Institute of Biochemistry I, Semmelweis University, Medical School, Budapest, Hungary

The Possible Pitfalls of the Measurement of Intracellular Calcium Concentration of Lymphocytes with the Fluorescent Indicator Quin2

P. CSERMELY and J. SOMOGYI

Abstract

The measurement of intracellular calcium concentration of small cells became feasible and relatively easily accomplishable following the development of the fluorescent calcium chelating dye, quin2, by R. Y. TSIEN and his co-workers.

The present paper gives an experimental survey of the major possible pitfalls of the method such as: 1) the improper choosing of the concentration of quin2/AM, 2) the misinterpretation of the calibration procedure, 3) the accidental heavy metal traces in the medium, and 4) the uneven distribution of quin2 among the individual cells or within a certain cell. We report some original data on: 1) the distorting effect of heavy metal ions on the measurement and the use of chelex 100 resin to eliminate heavy metal traces from the mediums, 2) the negligible contribution of dead cells to the overall fluorescence signal demonstrated by flow-cytometry, and 3) fluorescence polarization of quin2 in lymphocytes.

Introduction

During the past ten to fifteen years, several methods have been developed for the highly sensitive detection of changes in calcium concentration in living cells. The different experimental approaches include measurements with fluorescent dyes, e.g., chlorotetracycline (1), metallochromic indicators, e.g. arsenazo-III (2), Ca^{2+}-sensitive microelectrodes (3) and photoproteins, e.g. aequorin (4–5). However, none of these methods are easily applicable for the measurement of intracellular calcium concentration (Ca_i) in small cells such as lymphocytes because of the qualitative, complementer-type signal (chlorotetracycline) or the membrane damage necessary

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Abbreviations: Ca_i = free cytoplasmic calcium concentration; *chelex 100* = divalent cation-chelating resin; *DMSO* = dimethyl sulfoxide; *DTPA* = diethylene-triamine-pentaacetic acid; *EGTA* = ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; *HEPES* = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; *PrI* = propidium iodide; *quin2 and quin2/AM* are the abbreviations of 2-((2-(bis(carboxymethyl)amino)-5-methylphenoxy)-methyl)-6-methoxy-8-(bis(carboxymethyl)amino)-quinoline tetrasodium salt and its tetra-acetoxymethyl ester, respectively, recommended by R. Y. Tsien; *TPEN* = N,N,N',N'-tetrakis(2-pyridylmethyl)ethylene-diamine; *Tris* = tris(hydroxymethyl)-amino-methane.

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The development of the fluorescent, Ca^{2+}-chelating dye, quin2 and its acetoxymethyl ester (6–8) has overcome most of these problems. As the method becomes more and more a routine measurement of a biochemical/cell biological laboratory, the importance of critical surveys discussing the possible pitfalls of the measurement increases. Our present report demonstrates as possible sources of error in the Ca_i determination: 1) the imperfect conditions of the quin2 loading incubation, 2) the improper calibration procedure, 3) the impurities of the medium, especially the presence of heavy metal ions and deals with the distribution of quin2 among cell populations and within a certain cell.

Materials and Methods

Materials

Quin2, quin2/AM, chelex 100, DTPA, EDTA, fluorescein-diacetate, HEPES, propidium iodide and trypan blue were obtained from Sigma Chemicals. ^{3}H-quin2/AM was an Amersham product. TPEN and the ionophores A23187 and ionomycin were from Calbiochem, X537-A from Hoffman La Roche. Digitonin was from Fischer Scientific; Triton X-100 and EGTA were from Serva. DMSO was a Fluka product; $CaCl_2$ was from Merck, $MgCl_2$ from BDH of high purity. All the other reagents used were from Reanal, Hungary, of analytical purity.

Medium for cells

Cells were isolated and stored in a modified Hanks' medium containing 143 mM NaCl, 1 mM Na_2SO_4, 5 mM KCl, 1 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose and 10 mM HEPES, pH 7.4. Usually the medium was treated with chelex 100 resin overnight at 4 °C to remove the accidental traces of heavy metals (standard medium). To 1 l of medium (without Ca^{2+} and Mg^{2+} ions at pH 7.4), 7 g of chelex 100 resin (wet weight, approx. 5 g of dry weight) were added. The final volume of the medium, the final concentration of Ca^{2+} and Mg^{2+} and the pH of 7.4 were set after the chelex 100 resin had been removed by filtration. The chelex 100 resin was activated prior to use by successive washing with HCl (0.1 M), H_2O, NaOH (0.1 M), H_2O, EDTA (1 mM) and H_2O.

Preparation of lymphocytes

Thymocytes were prepared using the methods of KLEIMAN et al. (9) and BÁNFALVI et al. (10). Rabbit (2 kg, male) or mouse (CLFP, 6–8 weeks old, male) thymus glands were teased into standard medium setting a final cell density of approx. 10^7 cells per ml. Tissue fragments were removed by filtration through four layers of sterile-washed cotton gauze. The cells were centrifuged at $400 \times g$ for 10 min at room temperature and washed two times with the same volumes of the standard buffer; then the cell density was set to 5×10^7 cells per ml. In the experiments, viability was never less than 95 % (usually more than 98 %) as judged with trypan blue exclusion.

