Scientific Report

regarding the implementation of the project PCE Ideas no. 342/2011 during the period December 2013 – December 2014

The present report structure follows the Work Plan proposed for the fourth stage of the project (Act Adiţional 1/2014). Supplementary to the objective proposed and fulfilled, we have performed more studies, that are continuing researches carried out in the third stage of the project and completing them. Nearly all the results currently reported have been communicated at national and international conferences and published in ISI journals, the resulting articles receiving awards from UEFISCDI at the 2014 competition. The report content is as follows:

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1. Assessment of quercetin ability to mediate calcium release in Jurkat lymphoblasts

INTRODUCTION

In a previous stage of the project (2013), which results have been published in a prestigious ISI journal [1] we have found that the flavonoid quercetin (3,3',4',5,7-pentahydroxyflavone; QC) can be used as an efficient fluorescent probe to distinguish with high sensitivity between the open and the closed conformation of the RyR3 channel in human leukemia Jurkat T-cells. Thus, we could quantify RyR3 activity in both intact and permeabilized cells and characterize the dependence of the open probability on the cytosolic concentration of Ca\(^{2+}\) or QC. The data presented in the above mentioned research also indicated that in situ, the RyR3 channel activity under physiological conditions is partially suppressed (hindered channel) whereas the channel is nearly fully activated upon exposure to high (~1 mM) concentrations of bulk cytosolic Ca\(^{2+}\) and subsequent chelation of Ca\(^{2+}\) (rectified channel).

Our current measurements unveil a second activating, low-affinity Ca\(^{2+}\) cytosolic site of the RyR3 receptor, which appears to be operational in the rectified but not in the hindered channel state. Nevertheless, the bell-shaped Ca\(^{2+}\)-dependence of the open probability (\(P_{\text{open}}\)) of the rectified channel [1] appeared to be remarkably similar to that observed in bilayer experiments [2-5], which underlines the idea that the steady state \(P_{\text{open}}\) data are not sufficient by themselves to infer the total number of regulatory sites.

Quercetin has been found to modulate the activity of the type 1 RyR (the skeletal muscle specific RyR isoform) in lipid bilayers [6] and to promote Ca\(^{2+}\) release from purified SR [7]. We determined previously that quercetin induced a strong Ca\(^{2+}\) release signal in intact Jurkat cells [1, 8] which appears to be mediated by RyR3 receptors [1]. QC can also inhibit reversibly the SR Ca\(^{2+}\)-ATP-ase and the uptake of Ca\(^{2+}\) ions by the sarcoplasmic reticulum [9-11]. This molecule is poorly fluorescent in aqueous solutions [12,13] but displays a specific fluorescence upon cellular internalization [12, 8], which appears to be connected with binding to mitochondrial proteins [14], ER ryanodine receptors [1, 8] or nuclear targets [15]. Previous modeling studies [16] based on steady \(P_{\text{open}}\) data obtained with the RyR1 isoform incorporated into artificial lipid bilayers [6] suggested that RyR1 has two distinct QC sites which are allosterically coupled with the activating and the inhibitory Ca\(^{2+}\) site, respectively. However, while the stimulatory effect of quercetin on RyR opening by Ca\(^{2+}\) could be well characterized, the available data [6] were not sufficient to make detailed inferences regarding the effect of quercetin on the inhibitory Ca\(^{2+}\) site [16]. Our current investigations indicate that RyR3 possesses three distinct QC binding sites [17]. One of the three sites stimulates RyR3 activation while the other two promote channel closing. Moreover, we show that in the case of the RyR3 receptor the Ca\(^{2+}\) and QC binding sites are also allosterically coupled and we confirm some of the QC binding parameters derived for RyR1 [16].

In this study, based on the previous results we are investigating into more detail the distinct modes of RyR3 activity through the assessment of its regulation by quercetin and Ca\(^{2+}\) in the rectified and hindered states of the channel both in open and closed conformations.

We have also proposed ourselves to study the effect of calcium chelator BAPTA/AM on the Ca\(^{2+}\)intracellular concentration after exposure to quercetin or menadione of Jurkat cells. Additionally to the objectives planned for the current phase of the project we present recent data concerning the antiproliferative effects and synergy of quercetin and menadione in human leukemia cells Jurkat T, extending the investigations carried out in the previous phase of treatments of long duration [18]. The results presented here have been partially published in ISI journals, the other ones being the object of a manuscript ion preparation.
MATERIALS AND METHODS.

Human leukemia Jurkat T-cell lymphoblasts (clone E6.1 from ECACC) were cultured in RPMI 1640 medium (Invitrogen 72400-021) containing Glutamax-I and 25 mM HEPES, and supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37°C in a humidified incubator with a 5% CO₂ atmosphere. Cell density, viability and morphology were examined under a phase contrast microscope. Viability was assessed by the trypan-blue exclusion test. Cell counting was performed using a Bürker-Türk haemocytometer. Unless stated otherwise, reagents were purchased from Sigma. Dihydrated quercetin (100 mM) and digitonin (50 mM) were dissolved in dimethyl sulfoxide (DMSO) and kept at -20°C for maximum 2 months. EGTA dissolved in NaOH was stocked at 0.75 M, pH 7.2/HCl, and kept at 18°C for up to 1 month. In some experiments quercetin was freshly diluted in DMSO to 2, 10 or 30 mM before the measurement. The extracellular-like solution (ECS) contained 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, 10 mM glucose, pH 7.2/NaOH. The saline solution used in long-term measurements with permeabilized cells (PCS) contained 140 mM KCl, 4 mM NaCl, 0.14 mM CaCl₂, 0.5 mM MgCl₂, 20 mM HEPES, pH 7.2/NaOH. When required, CaCl₂ solutions were freshly prepared at working concentrations of 50 mM, 1 M or 6 M (CaCl₂) in either ECS or PCS as needed. The level of free Ca²⁺ in solutions was calculated using the software WEBMAXCLITE v1.15. For assessment of QC and NADH fluorescence in intact or permeabilized cells, exponentially growing Jurkat cells were washed three times in ECS or PCS as indicated, resuspended in 2 ml ECS or PCS, counted and adjusted to the desired cell concentration by addition of ECS or PCS as necessary. The cells were then transferred to the spectrophotometer into a 2 ml quartz cuvette maintained at 37°C under continuous stirring. Cell viability assessed immediately before the fluorescence recordings was >90% in all cell samples. Fluorescence was recorded in a Horiba Jobin Yvon spectrophotometer equipped with two monochromators, by sequential excitation at 380 and 440 nm. The excitation pulses were repeated every 20.28 s. Integration time was 10 ms. Emission/excitation parameters were 540 nm/380 nm and 540 nm/440 nm for QC fluorescence, and 450 nm/380 nm and 495 nm/440 nm to estimate the interference with NADH and FLV fluorescence. Quercetin was always added after an initial pre-equilibration period (~45 min. of recording) when the fluorescence signal became stable. Cell permeabilization was achieved by addition of 35 μM digitonin for at least 15 min. In intact cell studies, cells were prepared in ECS and challenged with 50 μM QC. At the end of the measurement, digitonin was added to the cuvette for 15-20 min., and then 10 mM EGTA was added to calibrate the QC fluorescence signal. Qₘₐₓ was determined as the ratio F₃₈₀/F₄₄₀ obtained after an additional 20-30 min. of recording. Part of these experiments were performed in a similar manner with the difference that cell were preloaded with fura-2. For details please refer to ref. [1]. In permeabilized cell studies, cells were prepared in PCS. After ~30 min. of recording, cells were first permeabilized for 15 min. and then challenged with 5 μM QC. In experiments done with increasing QC levels up to 10 μM, rectification was achieved at the end of the measurement by addition of CaCl₂ for 15 min. to obtain ~0.75-2 mM bulk Ca²⁺, followed by addition of EGTA for 20-30 min. to obtain 100 nM Ca²⁺. Background fluorescence was determined in 2 ml of the appropriate saline solution with or without addition of corresponding agents, and subtracted from all data. Correction for NADH/FLV interference was performed for the QC fluorescence signals F₃₈₀ and F₄₄₀ according to the equation: F(t) = Fₜₑｃₑ(t) - Fₙₑᵣᵣₛ × Fᵢ(t)/Fᵢₑᵣᵣₛ, where F denotes the corrected value of F₃₈₀ or F₄₄₀ at time t, Fₜₑｃₑ is the recorded value of F₃₈₀ or F₄₄₀, Fᵢ(t) is the corresponding raw value of NADH and FLV intrinsic fluorescence with excitation at 380 nm or 440 nm, respectively, and Fᵢₑᵣᵣₛ is the 5 min.-average of Fᵢ before stimulation with QC. Data fitting in Figs. 2-3 was performed by minimizing the sum of normalized squares derived from each of the three (F₄₄₀ₑᵣᵣₛ, F₄₄₀ᶜₑᵈₑ and F₃₈₀ₑᵣᵣₛ) data sets, i.e., \[ \sum_{j=1}^{n_1} \left( \sum_{i=1}^{n_2} \left( \frac{y_{\text{fit},j} - y_{\text{exp},j}}{y_{\text{fit},j}} \right)^2 \right) / n_1 = \text{min} \] . where n₁, n₂ and n₃ are the numbers of data points in each set, and yᵢᵣᵣₛ and yₑᵣᵣₛ denote theoretical and experimental values of the variables, respectively. The fit shown in Fig. 5A was done using the program Origin, version 7.5. Unless otherwise stated, data are presented as median ± s.e.m. Confocal microscopy. Cells were co-incubated for 30 min. in complete medium at 37°C with the ER- and mitochondria-specific fluorescent
probes ER-Tracker Red (2 μM) and JC-1 (1 μg/ml), respectively. ER-Tracker Red binds to ATP-sensitive K⁺ channels which are prominent on ER, whereas JC-1 accumulates in active mitochondria as fluorescent aggregates that emit in orange-red. Cells were then washed twice with warm SS and resuspended in SS containing 10 μM quercetin, at ~10^6 cells/ml. After an additional 20-30 min. of incubation, the cells were immediately mounted on a microscope slide and sealed off by gluing the cover slip to the slide. Images were acquired with a Carl Zeiss LSM710 laser-scanning confocal microscopy system with spectral detection capabilities, using a Zeiss 63× 1.40NA immersion objective. Carl Zeiss Zen 2010 software version 6.0 was used for image acquisition and analysis, and some image processing was also done using LSM Image Browser (Zeiss). Excitation light was provided by 405, 445, 488, and 543 nm laser lines. Fluorescence emission was detected at 520-580 nm for quercetin (with excitation at 405 nm or 445 nm), 578-614 nm for JC-1 (with excitation at 488 nm) and 605-663 nm for ER-Tracker Red (with excitation at 543 nm). Distinct tracks were acquired for each of the analyzed dyes in order to minimize the spectral overlap of fluorescence emissions. The scanning speed was set at 1.58 microseconds/pixel, and the confocal aperture corresponded to a backprojected size of 1 Airy unit.

