Biochimica et Biophysica Acta, 1019 (1990) 159–165
Elsevier

BBABIO 43265

Effect of cyclosporin A on the membrane potential and Ca^{2+} level of human lymphoid cell lines and mouse thymocytes

György Vereb, Jr., György Panyi, Margit Balázs, László Mátyus, János Matkó and Sándor Damjanovich

Department of Biophysics, University Medical School of Debrecen, Debrecen (Hungary)

(Received 30 October 1989)
(Revised manuscript received 26 April 1990)

Key words: Cyclosporin A; Indo-1; Quin2; Membrane potential; (Human lymphoid cell lines); (Mouse thymocytes);

The effect of the immunosuppressive cyclosporin A (CsA) on the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and membrane potential of human B and T lymphoblastoid cells and mouse thymocytes was studied in order to reveal some features of the early stage of drug–cell interaction. Cytosolic free Ca^{2+} concentration of the cells was measured by spectrofluorimetry using indo-1 and quin2 fluorescent calcium indicators. Membrane potential was monitored in a flow cytometer with oxonol dye. CsA applied at 2–20 μg/ml final concentrations caused a dose-dependent, rapid, transient rise of $[Ca^{2+}]_i$ in all cell types. This effect could be blocked by chelating the extracellular Ca^{2+} with EGTA but was not sensitive to Ca^{2+} channel blockers verapamil and nifedipine or K^+ channel blocker 4-aminopyridine. A possible explanation for the calcium mobilizing effect of CsA is an ionophore-like mode of action at the cell membrane level. Besides directly interfering with mitogenic signals, the elevation of $[Ca^{2+}]_i$ could be responsible for an initial hyperpolarization observed in CsA-treated T lymphocytes. This hyperpolarization, however, was not detectable in B lymphoblastoid cells. A further difference between B and T cells was the diverse pattern of depolarization following CsA treatment. This variance in the behaviour of T and B lymphocytes and the diversity of membrane transport systems in its background could account for the different final outcome of the drug–cell interaction.

Introduction

Cyclosporins, a family of cyclic endecapeptide antibiotics, were introduced by Borel and co-workers in 1976 as strong inhibitors of allograft rejections in solid organ transplantation [1,2]. Cyclosporin A, the variant discovered first and studied to the greatest extent, exerts a selective inhibitory effect on cytotoxic, helper, and delayed type hypersensitivity T lymphocyte function, while suppressor T cells are relatively unaffected [3–6]. Despite widespread investigations, the cellular and molecular mechanisms of this effect have not yet been clarified.

Owing to its lipophylic character, CsA is incorporated into the cell membrane rather than binding to a specific cell-surface receptor [7]. This alone can affect the viscosity of the membrane [8,9], and thus might influence the regulation of the immune system [10,11]. In addition, correlation of viscosity, transmembrane potential, cytosolic calcium concentration and membrane protein dynamics and function have been demonstrated in several cases [11]. All of these have an important role in the regulation of cell metabolism and proliferation, and the modulation of transmembrane signalling during lymphocyte activation. In turn, the elucidation of the changes in these properties upon the effect of CsA could be a key to finding the mechanism of action and selectivity of the drug.

During lymphocyte activation the minimum 2-fold rise of $[Ca^{2+}]_i$ is indispensable [12], although not sufficient [13]. This rise can be prevented by CsA after a 30 min preincubation according to Gelfand and co-workers [14], but many investigators reported findings con-

Abbreviations: CsA, cyclosporin A; $[Ca^{2+}]_i$, cytosolic free calcium concentration; $DiBaC_4(3)$, (bis(1,3-dibutylbarbituricacid-(5)-trimetheneoxonol); $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; DMSO, dimethylsulfoxide; FCS, fetal calf serum (heat inactivated); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IL-2, interleukin 2; indo-1/AM, indo-1 acetoxymethylester; PK-C, protein kinase C; quin2/AM, quin2-acetoxymethylester.

Correspondence: Gy. Vereb, Jr., Department of Biophysics, University Medical School of Debrecen, P.O.B. 3, H-4012 Debrecen, Hungary.

tradictory to this [15–17]. Also, there were clues suggesting that the action of CsA may not only take place via interference with normal activation pathways [18–20] but that the drug could have effects of its own. We have shown earlier that mouse [21] and human [22] lymphocytes are depolarized by CsA in a dose-dependent manner as revealed by $DiOC_6(3)$. Also, the level of cytosolic free calcium, detected by quin2, was increased by 2 μg/ml CsA [22], the uppermost limit of therapeutic blood level [23].