Quin2 loading

Unless otherwise indicated, cells were loaded by incubation with quin2/AM at a final concentration of 20–30 μM from a 20 mM DMSO stock solution in standard medium at a Ca^{2+} concentration of 0.5 mM. 5×10^7 cells per ml were incubated at 37 °C for 20 min, then diluted

tenfold with standard medium (Ca^{2+} concentration: 0.5 mM) and incubated further at 37 °C for 40 min. After incubation, the cells were centrifuged at $400 \times g$ for 10 min at room temperature, gently suspended in standard medium setting the final cell density to 5×10^6 cells per ml and kept at room temperature. The amount of extracellular quin2 was routinely checked by the method of Hesketh et al. (11) adding $MnCl_2$ at a final concentration of 10 µM to intact quin2 loaded lymphocytes in a separated 2 ml aliquot of the cell suspension. However, the relative amount of the extracellular quin2 never exceeded 5 % of the total fluorescence, but if the value was greater than 2 %, the cells were centrifuged and resuspended again (at a cell density of 5×10^6 cells per ml) immediately before the measurements in order to reduce the amount of the extracellular quin2 below 2 %.

Fluorescence measurements

The quin2 fluorescence was recorded with a Varian SF-330 (U.S.A.) or with a Jobin Yvon Spectrofluo JY3 (France) spectrofluorometer at an excitation-emission wave length pair of 339 nm and 492 nm with 4 and 10 nm slits, respectively. Samples were examined in 1-cm square quartz cuvets at room temperature. The results were corrected for the dilution of the sample. Appropriate (only DMSO incubated) controls were rountinely examined, and the observed changes in autofluorescence, light scattering were taken into correction. The fluorescence of the Ca-quin2 complex after permeabilization (100 % fluorescence) was rountinely checked by the addition of 100 µM Ca-DTPA or 250 µM Ca-EGTA complex to eliminate the quenching effect of the possible traces of heavy metal ions.

Fluorescence polarization measurements were made using polarization filters (Oriel Co., Stamford, CT, U.S.A., No. 2732) by the general procedure of Chen and Bowman (12). The grating factor (G, which is the ratio of the fluorescence intensities measured at horizontal-vertical or horizontal-horizontal filter positions) was 0.90 in our experiments.

Flow cytometric measurements

The flow cytometric analysis was done with a FACS II (Becton and Dickinson) instrument equipped with a light source of a Spectra Physics 2000 krypton-argon laser. The 351.1–363.8 nm UV multiline was used as an excitation beam at an energy output of 160 mW. The cell density of the sample was $2–3 \times 10^6$ cells per ml in standard medium. Prior to measurements, the cells were passed through a 25 µm mesh to remove the possible aggregations. In one experiment, 10^5 cells (in approx. 50 µl) were examined with a duration of approx. 5 min. The sample was injected into a laminar stream of 0.15 M NaCl. During measurements, the low angle light scattering, blue fluorescence (in between the half transmission points of 408 and 550 nm) and red fluorescence (more than 650 nm) were detected.

Results and Discussion

Loading – the intracellular concentration of quin2

As it is shown in Figure 1A, there is a relatively narrow concentration range (from 20 to 40 µM of quin2/AM) where the amount of entrapped quin2 is a linear function of the total quin2/AM concentration. The unproportionally small loading efficiency in our experiments at 5–10 µM quin2/AM concentration might be explained with the non-specific binding of the hydrophobic acetoxymethyl ester to membranes, walls of the reaction vessel, etc. The small decrease in the intracellular quin2 concentration after the linear range shown in Figure 1A may be due to some (yet not clearly defined) toxic effects of the intracellular ester hydrolysis (11, 13–15).

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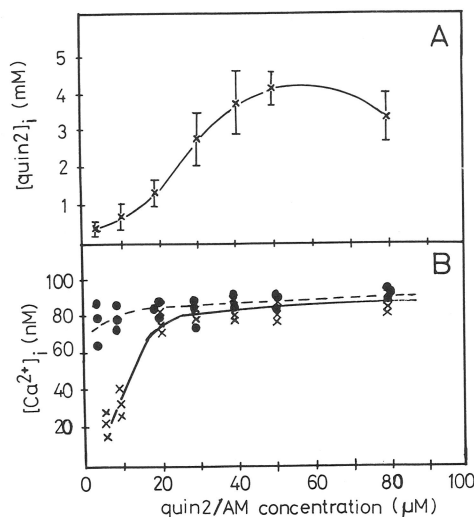45–60 m
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Fig. 1. The intracellular quin2 (A) and calcium (B) concentration as the function of the quin2/AM concentration. Rabbit thymocytes were loaded with quin2 as described in «Materials and Methods» at the quin2/AM concentration indicated. Intracellular quin2 concentration was measured using ^{3}H-quin2/AM. The intracellular volume of rabbit thymocytes was taken as 158 fl (28). The intracellular calcium concentration was calculated according to TSIEN et al. (13). *Panel A:* intracellular quin2 concentration (x). The data are means ± SD of three separate experiments. *Panel B:* intracellular calcium concentration in the absence (x—x) or presence (● – – – ●) of 20 µM TPEN. Data are from three separate experiments.

