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A comparative study of the calcium system in memory T cells and naive T cells

Alla Sigova[a], Elena Dedkova[a], Valery Zinchenko[a,*], Ivan Litvinov[b]

[a] *Institute of Cell Biophysics, Russian Academy of Sciences, Laboratory of Intracellular Signaling, 142292 Pushchino, Moscow region, Russia*
[b] *Shemjakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Laboratory of Immunochemistry, 117871, Miklukho-Maklaya street 16/10, Moscow V-437, Russia*

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Abstract The comparative analysis of responses of memory and naive T lymphocytes to Ca^{2+}-mobilizing agents, namely Con A, thimerosal, thapsigargin and ionomycin, was carried out. The effect of these agents on both types of T cells differed qualitatively and quantitatively. The lack of intracellular Ca^{2+} stores in memory T cells was shown. Ca^{2+}-mobilizing agents did not induce influx of Ca^{2+} in memory T cells from outside and this was the reason for their stability to Ca^{2+} ionophores. It was also shown that memory T cells were resistant to the 'Ca^{2+} paradox'.

Key words: Immunological memory; T lymphocyte; Ca^{2+} transport; Ionophore

1. Introduction

An important role in the generation of long-lasting protective immunity is held by memory T cells [1]. Although the phenomenon of immunological memory was shown many years ago little is known about the development and peculiarities of memory T cells. By using flow cytometry, Ishida and Chused [2] have recently demonstrated that murine splenic T lymphocytes contain a minor subpopulation of ionomycin-resistant cells, and $[Ca^{2+}]_i$ in this T cell subset remained approximately constant after the addition of ionomycin. When splenic T lymphocytes were exposed to graded doses of ionomycin this T cell subset became denser and could be separated on ionomycin-containing Percoll step gradients [2]. Cells recovered from such a gradient and washed to remove the ionomycin kept their viability. Miller et al. [3] have shown that the ionomycin-resistant cell population is enriched in cells that express $CD44^{high}$ and $CD45RB^{low}$, and thus appears to consist largely of memory T cells. The ionomycin-resistant T cell subset can, however, respond well to an Ag against which the donor mice have produced a strong memory T cell immune response [3]. It has also been shown by using limiting dilution analysis that the Ag-specific helper memory T cells were found predominantly in the ionomycin-resistant fraction of the spleen T lymphocytes [3]. Recently, using adoptive cell transfer and following infection challenge, we have shown that ionomycin-resistant T cells can transfer the resistance to *Neisseria meningitidis* and *Mycobacterium tuberculosis* infections [4,5]. Taking together these experiments have led to the suggestion that these 'ionomycin-resistant' T cells might correspond to memory T cells. Changes in resistance to calcium signal development may represent a fundamental distinction between naive and memory T cells, and could contribute to differences in activation requirements between these two cell subsets.

The main aim of the present work was to study the differences and peculiarities in calcium homeostasis between naive and memory T cells.

2. Materials and methods

CBA mice were maintained under conventional conditions at the animal facilities of the Institute of Bioorganic Chemistry RAS and were used at ages between 8–45 weeks. Most experiments were performed on females, although no sex-related differences were found. T lymphocytes were negatively selected from splenocytes by two rounds of panning on Petri dishes, that were previously coated with 5 mg/ml of rabbit anti-mouse Ig (Dako).

To obtain ionomycin-resistant T cells the purified T lymphocytes were incubated at $20–50\times10^6$ cells/ml in RPMI 1640 containing 1% FCS and 4 mM ionomycin for 30 min at 37°C. This cell suspension was loaded onto a Percoll density gradient (40, 50, 60, 70, 80 and 90%, 2 ml per step) and centrifuged at $2000\times g$ for 20 min at room temperature. Cells were collected from the interfaces and washed twice in RPMI 1640 (containing 5% FCS).

