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Changes in Intracellular Free Calcium Concentration in Resting Lymphocytes at the Borderline of Life

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Intracellular free Ca^{2+} plays a key role in the regulation of intracellular processes. The best known examples of this regulation are connected with the activity of muscle fibers, liberation of neurotransmitter substances from the vesicles, and with mitosis [1–3]. In addition to this, evidence is accumulating to show that the calcium ion is an essential common component of several intracellular enzyme reactions [4].

Calcium ions influence the intracellular biochemical events in two ways: on the one hand, they act as 'second messengers', transmitting certain signals coming to the cell from the extracellular space in the form of electrical or chemical stimuli, and on the other hand, the calcium is a charge carrier. The ionic current passing through the calcium channel of the plasma membrane alters the membrane potential and by that influences the intracellular biochemical machinery [5].

The Ca^{2+} level of cytosol is regulated in many ways by different mechanisms. First of all, the calcium-binding proteins and the calcium pump, exchange and channel systems of the plasma membrane, endoplasmic (sarcoplasmic) reticulum and mitochondria belong to them [6].

The age-related changes in the immune system are among the processes subjected to the most detailed studies on ageing in gerontology. The age-

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related decrease in immune functions is a factor of paramount importance in the diseases common in old age [7]. Deterioration of the mitotic activity of lymphocytes plays an important role in the diminution of the immune capacity. This is based on a defective transfer of signals for mitosis, decrease in protein synthesis, enzyme induction and in the quantity of cofactors of enzymatic reactions [8]. The investigations seemed to be justified also by the decisive role attributed to both the lymphocytes and the intracellular calcium in the ageing process [8, 9]. Cytosolic free Ca^{2+} concentration is one of the basis intracellular cofactors of lymphocytic metabolism [10]. There is at present scanty evidence in the literature concerning ageing-induced changes in the latter [11]. Therefore we have undertaken to collect data in this field, using the unique opportunity offered by the studies on the centenarians living in Hungary.

Material and Methods

Lymphocytes were separated from the peripheral blood of centenarians using the method of Böyum [12]. For measuring cytosolic free calcium, cells were loaded with fura-2/AM or with quin2/AM and measured by the method of Tsien [13], Grynkievicz et al. [14] and Pollock et al. [15] respectively (fig. 1, 2). A more detailed analysis of the potential sources of error occurring with measurements of intracellular calcium concentrations in lymphocytes can be found in a previous paper [18].

Results

In our investigations only a smaller but representative group of the centenarian population has been involved. In table 1 the data of lymphocytes from female donors are presented. The mean age of the 18 test subjects was 101±1 year. The youngest was in her 100th year, the oldest was 104 years old. The intracellular free calcium concentration of lymphocytes was 59±16 n*M*. The range shows that the group was relatively homogeneous in nature, the usual range of the means being much higher with most of the laboratory parameters in the elderly and in general with the advance of age. The lowermost intracellular calcium ion contents $(Ca^{2+})_i$ value measured was 30.5 n*M*; the highest was 89 n*M*. Even this latter one is far below the mean physiological value in adults (120 n*M*) [17–20]. Thus, at the borderline of age the intracellular Ca^{2+} concentration is reduced to half of the normal adult value with ageing. However, within that low free calcium level no correlation can be demon-

