[6]-Gingerol Induces Ca^{2+} Mobilization in Madin-Darby Canine Kidney Cells

Chung-Yi Chen,[†] Ching-Hsein Chen,[§] Chiu-Hu Kung,[‡] Shih-Hsing Kuo,[†] and Soong-Yu Kuo*[,†]

School of Medicine and Health Sciences, Fooyin University, Kaohsiung County 83101, Taiwan, Republic of China, Department of Medical Technology, Fooyin University, Kaohsiung County 83101, Taiwan, Republic of China, and Graduate Institute of Biomedical and Biopharmaceutical Sciences, College of Life Sciences, National Chiayi University, Chiayi 60004, Taiwan, Republic of China

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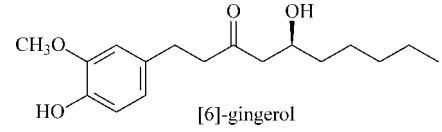
[6]-Gingerol, a major phenolic compound derived from ginger (*Zingiber officinale*), is a potential chemopreventive compound that can induce stress in cancer cells and cause apoptotic cell death. This study examines the early signaling effects of [6]-gingerol on renal cells. It was found that [6]-gingerol caused a slow and sustained rise of $[Ca^{2+}]_i$ in a concentration-dependent manner. [6]-Gingerol also induced a $[Ca^{2+}]_i$ rise when extracellular Ca^{2+} was removed, but the magnitude was reduced by 80%. Depletion of intracellular Ca^{2+} stores with CCCP, a mitochondrial uncoupler, did not affect the action of [6]-gingerol. In a Ca^{2+}-free medium, the [6]-gingerol-induced $[Ca^{2+}]_i$ rise was partially abolished by depleting stored Ca^{2+} with thapsigargin (an endoplasmic reticulum Ca^{2+} pump inhibitor). The elevation of [6]-Gingerol-caused $[Ca^{2+}]_i$ in a Ca^{2+}-containing medium was not affected by modulation of protein kinase C activity. The [6]-gingerol-induced Ca^{2+} influx was blocked by nicardipine. U73122, an inhibitor of phospholipase C, abolished ATP (but not [6]-gingerol)-induced $[Ca^{2+}]_i$ rise. These findings suggest that [6]-gingerol induces a significant rise in $[Ca^{2+}]_i$ in MDCK renal tubular cells by stimulating both extracellular Ca^{2+} influx and thapsigargin-sensitive intracellular Ca^{2+} release via as yet unidentified mechanisms.

Ginger (*Zingiber officinale* L., Zingiberaceae) is a common condiment for various foods and beverages. Ginger also has a long history in traditional medicine. The underground stems or rhizomes of this plant have been used as a medicine in East Asian, Indian, and Arabic herbal traditions since ancient times.[1] In mainland China, the rhizomes of ginger have been used in oriental medicine for the treatment of the common cold, disorders of the gastrointestinal tract, neuralgia, rheumatism, colic, and motion discomfort.[2,3] The nonvolatile pungent ingredients from ginger include gingerol, shoagol, and zingerone. Recently, several population-based studies have shown that persons in Southeast Asian countries have a much lower risk of colon, gastrointestinal, prostate, breast, and other cancers than those in European and American countries.[4] It is believed that constituents of their diet may play important roles in cancer prevention. Indeed, some phenolic substances present in fruit and vegetables, and in medicinal plants, have potential cancer chemopreventive activities, as supported by both in vitro and in vivo experiments.[2,5–7] These agents are known to have the ability to suppress the transformative, hyperproliferative, and inflammatory processes of carcinogenesis.