The $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} probe, Fura-2. The cells were incubated for 40 min at 37°C with 1.5 μM acetoxymethyl ester of the probe, then cells were washed twice with fresh medium and suspended at the same concentration in the dye-free medium. The cells were then used within 1 h. To monitor $[Ca^{2+}]_i$, the cells were placed into the spectrofluorometer cuvette (300 μl) and kept at 37°C. The $[Ca^{2+}]_i$ was estimated as described in [6]. The wavelengths of excitation and registration for Fura-2 were 337 nm and 510 nm, respectively.

3. Results

To investigate the differences between naive and memory T cells, Ca^{2+}-dependent systems of intracellular signaling in these cells were compared. It has been demonstrated that spleen T cells can be subdivided into two subsets: naive (80–90%) and memory T cells (10–20%) [16]. The lack of a direct method for selection of naive T cells did not allow us to compare independently their properties with memory T cells. In this case spleen T cells from young mice containing a minimal part of memory T cells were used as naive T cells. Memory T cells were obtained by ionomycin treatment of splenic T cells as described in Section 2.

Because of the separation method used $[Ca^{2+}]_i$ in naive and memory T cells might be different. The estimated initial $[Ca^{2+}]_i$ was similar in both T cell types comprising about 130 ± 20 nM. Therefore, differences between naive and memory T cells could not be associated with their resting $[Ca^{2+}]_i$.

*Corresponding author. Fax: (7) (0967) 790509.
E-mail: zinchenko@venus.iteb.serpukhov.su

Abbreviations: Con A, concanavalin A; $[Ca^{2+}]_i$, concentration of Ca^{2+} ion in cytoplasm; ER, endoplasmic reticulum; PMA, phorbol 12-myristate 13-acetate; FCS, fetal calf serum; PM, plasma membrane

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An increase of $[Ca^{2+}]_i$ is thought to play a critical role in the entry of resting T lymphocytes into the activation state. It has been supposed that the differences between naive and memory T cells could be revealed by various agents that induce $[Ca^{2+}]_i$ changes. In this way memory and naive T cells were compared by their sensitivity to ionomycin, thapsigargin, thimerosal and Con A.

Naive and memory T cells were examined with different concentrations of ionomycin (Fig. 1). Ionomycin concentrations lower than 10^{-9} M did not increase $[Ca^{2+}]_i$ in either cell type. Above this range an identical concentration of ionophore induced a much higher response in naive T cells than in memory T cells. For example the addition of 10^{-7} M ionomycin to naive T cells increased $[Ca^{2+}]_i$ to 2600 ± 40 nM, while the same dosage of ionomycin raised $[Ca^{2+}]_i$ in memory T cells only to 160 ± 6 nM. Therefore, memory T cells are resistant to ionomycin action and these results agree well with the data obtained by Ishida and Chused [2].

The $[Ca^{2+}]_i$ level attained in cells exposed to limiting doses of ionomycin is likely to represent a balance between the rate of Ca^{2+} influx across the PM, the rate of Ca^{2+} efflux from ER and mechanisms that sequester or extrude Ca^{2+} from cytoplasm. Low concentrations of ionomycin increase $[Ca^{2+}]_i$ by the activating of innate Ca^{2+}-transporting systems in PM and in ER [7,8]. In this case the response of cells to low doses of Ca^{2+} ionophores correlates with the availability of Ca^{2+} in intracellular stores. Therefore, the resistance of memory T cells to ionomycin-treatment could be explained by the absence of available intracellular Ca^{2+} stores in these cells. To confirm this hypothesis the measurement of Ca^{2+} mobilization by ionomycin (1 μM) from intracellular stores in T cells incubated in Ca^{2+}-free medium was performed. In this case the ionomycin-mediated rise of $[Ca^{2+}]_i$ correlates only with the amount of available Ca^{2+} in intracellular stores. The amplitudes of ionomycin-induced responses in both types of T cells were compared. Qualitative differences between naive and memory T cells were demonstrated. As shown in Fig. 2A, the estimated initial $[Ca^{2+}]_i$ was similar in both T cell types incubated in Ca^{2+}-free medium supplemented with 0.5 mM EGTA. The intracellular Ca^{2+} stores in naive T cells contained a high amount of Ca^{2+}. No responses to ionomycin-treatment in memory T cells were shown (Fig. 2A). The obtained data accounts for the absence of Ca^{2+} in ER in memory T cells. It is well known that Ca^{2+}-ATPase carries out Ca^{2+} transport inside ER. The peculiarities in activity of ER Ca^{2+}-ATPase in naive and memory T cells can be analyzed by thapsigargin.

