Dual effects of tetrandrine on cytosolic calcium in human leukaemic HL-60 cells: intracellular calcium release and calcium entry blockade

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1 Tetrandrine (TET, a Ca^{2+} antagonist of Chinese herbal origin) and thapsigargin (TSG, an endoplasmic reticulum Ca^{2+} pump inhibitor) concentration-dependently mobilized Ca^{2+} from intracellular stores of HL-60 cells, with EC_{50} values of 20 μM and 0.8 nM, respectively. After intracellular Ca^{2+} release by 30 nM TSG, there was no more discharge of Ca^{2+} by TET (100 μM), and *vice versa*.

2 Pretreatments with 100 nM rauwolscline (α_2 -adrenoceptor antagonist), 100 nM prazosin (α_1 -adrenoceptor antagonist), 10 nM phorbol myristate acetate (PMA, a protein kinase C activator) or 100 nM staurosporine (a protein kinase C inhibitor) had no effect on 100 μM TET-induced intracellular Ca^{2+} release.

3 After intracellular Ca^{2+} release by 30 nM TSG in Ca^{2+} -free medium, readmission of Ca^{2+} caused a substantial and sustained extracellular Ca^{2+} entry. The latter was almost completely inhibited by 100 μM TET (IC_{50} of 20 μM) added just before Ca^{2+} readmission. In Ca^{2+} -containing medium, 30 nM TSG caused a sustained phase of cytosolic Ca^{2+} elevation, which could be abolished by 100 μM TET. TET was also demonstrated to retard basal entry of extracellular Mn^{2+} and completely inhibit TSG-stimulated extracellular Mn^{2+} entry.

4 TSG-induced extracellular Ca^{2+} entry was insensitive to the L-type Ca^{2+} channel blocker, nifedipine (1 μM), but was completely inhibited by the non-selective Ca^{2+} channel blocker La^{3+} (300 μM). Depolarization with 100 mM KCl did not raise the cytosolic Ca^{2+} level.

5 These data suggest that (a) TET and TSG mobilized the same Ca^{2+} pool and TET-induced intracellular Ca^{2+} release was independent of protein kinase C activity and α -adrenoceptor activation, and (b) TET blocked the voltage-insensitive Ca^{2+} entry pathway activated by TSG. These dual effects on HL-60 cells were also observed with hernandezine (HER), a TET-like compound and in another cell type, murine B lymphoma M12.4 cells.

Keywords: Tetrandrine; thapsigargin; hernandezine; calcium antagonist; cytosolic calcium; calcium release; human leukaemia; HL-60 cells

Introduction

Tetrandrine (TET), a *bis*-benzylisoquinoline alkaloid isolated from the Chinese medicinal herb *Stephania tetrandra*, has various pharmacological actions. It has been demonstrated clinically (Gao *et al.*, 1965) and in experimental animals (Qian *et al.*, 1983) to have a hypotensive effect, which in part could be due to its Ca^{2+} antagonistic properties (Jia *et al.*, 1985; Fang & Jiang, 1986). It has also been shown to cause suppression of fibroblast proliferation (Reist *et al.*, 1993) and collagen synthesis (Liu *et al.*, 1983), which might account for its anti-silicotic effect (Li *et al.*, 1981; Yu *et al.*, 1983). The anti-inflammatory effect of TET is most likely attributable to its inhibitory effects on immune cells such as B cells, mast cells, monocytes and neutrophils (Seow *et al.*, 1986; 1988; Teh *et al.*, 1988; Ferrante *et al.*, 1990). Not surprisingly, its various pharmacological actions are paralleled by its multiple modulatory effects at the molecular or signal transduction level. For instance, its Ca^{2+} antagonistic properties have been related to its inhibition of L-type and T-type Ca^{2+} channels (King *et al.*, 1988; Liu *et al.*, 1991; 1992; Rossier *et al.*, 1993), and receptor-mediated Ca^{2+} entry (Kwan *et al.*, 1992). In human lymphocytes, TET caused not only attenuation of extracellular Ca^{2+} entry, but also reduction of inositol trisphosphate production (Ioannoni *et al.*, 1989). There has been evidence that TET might bypass the plasma membrane receptor-effector coupling and act directly on intracellular messengers. For example, in human lymphocytes, protein

kinase C (PK-C) activity stimulated with phorbol myristate acetate (PMA), a direct activator of PK-C, was inhibited by TET, suggesting that TET could possibly exert a direct inhibition on this intracellular signalling molecule (Ioannoni *et al.*, 1989).