1.1. Characterization of the in situ regulation of RyR3 receptor by Ca²⁺ and quercetin; the determination of the binding parameters of the specific binding sites in the open/closed conformation of the channel

RESULTS

1.1.1. Discrimination between open and closed channel conformations by dual excitation fluorescence measurements

In a previous work [1, report 2013] we have shown that the ratio \( Q = F_{380}/F_{440} \) of the quercetin-specific cellular fluorescence emitted at 540 nm upon excitation at 380/440 nm reflects the open probability of the endoplasmic reticulum RyR3/Ca²⁺ release channel in both intact and permeabilized Jurkat cells. The results presented therein indicate that F380 and F440 quantify the binding of quercetin to the open Ca²⁺ release channels, and to channels found in either open or closed configuration, respectively. Thus, F440 represents the additive contribution of the fluorescence of the QC-liganded closed channels, which displays a specific excitation band at 440 nm, and that of a residual fluorescence emitted by the QC-bound open channels, which is characterized by an excitation maximum centered on 380 nm [1, 8].

Some representative images of the cellular distribution of these two fluorescent species of quercetin observed by confocal microscopy are provided in Fig. 1. Both QC fluorescent signals colocalize with the endoplasmic reticulum (Fig. 1F-G), which appeared as a widely spread thin layer enfolding the mitochondria (Fig. 1A-C), whereas mitochondria could be distinguished as individual entities of several hundreds of nanometers in diameter (Fig. 1B). Both fluorescent species of quercetin exhibited similar distributions over the ER, with some dense regions in close proximity (<0.5 μm) to the mitochondria (Fig. 1D-E). In addition, a somewhat higher fraction of open RyR3 channels seemed to be located on the outer shell of the ER surrounding the mitochondria (Fig. 1H), suggesting that under the current conditions RyR3s that were closer to the plasma membrane were more prone to opening. A small fraction of quercetin fluorescence seemed to originate from the nucleus (Fig. 1D-E), which could derive from quercetin bound to ryanodine receptors present in the nuclear membranous system.

In a previous work [1] we have shown that normalization of the QC fluorescence ratio \( Q \) to its maximal value, \( Q_{\text{max}} \), can provide a quantitative means to assess the open probability of RyR3 in situ \( (P_{\text{open}} = Q/Q_{\text{max}}) \).
Fig. 1. Colocalization of fluorescent quercetin with the ER. Confocal microscopy was carried out as described under “Materials and Methods”. In A-G, fluorescence of ER-Tracker Red, quercetin (excited at 405 nm) and JC-1 is presented in blue, green and red, respectively. Individual scans for each dye are displayed in A, B and D. Panels C, E and F present overlay images of two selected dyes. Panel G is obtained by superimposing the fluorescent signals from all three dyes (ER-Tracker Red, JC-1, and QC excited at 405 nm). In panel H fluorescent quercetin excited at 405 nm and 445 nm is shown in blue and red, respectively.

Ideally, $Q_{\text{max}}$ represents the ratio between the maximal value of the open channel fluorescence that can be reached upon excitation at 380 nm, and the fluorescence emitted by the fully activated open channel upon excitation at 440 nm. The data indicated that $Q_{\text{max}}$ is fully determined by two parameters, namely the width of the 380 nm excitation band, and the wavelength difference between the two excitation bands. The former parameter appeared to
depend on the cell concentration in the sample, but in a given sample it did not change with the treatment conditions [1].

To discriminate between the fluorescence emitted by the channel in the open or in the closed conformation, we should first point out that when all the RyR3 channels in a given cell sample are fully activated, the ratio F380/F440 is equal to $Q_{\text{max}}$. Based on the invariability of the 380 nm excitation band width with the treatment conditions ([1], Table 1 therein), an important implication with general applicability to any given cell sample is that the ratio:

$$F_{440,\text{open}} = \frac{F_{380}}{Q_{\text{max}}}$$

(1)

represents the contribution to F440 of the channels found in open configuration, and the corresponding contribution of the channels in closed configuration is then

$$F_{440,\text{closed}} = F_{440} - F_{440,\text{open}}$$

(2)

Data analysis indicates that $F_{440,\text{open}}$ and $F_{440,\text{closed}}$ are proportional to the number of QC-ligated open and closed channels rather than to the total number of QC molecules bound to the open and the closed channels, respectively. Consequently, the fraction of channels found in open or closed conformation can be readily determined by dividing $F_{440,\text{open}}$ or $F_{440,\text{closed}}$ to the maximal F440 value, $F_{440,\text{max}}$. As discussed below, this upper bound could be obtained at saturating levels of quercetin (>300 μM) and it appeared to be invariant at activating or inhibitory bulk concentrations of cytosolic Ca$^{2+}$, [Ca$^{2+}$]$_{\text{bulk}}$.

1.1.2. RyR3 regulation by Ca$^{2+}$ in open/closed conformation

Using the conformation sensitive fluorescent approach described above, we investigated in more detail the unexpected mechanism of channel rectification revealed by our previous measurements [1, report 2013]. We have scrutinized the molecular properties of this unique facet of RyR3 regulation by assessing the dependence of the fluorescence emitted by the QC-ligated channel on [Ca$^{2+}$]$_{\text{bulk}}$ (Fig. 2A-B) in the closed or open configurations of the channel, in either hindered or rectified states. To this end, by using quercetin (5 μM) as a RyR fluorescent probe, we assessed $F_{440,\text{open}}$ and $F_{440,\text{closed}}$ in permeabilized cell suspensions at various decreasing Ca$^{2+}$ levels which were controlled via stepwise addition of CaCl$_2$ or EGTA in sequences of ~7-10 min. duration (see also Fig. 4 in [1]). To describe the quasi-steady state at each agonist level, average fluorescence values were calculated over the final 4-5 min. of each recording step. To obtain the rectified channel state, cells were first permeabilized for 15 min. in PCS and then challenged with 5 μM QC, after which the level of Ca$^{2+}$ was first brought within the range 0.75 - 2 mM, and then EGTA was added sequentially. Maximal channel activation could be achieved in a final [Ca$^{2+}$]$_{\text{bulk}}$ ranging between approximately 100 nM and 100 μM ([1], Fig. 2D here). The hindered channel state was obtained when [Ca$^{2+}$]$_{\text{bulk}}$ was either increased (by addition of CaCl$_2$) or decreased (by addition of EGTA) from an initial level lying between 1 and 140 μM ([1], Fig. 2D here).

The hindered channel in open configuration (Fig. 2A) appeared to be regulated by a class of high-affinity activating Ca$^{2+}$ sites ($A_1$) and a class of low-affinity inhibitory Ca$^{2+}$ sites ($I$) (discussed below). A surprisingly different picture could be observed with the open rectified channel, which revealed a second class ($A_2$) of activating Ca$^{2+}$ sites of low affinity, while no apparent inhibitory site could be distinguished (Fig. 2A). In closed conformation, all three classes of Ca$^{2+}$ sites appeared to be operational in both hindered and rectified channels (Fig. 2B).

To substantiate these conclusions, we first analyzed the $F_{440,\text{open}}$ data obtained with the hindered channel (Fig. 2A), which were fitted to the equation:

$$y = F_{\text{Om}} \times \frac{x^{HA1}}{(x^{HA1} + K_{dA1}^{HA1})} \times \frac{K_{dI}^{HI}}{(x^{HI} + K_{dI}^{HI})}$$

(3)

where $y$ represents $F_{440,\text{open}}$ and $x$ the concentration of cytosolic free Ca$^{2+}$. The best fit parameters were: $F_{\text{Om}} = 30385$, $K_{dA1} = 10.8$ pM, $HA1 = 0.49$, $K_{dI} = 807$ mM, $HI = 0.26$. In Eq.
3, $K_{dA1}$ and $HAI$ represent the dissociation constant and Hill coefficient of the high affinity activating Ca$^{2+}$ site, $A1$, and $K_{dI}$ and $HI$ represent the dissociation constant and Hill coefficient of inhibitory Ca$^{2+}$ site, $I$, in the open conformation of the receptor.

Several lines of evidence discussed below suggest that F380 and F440 are proportional to the number of quercetin-ligated RyR3 channels rather than to the number of QC molecules bound to these channels. In addition, $P_{open} = Q/Q_{max} = F440_{open}/F440$. Then the fluorescence intensity emitted by the QC-bound channels in the open or in the closed conformation can be expressed as:

$$F440_{open} = a N_{QO} = a N_Q P_{open} = a N_Q \tau_Q/(\tau_Q + \tau_C) \quad (4)$$

$$F440_{closed} = a N_{Qc} = a N_Q (1 - P_{open}) = a N_Q \tau_C/(\tau_Q + \tau_C) \quad (5)$$

where $a = $ constant, $N_Q$ represents the number of RyR3 molecules that bind QC at a certain concentration of QC (see also the next section), $N_{QO}$ and $N_{QC}$ represent the number of QC-bound open and QC-bound closed channels, respectively, and $\tau_Q$ and $\tau_C$ represent the mean open- and closed-dwell time of the QC-bound channel, respectively. Eqs. 4 and 5 share the same constant $a$ since $a$ is determined by the incident flux of photons and by the spectrofluorimeter characteristics, but it may also depend on the absorptivity and the quantum yield of the fluorophore (QC) and that of the RyR3 protein. Hence, this result indicates that the probability for the RyR3-QC complex to fluoresce upon being excited at 440 nm is the same in both open and closed conformations. After summing up Eqs. 4 and 5 we obtain:

$$F440 = a N_Q \quad (6)$$

So, normalizing F440 to the maximal value obtained at saturating levels of quercetin (discussed below in the following section), which is:

$$F440_{max} = a N \quad (7)$$

where $N$ represents the total number of functional RyR3 molecules in a cell, we obtain the fraction of QC-bound RyR3 molecules at a given level of cytosolic Ca$^{2+}$.

