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Activity of olive oil phenols on lymphomonocyte cytosolic calcium

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Abstract

Phenols, present in the Mediterranean diet, have antioxidant properties and are free radical scavengers; however, the molecular mechanisms of their beneficial effects are not yet fully understood.

The level of cytosolic calcium ($[Ca^{2+}]_i$) is an important signal also in nonexcitable cells, including immune cells, and regulates fundamental processes.

In this paper, we determine $[Ca^{2+}]_i$ in human lymphomonocytes incubated with two olive oil phenols: 3,4-(dihydroxyphenyl)ethanol and *p*-(hydroxyphenyl)ethanol.

Both tested phenols increase $[Ca^{2+}]_i$ in a dose-dependent way. This effect is antagonized by nifedipine and is noticeable both in the presence and in the absence of calcium in the extracellular medium.

Keywords: Cell calcium; Nifedipine; Olive oil phenols; Lymphomonocytes; Human

1. Introduction

Typical components of the Mediterranean diet such as olive oil and red wine contain high concentrations of phenols that may have important antioxidant roles. The main phenols identified in extra-virgin olive oil belong to different classes: simple phenols such as 3,4-(dihydroxyphenyl)ethanol (3,4-DHPEA) and *p*-(hydroxyphenyl)ethanol (*p*-HPEA) and secoiridoids (oleuropein, the aglycone of ligstroside and their respective decarboxylated dialdehyde derivatives) [1]. Epidemiological studies suggest that phenols reduce the incidence of coronary heart disease [2].

The mechanisms responsible for the effects of phenols have not yet been fully clarified. Antioxidant activity [3] and free radical scavenging [4] may be relevant in this connection. Yet, other possibilities have been put forward. For instance, it has been reported that red wine phenols increase cytosolic calcium ($[Ca^{2+}]_i$) in bovine endothelial cells, similarly to bradykinin and to ATP (adenosine triphosphate) [5].

Cytosolic calcium is an important signal also in nonexcitable cells, including immune cells, and regulates fundamental cellular processes such as activation, growth and differentiation [6,7]. The increase of $[Ca^{2+}]_i$ may result from mobilization from either an intracellular store or an extracellular medium or from both [6].

Some dihydropyridine derivatives, which specifically bind with high affinity to the α1C chain of L-type channels [8,9], regulate the functional state of the channel either opening or closing it. Nifedipine blocks the opening of the channel, thus inhibiting the calcium flux through it [9,10].

The aim of this work was to ascertain whether 3,4-DHPEA and *p*-HPEA, contained in olive oil, may exert some effects on $[Ca^{2+}]_i$ in human lymphomonocyte populations where the ion regulates many cellular processes [11,12]. We found that phenols increase the level of $[Ca^{2+}]_i$ in human lymphomonocytes, both in the presence or in the absence of calcium in the external medium, and that this effect is reduced by nifedipine treatment.

2. Materials and methods

2.1. Materials

The 3,4-DHPEA was extracted from extra-virgin olive oil using the procedure reported in a previous paper [13] and

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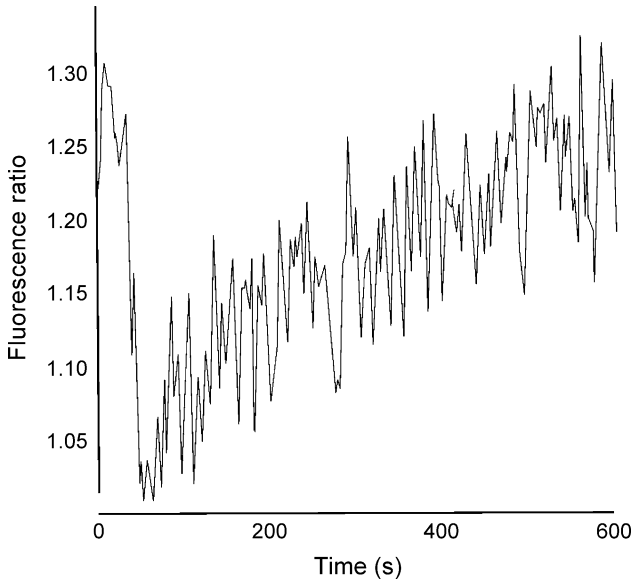

Fig. 1. Variations of fluorescence ratio in human lymphomonocytes in the presence of 50 μM of nifedipine. The figure was drawn from the output of the measuring system to illustrate the behavior of the phenomenon.

stored at −25°C under N_2. The *p*-HPEA was obtained from Janssen Chemical (Beerse, Belgium). Nifedipine was produced by Sigma Chemical (St. Louis, Mo, USA).

