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Specific interaction between tetrandrine and *Quillaja* saponins in promoting permeabilization of plasma membrane in human leukemic HL-60 cells

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Abstract

Spontaneous Ni^{2+} entry (leak), measured as fluorescence quench in fura-2-loaded HL-60 cells at the excitation wavelength of 360 nm, was strongly inhibited by tetrandrine (TET, 100 μM), a Ca^{2+} antagonist of Chinese herbal origin. Exposure of the cells for 5 min to saponins from *Quillaja saponaria* (QS, 30 μg/ml), surfactants well known to permeabilize the plasma membrane by complexing with cholesterol, promoted Ni^{2+} entry without causing fura-2 leak-out. Unexpectedly, TET caused an immediate (within 2.5 min) augmentation of QS-promoted Ni^{2+} entry; and a 5-min treatment with both TET and QS resulted not only in an enhanced Ni^{2+} entry, but also a fura-2 leak-out. Ginseng saponins (100 μg/ml) alone or together with TET did not cause such a permeabilization. Permeabilization induced by 1–3 μM digitonin, another cholesterol-complexing glycoside, could not be enhanced by TET. TET did not affect permeabilization induced by Triton X-100 (0.01%), a detergent which non-specifically disrupts the hydrophobic interaction at the plasma membrane. TET also did not enhance Ni^{2+} entry triggered by ionomycin (0.35 μM) or SK&F 96365 (20 μM). Further, it did not augment Ni^{2+} entry when the plasma membrane fluidity was modulated by changes of temperature (27–47°C) or treatment with 5% ethanol. This QS-promoted Ni^{2+} entry could not be amplified by other lipophilic Ca^{2+} antagonists, such as diltiazem (100 μM) and verapamil (100 μM). The results hence indicate that TET enhanced Ni^{2+} entry (or permeabilization) elicited by QS treatment, but not other perturbations of the plasma membrane. We suggest that pore formation at the plasma membrane, a consequence of QS-cholesterol interaction, can be specifically enhanced by TET. Also, a comparative study of the effects of TET and its very close analogues, hernandezine and berbamine, reveals that the methoxyl group at the R_2 position of TET appears to be crucial in enhancing QS-promoted Ni^{2+} entry.

Keywords: Saponin; Tetrandrine; Ni^{2+}; Mn^{2+}; HL-60 cell

1. Introduction

Tetrandrine (TET), a bis-benzylisoquinoline alkaloid isolated from the Chinese medicinal herb *Stephania tetrandra* (Fig. 1), has been demonstrated to be an effective hypotensive and antisilicotic drug in animal experiments and clinical studies [1–4]. The

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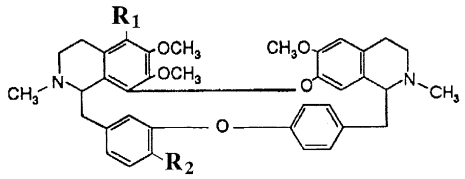

R_1	R_2	
H	OCH_3	Tetrandrine
H	OH	Berbamine
OCH_3	OCH_3	Hernandezine

Fig. 1. Chemical structures of tetrandrine, hernandezine and berbamine.

hypotensive effect of TET is believed to be due to its blockade of L-type voltage-operated Ca^{2+} channels (VOCC) [5,6]. TET also inhibits T- and N-type VOCC in a number of excitable tissues [5,7–9]. More recently, TET was shown to act on Ca^{2+} channels not opened by depolarization. For instance, TET inhibits the agonist-activated Ca^{2+} entry in vascular smooth muscles [10,11] and blocks Ca^{2+} entry activated by thapsigargin (an endoplasmic reticulum Ca^{2+} pump inhibitor) as a result of intracellular Ca^{2+} store depletion in HL-60 cells, 3T3 fibroblasts, rat glioma C6 cells and parotid acinar cells [12,13]. TET also affects targets other than Ca^{2+} channels, such as Ca^{2+}-activated K^+ channels, Na^+,K^+-ATPases and protein kinase C [5,14,15]. These observations may explain the modulatory effects of TET on many biological responses in vitro and in vivo [5], but on the other hand, they cast doubt on the specificity of TET as a Ca^{2+} antagonist.

Emptying of the intracellular Ca^{2+} store following agonist stimulation or thapsigargin treatment activates the entry of Ca^{2+} and Mn^{2+} (a surrogate ion for Ca^{2+}) through plasmalemmal channels in many cell types including HL-60 cells [12,13,16,17]. It was previously shown in our laboratory that such store-operated divalent cation entry in HL-60 cells could be strongly inhibited by TET [12]. More recently, we also identified the chemical moiety of TET which was crucial for the above-mentioned inhibitory action [18]. In this work, using the same cell system (HL-60), we tested the specificity of TET as a Ca^{2+} antagonist: if TET blocks the divalent cation entry by acting specifically on Ca^{2+} channels, then it presumably would not affect cation influx that is promoted by *Quillaja* saponins (QS). The latter are plant glycosidic compounds which form pores at the plasma membrane by causing aggregation of cholesterol [19,20]. Unexpectedly we observed that TET accelerated the entry of Ni^{2+} which was promoted by QS. However, TET did not accelerate the influx of Ni^{2+} when the fluidity of the plasma membrane was modulated or when the hydrophobic association in the plasma membrane was non-specifically disrupted. We here show evidence that TET-QS interaction bears a stringency in structural requirements. The TET-QS interaction will also be discussed in the context of their frequent uses in basic research and their potential therapeutic values.