On the other hand, high concentrations of intracellular quin2 are chelating the intracellular Ca^{2+} ions, hence damping the possible Ca^{2+} signals and probably exhausting the intracellular Ca^{2+} pools (14).

The intracellular free calcium concentration in Figure 1B is around 80 nM at quin2/AM concentrations higher than 20 µM in rabbit thymocytes. This value is close to the ones measured in mouse thymocytes. The Ca_i values sharply decrease at lower intracellular quin2 concentrations than 1 mM. In the presence of TPEN, the potent cell permeable heavy-metal chelator, the Ca_i values increase to the range of 80 nM. This observation (in agreement with the findings of ARSLAN et al. (16)) shows that the intracellular heavy metal (Zn, Fe, Cu, Mn) ions effectively quench the fluorescence of the Ca-quin2 complex at low intracellular quin2 concentrations.

Figure 2 shows the time dependence of quin2/AM hydrolysis. The amount of intracellular quin2 can be calculated from the shift in the emission maximum of 425 nm characteristic to quin2/AM to 492 nm characteristic to quin2 during hydrolysis (13). Panel A shows that after 45–60 min the hydrolysis is practically complete. Since the final intracellular quin2 concentration was 1.5–2.0 mM in the experiments of Figure 2A, at least 30 min of incubation is necessary to reach the 1 mM intracellular quin2 concentration, i.e. the plateau in Ca_i in Figure 1B (compare Figs. 1A, 1B and 2A).
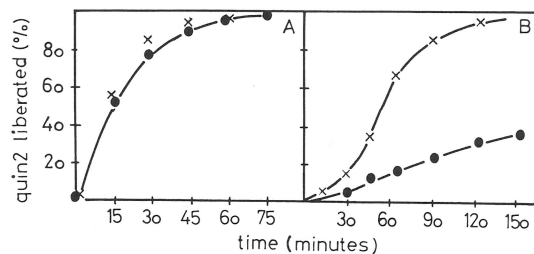
Fig. 2. The time course of the hydrolysis of quin2/AM at different cell concentrations. Rabbit thymocytes were incubated with quin2/AM at different cell concentrations as described in «Materials and Methods». At time zero, the quin2/AM concentration was 25 μM. At the times indicated, aliquots were taken and their fluorescence was measured. Excitation wave length was 339 nm; the emission was recorded at 425 and 492 nm. From the ratio to these two fluorescences, the percentage of quin2 liberated was calculated assuming a ratio of 4.0 and 0.6, corresponding to quin2/AM and quin2, respectively. In *panel A,* the quin2 loading was done at a cell density of 5×10^6 cells per ml (●), 5×10^7 cells per ml (x). In *panel B,* the cells were lysed prior to addition of quin2/AM with 100 μM digitonin and the hydrolysis of quin2/AM was followed at cell densities of 5×10^6 cells per ml (●) or 5×10^7 cells per ml (x). The data are representative of three identical experiments.

Figure 2A shows furthermore that the time course of the quin2 loading and the final loading efficiency depend only on the relative amount of quin2/AM per lymphocyte and that the dilution has no significant effect on the kinetics of the process. There was no change in the conversion rate of quin2/AM if the cells were diluted from 5×10^7 cells per ml to 5×10^6 cells per ml after 20 min of incubation (not shown). If the cells were lysed with digitonin prior to the addition of quin2/AM (Fig. 2, panel B), the rate of hydrolysis was considerably lower than in experiments performed with intact cells. In addition, after cell lysis the conversion rate of quin2/AM to quin2 was significantly lower at a «cell density» of 5×10^6 cells per ml than at a tenfold higher «cell concentration». The increase of the cell density also changes the digitonin per cell ratio. This change – in this concentration range – does not influence the kinetics of the hydrolysis because very similar results can be obtained if the experiments of Figure 2B are repeated with 10 μM or 1 mM digitonin (not shown). What can be the explanation for the observed differences? The digitonin-induced esterase denaturation seems to be an unlikely explanation because the difference in the rate of hydrolysis at different «cell densities» is stable at a wide range of detergent concentration. Upon digitonin permeabilization, a significant dilution of the quin2/AM hydrolysing esterases may occur. If the quin2/AM concentration in the vicinity of esterases remained essentially unchanged during permeabilization, the dilution of the esterases may explain the fall in the rate of hydrolysis. On the other hand, if the uptake of quin2/AM were the rate limiting step of the process, the rate of hydrolysis should increase rather than decrease upon permeabilization, i.e., abolishing the barrier which limited the access of quin2/AM for rapid hydrolysis. Therefore, these

observations support the suggestion of RINK and POZZAN (14) that the rate-limiting step is hydrolysis rather than uptake in the loading process.