Regarding the great importance of membrane potential and $[Ca^{2+}]_i$ in the regulatory processes, our aim was to elucidate the immediate effect of CsA on these factors also with respect to their possible correlation. Our experiments were performed on human T and B lymphoblastoid cell lines and mouse thymocytes applying the oxonol derivative $DiBaC_4(3)$, less toxic than $DiOC_6(3)$, for detecting the membrane potential, and indo-1, a fluorescent Ca^{2+} chelator more sensitive than quin-2, for the determination of $[Ca^{2+}]_i$.

Materials and Methods

Cells. Thymic lymphocytes were prepared from freshly killed 3–4-week-old BALB/c mice. After filtration and Ficoll gradient centrifugation (1070 g/l, 10 min, $3000 \times g$) they were suspended in buffer A containing 10 mM Hepes (Fluka AG), 140 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose at pH 7.4. JY cells, a human B lymphoblastoma line; HUT-78 cells, a human T lymphoma line and P3HR-1 cells, which are human Epstein-Barr virus genome-positive lymphoblastoid B cells, were cultured in RPMI-1640 medium (Sigma), supplemented with 10% FCS, 2 mM L-glutamine, penicillin-G and streptomycin at 37 °C in 5% CO_2 atmosphere. Cells harvested in the logarithmic phase were washed twice in buffer A. Viability was above 95% as routinely tested by propidium iodide exclusion.

Measurement of cytosolic Ca^{2+} concentration. $[Ca^{2+}]_i$ was measured using the method described in Ref. 24. Lymphocytes ($4 \cdot 10^7$ cells/ml) were suspended in buffer A and incubated with 10 μM quin2/AM for 30 min at 37 °C and for another 30 min after a 10-fold dilution. Indo-1/AM was applied similarly but at a concentration of 1 μM and only for 30 min. Cells were washed twice, resuspended in buffer A at a concentration of 10^6 cells/ml and stored on ice before fluorescence measurements took place in a 1×1 cm rectangular quartz cuvette held at 37 °C. Fluorescence signals were recorded in a Hitachi MPF-4 fluorescence spectrophotometer. Excitation and emission wavelengths were 339 and 490 nm for quin2, 340 and 390 nm for indo-1, with 5 and 10 nm slits, respectively. Calibration for each sample was performed as described earlier [25–27].

Determination of the membrane potential in a flow cytometer. Flow cytometric membrane potential measurements were carried out in a modified FACS III Becton-Dickinson flow cytometer. 10^6 cells/ml in buffer A were incubated at 37 °C with 150 nM final concentration of oxonol ($DiBaC_4(3)$), (Molecular Probes, U.S.A.) for 3 min and the fluorescence histograms were taken thereafter in the flow cytometer.

The laser was tuned to 488 nm with 160 mW constant output. The flow rate of cells was 500/s to get better resolution. Forward-angle light scatter (FALS) and right-angle fluorescence (using a 520 nm long-pass glass filter) were measured simultaneously for each cell. Each histogram contains data from 10^5 cells. The fluorescence signal collection was restricted to live cells by gating on FALS. Different extracellular K^+ concentrations ranging from 3 mM to 140 mM were set to obtain a calibration curve of fluorescence intensity versus membrane potential. Gramicidin (2 μg/ml) was used to reach total depolarization of the membrane. CsA was added to oxonol equilibrated cells immediately before analysis or 10–60 min prior to addition of oxonol, when indicated.

The system was calibrated with standard samples of fixed chicken erythrocytes in order to obtain comparable results.

Cyclosporin A. Cyclosporin A, a generous gift from Prof. J.F. Borel (Sandoz, Switzerland), was dissolved in DMSO at a concentration of 10 mg/ml. The final concentration of DMSO in the samples never exceeded 0.5%.

DMSO and 4-aminopyridine were from Merck (Darmstadt, F.R.G.). Ionomycin from Calbiochem (San Diego, CA), all other chemicals were from Sigma unless otherwise indicated.

