_0$ is equal with the value of panel B corresponding to the appropriate $[Ca^{2+}]_i$. Horizontal arrow indicates the point where $[Ca^{2+}]_0 =$ 1.2 mM in both curves. Vertical arrows indicate the intracellular calcium concentrations characteristic of lymphocytes from "old" and "adult" donors, respectively. *Panel B:* $[Ca^{2+}]_i$ as the function of the Ca^{2+} concentration of the medium ($[Ca^{2+}]_0$). (Panel B is reproduced by permission from (15)).

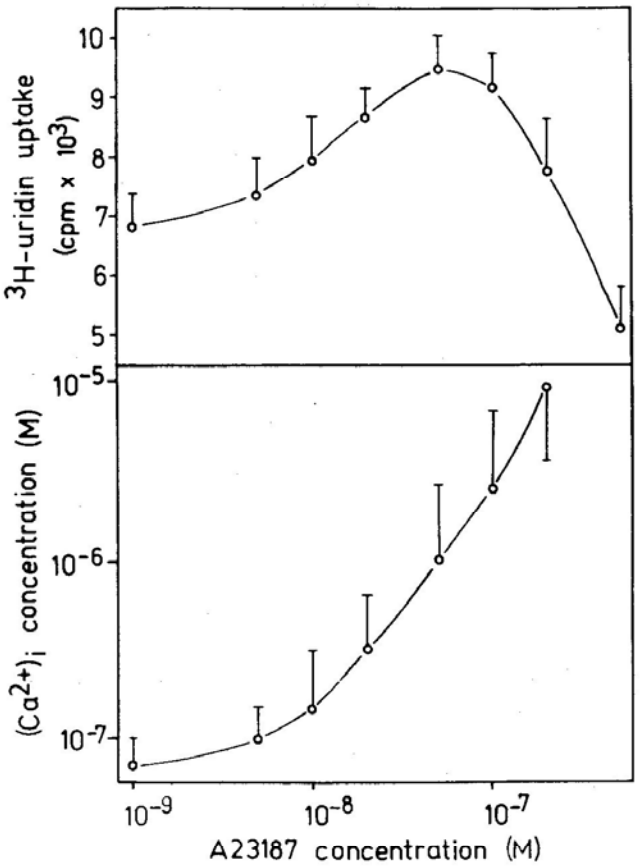

Fig. 4. Correlation of intracellular calcium concentration with the activation of lymphocytes. The uptake of ^{3}H-uridine and the intracellular Ca^{2+} concentration of lymphocytes was measured as described in Material and methods. For the measurement of $[Ca^{2+}]_i$ the indicator fura-2 was applied. Intracellular Ca^{2+} concentration was changed by addition of the Ca-ionophore A23187 at the different final concentrations indicated. Data are means ± SD of three individual experiments.

Discussion

The activation of lymphocytes is linked to the increase in intracellular calcium concentration in many ways. Mitogens increase the $[Ca^{2+}]_i$ (25), increase in extracellular calcium concentration causes both an increase in $[Ca^{2+}]_i$ and mitogenesis (15, 28, 29). Several other cellular processes involving the microfilamental system, such as the chemotaxis of leukocytes or the growth of the axon, are also activated by an increase in $[Ca^{2+}]_i$ (33).

Hammerschlag et al. (34) and Ochs et al. (35) observed a 60% decrease in the transport function of axons after the decrease of the neuronal $[Ca^{2+}]_i$. Fibroblasts derived from Alzheimer donors showed a 70% decrease in $[Ca^{2+}]_i$ compared with a control group of the same (old) age. The decrease of $[Ca^{2+}]_i$ was higher (81%) if the Alzheimer fibroblasts

were compared with cells from an adult (35–55 years) control group. This shows that aging may also diminish the fibroblastic $[Ca^{2+}]_i$. The Alzheimer fibroblasts showed a markedly reduced cell growth which could be completely restored after addition of the calcium ionophore A23187 (24). There is also evidence which suggests that the decrease in $[Ca^{2+}]_i$ correlates with the decrease of proliferation in lymphocytes (6, 36, 37).

There are relatively few and partly contradictory data in the literature about *changes in the intracellular calcium homeostasis in aging*. Miller & Simons (38) showed that stimulated T lymphocytes from aged rodents have a lower cytosolic free calcium than those from young animals. The mitogenic response of aged human lymphocytes is more sensitive to the decrease of extracellular calcium concentration than that of the control, "adult" cells (9). The block of calcium channels prevents mitogenesis to a higher degree in aged lymphocytes than in adult cells (39). Both findings may indicate that in aged lymphocytes the intracellular calcium signal is significantly attenuated, hence these cells have greater dependency on extracellular calcium. On the other hand, these results may relate to our data indicating a significant decrease in the intracellular calcium concentration in the resting state, since in aging it is a general compensation phenomenon that the aged cell has increased sensitivity to lacking compounds, e.g.: Ca^{2+}.

It has recently been discussed (6, 40) whether the change in calcium homeostasis might be a general characteristic of aging and age-related disorders. According to biochemical evidence aging decreases cytosolic free calcium concentration in lymphocytes, while electrophysiological findings suggest an elevated intracellular free calcium concentration in neurones. With aging the total cell calcium content of lymphocytes increases, but calcium availability (i.e., cytosolic free Ca^{2+}) is diminished. Compounds that increase calcium availability ameliorate age-related deficits in cell function.

The immune function markedly decreases with aging (1, 2, 7, 8, 10, 11). T lymphocytes of aged donors contain certain cell populations which have a high, "normal" mitogenic response, while other cell populations have almost totally lost their ability to proliferate. Aging causes a shift between these two populations, increasing the amount of the latter, the non-responsive cells (36, 37).

Some evidence has demonstrated that lymphocyte activation requires and provokes elevation of cytosolic free Ca^{2+}. However, it is clear that the decline of immune function in aging can not only be due to the decrease in intracellular calcium concentration (41–44). However, calcium availability may be significantly reduced by the decrease in the intracellular calcium concentration in the resting state, which is the major finding of the present report. Consequently, the reduced calcium availability may induce various adaptation-like changes in the overall metabolism of the cell.

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