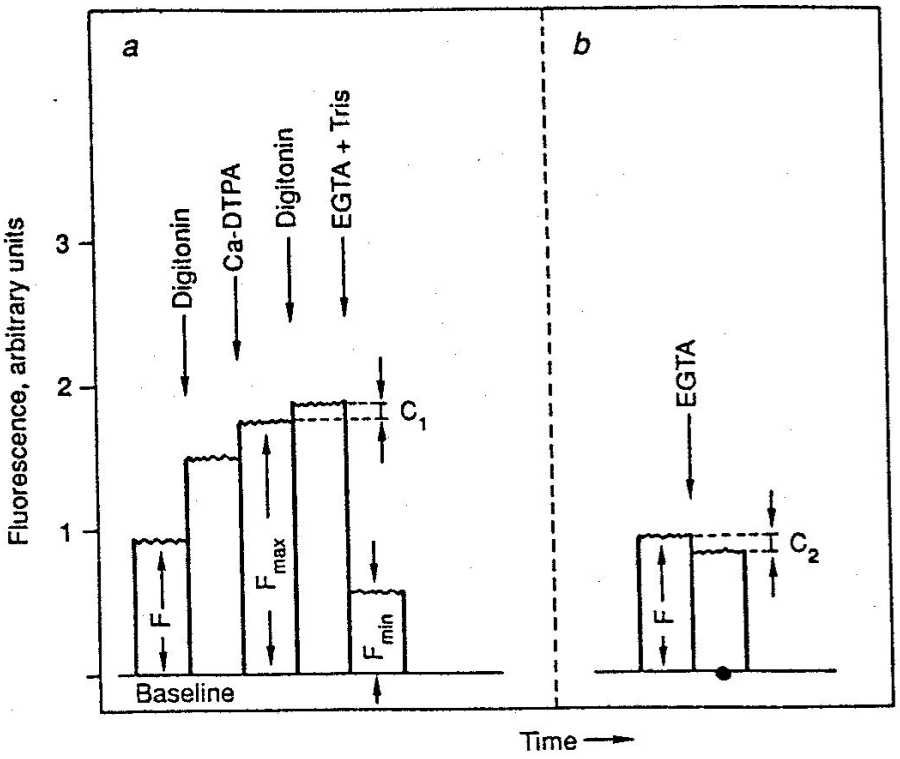

Fig. 1. The procedure of intracellular calcium concentration measurement with quin2 (sketch). *a* F denotes the fluorescence of the sample, F_{max} is measured after the addition of digitonin at a final concentration of 10 μM. The Ca-DTPA complex is used at a final concentration of 100 μM to strip out heavy metal ions from their quin2 complexes. The second addition of digitonin gives a correction (C_1) for the aspecific fluorescence increase caused by the detergent. EGTA (5 mM) and Tris base (20 mM) is used to set the autofluorescence of the sample (F_{min}). *b* An aliquot of the sample is used for the determination of the fluorescence of extracellular quin2 (C_2) by the addition of EGTA (100 μM).

strated between the years (100–104) lived and the measure of intracellular free calcium concentration.

Table 2 shows the results for the male subjects. The mean age of the 13 men tested was 101±1 year, i.e. it was the same as with the females. As in the case of women, the youngest man was in his 100th year, and the oldest was 104 years old. The intracellular free calcium concentration of the lymphocytes was 61±13 nM, somewhat higher than in the female group. The value of standard deviation (SD) was better than in the female group. This suggests that the male group was more homogeneous. The lowest value measured was 37.5 nM, the highest 78.5 nM. The changes were similar with the two sexes and it seems that at $(Ca^{2+})_i$ values lower than 30 nM the lymphocytes are not viable anymore.

Two cases have been excluded from the averaging. In samples 115 and 120 the postseparation lymphocyte count was pathologically high. Likewise, the

