Stimulation of Sarcoplasmic Reticulum Ca^{2+}-ATPase by Gingerol Analogues

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We have reported previously that [8]-gingerol increased Ca^{2+}-ATPase activity.[1,2] Here we synthesized gingerol related compounds (AP-004, AP-005 and AP-015) and investigated the effects of gingerols ([6]-gingerol, [8]-gingerol and [10]-gingerol) and the synthesized compounds on the Ca^{2+}-ATPase activity of sarcoplasmic reticulum (SR). The Ca^{2+}-ATPase activity and the Ca^{2+}-pumping activity increased by these compounds in a concentration-dependent manner. It is probable that both the *o*-methoxyphenol and hydrocarbon chain in the molecule of gingerol analogues are necessary for the activation of the Ca^{2+}-pumping ATPase activity of SR.

Key words gingerol; Ca^{2+}-pumping ATPase; sarcoplasmic reticulum

In recent years there have been numerous efforts to find and develop novel cardiotonic agents that are more selective than either cardiac glycosides or catecholamines. These studies have produced several such agents, including amrinone, sulmazol, MDL 17043, BAY K 8644, isomazole and [8]-gingerol. The rhizome of ginger (*Zingiber officinale* ROSCOE) has been used not only as a seasoning spice but also as a traditional medicine. Gingerols ([6]-gingerol, [8]-gingerol and [10]-gingerol), which have been identified as major constituents of the rhizome of ginger,[3] have been shown to have various pharmacological effects such as inhibition of the biosynthesis of prostaglandins,[4] anti-hepatotoxic activity in primary cultured rat hepatocytes[5] and the inhibition of gastric movements in rats.[6] [8]-Gingerol has been shown to induce inotropic action on isolated atria[7] and to activate Ca^{2+}-ATPase pumping activity, indicating that gingerol is the first example of a cardiotonic agent that directly activates the Ca^{2+}-pump of sarcoplasmic reticulum (SR).[2]

MATERIALS AND METHODS

Isolation of Gingerols Gingerols ([6]-gingerol, [8]-gingerol and [10]-gingerol) were purified from the dried rhizome of ginger *Z. officinale* ROSCOE. The purification of gingerols was performed as previously reported.[7]

Synthesis of Compounds AP-004: To a solution of 4-hydroxy-3-methoxycinnamic acid (200 mg, 1.03 mmol), *n*-octylamine (140 mg, 1.08 mmol), *N*-hydroxybenzotriazole (HOBT) (189 mg, 1.23 mmol) and triethylamine (100 μl, 0.72 mmol) in dichloromethane (20 ml) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) (236 mg, 1.23 mmol) at 0—5 °C, and the mixture was stirred at the same temperature for 15 min and then at room temperature for 4 h. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in ethyl acetate. The solution was washed with sat. $NaHCO_3$, 1 N HCl and sat. NaCl successively, dried over anhydrous $MgSO_4$, and then filtered and concentrated under reduced pressure. The residue was purified by dry column flash chromatography (SiO_2/hexane : ethyl acetate = 1 : 1) to give 4-hydroxy-3-methoxycinnamic acid *n*-octylamide (AP-004) as a colorless amorphous solid (296 mg). AP-004: IR (KBr): 3269, 2926, 2854, 1655, 1589, 1516, 1464, 1429, 1271, 1209, 1159, 1124 cm^{-1}. High resolution FAB-MS: Found: 306.2077; Calcd: 306.2069. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.84—0.93 (3H, m), 1.22—1.40 (10H, m), 1.56 (2H, quint., J = 7.0 Hz), 3.37 (2H, dt, J = 5.8, 7.0 Hz), 3.91 (3H, s), 5.52—5.63 (1H, br), 5.86 (1H, s), 6.24 (1H, d, J = 15.7 Hz), 6.90 (1H, d, J = 8.3 Hz), 6.98 (1H, d, J = 1.9 Hz), 7.06 (1H, dd, J = 1.9, 8.3 Hz), 7.54 (1H, d, J = 15.7 Hz).

AP-005: To a solution of AP-004 (142 mg, 0.465 mmol) in methanol (6 ml) was added 10% Pd–C (30 mg),and the mixture was stirred vigorously under an atmospheric pressure of hydrogen at room temperature for 1 h. The reaction mixture was filtered *via* Celite and the catalyst was washed with methanol. The combined filtrate and washings were concentrated under reduced pressure. The residue was purified by TLC (SiO_2/hexane : ethyl acetate = 1 : 1) to give 3-(4-hydroxy-3-methoxyphenyl)propanoic acid *n*-octylamide (AP-005) as a colorless solid (119 mg). AP-005: mp: 92.0—94.5 °C. IR (KBr): 3521, 2924, 2852, 1549, 1454, 1439, 1363, 1265, 1227, 1149, 1030 cm^{-1}. High resolution FAB-MS: Found: 308.2188; Calcd: 308.2188. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.84—0.92 (3H, m), 1.17—1.35 (10H, m), 1.35—1.47 (2H, m), 2.42 (2H, t, J = 7.6 Hz), 2.89 (2H, t, J = 7.6 Hz), 3.19 (2H, dt, J = 5.6, J = 7.2 Hz), 3.86 (3H, s), 5.25—5.35 (1H, br), 5.52 (1H, s), 6.68 (1H, dd, J = 2.0, 8.0 Hz), 6.71 (1H, d, J = 2.0 Hz), 6.83 (1H, d, J = 8.0 Hz).

