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(-)-Epicatechin and related procyanidins modulate intracellular calcium and prevent oxidation in Jurkat T cells

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Abstract
This study investigated the effects of (-)-epicatechin (EC), its oligomers, dimer B2 (B2) and trimer C1 (C1), on calcium-dependent cell oxidation. Jurkat T cells were subjected to a Ca^{2+} mobilization challenge by replacing Na^+ with K^+ in the incubation media. A $249 \pm 38\%$ increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was observed and that effect was prevented by the presence of inhibitors of Ca^{2+} mobilization and entrance. The pre-incubation of the cells in the presence of EC, B2 or C1 prevented K^+-mediated increase in $[Ca^{2+}]_i$. IC_{50} were 10, 24 and 196 nM for EC, B2 and C1, respectively. Cell membrane depolarization was affected by K^+, but neither inhibitors of Ca^{2+} mobilization nor EC, B2 or C1 modified the increase in membrane potential. An $84 \pm 7\%$ increase in cell oxidants was observed after cell exposure to K^+. This increase was prevented by the inhibition of Ca^{2+} mobilization, NADPH oxidase and protein kinase C, as well as by 10 nM EC, 10 nM B2 or 100 nM C1. In addition, EC and B2 (100 nM) significantly inhibited the activation of the $[Ca^{2+}]_i$-regulated transcription factor NFAT. These results indicate that EC and related oligomers, assayed at physiologically achievable concentrations, can modulate $[Ca^{2+}]_i$ and then prevent cell oxidation and other Ca^{2+}-regulated events.

Keywords: *Oxidation, antioxidant, procyanidin, flavonoid, flavanol, inflammation, membrane interactions, gastrointestinal tract, calcium channels, cocoa*

Abbreviations: *BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester; B2, dimer B2; [Ca2+]i, intracellular Ca2+ concentration; C1, trimer C1; DCF-DA, 5-(and-6)-carboxy-2′, 7′-dichloro-dihydrofluorescein-diacetate; DiBaC4(3), bis-(1,3-dibutylbarbituric acid)trimethine oxanol; DPI, diphenyleniodonium chloride; FURA-2AM, 1-[6-amino-2-(5-carboxy-2-oxazolyl) -5-benzo-furanyloxy]-2-(2-amino-5-methylphenoxy)-ethane-N,N,N′,N′-tetraacetic acid pentaacetoxymethyl ester; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide; PI, propidium iodide; ROS, reactive oxygen species; Ro32-0432, bisindolylmaleimide XI hydrochloride; SKF 96365, 1-(β-(3-(4-methoxyphenyl)propoxy)-4-methoxyphenethyl)-1H-imidazole hydrochloride; TMB-8, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride; U-73122, 1-[6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione.*

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Introduction

Intracellular calcium ($[Ca^{2+}]_i$) is involved in the regulation of a number of cellular processes, but excess $[Ca^{2+}]_i$ leads to cell injury and death by both apoptosis and necrosis [1]. In inflammatory processes, sustained $[Ca^{2+}]_i$ increase is a key event for the activation of inflammatory cells including T lymphocytes, basophils, macrophages and denditric cells [1–5]. In T-lymphocytes increased $[Ca^{2+}]_i$ results in the activation of transcription factors, e.g. NFAT, NFκB and c-Jun N-terminal kinase [2–6]. NFAT is a transcription factor highly dependent on extracellular Ca^{2+} influx and involved in the anti-inflammatory response of T-cells [2–7]. In parallel to transcription factor regulation, excess of $[Ca^{2+}]_i$ promotes the cell production of reactive oxygen species (ROS) mainly through the activation of NADPH oxidase [8] and nitric oxide synthase [9], as well as through mitochondrial dysfunction [10]. In states of chronic inflammation, high levels of $[Ca^{2+}]_i$ and increased ROS production should be controlled to minimize the consequent tissue damage.

The flavanol (-)-epicatechin (EC) and its related oligomers, the procyanidins, are widely distributed in plants and can be present in high concentrations in plant foods commonly found in human and animal diets [11]. The involvement of flavanols and procyanidins in modulating inflammation and oxidative stress has provided mechanistic explanations for their beneficial effects for human health including prevention of cardiovascular disorders [12–15] and cancer [16,17].

In vitro studies have shown that EC and cocoa-derived procyanidins can modulate the inflammatory response by increasing the expression of anti-inflammatory cytokines and by decreasing those with pro-inflammatory activity in different cell types ([18] and references therein). We have previously demonstrated that EC and B2 dimer (B2) can protect T-cells from oxidation and inhibit NF-κB activation, preventing the expression of pro-inflammatory cytokines [19]. In intestinal cells, procyanidins, i.e. hexamers, have been shown to interact with cell plasma membrane and prevent oxidant production, loss of epithelia permeability [20] and NF-κB activation [21]. These effects may be partially ascribed to the ability of monomers and dimeric procyanidins to be taken up into cells and/or the ability of higher procyanidins to interact with cell membranes and trigger anti-inflammatory responses.