The phenolic compounds derived from ginger possess many interesting pharmacological and physiological activities. Of these, [6]-gingerol [1-(4′-hydroxy-3′-methoxyphenyl)-5-hydroxy-3-decanone], the major pungent principle of ginger, has potential anti-inflammatory, antioxidant, anticarcinogenic, and antimutagenic activities.[8–10] Evidence reveals that [6]-gingerol exerts an inhibitory effect on DNA synthesis and causes apoptosis in human promyelocytic leukemia (HL-60) cells.[11] In vitro, [6]-gingerol inhibited both the VEGF- and bFGF-induced proliferation of human endothelial cells and caused cell-cycle arrest in the G_1 phase.[12] This compound also induced growth arrest in the G_1 phase and was cytotoxic to two human pancreatic cancer cell lines, HPAC, expressing wild-type (wt) p53, and BxPC-3, expressing mutated p53.[13]

The present study was aimed to explore the effect of [6]-gingerol on $[Ca^{2+}]_i$ in renal tubular cells. Madin-Darby canine kidney (MDCK) cells have properties akin to human renal tubular cells and have been used successfully as a model for renal research.[14–17] Many endogenous and exogenous agents can stimulate MDCK cells by causing a $[Ca^{2+}]_i$ increase, such as ATP,[14] bradykinin,[15] despramine,[16] and methylglyoxal.[17] The inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} store releases Ca^{2+} into the cytosol when cells are stimulated by endogenous agents such as ATP[14] and bradykinin.[15] However, exogenous agents can release Ca^{2+} from IP_3-insensitive stores,[14] and Ca^{2+} release may induce Ca^{2+} influx across the plasma membrane via the process of store-operated Ca^{2+} entry.[18]

Using fura-2 as a fluorescent Ca^{2+} indicator, the present study has shown that [6]-gingerol induced a significant and prolonged $[Ca^{2+}]_i$ increase in MDCK cells. The concentration–response relationship, the Ca^{2+} sources of the Ca^{2+} signal, and the role of phospholipase C in the signal have been investigated.



[6]-gingerol

[6]-Gingerol at concentrations between 5 and 20 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner in the presence of extracellular Ca^{2+}. Figure 1A shows typical recordings of the $[Ca^{2+}]_i$ rise induced by 5–20 μM [6]-gingerol. At a concentration of 0.1 μM, [6]-gingerol had no effect on $[Ca^{2+}]_i$ (i.e., equivalent to baseline, 0 μM). The $[Ca^{2+}]_i$ rise induced by 5–20 μM [6]-gingerol comprised an immediate rise and a sustained phase in 250 s. At a concentration of 10 μM, the $[Ca^{2+}]_i$ rise had a net value of 295 $\pm$ 2 nM at 250 s. Figure 1C (filled circles) shows the concentration–response curve of the [6]-gingerol-induced response.

Experiments were performed to evaluate the relative contribution of extracellular Ca^{2+} entry and Ca^{2+} release from stores in the [6]-gingerol response. Figure 1B shows that removal of extracellular Ca^{2+} largely suppressed the [6]-gingerol-induced $[Ca^{2+}]_i$ elevation. The concentration–response relationship of [6]-gingerol-induced $[Ca^{2+}]_i$ rise in the presence and absence of extracellular Ca^{2+} is shown in Figure 1C. Ca^{2+} removal inhibited the $[Ca^{2+}]_i$ rise caused by 20 μM [6]-gingerol by 78% as the maximum value ($n = 5$; $p < 0.05$).

To investigate the possible mechanisms of this calcium rise under calcium-free conditions, several inhibitors of intracellular Ca^{2+} storage were used. Carbonylcyanide *m*-chlorophenylhydrazone

* Corresponding author. Tel: +886-7-7811151-6200. Fax: +886-7-7834548. E-mail: mt100.sykuo@ msa.hinet.net.
[†] School of Medicine and Health Sciences, Fooyin University.
[‡] Department of Medical Technology, Fooyin University.
[§] National Chiayi University.