It is clear from these considerations that *there is a very narrow range in the intracellular quin2 concentration (in the case of lymphocytes from 1 mM to 3–4 mM) where the evaluation of the experiments is most reliable.* The loading efficiency depends mainly on the quin2/AM concentration and on the time of incubation. These parameters must be accurately optimized in the case of each new cell type that is tested.

Calibration – fluorescence artifacts

Quin2, according to its fluorescence intensity, indicates Ca_i; hence a calibration procedure is needed after each experiment. We had to expose the intracellular quin2 to at least two different defined Ca^{2+} concentrations by making the cell membrane permeable to extracellular Ca^{2+}. In Figure 3, we show the effect of increasing concentrations of ionomycin, A23187, X537-A (lasalocid-A), digitonin and Triton X-100 on the intracellular calcium concentration of lymphocytes. From our data, it can be concluded that: 1) X537-A is not suitable for permeabilization in this case, since at the minimal effective concentration (i.e., the minimal concentration which allows to saturate the intracellular quin2 with Ca^{2+}) of this ionophore, its auto-fluorescence is comparable or exceeds the overall signal; 2) the minimal effective concentration of ionomycin, A23187, digitonin and Triton X-100 are 50 nM, 200 nM, 5 μM and 200 μM (0.012 %), respectively in standard
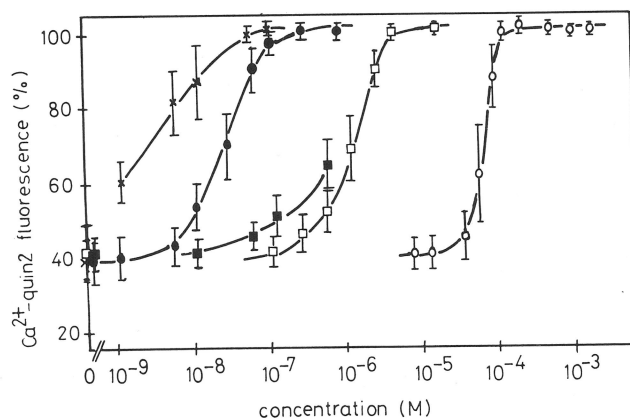

Fig. 3. Permeabilization of lymphocytes by different ionophores and detergents. Rabbit thymocytes were loaded with quin2 as described in «Materials and Methods». Various concentrations of ionomycin (x), A23187 (●), X537-A (■), digitonin (□) and Triton X-100 (○) were added to the lymphocytes. The quin2 fluorescence was measured at an intracellular quin2 concentration of 3 mM in 5×10^6 cells per ml of standard medium ($Ca_{out} = 1$ mM). The percentage of the Ca-quin2 complex was calculated using the calibration method of TSIEN et al. (13). The calculations were made from steady-state values (measured usually after 3–5 min). The average molecular weight of Triton X-100 was taken as 620 D. The data are means ± SD of three separate experiments.

medium at a cell density of 5×10^6 cells per ml. We must note that the minimal effective concentrations strongly depend on the cell density and on the type of the medium. In mediums supplemented with bovine serum albumin or serum, the minimal effective concentrations might be ten- or even one hundredfold higher because of the distribution between serum proteins and the cell membrane (not shown).
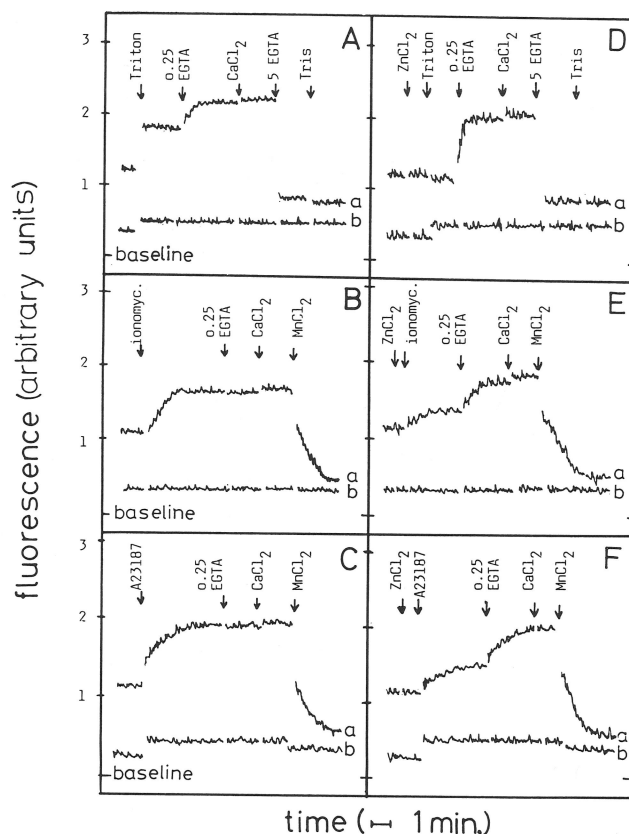
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Fig. 4. The comparison of three calibration methods of Ca_i and the effect of heavy metal (Zn^{2+}) traces on the different methods. The fluorescence of the same batch of quin2-loaded mouse thymocytes was recorded at an intracellular quin2 concentration of 2 mM in 5×10^6 cells per ml of standard medium (panels A–C). At panels D–F, 1 µM $ZnCl_2$ was added to the medium at the beginning of the measurements. At the arrows, different additions were made: 0.05 % Triton X-100, 50 nM ionomycin, 200 nM A23187, 0.25 or 5 mM (as indicated) EGTA, 0.25 mM $CaCl_2$, 20 mM Tris, 10 µM $MnCl_2$. Traces «a» show the fluorescence signal of quin2 loaded lymphocytes, traces «b» represent the autofluorescence of a non-loaded cell suspension at the same cell density. The traces are representative of three identical experiments. Panel A: calibration with Triton X-100; panel B: calibration with ionomycin; panel C: calibration with A23187; panel D: calibration with Triton X-100 in the presence of 1 µM Zn^{2+}; panel E: calibration with ionomycin in the presence of 1 µM Zn^{2+}; panel F: calibration with A23187 in the presence of 1 µM Zn^{2+}.