From this evidence and the extensive literature showing that flavanols and procyanidins prevent cell oxidation [22], it is possible to infer that these compounds can provide an anti-inflammatory protection by mechanisms related to the control of tissue oxidation. However, the low levels of flavanols and procyanidins present in tissues are not consistent with an oxidant scavenging action. In this work, we investigated the hypothesis that EC and select dimer- and trimer-enriched fractions from cocoa could modulate $[Ca^{2+}]_i$ and, subsequently, Ca^{2+}-dependent oxidant production and NFAT activation.

Materials and methods

Chemicals

(-)-Epicatechin (purity 89%) was obtained from Sigma (St. Louis, MO). (-)-Epicatechin-(4$\beta \rightarrow$8)-epicatechin dimer (B2) (purity > 99%) was obtained from Extrasynthese (Genay, France). Epicatechin-(4$\beta \rightarrow$8)-epicatechin-(4$\beta \rightarrow$8)-epicatechin trimer (C1) was isolated from cocoa (purity > 94.8%) by Mars Incorporated (Hackettstown, NJ). The fluorescent probes 5-(and-6)-carboxy-2′;, 7′-dichloro-dihydrofluorescein-diacetate (DCF-DA); bis-(1,3-dibutylbarbituric acid)trimethine oxanol $DiBaC_4(3)$; propidium iodide (PI) and 1-[6-amino-2-(5-carboxy-2-oxazolyl) -5-benzo-furanyloxy]-2-(2-amino-5-methylphenoxy)-ethane–*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxymethyl ester (FURA 2-AM) were purchased from Molecular Probes Inc. (Eugene, OR). 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8); 4-hydroxy-3-methoxyacetophenone (apocynin); 1-[6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione (U-73122); 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM); diphenyleniodonium chloride (DPI); 1-(β-(3-(4-methoxyphenyl)propoxy)-4-methoxyphenethyl)-1H-imidazole hydrochloride (SKF 96365); and bis-indolylmaleimide XI hydrochloride (Ro32-0432) were obtained from Calbiochem (La Jolla, CA). RPMI 1640 medium, streptomycin, penicillin and foetal bovine serum were purchased from Gibco BRL (Grand Island, NY). The reagents for the EMSA assays were obtained from Promega (Madison, WI). The protease inhibitor cocktail was obtained from Roche Applied Science (Mannheim, Germany). The oligonucleotide containing the consensus sequence for NFAT was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) and all other reagents were from the highest quality available and were purchased from Sigma (St. Louis, MO).

Cell culture

Human leukaemia T-cells (Jurkat) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Jurkat T cells were cultured at 37°C in a 5% CO_2 atmosphere, in RPMI 1640 medium, supplemented with 2 mM L-glutamine,

10% (v/v) foetal bovine serum and penicillin (50 units/ml) and streptomycin (50 μg/ml).

Incubations

Jurkat T cells (2×10^5 cells/0.2 ml) cultured in 96-well plates were added with EC, B2, C1 (0.01–10 μM final concentration) or calcium modulators and pre-incubated for 60 min at 37°C in a 5% CO_2 atmosphere. Cells were then centrifuged for 10 min at $800 \times g$ at 4°C, supernatants were discarded and cells exposed to fluorescent probes during 30 min. Cells were centrifuged as previously indicated, resuspended with 0.2 ml of either Na-medium (140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM HEPES, 1 mM NaH_2PO_4, 5.5 mM glucose, pH 7.4) or K-medium (5 mM NaCl, 140 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM HEPES, 1 mM NaH_2PO_4, 5.5 mM glucose, pH 7.4). Both media were added with the same amount of EC, B2 or C1 used in the initial pre-incubation. After 15 min of incubation at 37°C, samples were centrifuged for 10 min at $800 \times g$ at 4°C, the supernatant was discarded and cells were disrupted by incubation for 20 min in the presence of 0.1% (v/v) Igepal in PBS. Cell viability, evaluated as MTT reduction assay [23], was > 90% for all the experimental conditions assessed. When necessary, the samples were subsequently added with 25 μM PI to evaluate the DNA content. After 20 min of incubation at room temperature in the dark, PI fluorescence at 590 nm ($\lambda_{excitation}$: 538 nm) was measured.

Determination of $[Ca^{2+}]_i$

To determine $[Ca^{2+}]_i$, after incubation in the presence of EC, B2, C1 or calcium modulators, cells were exposed to 1 μM Fura 2AM for 30 min at 37°C. The non-incorporated probe was removed by washing cells twice with warm PBS. Cells were treated as indicated above and, after cell disruption, the fluorescence (510 nm) of the Ca^{2+}-Fura 2 complex ($\lambda_{excitation}$: 340 nm) and of free Fura 2AM ($\lambda_{excitation}$: 380 nm) was determined in a SFM-25 espectrofluorometer (Kontron Instruments sPA, Milan, Italy). $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. [24].

Evaluation of plasma membrane potential

After cell pre-incubation for 60 min with EC, B2, C1 or calcium modulators, the cells were treated as indicated above and incubated for 30 min at 37°C in the presence of 13 μM DiBacC4(3) and then for 15 min in Na- or K-medium. The fluorescence of DiBacC4(3) is triggered when the dye enters the cell membrane as a result of membrane depolarization. After incubation, samples were centrifuged at $800 \times g$ for 10 min, disrupted as indicated and the fluorescence of DiBacC4(3) determined at 516 nm ($\lambda_{excitation}$: 493 nm). DNA content in the samples was determined as described above.