Results

Determination of cytosolic free calcium concentration with quin2 and indo-1 fluorescent chelators

HUT-78 cells were loaded with acetoxymethylester derivatives of quin2 or indo-1 dyes. Fig. 1 shows a typical measurement of cytosolic calcium level in the presence of CsA. The calibration with ionomycin and Mn^{2+} indicates that 2 μg/ml CsA increases the cytosolic Ca^{2+} level by approx. 70 nM. The transiently increased cytosolic calcium level returns to the resting level with a half-life of less than 1 min. The applied concentration of manganese chloride (0.4 mM) was optimal for maximal quenching of fluorescence without observable increase in light scattering.

Fig. 2 shows the effect of 2 μg/ml CsA on the $[Ca^{2+}]_i$ of three different cell types as measured by indo-1 (panel A) and quin2 (panel B). Human T (HUT-78), and B (P3HR-1) lymphoblastoid cells and mouse (BALB/c) thymocytes were compared. The transient

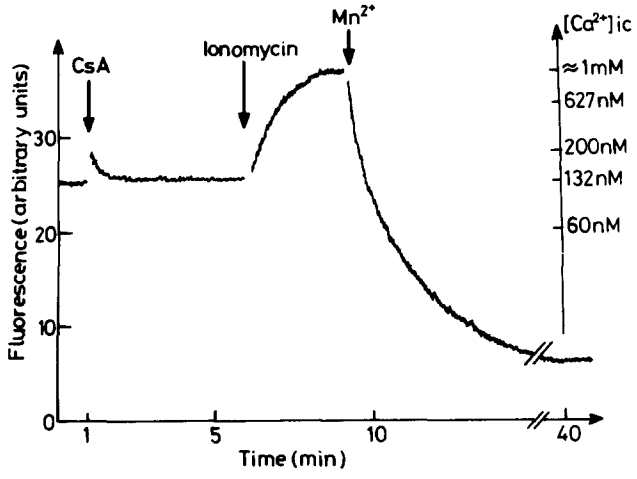

Fig. 1. Measurement of cytosolic free calcium level of HUT-78 lymphocytes. Cells were loaded with quin2 fluorescent chelator as described in Materials and Methods. The trace shows the fluorescence intensity (excitation: 339 nm; emission 492 nm) of $2 \cdot 10^6$ cells in 2 ml buffer A in a 1×1 cm rectangular cuvette thermostated at 37°C. Arrows indicate the addition of CsA (10 μg/ml), ionomycin (1.5 μg/ml) and $MnCl_2$ (0.5 mM). The cytosolic calcium concentration ($[Ca^{2+}]_i$) shown on the right ordinate was calculated according to the equations described earlier [25–27].

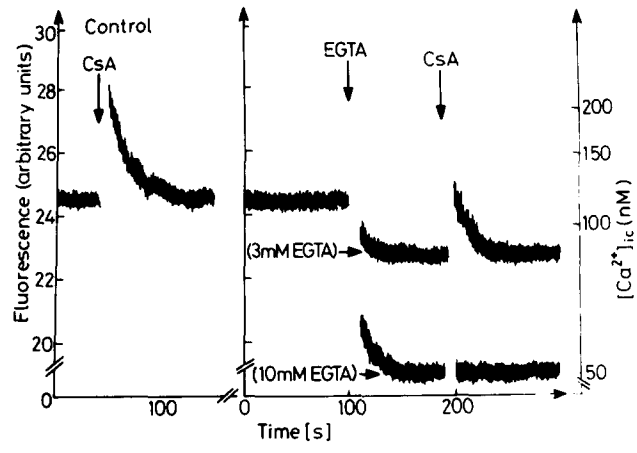

Fig. 3. Effect of CsA on cytosolic Ca^{2+} level of HUT-78 cells in the presence and absence of EGTA. The loading of cells with indo-1, and the measurement of $[Ca^{2+}]_i$ was carried out as described in Materials and Methods. The left panel shows the fluorescence intensity (left axis) and the $[Ca^{2+}]_i$ (right axis on the right panel) of a representative sample before and after addition of 2 μg/ml CsA (addition of CsA is indicated by an arrow). The right panel shows the same parameters ($[Ca^{2+}]_i$ and fluorescence intensity) of a representative sample treated with EGTA. Addition of EGTA (3 and 10 mM) and CsA (2 μg/ml) is indicated by arrows. The buffer contained 1 mM Ca^{2+}. $[Ca^{2+}]_i$ values were calculated according to the equations described earlier [25–27].

rise of cytosolic calcium concentration caused by CsA could be detected in all cell types using indo-1, while quin2 revealed similar change only in T cells. Control experiments with DMSO, the solvent of CsA, were negative.