2. Materials and methods

2.1. Cell culture

HL-60 cells obtained from American Type Culture Collection, Maryland, were maintained in RPMI 1640 medium (Sigma Chem. Co.) supplemented with 10% fetal calf serum, 5 μg gentamycin/ml and penicillin/streptomycin (100 U/ml, 100 μg/ml) in a humidified atmosphere with 5% CO_2 at 37°C. The cells were split in a 1:5 ratio every two or three days.

2.2. Materials

QS (product number S2149 and S7900), ionomycin, Triton X-100, verapamil, thapsigargin, digitonin, filipin and fura 2-AM were obtained from Sigma Chemical Co., St. Louis, USA. S2149 and S7900 QS are both from *Quillaja* bark but are from different primary sources. Unless otherwise stated, S2149 QS was used in all the experiments. Saponins (number 7695, plant source unknown) were also purchased from E. Merck (Darmstadt, Germany). Ginseng saponins were from Sanchakou Ginsenoside Company (Heilongjiang, China). Diltiazem was from Calbiochem (La Jolla, CA). Fetal calf serum was purchased from Gibco BRL (Gaithersburg, MD, USA). SK&F 96365 was from BIOMOL Research Lab., Plymouth Meeting, PA, USA. Tetrandrine

(TET) (purity > 98%) was purchased from Aldrich (Milwaukee, WI, USA). Hernandezine (HER) was a generous gift from Professor G.Z. Liu of the China-Japan Friendship Hospital (Beijing, China). Both TET and HER were dissolved in 0.1 N HCl to yield a stock solution of 30 mM. The amount of vehicle added to the reaction medium did not affect the medium pH or the fluorescence reading. Berbamine (BER), kindly provided by Professor M.R. Rao (Department of Cardiovascular Pharmacology, Nanjing Medical College), was dissolved in distilled water to yield a stock solution of 30 mM.

2.3. Measurement of $[Ca^{2+}]_i$ and divalent cation influx

The methods used to measure $[Ca^{2+}]_i$ and the influx of divalent cations were as described previously [12,21]. Hank's buffered saline solution (HBSS), composed of (mM) NaCl 138, KCl 5.3, $MgSO_4$ 0.8, $CaCl_2$ 1.2, KH_2PO_4 0.44, Na_2HPO_4 0.34, glucose 5, and Hepes (N-[2-hydroxyethyl]-piperazine-N′-2-ethane sulfonic acid) 25, buffered at pH 7.4, was used for washing the cells. HL-60 cells grown to a density of around 1.5×10^6/ml were harvested, washed once ($200 \times g$, 5 min) in Ca^{2+}-containing HBSS and resuspended in RPMI 1640 medium at a cell density of about 2×10^7/ml. Fura 2-acetoxymethyl (AM) ester was added to this cell suspension at a final concentration of 5 μM. The cell suspension was then incubated at 37°C for 45 min. Thereafter, the fura 2-loaded cells were washed twice ($200 \times g$, 5 min) in Ca^{2+}-containing HBSS and resuspended in the same buffer solution at a cell density of 2.8×10^6/ml. Before experimentation of each sample, 1 ml of this cell suspension was centrifuged ($200 \times g$, 5 min) and the cell pellet was resuspended in 2 ml Ca^{2+}-containing (for $[Ca^{2+}]_i$ measurement) or Ca^{2+}-free HBSS (for measurement of divalent cation influx). This 2-ml cell suspension was transferred to a quartz cuvette which contained a mini-stirrer, and warmed at 37°C (except in experiments shown in Fig. 9) for about 3 min before addition of drugs or divalent cations. Fura-2 fluorescence was measured by a Hitachi F-4000 fluorescence spectrophotometer. For $[Ca^{2+}]_i$ measurement, the excitation and emission wavelengths were set at 340 nm and 500 nm respectively. Fluorescence was not calibrated into $[Ca^{2+}]_i$ but instead expressed in arbitrary units because of the possible fura-2 leak-out of the cells after prolonged QS treatment (see Fig. 3). For the measurement of Mn^{2+} and Ni^{2+} influx, the excitation and emission wavelengths were set at 360 nm and 500 nm respectively. At 360 nm excitation wavelength, the fura-2 fluorescence is insensitive to Ca^{2+} but quenchable by heavy metals such as Mn^{2+} and Ni^{2+} [22].