Evaluation of cell oxidants

After pre-incubation with EC, B2, C1 or calcium modulators, cells were exposed to 30 μM DCF-DA for 30 min at 37°C. This probe crosses the cell membranes, being de-acetylated inside the cells, and reacts with oxidants to give a fluorescent product (DCF). The non-incorporated probe was eliminated by washing the cells twice with warm PBS. Cells were then incubated in Na- or K-medium for 15 min. At the end of the experiment, cells were disrupted and the fluorescence of the oxidized probe was measured at 525 nM ($\lambda_{excitation}$: 475 nM). DNA content in the samples was determined as described above.

Evaluation of NFAT binding activity

After cell pre-incubation for 60 min with EC, B2, C1 or calcium modulators and incubation for 15 min in Na- or K-medium, cells were rinsed with PBS and centrifuged at $800 \times g$ for 10 min at 4°C. Nuclear fractions were prepared as described [25]; briefly the pellet was lysed in 100 μl of 20 mM Hepes buffer, pH 7.9, containing 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 1% (v/v) Igepal, 20% (v/v) glycerol, 5 mm dithiothreitol and EDTA-free protease inhibitors. Samples were kept on ice for 30 min and then debris precipitated by centrifugation at $10\,000 \times g$ for 15 min at 4°C. Protein concentration was determined by the method of Bradford [26] and supernatant samples stored at −80°C until assayed. For the EMSA, the oligonucleotide containing the consensus sequence for NFAT was end-labelled with [γ^{32}P]ATP using T4 polynucleotide kinase and purified using Chroma Spin-10 columns. Samples were incubated with the labelled oligonucleotide (20 000–30 000 cpm) for 20 min at room temperature in 1X binding buffer [5X = 50 mM Tris-HCl buffer, pH 7.5, containing 20% (v/v) glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiotreitol, 250 mM NaCl and 0.25 mg/ml poly(dI-dC)]. Products were separated by electrophoresis in a 6% (w/v) non-denaturing polyacrilamide gel using 0.5X TBE (45 mM Tris/borate, 1 mM EDTA) as the running buffer. The gels were dried and the radioactivity quantified in a Phosphoimager 840 (GE Healthcare, Piscataway, NJ).

Statistical analysis

One-way analysis of variance (ANOVA) followed by Fisher's PLSD (protected least square difference) test and *t*-test were performed using the routines available in StatView 5.0 (SAS Institute, Cary, NC). A *p*-value < 0.05 was considered statistically significant. Values are shown as means ± SEM.