AP-015: To a solution of 5-hydroxy-1-(4-benzyloxy-3-methoxyphenyl)-3-dodecanone (100 mg, 0.243 mmol), which was prepared as previously reported by Tsuge *et al.*,[8] in ethanol (5 ml), was added a solution of hydroxylamine hydrochloride (20.0 mg, 0.288 mmol) in water (0.5 ml) at 0—4 °C, and the mixture was stirred at room temperature for 30 min. Pyridine (160 μl, 1.99 mmol) was added to the mixture and the resulting reaction mixture was dissolved in ethyl acetate. The solution was washed with 1 N HCl, water and sat. NaCl, then dried over Na_2SO_4, filtered and concentrated under reduced pressure.

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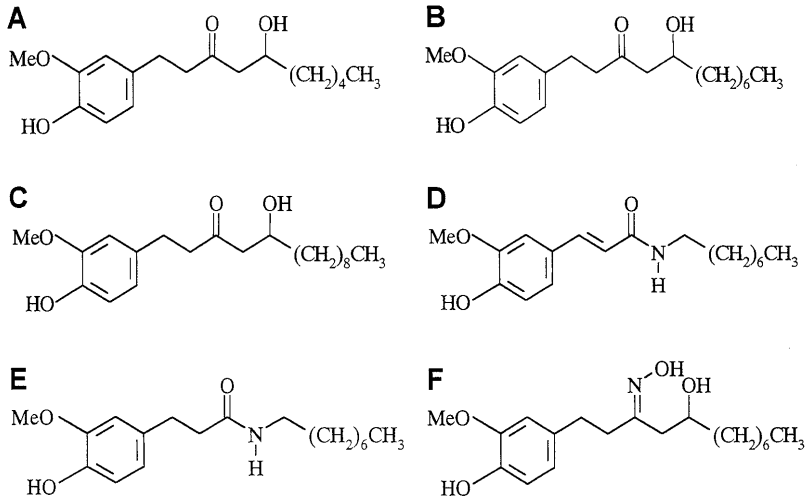

Fig. 1. The Chemical Structures of Gingerols and Their Synthesized Compounds
6-Gingerol (A); 8-gingerol (B); 10-gingerol (C); AP-004 (D); AP-005 (E); AP-015 (F).

The residue was purified by TLC (SiO_2/benzene : ethyl acetate = 5 : 1) to give (*Z*)-oxime and (*E*)-oxime as colorless solids (29.2 mg and 52.4 mg, respectively), and a mixture of the two (12.7 mg). (*Z*)-oxime: mp: 64.5—68.0 °C. FAB-MS: 428. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.824—0.91 (3H, m), 1.22—1.65 (12H, m), 2.29—2.37 (1H, br), 2.40 (1H, dd, J=3.6, 13.4 Hz), 2.51—2.58 (2H, m), 2.67 (1H, dd, J=9.3, 13.4 Hz), 2.75—2.83 (2H, m), 3.84—3.97 (1H, m), 3.88 (3H, s), 5.12 (2H, s), 6.66 (1H, dd, J=1.9, 8.0 Hz), 6.74 (1H, d, J=8.0 Hz), 7.26—7.45 (5H, m). (*E*)-oxime: mp: 78.5—80.5 °C. FAB-MS: 428. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.824—0.92 (3H, m), 1.22—1.65 (12H, m), 2.20 (2H, d, J=6.7 Hz), 2.61 (2H, dd, J=6.1, 9.2 Hz), 3.20—3.35 (1H, br), 3.82—3.95 (1H, m), 3.88 (3H, s), 5.12 (2H, s), 6.67 (1H, dd, J=2.2, 8.3 Hz), 6.75 (1H, d, J=2.2 Hz), 6.80 (1H, d, J=8.3 Hz), 7.25—7.47(5H, m).