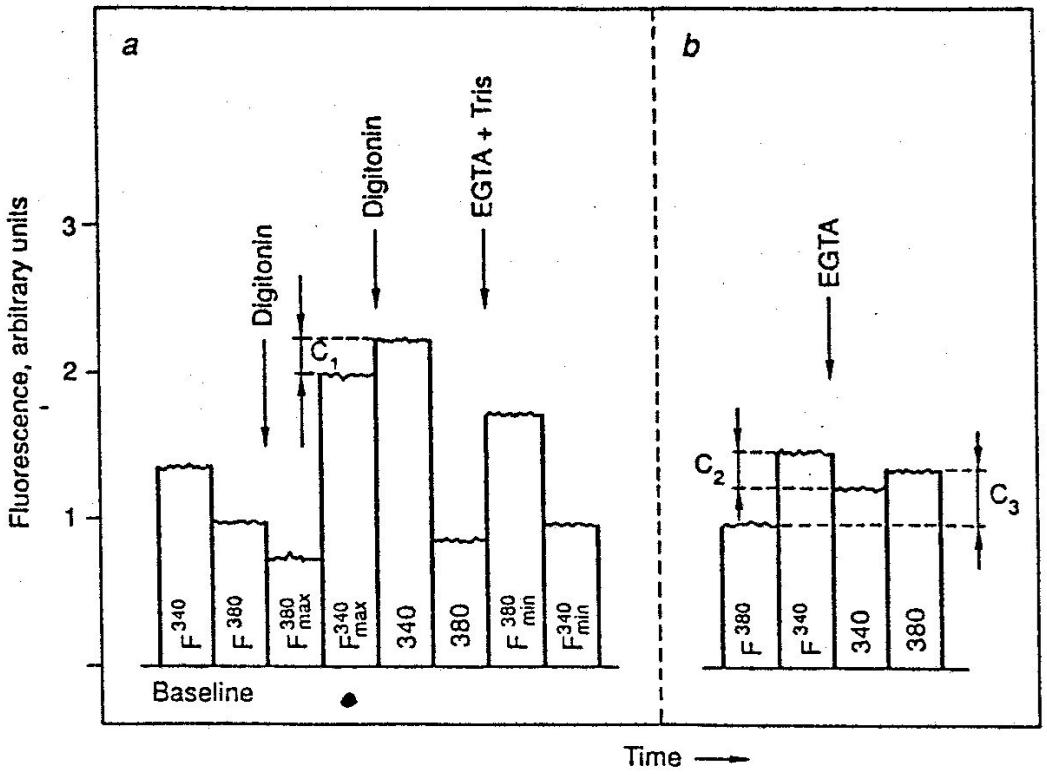

Fig. 2. The procedure of intracellular calcium concentration measurement with fura-2 (sketch). See figure 1 for the details of the procedure and the final concentrations of different chemicals used. In the measurement of fura-2 fluorescence the dual excitation wavelength for free fura-2 was 380 nm, while for the Ca-fura-2 complex 340 nm. C_1 = correction for the aspecific fluorescence of digitonin; C_2 = correction for the fluorescence of extracellular fura-2 at 340 nm; C_3 = the same correction at 380 nm.

cytosolic free calcium values was 10-fold the normal. Summarizing (fig. 3), the lymphocytic intracellular free calcium concentration mesured in the centenarians was 60±15 n*M*, thus, as it has already been mentioned, about half of the value typical for the adult age group (fig. 3a). The difference between the two age groups is significant at the 0.001 level. In spite of the fact that we are dealing with an old age group, the SD may be said to be rather good. Our data, i.e. the fall to half of the adult value of the cytosolic free calcium level in peripheral lymphocytes, are in good agreement with the observations made by Peterson et al. [21] who demonstrated a similar tendency in human fibroblasts.

Since there is scanty evidence in the literature concerning changes in calcium ion concentration in ageing, we enhanced the reliability of the measurements made in the centenarians by using two kinds of indicator. One of them was quin2, the other was fura-2, this new member of the family of

Table I. Cytosolic free calcium concentration in lymphocytes of females

Code No.	Age	$(Ca^{2+})_i$	Indicator	Health status	Results of lymphocyte studies[a]
44	100.5	46,5	fura-2	D, Tu	L
45	102	58,5	fura-2	D	
46	104	73	quin 2	D, Tu	L, foamy Mi, My
48	100.5	80,5	fura-2	H	L, My
49	100.5	76	fura-2	H	My, dense substance, foamy Mi
51	100	34,5	fura-2	H	L, giant Mi
53	100.5	46,5	fura-2	H	
55	103	64,5	fura-2	H	L
56	100	73	fura-2	H	My
67	100	47,5	fura-2	D	
80	100	89	fura-2	H	
89	100	30,5	quin 2	D, Tu	
90	100	57	quin 2	H	L
100	99.5	49	quin 2	H	L, My
111	102	62,5	fura-2	D	L
114	101	62,5	fura-2	H	
115	101	534[b]	quin 2	D	giant Mi
119	101	49	quin 2	H	L

H = healthy; D = diseased; Tu = tumorous; L = lipofuscin; My = myelin structure; Mi = mitochondrium.

