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Preliminary studies concerning the correlation between the quercetin fluorescence and its ability to trigger Ca^{2+} release from the Jurkat T cells

INTRODUCTION

A wide number of studies performed in the last decades have evidenced the beneficial effects of natural flavonoids on human health. The natural flavonoids are frequently found in fruits, vegetables and tea and it was observed that they possess cardioprotective, anticancer, antiinflammatory and antialergic properties [1, review and the references therein]. Among these flavonoids we have chosen to study the epigallocatechin 3-gallate (EGCG) and quercetin (QC; 3,5,7,3',4'-pentahydroxyflavone), namely the way they are interacting with the human leukaemia cells, the Jurkat T lymphoblasts. It has been shown that these two flavonoids can inhibate the cell proliferation and induce the apoptosis in various types of cancer [1-7]. Both EGCG and QC can exert a dual effect, pro- and anti-oxidant, depending on the dose and the duration of the treatment and numerous studies indicate that the malignant cells are more susceptible than the normal ones to the cytotoxicity of these two flavonoids [3,5-7]. This property recommends the use of the two flavonoids either in the prevention of various cancer types, in particular leukaemia, or in order to increase the chemotherapy efficience in the treatment of these illnesses. As, so far, the available data concerning the effects of these compounds on the cell cycle and the apoptosis/necrosis in Jurkat T cells are extremely limited and inhomogenous, we have proposed ourselves to undertake a series of studies with the aim to elucidate the mechanisms underlying the interaction of the two flavonoids with leukaemia cells, alone or in combination with chemotherapeutical agents. This kind of research has been initiated in a previous project (Ideas no. 1138/2009) and the results we have obtained prompted us to continue this field of research and, moreover, they have suggested the approach of a new line of research, namely the attempt to elucidate the role of the calcium release channels from ER/SR in the induction of cellular apoptosis. Among the results obtained in the previous project (published in 7 ISI papers and 9 BDI papers) we would like to mention those that evidence the role of QC and EGCG in the induction of apoptosis in Jurkat T cells, the combined effects with those of the chemotherapeutic agent menadione (MD) or the effect of the mitochondrial respiration inhibitor rotenone [4,8,9]. An extremely interesting result, that, as previously mentioned, will constitute a new line of research refers to the triggering of a calcium signal following the addition of 50 μM QC in Jurkat cells[10].

In the first phase of the project we have proposed ourselves to examine the way in which quercetin, which presents a weak fluorescence in aqueous solutions, modifies its fluorescence when enetring the Jurkat cells and to examine more closely the correlation between the quercetin application and the resulted calcium signal. It has been shown that QC binds itself to cellular proteins unidentified yet and is thus accumulated in large quantities in the cytosol and the mitochondria of Jurkat T cells [10,11].

MATERIALE AND METHODS

` ***Cell cultures.*** Human leukemia Jurkat T-cell lymphoblasts were cultured in MegaCell RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37°C in a humidified incubator with a 5% CO_2 atmosphere. Dihydrated quercetin (Sigma) and fura-2/AM (Invitrogen) were dissolved in dimethyl sulfoxide (DMSO) and kept at -20ºC. Unless stated otherwise, reagents were purchased from Sigma. Cell density, viability and morphology were examined under a phase contrast microscope. Viability was assessed by the trypan-blue exclusion test. Cell count was performed with the use of a haemocytometer.

Saline solutions. The extracellular-like solution (ECS) contained 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, 10 mM glucose, pH 7.2/NaOH. The intracellular-like solution (ICS) contained 140 mM

KCl, 4 mM NaCl, 0.14 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 mM HEPES, pH 7.2/NaOH. A variant of ICS (ICSM) did not contain $MgCl_2$. When required, $CaCl_2$ and $MgCl_2$ solutions were freshly prepared at working concentrations of 50 mM, 1 M or 6 M ($CaCl_2$), and 4 M ($MgCl_2$) in either ICS or ECS as needed. The level of free Ca^{2+} in solutions was calculated using the software WEBMAXCLITE v1.15.