To a solution of (*Z*)-oxime (25.0 mg, 0.0585 mmol) in methanol (5 ml) was added 10% Pd–C (10 mg), and the mixture was stirred vigorously under an atmospheric pressure of hydrogen at room temperature for 1 h. The reaction mixture was filtered *via* Celite and the catalyst was washed with methanol. The combined filtrate and washings were concentrated under reduced pressure. The residue was purified by TLC (SiO_2/hexane : ethyl acetate = 3 : 2) to give 5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-3-dodecanone (*Z*)-oxime (AP-015) as a colorless powder (16.7 mg). AP-015: mp: 89.0—92.5 °C. IR (KBr): 3230, 3274, 2926, 2854, 1518, 1452, 1273, 1254, 1039 cm^{-1}. High resolution FAB-MS: Found: 338.2320; Calcd: 338.2331. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.84—0.92 (3H, m), 1.21—1.70 (12H, m), 2.22—2.36 (1H, br), 2.40 (1H, dd, J=3.6, 13.3 Hz), 2.50—2.58 (2H, m), 2.67 (1H, dd, J=9.1, 13.3 Hz), 2.75—2.82 (2H, m), 3.82—3.98 (1H, m), 3.87 (3H, s), 5.44—5.57 (1H, br), 6.66—6.71 (2H, m), 6.83 (1H, d, J=8.6 Hz).

Gingerols and synthesized compounds were dissolved in dimethyl sulfoxide, of which the final concentration was kept at 1% (v/v) in all experiments.

Preparation of a Light Fraction of Fragmented SR (LSR) LSR was prepared from rabbit (male, 2 to 3 kg) skeletal muscle by the method of Mori *et al.*[9]

Preparation of Cardiac SR (CSR) CSR was prepared from pig (male, 80 to 100 kg) cardiac muscle by the procedure of Sumida *et al.*[10]

Measurement of the Ca^{2+}-ATPase Activity The Ca^{2+}-ATPase activity was measured using skeletal muscle LSR but not CSR, because the CSR fraction contains a respectable amount of mitochondria. The reaction procedure was the same as described previously.[11] The enzyme reaction mixture was as follows: 50 mM MOPS–KOH buffer (pH 7.0), 90 mM KCl, 5 mM $MgCl_2$, 0.75 mM $CaCl_2$, 1 mM EGTA, 0.05 mg LSR, 1 mM ATP and drugs for Ca^{2+}-ATPase. The amount of inorganic phosphate liberated during the 5 min incubation was determined by the malachite green method described by Chan *et al.*[11]

Measurement of the Extravesicular Ca^{2+} Concentration of CSR The extravesicular Ca^{2+} concentration of CSR suspension was measured at 30 °C with a Ca^{2+} electrode prepared as described previously.[12] The Ca^{2+} electrode showed a Nernstein response (slope, 27—29 mV/pCa unit) in the calibration solutions containing Ca^{2+}–EGTA between pCa 3 and 6.5. The assay mixture contained 90 mM KCl, 0.05 mM $CaCl_2$, 0.25 mM $MgCl_2$, 2 mM NaN_3, 0.5 mM ATP, an ATP-regenerating system (5 mM creatine phosphate and 0.1 mg/ml creatine kinase), 1.5 mg/ml CSR and 50 mM MOPS–KOH buffer (pH 7.0).

RESULTS AND DISCUSSION

An important problem to be elucidated in the excitation–contraction coupling of skeletal and cardiac muscles concerns the regulation of SR function. Ca^{2+}-ATPase in the SR membrane plays a key role in muscle relaxation by energized Ca^{2+}-pumping from the cytoplasm into the lumen of SR. Gingerol may provide a valuable chemical tool for studies aimed at clarifying the regulatory mechanisms of SR Ca^{2+}-pumping systems, as well as the causal relationship between the Ca^{2+}-pumping activity of SR and muscle contractility.

We have previously reported that [8]-gingerol increased Ca^{2+}-ATPase activity.[1,2] As shown in Fig. 2, the SR Ca^{2+}-ATPase activity of skeletal muscle was increased by

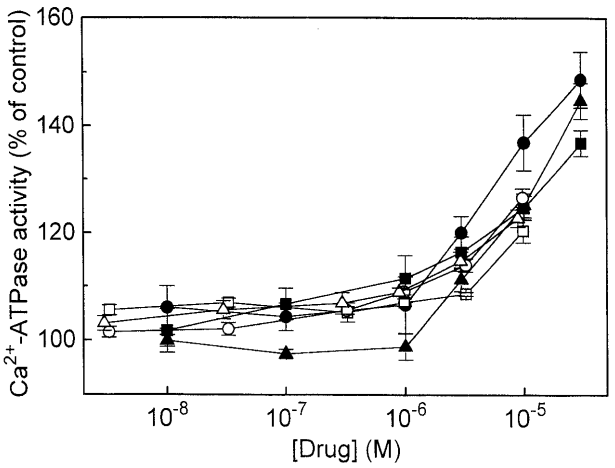

Fig. 2. The Concentration–Response Curves for Gingerols and Their Synthesized Compounds on the Activities of Ca^{2+}-ATPase from Skeletal SR