2.4. Measurement of fura-2 leak-out

Fura 2-loaded HL-60 cells were washed and prepared as described above. Before experimentation of each sample, 1 ml of cell suspension was centrifuged ($200 \times g$, 5 min) and the cell pellet was resuspended in 2 ml Ca^{2+}-free HBSS. This 2-ml cell suspension was transferred to a quartz cuvette which contained a mini-stirrer, and warmed at 37°C for about 3 min. Fluorescence was measured as described above (excitation and emission wavelengths at 360 nm and 500 nm respectively). Cells were treated with QS for around 1 min and then TET (various combinations of concentrations) for 2.5 or 5 min (Fig. 3). Subsequently the cells were retrieved from the reaction cuvette and rapidly centrifuged ($500 \times g$, 50 s). After that, the supernatant was discarded and the cell pellet was resuspended again in 2 ml Ca^{2+}-free HBSS. Fluorescence was measured again and expressed as a percentage of that before treatments with QS and TET (Fig. 3, *y*-axis). These values indicate the amount of fura-2 remaining in the cytosol. Control samples (i.e., without drug treatment) were processed in the same manner, and the fluorescence in the control samples dropped by some 15%, which was most likely due to spontaneous fura-2 leak-out and also cell loss during the cell retrieval and centrifugation processes. The differences between the controls and the drug-treated groups therefore represent the proportions of fura-2 leak-out caused by drug treatment.

2.5. Statistical analysis

Results are expressed as mean $\pm$ S.E.M. The Student's paired *t*-test was employed and differences were considered significant when $P < 0.05$.

3. Results

3.1. Effects of TET on QS-induced permeabilization to Ca^{2+} and fura-2

The plateau phase of the thapsigargin-induced Ca^{2+} response in Ca^{2+}-containing medium, which was due to sustained Ca^{2+} entry activated by Ca^{2+} store depletion (capacitative Ca^{2+} entry), was substantially inhibited by 100 μM TET (Fig. 2a) [12,18]. If TET blocks Ca^{2+} entry by specifically interacting with Ca^{2+} channels, it presumably would not affect Ca^{2+} entry promoted by QS. The latter acts by permeabilizing the plasma membrane via complexing with cholesterol [19,20]. QS at 30 μg/ml, a concentration known to permeabilize selectively the plasma membrane [23–25], induced a sustained elevation in fluorescence (Fig. 2b). As fura-2 would possibly leak out of the cells after QS-induced permeabilization (see below), such elevation in fluorescence could be due to both an elevation in $[Ca^{2+}]_i$ and saturation of leaked fura-2 with extracellular Ca^{2+}. Therefore, fluorescence was not calibrated into $[Ca^{2+}]_i$. Addition of TET did not suppress such QS-induced fluorescence elevation, but instead caused a further rise in fluorescence. The latter could be explained by the fact that TET alone induced a rise in fluorescence (Fig. 2c), which we have already shown in HL-60 cells to be due to Ca^{2+} release from the intracellular stores [12]. This further suggests that QS at 30 μg/ml did not permeabilize the intracellular Ca^{2+} store which could be depleted by subsequent addition of TET. Surprisingly, QS induced a larger elevation in fluorescence in the presence than in the absence of TET pretreatment (compare Fig. 2c and Fig. 2b; at 12 min after QS exposure, $51.3 \pm 5.6\%$ versus $39.8 \pm 4.9\%$ above pre-treatment level; $n = 4$; $P < 0.05$), suggesting that TET could enhance QS-induced permeabilization to Ca^{2+} or leak of fura-2 or both.

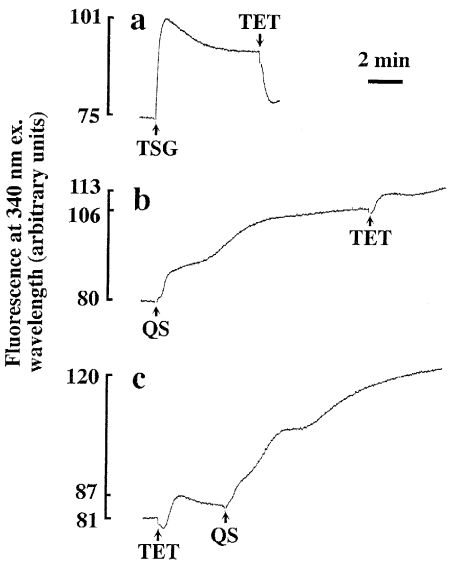

Fig. 2. Effects of TET on TSG-activated Ca^{2+} entry and QS-induced permeabilization. HL-60 cells in Ca^{2+}-containing HBSS were stimulated with (a) 30 nM TSG and (b) 30 μg/ml QS and at the sustained phase of fluorescence elevation, 100 μM TET was added. (c) Same conditions as in (b), and QS was added after TET. The *y*-axis represents fura-2 fluorescence at 340 nm excitation wavelength. Similar traces were obtained in 3 other separate experiments.

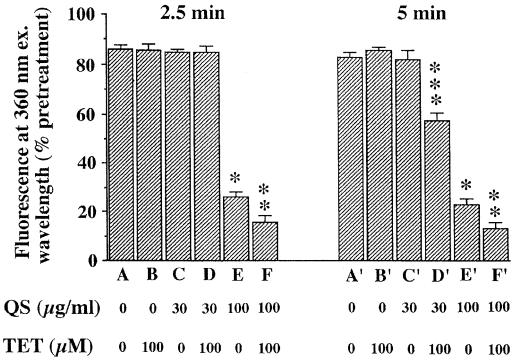

Fig. 3. Effects of QS and TET on fura-2 leak-out. Experimental details are described in Section 2. Pretreatment fluorescence values (arbitrary units) from A to F′ are, respectively, 46.7 ± 6.1, 47.8 ± 6.4, 48.9 ± 5.9, 49.8 ± 4.8, 53.2 ± 2.2, 50.6 ± 4, 51.8 ± 0.5, 52.5 ± 1.4, 53.1 ± 0.5, 52.3 ± 0.2, 52.9 ± 1.4 and 50.8 ± 2.5. Results are the mean $\pm$ S.E.M. of 3–4 separate experiments. * E and E′ are significantly ($P < 0.05$) different from A and A′ respectively. ** F and F′ are significantly ($P < 0.05$) different from E and E′ respectively. *** D′ is significantly ($P < 0.05$) different from C′.