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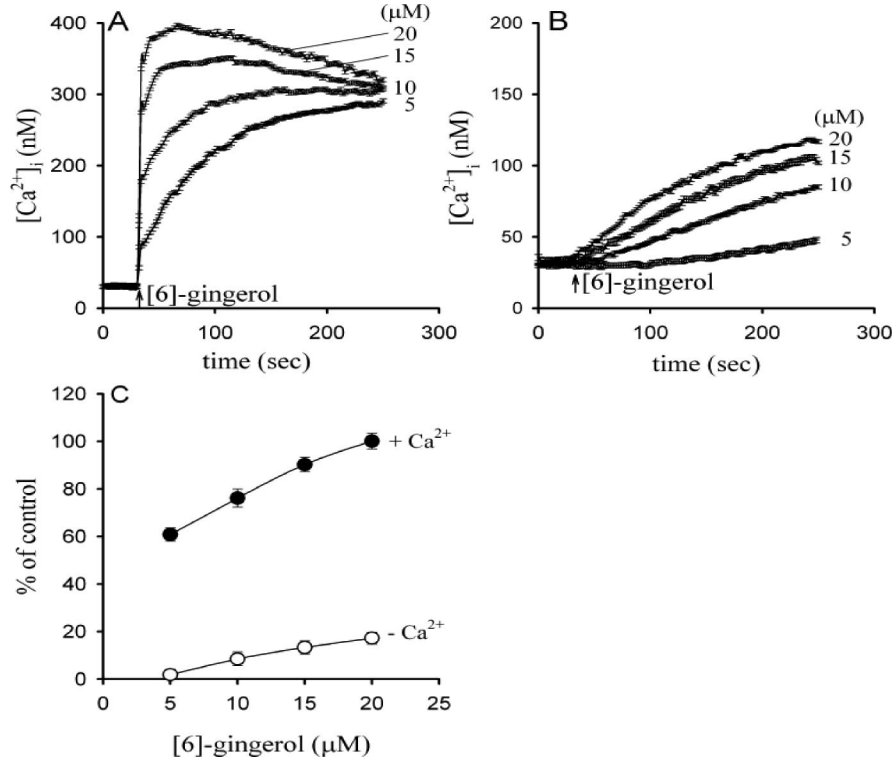

Figure 1. Effects of [6]-gingerol on $[Ca^{2+}]_i$ in MDCK cells. (A) Concentration-dependent effects of [6]-gingerol, with the concentration of the reagent indicated. Experiments were performed in Ca^{2+}-containing medium. [6]-Gingerol was added at 30 s and was present throughout the measurements for 250 s. (B) Effect of extracellular Ca^{2+} removal on [6]-gingerol-induced $[Ca^{2+}]_i$ elevation. The concentration of [6]-Gingerol is indicated. (C) Concentration–response plots of [6]-gingerol-induced $[Ca^{2+}]_i$ rises in Ca^{2+}-containing medium (filled circles) and Ca^{2+}-free medium (open circles). The data are presented as the percentage of control, which is the net $[Ca^{2+}]_i$ rise induced by 20 μM [6]-gingerol in Ca^{2+}-containing medium. Data are mean SEM of five experiments (*$p < 0.05$ compared to open circles).

(CCCP) is a mitochondrial uncoupler and has been shown to release Ca^{2+} from mitochondria in different cells.[17,19] Thapsigargin is an endoplasmic reticulum Ca^{2+} pump inhibitor.[20] The role of the endoplasmic reticulum Ca^{2+} stores in the observed [6]-gingerol-induced $[Ca^{2+}]_i$ elevation was examined because previous studies have shown that these stores play a key role in Ca^{2+} release in MDCK cells.[20] Figure 2A shows that, in Ca^{2+}-free medium, application of 1 μM thapsigargin caused a $[Ca^{2+}]_i$ rise that comprised an initial increase and a gradual decay toward baseline. The net maximum $[Ca^{2+}]_i$ value was 216 ± 4 nM ($n = 5$). After depleting the endoplasmic reticulum Ca^{2+} store with thapsigargin, addition of 20 μM [6]-gingerol did not induce a $[Ca^{2+}]_i$ rise, as shown in Figure 1A. Conversely, Figure 2B shows that after preincubation with [6]-gingerol (20 μM) for 220 s subsequent addition of 1 μM thapsigargin induced a $[Ca^{2+}]_i$ rise with a net value of 68 ± 3 nM ($n = 5$), which was 32% ($p < 0.05$), smaller than the control thapsigargin response shown in Figure 2A.