Recently, Kwan & Wang (1993) observed that TET relaxed KCl-induced contraction in canine saphenous and mesenteric veins, but after washout, a transient contraction unexpectedly followed. This contraction upon washout of KCl was not observed with nifedipine or sodium nitropruside as the relaxants. In rat alveolar type II cells, TET and chemically related compounds were shown to raise cytosolic free calcium concentration ($[\text{Ca}^{2+}]_i$) slightly (Miles *et al.*, 1993). Both contraction and $[\text{Ca}^{2+}]_i$ elevation induced by TET were observed in Ca^{2+} -containing medium, and therefore, whether the contraction and $[\text{Ca}^{2+}]_i$ elevation were due to interacellular Ca^{2+} release or extracellular Ca^{2+} entry is unknown. Nevertheless, these two novel phenomena cast serious doubt on TET being a selective and effective Ca^{2+} antagonist. In this work we investigated the effects of TET on $[\text{Ca}^{2+}]_i$ homeostasis of human leukaemic HL-60 cells. We describe here two novel effects of TET, namely, blockade of extracellular Ca^{2+} entry induced by thapsigargin (TSG, a selective endoplasmic reticulum Ca^{2+} pump inhibitor (Thastrup *et al.*, 1990; Lytton *et al.*, 1991)) and stimulation of Ca^{2+} release from intracellular stores. Entry of extracellular Ca^{2+} and release of Ca^{2+} from intracellular stores into the cytosol will hereafter be, for the sake of simplicity, referred to as Ca^{2+} entry and Ca^{2+} release, respectively. We have characterized the pharmacological properties of the Ca^{2+} entry

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pathway that TET blocked and partially delineated the mechanism of TET-induced Ca^{2+} release. We have also shown that hernandezine (HER), a chemically related compound of TET, behaved very similarly to TET in HL-60 cells.

Methods

Cell culture

HL-60 cells and M12.4 cells obtained from American Type Culture Collection, Maryland, U.S.A., were maintained in RPMI 1640 medium (Sigma), supplemented with 10% foetal calf serum, 5 μg gentamycin ml^{-1} and penicillin/streptomycin (100 u ml^{-1} , 100 $\mu\text{g ml}^{-1}$) 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.

$[\text{Ca}^{2+}]_i$ measurement

The method of measuring $[\text{Ca}^{2+}]_i$ was modified from the protocol described by Lambert & Nahorski (1990). Hank's buffered saline solution (HBSS), composed of (mM): NaCl 138, KCl 5.3, MgSO_4 0.88, 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 sulphonic acid) 25 buffered at pH 7.4, was used for cell washing. HL-60 cells or M12.4 cells grown to a density of around $1.5 \times 10^6 \text{ ml}^{-1}$ were harvested, washed once in Ca^{2+} -containing HBSS and resuspended in serum-free RPMI 1640 medium at a cell density of about $2 \times 10^7 \text{ ml}^{-1}$. 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 two times in Ca^{2+} -containing HBSS and resuspended in the same buffer solution at a cell density of $2.8 \times 10^6 \text{ ml}^{-1}$. Before experimentation on each sample, 1 ml of this cell suspension was centrifuged and the cell pellet was resuspended in 2 ml Ca^{2+} -free or Ca^{2+} -containing HBSS. Ca^{2+} -free HBSS was used when Ca^{2+} release from intracellular stores was to be detected. Ca^{2+} -containing HBSS was used when thapsigargin was to induce a sustained Ca^{2+} entry. This 2-ml cell suspension was then transferred to a quartz cuvette which contained a mini stirrer, and warmed at 37°C for about 3 min before addition of agonists or drugs. Fura 2 fluorescence was detected with a Hitachi F-4000 fluorescence spectrophotometer. Excitation and emission wavelengths were set at 340 nm and 500 nm respectively. For calibration, F_{max} was obtained by lysing the cells with 0.1% Triton X-100 and F_{min} by chelating Ca^{2+} with 20 mM EGTA. $[\text{Ca}^{2+}]_i$ was calculated according to Grynkiewicz *et al.* (1985). The basal $[\text{Ca}^{2+}]_i$ ranged from 70–110 nM.