Effect of CsA on the cytosolic calcium concentration of HUT-78 and P3HR-1 cells as revealed by indo-1

Addition of 3 and 10 mM EGTA to the cell suspensions containing 1 mM Ca^{2+} decreased the fluorescence signal, thereby indicating a 30% and 65% decrease of

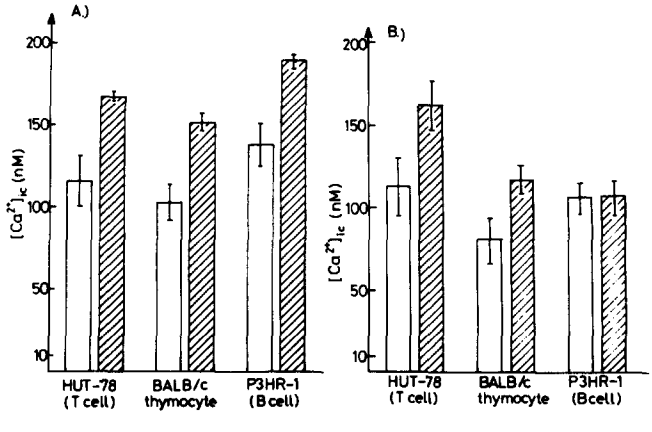

Fig. 2. Effect of CsA on cytosolic Ca^{2+} level of different cell types. The loading procedure for indo-1/AM or quin2/AM and the measurement of $[Ca^{2+}]_i$ was carried out as described in Materials and Methods. The peaks of transient increases in $[Ca^{2+}]_i$ induced by 2 μg/ml CsA (shaded columns) and the resting $[Ca^{2+}]_i$ (empty columns) are displayed as revealed by indo-1 (A) and quin2 (B) indicator. The error bars indicate the standard deviations ($n = 5–10$).

the $[Ca^{2+}]_i$, respectively. Consecutive administration of CsA revealed that the effect of the drug could be diminished by chelating the extracellular calcium with 3 mM EGTA and completely abolished by 10 mM EGTA. A representative measurement on HUT-78 cells is shown in Fig. 3. Restoring the 1 mM extracellular calcium concentration by excess Ca^{2+} totally restituted the resting level of cytosolic Ca^{2+} concentration and the effect of CsA was observable again (data not shown).

Dose dependence of the effect of CsA on the cytosolic Ca^{2+} is presented in case of HUT-78 and P3HR-1 cells in Fig. 4. The correlation coefficients of linear regressions were 0.985 and 0.974, respectively. The presence of 10^{-5} M verapamil or nifedipine, blockers of voltage-sensitive Ca^{2+} channels, or 10^{-4} M K^+-channel blocker 4-aminopyridine did not influence the effect of cyclosporin, either when added together with the drug, or after preincubation for 5 to 50 min. 50 μM La^{3+} or 1 mM Ni^{2+} applied 2 min before adding CsA diminished the peak of $[Ca^{2+}]_i$ by 70%, resulting in a value not significantly different from resting levels. Increasing amounts of DMSO did not change the $[Ca^{2+}]_i$ (data not shown).

Repeated administration of 10 μg/ml CsA to the same sample caused the rapid transient elevation of the $[Ca^{2+}]_i$ each time (Fig. 5). The equilibrium concentration of Ca^{2+} was reached at a higher level after each application of the drug. Addition of CsA after membrane permeabilization by ionomycin had no effect on the fluorescence intensity, indicating that the increased

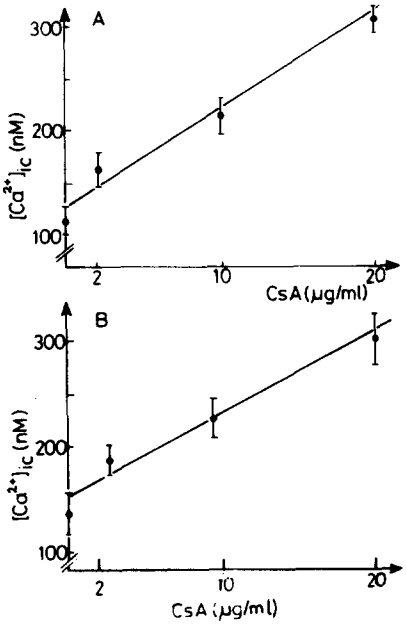

Fig. 4. Dose-dependent effect of CsA on $[Ca^{2+}]_i$. HUT-78 (A) and P3HR-1 (B) cells were loaded with indo-1 as described in Materials and Methods. CsA was added at final concentrations of 2, 10 and 20 μg/ml. The average peak values of calcium transients are shown with the error bars representing the standard deviation ($n = 5$–10). The correlation coefficient of linear regression was 0.985 for HUT-78 and 0.974 for P3HR-1 cells.