Figure 2C shows that after depleting the mitochondrial Ca^{2+} store with CCCP (2 μM) addition of 20 μM [6]-gingerol induced a $[Ca^{2+}]_i$ increase that was indistinguishable in kinetics and magnitude from the control response shown in Figure 1B. Conversely, Figure 2D shows that after preincubation with [6]-gingerol (20 μM) for 250 s subsequent addition of 2 μM CCCP induced a $[Ca^{2+}]_i$ rise with a net value of 262 nM ($p < 0.05$), which was the same as the control CCCP response shown in Figure 2C. Thus, it appears that mitochondrial Ca^{2+} stores may not be involved in [6]-gingerol-induced Ca^{2+} release.

Previous studies have shown that in MDCK cells stored Ca^{2+} can be released by pathways dependent or independent of phospholipase C activity.[21,22] Thus, an effort was made to explore whether phospholipase C played a role in gingerol-induced Ca^{2+} release. Figure 3A shows that in Ca^{2+}-free medium addition of 2 μM U73122 to suppress phospholipase C activity[21] did not alter basal $[Ca^{2+}]_i$ but abolished the $[Ca^{2+}]_i$ rise induced by ATP (10 μM) ($n = 5$), a phospholipase C-dependent Ca^{2+} mobilizer.[22] The control ATP (10 μM)-induced $[Ca^{2+}]_i$ had a net peak value of 112 ± 3 nM ($n = 5$) (Figure 3B). Conversely, U73343 (10 μM), an inactive U73122 analogue,[21] did not affect ATP-induced $[Ca^{2+}]_i$ elevation (not shown). This suggests that U73122 effectively suppressed phospholipase C activity. Figure 3A further shows that 20 μM gingerol added after ATP induced a $[Ca^{2+}]_i$ rise that was similar to the control [6]-gingerol response shown in Figure 1B ($n = 5$).

To explore the pathways underlying [6]-gingerol-induced Ca^{2+} entry, the effects of several L-type Ca^{2+} entry blockers on [6]-gingerol-induced $[Ca^{2+}]_i$ rise were evaluated. Figure 4 shows that in Ca^{2+}-containing medium pretreatment with 1 μM nicardipine inhibited 20 μM [6]-gingerol-induced $[Ca^{2+}]_i$ elevation by 70% ($n = 5$; $p < 0.05$). However, the Ca^{2+} influx was not affected by 1 μM diltiazem, nifedipine, or verapamil ($n = 5$, Figure 4).

The role of protein kinase C in [6]-gingerol-induced $[Ca^{2+}]_i$ elevation was investigated. It was shown that the 20 μM [6]-gingerol-induced $[Ca^{2+}]_i$ elevation was not altered by pretreatment with 10 nM phorbol myristate acetate (a protein kinase C activator) or 2 μM GF109203 X (a protein kinase C inhibitor) ($n = 5$; data not shown).