Two ways of calibration of the fluorescent quin2 signal have been developed. The method of TSIEN et al. (8, 13) first exposes quin2 to the extracellular (1 mM) Ca^{2+} concentration by detergent-(Triton X-100 or digitonin)-permeabilization, than lowers the free Ca^{2+} concentration below 1 nM EGTA and subsequent alkalinization. In the method of HESKETH et al. (11), the maximal quin2 fluorescence is produced via ionophore-permeabilization of the plasma membrane and the «autofluorescence»-level is measured in the presence of Mn^{2+} ions which quench the quin2 fluorescence forming the practically non-fluorescent Mn-quin2 complex.

In Figure 4, these calibration methods are presented. Besides Triton X-100 (panels A, D) and ionomycin (panels B, E), the permeabilization with the more widely available A23187 (panels C, F) was also examined. In Figure 4D–F, 1 µM $ZnCl_2$ was added to the medium to demonstrate the effect of heavy metal traces to the calibration procedure. Essentially identical results were obtained when 0.05 % Triton X-100 was changed to 20 µM digitonin or when 0.25 mM EGTA was substituted by 0.1 mM DTPA (not shown). In panels B–C, E–F the increase of the Mn^{2+} concentration up to 0.5 mM did not cause a further decrease in the quin2 fluorescence signal

Table 1. The calculated intracellular Ca^{2+} concentrations obtained by different methods of calibration

calibration methods	intracellular Ca^{2+} concentration (nM)[a]			
	without any correction	Ca-EGTA corrected	autofluorescence corrected	Ca-EGTA plus autofluorescence corrected
«Triton X-100»[b]	70 ± 10	45 ± 4	132 ± 21	76 ± 11
«Triton X-100» + 1 µM $ZnCl_2$	n.i.[c]	50 ± 5	n.i.	85 ± 9
«ionomycin»	83 ± 8	83 ± 8	83 ± 8	83 ± 8
«ionomycin» + 1 µM $ZnCl_2$	306 ± 21	89 ± 7	306 ± 21	89 ± 7
«A23187»	63 ± 8	63 ± 8	80 ± 10	80 ± 10
«A23187» + 1 µM $ZnCl_2$	141 ± 13	59 ± 7	280 ± 25	81 ± 8

[a] The values were calculated from the experiments described in Figure 4 using the equation $Ca_i = 115 (F-F_{min})/(F_{max}-F)$ (nM) in case of Triton X-100 permeabilization (Tsien et al.; 8, 13) and the equation $Ca_i = 115(F-F_{min}-0.16(F_{max}-F_{min}))/(F_{max}-F)$ (nM) in the case of ionophore permeabilization (Hesketh et al.; 11), respectively. In the equations, F is the initial quin2 fluorescence of the sample. In the first column («without any correction»), F_{max} is the value observed after permeabilization, F_{min} is the final fluorescence. In the second column («EGTA corrected») F_{max} is the fluorescence after the addition of 0.25 mM EGTA and 0.25 mM $CaCl_2$ (F_{min} is the same as before). In the last two columns the F_{max} and F_{min} values of the first two columns are corrected for the autofluorescence (traces «b» in Fig. 4.). The values are means ± SD of three separate experiments.
[b] The different methods are named after the agent used for permeabilization.
[c] Not interpretable.

(not shown). Table 1 shows the Ca_i values calculated from the data of Figure 4.