In experiments under Ca^{2+} -free conditions, about 30 s before agonist or drug stimulation, a small amount of EGTA was added to the cell suspension to chelate contaminating Ca^{2+} to maintain a stable baseline which was important to help interpreting whether the Ca^{2+} release was transient or exhibiting a plateau phase. The amount of EGTA (22.5–100 μM) added to each sample in a given experiment was the same, but might vary in separate experiments, depending on the level of contaminating Ca^{2+} .

In experiments when La^{3+} was used to block Ca^{2+} entry, phosphate salts were omitted from the HBSS and the concentration of HEPES was reduced to 8 mM in order to avoid precipitation upon La^{3+} addition. Also, 1% foetal calf serum was included in the reaction medium to avoid La^{3+} -induced cell aggregation.

Mn^{2+} entry experiments

At 360 nm excitation wavelength, fura 2 fluorescence is insensitive to Ca^{2+} but quenchable by Mn^{2+} . Therefore, at this

wavelength, a decrease in fura 2 fluorescence upon addition of Mn^{2+} indicated Mn^{2+} entry from the extracellular space into the cytosol (hereafter simply referred to as Mn^{2+} entry), and this protocol has been used as a means to detect divalent cation entry, which is suggestive of Ca^{2+} channel opening (Merritt *et al.*, 1989). In this work, cell suspension in Ca^{2+} -free medium (without EGTA supplementation) was prepared as described above, and after a 3-min warming at 37°C, 0.5 mM Mn^{2+} was added for 1–1.5 min before the cells were exposed to pharmacological agents. Excitation and emission wavelengths were set at 360 nm and 500 nm respectively.

Materials

ATP, nifedipine, prazosin, rauwolscline, thapsigargin, phorbol myristate acetate, and fura 2-AM were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. CPA and staurosporine were purchased from Calbiochem, Richmond, CA, U.S.A. Foetal calf serum was purchased from Gibco BRL (Gaithersburg, MD, U.S.A.). TET (purity > 98%) from Jing-Hua Pharmaceutical Company (Zhejiang, China) and Aldrich (Milwaukee, WI, U.S.A.) were tested in preliminary experiments and both exhibited the dual effects of intracellular Ca^{2+} release and blockade of Ca^{2+} entry. Subsequently in all the experiments shown in this work, TET from the Jing-Hua Pharmaceutical Company was employed. 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.

Statistical analysis

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

Results

Relationship between the TET- and TSG-releasable Ca^{2+} pool

Both TET and TSG concentration-dependently released intracellular Ca^{2+} of HL-60 cells (Figure 1a,b). It is noteworthy that there was a plateau phase of the TET-induced Ca^{2+} release, which was distinct from the transient TSG-induced Ca^{2+} release (see the Discussion section). The concentration-response curves are shown in Figure 1c. TSG was more efficacious than TET ($[\text{Ca}^{2+}]_i$ elevation of 110 nM vs 49 nM). Furthermore, TSG was much more potent than TET (EC_{50} value of 0.8 nM vs 20 μM).

We then investigated the relationship between the TET- and TSG-sensitive intracellular Ca^{2+} pool(s). It can be demonstrated that during the time course of the TET-induced Ca^{2+} release, the Ca^{2+} release by 30 nM TSG was progressively inhibited so that after around 20 min, TSG did not cause further Ca^{2+} release (Figure 2a–d). Similarly, TET could release no more Ca^{2+} after the cells had been challenged with 30 nM TSG (Figure 2e). A prolonged treatment with a suboptimal concentration (0.6 nM) of TSG also substantially inhibited TET-induced Ca^{2+} release (Figure 2f). The Ca^{2+} pool dischargeable by cyclopiazonic acid (CPA), another microsomal Ca^{2+} pump inhibitor chemically unrelated to TSG (Mason *et al.*, 1991; Kwan & Deng, 1991), exactly overlapped with the TSG-sensitive pool (Leung *et al.*, 1994). TET could release only a very small amount of Ca^{2+} after prolonged treatment with a suboptimal concentration (3 μM) of CPA (Figure 2g). Taken together, the results suggest the the TET-sensitive Ca^{2+} pool exactly overlapped with the TSG/CPA-dischargeable Ca^{2+} pool.