fluorescence was due to higher $[Ca^{2+}]_i$. A similar pattern was obtained when 2 ng/ml ionomycin was applied instead of 10 μg/ml CsA. Adequate controls with DMSO were negative (data not shown).

JY human lymphoma cells and BALB/c mouse thymocytes responded to cyclosporin A in a manner not significantly different from that observed in HUT-78 and P3HR-1 cells.

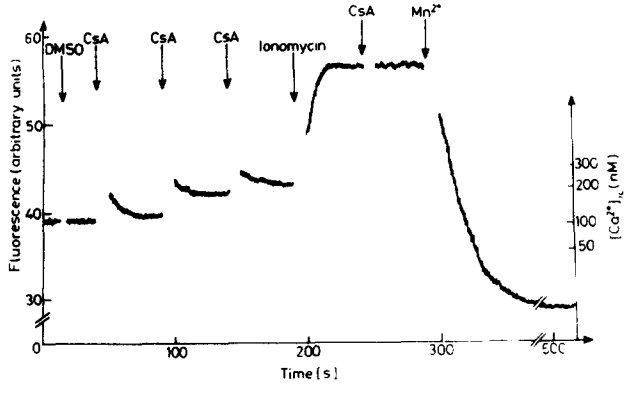

Fig. 5. Effect of repeated addition of CsA to the same sample. The trace shows the fluorescence intensity (left axis) and the calculated $[Ca^{2+}]_i$ (right axis) of a representative sample. P3HR-1 cells were loaded with indo-1 as described in Materials and Methods. DMSO (2 μl), ionomycin (1.5 μg/ml), and $MnCl_2$ (0.5 mM) were added as indicated by arrows. For CsA each arrow indicates a 10 μg/ml increase in its concentration.

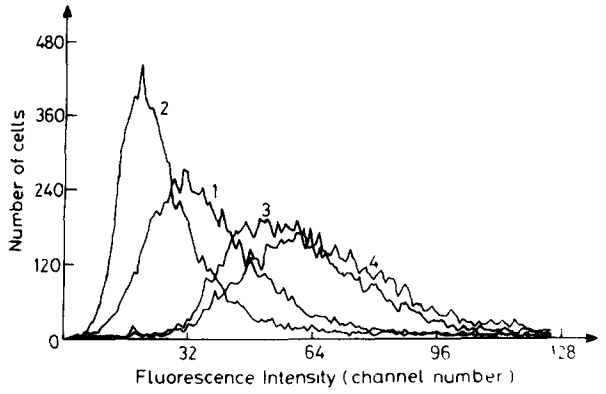

Fig. 6. Immediate effect of CsA on the membrane potential of HUT-78 cells. The figure shows the fluorescence intensity distributions of control (1) and cyclosporin A (10 μg/ml) -treated (2) cells and the fluorescence distribution after addition of 80 mM K^+ (3) followed by 10 μg/ml gramicidin (4). Each histogram contains data from 10^5 cells. Samples were equilibrated with oxonol as described in Materials and Methods. CsA and other chemicals were added immediately before analysis.

Immediate effect of CsA on the membrane potential of human B and T lymphoblastoid cells

10 μg/ml CsA was administered to 10^6 HUT-78 cells in 1 ml buffer A after their equilibration with 150 nM oxonol ($DiBaC_4(3)$) for 3 min at 37°C. Fluorescence histograms of 10^5 cells were taken immediately afterwards. A representative distribution is shown in Fig. 6. Identical experiments were performed with DMSO, the solvent of CsA. The distributions obtained here were the same as those for the untreated cells (Fig. 6). Since oxonol is an anionic dye, the immediate decrease of fluorescence (the shift of the curve to the left) indicates a hyperpolarization of the membrane upon the effect of 10 μg/ml CsA. Viability of the cells was demonstrated by subsequent addition of 80 mM K^+ and 2 μg/ml gramicidin resulting in the increase of fluorescence (shift to the right), the sign of the expected depolarization (Fig. 6).