Heavy metal ions have a hundred or thousand times higher affinity for quin2 than Ca^{2+}, forming practically non-fluorescent complexes with quin2 (11); therefore, they effectively quench the quin2 fluorescence even in submicromolar concentrations. Heavy metal traces can be present in various components of the medium, e.g., serum, or agents of biological origin (antibodies, lectins, etc.). Ten ppm of heavy metal contamination in the inorganic salts (especially in NaCl as the major component) can totally distort the calibration procedure as it is shown in Figure 4D–F or in the first column of Table 1. The effect of intracellular heavy metal ions (especially at low intracellular quin2 concentration) might also be significant (rise of trace «a» in Fig. 4A after the addition of 0.25 mM EGTA and rise of the Ca_i after TPEN addition in Fig. 1B).

The fluorescence of the Ca-quin2 complex after permeabilization (100 % fluorescence) can be corrected by the addition of Ca-EGTA or Ca-DTPA. Both chelators have several magnitudes higher affinity for heavy metal ions than for Ca^{2+}, and their affinity constants for heavy metal ions are comparable to or higher than those of quin2. As a result, they effectively reconstitute the 100 % quin2 fluorescence. The effect of Ca-EGTA correction on the numerical values of Ca_i is shown in the second column of Table 1. The methods using ionomycin or A23187 for permeabilization are less sensitive for heavy metal contamination than the «detergent method». This can be explained by the fact, that zinc has three magnitudes higher affinity for Ca-ionophores than Ca^{2+} itself ((17) a part of the ionophores is «plugged» in the presence of zinc ions). Thus, using ionophores the intracellular quin2 is exposed to zinc ions to a lesser extent than in the case of detergent permeabilization.

Other drawbacks of the calibration procedure are the autofluorescence of certain compounds (e.g. A23187) or the changes in fluorescence/light scattering during permeabilization with detergents. In Figure 4 traces «b» represent the autofluorescence of the DMSO treated control sample at the same final cell density. In Figure 4, panel C and F traces «b» show that manganese quenches the autofluorescence of A23187, this is in agreement with previous results (18). The third column in Table 1 shows the Ca_i values after autofluorescence correction. In the «detergent method», this correction causes major differences, in the case of A23187 permeabilization, the differences are smaller. In the «ionomycin method» there is no difference between the data, since ionomycin is non-fluorescent.

As a summary, we can say that the elimination of heavy metal traces from the medium prior measurements is highly suggested. The use of chelex 100 resin for this purpose might not cause any cell membrane damage, since the chelator is filtered out from the medium before the addition of the cells. However, a further correction of the 100 % quin2 fluorescence is necessary by using Ca-EGTA or Ca-DTPA complexes to eliminate the effect of

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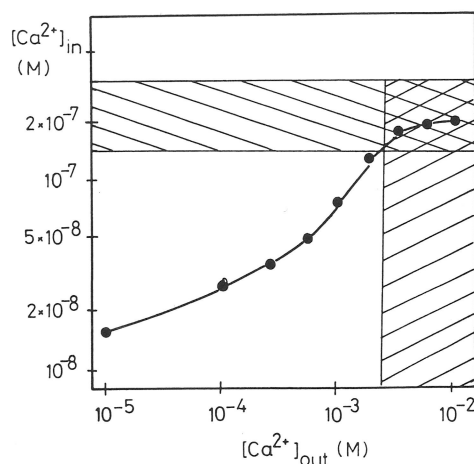findin
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Fig. 5. The dependence of the intracellular calcium concentration on the calcium concentration of the medium. Before the Ca_i determination, the cells were washed two times with standard medium with no Ca^{2+} added at a cell density of 5×10^6 cells per ml. The calcium concentration of standard medium was varied between 10^{-5} and 10^{-2} M by serial additions of 0.01 or 0.1 M $CaCl_2$ in standard medium as described. The free calcium concentration of the medium was checked by Ca^{2+}-sensitive electrode (Orion). The fluorescence signal was followed for at least 5–10 min after each addition, and the Ca_i was calculated from the final, steady-state value. The hatched areas represent the range of intracellular calcium concentrations occurring after the addition of mitogenic concentrations of Concanavalin A (determined in separate experiments, not shown) and the range of the mitogenic extracellular calcium concentration (WHITFIELD and co-workers; 19, 20). The experiment is a representative of two separate but identical experiments. Re-measurement of some data points after non serial addition of $CaCl_2$ gave essentially identical results.

intracellular heavy metal ions or the accidental heavy metal contamination in $MgCl_2$ or $CaCl_2$. Among the three methods tested (detergent – Triton X-100, digitonin –, ionomycin and A23187) the «ionomycin-method» is the least sensitive for different distortive effects. However after appropriate corrections (for the heavy metal content and the autofluorescence), *each method gives identical results* as it is shown in the last column of Table 1.

