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Role of Extracellular Calcium Ions at Early Stages of Human T-Lymphocyte Polyclonal Activation *in vitro*

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Abstract—In this work we comparatively analyzed interleukin-2 (IL-2) and interferon γ production (IFN-γ) and also CD69 and CD25 expression by activated T-cells depending on extracellular calcium concentration ($[Ca^{2+}]_e$), which was varied with EGTA. The expression of CD69 molecules on the surface of T-cells depended only on the presence of phorbol myristate acetate, occurred at $[Ca^{2+}]_e$ higher than 0.2 mM, and did not require the presence of ionomycin. The increase in $[Ca^{2+}]_e$ by itself cannot induce expression of CD25 and CD69 molecules by activated cells. The values of $[Ca^{2+}]_e$, at which maximal fractions of $CD3^+CD69^+(IL\text{-}2)^+$, $CD3^+CD69^+(IFN\text{-}\gamma)^+$, and $CD3^+CD25^+$ activated T-cells were reached, never coincided with mean values of $[Ca^{2+}]_e$ for healthy donors and were different from each other. So, there is different $[Ca^{2+}]_e$ dependence for initial stages of activated T-cells differentiation. The relation between T-cells activation parameters and their differentiation is discussed.

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Key words: human peripheral blood, ionomycin, PMA, cell activation, EGTA, IL-2, IFN-γ

The physiological role of calcium ions has been studied since the first work of Sidney Ringer published in 1883 [1]. In almost every work studying molecular mechanisms of T-cells activation, "calcium signal" is mentioned as one of the key stages. The role of extracellular calcium concentration ($[Ca^{2+}]_e$) has been shown for the earliest stages of T-cells activation [2] and their proliferation stage [3-5]. Intermediate activation stages are characterized much less well.

Checking the role of $[Ca^{2+}]_e$ in T-cell response is technically quite simple: for this purpose either medium without calcium ions, Ca^{2+} binding with EGTA, or their combination is used. The advantage of these methods is reversibility: cells practically always can be switched to another medium by a series of washes or the necessary amount of calcium ions can be added to the same medium.

It is most likely that not all stages of T-cells activation are dependent on $[Ca^{2+}]_e$. For example, studying T-lymphocyte activation by monoclonal antibodies (mAb) to CD3 molecule, which is closely linked with T-cell antigen receptor, showed the existence of EGTA-sensitive and EGTA-insensitive signals [6-8]. In this work the dependence of stages of polyclonal T-cell activation on $[Ca^{2+}]_e$, which was varied using EGTA, was studied. Most attention was given to the appearance of CD69 and CD25 molecules on the cell surface and to the production of interleukin-2 (IL-2) and interferon γ (IFN-γ) by T-cells. Most of the experiments were done on whole blood.

MATERIALS AND METHODS

Reagents. Hanks' and RPMI-1640 media, L-glutamine, fetal bovine serum (FBS), $NaHCO_3$, and trypan blue were from Gibco (UK); brefeldin A, phosphate-buffered saline (PBS), dimethylsulfoxide, and MTT from ICN (USA); EGTA, phorbol myristate acetate (PMA), and paraformaldehyde from Sigma (USA); ionomycin

Abbreviations: $[Ca^{2+}]_e$, extracellular calcium concentration; $[Ca^{2+}]_i$, intracellular calcium concentration; CD, cell differentiation cluster; ConA, concanavalin A; FBS, fetal bovine serum; HPB, human peripheral blood; IFN-γ, interferon γ; IL-2, interleukin-2; IR, ionomycin-resistant cells; mAb, monoclonal antibodies; PBS, phosphate-buffered saline; PHA, haricot bean phytohemagglutinin; PMA, phorbol myristate acetate.
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and heparin from Calbiochem (USA); Ficoll-Paque from Pharmacia (Sweden). For all pharmacologically active substances, we first determined optimal concentrations at which maximum effect was demonstrated at minimum toxicity during the whole experiment. Plastic ware was from Nunc (Denmark). All mAb were from Immunotech (France).

Donor blood. Basic blood analysis of all donors and cell immune status defined with mAb combinations to CD3/CD19, CD3/CD4, CD3/CD8, CD45/CD14, (CD16 + CD56)/CD3, CD3/CD25, and CD3/HLA-DR corresponded to those of healthy people [9]. The values of $[Ca^{2+}]_e$ of all donors corresponded to the norm for healthy people. Basic blood analysis was done on an ACT 5 DIFF hematological analyzer (Beckman-Coulter, USA).

Mononuclear cells were separated from heparinized donor blood by centrifugation on Ficoll-Paque with buoyant density 1.077 g/cm^3 by the usual method. Mononuclear cell fraction was collected from the interphase boundary and washed with PBS twice by centrifugation.

Cells resistant to the action of ionomycin were separated by the method described in [10]. Mononuclear cells were incubated at 37°C in a humid atmosphere containing 5% CO_2 with ionomycin (2 μM) for 10 min in Hanks' solution with 10% FBS. After incubation with ionomycin, cells were washed twice with Hanks' solution with 10% FBS to remove the ionophore. Ionomycin-sensitive and ionomycin-resistant (IR) cells were separated by centrifugation on Ficoll-Paque with buoyant density 1.077 g/cm^3. All cells passing through the Ficoll-Paque layer were assumed to be sensitive to ionomycin. They normally did not survive. The cells that stayed on the interphase boundary are described as ionomycin-resistant. They were collected from the interphase boundary and washed with PBS with 5% FBS with collection by centrifugation.