The F440open data obtained with the rectified channel in open configuration (Fig. 2A) were well fitted by the equation:

$$y = F_{O1} \times x^{HA1}/(x^{HA1} + K_{dA1}^{HA1}) + (F_{O0} - F_{O1}) \times x^{HA2}/(x^{HA2} + K_{dA2}^{HA2}) \quad (8)$$

where $y$ represents F440open and $x$ the concentration of cytosolic free Ca$^{2+}$. $F_{O0}$ was set to 30385, the maximal value of F440open derived above (Eq. 3). The best fit parameters were: $F_{O1} = 17014$, $K_{dA1}^{HA1} = 319$ pM, $HA1 = 0.86$, $K_{dA2}^{HA2} = 1.09$ mM, $HA2 = 2.57$.

Eq. 8 describes the contribution of two populations of QC-ligated open channels, namely channels in the open state $O1$ and channels in the open state $O2$. These two states have either the $A1$ or the $A2$ Ca$^{2+}$ site occupied, respectively, which implies that $A1$ and $A2$ act as complementary sites, i.e., Ca$^{2+}$ cannot bind $A2$ unless $A1$ is free, and vice versa. In addition, binding of Ca$^{2+}$ to $A1$ or $A2$ opens the channel, and conversely, dissociation of Ca$^{2+}$ from any of the two sites shuts the channel.

Let $b_{A1}$ and $b_{A2}$ denote the Ca$^{2+}$-binding capacity of $A1$ and $A2$, respectively, which can be described as:

$$b_{A1} = x^{HA1}/(x^{HA1} + K_{dA1}^{HA1}) \quad (9)$$

$$b_{A2} = x^{HA2}/(x^{HA2} + K_{dA2}^{HA2}) \quad (10)$$

where $K_{dA1}$, $K_{dA2}$, $HA1$ and $HA2$ indicate dissociation constants and Hill coefficients of the two binding sites $A1$ and $A2$, respectively. The fluorescence emitted by all the QC-bound open channels is then

$$F440_{open} = a [N_{Qom} b_{A1} P_{O1} + N_{Qom} b_{A2} (1 - P_{O1})] \quad (11)$$

where $N_{Qom}$ represents the maximal number of open channels that can bind QC at a cytosolic level of 5 µM QC (see also the next section), and $P_{O1}$ is the probability that a QC-bound open channel is in the open state $O1$, so

$$P_{O1} = \tau_{O1}/(\tau_{O1} + \tau_{O2}) \quad (12)$$
where $\tau_{O1}$ and $\tau_{O2}$ represent the average dwell time a channel spends in the open state O1 and the dwell time spent in the open state O2, respectively. In other words, $\tau_{O1}$ and $\tau_{O2}$ represent the mean time a QC-bound open channel has the site A1 or the site A2 occupied by Ca$^{2+}$, respectively. After substituting Eqs. 9, 10 and 12 into Eq. 11 we obtain Eq. 8, where

$$F_{Om} = a N_{QOm}$$  \hspace{1cm} (13)
$$F_{O1} = F_{Om} \times P_{O1}$$  \hspace{1cm} (14)

At saturating levels of Ca$^{2+}$, when $b_{A1} = b_{A2} = 1$, the relative abundance of the two populations of channels in open states O2 and O1, respectively, is equal to $P_{O1}/(1 - P_{O1}) = \tau_{O1}/\tau_{O2}$. Using $P_{O1} = F_{O1}/F_{Om} = 0.560$, we obtain $\tau_{O1}/\tau_{O2} = 1.27$. Since $\tau_{O1}$ and $\tau_{O2}$ are the inverse of the corresponding rate constant of Ca$^{2+}$ dissociation from A1 and A2, respectively, it means that Ca$^{2+}$ dissociates from A1 1.27 times slower than it dissociates from A2. It should be mentioned that the data obtained with the hindered channel could not be fitted reasonably by Eq. 8. So, our results suggest that the rectified channel in the open conformation possesses two activating Ca$^{2+}$ sites which are both accessible to cytosolic Ca$^{2+}$, whereas the inhibitory Ca$^{2+}$ site seems to be shielded somehow. In the hindered channel found in open conformation, only the high affinity activating Ca$^{2+}$ site and the inhibitory site Ca$^{2+}$ appear to be exposed and functional.

The data of F440$_{\text{closed}}$ obtained with the hindered channel in closed state (Fig. 2B) exhibited an inverted bell-shaped Ca$^{2+}$-dependence with a non-negligible plateau over a large range of [Ca$^{2+}$]$_{\text{bulk}}$, which is a clear result of Ca$^{2+}$ binding to the two activating sites and the inhibitory site, respectively. In the low-level Ca$^{2+}$ domain ($< 0.01 \mu M$), A2 and I are unoccupied and Ca$^{2+}$ binding to A1 decreases the number of QC-bound closed channels. Within the plateau region ($\sim 0.01$-$100 \mu M$ Ca$^{2+}$), A1 is saturated while A2 and I are unoccupied. Finally, at $>10 \mu M$ Ca$^{2+}$, A1 is saturated and Ca$^{2+}$ can bind to both A2 and I. The dominant effect of the inhibitory site to close the channel leads to the increased fluorescence emission observed within the high-concentration Ca$^{2+}$ domain. Consequently, the data were fitted to the equation:

$$y = F_{\text{cm}} \left[1 - \frac{P_{d1} \times x^{HA1}/(x^{HA1} + K_{dA1}^{HA1}) + (1 - P_{d1}) \times x^{HA2}/(x^{HA2} + K_{dA2}^{HA2})}{K_{dH}^{HI}/(x^{HI} + K_{dH}^{HI})}\right]$$  \hspace{1cm} (15)

with $F_{\text{cm}} = 356096$, $P_{d1} = 0.907$, $K_{dA1}^{HA1} = 6.82$ pM, $HA1 = 0.93$, $K_{dA2}^{HA2} = 13.2$ mM, $HA2 = 0.68$, $K_{dH} = 4.095$ M, $HI = 0.34$. The significance of the parameters related to the activating Ca$^{2+}$ sites A1 and A2 is similar to that of the corresponding parameters appearing in Eq. 8. Thus, $P_{d1}$ and $(1 - P_{d1})$ represent the probability that a QC-bound closed channel having both sites A1 and A2 unoccupied, binds Ca$^{2+}$ to A1 and to A2, respectively. So, the ratio of the corresponding association rates is $P_{d1}/(1 - P_{d1}) = 9.75$, which means that Ca$^{2+}$ associates with A1 10 times faster than it associates with A2. In addition, similarly to Eq. 13, $F_{\text{cm}} = a N_{\text{QC}}$, where $N_{\text{QC}}$ represents the maximal number of closed channels that can bind QC at a cytosolic level of 5 $\mu M$ QC. It should also be noted that Eq. 15 supports the notion that the inhibitory Ca$^{2+}$ site, which is responsible for the rising right side of the curve, functions independently of the two activating sites.

The data of F440$_{\text{closed}}$ obtained with the rectified channel in closed state were qualitatively similar to those obtained with the hindered channel (Fig. 2B). However, the plateau was significantly lower than before, indicating that site A2 has only a minor effect on F440$_{\text{closed}}$. In addition, the fit based on Eq. 15 could not yield realistic values of $HA2$, indicating that site A2 has a low affinity which should be at least comparable with the affinity of site I. Consequently, the data were fitted to the equation:

$$y = F_{\text{cm}} \left[1 - P_{d1} \times x^{HA1}/(x^{HA1} + K_{dA1}^{HA1}) \times K_{dH}^{HI}/(x^{HI} + K_{dH}^{HI})\right]$$  \hspace{1cm} (16)

with $F_{\text{cm}} = 356843$, $P_{d1} = 0.9807$, $K_{dA1}^{HA1} = 108.0$ pM, $HA1 = 0.93$, $K_{dH} = 4.84$ mM, $HI = 1.54$. 

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Fig. 2. RyR3 in situ regulation by cytosolic Ca$^{2+}$. Quercetin fluorescence was monitored in permeabilized Jurkat cells, as described in the text (see also Fig. 4 in ref. [1]) (rectified channel: $n = 24$ experiments, 150 data points; hindered channel: $n = 31$ experiments, 195 data points) A, Ca$^{2+}$-dependence of the fluorescence emitted by QC-bound channels in open conformation (F440$^\text{open}$). The data were fitted to Eq. 3 (hindered channel) or Eq. 8 (rectified channel). B-C, Ca$^{2+}$-dependence of the fluorescence emitted by QC-ligated channels in closed conformation (F440$^\text{closed}$). Data in B were fitted to Eq. 15 (hindered channel) or Eq. 16 (rectified channel). In C, the full fitting curves are shown. In A-B, data from samples with similar cell densities were used. Accordingly, the median ± s.e.m. of $Q_{\text{max}}$ was 7.85 ± 1.36 in all $n = 24$ rectified-channel determinations, and 7.34 ± 1.03 in $n = 22$ hindered-channel experiments performed with increasing [Ca$^{2+}$]$_{\text{bulk}}$ before calibration. When rectification was not achievable ($n = 9$), $Q_{\text{max}}$ was calculated using the calibration curve described in Fig. 1D in ref. [1]. D. The open probability ($P_{\text{open}}$), and E-F, the fraction of QC-bound channels depend biphasically on Ca$^{2+}$. In D, $P_{\text{open}}$ was calculated as the ratio F440$^\text{open}$(F440$^\text{open}$ + F440$^\text{closed}$) obtained from experiments described in A-B, plus other experiments with significantly different cell densities in the samples (rectified channel: $n = 45$ experiments, 205 data points; hindered channel: $n = 42$ experiments, 238 data points). The data shown in D are equivalent to those obtained in Fig. 4C,D in [1], but are fitted to the corresponding functions based on Eqs. 3 and 15 (hindered channel) or Eqs. 8 and 16 (rectified channel). In E, total fluorescence F440 was divided to the maximal value F440$_{\text{max}}$ indicated in Fig. 3A. Fitting curves in E-F were obtained by summing up the corresponding open- and closed-conformation curves shown in A and B-C, respectively, followed by normalization to F440$_{\text{max}}$. All fluorescence signals were corrected for NADH/FLV interference.