Assessment of intracellular [Ca^{2+}]$_i$ was performed according to the method of Grynkiewicz et al. [12] as described in [10] with minor modifications. Exponentially growing Jurkat cells were washed twice in ECS. The cells were then incubated with 4 μM fura-2/AM for 10-15 min. in the dark at room temperature (24-25ºC) with occasional agitation, then washed twice in ECS, resuspended in 2 ml ECS, counted and adjusted to the desired cell concentration by addition of ECS as necessary. Cells were then incubated at 37ºC for an additional 45 min. for complete de-esterification of the calcium indicator. The cells were then transferred to the spectrofluorimeter into a 2 ml quartz cuvette maintained at 37ºC under continuous stirring. Cell viability assessed immediately before the fluorescence recordings was >90% in all cell samples. Fluorescence was recorded with a Horiba Jobin Yvon spectrofluorimeter, by sequential excitation at 340, 380 and 440 nm. The excitation pulses were repeated every 20.28 s. Integration time was 10 ms. Emission/excitation parameters were 495 nm/340 nm and 495 nm/380 nm for fura-2, 560 nm/380 nm and 560 nm/440 nm for QC, 420 nm/380 nm for NADH and 470 nm/440 nm for flavins (FLV). Quercetin was added after an initial pre-equilibration period (~45 min. of recording) when the fluorescence signal became stable. The cytosolic Ca^{2+} concentration, $[Ca^{2+}]_{cyt}$, was calculated from the equation [Grynkiewicz]:

$$[Ca^{2+}]_{cyt} = \beta K_d (R - R_{min})/(R_{max} - R) \quad (1)$$

where $R = F_{340}/F_{380}$ represents the ratio of the fluorescence emission at 340 nm and 380 nm excitation, respectively, R_{min} is the ratio F_{340}/F_{380} obtained in a nominally Ca^{2+}-free solution, R_{max} is F_{340}/F_{380} obtained when the Ca^{2+} indicator is saturated with Ca^{2+}, β is the ratio between F_{380} in the Ca^{2+}-free solution and F_{380} at saturation, and $K_d = 0.225$ μM is the Ca^{2+}-dissociation constant of the indicator. F_{340} and F_{380} were corrected for autofluorescence. Calibration for R_{max} was performed at the end of the measurement by addition of 35 μM digitonin for 15-20 min. The degree of cell permeabilization was 100% as evaluated by trypan blue exclusion tests in separate determinations. Then 10 mM EGTA was added to calibrate the QC fluorescence signal in 15 nM Ca^{2+}. After 15-20 min., other 10 mM EGTA were added to reach a final concentration of 7 nM free Ca^{2+} and R_{min} was evaluated after an additional 15-20 min. The control cells have been treated in the same manner only instead of calcium indicator an equal amount of DMSO has been added.

Fluorescence spectroscopy. Cell suspensions were prepared at a density of ≈10^6 cells/ml in ECS after three washes in ECS. 2 ml of suspension were transferred to the quartz cuvette under continuous stirring. Fluorescence recordings were done at 37ºC in a Horiba Jobin Yvon spectrofluorimeter, as described above for fura-2 loaded cells. Recordings were briefly interrupted for about 4 min. to collect fluorescence spectra at indicated parameters. The first spectrum was recorded after a pre-equilibration period of 45 min. Excitation/emission spectra were collected before and after addition of 50 μM quercetin at indicated times. After 1 h of exposure to QC, 35 μM digitonin was added to the cuvette. Fluorescence spectra were collected after 15 min., then 10 mM EGTA was added and a new series of spectra were recorded after an additional 15 min. In this final step, the calculated free Ca^{2+} concentration in solution was 15 nM. For ***autofluorescence measurements***, separate control cell samples were treated in the same manner with the exception that an equal amount of DMSO was added instead of fura-2 or instead of quercetin, respectively.

RESULTS AND DISCUSSION

On the basis of previously obtained preliminary results [10], we have repeated into more detail the experiments concerning the induction of a calcium signal following the entrance of quercetin in the intact Jurkat T cells. Indeed, the fluorescence measurements of the fura 2/AM loaded cells indicate a sustained calcium release from the intracellular stores, generating thus a bi-phasic Ca^{2+} signal evoked by 50 μM QC (Fig. 1a). In order to establish that this was really a true calcium signal and not an artifact, we have used an inhibitor of calcium channels, namely ruthenium red (RR). As it can be noticed in the Fig. 1b,in the same experimental conditions, the calcium signal is inhibited. Moreover, RR is an specific inhibitor for the ryanodinic receptor (RyR) and not of the inositol trisphosphate receptor (IP3).