[a] See Beregi et al.: Studies on Lymphocytes [this volume].

[b] Pathological value, not included in calculating the mean.

calcium indicators, which has several advantages over quin2 [14]. It was necessary to compare the measurements with fura-2 with those made with quin2 because prior to the advent of the former the measurements had been made usually with quin2. Figure 3b compares the results of measurements made with the two different calcium indicators. Although somewhat higher calcium levels were measured with fura-2, essentially the results obtained by the two methods were identical. Figure 3c shows that no difference was demonstrated in intracellular free calcium concentration between the two sexes, the values for males being closely similar to those for females.

It remains to be seen what significance might be attributed to this low calcium level from the point of view of the mechanism of ageing. On the one

Table 2. Cytosolic free calcium concentration in lymphocytes of males

Code No.	Age	$(Ca^{2+})_i$	Indicator	Health status	Results of lymphocyte studies[a]
34	101	73	fura-2	H	giant Mi, My
47	104	55	fura-2	D	
52	101.5	47	fura-2	H	L
54	100	37.5	fura-2	H	giant Mi, My
57	100	72	fura-2	H	L
70	102	50	fura-2	D, Tu	L
71	100	67	fura-2	H	L
72	100	53	fura-2	H	
73	102	63.5	fura-2	H	My
112	99.5	78.5	fura-2	H	L, giant Mi, My
113	100	75.5	fura-2	H	L
117	100	65.5	fura-2	H	L, giant Mi
120	103	565[b]	quin 2	D	L, My

[a,b] See respective footnotes (and abbreviations) in table 1.

hand, it is possible that the persons living longer than the average life span may thank their longevity right to this low calcium level. A lower Ca^{2+} level means namely a certain measure of protection against functional overloading. On the other hand, the results of biological investigations seem to indicate that intracellular free calcium levels of 30–60 n*M* represent the lower limit of lymphocytic reactivity, below which the lymphocytes cannot function anymore. The latter hypothesis is substantiated by the observations made by Whitney and Sutherland [22] and Abboud et al. [23], according to which activation of lymphocytes does not come about when the intracellular Ca^{2+} concentration sinks greatly below 100 n*M*.

As it has been mentioned already, the Ca^{2+} concentration in centenarian lymphocytes is 50% that of adults. At the same time, the extracellular (serum) ionized Ca concentrations are the same in both the old and adult individuals [24]. The serum *free* calcium level is a homeostatic parameter well controlled by the organism and shows essentially no changes with ageing. The decrease of total calcium level measured in ageing is due not to the free ionized, but rather to the protein-bound calcium. This explains why the difference between intracellular and extracellular calcium concentrations is greater in the lymphocytes from aged subjects.