We investigated the enhancement by TET of QS-induced permeabilization by checking the fura-2 leakage. Neither 100 μM TET nor 30 μg/ml QS on its own stimulated fura-2 leakage throughout the 5-min time course (Fig. 3A,B,C,A′,B′,C′). Exposure to both 100 μM TET and 30 μg/ml QS did not affect the fura-2 leakage during the first 2.5 min but induced a significant ($P < 0.05$) leakage after 5 min. (Fig. 3D,D′). A 2.5-min treatment was sufficient

($P < 0.05$) for 100 μg/ml QS alone to cause a substantial fura-2 leak-out, which was significantly ($P < 0.05$) augmented by TET (Fig. 3E,F). Further incubation only marginally increased the leak-out of fura-2 (Fig. 3E′,F′).

3.2. Effects of TET on QS-induced permeabilization to Ni^{2+} and Mn^{2+}

Another approach to study the effect of TET on QS-induced permeabilization was to examine the entry of divalent cations, such as Ni^{2+} and Mn^{2+}, which quench the fura-2 fluorescence [12,21,22]. Addition of Ni^{2+} caused a gradual quenching, indicating that there was a small spontaneous Ni^{2+} entry (leak) into the cytosol (Fig. 4a; quenching rate after HCl treatment was 4.4 ± 1 arbitrary fluorescent units/4 min; $n = 4$). The basal Ni^{2+} entry was blocked by TET (Fig. 4b; quenching rate after TET treatment was 0.3 ± 0.3 arbitrary fluorescent units/4 min; $n = 4$). Such blockade was statistically significant ($P < 0.05$). QS (30 μg/ml) markedly accelerated the quenching (Fig. 4c). On the basis of the following observations, such acceleration of quenching indicated that QS increased the rate of Ni^{2+} entry: (a) QS (or TET alone or in combination) did not affect the fluorescence (360 nm excitation) of fura-2 acid (not shown) (b) QS (or TET alone or in combination) did not affect the fluorescence of fura-2-loaded cells (see Fig. 6) (c) QS (30 μg/ml) did not cause fura-2 leak-out even after 5 min (see Fig. 3C,C′). Addition

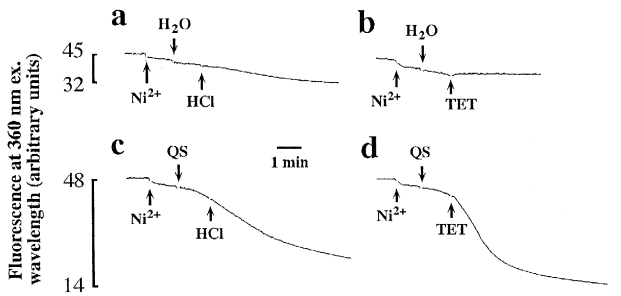

Fig. 4. Effects of QS and TET on Ni^{2+} entry. After exposure of HL-60 cells in Ca^{2+}-free HBSS to 1.2 mM Ni^{2+}, QS (30 μg/ml) or water (sham treatment) was added for around 1 min before further treatment with TET (100 μM) or HCl (solvent control). The *y*-axis represents fura-2 fluorescence at 360 nm excitation wavelength. Similar traces were obtained in at least 3 other separate experiments.

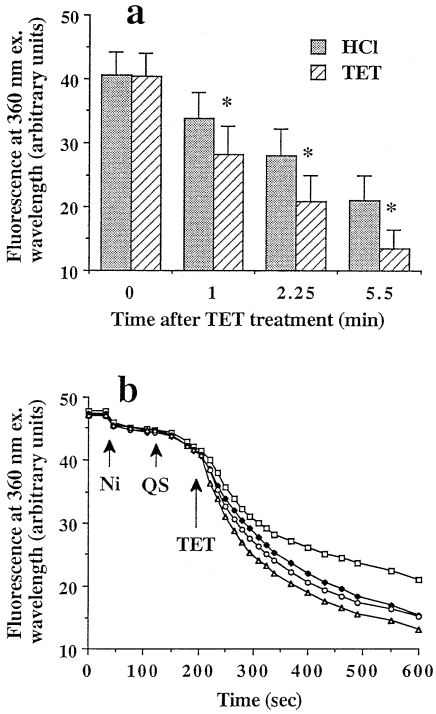

Fig. 5. Effects of QS and TET on Ni^{2+} entry (quantitative results of Fig. 4c,d). (a) The fluorescence changes after HCl/TET treatment (Fig. 4c,d) was quantified at several time points. The values at the zero time point represent the fluorescence just before HCl/TET treatment. Results are the mean $\pm$ S.E.M. of 7 separate experiments. * indicates statistical significance ($P < 0.01$). (b) Concentration-dependent effects of TET on QS-induced Ni^{2+} entry (using the protocol shown in Fig. 4d). Symbols used: square, solvent (HCl); diamond, circle, triangle represent 30, 60 and 100 μM TET, respectively. Results are the mean of 4 separate experiments; for clarity, S.E.M. are not shown.