The same phenomenon could be observed in experiments with mouse thymocytes. On the other hand, when performing the same experiments on B lymphoblastoid JY cells, the immediate hyperpolarizing effect of CsA did not appear (data not shown).

Time-resolved effect of CsA on the transmembrane potential of T and B lymphoblastoid cells

HUT-78 cells were incubated with 10 μg/ml CsA. At 10, 20, 30 and 60 min histograms of 10^5 cells were registered following a 3 min equilibration with 150 nM oxonol. In Fig. 7 a representative distribution belonging to each stage of sampling shows that the membrane potential, although still slightly hyperpolarized at 10 min, is almost completely depolarized at 20 min as best demonstrated by checking against the distribution of the gramicidin-treated sample shown in Fig. 6. How-

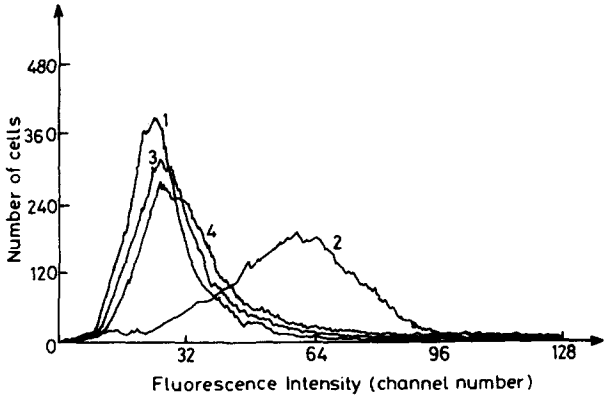

Fig. 7. Effect of preincubation with cyclosporin A on the membrane potential of HUT-78 cells. The curves represent the fluorescence intensity distributions of samples (10^5 cells) preincubated with 10 μg/ml CsA for 10 (1), 20 (2), 30 (3) and 60 (4) min and equilibrated with oxonol for 3 min as described in Materials and Methods. The fluorescence signal amplification was the same as in Fig. 6.

ever, the histograms belonging to 30 and 60 min indicate an almost completely restored resting membrane potential by then. Control experiments with DMSO, the solvent of CsA, were negative.

JY cells were treated according to the same protocol. While immediate hyperpolarization upon addition of CsA was not detected, a considerable depolarization of the membrane developed within 10 min without significant change during the 60 min time interval. This depolarization could be further increased by 2 μg/ml gramicidin as demonstrated by mean fluorescence values (in arbitrary units) for the control (42.0 ± 2.3), the 20 min (81.2 ± 5.8) and the gramicidin-treated (178.0 ± 6.5) samples.

Discussion

A prerequisite to understanding how CsA acts on lymphocytes is to determine the sequence of changes in functional parameters indicating the drug-cell encounter. Various biochemical changes have been shown to occur in lymphocytes as a consequence of CsA treatment. However, in most cases the studies took place several hours or days following the administration of the drug. From these investigations we know that the immune response of different subpopulations of B and T lymphocytes is selectively affected by CsA [28,29], the IL-2 production of T_{helper} cells is blocked at the level of mRNA synthesis [30] while the formation of IL-2 receptors is not influenced [31,14], the sensitive step of blast transformation is within 6–8 h after mitogenic induction [13], CsA inhibits the clonal proliferation of T_{killer} cells [32] and the antigen dependent lymphokin production of T_{DTH} cells is blocked, too [5,6].

However, comparatively less is known about the immediate effects of CsA. Even at the level of $[Ca^{2+}]_i$ regulation, where fast and transient changes have profound effects, mainly the interference of CsA with normal activation processes after preincubation with the drug was studied [14–20] and yet little attention was paid to immediate early action of CsA [21,22].

In the preliminary steps of our investigation two fluorescent calcium chelators were compared with respect to their capacity of revealing immediate changes of $[Ca^{2+}]_i$ caused by a therapeutic dose (2 μg/ml) of CsA. When using quin2 as indicator, the transient rise could be shown only in case of T lymphoma cells and mouse thymocytes, but not in B lymphoid cells. However, indo-1 enabled us to detect this effect in all of these cell types.