Figure 1 Ca^{2+} release elicited by TET and TSG. HL-60 cells in EGTA-supplemented Ca^{2+} -free HBSS were challenged with (a) various concentrations of TET (3, 10, 30 and 100 μM ; from bottom trace to top), or (b) various concentrations of TSG (0.23, 0.6 and 30 nM; from bottom trace to top). Similar traces were obtained in at least 2 other separate experiments. Concentration-response curves of TSG ($\square$) and TET ($\circ$, peak; $\bullet$, plateau phase) are shown in (c). Each point is the mean $\pm$ s.e. of 3–4 separate experiments. For abbreviations in this and subsequent legends, see text.

Roles of PK-C and α -adrenoceptors in TET-induced Ca^{2+} release?

There have been reports that suggest TET might affect PK-C activity (Ioannoni *et al.*, 1989; Kwan & Wang, 1993). We therefore tested whether TET released intracellular Ca^{2+} by activating or inhibiting PK-C. A brief treatment (3 min) with a PK-C activator, phorbol myristate acetate (PMA) (3 nM) caused a substantial ($85 \pm 6.7\%$; $n = 4$; $P < 0.05$) inhibition of ATP-induced Ca^{2+} release (Figure 3a,b). This inhibition could be readily reversed by a 5-min exposure to a PK-C inhibitor staurosporine (100 nM) before PMA treatment (Figure 3c). This indicates that staurosporine could significantly inhibit PK-C activity. Staurosporine did not qualitatively or quantitatively affect TET-induced Ca^{2+} release (Figure 3d,e). PMA (10 nM) caused almost complete ($97.7 \pm 0.3\%$; $n = 3$; $P < 0.05$) inhibition of ATP-induced Ca^{2+} release, but did not significantly suppress TET-induced Ca^{2+} release ($87.5 \pm 7.6\%$ of the control value; $n = 3$; $P > 0.05$). These results suggest that TET-induced Ca^{2+} release was independent of PK-C activity. There has been evidence that TET could activate α_2 -adrenoceptors in canine saphenous and mesenteric vein smooth muscles (Kwan & Wang, 1993). However, we can rule out the possible involvement of α_2 -adrenoceptors in TET-induced Ca^{2+} release, since rauwolscine, an α_2 -adrenoceptor antagonist, did not affect TET-induced Ca^{2+} release ($95.9 \pm 10.9\%$ of the control value; $n = 3$; $P > 0.05$). Prazosin, an α_1 -adrenoceptor antagonist also failed to affect TET-induced Ca^{2+} release ($102.1 \pm 7.1\%$ of the control value; $n = 3$; $P > 0.05$).

TSG-induced Ca^{2+} entry could be blocked by TET, La^{3+} but not nifedipine

In a number of cells, depletion of intracellular Ca^{2+} stores stimulates Ca^{2+} entry into the cytosol (for reviews see Putney 1986; 1990). This 'capacitative Ca^{2+} entry' (CCE) also operates in HL-60 cells (Demaurex *et al.*, 1992; Alonso-Torre *et al.*, 1993). Emptying of intracellular Ca^{2+} stores by suboptimal concentrations of TSG or CPA induced a sustained Ca^{2+} entry upon Ca^{2+} readmission (Figure 4a,b). However, as shown in Figure 4c, TET did not induce any CCE (upper

Figure 2 Relations of the TET-, TSG- and CPA-releasable pools. HL-60 cells in EGTA-supplemented Ca^{2+} -free HBSS were challenged with 100 μM TET and at different time points during the TET-induced Ca^{2+} release, 30 nM TSG was added (a–d). The lower trace in (d) was the baseline of the control (without any treatment with pharmacological agents). After Ca^{2+} release by 30 nM TSG (e), 0.6 nM TSG (f) or 3 μM CPA (g), 100 μM TET was added. Similar traces were obtained in 2 other separate experiments.