of TET accelerated QS-induced quenching (Fig. 4d). The acceleration was immediate and at 1 min the QS-induced quenching was enhanced by 65% in the presence of TET (Fig. 5a). Enhancement by TET of the QS-induced quenching at earlier time points (1 and 2.25 min) (Fig. 5a) was most likely due to an enhanced Ni^{2+} entry but not to an increased fura-2 leak-out (since there was no fura-2 loss when cells were treated with QS (30 μg/ml) and TET for 2.5 min (Fig. 3D)). However, such treatment for 5 min resulted in a significant fura-2 loss (Fig. 3D′), which would in part contribute to the enhancement by TET of QS-induced quenching at later time points (e.g., 5.5 min) (Fig. 5a). Fig. 5b shows that TET (30–100 μM) concentration-dependently enhanced the QS-induced quenching.

QS of another product number (S7900) from Sigma, and hemolytic saponins (of unknown plant source) from E. Merck, were also tested. At 30 μg/ml, they both promoted Ni^{2+} entry, which was further accelerated by TET (results not shown but were similar to those shown in Fig. 4c,d). Saponins from ginseng (i.e., ginsenosides) have been shown to be non-hemolytic even at 200 μg/ml [25]. We observed that ginseng saponins, even at 100 μg/ml, did not promote Ni^{2+} entry; and subsequent addition of TET actually retarded the Ni^{2+} leak (not shown but similar to that shown in Fig. 4b).

Results consistent with those in Fig. 4 were observed if Ni^{2+} was added after treatment with QS or/and TET (Fig. 6). TET pretreatment inhibited Ni^{2+} entry, while QS accelerated it. With QS and TET pretreatment, Ni^{2+} addition resulted in a sudden and huge drop in fluorescence followed by a gradual quench. As treatment with both QS and TET for 5 min resulted in fura-2 leakage (Fig. 3), the quenching by Ni^{2+} of extracellular leaked fura-2, together with the influx of Ni^{2+} into the cytosol, could account for the initial rapid quench of fluorescence.

To test the ion specificity of the effect of QS and TET, the entry of Mn^{2+}, which also quenches the fura-2 fluorescence, was also studied under similar experimental conditions. Addition of Mn^{2+}, like Ni^{2+}, also caused a gradual fall in fluorescence, suggesting Mn^{2+} entered the cytosol spontaneously (Fig. 7a). The cells appeared to be much more permeable to Mn^{2+} than to Ni^{2+}, therefore a lower concentration of Mn^{2+} (0.2 mM) was used in order to have a quenching rate comparable to that of Ni^{2+}. TET

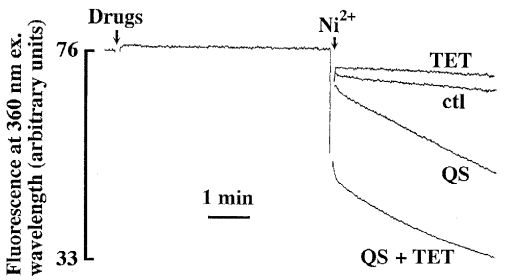

Fig. 6. Effects of QS and TET on Ni^{2+} entry. HL-60 cells in Ca^{2+}-free HBSS were treated with HCl (solvent control, ctl), 100 μM TET, 30 μg/ml QS or 100 μM TET plus 30 μg/ml QS before 1.2 mM Ni^{2+} addition. The *y*-axis represents fura-2 fluorescence at 360 nm excitation wavelength. Similar traces were obtained in 2 other separate experiments.

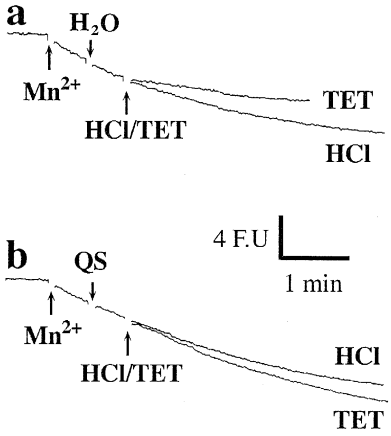

Fig. 7. Effects of QS and TET on Mn^{2+} entry. After exposure of HL-60 cells in Ca^{2+}-free HBSS to 0.2 mM Mn^{2+}, water (sham treatment) (a) or QS (30 μg/ml) (b) was added for around 0.5 min before further treatment with TET (100 μM) or HCl (solvent control). The vertical bar represents 4 fura-2 fluorescence units at 360 nm excitation wavelength. Similar traces were obtained in 2 other separate experiments.

strongly retarded the basal Mn^{2+} entry (Fig. 7a), as was observed previously [12]. Mn^{2+} entry was not accelerated upon the addition of QS (compare lower trace of Fig. 7a and upper trace of Fig. 7b). The Mn^{2+}-induced quenching rate in the presence of QS was not much affected by the addition of TET for 2 min, yet the difference appeared to become more conspicuous after 2.5 min (Fig. 7b). This is consistent with the observation that after 2.5 min, fura-2 leaked out (Fig. 3D′) and thus contributed to an enhanced quenching.