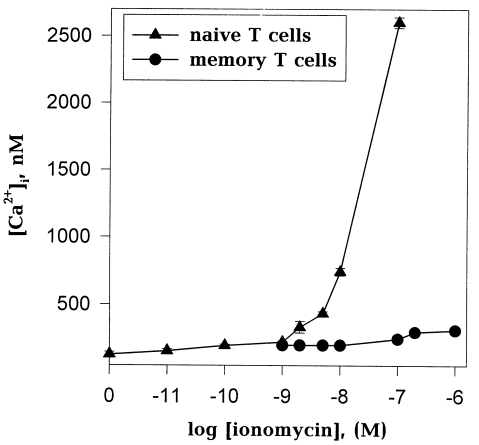

Fig. 1. The level of intracellular calcium in naive T cells and in memory T cells vs. ionomycin concentration.

Thapsigargin inhibition of Ca^{2+}-ATPase in ER finally leads to induction of Ca^{2+} influx from outside [11]. The effects of thapsigargin on $[Ca^{2+}]_i$ in naive and memory T cells were compared. In naive T cells 10^{-7} M thapsigargin led to a rapid increase of $[Ca^{2+}]_i$ (Fig. 2B). No influence of the same dosage of thapsigargin on $[Ca^{2+}]_i$ in memory T cells was observed. The lack of effect of thapsigargin on $[Ca^{2+}]_i$ in memory T cells suggests the hypothesis that the absence of intracellular Ca^{2+} stores regulated Ca^{2+} influx in these cells.

The phenomenon of the 'Ca^{2+} paradox', known for some types of cells, is characterized by a rapid and redundant Ca^{2+} influx in response to the addition of extracellular Ca^{2+} to cells previously incubated in Ca^{2+}-free medium with EGTA [12,13]. The molecular mechanisms of this phenomenon are still unclear. It has been shown only that the incubation of T cells in Ca^{2+}-free medium leads to exhausted intracellular Ca^{2+} stores and to open Ca^{2+} and Ca^{2+}-dependent K^+ channels [24]. Subsequent addition of Ca^{2+} (2 mM) may rapidly increase Ca^{2+} influx across the PM. As shown in Fig. 2C, the estimated initial $[Ca^{2+}]_i$ was similar in both T cell types incubated in Ca^{2+}-free medium with 0.5 mM EGTA. Subsequent addition of 2 mM Ca^{2+} to EGTA-treated naive T cells increased $[Ca^{2+}]_i$ from 130 nM to 1240 nM. This rise of $[Ca^{2+}]_i$ in naive T cells induces the subsequent rapid death of these cells. In contrast, subsequent addition of 2 mM Ca^{2+} to EGTA-treated memory T cells did not increase $[Ca^{2+}]_i$. These T cells kept their vitality after the Ca^{2+} addition. It is likely that in memory T cells the system of Ca^{2+} entrance could also be reduced. To confirm this hypothesis the permeability of PM to Ca^{2+} in naive and memory T cells was tested in another way.

It was shown that the SH-reagent thimerosal induced the rise of $[Ca^{2+}]_i$ in T cells by inhibiting PM Ca^{2+}-ATPases and opening Ca^{2+} channels [14]. As shown in Fig. 2D, thimerosal (60 μM) increased $[Ca^{2+}]_i$ about 10-fold from the initial level in naive T cells. In contrast the same dosage of thimerosal increased Ca^{2+} influx in memory T cells insignificantly. Therefore, the PM of memory T cells has altered the system of Ca^{2+} entrance. One may assume that in memory T cells not only the intracellular Ca^{2+} stores are absent but the system of Ca^{2+} entrance could also be reduced. It is interesting to note that the low permeability of Ca^{2+} channels of PM may be the second reason for stability of memory T cells to Ca^{2+} ionophore action.