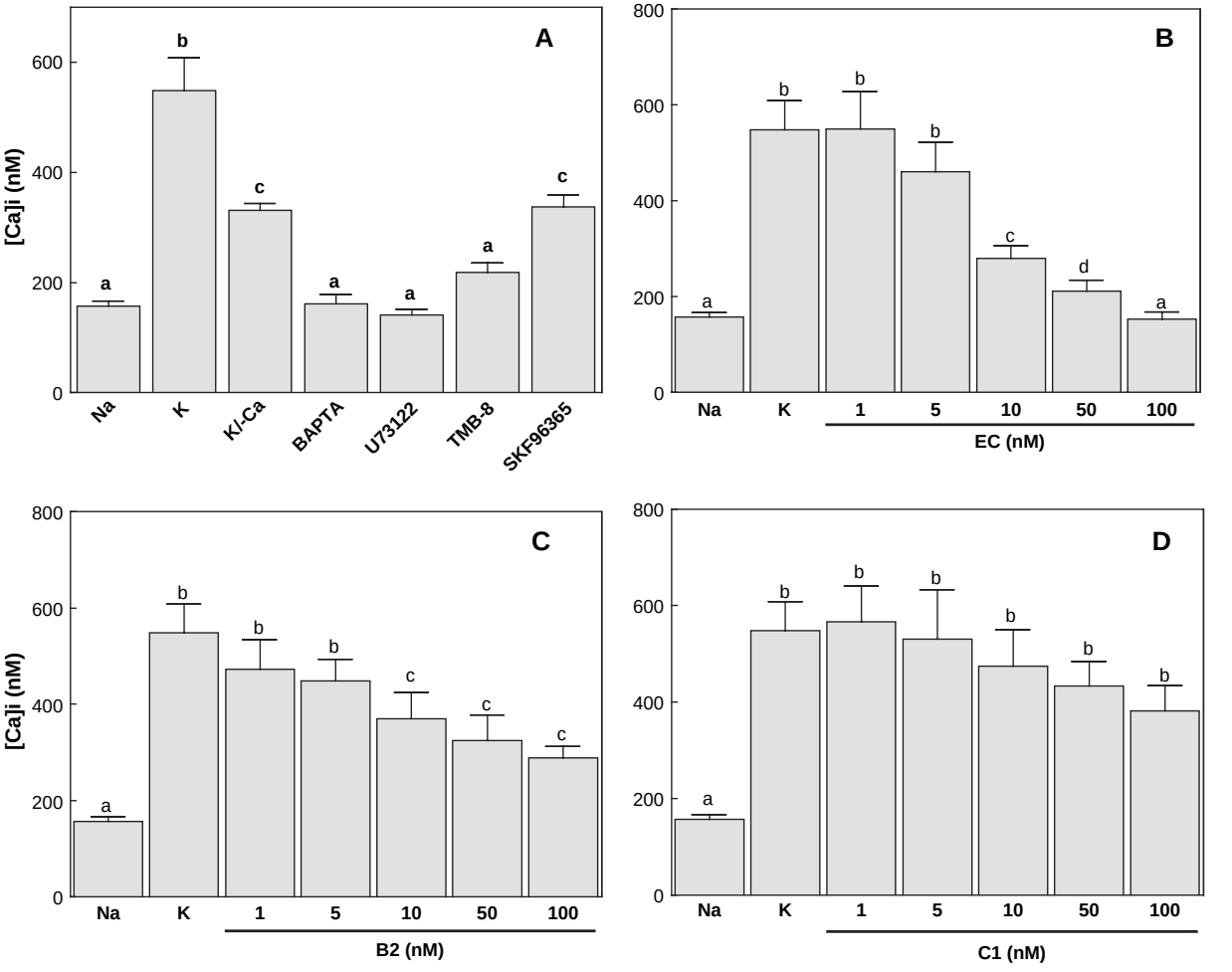

Figure 1. Effects of modulators of $[Ca^{2+}]_i$, EC, B2 and C1 on $[Ca^{2+}]_i$. Jurkat T cells were pre-incubated for 60 min at 37°C in the presence of BAPTA-AM (10 μM), U73122 (3 μM), TMB-8 (25 μM) or SKF-96365 (30 μM) (A) or 1–100 nM of EC, B2 or C1 (B–D). Subsequently cells were exposed to the fluorescent probe Fura 2AM (1 μM) for 30 min and finally submitted to a 15-min incubation in media containing 140 mM Na^+ (Na), 140 mM K^+ (K) or 140 mM K^+ without added calcium (K (-Ca)). $[Ca^{2+}]_i$ was evaluated from changes in Fura-2 fluorescence, as described in Materials and methods. Values are shown as means ± SEM of at least five independent experiments. Values having different letters are significantly different ($p < 0.05$).

Results

Effects of modulators of $[Ca^{2+}]_i$, EC, B2 and C1 on K^+-mediated changes in $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ in Jurkat T cells was evaluated using the fluorescent probe Fura 2AM ($n = 5$ independent experiments). $[Ca^{2+}]_i$ increased linearly from 0 to 15 min of incubation reaching values of 547 ± 59 and 157 ± 9 nM when cells were incubated in 140 mM K^+ (K-medium) or Na+ (Na-medium), respectively (Figure 1; $p < 0.01$). The increase in $[Ca^{2+}]_i$ generated by K-medium was prevented ($p < 0.01$) by pre-incubating the cells for 1 h at 37°C in the presence of BAPTA-AM (10 μM) or the phospholipase C inhibitor U73122 (3 μM) (Figure 1A). The pre-incubation for 1 h at 37°C in the presence of 25 μM TMB-8 (an inhibitor of Ca^{2+} release from the endoplasmic reticulum) or 30 μM SKF-96365 (an inhibitor of store-operated calcium channels) prevented the K^+-mediated increase of $[Ca^{2+}]_i$ by 84 ± 5 and 54 ± 6% ($p < 0.05$), respectively (Figure 1A). The concentration of the Ca^{2+}-inhibitors and the incubation times used were chosen based on previously published data [27–30]. When incubated in K-medium in the absence of calcium the $[Ca^{2+}]_i$ was inhibited by 42 ± 3%. To evaluate whether EC, B2 or C1 could affect the K^+-mediated increase in $[Ca^{2+}]_i$, cells were pre-incubated for 60 min in the presence of each of these compounds. EC, B2 or C1 inhibited $[Ca^{2+}]_i$ increase in a concentration-dependent manner (Figure 1B–D). The calculated IC_{50} were 10, 24 and 196 nM for EC, B2 and C1, respectively. EC prevention of K^+-mediated $[Ca^{2+}]_i$ increase was significant ($p < 0.05$) at concentrations as low as 10 nM (Figure 1B). At 100 nM, EC and B2 inhibited 104 ± 4 and 68 ± 6% of K^+-mediated $[Ca^{2+}]_i$ increase, respectively;