Qualitative analysis of T-cell proliferation response to mitogenic stimulus. Separated mononuclear cells were analyzed by a standard protocol using MTT [11]. (The use of MTT for analyzing cell response to mitogenic stimulus does not provide strict quantitative estimation.) Cells were cultivated in 96-well plates in a humid atmosphere with 5% CO_2 for 72 h at 37°C in clear RPMI-1640 medium with L-glutamine, $NaHCO_3$, and 10% FBS. For T-cell stimulation we used: concanavalin A (ConA) (2.5 μg/ml), haricot bean phytohemagglutinin (PHA) (5 μg/ml), and mixture of PMA (50 ng/ml) with ionomycin (2 μM). The number of live cells and value of stimulation index was by determined by the method described in [11]. Optical density was measured using a Multiscan Plus plate reader (LKB, Finland) at 540 nm. The optical density of wells with cells without stimulation was taken as the control.

Flow cytofluorimetry. For cell phenotypic analysis, EPICS XL MCL flow cytofluorimeter (Coulter, USA) was used. Necessary logical restrictions were introduced into histograms of cell distribution by small-angle and 90° light scattering to correctly exclude from analysis all particles that were not in congruence with living lymphocytes by size and granularity. Propidium iodide staining and separated cells sensitive to ionomycin were used for precise localization of dead lymphocytes. The borders of "lymphocyte window" were also controlled by staining of cells with mAb to CD45/CD14. The data were analyzed using the WinMDI program (USA). In each sample, we analyzed not less than 10^4 cells. We used mAb for isotypic control in experiments. The use of flow cytometry allows us to analyze T-cell populations in human peripheral blood (HPB). Thus, further we do not mentioned specifically that "T-cells" means "T-cells as components of HPB".

T-Cells were activated by PMA (50 ng/ml) with ionomycin (2 μM) in 100 μl of heparinized blood. Added substances together constituted less than 7% of the final volume. Specific amounts of dimethylsulfoxide and PBS were added to control samples. Before stimulation, cells were incubated with EGTA for 30 min in 5% CO_2 atmosphere at 37°C. Intracellular molecules IL-2, IFN-γ, and CD69 were evaluated with an Immunotech kit for their determination according to the producer's instructions.

RESULTS

A mixture of PMA and ionomycin is used for T-cell activation [12-14]. Both substances interact directly with cell signal systems bypassing antigen receptor. In this combination ionomycin is responsible for changing intracellular calcium concentration ($[Ca^{2+}]_i$), and PMA – for protein kinase activation. The emergence of CD69 molecules on the surface of T-cells is recognized as a phenotypic sign of early stages of T-cell activation [13]. Further stages are characterized by the expression of CD25 molecules [13]. The production of IL-2 and IFN-γ by T-cells can be used as functional criterion of their activation. This criterion was used for analyzing the effect of $[Ca^{2+}]_e$ on T-cell activation by PMA with ionomycin. The shape of the titration curve of HPB with EGTA (Fig. 1) is a starting point for analyzing all results, and all obtained dependencies were compared with it. The expected result before the beginning of the work was similarity of shape of the curve for T-cell activation versus $[Ca^{2+}]_e$ with the above curve, but in fact this was not observed. Adding 1 mM EGTA decreased $[Ca^{2+}]_e$ to its lower limit in normal conditions in HPB (0.38 mM). The lowest $[Ca^{2+}]_e$ value in HPB (0.25 mM) was described for patients with acute sepsis [15, 16]. Thus, lower $[Ca^{2+}]_e$ values are considered to be non-physiological.

The absence of ionomycin does not influence the ability of PMA to increase the share of $CD3^+CD69^+$ cells among T-lymphocytes from 1-2% in HPB of healthy

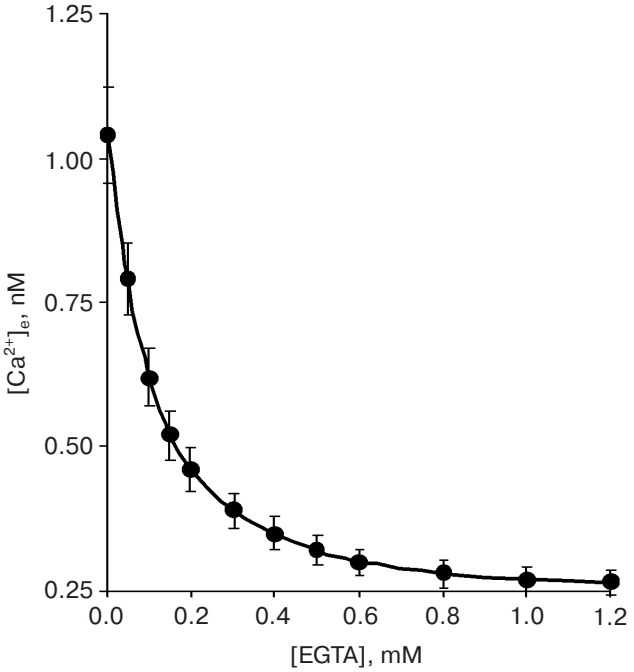

Fig. 1. Typical titration curve of healthy donor HPB with increasing concentrations of EGTA. Concentration of extracellular calcium ions was measured with a calcium-selective electrode. One of 20 experiments is shown.