The mitogenic plant lectin Con A is commonly used to demonstrate the activation capacity of T cells. Con A induces an increase $[Ca^{2+}]_i$ in T cells and this rise plays an important role in the activation processes [9]. Both naive and memory T cells were examined for their ability to generate a Ca^{2+} signal in response to mitogenic dosage of Con A. In agreement with the published results [10], it was shown that splenic T cells exposed to Con A rapidly increase their $[Ca^{2+}]_i$ (Fig. 2E). In contrast, no change in $[Ca^{2+}]_i$ in response to the same dosage of Con A in memory T cells was demonstrated (Fig. 2E). Thus, another difference between T cell subpopulations was the absence of Con A-induced $[Ca^{2+}]_i$ increase in memory T cells.

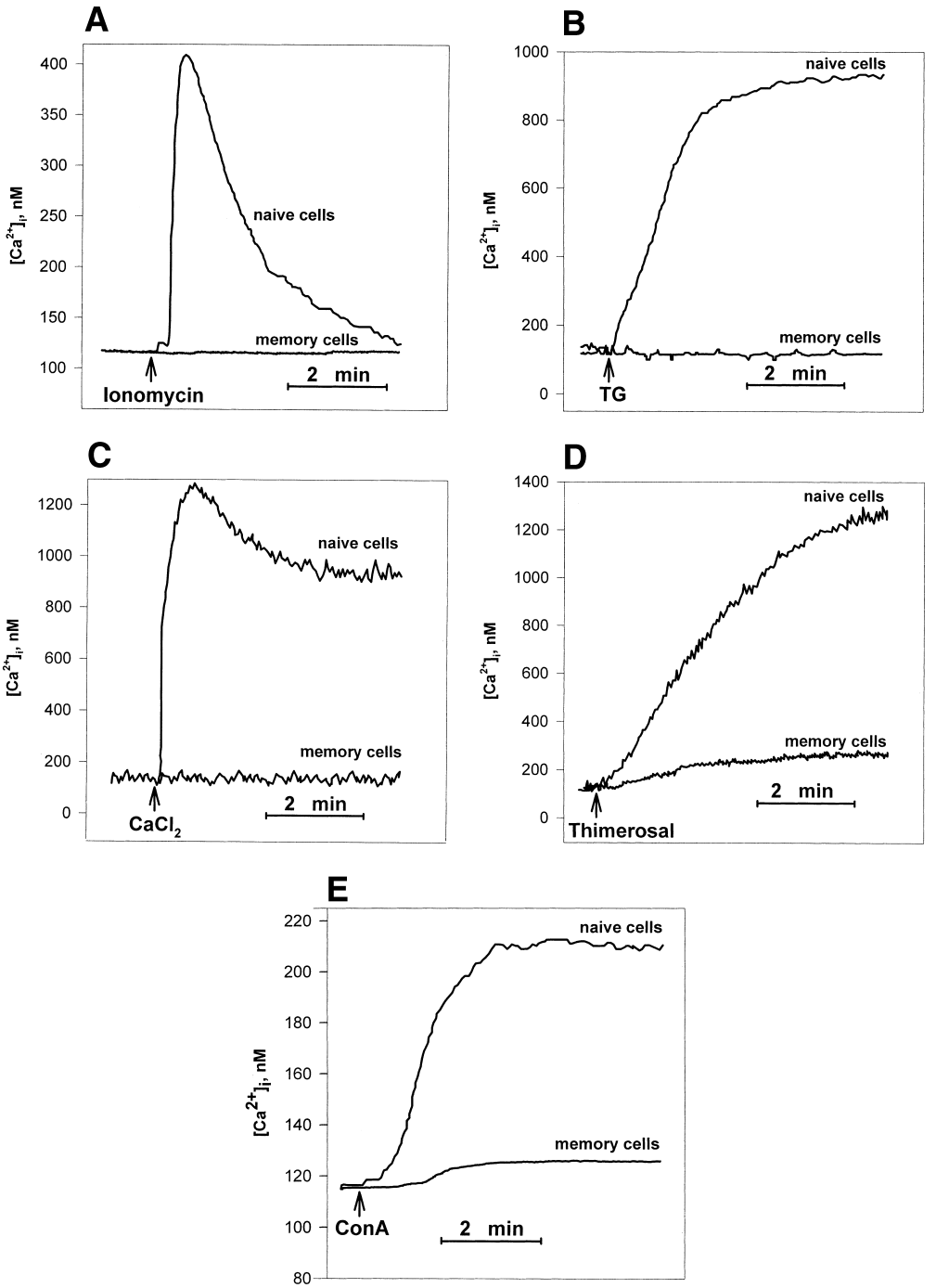

Fig. 2. Kinetics of typical calcium responses in naive and memory T cells. A: Ionomycin-induced $[Ca^{2+}]_i$ rise in the calcium-free medium. The arrow indicates the addition of 1 μM ionomycin. B: Thapsigargin-induced $[Ca^{2+}]_i$ elevation in memory and naive T cells in calcium-containing medium. The arrow indicates the addition of 10 nM thapsigargin. C: The effect of 2 mM $CaCl_2$ addition to naive and memory T cells after 40 min incubation in the calcium-free medium with 0.5 mM EGTA. The arrow indicates the addition of 2 mM $CaCl_2$. D: Effect of thymerosal treatment on $[Ca^{2+}]_i$ in memory and naive T cells. The arrow indicates the addition of 60 μM thymerosal in calcium-containing medium. E: $[Ca^{2+}]_i$ response to Con A in memory and naive T cells in calcium-containing medium. The arrow indicates the addition of 10 μg/ml Con A.

4. Discussion

Here we present the results of investigations designed to determine what types of differences in Ca^{2+} signal-generating systems of intracellular signaling between naive and memory T cells exist. T cells from young mice containing a minimal part of memory T cells were used as naive T cells. Memory T cells were obtained by the ionomycin-treatment of splenic T cells as described by Miller et al. [3].