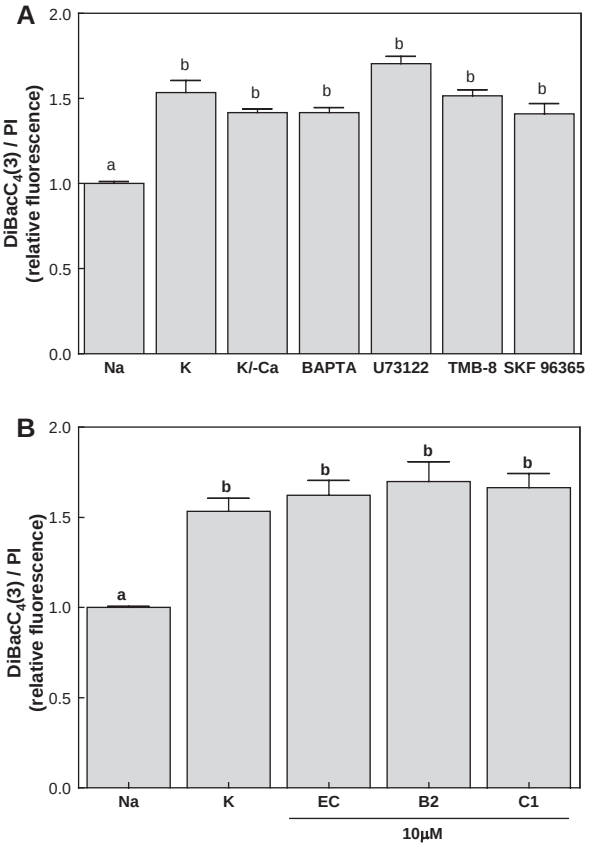

Figure 2. Effects of modulators of $[Ca^{2+}]_I$, EC, B2 and C1 on cell membrane potential. Jurkat T cells were pre-incubated for 60 min at 37°C in the presence of TMB-8 (25 μM), BAPTA-AM (10 μM), SKF-96365 (30 μM) or U73122 (3 μM) (A) or 10 μM of EC, B2 or C1 (B). Subsequently cells were exposed to the fluorescent probe $DiBacC_4(3)$ (13 μM) for 30 min and finally submitted to a 15-min incubation in media containing 140 mM Na^+ (Na), 140 mM K^+ (K) or 140 mM K^+ without added calcium (K (-Ca)). Membrane potential was evaluated by the intracellular content of the fluorescent probe $DiBacC_4(3)$ and referred to DNA content in the samples, as described in Materials and methods. Values are shown as means ± SEM of at least four independent experiments. Values having different letters are significantly different ($p < 0.05$).

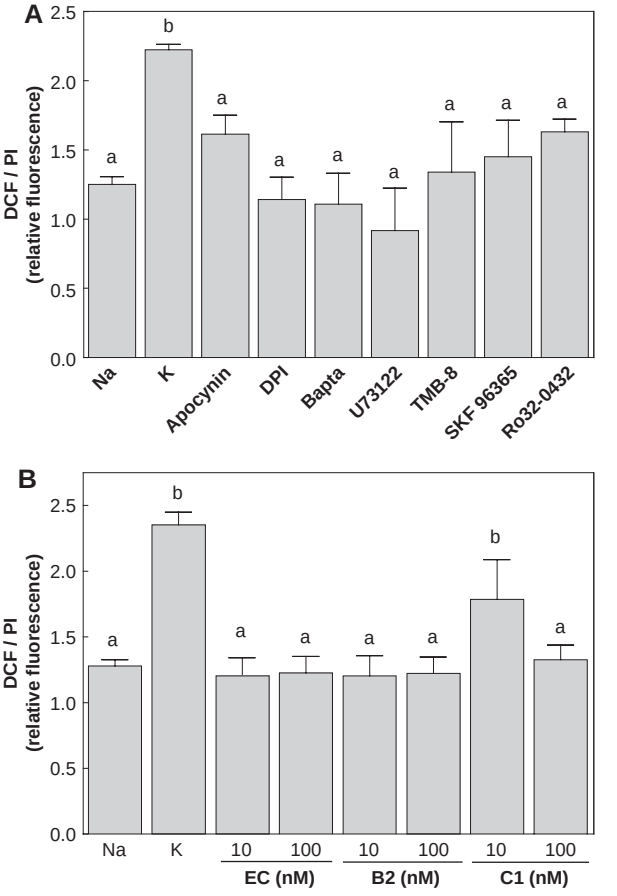

Figure 3. Effects of modulators of $[Ca^{2+}]_I$, EC, B2 and C1 on cell oxidants. Jurkat T cells were incubated for 60 min at 37°C in the presence of BAPTA 2AM (10 μM), U73122 (3 μM), TMB-8 (25 μM), SKF-96365 (30 μM), apocynin (100 μM), DPI (0.5 μM) or Ro32-0432 (1 μM) (A); or 10 or 100 nM EC, B2 or C1 (B). Subsequently cells were exposed to the fluorescent probe DCF-DA (30 μM) for 30 min and finally submitted to a 15-min incubation in media containing 140 mM Na^+ (Na) or 140 mM K^+ (K). Cell oxidants production was evaluated as DCF fluorescence and referred to DNA content in the samples, as described in Materials and methods. Values are means ± SEM of at least four independent experiments. Values having different letters are significantly different ($p < 0.05$).

but the inhibitory effect of 100 nM C1 was non-significant.

Effects of modulators of $[Ca^{2+}]_i$, EC, B2 and C1 on K^+-mediated cell membrane potential

The extent of cell plasma membrane depolarization was evaluated using the fluorescent probe $DiBacC_4(3)$. After a 15 min exposure to K-medium, $DiBacC_4(3)$ fluorescence was 53 ± 7% higher ($p < 0.05$) than in cells exposed to Na-medium (Figure 2A). Pre-incubation of the cells with BAPTA-AM, U73122, TMB-8 or SKF-96365 did not affect K^+-mediated cell membrane potential (Figure 2A). Similarly, pre-incubation of the cells in the presence of EC, B2 or C1 (0.1–10 μM) did not affect K^+-mediated increase of $DiBacC_4(3)$ fluorescence. Figure 2B shows the absence of effects of EC, B2 and C1 on $DiBacC_4(3)$ fluorescence at the highest concentration tested, i.e. 10 μM.