donors up to nearly 100% level. (No differences were found in the effect of PMA on $CD3^+CD8^+$ and $CD3^+CD8^-$ T-cells.) During the first 2 h of cell incubation with PMA, the share of $CD3^+CD69^+$ T-cells and the density of CD69 molecules on them (Fig. 2, a and b) increased to a maximum, where they stayed for more than 12 h. In the presence of brefeldin A, an inhibitor of protein exit from the Golgi apparatus, CD69 molecules are accumulated in cells [13]. The kinetics of this process has a shape similar to that shown on Fig. 2, a and b (data not presented). The decrease of number of CD4 molecules on T-cells is always registered in parallel with that process (Fig. 2c). The effect of PMA on T-cells was slightly dependent on $[Ca^{2+}]_e$ decrease achieved using EGTA. PMA alone did not induce IL-2 and IFN-γ production in T-cells and expression of CD25 molecules on them. Comparable results were obtained for five donors, and they are in good accordance with data for healthy people [12-14]. So, the synthesis of CD69 molecules by T-cells first of all reflects successful activation of protein kinases C in them, and is almost independent from the presence of ionomycin or $[Ca^{2+}]_e$ value in the physiological range.

Ionomycin imitates well one of the early stages of T-cell activation. It quickly releases the calcium depot inside T-cells and brings further sharp increase in $[Ca^{2+}]_i$ through the entering of this ion from outside [3]. This chain of events has been known for a long time as a crucial point of Ca^{2+}-dependent reactions in T-cell activation *in vivo* [17, 18]. Ionomycin by itself has a cytotoxic activity on T-cells [10, 19-21]. (Naive $CD4^+CD45RA^+CD45R0^-$ T-cells are sensitive to ionomycin, and $CD4^+CD45RA^-CD45R0^+$ memory T-lymphocytes are resistant to it [10].) In the absence of PMA, ionomycin does not increase the share of $CD3^+CD69^+$ and $CD3^+CD25^+$ cells in the population or their production of IL-2 and IFN-γ (data not provided). Brefeldin A did not inhibit its action on T-cells (data not provided). The following tests were used for analyzing the effect of ionomycin on T-cells: reaction flow in medium with Ca^{2+}, its sensitivity to Ca^{2+} removal with EGTA, and restoration of the process after $[Ca^{2+}]_e$ was brought back to normal conditions. (This approach provides almost total reversibility of the changes.) The incubation of T-cells in Hanks' medium with 4 mM EGTA for 1 h at 37°C did not influence their vitality or composition. Further adding of ionomycin (5 μM) did not influence their vitality (data not provided). (Adding 5 mM EGTA

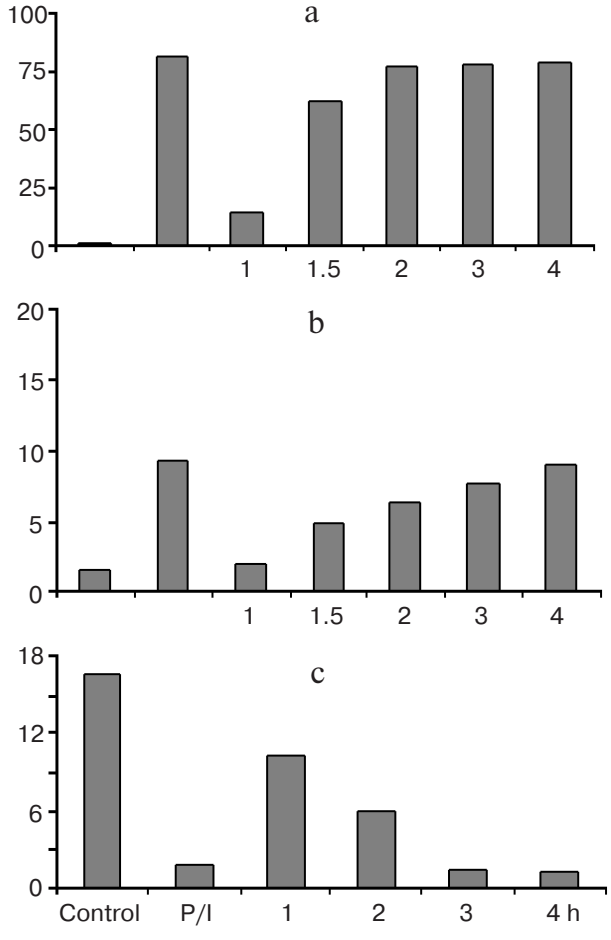

Fig. 2. Changes in HPB T-cell population in response to PMA within the first 4 h of incubation. a) Share of $CD3^+CD69^+$ cells in percent of the total number of $CD3^+$ T-lymphocytes; b) density of CD69 molecules on the surface of $CD3^+$ T-cells in arbitrary units (MEAN); c) density of CD4 molecules on T-cells in arbitrary units (MEAN). P/I, mixture of PMA with ionomycin.

"washes out" calcium ions only from activated T-cells [22].) Similar results were obtained for five donors and are in good accordance with data on the effect of ionomycin on T-cells depending on $[Ca^{2+}]_e$ value [23]. Therefore, calcium ions are necessary in the outer medium for ionomycin cytotoxic activity on T-cells.

For the same cell samples, we evaluated on a qualitative level their ability for proliferative response to ConA, PHA, and a mixture of PMA with ionomycin (Fig. 3). Incubation of cells in Ca^{2+} free medium even after adding ionomycin did not influence the ability of ConA to stimulate their proliferation. For IR T-cells the absence of response to ConA (stimulation index was not higher than 1.03 ± 0.05) is in good accordance with the earlier obtained data [20]. It is likely that all T-cells retain their ability to respond to ConA after exposure to ionomycin in calcium-free medium. The response of the IR population to other stimuli was significantly higher than that of common T-cells (Fig. 3). Similar results were obtained for five donors and are in good accordance with data for healthy people [9, 24]. Therefore, all T-cells retain their ability to response to mitogenic stimuli after incubation with EGTA.