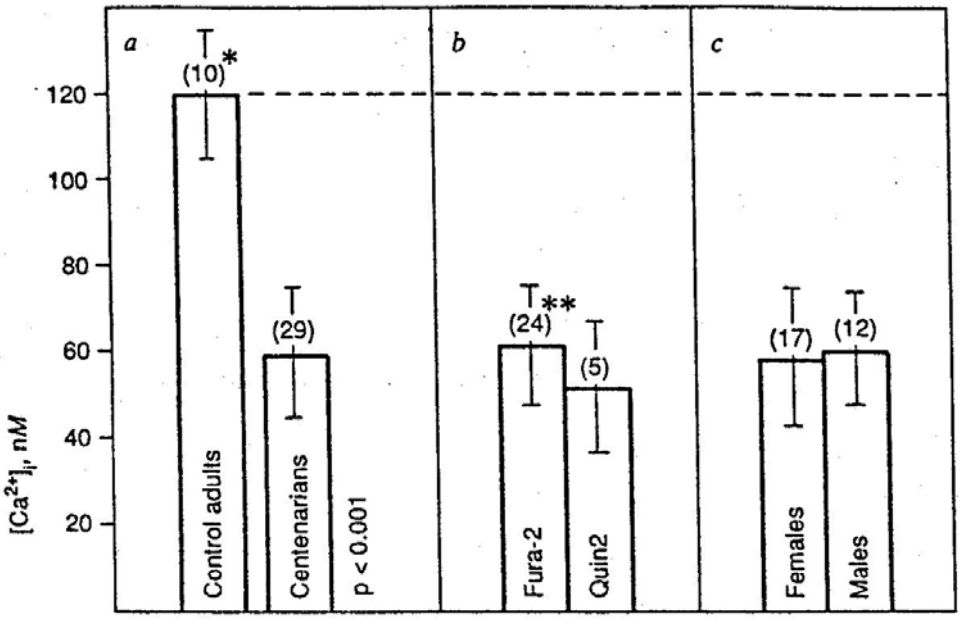

Fig. 3. Cytosolic free calcium concentration in lymphocytes of centenarians. *a* Comparison of the adult results with those of the centenarian age group. *b* Comparison of the values measured with fura-2 indicator with those measured with quin2 calcium indicator. *c* Comparison of male and female sample values. Isolation of peripheral blood lymphocytes and measurement of their intracellular calcium concentration was performed as described in the text. The level of significance (p) was determined using Student's t test: *The number of adult samples was not increased in these experiments, since the average intracellular calcium concentration correlates with both our earlier data and the values given in the literature very well. **The number of experiments with fura-2 was greater since this indicator has much better properties in many respects [see 14] than quin2. A few experiments with quin2 were also included for sake of comparison with the majority of the data in the literature.

At any rate, the decreased $(Ca^{2+})_i$ level has many consequences. Activation of lymphocytes is accompanied by changes in the plasma membrane potential [25, 26]. According to published evidence [14–17] and our own findings, the lymphocytic plasma membrane maintains at rest a 10,000-fold (10^4) Ca^{2+} concentration gradient, since the normal value of the serum ionized calcium concentration $(Ca^{2+})_o$ is 1.05–1.3 m*M*, and in the sample taken from an adult person the cytosolic free Ca^{2+} concentration $(Ca^{2+})_i$ was 120 n*M*. This difference in concentration represents a great diffusion power and creates at the same time a substantial membrane potential component (Nerst potential) of Ca^{2+} origin.

Discussion

According to our measurements, the cytosolic free calcium content of the lymphocytes obtained from centenarians was half of the value for adults. This decrease means at the same time a greater difference in concentration, thus a greater diffusion power and a higher membrane potential component of Ca^{2+} origin. The lymphocytes from centenarians may use this greater diffusion and electrical driving force for counteracting the age-induced deterioration of calcium homeostasis (drain it), for instance to improve the sodium-calcium or hydrogen-calcium exchange, the activity of the calcium pumping ATPase, or the conductivity of the calcium channel.

The question is: How does the cytosolic Ca^{2+} level influence the function of lymphocytes? In recent years evidence has been accumulating regarding the Ca^{2+}-dependent mitogenic signal transfer system. A prerequisite of lymphocyte activation is an elevation of intracellular free Ca^{2+} level. In response to the increased calcium concentration, several key enzymes of the lymphocytes are activated [27]. Upon binding the calcium ion the spatial structure of the target protein (enzyme molecule) is changed, changing thereby also its activity. In this context the activity level of the cell is dependent also on the intracellular free calcium ion concentration. Corresponding to the function and energy demand of the cell the intracellular Ca^{2+} level is changing continuously, of course within the narrow physiological limits [28].