This study is the first to investigate the effects of [6]-gingerol on $[Ca^{2+}]_i$ in renal tubular cells. The results obtained suggest that [6]-gingerol causes a significant concentration-dependent, sustained $[Ca^{2+}]_i$ rise in MDCK cells. In Ca^{2+}-containing medium, the $[Ca^{2+}]_i$ rise induced by [6]-gingerol was sustained without a decay during the 5 min of measurements. Sustained $[Ca^{2+}]_i$ elevations are thought to alter many cellular functions.[24] [6]-Gingerol may affect cell physiology significantly by changing Ca^{2+} signaling and stimulating Ca^{2+}-coupled bioactive molecules. The results show that the $[Ca^{2+}]_i$ rise was contributed to by both intracellular Ca^{2+} release and

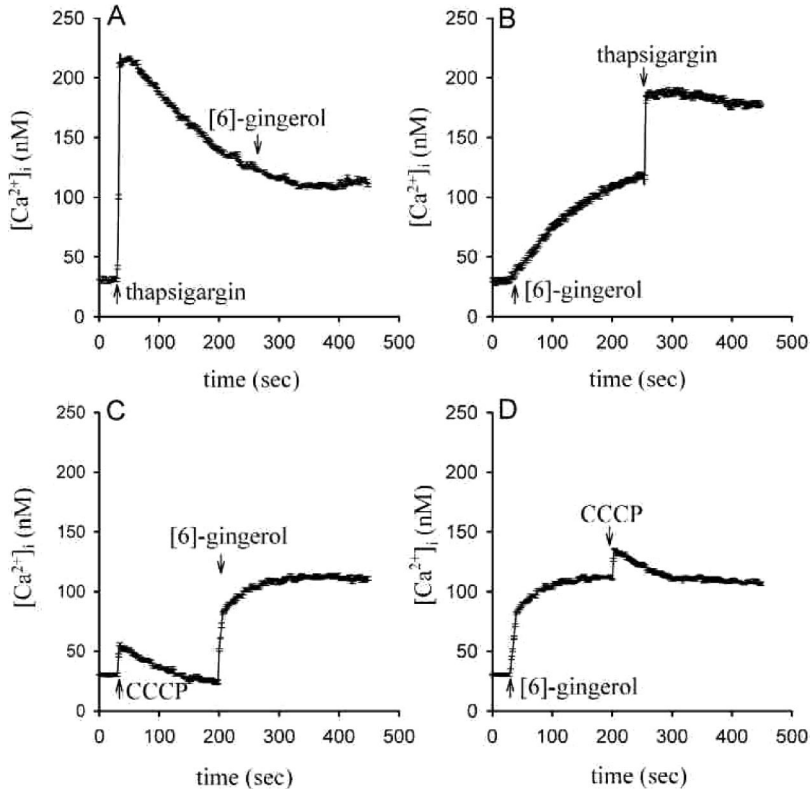

Figure 2. Intracellular sources of [6]-gingerol-induced $[Ca^{2+}]_i$ elevation. All experiments were performed in Ca^{2+}-free medium. Reagents were applied at the times indicated by arrows. The concentration of reagents were 2 μM CCCP, 20 μM [6]-gingerol, and 1 μM thapsigargin. Data are mean SEMs of five experiments.

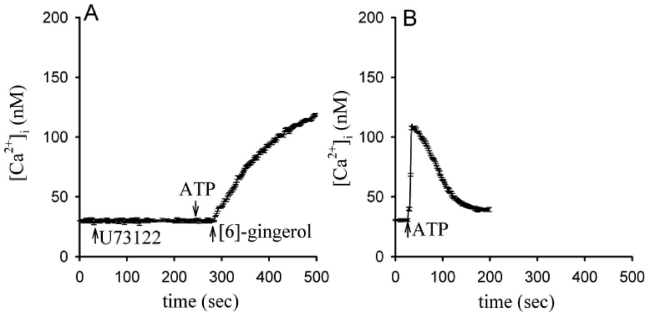

Figure 3. Effect of U73122 on [6]-gingerol-induced $[Ca^{2+}]_i$ elevation. (A) U73122 (2 μM), ATP (10 μM), and [6]-gingerol (20 μM) were added at 30, 250, and 280 s, respectively. (B) ATP (10 μM) was added at 30 s. All experiments were performed in Ca^{2+}-free medium. Data are mean SEMs of five experiments.

extracellular Ca^{2+} influx, because the signal was largely suppressed by removal of extracellular Ca^{2+}.