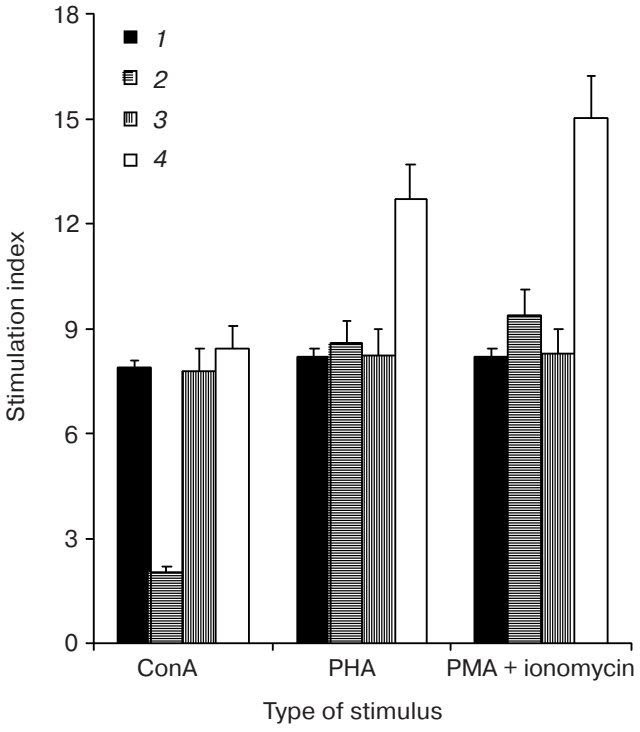

Fig. 3. Comparative analysis of the ability of HPB mononuclear cell population and its IR fraction for proliferative response to polyclonal mitogenic stimuli before and after exposure to ionomycin depending on a mode of cell incubation with EGTA. Results of one of three independent experiments on HPB of healthy donors are shown. Designations: *1*) original population of HPB mononuclear cells in Hanks' medium; *2*) separated IR population of HPB cells in Hanks' medium; *3*) original population of HPB mononuclear cells incubated in PBS with 5 mM EGTA; *4*) population of HPB mononuclear cells incubated in PBS with 5 mM EGTA, after exposure to 2 μM ionomycin.

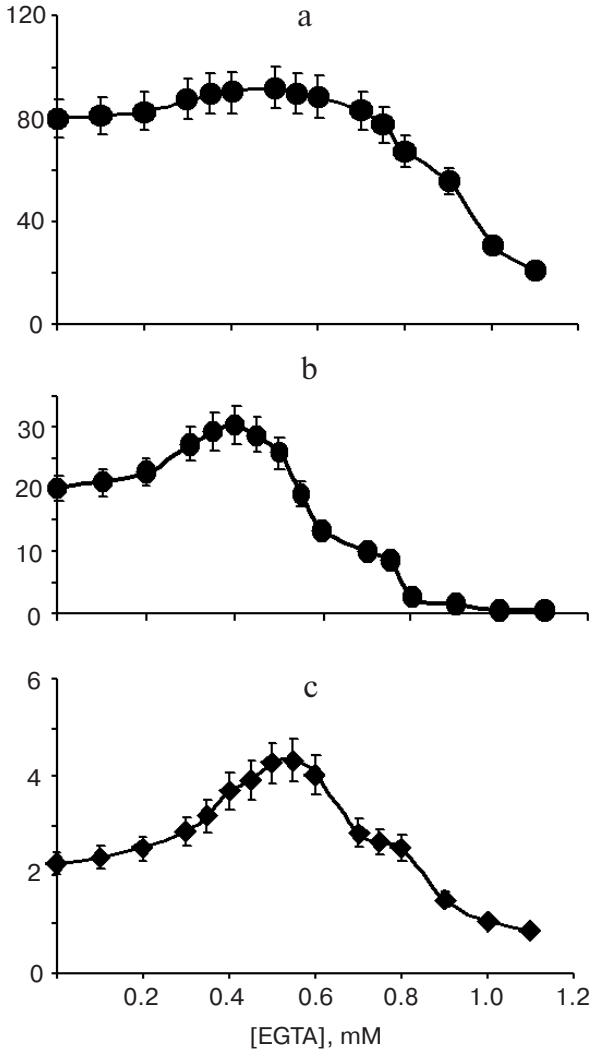

Fig. 4. Dependence of the shares of $CD3^+CD69^+$ T-cells (a) and $CD3^+CD69^+(IL\text{-}2)^+$ T-cells (b) and the number of IL-2 molecules per cell evaluated by the MEAN parameter (c) on $[Ca^{2+}]_e$.

PMA with ionomycin had a qualitatively different influence on T-cells than each of the reagents alone. T-Cells activated by a mixture of PMA with ionomycin synthesized molecules of IL-2, IFN-γ, CD69, and CD25. The decrease in number of CD4 molecules on the surface of T-cells under the influence of PMA was not abolished by ionomycin (data not provided). Similar results were obtained for 10 donors and are in good accordance with data for healthy people [12-14].

The T-cell activation by PMA with ionomycin (Fig. 4a) was studied depending on $[Ca^{2+}]_e$. Decreasing $[Ca^{2+}]_e$ to the lower limit of normal condition for healthy people ($[EGTA] \leq 0.4$ mM) is accompanied by the appearance of wide maximum of the share of $CD3^+CD69^+$ T-cells in the population. Only at $[Ca^{2+}]_e$ values lower than 0.25 mM, a

decrease in the share of $CD3^+CD69^+$ T-cells and in density of CD69 molecules on them was noted. Comparable results were obtained for 10 donors. It is most likely that the appearance of $CD3^+CD69^+$ T-cells is not inhibited by decreasing $[Ca^{2+}]_e$ to the limit of its physiological values.