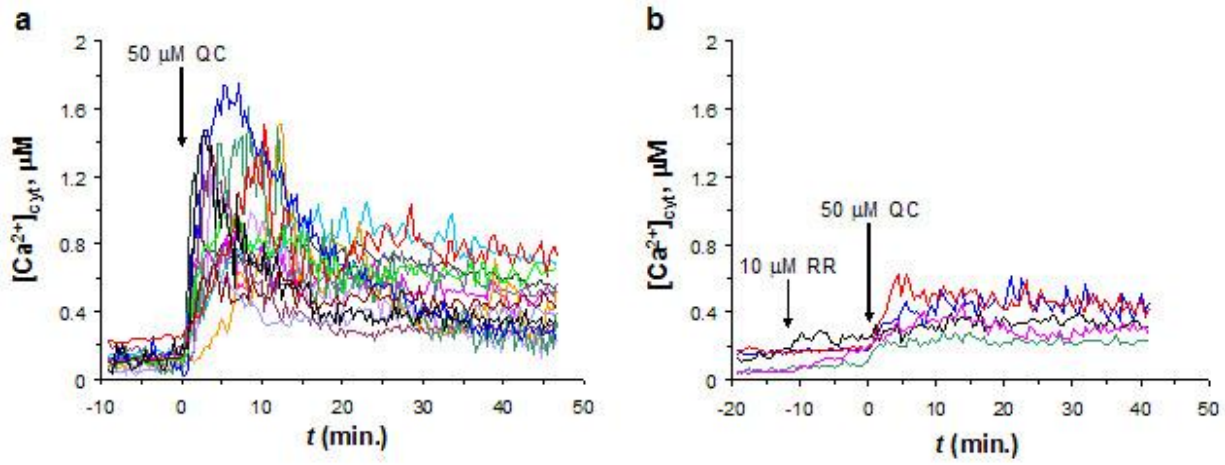

Fig. 1. Ca^{2+} and QC fluorescence signals evoked by 50 μM QC in Jurkat cells. **a-b**, Individual traces of Ca^{2+} signals induced by 50 μM QC in fura-2 loaded Jurkat cells in the absence of ruthenium red (RR) in 14 separate experiments (**a**) or in the presence of 10 μM RR in 5 independent experiments (**b**). The signals have been calibrated at the end of each experiment. The emission was collected at 420 nm with excitation at 360 nm. The signal was background-corrected.

The fluorescence spectra measured in a previous work [10], evidenced a decrease in the NADH level in intact Jurkat T cells exposed at 50 μM QC. We took a step further in this research and we measured the NADH level when the cellular calcium concentration is modified, respectively, we compared the evolution of this level in intact cells, in cells permeabilized with digitonin and then after adding EGTA. We found out that, in all the cases, the NADH level invariably decrease. (Fig. 2).

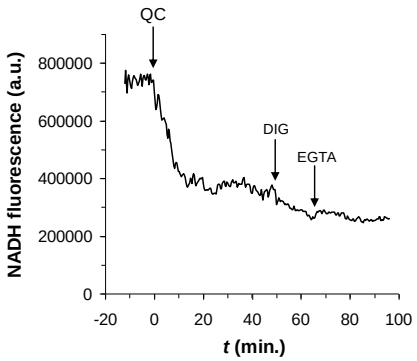

Fig. 2. The time course of NADH fluorescence after addition of 50 μM QC, 35 μM digitonin (DIG) and finally 10 mM EGTA to Jurkat cell suspensions.

Farther, we have examinated, developing the previous research line [10] the way in which the quercetin fluorescence is modified depending on the calcium concentration in the cells.

In our previous studies [10] we have observed that the wavelengths at which the excitation spectra of the intracellular quercetin present maxima are found around 389 nm, and 449 num respectively. The emission spectrum presents a maximum around 540 nm. We have proposed ourselves to examine into more detail the evolution of these spectra as a function of calcium concentration in the cell.