Effects of modulators of $[Ca^{2+}]_I$, EC, B2 and C1 on K^+-mediated increase in cell oxidants

Intracellular oxidants levels were next evaluated by the fluorescence of DCF. Incubating the cells in K-medium generated 84 ± 7% higher DCF fluorescence than cells incubated in Na-medium ($p < 0.01$) (Figure 3A). Pre-incubation of cells with BAPTA-AM, U73122, TMB-8 and SKF-96365 prevented the K^+-dependent increase of cell DCF fluorescence ($p < 0.05$). Given the known capacity of Ca^{2+} to activate NADPH oxidase either through a direct activation or via PKC, two NADPH oxidase inhibitors (DPI and apocynin) and one PKC inhibitor

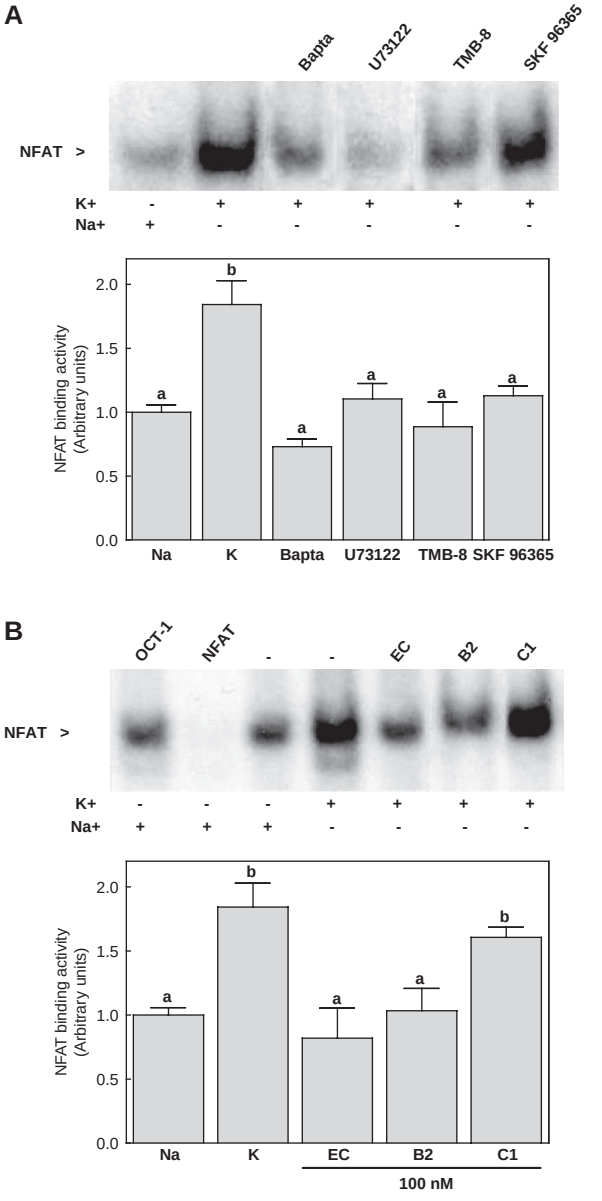

Figure 4. Effects of modulators of $[Ca^{2+}]_I$, EC, B2 and C1 on NFAT activation. Jurkat T cells were pre-incubated for 60 min at 37°C in the presence of TMB-8 (25 μM), BAPTA 2AM (10 μM), SKF-96365 (30 μM) or U73122 (3 μM) (A); or 100 nM of EC, B2 or C1 (B). Subsequently cells were exposed to a 15-min incubation in media containing 140 mM Na^+ (Na) or 140 mM K^+ (K). NFAT-DNA binding was evaluated by EMSA in the nuclear fractions, as described in Materials and methods. The specificity of the band was checked by incubating samples with a 100-molar excess of the non-related sequence OCT-1 or with unlabelled NFAT. Values are shown as means ± SEM of four independent experiments. Values having different letters are significantly different ($p < 0.05$).

(Ro32-0432) were tested by pre-incubating the cells in the presence of these compounds. DPI (0.5 μM), apocynin (100 μM) and Ro32-0432 (1 μM) prevented the K^+-mediated increase of DCF fluorescence ($p < 0.05$) (Figure 3A). At 10 nM concentration, EC and B2 completely inhibited the K^+-induced increase of DCF fluorescence (Figure 3B). Likewise, pre-incubation with C1 inhibited K^+-induced increase of DCF fluorescence, but at 100 nM ($p < 0.05$).