Significantly, $F_{\text{Cm}}$ obtained here was virtually equivalent to the corresponding value obtained with the hindered channel. Moreover, since $P_{\text{C1}}$ is very close to 1, we can assume
that the estimated values of $K_{dl}$ and $HI$ should reflect fairly well the actual features of the inhibitory Ca$^{2+}$ site. So, the ratio of the on-binding rates corresponding to sites $A1$ and $A2$ is $P_{C1}/(1 - P_{C1}) = 50.8$, which means that Ca$^{2+}$ binds to $A1$ 51 times faster than it binds to $A2$. The overall Ca$^{2+}$-dependence of F440$_{closed}$ predicted by Eqs. 15 and 16 for both hindered and rectified channels in the presence of 5 µM quercetin is illustrated in Fig. 2C. Interestingly, the maximal value $F_{Cm}$ appears to be virtually the same in either hindered or rectified channel states.

The results obtained here are consistent with the findings that the RyR3 channel incorporated into lipid bilayers exhibits two distinct open dwell times and two or three distinct closed dwell times [5]. Moreover, the ratios of the two open or closed dwell times obtained here are in good agreement with the ratios reported therein. Our data indicate that the hindered channel in situ has a single open dwell-time distribution and a double closed dwell-time distribution, whereas the open and the closed dwell-time distributions of the rectified channel in situ display two distinct components each. These findings, together with the fact that the RyR3 characteristic shape of the Ca$^{2+}$-dependence of $P_{open}$ reported in bilayer experiments [2-5] was observed in our assays with the rectified but not with the hindered channel ([1], Fig. 2D here), support the idea that the RyR3 channel assumes a different configuration in an artificial membrane environment, which appears to correspond to the rectified mode observed in our experiments.

In addition, this type of conformation selective fluorescence measurements reinforced the idea of RyR regulation by allosteric coupling of Ca$^{2+}$ and QC binding sites as proposed for the RyR1 isoform [16], which was clearly demonstrated by the marked Ca$^{2+}$-dependence of the fraction of QC-recruited channels (Fig. 2E-F). Moreover, as discussed before [1], the Ca$^{2+}$-dependence of $P_{open}$ derived from the F440$_{open}$ and F440$_{closed}$ fluorescence data described above (Fig. 2D) apparently indicates the existence of one major activating Ca$^{2+}$ site of high affinity and one inhibitory Ca$^{2+}$ site of low affinity, in both hindered and rectified channel states. It is hence straightforward that the differential assessment of quercetin bound to RyR3 in open or closed conformation can provide more exhaustive insights into the regulation of channel activity by various ligands.

1.1.3. RyR3 regulation by quercetin in open/closed conformation

The dependence of F440$_{open}$ and F440$_{closed}$ on the flavonoid level (Fig. 3A-B) uncovered interesting features of RyR3 channel in situ regulation by quercetin. In order to better understand how we have obtained the equations that allowed us to calculate the dissociation constants and the Hill coefficients, we have reproduced here some of the figures obtained from the experiments carried out in the previous phase of the project. We studied here the hindered channel case because it presents more physiological interest, at variance with the rectified channel situation which appears to be closer to that manifested in artificial lipid bilayers [1].

We studied here the hindered channel case because it presents more physiological interest, at variance with the rectified channel situation which appears to be closer to that manifested in artificial lipid bilayers [1]. The current results indicate that in the closed conformation of the hindered receptor two distinct classes of QC inhibitory binding sites ($IQ1$ and $IQ2$) are functional, and no activating QC binding site is available for binding, whereas in the open conformation an activating site ($AQ$) seems to operate. It should be stressed here that at this point, we cannot distinguish unequivocally between the modes in which quercetin acts to promote either the opening or the closing of the channel (i.e., directly or by allosteric
modulation). However, we refer to the activating or inhibitory QC sites in correspondence with their stimulatory or inhibitory effect on channel activity at a fixed Ca\(^{2+}\) concentration.

Fig. 3. RyR3 in situ regulation by quercetin. QC-dependence of the fluorescence emitted by QC-liganded channels in open or in closed conformation (open and closed circles, respectively) in the presence of 80 nM cytosolic Ca\(^{2+}\) (A) or 1.2 mM cytosolic Ca\(^{2+}\) (B). Data were obtained as median ± s.e.m. from 8-16 (A) or 4-12 (B) determinations similar to those described in Fig. 5A in ref. [1]. Shortly, F380 and F440 fluorescence signals were recorded in permeabilized cells at increasing levels of cytosolic QC adjusted by sequential addition of quercetin. All samples had similar cell densities. The median ± s.e.m. of \( Q_{\text{max}} \) was (A) 7.41 ± 1.27 in \( n = 8 \) experiments, or (B) 7.26 ± 1.47 in \( n = 7 \) experiments in which \( Q_{\text{max}} \) could be assessed after rectification in a final level of 100 nM Ca\(^{2+}\) and 10 \( \mu \)M QC. In the remaining experiments \( Q_{\text{max}} \) was calculated using the standard calibration curve (Fig. 1D in ref. [1]). The closed- and open-conformation data were fitted to Eq. 17 and Eq. 22, respectively (curves). All fluorescence signals were corrected for NADH/FLV interference.

Fig. 4. Cytosolic QC-dependence of (A) RyR3 open probability and (B) the fraction of QC-bound channels as derived from data shown in Fig. 3A and B. In A, \( P_{\text{open}} \) was calculated as the ratio F440\(_{\text{open}}\)/F440. The results are equivalent to those obtained in Fig. 5C,D in [1], but the current fit is based on Eqs. 17 and 12. In B, total fluorescence F440 was divided to the corresponding F440\(_{\text{max}}\) indicated in the text. Continuous curves shown for 80 nM or 1.2 mM Ca\(^{2+}\) were calculated by summing up the two fitting curves shown in A or B, respectively, and then by dividing the result to the corresponding F440\(_{\text{max}}\). All fluorescence signals were corrected for NADH/FLV interference.

To substantiate the conclusions mentioned above, we first found that the closed conformation fluorescence data were well fitted by the equation:

\[
y = F440_{\text{max}} \times \left( P_{C1'} \frac{H1}{H1 + Kd1} + (1 - P_{C1'}) \frac{H2}{H2 + Kd2} \right)
\]

(17)

where \( y \) represents F440\(_{\text{closed}}\) and \( x \) the concentration of cytosolic free QC. The parameters derived from the best fit are F440\(_{\text{max}}\) = 647652.3, \( P_{C1'} = 0.0615, K_{d1} = 3.08 \mu\text{M}, H1 = 0.78, K_{d2} = 202.0 \mu\text{M} \) and \( H2 = 1.67 \) in the presence of 80 nM cytosolic Ca\(^{2+}\) (Fig. 4A), and
F440\textsubscript{max} = 612816.6, P\textsubscript{C1'} = 0.0934, K\textsubscript{d1} = 3.43 \mu M, H1 = 0.87, K\textsubscript{d2} = 127.4 \mu M and H2 = 1.58 in the presence of 1.2 mM cytosolic Ca\textsuperscript{2+} (Fig. 3B).

Eq. 17 describes the contribution of two populations of QC-ligated closed channels (namely, channels in closed state C1', and channels in closed state C2'). These two states have either the IQ1 or the IQ2 inhibitory site occupied, respectively, which means that IQ1 and IQ2 act as complementary sites, i.e., QC cannot bind IQ2 unless IQ1 is free, and vice versa. Let b\textsubscript{IQ1} and b\textsubscript{IQ2} denote the QC-binding level of IQ1 or IQ2, respectively, which can be described as:

\begin{align*}
\textit{b_{IQ1} = x_{1}^{H1}/(x_{1}^{H1} + K_{d1}^{H1})} \\
\textit{b_{IQ2} = x_{2}^{H2}/(x_{2}^{H2} + K_{d2}^{H2})}
\end{align*}

(18)  
(19)

where K\textsubscript{d1}, K\textsubscript{d2}, H\textsubscript{1} and H\textsubscript{2} indicate dissociation constants and Hill coefficients of the two binding sites, respectively. The fluorescence emitted by all closed channels is then

\[ F440_{closed} = a \left[ N \cdot b_{IQ1} \cdot P_{C1'} + N \cdot b_{IQ2} \cdot (1 - P_{C1'}) \right] \]

(20)

where N represents the total number of functional RyR3 molecules in a cell, P\textsubscript{C1'} is the probability that a closed channel is in the closed state C1', so

\[ P_{C1'} = \tau_{C1'}/(\tau_{C1'} + \tau_{C2'}) \]

(21)

where \tau\textsubscript{C1'} and \tau\textsubscript{C2'} represent the average dwell time a closed channel spends in the closed state C1' and in the closed state C2', respectively. Substituting Eqs. 18, 19 and 21 into Eq. 20 and using Eq. 7, we obtain Eq. 17.

At saturating levels of QC, when b\textsubscript{IQ1} = b\textsubscript{IQ2} = 1, the relative abundance of the two populations of closed channels found in states C2' and C1', respectively, is equal to (1 - P\textsubscript{C1'})/P\textsubscript{C1'} = \tau_{C2'}/\tau_{C1'}. Using the P\textsubscript{C1'} value obtained from the fit, we obtain \tau_{C2'}/\tau_{C1'} = 9.7 in the presence of 1.2 mM cytosolic Ca\textsuperscript{2+} and \tau_{C2'}/\tau_{C1'} = 15.3 in the presence of 80 nM cytosolic Ca\textsuperscript{2+}, suggesting that Ca\textsuperscript{2+} affects the rate of QC binding to at least one inhibitory QC site.

An alternative explanation for the double-component appearance of the QC-dependence of F440\textsubscript{closed} is that a different RyR isoform may contribute to the observed fluorescence emission. However, this appears to be highly unlikely because the Ca\textsuperscript{2+}-dependence of P\textsubscript{open} obtained at the low level of 5 \mu M QC and also at 50 \mu M QC (not shown) is specific for the RyR3 isoform. More importantly, the relative contribution of the two inhibitory QC sites appears to be reversed in open conformation, with an estimated dwell-time ratio \tau_{C1'}/\tau_{C2'} of 1 or 6.2 in favor of the high-affinity site (see below). Consequently, these data strongly support the idea that the ryanodine receptor type 3 possesses at least two functionally distinct QC inhibitory sites.

It should be stressed that the F440\textsubscript{max} values obtained with 80 nM or 1.2 mM Ca\textsuperscript{2+} were closely similar. Consequently, we consider that F440\textsubscript{max} is reached when all RyR3 receptors have bound quercetin, an idea that is also strengthened by the fact that under this assumption we obtained consistent results regarding the cytosolic level of free quercetin and a good agreement between the diffusional limit of the cytosolic level of free QC (43.3 \mu M) and the level applied in the extracellular solution (50 \mu M).