2.2. Preparation of lymphomonocytes

Human lymphomonocytes were prepared as described in Ref. [14]. Briefly, human blood was diluted with 0.9% NaCl (1:1, by volume) and was then layered (6 ml) on 3 ml of Lymphocyte-H (Cedarlane Laboratories, Hornby, Canada). It was subsequently centrifuged at room temperature for 20 min at 800×*g*. Lymphocytes were collected at the interface and concentrated by centrifugation (20 min at 400×*g*). Cells were then suspended in phosphate-buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1,5 mM KH_2PO_4, pH 7.4) to have about 30×10^6 cells/ml. This preparation was used for further procedures.

2.3. Cytosolic calcium determination

FURA-2 AM (2 μl of a 2 mM solution in DMSO) was added to lymphomonocyte suspensions (about 30×10^6 cells) and incubated for 60 min at 37°C in the dark. Cells were then harvested by centrifugation at 600×*g* for 10 min and were finally suspended in 1 ml of PBS.

The concentration of calcium was determined as described in Refs. [15,16]. Briefly, 50 μl of PBS suspension was transferred to a cuvette containing 3 ml of the same buffer. Fluorescence was measured with a Perkin-Elmer LS 50 B spectrophotofluorimeter equipped with a double excitation system (ex. 336 and 380 nm; em. 510 nm). Slit widths were set at 1.5 nm for excitation and 3 nm for emission. Cytosolic calcium concentrations were calculated as reported [17].

Cytosolic calcium was first determined on control cells, then on cells exposed to 60–240 μM of 3,4-DHPEA, 60–240 μM of *p*-HPEA and/or 25 or 50 μM of nifedipine.

3. Results

Cytosolic calcium was estimated with the FURA-2 AM method by measuring the ratio of fluorescence at 510 nm after excitation at 336 and 380 nm [17]. Fig. 1 shows a typical time course of fluorescence ratio after the addition of 50 μM of nifedipine. The results obtained with nifedipine 25 μM were similar although less pronounced. Therefore, according to the literature, nifedipine produced a decrease of $[Ca^{2+}]_i$ [10].

The effect of nifedipine was rapid; in a few seconds, the $[Ca^{2+}]_i$ decreased consistently. Yet, it increased again with time to reach the values of untreated cells in about 10 min, following the pattern shown in Fig. 1.

We show (Fig. 2) a typical pattern of fluorescence ratio variation upon the addition of *p*-HPEA at different concentrations (60, 120 and 240 μM). A similar behavior was found upon the addition of 3,4-DHPEA. Therefore, olive oil phenols induced a dose-dependent increase of $[Ca^{2+}]_i$ in

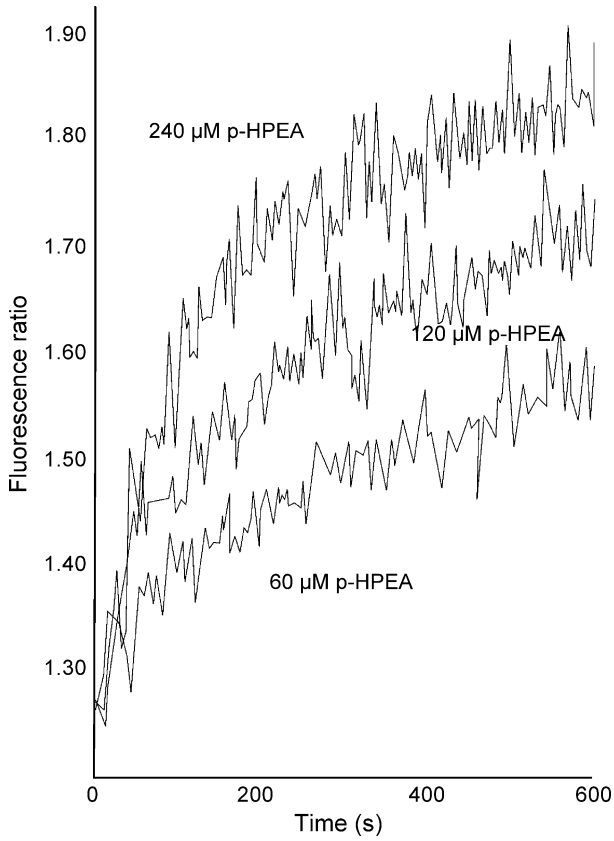

Fig. 2. Variations of fluorescence ratio in human lymphomonocytes in the presence of *p*-HPEA. The sample contained 60 to 240 μM of *p*-HPEA. The figure was drawn from the output of the measuring system to illustrate the behavior of the phenomenon. The 3,4-DPHEA had a similar behavior (not illustrated in the figure).