Effects of modulators of $[Ca^{2+}]_I$, EC, B2 and C1 on K^+-mediated NFAT activation

NFAT activation is an important Ca^{2+}-dependent event involved in inflammation. NFAT-DNA binding activity was evaluated by EMSA in nuclear fractions (Figure 4). The specificity of the NFAT band was checked by incubating the nuclear fractions with a 100-fold molar excess of unlabelled oligonucleotide for OCT-1 or NFAT. After exposing cells to K-medium, NFAT-DNA binding activity was increased by 84 ± 4% ($p < 0.001$) relative to cells exposed to Na-medium (Figure 4A). Pre-incubation with BAPTA-AM, U73122, TMB-8 or SKF-96365 inhibited K^+-mediated NFAT activation ($p < 0.05$). EC and B2 at 100 nM concentration completely prevented K^+-mediated NFAT activation (Figure 4B) ($p < 0.05$). C1 (100 nM) did not cause a significant inhibition of K^+-mediated NFAT activation.

Discussion

The present results demonstrate that EC and B2, and to a minor extent C1, prevented cell oxidant increase through the inhibition of Ca^{2+}-mediated events in Jurkat T cells. Importantly, this inhibition occurred at concentrations of EC and B2, at which lymphocytes can be physiologically exposed, after consumption of flavanol-containing foods.

As suggested by the results obtained using different modulators of $[Ca^{2+}]_i$ homeostasis, in the present model the increase in $[Ca^{2+}]_i$ occurs as the result of both the mobilization from intracellular stores and the influx of extracellular Ca^{2+}. The incubation in the presence of EC, B2 or C1 prevented the increase in $[Ca^{2+}]_i$, EC being the most effective inhibitor as indicated by the lower IC_{50}. The calculated IC_{50} values are consistent with an inverse association between the degree of EC oligomerization and the capacity to prevent the K^+-mediated $[Ca^{2+}]_i$ increase. The range of concentrations of EC, B2 and C1 that were tested (centred in 10–100 nM) was chosen based on the concentration of free EC (< 100 nM) and dimers (41 nM) found in human plasma after the ingestion of a procyanidin-rich meal [13,31]. Importantly, these concentrations guarantee a negligible artifactual production of hydrogen peroxide, by the interaction of EC, B2 and C1, with the cell culture medium [32].

Concerning the mechanism of action of EC, B2 and C1 in preventing $[Ca^{2+}]_i$ increase, it is possible to speculate that given the capacity of these

compounds to adsorb to membranes and proteins, they could primarily interact with the extracellular leaflet of the bilayer and regulate membrane components that participate in the modulation of calcium fluxes, e.g. calcium channels. This interaction is also supported by: (i) the incubation time and the concentrations used would not allow the build-up of significant intracellular concentration of these compounds [19,33]; and (ii) although EC and B2 could be transported into cells, based on the molecular size, the transport of C1 across the cell membrane is unlikely to occur. In the current conditions, the decrease in cell membrane potential caused by high extracellular K^+ was not modified by regulators of Ca^{2+} mobilization, or by EC, B2 or C1. This indicates that the prevention of the initiation of Ca^{2+} mobilization was not driven by changes in membrane potential, as previously observed when some of these compounds were assayed at concentrations higher than those used in the present experiments [34,35].

Given the capacity of Ca^{2+} to trigger cell oxidation and the claimed antioxidant action of flavonoids, we next investigated the potential action of EC, B2 and C1 on cell oxidant levels. The observed increase in cell oxidants was dependent on $[Ca^{2+}]_i$, as indicated by the results showing that the regulators of Ca^{2+} mobilization assayed prevented the increase in oxidant production. The increase in cellular oxidant levels was also prevented by the PKC inhibitor, Ro32-0432, and by two NADPH-oxidase inhibitors, a general flavoprotein inhibitor (DPI) and a specific inhibitor of NADPH oxidase assemble (apocynin). These results suggest that the activation of NADPH oxidase may be responsible for the increase in cell oxidants following exposure to the K-medium. It should be noted that the NADPH oxidases present in Jurkat cells are not fully characterized. Recently, the presence of three isoenzymes (NOX1, NOX3 and NOX4) mRNA has been reported [36]. Unlike other isoenzymes, these NADPH oxidases do not bind Ca^{2+}, but are activated by Ca^{2+}-regulated PKC [37]. In the present conditions, the high $[Ca^{2+}]_i$ would trigger a PKC-dependent NADPH oxidase activation, as supported by the inhibition of cell oxidant increase by Ro32-0432. Cell pre-incubation in the presence of EC or B2, at concentrations as low as 10 nM, prevented the increase in intracellular oxidant production, likely through the inhibition of Ca^{2+}-dependent PKC-mediated NADPH oxidase activation. In addition, the influence of $[Ca^{2+}]_i$ on DCF oxidation ($r^2 = 0.60$, $p < 0.01$) suggests a regulation of intracellular oxidants by $[Ca^{2+}]_i$ in which 350 nM $[Ca^{2+}]_i$ appears as the lowest concentration at which the oxidant increase was observed.