While we cannot entirely rule out the idea that the brief exposure to ionomycin might induce some long-lasting change in cell behavior that alters subsequent responsiveness (up or down), and that the variations in responsiveness we see might not reflect intrinsic differences between naive and memory T

cells themselves, we have several reasons for discounting this. Firstly, the ionomycin-exposed T cells respond strongly to specific antigens under culture conditions [3,4,16,23]. The ionomycin-resistant T cells are able to confer resistance to *Neisseria meningitidis* [5] and *Mycobacterium tuberculosis* infections in mice [4]. Furthermore, the $CD44^{low}$ naive T cells are more sensitive to ionomycin [25], and might therefore be supposed to be more likely to be damaged by exposure to this agent, and yet are found to contain most Con A-responsive cells.

In the present study Ca^{2+} signal-generating systems of intracellular signaling for naive and memory T cells were analyzed. The initial $[Ca^{2+}]_i$ and the influence of Ca^{2+}-mobilizing agents, namely Con A, thimerosal, thapsigargin and ionomycin, on naive and memory T cells were compared.

As mentioned above, memory T cells were obtained by ionomycin treatment of spleen T cells. It was supposed that ionomycin exposure during isolation may result in an altered $[Ca^{2+}]_i$ in memory T cells. But the estimated initial $[Ca^{2+}]_i$ was similar in naive and memory T cells. Therefore, differences between naive and memory T cells could not be associated with their $[Ca^{2+}]_i$.

It has been supposed that the differences between naive and memory T cells could be revealed by the action of Ca^{2+}-mobilizing agents. Our data on the ionomycin resistance of memory T cells (Fig. 1) agree well with the results obtained by Ishida and Chused [2]. Various types of cells differ in sensitivity to Ca^{2+} ionophores [18–20]. Such differences in sensitivity can be attributed to the absence of activation of innate cellular Ca^{2+}-transporting systems by Ca^{2+} ionophores [7,8]. The sensitivity of cells to ionophores correlates with the availability of the intracellular Ca^{2+} stores and conductivity of PM Ca^{2+} channels.