3.3. Effect of TET on membrane permeabilization to Ni^{2+} in the presence of other drugs which acted on the membrane or ion channels

Could TET enhance plasma membrane permeability to Ni^{2+} induced by perturbations other than QS? In the presence of Ni^{2+}, the permeabilization by non-specific detergent, Triton X-100 (0.01%) immediately caused quenching (Fig. 8). The concentration of Triton X-100 was selected such that the rate of fluorescence quench by the introduction of Ni^{2+} remained similar to that found in the experiments using 30 μg/ml QS. We did not check whether such permeabilization involved Ni^{2+} entry alone or Ni^{2+} entry plus fura-2 leak-out. We observed that Triton

X-100 at such low concentration did not cause cell lysis but resulted in cells completely stained with Trypan blue. Treatment with TET for 6.5 min did not significantly affect the Triton-induced permeabilization.

Addition of ionomycin (0.35 μM) accelerated Ni^{2+} entry; subsequent treatment with TET did not further increase the Ni^{2+} entry rate, but instead slightly retarded Ni^{2+} entry (data not shown). In a recent report, we showed that while SK&F 96365 (a newly developed non-specific Ca^{2+} channel blocker) behaved as a Ca^{2+} antagonist at low concentrations (3 μM causing 70% suppression of thapsigargin-activated Ca^{2+} entry in HL-60 cells), high concentrations of this drug (16–100 μM) actually promoted Ni^{2+} entry [21]. Here we did not observe any potentiating effect of TET on Ni^{2+} entry promoted by 20 μM SK&F 96365 (results not shown).

3.4. The effect of TET on the basal Ni^{2+} influx upon modification of the plasma membrane fluidity

Membrane fluidity has been known to be positively related to temperature [26]. At 37°C, Ni^{2+} entry was retarded by TET (Fig. 4a,b). TET also retarded Ni^{2+} entry at either a lower (27°C) or a higher (47°C) temperature (Fig. 9). Therefore TET did not enhance (indeed inhibited) Ni^{2+} entry, although the fluidity of the plasma membrane was modulated by changes of temperature, which affected the basal leak to Ni^{2+}.

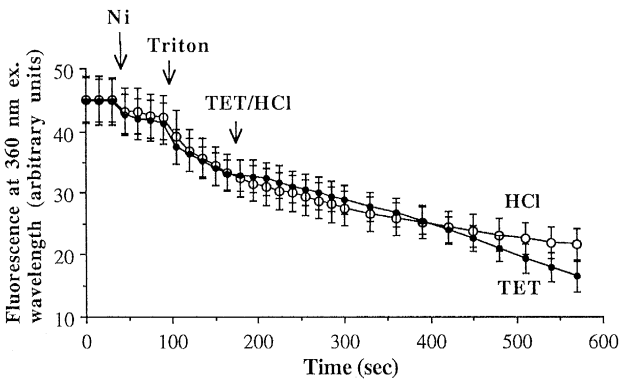

Fig. 8. TET did not affect Triton X-100-induced permeabilization. After exposure of HL-60 cells in Ca^{2+}-free HBSS to 1.2 mM Ni^{2+}, Triton X-100 (0.01%) was added before further treatment with TET (100 μM) or HCl (solvent control). The *y*-axis represents fura-2 fluorescence at 360 nm excitation wavelength. Results are the mean ± S.E.M. of 4 separate experiments.

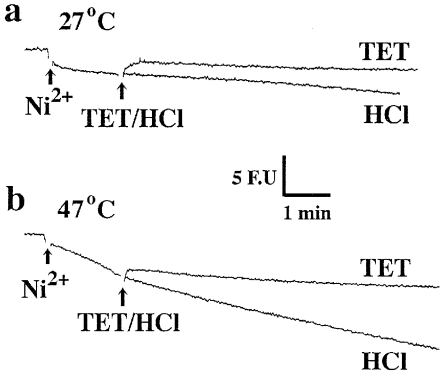

Fig. 9. Effect of TET on Ni^{2+} entry at different temperatures. HL-60 cells in Ca^{2+}-free HBSS were equilibrated at 27°C (a) or 47°C (b) for around 4 min. Subsequently the cells were exposed to 1.2 mM Ni^{2+} and further treated with HCl (solvent control) or 100 μM TET. The vertical bar represents 5 fura-2 fluorescence units at 360 nm excitation wavelength. Similar results were obtained in another experiment.

Furthermore, ethanol has been known to cause fluidization of the plasma membrane [27], and TET did not cause any acceleration of Ni^{2+} entry when the cells had been treated with 5% ethanol (results not shown).