The low concentrations (10 nM) of EC and B2 necessary to achieve the complete prevention of oxidation do not underscore a direct free radical scavenging action of these compounds. The amount of oxidants that can be generated in cells subjected to an oxidant challenge within 15 min can be estimated as 6×10^8 oxidant species [38]. Considering that the maximum number of molecules of EC, B2 or C1 that can be incorporated into cells after 15 min of incubation can be estimated as 5×10^6 (Sagdicoglu and Fraga, unpublished), only 1% of oxidants would be scavenged (assuming maximal affinity and diffusion controlled rate). In addition, the possibility of an inhibitory effect of NADPH oxidase by apocynin-like EC and B2 metabolites as observed in HUVEC cells [39,40] is unlikely to occur under the current experimental conditions. The reported IC_{50} for HUVEC NADPH oxidase inhibition were in the micromolar order and determined after 24 h of incubation, while in the present conditions nanomolar concentrations of EC and B2 inhibit oxidant production. Finally, it is important to point out that $[Ca^{2+}]_I$ and mitochondrial ROS production are highly related [10]. In the present cell model, the incomplete inhibition of cell oxidant production by NADPH and PKC inhibitors could be related to the contribution of other sources of ROS, e.g. mitochondria. The complete inhibition of cell oxidation increase by EC and B2 reinforces the concept that the prevention of oxidation by these compounds was secondary to the inhibition of $[Ca^{2+}]_i$ increase and not to other intracellular events, such as the scavenging of mitochondria generated ROS.

An increase in $[Ca^{2+}]_i$ is a key signal that modulates the expression of several proteins associated with inflammation. In lymphocytes, $[Ca^{2+}]_i$ stimulates NFAT activation and the consequent transcription of pro-inflammatory cytokines [41]. In the present experimental conditions, cell exposition to K-medium leads to a $[Ca^{2+}]_i$-dependent NFAT activation. The dependence on Ca^{2+} is suggested by the prevention of NFAT activation by regulators of Ca^{2+} mobilization. EC and B2 (100 nM) inhibited K^+-mediated NFAT activation, indicating a potential action of the compounds controlling undesirable Ca^{2+} uptake. The lack of a significant effect of C1 on NFAT activation suggests that $[Ca^{2+}]_i$ of $\sim$350 nM is necessary to trigger NFAT activation. This is in line with the fact that the activation of NFAT requires high levels of $[Ca^{2+}]_I$, as that provided by the opening of CRAC channels [7]. From the obtained results, it can not be precluded that the inhibition of NFAT activation could be in part surrogated to the decrease in oxidants mediated by EC, B2 and C1, as was observed in Hodgkin's lymphoma cells [42].

The effects of the assayed flavanol and procyanidins are comparable with the findings in rat lenses showing that the flavonol quercetin inhibited by 90% the H_2O_2-dependent Ca^{2+} uptake [43]. Quercetin

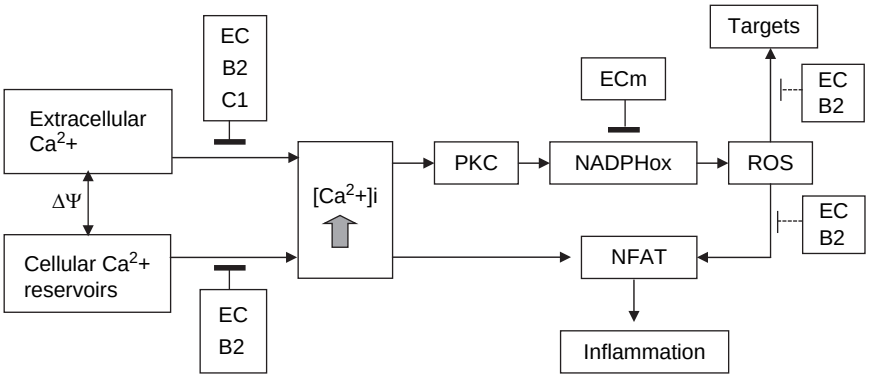

Figure 5. Sequence of events leading to calcium-mediated ROS modulation by EC, B2 and C1 in Jurkat T cells. Dashed lines indicate unfeasible inhibitory effects due to low cellular concentrations of flavanols and procyanidins. ECm = (-)-epicatechin metabolites; ΔΨ = changes in cell membrane potential.

differs from EC in the substitution at position 4 of ring C, where it has a keto group instead of hydrogen. Interestingly, epigallocatechin gallate, which has a gallic acid moiety in the same position, stimulates Ca^{2+} mobilization *per se* [44]. Similarly, EC oligomerization, which also occurs at carbon 4 of C ring, decreases EC ability to modulate Ca^{2+} mobilization. Together, these results support the hypothesis that substitutions in the 4 position of the B ring influence the nature of interactions of flavonoids with cell membrane components and, thus, define some of their biological effects.

In summary, the present results demonstrate that EC and B2, and to a lesser degree C1, assayed at concentrations at which lymphocytes can be physiologically exposed, influence $[Ca^{2+}]_i$ and, consequently, $[Ca^{2+}]_i$-dependent cell oxidation (Figure 5). Further studies are necessary to identify how mechanisms involved in Ca^{2+} mobilization and uptake could be finally regulated by EC and related compounds. The relevance of the observed results for other types of cells and for flavonoid-metabolites also remains to be studied, as well as the importance of the proposed mechanisms for human health. Nevertheless, these results provide a mechanism supporting the concept that the incorporation in the diet of foods containing relatively high amounts of flavanols and procyanidins, e.g. cocoa, could provide health benefits to individuals with chronic and 'silent' inflammatory and other pro-oxidative conditions.

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