Regarding the Ca^{2+} depositories of the [6]-gingerol response, the stored Ca^{2+} in the mitochondria do not appear to play a significant role since depletion of mitochondrial Ca^{2+} with CCCP did not affect [6]-gingerol-induced Ca^{2+} release. The thapsigargin-sensitive endoplasmic reticulum store appears to play a crucial role because the [6]-gingerol-induced Ca^{2+} release was partly abolished by depletion of the endoplasmic reticulumic Ca^{2+} store with thapsigargin, and, conversely, pretreatment with thapsigargin also inhibited [6]-gingerol-induced Ca^{2+} release. The endoplasmic reticulum is one of the major Ca^{2+} stores where various proteins and lipids are synthesized and modified.[25,26] Perturbation of endoplasmic reticulum Ca^{2+} homeostasis, protein misfolding, or oxidative stress can lead to cell death.[26,27] Evidence reveals that reactive oxygen species (ROS) and the oxidation–reduction (redox) state play significant roles in many cytotoxic pathways caused by frequently used antitumor drugs or environmental toxicants.[28] A rise in $[Ca^{2+}]_i$ induced by oxidants may activate Ca^{2+}-dependent enzymes such as proteases, nucleases, and phospholipases to facilitate mitochondrial oxidative stress leading to cytotoxicity.[29,30] Exactly how [6]-gingerol releases Ca^{2+} stored in the endoplasmic reticulum is unclear, but the process seems to be independent of phospholipase C activity because suppression of this protein did not affect [6]-gingerol-induced Ca^{2+} release. Because [6]-gingerol and thapsigargin share the same Ca^{2+} stores, [6]-gingerol may very likely release Ca^{2+} in a manner similarly to thapsigargin by inhibiting the endoplasmic reticulum Ca^{2+} pump.

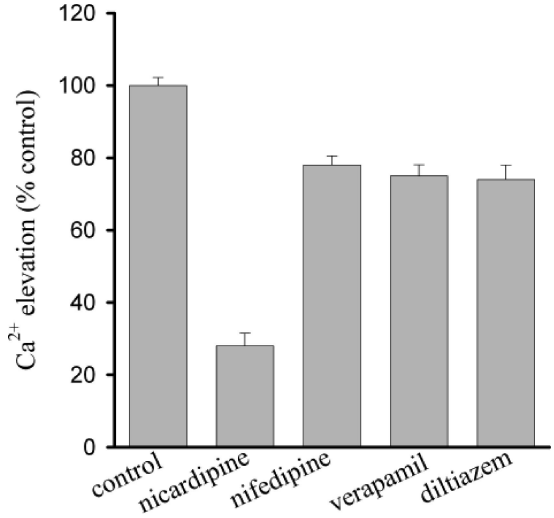

Figure 4. Effect of Ca^{2+} blockers on [6]-gingerol-induced $[Ca^{2+}]_i$ elevation. All experiments were performed in Ca^{2+}-containing medium. The data are presented as the percentage of the control, which is the net area under the curve (30–250 s) of the $[Ca^{2+}]_i$ rise induced by 20 μM [6]-gingerol. Data are mean SEMs of five experiments (*p < 0.05 compared to control).

The [6]-gingerol-induced Ca^{2+} influx appears to be via a pathway sensitive to nicardipine, since nicardipine-sensitive [6]-gingerol-induced Ca^{2+} influx was not controlled via conventional L-type Ca^{2+} channels, as it was not inhibited by diltiazem and verapamil. This is consistent with a previous study showing that MDCK cells are nonexcitable.[31]