In order to better characterize the fluorescent properties of the intracellular quercetin, we have monitored the modifications of the fluorescence spectra when the intact Jurkat cells are exposed to 50 μM QC and after the cell permeabilization by digitonin. Fig. 3 illustrates an example of emission fluorescence spectra of QC in intact cells and in permeabilized Jurkat T cells, obtained on the excitation with 380 nm and 440 nm respectively. All the spectra present a prominent maximum at ~535-540 nm specific to the tautomeric forms of quercetin [13].

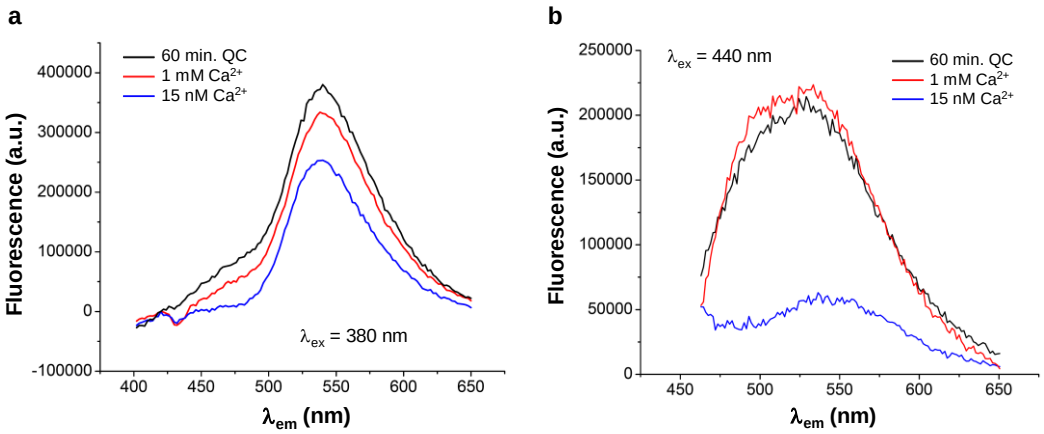

Fig. 3. Fluorescence emission spectra of QC in intact and permeabilized Jurkat cells. The excitation was done at 380 nm (**a**) and 440 nm (**b**). The spectra were recorded at 60 min. after exposure of the intact cells to 50 μM QC (black line), after the permeabilization in 1 mM Ca^{2+} (red line) or in 15 nM Ca^{2+} (blue line). These results have been obtained by averaging in 4 independent representative experiments. .