The open conformation data (Fig. 3A-B) were fitted to the equation:

\[ y = F440_{max} \cdot x_{1}^{H1}/(x_{1}^{H1} + K_{d1}^{H1}) \cdot [P_{C1'} \cdot K_{d1}^{H1}/(x_{1}^{H1} + K_{d1}^{H1}) + (1 - P_{C1'}) \cdot K_{d2}^{H2}/(x_{2}^{H2} + K_{d2}^{H2})] + (1 - P_{C1'}) \cdot K_{d2}^{H2}/(x_{2}^{H2} + K_{d2}^{H2}) \]

(22)

where y represents F440\textsubscript{open} and x the concentration of cytosolic free QC. In experiments conducted with 80 nM Ca\textsuperscript{2+}, F440\textsubscript{max} was set to 647652.3 as derived from the closed conformation case discussed above, and the corresponding parameters obtained from the best fit were K\textsubscript{d} = 411.2 \mu M, H = 0.75, P\textsubscript{C1'} = 0.500, K\textsubscript{d1} = 10.11 \mu M, H1 = 0.55, K\textsubscript{d2} = 88.2 \mu M and H2 = 3.60. With 1.2 mM Ca\textsuperscript{2+}, F440\textsubscript{max} was set to 612816.6, and best-fit parameters were K\textsubscript{d} = 17.0 \mu M, H = 1.14, P\textsubscript{C1'} = 0.861, K\textsubscript{d1} = 61.8 nM, H1 = 0.69, K\textsubscript{d2} = 199.8 \mu M and H2 = 1.45. In Eq. 22, the term in the brackets describes the quenching effect of the two inhibitory sites IQ1 and IQ2 as discussed above, whereas the first term, \( x_{1}^{H1}/(x_{1}^{H1} + K_{d1}^{H1}) \), illustrates the
activation of the channel promoted by QC binding to an activating QC site \((AQ)\), which operates independently of the two QC inhibitory sites. Here \(K_d\) and \(H\) denote dissociation constant and Hill coefficient of site \((AQ)\). Conversely, the channel closes upon QC dissociation from \((AQ)\). The description provided by Eq. 22 is consistent with the existence of two open states of the channel, \(O1'\) and \(O2'\), which have the \((AQ)\) site bound and either \((IQ)1\) or \((IQ)2\) unbound, respectively. The characteristic parameters of the two inhibitory QC binding sites in open and closed conformations derived above indicate a significant change in the affinity and the number of QC binding molecules. Thus, the data indicate that in the presence of 80 nM cytosolic Ca\(^{2+}\), \(K_{d1}\) increases three times whereas \(H_1\) decreases from 0.78 to 0.55 upon channel opening. Under the same conditions, \(K_{d2}\) decreases 2.3 times whereas \(H_2\) increases from 1.7 to 3.6, implying that in open conformation QC binds to \((IQ)1\) with an overall lower affinity and with negative cooperativity, and binds to \((IQ)2\) with increased affinity and higher cooperativity as compared with the closed conformation. Thus, it appears that 4 molecules of quercetin bind cooperatively to the tetramer receptor at the site \((IQ)2\) in open conformation. It should be noted that in the presence of 1.2 mM cytosolic Ca\(^{2+}\) the binding parameters of site \((IQ)2\) \((K_{d2} \approx 160 \mu M, H_2 \approx 1.5)\) in the open conformation of the channel are similar to those obtained in the closed conformation, whereas the affinity of \((IQ)1\) increases 5 times in the closed conformation but the number of QC molecules needed to bind \((IQ)1\) to promote channel closing remains \(\approx 1\) in both configurations. Using the best-fit parameters we can calculate the relative abundance of the two populations of open channels dwelling in states \(O1'\) and \(O2'\) at saturating levels of QC, \(P_{C1'}(1 - P_{C1'})\), which is 6.2 in the presence of 1.2 mM cytosolic Ca\(^{2+}\) and 1 in the presence of 80 nM cytosolic Ca\(^{2+}\). Like before, the difference between these two figures indicates an operative effect of Ca\(^{2+}\) on the dissociation of QC from the two inhibitory QC sites. Thus, binding of Ca\(^{2+}\) to its inhibitory \(I\) site appears to increase the probability of state \(O1'\) with respect to \(O2'\), by increasing the ratio of the QC association rates corresponding to sites \((IQ)1\) and \((IQ)2\), respectively.

It is worth mentioning that the binding parameters of \((IQ)2\) in both open and closed conformation are similar to those predicted for the QC-binding site that is allosterically coupled with the inhibitory Ca\(^{2+}\) site of the RyR1 receptor \((K_d = 86-210 \mu M, H = 4)\) [16]. Moreover, the affinity of site \((AQ)\) established here is also similar to that estimated for RyR1 \((K_d = 300 \mu M)\) in one of its four activity modes [16]. As a consequence of the manifested dual regulation by quercetin, \(P_{open}\) exhibited a biphasic dependence on the cytosolic level of quercetin at both tested concentrations of cytosolic Ca\(^{2+}\) (Fig. 4A).

At increasing QC levels, the fraction of QC-bound RyR3 channels increased progressively in two main stages which were apparently dominated by the binding of quercetin to the two inhibitory sites in the closed conformation of the channel (Fig. 4B). The fractions of QC-ligated channels at the two concentrations of cytosolic Ca\(^{2+}\) appeared to be significantly different in the domain 2 - 200 \(\mu M\) QC (Fig. 4B), with a lower fraction of QC-bound channels at 80 nM Ca\(^{2+}\).

1.2. Estimation of the cytosolic quercetin concentration and of the Ca\(^{2+}\) release flux in intact cells exposed to quercetin

RESULTS

1.2.1. The intracellular level of free quercetin

Revisiting the QC fluorescence data obtained in intact Jurkat cells exposed to 50 \(\mu M\) QC (see Fig. 1 in [1]), we remark that we can now assess the kinetics of RyR3 recruitment by
quercetin and provide the first estimation, to our knowledge, of the cytosolic level of free quercetin following cell exposure to QC.

To estimate the fraction of QC-recruited channels and the Ca\(^{2+}\) release flux during exposure of intact cells to 50 \(\mu\)M QC, the fraction of QC-bound channels \((f_b)\) was calculated at each instant as:

\[
f_b = \frac{F_{440}}{F_{440_{\text{max}}}}
\]

where \(F_{440_{\text{max}}} = 647652.3\) as discussed above. \(F_{380}\) and \(F_{440}\) were measured in 19 separate experiments as described in [1] (see Fig. 1 therein). The corresponding traces of \(P_{\text{open}}\) and \(f_b\) are displayed in Fig. 5A and Fig. 5B, respectively. We can then use the relationship between \(f_b\) and \([QC]_{\text{cyt}}\) established above (Fig. 4B) to extract the corresponding \([QC]_{\text{cyt}}\) at each time step. According to these calculations, QC uptake has two distinct phases on different timescales. The initial rapid phase (time constant 1.04 min.) most likely reflects a transport process through the plasma membrane since the corresponding saturation level (43.3 \(\mu\)M) is close to the concentration of QC applied in the external medium (50 \(\mu\)M). The time constant (34.5 min.) characterizing the second phase indicates a slow process of QC accumulation inside the cytosol which may be due in part to the flavonoid release from the mitochondria. Consistent with this, Fiorani et al. have determined that quercetin uptake in Jurkat cells is complete within less than 1 min. and that large quantities of QC are retained in bound form inside the cells, particularly inside their mitochondria [14]. However, intramitochondrial quercetin appeared to be available for subsequent redistribution to the cytosol, on a similar slow timescale as described here. Hence, under our conditions, after the initial uptake phase QC seems to be gradually released from intracellular organelles, slowly raising the cytosolic level to 80.1 \(\mu\)M free QC (Fig. 6A). A particular implication of these findings is that the decay phase of the Ca\(^{2+}\) signal evoked by quercetin in intact Jurkat cells (Fig. 1A in ref. [1]) is essentially driven by the slow increase in the cytosolic level of free quercetin which inhibits progressively more RyR3s.

**Fig. 5.** A, Time course of the open probability, and B, of the fraction of QC-bound RyR3s after addition of 50 \(\mu\)M QC to Jurkat cell suspensions. In A and B, data represent average from 19 experiments conducted as described in ref. [1] (Fig. 1 therein). In B, F440 obtained from these recordings was normalized to F440\(_{\text{max}}\) derived from data in Fig. 3A.

It is to be mentioned that a closely similar time course of quercetin uptake (Fig. 6A, inset) was recently determined in a different (HepG2) cell line by monitoring the increase in the fluorescence of intracellular quercetin excited at 488 nm [15]. Based on findings that QC uptake was inhibited by the nonspecific ATPases/phosphatases inhibitor sodium orthovanadate as well as by decreasing the temperature to 4\(^\circ\)C, the observed kinetics was attributed to a quercetin influx mediated by an active transport mechanism [15].
Fig. 6. Recruitment of RyR3s by quercetin and flavonoid uptake in intact Jurkat cells. A. Kinetic profile of the cytosolic level of free QC in intact cells challenged with 50 μM QC at \( t = 0 \). In A, \([QC]_{cyt}\) was estimated by numerical correspondence between the data shown in 3B and the fitting curve displayed in Fig. 4A with 80 nM Ca\(^{2+}\). The data were fitted to the function \( y = y_m - A \exp(-t/\tau_1) - (y_m - A) \exp(-t/\tau_2) \), with \( y_m = 81.9 \) μM, \( A = 43.3 \) μM, \( \tau_1 = 1.04 \) min. and \( \tau_2 = 34.5 \) min. Inset: Data in A were normalized to \( y_m \) and compared to results obtained in a different cell line by a different method [26]. Fluorescence data shown in Fig. 1C in [26] were normalized to a maximal value (43.2), which was derived by least-square fit of the respective data to our normalized curve. B. Relative Ca\(^{2+}\) release flux in intact cells challenged with 50 μM QC (arrow). Relative fluxes are presented for QC-free (blue) and QC-bound (red) RyR3s. The total release flux (black curve) is obtained as the sum of QC-free and QC-ligated RyR3 fluxes.