Figure 3 Effects of PMA and staurosporine (STA) on ATP- and TET-induced Ca^{2+} release. HL-60 cells in EGTA-supplemented Ca^{2+} -free HBSS were stimulated with $10 \mu M$ ATP (a); in (b), $10 \mu M$ ATP stimulation was preceded by $3 nM$ PMA treatment; and in (c), the inhibitory effect of PMA on ATP stimulation was reversed by $100 nM$ STA. $100 nM$ STA pretreatment did not affect TET-induced Ca^{2+} release (d and e). Similar traces were obtained in 2 other separate experiments.

Figure 4 TET did not induce capacitative Ca^{2+} entry. HL-60 cells in EGTA-supplemented Ca^{2+} -free HBSS were treated with $0.6 nM$ TSG (a), $3 \mu M$ CPA (b), or $100 \mu M$ TET (c, upper trace) for approximately 12 min before readmission of $1.2 mM$ Ca^{2+} . In (c, lower trace), when $1.2 mM$ Ca^{2+} was readmitted to the control sample which had not been treated with any pharmacological agent, there was an increase in fluorescence. Similar traces were obtained in 2 other separate experiments.

trace): after TET-induced Ca^{2+} release, the increase in fluorescence upon Ca^{2+} readmission was unlikely to be Ca^{2+} entry, and was most possibly due to the saturation of leaked fura-2 with extracellular Ca^{2+} because such increase in fluorescence upon Ca^{2+} readmission was also observed even when there had not been any pretreatment (lower trace). The absence of CCE after TET-induced Ca^{2+} release suggests that TET might have blocked the CCE itself induced.

Results in Figure 5 show that TET blocked CCE induced by TSG. After Ca^{2+} release by TSG, addition of Ca^{2+} caused substantial and sustained Ca^{2+} entry, which could be abolished by TET pretreatment before Ca^{2+} readmission (Figure 5a,b). The fluorescence increase upon Ca^{2+} readmission in Figure 5a, as just explained, was most likely due to saturation of leaked fura-2 with Ca^{2+} (see Figure 5c). Using the protocol shown in Figure 5a, we obtained the concentration-inhibition curve for TET and estimated the IC_{50} of TET to be around $20 \mu M$ (Figure 6). That TET was able to inhibit CCE induced by TSG could also be observed if TET was added together with TSG (Figure 5d). In Ca^{2+} -containing medium, TSG caused a tonic elevation of $[Ca^{2+}]_i$ (Figure 5e). This tonic phase, presumably due to sustained Ca^{2+} entry, was abolished by TET. The biphasic $[Ca^{2+}]_i$ elevation induced by TSG in Ca^{2+} -containing medium was converted into a monophasic one if TET was added together with TSG (Figure 5f). The ability of TET to block TSG-induced CCE could also be demonstrated using the Mn^{2+} entry protocol (Figure 7). Addition of Mn^{2+} caused a gradual quench of fluorescence, which reflected basal Mn^{2+} entry. Treatment with TSG accelerated Mn^{2+} entry. TET substantially reduced basal Mn^{2+} entry and inhibited subsequent TSG-accelerated Mn^{2+} entry. Basal Mn^{2+} entry was also substantially reduced if TET was added before Mn^{2+} (not shown).

The Ca^{2+} entry pathway activated by TSG-induced Ca^{2+} pool emptying, which have just been shown to be TET-sensitive, were pharmacologically characterized. The tonic phase of $[Ca^{2+}]_i$ elevation induced by TSG in Ca^{2+} -containing medium was not affected by the selective L-type Ca^{2+} channel antagonist nifedipine (NIF), but was abolished

Figure 5 Blockade of TSG-induced Ca^{2+} entry by TET. Concentrations of TSG, TET, and Ca^{2+} used were 30 nM, 100 μM and 1.2 mM respectively. HL-60 cells in EGTA-supplemented Ca^{2+} -free HBSS were challenged with (a) TSG and then TET, (b) TSG and then vehicle (Veh; 6.7 μl 0.1 N HCl), or (d) TET and TSG simultaneously, before Ca^{2+} readmission. In (c), when Ca^{2+} was readmitted to the control sample which had not been treated with any pharmacological agent, there was an increase in fluorescence. Cells in Ca^{2+} -containing HBSS were exposed to TSG (e) or a combination of TSG and TET (f). In the former case, TET was added during the TSG-induced sustained $[\text{Ca}^{2+}]_i$ elevation. Similar traces were obtained in 2 other separate experiments.