6-Gingerol (■); 8-gingerol (●); 10-gingerol (▲); AP-004 (□); AP-005 (○); AP-015 (△). Reaction mixture (final volume, 0.5 ml) was as follows: 50 mM MOPS–KOH buffer (pH 7.0), 90 mM KCl, 5 mM $MgCl_2$, 0.75 mM $CaCl_2$, 1 mM EGTA, 0.0065 mg/ml LSR, 0.3 mM ATP and gingerols, and the synthesized compounds for Ca^{2+}-ATPase. The amount of inorganic phosphate liberated during the 5 min incubation was determined by the malachite green method described by Chan *et al.*[11]

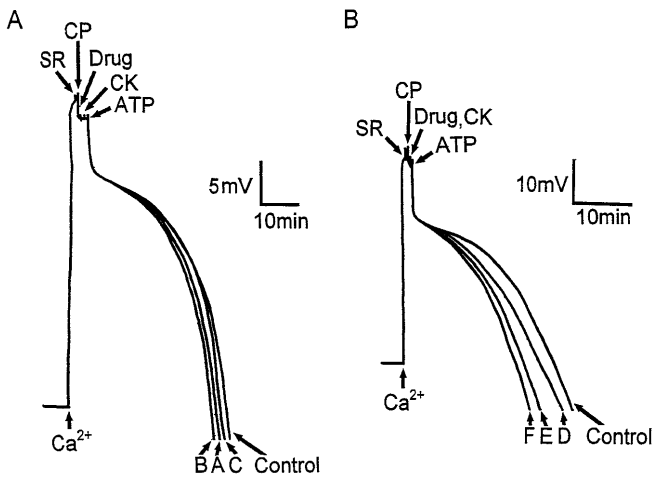

Fig. 3. Stimulatory Effect of Gingerols (A) and Their Synthesized Compounds (B) on the Ca^{2+}-Pumping Activity of CSR

6-Gingerol (A); 8-gingerol (B); 10-gingerol (C); AP-004 (D); AP-005 (E); AP-015 (F). The extravesicular Ca^{2+} concentration was monitored with a Ca^{2+} electrode in a mixture containing 1.5 mg/ml HSR, 0.5 mM ATP, 0.05 mM $CaCl_2$, 0.5 mM $MgCl_2$, 90 mM KCl, ATP-regenerating system, *i.e.* 5 mM creatine phosphate (CP) and 0.1 mg/ml creatine kinase (CK), and 50 mM MOPS–KOH buffer (pH 7.0). At the begining of each experiment, 0.05 mM $CaCl_2$ was added before the application of CSR, CP, drugs, CK or ATP. Each trace was superimposed. The vertical calibration bar indicates a response for a voltage change of 10 mV (27—29 mV corresponds to 1 pCa unit).

gingerols and their synthesized compounds in a concentration-dependent manner. Both the natural compounds and the synthesized compounds with an *o*-methoxy phenol and hydrocarbon chain in the molecule are able to activate the Ca^{2+}-ATPase activity.

The Ca^{2+}-pumping ATPase activity of CSR can be visualized directly by monitoring the extravesicular Ca^{2+} concentration of CSR with our sensitive Ca^{2+} electrode. A tracing of the Ca^{2+}-pumping activity in Fig. 3 is representative of three experiments. As shown in Fig. 3, upon the addition of ATP, the free Ca^{2+} concentration decreased promptly due to the formation of Ca·ATP complexes, and further decreased gradually due to the active Ca^{2+} uptake by CSR. The earlier part of the Ca^{2+} uptake was almost linear in an antilogarithmic plot. When the Ca^{2+} concentration was reduced to submicromolar levels, the Ca^{2+} uptake was slowed.

The effects of gingerols and their synthesized compounds were examined using this system on the Ca^{2+}-pumping activity. The slope of the Ca^{2+} uptake profile suddenly steepened just after the application of gingerols and their synthesized compounds. No such influence was observed after the addition of the vehicle solvent. After pretreatment with gingerols and the synthesized compounds, the slope of the time course curve of the Ca^{2+} uptake was much steeper than that of the control. These results suggest that all these compounds activate the Ca^{2+}-pumping activity of CSR. Both an *o*-methoxy phenol and hydrocarbon chain in the molecule are probably necessary for the activation not only of Ca^{2+}-ATPase but also for the Ca^{2+}-pumping activity of SR by gingerol analogues.

The level of mitochondrial contamination of skeletal muscle LSR is considerably less than in CSR. Therefore, the present study was undertaken using the Ca^{2+}-pumping system of pig CSR as well as skeletal muscle LSR from different species. Further isolation, synthesis and examination of detailed pharmacological actions of these compounds are under way.

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