Fig. 7. A. Normalized signals of \([Ca^{2+}]_{cyt}\) and global \( P_{\text{open}} \) of RyR3s elicited in Jurkat cells exposed to 50 μM QC. To facilitate comparison, the \([Ca^{2+}]_{cyt}\) data taken from Fig. 1A in ref. [16] were corrected for baseline (122.0 nM) and then normalized to the maximal increase (879.8 nM - 122.0 nM). Similarly, the relative increase of global \( P_{\text{open}} \) was calculated as \( (y - 0.2905)/(y_{\text{max}} - 0.2905) \), where the \( y \) time series was taken from panel D (“total”) and \( y_{\text{max}} = 0.3379 \) is the peak value of \( y \). B. Pearson-correlation and G, cross-correlation between global \( P_{\text{open}} \) and \([Ca^{2+}]_{cyt}\) as a function of the delay between the two signals. Data from B recorded after stimulation with QC (\( t > 0 \)) were used. The delay for which the maximum of each function is reached is indicated by arrows.

Thus, in addition to a passive lipophilic polyphenol diffusion [19], an energy-dependent uptake also seems to occur [14, 20]. Nevertheless, under our conditions the fast QC uptake phase is most likely attributable to passive diffusion through the plasma membrane, which may require a physiological temperature range to manifest, whereas the slow QC accumulation phase is probably sustained by an active transport mechanism in combination with a slow redistribution from the mitochondria.

1.2.2. RyR3 density and the Ca\(^{2+}\) release flux

To estimate the RyR3-mediated Ca\(^{2+}\) release flux evoked by 50 μM QC in intact Jurkat cells, we consider a constant release current through individual RyR3 channels, an
assumption supported by data obtained from experiments with rat ventricular myocytes [21] or with mammalian skeletal muscle fibers [22]. The total release current \( i \) is then:

\[
i = N_i \left[ f_b P_{\text{open}}^b + (1 - f_b) P_{\text{open}}^f \right]
\]

where \( N \) is the total number of functional RyR3s inside an average size cell, \( i_0 \) is the unitary RyR3 current, \( f_b \) and \((1 - f_b)\) represent the fractions of QC-bound and QC-free channels, respectively, and \( P_{\text{open}}^b \) and \( P_{\text{open}}^f \) are the open probabilities of QC-bound and QC-free channels, respectively. The \( P_{\text{open}} \) and \( f_b \) kinetic data are taken from Fig. 5A and 5B, respectively.

Our previous investigations [1] indicate that the \( \text{Ca}^{2+} \) release signal evoked by 50 \( \mu \text{M} \) QC in intact Jurkat cells did not lead to inactivation nor removed the basal inhibition of RyR3s in intact functioning cells, since the RyR3s could be fully activated via rectification at the end of the measurements. This observation, in conjunction with the noticeable uniformity of \( P_{\text{open}} \) over a wide range of physiological cytosolic \( \text{Ca}^{2+} \) (Fig. 4D in [1]), strongly support the assumption that during exposure to QC, when \([\text{Ca}^{2+}]_{\text{cyt}}\) varies between ~0.1 and ~1 \( \mu \text{M} \) (Fig. 1A in [1]), \( P_{\text{open}}^f \) does not vary significantly. So, we can consider that \( P_{\text{open}}^f = P_{\text{open}}^{\text{rest}} = 0.2905 \) (the “initial” value indicated in Fig. 1F in [1]). Consequently,

\[
i = i_{\text{max}} f_b P_{\text{open}}^b + (1 - f_b) P_{\text{open}}^{\text{rest}}
\]

where \( i_{\text{max}} = N i_0 \) is the maximal release current established when all RyR3s are maximally activated (i.e., all channels have \( P_{\text{open}} = 1 \)). After normalization to \( i_{\text{max}} \), we obtain the total relative release flux \((i/i_{\text{max}})\), the relative release flux through QC-bound channels \((f_b P_{\text{open}}^b)\) and the relative release flux through QC-unbound channels \(((1 - f_b) P_{\text{open}}^{\text{rest}})\) which are depicted in Fig. 6B. The global \( P_{\text{open}} \) of RyR3s during exposure to QC represents the average of \( P_{\text{open}} \) weighted over the two distinct populations of QC-ligated and QC-unliganded channels and is equal to \( f_b P_{\text{open}}^b + (1 - f_b) P_{\text{open}}^{\text{rest}} \). The relative increase in global \( P_{\text{open}} \) is displayed in Fig. 7A. A rough estimation of \( N \) can be derived by assuming an average volume of Jurkat cells \( V_{\text{cel}} = 4\pi r^3/3 = 796 \text{ fl} \), where \( r = 5.75 \mu \text{m} \) is the average radius of Jurkat cells [23, 24]. The RyR density has been estimated according to the calculations in [25], using values of the maximal number of ryanodine binding sites \( (B_{\text{max}}) \) assessed in Jurkat membranes [26]. Compared with other cell types [27-33], human Jurkat cells appear to present a relatively low capacity of binding ryanodine [26]. The maximal number of specific ryanodine binding sites determined in Jurkat membranes, \( B_{\text{max}} = 66 \text{ fmol per mg protein} \) [26], translates to a cellular RyR3 density \( D = B_{\text{max}}/c \times N_A \times \rho \), where \( N_A \) is the Avogadro’s number, \( \rho = 1.05 \times 10^{-12} \text{ g/fl} \) is the cellular density assumed to be equal to the density of human CEM T-lymphoblastoid cells [34] and to that of myocardium [35], and \( c \) is a conversion factor that relates \( B_{\text{max}} \) assessed in membrane preparations to \( B_{\text{max}} \) assessed in wet tissue. The protein content of rat heart homogenates has been determined to be 106 mg protein per 1 g of wet tissue [32], which provides \( c = 9.4 \). Similar values \( c = 12.1 \) and 5.8 can be determined from measurements of \( B_{\text{max}} \) in mouse myocardium [28] and in adult rat heart [31], respectively, by comparing the ryanodine binding capacity of crude homogenates to that of SR microsomes. Therefore, an average conversion factor \( c = 9 \) has been assumed in the current calculations. We obtain then \( D = 4.64 \text{ receptors/fl} \) and \( N = DV_{\text{cel}} = 3693 \).

From data shown in Fig. 5B, the fraction of QC-recruited channels at the moment when the peak of the global release rate is reached \((t_m = 60.83 \text{ s})\) is \( f_{\text{bm}} = 0.185 \), which means that \( N_{\text{bm}} = f_{\text{bm}} N = 683 \) channels are recruited by quercetin at this time. The open probability of these channels is \( P_{\text{open}}^{\text{bm}} = 0.530 \) (Fig. 5A). The maximal increase in the cytosolic concentration of \( \text{Ca}^{2+} \) during stimulation with quercetin is \( \Delta[\text{Ca}^{2+}]_{\text{cyt}} = 758 \text{ nM} \) (Fig. 1A in [1]). Numerical simulations of \( \text{Ca}^{2+} \) release in the presence of endogenous and exogenous mobile and fixed buffers predict that this increase would require a release current \( i_q = \Delta[\text{Ca}^{2+}]_{\text{cyt}}/r \) where \( r = 0.909 \text{ nM/pA} \) is a rough estimate of the cytosolic \( \text{Ca}^{2+} \) increase per 1
pA of release current [36] (e.g., Fig. 11C therein). Consequently, the unitary current of QC-recruited channels is \( \bar{i}_{0} = \frac{iQ/N_{m}}{P_{open}^{bm}} = 0.230 \) pA.

It is generally considered that ryanodine binds the RyR receptor in the open conformation, therefore estimation of RyR densities from ryanodine binding data should be viewed with caution. Zhou et al. [25] estimated a ten-fold difference between the RyR densities derived from biochemical studies of ryanodine binding and those derived from morphometry of EM images of mammalian or amphibian skeletal muscle. Hence, considering \( D' = 10D \) as an upper bound for the RyR3 density in Jurkat cells, we obtain an approximate range \( i_0 = 0.023 - 0.230 \) pA for the \( \text{Ca}^{2+} \) current carried by a single RyR3 in situ, and the corresponding range of the total number of RyR3 receptors in a cell, \( N = 3693 - 36931 \). The highest release current that can be produced when all RyR3s in the cell are open is \( i_{max} = N \bar{i}_0 = i_Q N_{m}^{bm} = 849 \) pA, irrespective of the value of \( N \). The global RyR3-release current in resting cells can be roughly evaluated to be \( \bar{i}_{ext} = P_{open}^{rest} i_{max} = 247 \) pA, i.e. 29.05% of the maximal release capacity established when all RyR3 channels are open. Upon stimulation with 50 \( \mu \)M QC, the peak release flux observed at \( t_m \) is \( i_{m} = P_{open}^{m} i_{max} = 287 \) pA, where \( P_{open}^{m} = 0.338 \) is the value of global \( P_{open} \) at that moment (Fig. 6B, curve denoted as “Total”), whereas the final steady current is \( \bar{i}_{s} = P_{open}^{s} i_{max} = 265 \) pA, where \( P_{open} = 0.312 \) is the asymptotic value of global \( P_{open} \) derived from exponential fit to the decay phase data shown in Fig. 6B (curve denoted as “Total”). It should be noted that \( i_{max}, i_{m} \) and \( i_{s} \) do not depend on \( N \).

Our current results indicate that Jurkat cells possess an intrinsically high capacity of \( \text{Ca}^{2+} \) mobilization from intracellular stores via RyR3 activation, hence even small increases in the release flux can generate significant cytosolic \( \text{Ca}^{2+} \) overload. As a consequence, the correlation between global \( P_{open} \) and \([\text{Ca}^{2+}]_{cyt}\) was very strong (Fig. 7A-C). Pearson- and cross-correlation analysis indicates a delay of 0.68-2.36 min. between the global release flux and the cytosolic \( \text{Ca}^{2+} \) signal. In comparison with the corresponding value of 2.37-3.38 min. extracted from the correlation between \([\text{Ca}^{2+}]_{cyt}\) and the open probability of QC-recruited RyR3 channels (Fig. S3 in [1]), this figure should be closer to the characteristic time of cytosolic \( \text{Ca}^{2+} \) clearance in intact Jurkat cells.

Finally, we should stress that a large pool of data obtained in our studies (current paper and [1, 17]) were better explained when we considered that F440\(_{open}\) and F440\(_{closed}\) are proportional to the number of QC-ligated open and closed channels rather than to the total number of QC molecules bound to the open and the closed channels, respectively. For example, this approach provided a more consistent and realistic description of the data regarding the QC-dependence of F440\(_{open}\) and F440\(_{closed}\), as well as the data on the maximal fluorescence attained in various states or on the estimated level of cytosolic quercetin in intact cells exposed to 50 \( \mu \)M QC. Collectively, all our results presented here and in the previous paper suggest [1] that the RyR3 protein may act as an energy donor in the RyR3:QC complex, by transferring its excitation energy to the flavonoid molecule via the mechanism of Förster resonance energy transfer. The practical utility of this particular property of quercetin that allows the direct assessment of ryanodine receptor activity is straightforward.