Figure 5 shows the effect of the calcium concentration in the medium on the intracellular Ca^{2+} concentration. Ca_i markedly increases if the extracellular Ca^{2+} concentration is raised. As the Ca^{2+} concentration of the medium exceeds 1.5 mM, reaching that range which was reported to be mitogenic for thymocytes by WHITFIELD and co-workers (19, 20), the intracellular calcium concentration elevates to the range which can be provoked by mitogens, e.g. Concanavalin A (hatched areas in Fig. 5). This finding is in agreement with earlier reports (11, 13, 15, 21). The phenomenon was also demonstrated in other cell types, such as in bovine parathyroid cells (22) and rat pituitary (GH_4C_1) cells (23).

These results draw the attention to the importance of the proper choice of the extracellular calcium concentration in the experiments. A difference between the Ca^{2+} concentration of the RPMI-1640 medium (0.42 mM) and

the Eagle's basal medium (1.8 mM) can cause a difference of approx. 100 nM in Ca_i of T lymphocytes. This might cause substantial differences in the activities of several Ca^{2+}-dependent enzymes.

Average of the cell populations – viability

The measurement of the quin2 fluorescence in a spectrofluorometer can yield only an average signal, i.e. an average Ca_i of different cell populations which are present in the sample. From this point of view, the presence of dead cells might be particularly dangerous in the evaluation of the quin2 measurements. The increased permeability of the cell membrane to Ca^{2+}
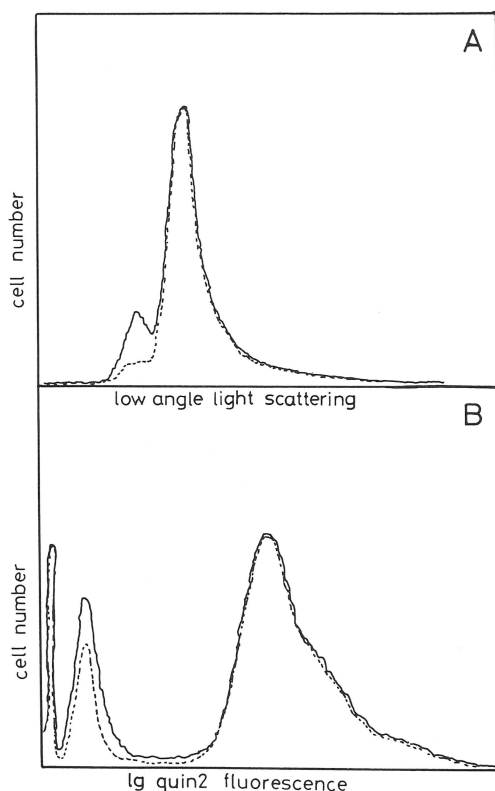

Fig. 6. The contribution of propidium iodide-negative (viable) cells to the low angle light scattering (A) and quin2 fluorescence (B) as judged by flow-cytometry. Rabbit thymocytes were loaded with quin2 and stained with propidium iodide. (To 2 ml of sample, 50 μl of propidium iodide were added from a 0.5 % w/v stock solution in 0.15 M NaCl). The intracellular quin2 concentration was 3 mM. The flow cytometric analysis was done as described under «Materials and Methods». *Panel A* shows the low angle light scattering, while *panel B* shows the quin2 fluorescence in a logarithmic scale. The distribution of the whole cell population is marked with a continuous line, while the distribution of the propidium iodide negative (viable) cells is marked with a dashed line.

ions is one of the first steps of the cell death. Therefore, a very small number of dead cells with a Ca_i of 1 mM might represent a significant portion of the overall fluorescence signal. This was the reason why we examined the contribution of dead cells to the quin2 fluorescence by flow cytometry.

Figure 6 shows the low angle light scattering (panel A) and the quin2 fluorescence (panel B) of rabbit T lymphocytes (solid line). Propidium iodide (PrI) is used as a fluorescent marker of dead cells (25), since the cell membrane of the living cell is impermeable to PrI which has practically no fluorescence in aqueous medium. In Figure 6, the dashed lines show the contribution of propidium iodide impermeable – living – cells to the overall signal. The fact that PrI-positive cells (shown as the difference between the dashed and solid lines) correspond to the dead cells is supported by the little low angle light scattering of this cell population (Fig. 6A).

The dead (PrI-positive) cells represented a portion of 4 % of the total cell population in the experiment shown in Figure 6. From the logarithmic distribution of quin2 fluorescence in panel B, it can be calculated that their contribution to the overall fluorescence signal is less than 1 %. Thus, dead cells do not seem to accumulate significant quantities of quin2. This result is in agreement with the expectations, since the activity of esterases is known to be diminished upon cell death (24). Therefore, we can conclude that a minor population of dead cells does not disturb the quin2 measurements in fluorometers.

The major peak of the quin2 fluorescence in panel B has an asymmetric character. This pattern is in agreement with the published results of RANSOM et al. (26) of an asymmetric quin2 fluorescence distribution of mouse splenic T lymphocytes. The asymmetry might arise from multimodal distribution where at least three subpopulations with different Ca_i can be distinguished. However, different amounts of quin2 in different cell populations can also yield the same multimodal distribution with identical Ca_i in the cells. This possibility does not seem to be very likely because the unimodal – gaussian – distribution of quin2 fluorescence of mouse splenic B lymphocytes (26) or that of cloned 9H5 T lymphocytes (27) argues against the differential quin2 distribution as a general phenomenon.