The synthesis of IL-2 and IFN-γ by activated T-cells depends on $[Ca^{2+}]_e$ in a different way than the expression of CD69 molecules (Fig. 4a). From 20 to 35% of $CD3^+CD69^+$ T-cells synthesized IL-2 after activation depending on the donor. The maximum position of the share of $CD3^+CD69^+(IL\text{-}2)^+$ T-cells coincided with the lower limit of $[Ca^{2+}]_e$ in normal condition for healthy people (Fig. 4b). Besides, on increasing [EGTA] up to 0.4 mM the share of $CD3^+CD69^+(IL\text{-}2)^+$ T-cells significantly increased by more than one third on average (Fig. 4b). Further increase in [EGTA] led to a sharp decrease in the share of $CD3^+CD69^+(IL\text{-}2)^+$ T-cells. The bell-shaped curve of this dependence points to cooperativeness of this process. The dependence of a number of IL-2 molecules per T-cell expressed in MEAN values on $[Ca^{2+}]_e$ (Fig. 4c) generally coincides by shape with the curve in Fig. 4b, but its maximum is in the region of $[Ca^{2+}]_e$ below the normal limit. Comparable results were obtained for seven donors. It is most likely that the production of IL-2 by activated $CD3^+CD69^+$ T-cells has its maximum at the border of normal condition and pathology and sharply decreases at further decrease in $[Ca^{2+}]_e$ values.

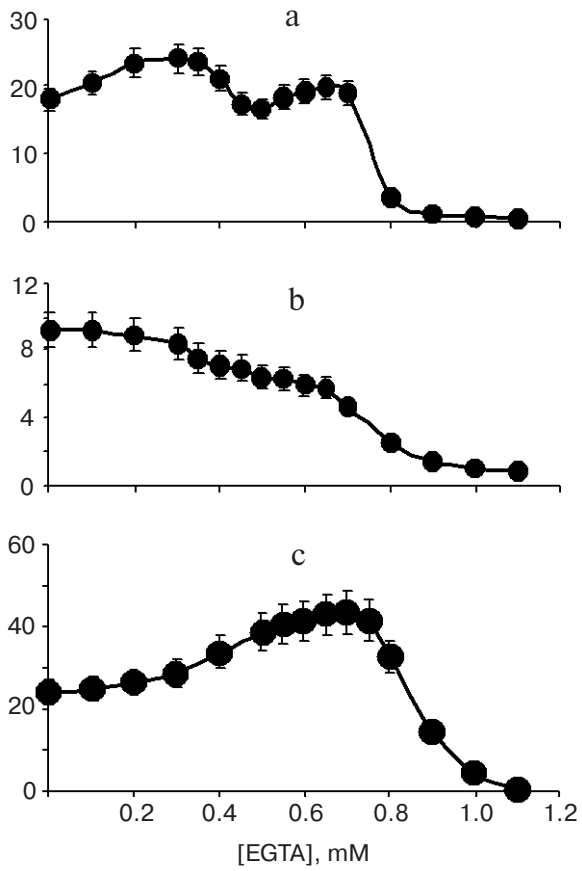

Fig. 5. Dependence of the share of $CD3^+CD69^+(IFN\text{-}\gamma)^+$ T-cells (a), of the number of IFN-γ molecules per cell evaluated by the MEAN parameter (b), and the share of $CD3^+CD69^+(IFN\text{-}\gamma)^+$ cells in the fraction of IR T-cells (c) on $[Ca^{2+}]_e$.

As in the case with IL-2, only 15-25% of $CD3^+CD69^+$ T-cells after activation synthesized IFN-γ, and this share depended on the particular donor. The dependence of the share of $CD3^+CD69^+(IFN\text{-}\gamma)^+$ T-cells on $[Ca^{2+}]_e$ is quite complicated—it has two maxima of almost equal intensity. The first is situated within the normal $[Ca^{2+}]_e$ range, but close to its lower limit. The second is in the region between the lower normal limit and minimum physiological values for $[Ca^{2+}]_e$. The location of the minimum between them most often coincides with the lower normal limit. When $[Ca^{2+}]_e$ value was decreased below the lower physiological limit, there were no $CD3^+CD69^+(IFN\text{-}\gamma)^+$ T-cells left in the population. The curve of the dependence of a number of IFN-γ molecules per cell on $[Ca^{2+}]_e$ (Fig. 5b) does not coincide by shape with the curve in Fig. 5a. For this parameter, a quite monotonous decrease with increase in [EGTA] and, correspondingly, $[Ca^{2+}]_e$ decrease below the lower physiological value (0.25 mM) was observed. Similar results were obtained for seven donors. It is most likely that different populations of $CD3^+CD69^+$ T-cells have various abilities to produce IFN-γ depending on $[Ca^{2+}]_e$.

The most probable candidate for the role of T-cells producing IFN-γ at $[Ca^{2+}]_e$ below physiological limit is memory IR T-lymphocytes. To check this suggestion, separated IR cells were activated by PMA with ionomycin at different $[Ca^{2+}]_e$ values (Fig. 5c). For this fraction, enriched with memory T-lymphocytes, we got a broad bell-shaped curve of the dependence of the share of $CD3^+CD69^+(IFN\text{-}\gamma)^+$ T-cells on $[Ca^{2+}]_e$ with a maximum below normal limit for healthy people. Comparable results were obtained for five donors. The second maximum is probably related to T-cells with a modified system of Ca^{2+}-signaling [19-21].