1.3. Estimation of the kinetic variations of intracellular \( \text{Ca}^{2+} \) concentration after exposure to quercetin or menadione in the presence of calcium chelator BAPTA/AM

In our previous studies we showed that quercetin (QC) induces a strong, biphasic \( \text{Ca}^{2+} \) release signal in Jurkat T cells, which is mediated by activation of ryanodine receptors (RyRs) of the endoplasmic reticulum (ER). Here we have investigated to what extent this calcium signal is affected by the reduction in calcium concentration of the intracellular medium (by incubation with the calcium chelator BAPTA/AM). Shortly, Jurkat cells were washed twice in
standard saline (SS) containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 20 mM HEPES, 10 mM glucose (pH 7.4/NaOH), then incubated for 10 min. with the calcium indicator fura-2/AM (4 µM) in the dark at 23°C, washed twice in SS and incubated with 50 µM BAPTA/AM for 20 min. in the dark at 23°C, centrifuged and resuspended in SS. The cell sample (1.5 ml) was then measured with a Horiba Jobin-Yvon spectrofluorimeter at 37°C under continuous agitation, as previously described [1, 17, raport 2013].

In the absence of BAPTA/AM, 50 µM QC added to Jurkat cell suspensions evoked a consistent increase in the cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{cyt}$), which peaked at 878 nM within 4.5 min. after extracellular addition of quercetin (Fig. 8A). This increase was substantially reduced in the presence of BAPTA/AM (Fig. 8A). Under otherwise similar conditions, the resting level of cytosolic Ca$^{2+}$ was 122 nM and 48 nM in the absence or the presence of BAPTA/AM, respectively. Addition of quercetin (50 µM) to BAPTA/AM-containing cells raised [Ca$^{2+}$]$_{cyt}$ to a relatively steady level of only 167 nM.

The RyR open probability ($P_{open}$) displayed a bimodal kinetic variation after addition of quercetin, in the absence, as well as in the presence of the calcium chelator (Fig. 8B). However, in BAPTA/AM-loaded cells there was a rapid but transient, full activation of the ryanodine receptors after QC addition. This initial phase, which lasted for several minutes, was followed by a progressive, slow decrease that was virtually identical to the corresponding phase observed in the absence of BAPTA/AM. Nevertheless, the initial increase in $P_{open}$ was consistently lower in the absence of BAPTA/AM, when the maximal $P_{open}$ was 0.55, as compared to the corresponding peak of 1.00 obtained with BAPTA/AM-loaded cells. These results are consistent with the characteristic property of Ca$^{2+}$ release channels to be inhibited by high levels of Ca$^{2+}$ that develop within the microdomain surrounding the channel mouth during the release flux, so that the prime effect of the calcium chelator would be to reduce the Ca$^{2+}$ gradient at the release site and thus to prolong the opening dwell time of the channel. However, the ending part of the $P_{open}$ trace shown in Fig. 8B is actually shaped by the inhibitory effect of quercetin, which reaches high, inhibitory levels at these exposure times [1, 17]. In consequence, the final decrease of $P_{open}$ is expected to be similar in the absence or the presence of BAPTA/AM. Clearly, our current results verify this assertion.

Having in view that our previous results indicated that one major component of the QC-induced calcium release (representing about one half of the total flux) is mediated by the ryanodine receptors, current data support the idea that only a minor part of the QC-induced [Ca$^{2+}$]$_{cyt}$ increase may be associated to a calcium influx through the plasma membrane. A prime candidate for this mechanism is the store-operated calcium entry, the main Ca$^{2+}$ entry route that is activated by depletion of the ER calcium stores. It can be concluded then that the remaining Ca$^{2+}$ flux participating in the calcium signal evoked by quercetin is most likely transported through the inositol trisphosphate receptors (IP$_3$Rs) of the ER.

Exposure of Jurkat cells to menadione (MD) induced only a marginal calcium response. Thus, following addition of 100 µM MD, [Ca$^{2+}$]$_{cyt}$ increased from a resting level of 140 nM to a steady value of 187 nM within about 15 min. from exposure (Fig. 9). Results obtained with different levels of exposure (50 µM and 250 µM MD) confirmed the low capacity of menadione to induce calcium release in this cell line (not shown). In consequence, the effect of the calcium chelator BAPTA/AM presented no further relevance and therefore was not evaluated.

However, the combination quercetin-menadione still retained a strong Ca$^{2+}$ release ability, which was partially reduced as compared to the release capacity of quercetin applied alone (Fig. 10). Thus, in the absence of BAPTA/AM, QC (50 µM) and MD (100 µM) added simultaneously to the cell suspension raised the cytosolic calcium concentration from 182 nM to 479 nM within 1 min. from addition, and there was no further decrease in [Ca$^{2+}$]$_{cyt}$.\
Fig. 8. Kinetic variations of the cytosolic Ca$^{2+}$ concentration (A) and of the open probability of ryanodine receptors, $P_{\text{open}}$ (B) induced by 50 μM quercetin in fura-2 labeled Jurkat cells, loaded (+BAPTA/AM) or not (-BAPTA/AM) with the calcium chelator, BAPTA/AM.

Fig. 9. Menadione (100 μM) induces a minor increase in the cytosolic Ca$^{2+}$ concentration of fura-2 labeled Jurkat cells.

Fig. 10. Kinetic variations of the cytosolic Ca$^{2+}$ concentration induced by 50 μM quercetin alone (QC) or in combination with 100 μM menadione (QC+MD) in fura-2 labeled Jurkat cells, in the presence or the absence of the calcium chelator, BAPTA/AM.
In BAPTA/AM-loaded cells challenged with the same QC/MD combination, \([\text{Ca}^{2+}]_{\text{cyt}}\) increased from 69 nM to a steady level of only 147 nM, displaying clear similarities to the corresponding \([\text{Ca}^{2+}]_{\text{cyt}}\) trace obtained with cells exposed to QC alone (Fig. 8A).

1.4. DISCUSSION

It is widely recognized that the ryanodine receptor type 3, RyR3, is expressed at relatively low levels in mammals, but its functional role has remained unclear [18, 37, 38, 39-41]. In T-cells, the immune response elicited by stimulation of the T-cell receptor/CD3 (TCR/CD3) complex appears to involve a long-lasting increase in cytosolic \(\text{Ca}^{2+}\), in which RyR3 plays a critical role [26]. The physiological implications of this involvement are important because T-cells require the sustained \(\text{Ca}^{2+}\) signal for clonal expansion, hence for generating a functional immune response [26]. Results from our laboratory presented elsewhere [1, 8] suggest that RyR3 may act as an important player in the apoptotic program triggered by quercetin in Jurkat T-cells [42, 43], in which the sustained cytosolic \(\text{Ca}^{2+}\) overload produced by RyR3 activation can act synergistically with NADH hyperoxidation to trigger mitochondrial pore opening [44]. In favor of this idea, we found that treatments with quercetin applied for 1 hour decreased the cellular content of NADH [1, 8, 43], induced significant apoptosis and decreased clonogenic survival in Jurkat cells, in a dose-dependent manner [42, 43].

From fluorimetric measurements we could estimate that the total \(\text{Ca}^{2+}\) release flux through RyR3s is roughly 247 pA in intact resting Jurkat cells, whereas the maximal release capacity established when all (~3693-36931) RyR3 channels are open was tentatively evaluated as 849 pA. After a fast activation phase, 50 µM QC applied in the extracellular medium seemed to evoke a fairly constant current through QC-recruited channels (~93 pA). However, the overall RyR3-mediated release current increased in 1 min. with 40 pA and then decayed with a time constant of 18.9 min. (not shown) to a slightly elevated value of 265 pA which appears to be sufficient to sustain the elevated level of cytosolic \(\text{Ca}^{2+}\) observed in our previous experiments [1].

Our current estimations indicate that the unitary RyR3 current is likely to reside within the range 0.023-0.230 pA, which appears to be lower than previous estimates for the RyR2 current in rat cardiac myocytes (1.2 pA) [21], and lower than the single-channel current (1-4 pA) assessed for all RyR isoforms in lipid bilayers at non-physiological high levels of luminal \(\text{Ca}^{2+}\) [3, 45, 46]. However, our estimate is near to the characteristic value determined with mammalian skeletal muscle fibers (0.5 pA) [47], or with the mammalian RyR2 or amphibian skeletal muscle RyR channel incorporated into planar bilayers under near-physiological conditions (0.26-0.66 pA) [45, 46]. Since Jurkat cells have ~400 µM \(\text{Ca}^{2+}\) inside their ER [48], a linear extrapolation from these electrophysiological measurements suggests a reference RyR current of ~0.21 pA in situ. Consistent with this, it has been concluded that the \(\text{Ca}^{2+}\) current carried by RyR in intact cells must be considerably <0.6 pA [45]. Interestingly, our estimate agrees well with the single-channel current (~0.15 pA) transported by the related receptor IP3R type 3 embedded in the nuclear outer membrane, under physiological conditions [49].

In conclusion, by measuring the dual-excitation specific fluorescence of quercetin-loaded cells we have identified novel mechanisms of RyR3 regulation by cytosolic \(\text{Ca}^{2+}\) and quercetin in open and closed channel configurations, and we could estimate the RyR3-mediated \(\text{Ca}^{2+}\) release flux induced by quercetin in intact Jurkat cells. Our results support the idea that the RyR3 channel assumes different configurations in the native vs. an artificial
membrane environment, which correspond to the hindered and the rectified channel states observed in our experiments, respectively [1]. In addition to the generally recognized high-affinity Ca$^{2+}$ activating site, the data indicate that the RyR3 receptor possesses a second, low-affinity activating Ca$^{2+}$ site, which appears to be operational in the rectified but not in the hindered channel. The data also provide meaningful information regarding the cytosolic level of free quercetin in intact Jurkat cells exposed to quercetin. The significant difference in the affinity and the number of Ca$^{2+}$ and quercetin binding molecules in open and closed conformations is consistent with the documented conformation change that takes place upon channel opening [50]. Thus, we could determine for the first time to our knowledge, that the cytosolic concentration of free quercetin in intact Jurkat cells exhibits an initial rapid phase (time constant 1.04 min.) probably reflecting diffusional transport through the plasma membrane, followed by a slow accumulation of free quercetin inside the cytosol (time constant 34.5 min.) which provided an up to 1.6-fold gradient between the cytosol and the extracellular medium. Together with the evidences presented in our preceding paper [1], we thus lend further support [17] for the use of quercetin-specific cellular fluorescence as a valuable tool in the assessment of the functional and molecular properties of the RyR3 calcium channel in situ, as well as in identifying the target proteins and cellular distribution of the flavonoid quercetin and eventually in a better characterization of its molecular functions.