Localization of quin2 in the cytosol

The measurement of fluorescence polarization in the intracellular quin2 can provide information whether quin2 freely diffuses in the cytosol or whether it is bound to certain intracellular membranes, proteins, organelles. Increasing rotational mobility (decreasing emission anisotropy) during detergent permeabilization would show some kind of intracellular immobilization. In Figure 7A, the hatched bar represents the emission anisotropy of cell bound quin2, while the double hatched bar represents the value after Triton X-100 permeabilization. The value is slightly diminished. The difference is more expressed if we compare it with the anisotropy of quin2 in standard medium at the appropriate concentrations. Before deter-
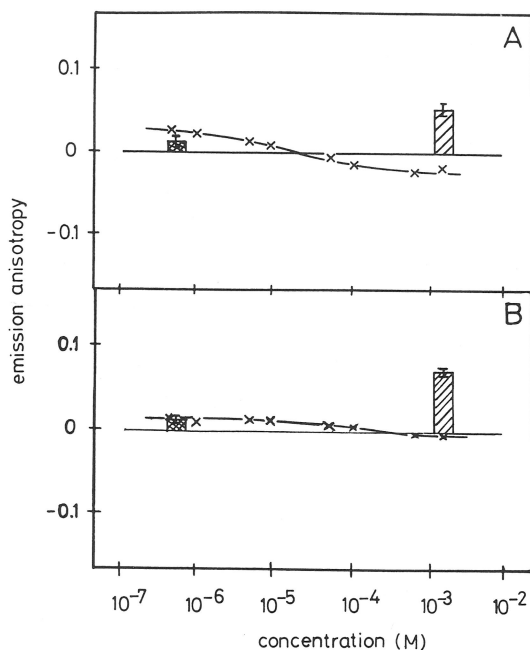
Fig. 7. The change in the emission anisotropy of quin2 (A) and fluorescein (B) fluorescence upon cell permeabilization. Fluorescence polarization measurements were done as described under «Materials and Methods». For the calculation of the emission anisotropy, we used the formula of Jablonski $r = (I_{vv} - G \times I_{vh})/(I_{vv} + 2G \times I_{vh})$ where I_{vv} and I_{vh} is the fluorescence intensity at vertical-vertical or vertical-horizontal filter positions, respectively, and G is the grating factor (see Chen and Bowman, 12). The data were corrected for the anisotropy changes of non-loaded cells. The points and the solid line represent the concentration dependence of the emission anisotropy of extracellular quin2 or fluorescein fluorescence in standard medium. The hatched and cross-hatched bars represent the emission anisotropy value of the intracellular quin2 or fluorescein concentration before and after permeabilization with 0.05 % Triton X-100 (± SD of 5 experiments), respectively. *Panel A:* emission anisotropy values of quin2. Cells were loaded with quin2 as described in «Materials and Methods». *Panel B:* emission anisotropy values of fluorescein. Cells were loaded with fluorescein diacetate at a concentration of 5×10^{-6} M. The incubation time was 30 min at 37 °C. The extracellular fluorescein was removed by centrifugation. The fluorescence was measured at the wave length pair of 496/520 nm with 2 nm bandwidths.

gent permeabilization, quin2 has an intracellular concentration in the millimolar range, while after permeabilization, assuming free distribution, its concentration diminishes to the micromolar range. Our data in Figure 7A show similar values for quin2 in standard medium and after permeabilization, while the anisotropy of the intracellular quin2 is different from the value of corresponding quin2 concentration in standard medium. The concentration dependence of the emission anisotropy of quin2 is not significantly different in the presence of cells or detergent permeabilized cells from the values measured in standard medium alone (data not shown). Identical changes can be measured in the fluorescence polarization of quin2

if we substitute Triton X-100 with digitonin at the permeabilization (not shown).

Our data are in agreement with the preliminary observations of TSIEN et al. (13), which also indicate a very small but positive polarization value suggesting some kind of immobilization. The observed changes in the emission anisotropy of quin2 might reflect an increased microviscosity in the cytoplasm or the transient attachment of quin2 to rotationally immobile sites.

To investigate the effect of increased intracellular microviscosity to the fluorescence polarization, we performed similar experiments with fluorescein diacetate (Fig. 7B). The data are very similar to those which we obtained with quin2. This suggests that at least a significant portion of the difference between the fluorescence polarization of cell bound and free quin2 is caused by unspecific effects, e.g., the effect of the cytoplasmic microviscosity.

Acknowledgements

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Dr. Péter Csermely, Institute of Biochemistry I, Semmelweis University, Medical School, P.O.B. 260, H-1444 Budapest, Hungary