The appearance of $CD3^+CD25^+$ T-cells is considered as a sign of the middle stage of T-lymphocyte activation [25]. Basic changes in the share of $CD3^+CD25^+$ cells were exhibited 24 h after the beginning of activation and are in good accordance with data for healthy people [13]. More than half of the activated T-cells expressed CD25 molecules. The dependence of the share of $CD3^+CD25^+$ T-cells on [EGTA] (Fig. 6) is very similar in shape to that described above for IFN-γ (Fig. 5a), but has a far less expressed local minimum. Also, it is "shifted" to the lower $[Ca^{2+}]_e$ values on the abscissa axis. The CD25 molecules appear on activated T-cells over a wide range of $[Ca^{2+}]_e$ concentrations with a poorly expressed maximum near the border of normal and non-physiological $[Ca^{2+}]_e$

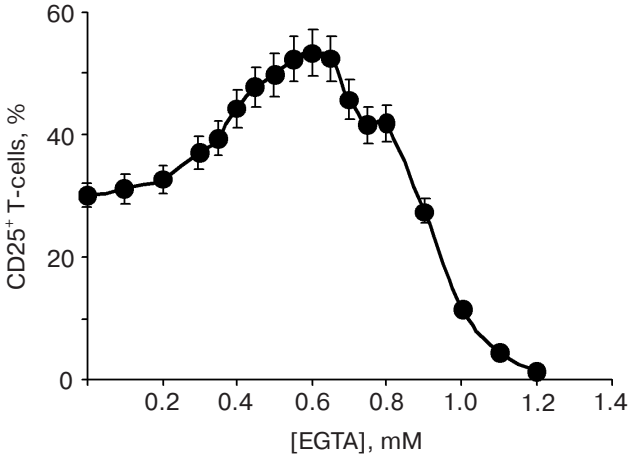

Fig. 6. Dependence of the share of $CD3^+CD69^+CD25^+$ T-cells activated by PMA with ionomycin on $[Ca^{2+}]_e$.

values. Similar results were obtained for five donors. So, this stage of T-cell activation is slightly dependent on $[Ca^{2+}]_e$.

DISCUSSION

The majority of works studying T-cell activation are done using mitogenic lectins, mixture of PMA and ionomycin, or mAb against CD3 *in vitro* [26, 27]. Equally often they use cell lines, like Jurkat, as T-cells, and just a few works have been done on whole blood or cells separated from it. The activation of T-cells *in vivo* proceeds in a microenvironment that is difficult to model under experimental conditions. (If even the presence of erythrocytes significantly influences the intensity of T-lymphocyte response on mAb to CD3 [28], then "participation" of other blood cells in this response is difficult to describe reliably.) The correspondence of principles determined for model systems *in vitro* with reactions taking place in an organism *in vivo* is widely discussed, but everybody understands the risk of presupposing their full identity. Thus, the majority of experiments in this work were done with HPB.

The use of PMA and ionomycin allows us to independently evaluate the role of each of two signals. PMA prevents cytotoxic action of ionomycin on naive $CD4^+$ T-cells. In contrast, ionomycin does not prevent the effect of PMA on CD4 molecules of T-cells. The mixture of PMA and ionomycin fully activates T-lymphocytes—it increases the share of $CD3^+CD69^+$ and $CD3^+CD25^+$ T-cells and their production of IL-2 and IFN-γ.

The sensitivity of T-cell activation to decreasing $[Ca^{2+}]_e$ to 0.1 mM and lower is recognized by most studies. However, there are conflicting opinions about the dependence of CD69 molecules expression by activated T-cells on $[Ca^{2+}]_e$, although in all works they have checked the sensitivity of this process to high doses of EGTA (5-10 mM). Most often data obtained on constantly dividing cells (thymocytes) [29, 30] contradict studies on cells separated from HPB, most of which are in the state of rest [12-14]. Results obtained in this study indicate independence of CD69 molecule expression by activated T-cells from $[Ca^{2+}]_e$ in the interval from 1.2 to 0.25 mM. First, PMA increases the share of $CD3^+CD69^+$ T-cells even in the absence of ionomycin. Second, the appearance of CD69 molecules on T-cells is inhibited only when $[Ca^{2+}]_e$ is decreased below 0.25 mM. Third, ionomycin has practically no influence on the growth of the share of $CD3^+CD69^+$ T-cells induced by PMA. It is most likely that the expression of CD69 molecules is a necessary but not sufficient condition of T-cell activation.

Activated T-cells produce IL-2 and IFN-γ in the range of $[Ca^{2+}]_e$ values from 1.2 to 0.25 mM. The maximal share of $CD3^+CD69^+(IL\text{-}2)^+$ T-cells is observed at significant $[Ca^{2+}]_e$ decrease from mid-normal values to pathology and takes place at about the border values between them (0.4-0.5 mM). The dependence of share of activated $CD3^+CD69^+(IFN\text{-}\gamma)^+$ T-cells on $[Ca^{2+}]_e$ is presented as a curve with two maxima that together cover a wider interval of $[Ca^{2+}]_e$ values than in case of $CD3^+CD69^+(IL\text{-}2)^+$ T-cells. The presence of two maxima probably reflects the existence of different subpopulations of IFN-γ-producing T-cells that have unequal sensitivity to $[Ca^{2+}]_e$. The first maximum is probably determined by naive T-cells. The second maximum is mainly connected with memory T-cells and cytotoxic T-lymphocytes. There are three arguments favoring this assertion. The second maximum is most expressed in the blood of elderly donors that have increasing with age number of memory T-cells [31]. For IR T-cells the maximum of the share of $CD3^+CD69^+(IFN\text{-}\gamma)^+$ cells is observed in the interval of $[Ca^{2+}]_e$ close to pathological. For separated $CD4^+CD45RA^-CD45R0^+$ memory T-cells there was also shown a decrease in the dependence of their proliferation and IL-2 production on $[Ca^{2+}]_e$ in response to mAb to CD3 molecules in comparison with naive $CD4^+CD45RA^+CD45R0^-$ T-cells of the same donor [32]. It is likely that the production of IFN-γ by memory T-cells has an optimum at lower $[Ca^{2+}]_e$ values compared to the naive precursors of these cells.