Figure 6 Concentration-dependent inhibitory effect of TET on TSG-induced Ca^{2+} entry. Using the protocol shown in Figure 5(a), various concentrations of TET were added after 30 nM TSG-induced Ca^{2+} release and before 1.2 mM Ca^{2+} readmission. The Ca^{2+} entry after TET treatment is expressed as a percentage of that of the control, in which only the vehicle (6.7 μl 0.1 N HCl) was added. Each point is the mean $\pm$ s.e. of 3 separate experiments.

by the non-selective Ca^{2+} channel blocker La^{3+} (Figure 8a). After TSG-induced Ca^{2+} release in Ca^{2+} -free medium, Ca^{2+} entry upon readmission of Ca^{2+} was not affected by prior treatment with NIF, but was abolished by La^{3+} (Figure 8b). Note that addition of La^{3+} after TSG-induced Ca^{2+} release in Ca^{2+} -free medium caused a fluorescence increase. This was

Figure 7 TET inhibited basal and TSG-stimulated Mn^{2+} entry. Addition of 0.5 mM Mn^{2+} to HL-60 cells in Ca^{2+} -free HBSS (without EGTA supplementation) caused a gradual quenching of fura-2 fluorescence at 360 nm excitation wavelength. Subsequently, cells were treated with vehicle (6.7 μl 0.1 N HCl) and then 30 nM TSG (lower trace), or 100 μM TET and then 30 nM TSG (upper trace). Similar traces were obtained in 2 other separate experiments.

most likely due to saturation of leaked fura-2 with La^{3+} , which has a fluorescence excitation spectrum qualitatively and quantitatively very similar to that of Ca^{2+} (Kwan & Putney, 1990).

Effects of TET and HER in HL-60 cells and M12.4 cells

The effects of hernandezine (HER), a compound chemically closely related to TET, on $[\text{Ca}^{2+}]_i$ homeostasis of HL-60 cells were examined. This compound, like TET, also induced a tonic phase of Ca^{2+} release and abolished the sustained TSG-induced $[\text{Ca}^{2+}]_i$ elevation in Ca^{2+} -containing medium (Figure 9a,b). Both TET and HER were able to exercise the dual effects of Ca^{2+} release and blockade of Ca^{2+} entry in another cell system (murine B lymphoma M12.4 cells) (Figure 9c-f). However, it is noteworthy that Ca^{2+} release induced by both compounds was less sustained and the

Figure 8 The TSG-induced Ca^{2+} entry pathway in HL-60 cells was inhibited by La^{3+} but insensitive to nifedipine (NIF). In (a), the TSG-induced sustained $[Ca^{2+}]_i$ elevation in Ca^{2+} -containing HBSS was insensitive to $1 \mu M$ NIF but abolished by $300 \mu M$ La^{3+} . In (b), cells in EGTA-supplemented Ca^{2+} -free HBSS were stimulated with $30 nM$ TSG. Subsequently, either $1 \mu M$ NIF or $300 \mu M$ La^{3+} was added before $1.2 mM$ Ca^{2+} readmission. The pattern and magnitude of Ca^{2+} entry in the control (no drug treatment) or after nifedipine treatment were nearly identical. Similar traces were obtained in at least 2 other separate experiments.