The probable explanation can be found in the different fluorescent properties of the two dyes. Indo-1 has a fluorescent quantum yield and molar absorption coefficient respectively 4- and 7-times that of quin2 [27]. The smaller concentration of indo-1 which is hence sufficient for loading, in conjunction with its smaller affinity to calcium, accounts for the weaker 'buffering' of fast calcium transients. Using other cell types and agents El-Moatassim [33], Tatham [34] and Bijsterbosch [35] came to similar conclusions. Given this background, the different membrane properties of B and T cells – mainly the delayed activation of calcium pumps in T cells [36] – may account for our observations.

Hence for further experiments the more advantageous indo-1 was used. To determine the source of calcium mobilized by CsA, effects of the drug on the resting $[Ca^{2+}]_i$ were examined in the absence of extracellular calcium. When EGTA was added to the cell suspensions, a dose-dependent decrease of $[Ca^{2+}]_i$ could be observed similarly to the findings of Kojima and Ogata [37]. When added to a buffer containing 1 mM Ca^{2+}, 3 mM EGTA partially, 10 mM EGTA totally abolished the transient rise of $[Ca^{2+}]_i$ caused by CsA. The restitution of the original extracellular calcium concentration restored the effect of CsA. We concluded that the calcium effect of the drug depends upon the availability of extracellular calcium and the source of at least a fraction of the mobilized calcium is likely to be the extracellular space. This is supported by the finding that incubation of Con A-activated lymphocytes with CsA causes additional $^{45}Ca^{2+}$ uptake from the extracellular space [38].

It was of importance to know the route by which this fraction enters the cell. The dose dependence of the effect of CsA on $[Ca^{2+}]_i$ could mean the dose-dependent activation of calcium channels or it could be due to calcium transport carried out by CsA itself. To block calcium channels, verapamil, nifedipine, La^{3+} and Ni^{2+} were used. As Chandy et al. [39] supposed the role of voltage-gated potassium channels in calcium transport, the K^+ channel blocker 4-aminopyridine was also applied. Verapamil, nifedipine and 4-aminopyridine had no effect on the the rise of $[Ca^{2+}]_i$ caused by CsA.

Also, repeated administration of CsA was able to induce the observed rise in $[Ca^{2+}]_i$ again and again. These facts, and the linear correlation between the dose of CsA and the detectable peak of calcium transient induced by it, support the suggestion that CsA may act as a slow ionophore itself. On the other hand, it is an alternative possibility that CsA, by perturbing the resting structure of the cell membrane or by binding to one of its potential targets, Cyclophylin [18], may open a verapamil-, nifedipine- and 4-aminopyridine-insensitive pathway directing a calcium flow from the extracellular space towards the cytoplasm. The finding that aspecific Ca^{2+} channel blockers, such as lanthanum and nickel, diminished the rise of $[Ca^{2+}]_i$ caused by CsA is neither for, nor against the idea of CsA acting as a weak ionophore, since competition of La^{3+} or Ni^{2+} for CsA's hypothetical calcium binding site cannot be ruled out.

The rapid decline of the elevated calcium concentration to the resting level is probably due to uptake into intracellular stores and active pumping mechanisms causing a net efflux. Supposing an ionophor effect of CsA, this can happen only in the case that CsA generates a small influx of calcium relative to its quantity. However, due to low solubility of the drug, it is impossible to try whether greater quantities of CsA would result in a sustained high $[Ca_i^{2+}]$, comparable to that caused by ionomycin. On the other hand, addition of a small (2 ng/ml) dose of ionomycin to the examined cells produces the same effect as 10 μg/ml CsA.

When CsA is repeatedly administered, the gradual elevation of the equilibrium calcium level could be caused by the depletion of resources for active transport. This premise is also in accordance with a possible ionophore-like effect.

In therapeutic ranges of CsA, the activation of Ca^{2+} transport systems by calcium influx and the increase in the amount of stored calcium owing to this are the effects that seem to be of importance, since the elevation in the equilibrium calcium concentration occurs only well above the upper limit of therapeutic dose. The activation of transport and accumulation of stored calcium could indeed play a role in decreasing the efficacy of mitogen-induced calcium influx, thus intervening with lymphocyte activation. This truncated calcium influx has been demonstrated by Gelfand et al. [14] when CsA (1 μg/ml) -treated human peripheral lymphocytes were stimulated with Con A. It should be noted that there were findings contradictory to this, but the contradictions could originate from using shorter incubation with CsA [15], or detecting Ca^{2+} levels 90 min after Con A stimulation [17].