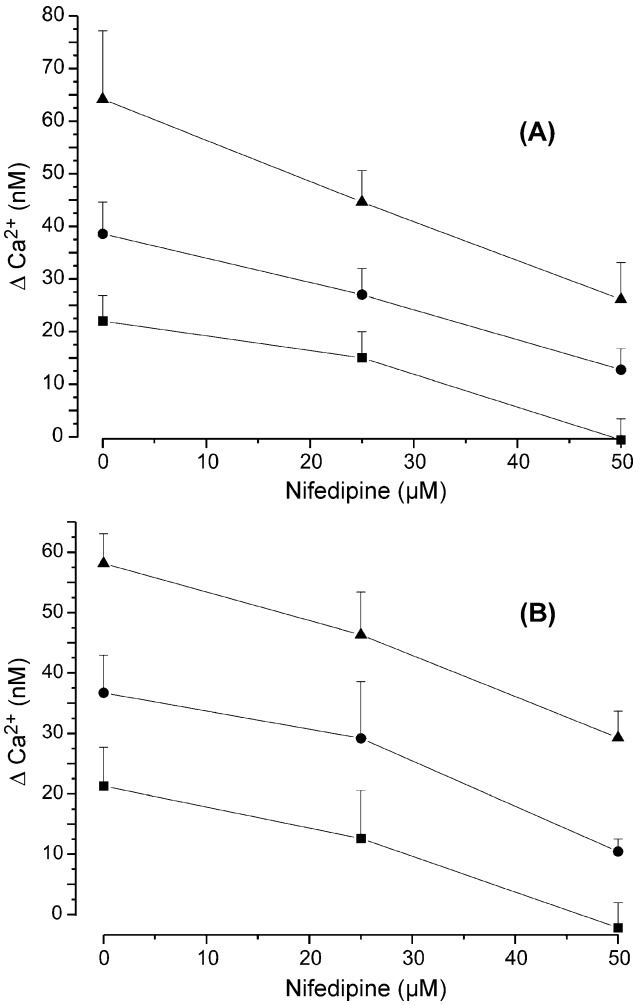

Fig. 3. Variations of $[Ca^{2+}]_i$ in human lymphomonocytes in the presence of 3,4-DHPEA (A) or of *p*-HPEA (B). Samples contained nifedipine (0 to 50 μM) and phenols (■–■, 60 μM; ●–●, 120 μM; ▲–▲, 240 μM). Cytosolic calcium in controls (neither nifedipine nor phenols) was 70±10 nM (10 determinations); results are expressed as the difference from the basal level. The results are the average of 10 experiments. Vertical bars represent the S.E.M. ANOVA: effects of nifedipine, 3,4-DHPEA and *p*-HPEA, $P<.001$.

human lymphomonocytes. The time course was roughly hyperbolic and reached a plateau after 10 min of incubation.

The experiments reported below were all made by measuring the values obtained 10 min after the addition of nifedipine and/or phenols (stable state for nifedipine and maximal effect for phenols). We tested the calcium-increasing effect of 3,4-DHPEA and of *p*-HPEA both in the presence of various concentrations of nifedipine and in the absence of the drug (Fig. 3). The two phenols showed a similar behavior [Fig. 3(A) and (B)]. Nifedipine (25 and 50 μM) decreased the effects of phenols. Therefore, the $[Ca^{2+}]_i$ increase was directly proportional to phenol concentration and inversely proportional to nifedipine concentration. The phenol-dependent increase of $[Ca^{2+}]_i$ diminished, but it was not entirely suppressed in these experimental conditions, unless nifedipine was 50 μM and phenols were 60 μM.

The variations of $[Ca^{2+}]_i$ are a complicated phenomenon. Nifedipine may either block the entry of calcium from the outside medium or hamper calcium transfer among intracellular compartments. In addition, the lymphocyte distribution of nifedipine-sensitive channels has not yet been clarified.

To distinguish between various possibilities, we performed a set of experiments omitting calcium in the extracellular medium (Fig. 4). In these conditions, the basal $[Ca^{2+}]_i$ levels were lower than those in the presence of external calcium (30 vs. 70 nM). Moreover, both phenols (Fig. 4) decreased their effect if calcium was omitted from the medium. Nifedipine further decreased the effect of olive oil phenols even in the absence of external calcium.