On the next activation stage, T-cells express CD25 molecules. This stage if supposed to be one of the main stages in the development of cell and humoral response [13-15]. After activation by a mixture of PMA and ionomycin, the growth of the share of $CD3^+CD69^+CD25^+$ T-cells takes place in a wider $[Ca^{2+}]_e$ range. According to published data [32], the influence of decreasing $[Ca^{2+}]_e$ to 0.1 mM and lower using EGTA is strongly dependent on the time of adding the chelator after the beginning of T-cell stimulation. The earlier from the beginning of induc-

tion $[Ca^{2+}]_e$ value was lower than 0.1 mM, the stronger it influenced the increase in the share of $CD3^+CD25^+$ T-cells in the population [23]. The presence of calcium ions in the outer medium is probably absolutely necessary for the induction of CD25 molecule synthesis *de novo* by naive T-cells during primary immune response. The expression of CD25 molecules on T-cells that had already past the induction stage (memory T-cells and cytotoxic T-lymphocytes) is significantly less dependent on EGTA [31-33]. For later activation stages, there was no significant effect of EGTA on the share of $CD25^+$ T-cells or the binding characteristics of IL-2 with its receptor [32, 34]. So, during differentiation of activated T-cells the sensitivity to $[Ca^{2+}]_e$ significantly decreases with each following stage, and this happens in parallel to the increase in T-lymphocyte resistance to the action of ionomycin [33].

The homeostasis parameters for HPB cells have been known for a long time, and they are assumed as most optimal for the functioning of the immune system. The $[Ca^{2+}]_e$ value in HPB is an important part of homeostasis. Upper 1.10 mM and lower 0.45 mM limits have been determined for it, and deviation from these values is considered as a sign of pathology [35]. Many acute and chronic infections, such as sepsis [15, 16, 36], AIDS or tuberculosis [37, 38], and also some leucosis [39], often bring significant deviations in $[Ca^{2+}]_e$ value in HPB from the norm.

The reactions of the immune system directed on elimination of factors generating them—against carriers of environmental influence and pathological conditions of the organism itself—have been named "immune response". So, the immune system should be enabled to make an effective response not only in the optimal state of the organism, most typical for healthy people, but also under pathological conditions. Some T-cell subpopulations might even specialize in functioning in such "non-physiological" pathological conditions. Otherwise, health and integrity of the organism as a whole would not be guarantied by the immune system.

A significant share of memory T-cell population has a modified system of calcium-dependent intracellular signalization and, at the same time, it has resistance to the action of ionomycin [10, 19, 20]. Earlier IR T-cells were described for mouse cervical node [19]. The use of laboratory animals gives a rare opportunity to make experiments by the method of adaptive transfer: separated and characterized populations of donor immune and intact (as a control) cells are transferred to naive syngeneic recipients, in which they check *in vivo* the properties of the transferred cells. (For obvious reasons it is not possible to obtain such data on people.) Only IR T-cells from spleen of mice, having a disease or being vaccinated, appeared to be able to adaptively transfer to naive recipients the protection from subsequent infection by *Mycobacterium tuberculosis* or *Neisseria meningitides* in corresponding models [40, 41]. The data directly suggest the ability of IR T-cells to accomplish basic functions of memory T-lymphocytes associated with recognition of the agent's antigen and fortify response to them *in vivo*, bringing effective protection from a pathogen. The majority of antigen-specific T-cells are also concentrated in the IR fraction [19].