Deepening of our understanding of the molecular pharmacology, influencing and controlling the intracellular free calcium level will lead to significant improvements in the treatment and prevention of calcium-related disorders. However, intracellular regulation involves extremely fine, complex, interdependent, multistage systems, which are not easy to influence at all. In spite of that, by means of electrophysiological studies, pharmacological and biochemical investigations we have now so-called 'Ca channel effectors' which are very promising in cardiovascular disorders, hypoxic-ischemic injury and impaired lymphocyte activation, respectively [29, 30].

In connection with our topics, Kennes et al. [31] found no age-dependent differences in the changes of the cytosolic free calcium level in the course of lymphocyte activation. In this case, however, the provision of a higher calcium level required for triggering off activation may be an important factor. In correlation with this fact, decrease in the availability of adequate amounts of Ca^{2+} from the intracellular stores or from the extracellular space, as well as the lower initial cytosolic free calcium level itself suggested by our own measurements may explain the reactivity decrease in old age.

Recently, in the investigations concerning aspects of the age-dependent mitotic activity decrease of lymphocytes, the changes in the transmission of the stimulating mitogenic signals and the interactions between the lymphocytic signal transmitter systems have been in the focus of interest [32].

One of the key steps in the activation process leading to mitosis is the production of interleukin-2 (IL-2) and IL-2 receptors [33]. It has been demonstrated in several laboratories that in the cells from elderly persons the secretion of the autocrine growth factors is markedly reduced [31]. Since this step in the activation process results from 4 to 8 h following stimulation, it may be assumed that the cause of the decrease should be looked for in an earlier stage of activation. Interest was then focussed upon the intracellular Ca^{2+} buffer systems and upon transmembrane Ca^{2+} transport, since the increase of free Ca^{2+} in the cytosole comes before IL-2 secretion. According to Miller [34], not the cellular depots, but the diminished Ca^{2+} influx is responsible for cytosolic Ca^{2+} deficit. Also Segal [35] demonstrated a decrease of the mitogen-induced $^{45}Ca^{2+}$ influx in old age in rat thymocytes.

Disorders in the mobilization of ionized calcium are blamed not only for the decrease in lymphocytic reactivity, but also for the age-dependent loss of sympathetic innervation sensitivity of the parotid gland of the rat [36]. Similarly, Ishikawa et al. [37] found in parotid cells that the ability of inositol triphosphate to directly stimulate calcium efflux is reduced by about 50% with increasing age. Then again, Gibson et al. [38] measured in 30-month-old mice 41–51% decreases of $^{45}Ca^{2+}$ incorporation in nervous tissues, in the cerebral cortex, striate body, hippocampus, cerebellum, midbrain and brain stem.

Data suggest that T-cell proliferation can be induced even without cell surface ligands by means of elevating intracellular calcium level with the Ca-ionophore A23187 and phorbol ester tumor promoter [10]. Miller [34] attained significant increases in cytosolic Ca^{2+}, protein kinase C activation and a great improvement of T-cell mitosis in old mice by a combination of the Ca-ionophore ionomycin and phorbol myristic acetate. However, others [39] could not demonstrate the same in human beings with the same activators.

The authors quoted have tried to find the missing link in the chain of events leading up to mitosis in the process of activation being injured in the course of ageing. In this connection, as mentioned earlier, it is an accepted fact that a necessary prerequisite of lymphocyte activation is an increase in the free calcium level in the cytosol. According to our measurements, in centenarians the availability of calcium is reduced. The cytosolic free calcium concentration

of their lymphocytes in resting state is decreased. This may be one of the causes of diminished ability of aged human lymphocytes to be activated in response to antigens and mitogens.

Summary

Intracellular free calcium concentration was determined in the peripheral lymphocytes of Hungarian centenarians by a fluorometric method using fura-2 and quin2 calcium indicators. The free calcium concentration of the lymphocytic cytosols from centenarians was greatly reduced in the resting state, to about half of the adult value. Our results were closely similar in men and women. There was no significant difference between values measured with various intracellular calcium indicators. It may be assumed that the decrease of immune capacity in centenarians is correlated, among others, with the decrease in the available free calcium in their lymphocytes. At the same time, the decrease of the free calcium level leads also to changes of adaptive nature.

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