The effect of nifedipine on $[Ca^{2+}]_i$ is highest 60 s after the addition of the drug. To further investigate the effects of phenols and their relationship with nifedipine channels, we measured $[Ca^{2+}]_i$ 60 s after the addition of both nifedipine and phenols (Fig. 5). The nifedipine-dependent decrease of

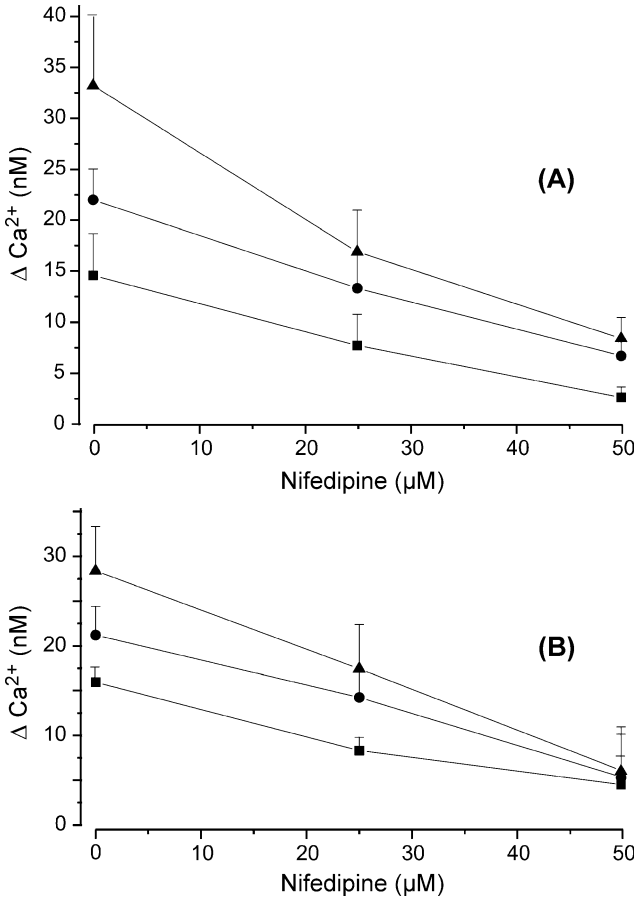

Fig. 4. Variations of $[Ca^{2+}]_i$ in human lymphomonocytes in the presence of 3,4-DHPEA (A) or of *p*-HPEA (B) and in the absence of calcium in the extracellular medium. Samples contained nifedipine (0 to 50 μM) and phenols (■–■, 60 μM; ●–●, 120 μM; ▲–▲, 240 μM). Cytosolic calcium in controls (neither nifedipine nor phenols) was 30±5 nM (10 determinations); results are expressed as the difference from the basal level. The results are the average of 10 experiments. Vertical bars represent the S.E.M. ANOVA: effects of nifedipine, 3,4-DHPEA and *p*-HPEA, $P<.001$.