3.5. Effects of TET on permeabilization induced by other cholesterol-complexing agents

It follows from the above results that TET could only enhance permeabilization induced by QS. We also tested whether TET could enhance permeabilization induced by other cholesterol-complexing agents, namely, filipin (a polyene antibiotic) and digitonin (a plant glycosidic compound) [20]. We were not able to use filipin as this agent on its own produced an intense fluorescence. Digitonin, at 1–3 μM, promoted Ni^{2+} entry at a rate similar to that promoted by 30 μg/ml QS, as shown in Fig. 4. However, digitonin-promoted Ni^{2+} entry was not further enhanced by 100 μM TET (not shown).

3.6. Effects of lipophilic Ca^{2+} channel blockers and TET analogues

Diltiazem and verapamil are lipophilic Ca^{2+} channel blockers structurally unrelated to TET. Here we did not observe any acceleration of QS-promoted Ni^{2+} entry by 100 μM of either agent (not shown).

HER and BER differ structurally from TET in a very minor manner (Fig. 1). These 2 compounds, like

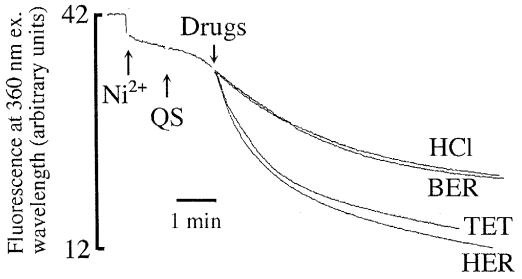

Fig. 10. BER did not enhance QS-induced Ni^{2+} entry. HL-60 cells in Ca^{2+}-free HBSS were exposed to 1.2 mM Ni^{2+} and 30 μg/ml QS before being challenged with HCl (solvent control), HER, TET or BER (all at 100 μM). The *y*-axis represents fura-2 fluorescence at 360 nm excitation wavelength. Similar traces were obtained in 2 other separate experiments.

TET, elicit Ca^{2+} antagonistic actions in a number of cell types [28–30], and they also block thapsigargin-activated Ca^{2+} entry in HL-60 cells [18]. Fig. 10 shows that TET and HER had a comparable potentiating effect on the QS action. However, the latter was not affected by BER.

4. Discussion

QS have been commonly used to selectively permeabilize the plasma membrane to probe into the intracellular events [23–25,31–33]. TET, a widely studied bis-benzylisoquinoline alkaloid, exerts multiple effects on plasmalemmal ion channels and receptors [5–15]. The major observation in this study that TET potentiated QS-induced increase in plasmalemmal permeability represents a novel finding, which is discussed below.

4.1. The permeability properties of pores formed by QS and TET

An exposure to 30 μg/ml QS for 5 min did not cause any fura-2 leakage in HL-60 cells; however, this treatment accelerated the entry of Ni^{2+} but not Mn^{2+} (Figs. 3, 4 and 7). It therefore appears that the pores formed by 30 μg/ml QS on the plasma membrane during the first 5 min were not large enough in size for fura-2 movement, but enough for Ni^{2+} to diffuse through; for Mn^{2+}, the pores were still poorly permeable. To the best of our knowledge, such a unique permeability property of the QS-formed pores to divalent cations has not been reported before. The sizes of QS-formed pores may well depend on the concentrations of QS used, as well as incubation time. Treatment of cells with 100 μg/ml QS for just 2.5 min could result in a substantial fura-2 leak-out (Fig. 3).

One novel action of TET reported here is the enhancement of QS-induced permeabilization. TET enhanced 30 μg/ml QS-promoted Ni^{2+} entry (without promoting fura-2 leak-out) at the first 2.5 min but thereafter they synergistically promoted fura-2 leakage (Figs. 3–5). It can therefore be envisaged that during the first 2.5 min TET enhanced the size of QS-formed pores to render Ni^{2+} much more permeant; thereafter, further incubation will develop to an extent that the pores were large enough to permit even fura-2 to diffuse out. If fura-2 leak-out had been rate-determining during the first 2.5 min, then one would expect similar fluorescence quench by added Ni^{2+} and Mn^{2+}. The augmentation by TET of QS-induced pore formation may well depend on the concentration of QS, as well as incubation time. Significant enhancement by TET of 100 μg/ml QS-promoted fura-2 leak-out began in the first 2.5 min, during which time 100 μg/ml QS itself already promoted a substantial fura-2 leakage (presumably due to a faster rate of pore formation) (Fig. 3). It therefore appears that enhancement by TET also depended on the rate of QS-induced pore formation.

4.2. Possible mechanism of the enhancement of QS action by TET

TET has been shown to inhibit L-, T- and N-type VOCC in several excitable tissues [6–9], and block non-voltage-gated Ca^{2+} channels in a number of non-excitable cells including HL-60 cells [12,13,18]. Whether TET is a selective Ca^{2+} antagonist remains controversial since it affects many types of Ca^{2+} channels and has modulatory effects on other targets such as Ca^{2+}-activated K^+ channels, Na^+,K^+-ATPases, α-adrenoceptors and protein kinase C [5,14,15,34]. QS are amphiphilic and thus act as surface-active agents on the plasma membrane [35,36]. Our observation that TET interfered with QS-induced permeabilization suggests that somehow

the effects of TET were strongly affiliated with the plasma membrane. The latter proposal is also consistent with the lipophilic nature of TET. The full recovery of KCl-induced vascular muscle contraction following TET inhibition required repeated wash-out over 2–3 h [10]. Further, TET has been shown to cause contraction in the venous smooth muscle via α-adrenoceptor activation even after wash-out [34]. Taken together, these findings suggest an association of TET with the plasma membrane itself, and therefore challenge the view that TET binds specifically to plasma membrane proteins, such as ion channels or pumps.