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REFERENCES

1. Ringer, S. (1883) *J. Physiol.*, **4**, 29-42.
2. Mills, G. B., Cheung, R. K., Grinstein, S., and Gelfand, E. W. (1985) *J. Immunol.*, **134**, 1640-1643.
3. Gelfand, E. W., Cheung, R. K., Mills, G. B., and Grinstein, S. (1988) *Eur. J. Immunol.*, **18**, 917-922.
4. Modiano, J. F., Kelepouris, E., Kern, J. A., and Nowell, P. C. (1988) *J. Cell. Physiol.*, **135**, 451-469.
5. Kerschbaum, H. H., and Cahalan, M. D. (1999) *Science*, **283**, 836-839.
6. Kagi, D., Ledermann, B., Burki, K., Zinkernagel, R. M., and Hengartner, H. (1996) *Annu. Rev. Immunol.*, **14**, 207-232.
7. Chakrabarti, R., Chang, J. Y., and Erickson, K. L. (1995) *J. Cell Biochem.*, **58**, 344-359.
8. June, C. H., Ledbetter, J. A., Rabinovitch, P. S., Martin, P. J., Beatty, P. G., and Hansen, J. A. (1986) *J. Clin. Invest.*, **77**, 1224-1232.
9. Reimann, K. A., Chernoff, M., Wilkening, C. L., Nickerson, C. E., Landay, A. L., and the ACTG immunology advanced technology laboratories (2000) *Clin. Diag. Lab. Immunol.*, **7**, 352-359.
10. Khaidukov, S. V., Holodenko, I. V., and Litvinov, I. S. (2003) *Cytology*, **45**, 249-254.
11. Niks, M., Otto, M., Busova, B., and Stefanovic, J. J. (1990) *Immunol. Meth.*, **126**, 263-271.
12. Testi, R., Phillips, J. H., and Lanier, L. L. (1989) *J. Immunol.*, **143**, 1123-1128.
13. O'Neil-Andersen, N. J., and Lawrence, D. A. (2002) *Clin. Diagn. Lab. Immunol.*, **9**, 243-250.
14. Godoy-Ramirez, K., Frank, K., Mahdavifar, S., Andersson, L., and Gaines, H. (2004) *J. Immunol. Meth.*, **292**, 1-15.
15. Chesney, R. W., McCarron, D. M., Haddad, J. G., Hawker, C. D., DiBella, F. P., Chesney, P. J., and Davis, J. P. (1983) *J. Lab. Clin. Med.*, **101**, 576-585.
16. Zaloga, G. P., and Chernow, B. (1987) *Ann. Int. Med.*, **107**, 36-49.
17. Partiseti, M., le Deist, F., Hivroz, C., Fischer, A., Korn, H., and Choquet, D. (1994) *J. Biol. Chem.*, **269**, 32327-32335.
18. Le Deist, F., Hivroz, C., Partiseti, M., Thomas, C., Buc, H. A., Oleastro, M., Belohradsky, B., Choquet, D., and Fischer, A. (1995) *Blood*, **85**, 1053-1062.
19. Miller, R. A., Flurkey, K., Molloy, M., Luby, T., and Stadecker, M. J. (1991) *J. Immunol.*, **147**, 3080-3086.
20. Sigova, A., Dedkova, E., Zinchenko, V., and Litvinov, I. (1999) *FEBS Lett.*, **447**, 34-38.

21. Bard, E., Colwill, R., L'Anglais, R., and Kaplan, J. G. (1978) *Can. J. Biochem.*, **56**, 900-904.
22. Wolf, C. H. J., Akerman, K. E. O., and Andersson, L. C. (1985) *J. Cell. Physiol.*, **123**, 46-50.
23. Greene, W. C., Parker, C. M., and Parker, C. W. (1976) *Cell. Immunol.*, **25**, 74-89.
24. Fagnoni, F. F., Vescovini, R., Passeri, G., Bologna, G., Pedrazzoni, M., Lavagetto, G., Casti, A., Franceschi, C., Passeri, M., and Sansoni, P. (2000) *Blood*, **95**, 2860-2868.
25. Jonuleit, H., Schmitt, E., Stassen, M., Tuettenberg, A., Knop, J., and Enk, A. H. (2001) *J. Exp. Med.*, **193**, 1285-1294.
26. Whitney, R. B., and Sutherland, R. M. (1972) *J. Cell. Physiol.*, **80**, 329-337.
27. Nusslein, H. G., Frosch, K. H., Woith, W., Lane, P., Kalden, J. R., and Manger, B. (1996) *Eur. J. Immunol.*, **26**, 846-850.
28. Song, S., Goodwin, J., Zhang, J., Babbitt, B., and Lathey, J. L. (2002) *Clin. Diagn. Lab. Immunol.*, **9**, 708-712.
29. Mascarenhas, S. R., Echevarria-Lima, J., Fernandez dos Santos, N., and Rumjanek, V. M. (2003) *Life Sci.*, **73**, 1037-1051.
30. Simms, P. E., and Ellis, T. M. (1996) *Clin. Diag. Lab. Immunol.*, **3**, 301-304.
31. Sallusto, F., Mackay, C. R., and Lanzavecchia, A. (2000) *Annu. Rev. Immunol.*, **18**, 593-620.
32. Schwinzer, R., and Siefken, R. (1996) *Transpl. Immunol.*, **4**, 61-63.
33. Khaidukov, S. V., and Litvinov, I. S. (2005) *Biochemistry (Moscow)*, **70**, 692-702.
34. Bard, E., Colwill, R., L'Anglais, R., and Kaplan, J. G. (1978) *Can. J. Biochem.*, **56**, 900-904.
35. O'Regan, S., Carson, S., Chesney, R. W., and Drummond, K. N. (1977) *Blood*, **49**, 345-353.
36. Burchard, K. W., Simms, H. H., Robinson, A., DiAmico, R., and Gann, D. S. (1992) *Arch. Surg.*, **127**, 265-272.
37. Sullivan, J. N., and Salmon, W. D., Jr. (1987) *South. Med. J.*, **80**, 572-576.
38. Beck, K. W., Schramel, P., Hedl, A., Jaeger, H., and Kaboth, W. (1990) *Biol. Trace Elem. Res.*, **25**, 89-96.
39. Yoshida, A., Ueda, T., Tanaka, Y., Imura, S., Konishi, F., Uchida, M., Kita, K., and Nakamura, T. (1991) *Rinsho Ketsueki*, **32**, 404-408.
40. Nikonenko, B. V., Khaidukov, S. V., Litvinov, I. S., Bocharova, I. V., and Apt, A. S. (2001) *Byul. Eksp. Biol. Med.*, **131**, 548-549.
41. Khaidukov, S. V., Agafonova, S. A., and Litvinov, I. S. (2006) *Membr. Biol. (Moscow)*, **23**, 32-41.