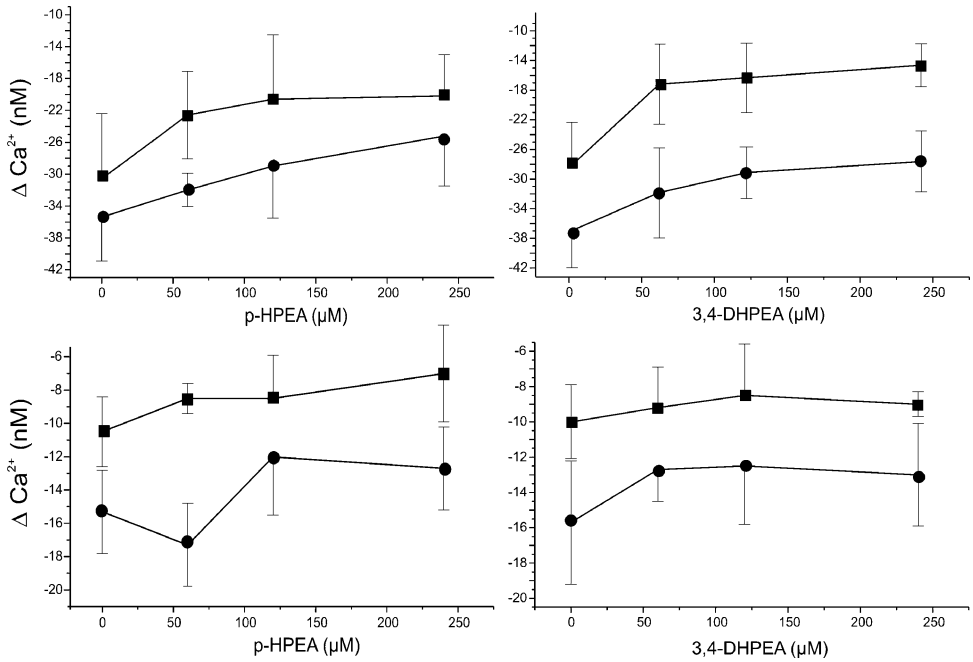

Fig. 5. The effect of *p*-HPEA and of 3.4-DHPEA on the decrease of calcium due to the presence of nifedipine. Reported values were obtained 60 s after the addition of both 25 (●) or 50 (■) μM of nifedipine and the indicated concentration of phenols. Upper row: the incubation medium contained 2 mM of Ca^{2+}. Cytosolic calcium in controls (neither nifedipine nor phenols) was 70±10 nM (10 determinations). Vertical bars represent the S.D. ANOVA: effects of nifedipine, 3,4-DHPEA and *p*-HPEA, $P<.001$. Lower row: no Ca^{2+} was added to the incubation medium. Measurements were made 60 s after the addition (when intracellular calcium was at its lowest concentration). Vertical bars represent the S.D. Cytosolic calcium in controls (neither nifedipine nor phenols) was 30±5 nM (10 determinations). ANOVA: effect of nifedipine, $P<.001$; effect of 3,4-DHPEA and *p*-HPEA, P=NS.

$[Ca^{2+}]_i$ was antagonized by both phenols, although 3,4-DHPEA seemed slightly more effective than *p*-HPEA. If cells were incubated in the absence of Ca^{2+} in the external medium, the decrease of $[Ca^{2+}]_i$ produced by the addition of nifedipine was lower than that in the presence of Ca^{2+} and the effect of phenols was also less pronounced.

Therefore, our data suggest that phenols act mainly on nifedipine-sensitive channels.

4. Discussion

Calcium is a common transduction signal in many cells. It activates a number of enzymes and processes such as cellular development and growth. It is generally believed that the ion is an essential messenger in T cell activation [6]. In B lymphocytes, calcium influx is related to cell activation [18].

Human lymphomonocytes treated with nifedipine show a rapid decrease of $[Ca^{2+}]_i$ (10 s; Fig. 1) both in the presence and in the absence of external calcium (not shown in the figure). Our findings on nifedipine effects agree with previous data [18] and indicate a similarity between calcium channels of lymphocytes and type L calcium channels of excitable cells. Calcium levels return to initial values in about 10 min after nifedipine addition. The cell can, therefore, compensate the initial decrease of $[Ca^{2+}]_i$ produced by the drug, thus indicating the activity of non-nifedipine-sensitive channels.

Both olive oil phenols tested in this work increased the $[Ca^{2+}]_i$ in lymphomonocytes (Fig. 2). The increase was dose dependent both for 3,4-DHPEA and for *p*-HPEA (Figs. 3 and 4). These results are in fair agreement with data obtained with wine phenols in bovine aortic endothelial cells [5].

The concentrations of phenols that we used (60–240 μM) are similar to those used by other authors (10^{-6}–10^{-4} M) [3] who demonstrated biological activities of these compounds aside from their known scavenger properties of superoxide and other reactive oxygen species.

The action of olive oil phenols and of nifedipine on $[Ca^{2+}]_i$ was different with regard to effect and time course. Although the $[Ca^{2+}]_i$ of lymphomonocytes appears to be in normal ranges 10 min after nifedipine treatment, the ability of these cells to respond to phenols is hampered by the drug (Figs. 3 and 4). This means that the effect of nifedipine on cell calcium movements lasts much longer than a few minutes and that it is covered by homeostatic mechanisms in living lymphomonocytes.

The variations of $[Ca^{2+}]_i$ in lymphomonocytes can be due to the entry of the ion from the external medium into the cell and/or to a release of calcium from intracellular stores. The absence of calcium in the external medium does not suppress the $[Ca^{2+}]_i$-increasing effect of olive oil phenols (Fig. 4). This indicates an activity of phenols on the release of calcium from intracellular stores. The comparison of $[Ca^{2+}]_i$ variations in the presence and in the absence of external calcium indicates an effect of phenols on both pathways. These conclusions are further supported by the experiment carried out 60 s after the

addition of phenols and of nifedipine (Fig. 5). The effect of nifedipine decreases if Ca^{2+} is omitted from the incubation medium, indicating that, in lymphomonocyte preparation, nifedipine-sensitive channels are located both on the plasma membrane and on the reticular membranes. The effects of phenols also diminish when Ca^{2+} is omitted, in agreement with the hypothesis that thyrosols act on nifedipine-sensitive Ca^{2+} channels. Therefore, we demonstrate that phenols act on nifedipine-sensitive channels, although an effect on different mechanisms of calcium transfer cannot be ruled out.

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