What is the action of TET on the plasma membrane? It is remarkable that TET could not accelerate permeability changes induced by other means of perturbing the plasma membrane. First, TET did not accelerate Ni^{2+} influx promoted by drugs that induce cation entry, such as ionomycin and SK&F 96365 (only at higher concentrations). Second, the failure of TET to potentiate Ni^{2+} entry upon ethanol treatment and temperature changes (factors known to modulate membrane fluidity) argues against the view that TET potentiated QS-induced Ni^{2+} entry via the modulation of membrane fluidity. Furthermore, permeabilization induced by Triton X-100 (a detergent that non-specifically dissociates hydrophobic interactions of phospholipids at the plasma membrane) was not affected by TET, suggesting that TET did not modify the interactions between Triton X-100 and the bulk plasmalemmal lipids. The above arguments seem to lead to the suggestion that TET enhanced a surface action unique to QS. Such an action might be aggregation of cholesterol, a well-known effect of QS which is believed to be the cause of QS-induced permeabilization [19,20]. The suggestion that TET enhanced QS-induced permeabilization by potentiating QS-triggered cholesterol aggregation is in concordance with our observation that ginseng saponins, being unable to aggregate cholesterol and induce permeabilization [25], still could not permeabilize cells even in the presence of TET (see the second part of Section 3). What may await future investigation to confirm the enhancement by TET of QS-triggered cholesterol aggregation is to use phospholipid- and cholesterol-containing artificial liposomes (instead of HL-60 cells), employing the protocols described in this report.

4.3. QS-TET interaction exhibits specificity

Both QS and digitonin are glycosidic compounds and have been well known to cause cholesterol aggregation [19,20]. However, TET could only enhance permeabilization induced by QS but not digitonin. It therefore appears that TET did not enhance cholesterol aggregation per se, but seemed to enhance cholesterol aggregation caused specifically by QS. Digitonin is a single glycoside while QS is a mixture of glycosidic compounds. Digitonin and QS differ remarkably in the lipophilic aglycon backbones and the amounts and types of sugar moieties [36,37]. Differences in the three-dimensional structures between the QS-cholesterol and digitonin-cholesterol complexes are to be expected. Indeed, freeze-fracture electron micrographs of *Xenopus* embryonic muscle cell membranes reveal different morphologies of QS-cholesterol complexes (irregular and rough appearance) and digitonin-cholesterol complexes (scalloped appearance) [20]. Our data therefore suggest a specificity in the molecular recognition between TET and QS-cholesterol complexes.

The specific nature of TET-QS interaction is also reflected by the inability of verapamil and diltiazem (lipophilic Ca^{2+} antagonists which are likely to partition into the plasma membrane lipid bilayer at high concentrations [38]) to mimic TET in enhancing QS-induced permeabilization. Furthermore, there is a stringent structural requirement – R_2 methoxyl group of TET (see Fig. 1 and Fig. 10) – for QS-TET interaction, suggesting that the interaction between the bulk lipophilic benzylisoquinoline framework of TET and QS was not sufficient to enhance QS action. It is interesting to note that the R_2 methoxyl group also appeared to be critical in many pharmacological actions of TET, such as immunosuppression [39–41] and Ca^{2+} entry blockade [18,42].

TET and QS together enhanced the entry of Ni^{2+} but not Mn^{2+}. This ion selectivity further lends support to the specific nature of the QS-TET interaction at the plasma membrane. Such ion selectivity is also reminiscent of the differential activation of cation entry by SK&F 96365 in HL-60 cells: at 30 μM, SK&F 96365 promoted the entry of Ni^{2+} but not Mn^{2+} [21]. Obviously, the subtle effects of TET and SK&F 96365 on plasmalemmal cationic fluxes warrant further investigation.

4.4. Therapeutic potential and experimental uses of QS and TET

In summary, our data indicate that TET on its own blocked divalent cation entry, but in the presence of QS, TET became a potentiator of QS-induced permeabilization. This may exemplify the complications in using compounds from natural sources due to drug interactions. There are a number of therapeutic potential and experimental uses of QS and TET. For example, QS, and its more refined preparation, Quil A, have been shown to be immunopotentiating [35,43] and provide potent immune adjuvanticity for viral antigens in animal models [36,44–46]. TET has been demonstrated to be an effective hypotensive and antisilicotic drug in animal experiments and clinical studies [1–4]. With respect to the employment of QS and TET as experimental tools, QS are extensively used in permeabilization studies, and there is an increasing interest in using TET as a pharmacological probe for various ion channels [5–13]. In view of such a wide spectrum of usages, the QS-TET drug interaction should be taken into consideration in future applications of these compounds.

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