In Ca^{2+}-free medium, [6]-gingerol-induced $[Ca^{2+}]_i$ elevation displayed a smaller $[Ca^{2+}]_i$ increase throughout the measurement period of 250 s. This suggests that Ca^{2+} influx contributed not only to the initial increase but also to the prolonged phase of the [6]-gingerol-induced $[Ca^{2+}]_i$ signal in the Ca^{2+}-containing medium. In nonexcitable cells, a possible Ca^{2+} influx pathway is a store-operated Ca^{2+} entry, a process triggered by depletion of Ca^{2+} stores.[31] This possibility was not explored due to the lack of selective pharmacological inhibitors for this Ca^{2+} influx.[32] Thus, it remains possible that Ca^{2+} entry mechanisms other than depletion-activated channels may be important in Ca^{2+} influx in nonexcitable cells.

Collectively, this study shows that in MDCK cells [6]-gingerol caused $[Ca^{2+}]_i$ elevations in a concentration-dependent manner by evoking phospholipase C-independent Ca^{2+} release from the endoplasmic reticulum and also by causing Ca^{2+} influx via a nicardipine-sensitive pathway. These effects may play a crucial role in the physiological action of [6]-gingerol.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were obtained in MeCN using a JASCO V-530 spectrophotometer. The IR spectra were measured on a Hitachi 260-30 spectrophotometer. ^1H (400 MHz, using $CDCl_3$ as solvent for measurement), ^{13}C (100 MHz), DEPT, HETCOR, COSY, NOESY, and HMBC NMR spectra were obtained on a Unity Plus Varian NMR spectrometer. LRFABMS and LREIMS were obtained with a JEOL JMS-SX/SX 102A mass spectrometer or a Quattro GC-MS spectrometer with a direct inlet system. Silica gel 60 (Merck, 230–400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.20 mm) were used for analytical TLC, and precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.50 mm) were used for preparative TLC. Spots were detected by spraying with 50% H_2SO_4 and then heating on a hot plate.

Plant Material. The roots of *Zingiber officinale* (ginger) were purchased from a local market in Kaohsiung, Taiwan, in July 2005, and were identified by Dr. Yen-Ray Hsui of the Division of Silviculture, Taiwan Forestry Research Institute, Taipei, Taiwan. A voucher specimen (Hsui-*Zo*-1) was deposited at Fooyin University.

Extraction and Isolation. The roots (4.1 kg) of *Z. officinale* were chipped, air-dried, and extracted repeatedly with $CHCl_3$ at room temperature. The combined $CHCl_3$ extracts were then evaporated and further separated into 14 fractions by column chromatography on silica gel with gradients of *n*-hexane−$CHCl_3$. Fraction 12, eluted with $CHCl_3$−MeOH (40:1), was subjected to silica gel column chromatography and yielded pure [6]-gingerol (92 mg), which was identified by spectroscopic data analysis and comparison with literature values.[33]

Cell Culture and Test Compound Treatment. MDCK cells were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium. The media were supplemented with 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were kept at 37 °C in 5% CO_2-containing humidified air.

Measurement of $[Ca^{2+}]_i$. Trypsinized cells (10^6/mL) were allowed to recover in the culture medium for 1 h before being loaded with 2 μM fura-2/AM for 30 min at 25 °C in the same medium. The cells were washed once with serum-free DMEM medium and resuspended in Ca^{2+}-containing medium (pH 7.4) containing (mM) NaCl, 140; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; HEPES, 5; and D-glucose, 5. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25 °C) with continuous stirring; the cuvette contained 1 mL of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer (Kyoto, Japan) by recording the excitation signals at 340 and 380 nm and the emission signal at 510 nm at 1 s intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 and 10 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as described previously assuming a K_d of 155 nM.[34] In experiments that were performed in the absence of extracellular Ca^{2+}, cells were bathed in Ca^{2+}-free medium in which $CaCl_2$ (2 mM) was substituted with 0.1 mM EGTA. Data are presented as means ± standard deviation (SD) and analyzed using one-way ANOVA with Scheffe's test. A p value of less than 0.05 was considered as statistically significant.

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