Another difference between memory and naive T cells was the absence of Con A-induced $[Ca^{2+}]_i$ increase in memory T cells. It is known that not all murine T cells can generate a Ca^{2+} signal in response to the mitogen Con A, and the proportion of non-responsive cells increases with the age of adult mice [15,16]. It was also shown that splenic T cells from old mice were relatively resistant to ionomycin [17]. (The resistance of memory T cells to ionomycin cannot be attributed to diminished membrane permeability to the Ca^{2+}-ionomycin complex [17].). These data are the indirect confirmation of the obtained results (Fig. 2E) that ConA cannot activate memory T cells.

The mechanism of Ca^{2+} signal transduction was then analyzed in more detail. Intracellular Ca^{2+} stores of ER are thought to play a critical role in the activation processes in T cells. The analysis of intracellular Ca^{2+} stores in naive and memory T cells was carried out in this way: first the measurement of ionomycin-mediated Ca^{2+} mobilization from intracellular stores in T cells incubated in Ca^{2+}-free medium was performed. No Ca^{2+} was found in intracellular stores in memory T cells. (However, the presence of a high amount of Ca^{2+} in the intracellular stores in naive T cells was demonstrated.) The absence of Ca^{2+} in ER can be explained by the alteration of Ca^{2+}-ATPase activity carrying out the Ca^{2+} transport into intracellular stores in memory T cells. Using thapsigargin to inhibit Ca^{2+}-ATPase in ER and induce subsequent Ca^{2+} influx from outside this supposition was confirmed. No influence of thapsigargin on $[Ca^{2+}]_i$ in memory T cells was observed (Fig. 2B). (In naive T cells 10^{-7} M thapsigargin led to a rapid increase of $[Ca^{2+}]_i$ (Fig. 2B).) The lack of effect of thapsigargin on $[Ca^{2+}]_i$ in memory T cells can also be explained by the absence of Ca^{2+}-ATPase in ER. This peculiarity of memory T cells can explain their resistance to ionomycin.

The absence of intracellular Ca^{2+} stores in memory T cells led to a breakdown of normal relations between ER and Ca^{2+}-transporting systems in the PM of memory T cells. In conditions of Ca^{2+} paradox in naive T cells intracellular Ca^{2+} stores become empty and Ca^{2+} channels in PM become open. The addition of extracellular Ca^{2+} leads to an abnormally high Ca^{2+}influx from outside into naive T cells (Fig. 2C). In contrast, under the same conditions no Ca^{2+} influx from outside into memory T cells was demonstrated (Fig. 2C). Therefore, in memory T cells during Ca^{2+} paradox PM Ca^{2+} channels either remain in a closed state or have a low permeability for Ca^{2+}. These suppositions were confirmed experimentally using thimerosal.

It has been found that thimerosal increases $[Ca^{2+}]_i$ in T cells by inhibiting the Ca^{2+}-ATPase in PM and opening Ca^{2+} channels. The fact that comparatively little thimerosal induced Ca^{2+} influx in memory T cells indicates that in memory cells not only the stores of intracellular Ca^{2+} are absent but the system of Ca^{2+} entrance could be also reduced. This assumption is confirmed by the resistance of memory T cells to the 'Ca^{2+} paradox'. The observed property of memory T cells may be technically used for selective isolation of this cellular subset, in the absence of added Ca^{2+} ionophore.

We showed that memory cells apparently have a more simple Ca^{2+} signal transduction system. It seems that $[Ca^{2+}]_i$ in memory T cells is regulated by the Ca^{2+}-transporting system of PM only. Studies on the activity of the PM calcium pumps and Ca^{2+} channels in memory cells may help to clarify the biochemical basis of signal transduction in these cells. The significance of the reduced Ca^{2+}-transporting systems in the physiology of memory cells is unknown. The cellular Ca^{2+}-signaling systems can unspecifically be activated by different physical and chemical factors [21,22]. Since memory T cells live for a long time (up to several years), the reduced Ca^{2+}-signaling system can lead to the increased stability of the cells to adverse effects of external factors.

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