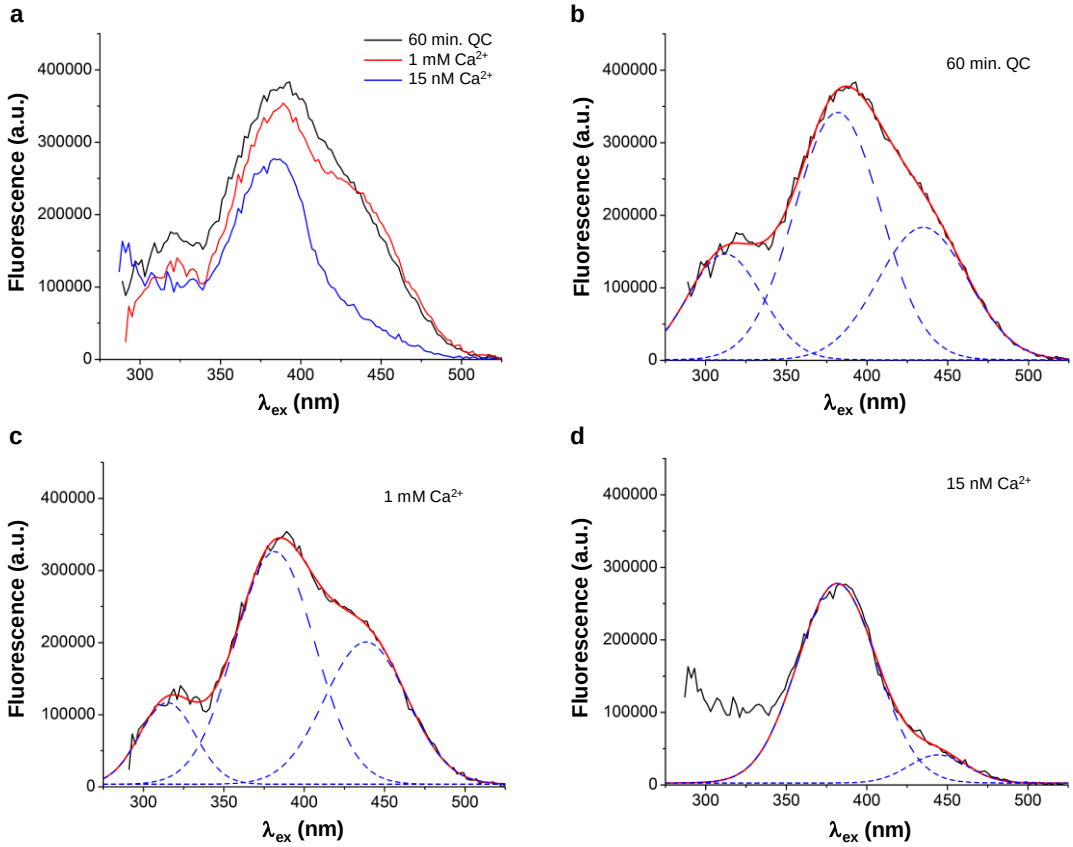

Fig. 4. Fluorescence excitation spectra of quercetin in intact and permeabilized Jurkat cells. Emission was collected at 540 nm. a, Spectra were taken 60 min. after exposure of intact cells to 50 μM QC (black trace), after permeabilization in 1 mM Ca^{2+} (red trace) or in 15 nM Ca^{2+} (blue trace). b-d, Gaussian deconvolution of the excitation spectra shown in a. The fitting curve (red) was obtained according to Eq. 4 with parameters provided in Table 1. Dashed blue curves represent the corresponding Gaussian components described by Eq. 4. In d, the first spectral component could not be determined. These results are representative of 4 independent experiments. Fluorescence was corrected for NADH/FLV interference.

Fig. 4 presents a representative set of excitation spectra of quercetin in intact and permeabilized cells, and the analysis by a Gaussian deconvolution. Our domain of interest lies in the two bands centered at ~380 nm and ~440 nm, respectively (Fig. 4, Table 1). The fluorescence data were fitted to the equation:

$$y = y_0 + A_1/w_1/(\pi/2)^{1/2} \exp\{-2[(\lambda_{ex} - \lambda_1)/w_1]^2\} + A_2/w_2/(\pi/2)^{1/2} \exp\{-2[(\lambda_{ex} - \lambda_2)/w_2]^2\} +$$

$$+A_3/w_3/(\pi/2)^{1/2} \exp\{-2[(\lambda_{ex} - \lambda_3)/w_3]^2\} \qquad (1)$$

where y represents the fluorescence emitted at 540 nm, y_0 represents a residual fluorescence component, A_1, A_2, A_3 and λ_1, λ_2, λ_3 are the amplitudes and wavelengths of the three excitation maxima, respectively. The parameters derived from the best fit are collected in Table S.

Our previous studies [10] have shown, in agreement with other studies in the literature [11], that quercetin accumulates inside the Jurkat cells and it binds to intracellular proteins, presenting a specific fluorescence with emission at ~540 nm.

Table 1. Fluorescence excitation parameters of intracellular quercetin

Conditions	y_0	A_1	w_1 (nm)	λ_1 (nm)	A_2	w_2 (nm)	λ_2 (nm)	A_3	w_3 (nm)	λ_3 (nm)
60 min. QC	765	8359997	45.7	312.1	22845346	53.4	381.8	12725602	55.6	434.8
1 mM Ca^{2+}	3570	4902427	34.5	314.9	20175523	49.9	381.6	12646700	51.1	438.4
15 nM Ca^{2+}	2751	n.d.	n.d.	n.d.	16938566	49.1	381.6	1753912	36.1	444.1

n.d., notdetermined

In the experiments performed at this stage of the project we have evidenced the specific mode in which the excitation and emission fluorescence spectra of quercetin are modified as a function of intracellular calcium concentration. Moreover, we could demonstrate that the addition of quercetin to intact Jurkat cells a calcium signal is evoked, and the inhibition with ruthenium red indicates the activation of a ryanodinic receptor. From the data presented here, we infer the possibility that the intracellular protein to which the quercetin binds might be the ryanodinic receptor from the endoplasmic reticulum. In the following research we poropse ourselves to study into more detail at which degree our hypothesis might be confirmed.

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