2. The antiproliferative effects and synergism of quercetin and menadione in human leukemia Jurkat T cells. Effects of long term (24 h) treatments

We have analyzed into more detail the effects of quercetin and menadione in leukemia Jurkat cells, by using the flow cytometry and extending the study on long term treatments. The results have been published in the ISI journal Leukemia Research [18].

2.1 Antiproliferative effects of MD and QC

Menadione applied for 24 h exhibited strong antiproliferative effects in Jurkat cells, as determined by flow cytometry (Fig. 8A). The half maximal effect concentration associated with induction of cell death within 24 h after drug removal was EC$_{50}$ = 14.7 µM and the Hill coefficient was $h = 5.2$. It should be noted that these figures are in agreement with previous reports from 24-h MD treatments of Hep G2 human hepatoblastoma cells [51]. Analysis of cell viability after combined treatments with 10-15 µM MD and 10-15 µM QC for 24 h confirmed the strong synergic interaction between the two agents (Fig. 11B). In particular, the combination MD:QC at concentrations 15 µM : 15 µM proved to be highly efficient in killing Jurkat cells, by inducing a fraction of 91.8 ± 6.0% dead cells 24 h after the treatment (Fig. 11B), and clonogenic survival of 1.4 ± 0.3% ($n = 3$). Moreover, the combination effect was 1.59 times stronger than the additive effect of the two agents applied alone (MD 15 µM: cell death rate 49.2 ± 9.6% and QC 15 µM: 16.9 ± 3.0%; so, by subtracting the non-specific cell death rate of 6.0 ± 3.7% obtained in control cells, one obtains the average MD- and QC-induced cell death rates of 43.2% and 10.9%, respectively, whereas the MD:QC combination produced 85.8% dead cells). In addition, the ratio of the combination effect to the additive effect was even higher (2.78) when taking into consideration the cell death rate assessed immediately after the treatment, indicating that addition of quercetin accelerates the cell death process. In a previous work we found by using a different, propidium-iodide based assay for detection of fragmented DNA, that both MD and QC induce cell death primarily through apoptosis in Jurkat cells [42,43]. Current investigations using the Annexin V/7-AAD assay confirmed that MD and QC-induced cell death evolves through the apoptotic pathway. For example, the combination MD:QC at
concentrations 15 μM: 15 μM produced 65.8 ± 10.3% dead cells within 24 h from drug addition (Fig. 11B), of which 30.9 ± 3.7% cells were found in early stages of apoptosis and 34.9 ± 4.5% cells were in late apoptosis/necrosis. In the same timeframe, menadione applied alone (15 μM) produced 19.7 ± 6.8% early apoptotic cells and 4.9 ± 1.4% late apoptotic/necrotic cells.

Subsequent evolution over the next 24 h (e.g., Fig. 11C) was thus consistent with a progressive migration of the cell populations through the intermediary quadrant Q2 (lower right) of early apoptotic cells, indicating that under our conditions cell death was mainly apoptotic. FSC/SSC (forward scatter, a cell size estimate, and side scatter, an estimate of cellular granularity) data provided additional information supporting this idea (e.g., Fig. 11C).
It should be mentioned that this behavior was observed in all current treatments. Due to the high value of the associated Hill coefficient, menadione at concentrations higher than EC_{50} (i.e., ≥20 µM) was very effective in inducing cell death within 24 h from drug removal (Fig. 11A). Therefore, the main effect of adding quercetin to the treatment was to accelerate this process. For instance, the combination MD:QC at concentrations 20 µM : 10 µM for 24 h produced a dead cell fraction of 93.1 ± 5.7% after 24 h from the treatment, which was not significantly different from the fraction of 87.0 ± 8.4% dead cells induced by MD alone (20 µM), whereas immediately after the treatment the corresponding death rates (50.1 ± 10.4% and 74.2 ± 10.8%, respectively) differed significantly and the combination-to-additive effect ratio was 1.43 (Fig. 11B).

2.2. Effects of MD and QC on Δψ_m

The primary Δψ_m disrupting effect of MD and QC observed in acute treatments (as discussed above) was found to persist during a longer exposure (6 h) to the two drugs applied alone or in combination.

In MD-treated cells, JC-1 red fluorescence was dose-dependently depressed, and double Annexin V/JC-1 labeling indicated that this effect preceded phosphatidylserine externalization (Fig. 12A), which occurs in the early stages of apoptosis. The cellular distribution of the two forms of the dye, namely the monomeric and the aggregate JC-1 form, displayed a complex response at increasing doses of menadione (Fig. 12B). However, the dominant feature observable in the JC-1 Green/JC-1 Red bivariate plot was the relocation of a significant fraction of events from quadrant Q4 (upper left) to Q1 (lower left), which was associated with a large population of depolarized mitochondria, as indicated by the JC1-Red/JC1-Green ratio (Fig. 12C). The dose-dependent MD-induced reduction of the Q4 population in JC-1 Green/JC-1 Red plots (a quantitative indicator of the polarized mitochondria population) provided an IC_{50} of 19.2 µM and a Hill coefficient of 1.56 (not shown). The JC-1 Red/Green fluorescence ratio displayed a progressive decrease with the MD level up to 20 µM, whereas at higher concentrations (40-100 µM) it exhibited a secondary, albeit quite small increase, which appeared to be due to a reduction of the monomeric JC-1 loading capacity of the cytosol. In agreement with the Annexin V determinations shown in Fig. 12A, one likely explanation for this finding may be related to loss of plasma membrane polarity during early apoptotic stages, which could affect staining of the cytosol with the cationic dye JC-1. The forward and side scatter data (Fig. 12D) further support the idea discussed above, indicating a gradual, dose-dependent transition to an apoptotic morphology (with reduced FSC, indicative of cell shrinkage, and enlarged SSC, indicative of increased granularity) during long-term exposure to menadione. After 6 h of exposure, quercetin at physiological levels of 10 µM and 15 µM proved to be a strong mitochondria-depolarizing agent, producing a median mitochondrial polarization of 13 ± 2% and 10 ± 3%, respectively, as assessed by flow cytometry determinations on JC-1 labeled cells. The Q4 cell fraction in the JC-1 Green/JC-1 Red plots was 63.0 ± 9.9%, 6.5 ± 3.3%, and 37.5 ± 11.2% after 6-h treatments with 15 µM MD, 15 µM QC, and their combination, respectively, suggesting that menadione may inhibit mPTP opening by quercetin, thereby weakening the depolarizing effect of the flavonoid. The data also show that loss of Δψ_m is not an apoptotic trigger per se, since QC (15 µM) applied alone for 24 h induced a relatively small fraction (16.9 ± 3.0%) of apoptotic cells 24 h after the treatment (Fig. 11B), even though the fraction of cells with polarized mitochondria assessed by the JC-1 Red/Green ratio was considerably low (less than 10%) both at 1 h and at 6 h after drug addition.
Fig. 12. Dose-dependent mitochondrial depolarization in Jurkat cells treated with menadione for 6 h. After treatment with DMSO (Control), 10, 15, 20, 40 or 100 μM MD, cells were double stained with Annexin V-FITC and JC-1 and analyzed by flow cytometry. (A) Annexin V-FITC/JC-1 Red density plots show as a major feature the migration of events from quadrant Q4 to Q1 and then Q3, indicating that loss of JC-1 aggregation inside the mitochondria precedes PS externalization. Cell fractions corresponding to each quadrant are specified. (B) JC-1 Green/JC-1 Red density plots indicate progressive reduction of the population of polarized mitochondria (quadrant Q4, with the corresponding event fraction shown in bold) at increasing MD concentrations. Both JC-1 Green and JC-1 Red intensities were normalized to FSC. (C) Histograms of the JC-1 Red /JC-1 Green ratio relative to control (“Polarization”) confirm the strong mitochondria-depolarizing effect of menadione. (D) FSC/SSC data are consistent with a gradual increase in the cell population with apoptotic morphology, in good correlation with the Annexin V data shown in A.
2.3. DISCUSSION

On investigating the antiproliferative effects of longer (24 h) treatments with MD, QC and their combination in Jurkat cells, we found a strong synergic interaction between MD and QC leading to efficient cell killing. Thus, the combination MD:QC at concentrations 15 μM : 15 μM proved to be highly effective, by inducing a high apoptotic fraction and a clonogenic survival of 1.2%. Moreover, addition of quercetin to the MD-treatment accelerated significantly the cell death process. Results also indicate that a MD:QC regimen at equimolar concentrations of 10 μM with treatment time of 24 h, which appears to be a clinically achievable (and safe) scheme, can inhibit Jurkat cell proliferation at efficient rate (the clonogenic survival obtained in this case was 10.1 ± 3.9%). Thus, current data suggest that the MD:QC combination administered at clinically relevant doses and rates could improve the outcome of conventional leukemia therapies, and therefore warrant the utility of additional studies in order to establish the therapeutic effects of this combination in different leukemia cell lines, as well as in animal models for leukemia.

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Dissemination

ISI papers

1. Irina Baran, Diana Ionescu, Alexandru Filippi, Maria Magdalena Mocanu, Adrian Iftime, Ramona Babes, Ioana Teodora Tofolean, Ruxandra Irimia, Alexandru Goicea, Valentin Popescu, Alexandru Dimancea, Andrei Neagu, Constanta Ganea, Novel insights into the antiproliferative effects and synergism of quercetin and menadione in human leukemia Jurkat T cells, Leukemia Research, 2014, Volume: 38 Issue: 7 Pages: 836-849 Published: JUL 2014 (IF 2.692) (is.0.950 )


4. Irina Baran, Constanta Ganea, RyR3 in situ regulation by Ca\textsuperscript{2+} and quercetin and the RyR3-mediated Ca\textsuperscript{2+} release flux in intact Jurkat cells, ARCHIVES OF BIOCHEMISTRY AND

Conferences


Awards

UEFISCDI awards 2014: papers 1, 3-5.

The contribution no. 9 has obtained 2nd prize at Craiova International Medical Students Conference.

Project manager,
Prof. Dr. Constanţa Ganea