Mizushima and co-workers [16] have found that CsA in vitro does not inhibit calcium-calmodulin dependent enzymes and protein kinase C, but it blocks thymocyte activation as measured by IL-2 production. Therefore they suggested that CsA inhibits late steps of lymphocyte activation. This finding does not exclude the possible blocking effect of CsA on yet unclear early steps in the mitogenic stimulation of lymphocytes, or even on late steps following kinase activation. For example, possible in vitro inhibition of PK-C by CsA was excluded by Mizushima and co-workers, but the effect of CsA on the translocation of the enzyme has never been checked. Translocation is a potentially important factor in PK-C function and may well be influenced by the early calcium effect of CsA through the increase in the amount of intracellularly stored Ca^{2+} or the change in calcium fluxes across membranous structures of the cell.

Besides activating calcium transport systems, the transient rise of $[Ca^{2+}]_i$ caused by CsA may have other regulatory effects. Tsien [40], Rink [41], Tatham [34] and Scharff [42] have reported evidence of the interconnection of membrane potential and $[Ca_i^{2+}]$ changes in different cell types. They equivocally claimed that the rise of $[Ca^{2+}]_i$ causes the hyperpolarization of cell membrane via opening calcium sensitive potassium channels, thereby shifting the membrane potential towards that of the potassium equilibrium. We found similar phenomena in case of the CsA-treated T-lymphoid line HUT-78 and BALB/c murine thymocytes. The rapid transient increase of $[Ca^{2+}]_i$ caused by the drug occurred contemporarily with hyperpolarization, although the latter had a longer duration. The B lymphoid P3HR-1 cells failed to react with hyperpolarization, even though the elevation of $[Ca^{2+}]_i$ was detectable. We assume that Ca^{2+}-sensitive potassium channels are not present at sufficiently large quantities in the membrane of this cell line. Ishida and Chused [36] reported evidence supporting this hypothesis, namely, they have shown the presence of these channels in T and their absence in B lymphocytes of mice.

When analyzing time-resolved changes of the membrane potential, a depolarization was found to follow the initial hyperpolarization caused by CsA. This depolarization has been shown earlier in other cell types [9,21,22]. The opening of potassium channels was the suggested mechanism of the phenomenon as based on $^{42}K^+$ release measurements on human peripheral blood lymphocytes. The lack of the initial hyperpolarization in these experiments could be explained by the applied membrane potential indicator, $DiOC_6(3)$, as it blocks calcium-dependent K^+ channels [43], which in turn could mask a slight initial shift towards the potassium equilibrium potential when $[Ca^{2+}]_i$ is increased.

In our experiments the depolarizing effect of CsA was not identical when the drug was applied to B and T cells. The latter were depolarized after longer preincubation, to a greater extent and their membrane potential was restored in 30 min, unlike that of B cells. This, together with the observed lack of Ca^{2+}-sensitive K^+ channels in B cells, implies that there must be at least two mechanisms of depolarization in CsA-treated

lymphocytes. For example, opening of sodium channels may be in effect [34] additional to that of potassium channels.

In summary, the effect of the immunosuppressive drug CsA on $[Ca^{2+}]_i$ and membrane potential was investigated. When comparing T and B lymphoid lines, CsA was found to cause a fast transient elevation of $[Ca^{2+}]_i$ in both cell types, for which an explanation suggesting a slow ionophore-like mode of action of the drug also arises. The result of this effect seems to be the activation of calcium transport systems, which in turn could interfere with mitogenic stimulation. Besides, we hold the rise of $[Ca^{2+}]_i$ responsible for the early hyperpolarizing effect of CsA in T lymphocytes. The consecutively evolving depolarization cannot, however, be uniformly regarded as a calcium-mediated effect of the drug. Rather, different mechanisms in the different cell types are suggested to participate in the event. This diversity in the behaviour of T and B cells together with the absence of calcium-dependent potassium channels in B lymphocytes, for which further evidence was found in this work, could account for the different final outcome of the drug–cell interaction.

Acknowledgements

This work was supported by grants from the Hungarian Acad. Sci. (OTKA 112) and OKKFT 1.1.4.2. to S.D. Dr G. Szabó Jr. is gratefully thanked for valuable discussions and J.F. Borel for kindly donating CsA. We also thank Á. Gara and A. Harangi for their skilful technical assistance.

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