Figure 9 The dual effects of HER in HL-60 cells and those of HER and TET in M12.4 cells. In (a), HL-60 cells in EGTA-supplemented Ca^{2+} -free HBSS were challenged with $100 \mu M$ HER. In (c) and (e), M12.4 cells in EGTA-supplemented Ca^{2+} -free HBSS were challenged with $100 \mu M$ TET and $100 \mu M$ HER, respectively. In (b), where HL-60 cells were suspended in Ca^{2+} -containing HBSS, $100 \mu M$ HER was added during the $30 nM$ TSG-induced sustained $[Ca^{2+}]_i$ elevation. In (d) and (f), where M12.4 cells were suspended in Ca^{2+} -containing HBSS, $100 \mu M$ TET and $100 \mu M$ HER, respectively, were added during the $3 nM$ TSG-induced sustained $[Ca^{2+}]_i$ elevation. Similar traces were obtained in 2 other separate experiments.

blockade of Ca^{2+} entry by both compounds was only partial in M12.4 cells compared to those in HL-60 cells.

Discussion

Our work describes two novel pharmacological actions of TET, namely, mobilization of intracellular Ca^{2+} and blockade of capacitative Ca^{2+} entry induced by TSG. It is unlikely that TET released intracellular Ca^{2+} by opening the ryanodine-sensitive channel (Ca^{2+} -induced Ca^{2+} release channel) since ryanodine (0.1–10 μM) and caffeine (10 mM) did not cause any Ca^{2+} release in HL-60 cells (results not shown). It also does not seem that TET induced Ca^{2+} release by activating the IP_3 receptor-channel because of the following reasons: (1) IP_3 -induced Ca^{2+} release (see, for example, the ATP-induced Ca^{2+} release shown in Figure 3) is characteristically very fast (peaking within seconds) and transient ($[\text{Ca}^{2+}]_i$ returning to basal level within 1–2 min). But the TET-induced Ca^{2+} release was relatively slow and long-lasting. (2) The IP_3 -releasable pool was only a very minor fraction of the TSG-sensitive pool (Leung *et al.*, 1994). It is demonstrated here that the TSG-sensitive pool could be discharged fully by TET. The latter phenomenon, together with the almost identical initial kinetics of Ca^{2+} release by TET, TSG (0.6 nM), and CPA (3 μM), suggest that TET might cause Ca^{2+} release by inhibiting the microsomal Ca^{2+} pump. If it is the case, the lower efficacy of TET than TSG suggests that TET could not fully inhibit the microsomal Ca^{2+} pump.

It is obvious that the TET- and TSG-dischargeable Ca^{2+} pool exactly overlapped. However, it is difficult to explain why there was a sustained phase of TET-induced Ca^{2+} release in HL-60 cells (but not in M12.4 cells), a phenomenon in contrast to the transient TSG-induced Ca^{2+} release, regardless of the concentration of TSG used. Given sufficient time, a suboptimal concentration (0.6 nM) of TSG could almost completely empty the TSG-sensitive pool: addition of TET afterwards did not release much Ca^{2+} . This indicates that most of the Ca^{2+} released was not recycled back into the pool, but was rather removed into the extracellular space (Ca^{2+} efflux). Given enough time, TET was also able to empty totally the TSG-sensitive pool, again implying that released Ca^{2+} was not recycled back into the pool. But in this case, $[\text{Ca}^{2+}]_i$ remained well above basal level, hinting that, to a certain extent, Ca^{2+} efflux was prevented. Therefore, TET might also inhibit Ca^{2+} efflux mechanism(s). If it is true that TET also inhibited Ca^{2+} efflux in HL-60 cells, it is puzzling why adding TET together with TSG (30 nM) did not produce a sustained phase of Ca^{2+} release. Also, it is interesting to note that after exposure to TET, the earlier TSG was added, the closer the $[\text{Ca}^{2+}]_i$ returned to basal level. We have not studied these phenomena further, and the only explanation we could offer is that perhaps TET does have a moderate inhibitory effect on Ca^{2+} efflux in HL-60 cells, but the high $[\text{Ca}^{2+}]_i$ raised by 30 nM TSG strongly activated Ca^{2+} efflux and dominated the inhibitory effect of TET.

Recently, it was shown that TET relaxed KCl-induced contraction in canine saphenous and mesenteric veins, and surprisingly, a transient contraction developed upon washout of KCl (Kwan & Wang, 1993). Such TET-induced contraction is believed to be mediated via α_2 -adrenoceptor activation since rauwolfscine (an α_2 -antagonist), but not prazosin (an α_1 -antagonist), abolished such contraction. However, our data do not suggest any involvement of α_2 -adrenoceptor in TET-induced Ca^{2+} release. Data from Kwan & Wang (1993) also suggest that TET-induced contraction may result from

the activation of the Na^+/H^+ exchanger, which could be a substrate of PK-C. It has been shown that TET could inhibit the activity of PK-C in human lymphocytes (Ioannoni *et al.*, 1989). Since we observed that the PK-C activator PMA and the PK-C inhibitor, staurosporine, did not have any significant effect on TET-induced Ca^{2+} release, we conclude that the latter is independent of PK-C activity.

That TET caused Ca^{2+} release, but did not induce any capacitative Ca^{2+} entry due to the emptying of the Ca^{2+} store, is not unexpected because TET exerts non-selective Ca^{2+} antagonistic actions: it blocks not only voltage-operated Ca^{2+} channels (King *et al.*, 1988; Liu *et al.*, 1991; 1992; Rossier *et al.*, 1993), but also voltage-insensitive Ca^{2+} entry (Kwan *et al.*, 1992). We show here in HL-60 cells that TET blocked the TSG-induced Ca^{2+} entry, which was via a La^{3+} -sensitive but nifedipine-insensitive Ca^{2+} -permeable pathway. We have also observed that there was no $[\text{Ca}^{2+}]_i$ elevation in response to high KCl-induced depolarization in Ca^{2+} -containing medium (not shown), indicating the lack of functional voltage-operated Ca^{2+} channels in HL-60 cells. Taken together, these data further confirm that TET is a non-selective Ca^{2+} antagonist.

Hernandezine (HER), a compound chemically very close to TET, and reportedly eliciting Ca^{2+} antagonistic effect in platelets (Ma *et al.*, 1991), also behaved similarly to TET in HL-60 cells. Furthermore, both compounds also blocked Ca^{2+} entry and caused Ca^{2+} release in M12.4 cells. Thus, the dual effects of TET and HER do not seem to exhibit cell-specificity. We are unable to tell whether TET-induced contraction in canine saphenous and mesenteric veins (Kwan & Wang, 1993) and slight $[\text{Ca}^{2+}]_i$ elevation in rat alveolar type II cells (Miles *et al.*, 1993) were due to internal Ca^{2+} release or not. It is because these phenomena were observed in Ca^{2+} -containing medium, therefore, the source of $[\text{Ca}^{2+}]_i$ elevation could be extracellular. But certainly TET does not cause Ca^{2+} release in all kinds of cells, for example, it did not release Ca^{2+} from intracellular stores of rat neutrophils (Hong *et al.*, 1989) and bovine adrenal glomerulosa cells (Rossier *et al.*, 1993).

Although TET, as a hypotensive agent, is inferior to the more selective and potent drugs like nifedipine and verapamil, it has been shown to be a clinically effective drug in the treatment of silicosis (Li *et al.*, 1981), a disease that hitherto has not been successfully under control by any conventional immunosuppressive drugs. Undoubtedly, the molecular mechanism(s) by which TET acts on fibroblasts, the key cells in the formation of silicosis, warrants future investigation. With respect to the basic pharmacological research, it has already been demonstrated that TET could block not only L- and T-type Ca^{2+} channels (King *et al.*, 1988; Liu *et al.*, 1991; 1992; Rossier *et al.*, 1993), but also receptor-mediated Ca^{2+} entry (Kwan *et al.*, 1992). Here we show that TET also (1) released intracellular Ca^{2+} , probably by inhibiting microsomal Ca^{2+} pumps, and (2) blocked voltage-insensitive Ca^{2+} entry activated by TSG. Therefore, although TET itself may have limitations as a pharmacological tool for investigation of $[\text{Ca}^{2+}]_i$ homeostatic mechanisms, it could well be a promising lead compound for the synthesis of analogues that may serve as selective pharmacological probes for voltage-insensitive receptor-mediated Ca^{2+} entry or microsomal Ca^{2+} pumps.

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