Fendiline-Induced Ca^{2+} Movement in A10 Smooth Muscle Cells

Yuk-Keung Lo[1,7], Jin-Shiung Cheng[1,7], Jue-Long Wang[2,7], Kam-Chung Lee[1,7], Kang-Ju Chou[1,7], Hong-Tai Chang[3,7], Kwong-Yui Tang[4,7] and Chung-Ren Jan[5,6]

[1]*Department of Medicine*
Kaohsiung Veterans General Hospital
[2]*Department of Rehabilitation*
Kaohsiung Veterans General Hospital
[3]*Department of Surgery*
Kaohsiung Veterans General Hospital
[4]*Department of Psychiatry*
Kaohsiung Veterans General Hospital
[5]*Department of Medical Education and Research*
Kaohsiung Veterans General Hospital
[6]*Department of Biology and Institute of Life Sciences*
National Sun Yat-sen University, Kaohsiung
[7]*School of Medicine*
National Yang-Ming University
Taipei 112, Taiwan, ROC

Abstract

The effect of fendiline, an anti-anginal drug, on cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$) in A10 smooth muscle cells was explored by using fura-2 as a Ca^{2+} indicator. Fendiline at concentrations between 10-50 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner with an EC50 of 20 μM. External Ca^{2+} removal reduced the Ca^{2+} signal by 75%. Addition of 3 mM Ca^{2+} increased $[Ca^{2+}]_i$ in cells pretreated with fendiline in Ca^{2+}-free medium. The 50 μM fendiline-induced $[Ca^{2+}]_i$ increase in Ca^{2+}-containing medium was inhibited by 10 μM of La^{3+}, nifedipine, or verapamil. In Ca^{2+}-free medium, pretreatment with 1 μM thapsigargin (an endoplasmic reticulum Ca^{2+} pump inhibitor) to deplete the endoplasmic reticulum Ca^{2+} store partly inhibited 50 μM fendiline-induced Ca^{2+} release; whereas pretreatment with 50 μM fendiline abolished 1 μM thapsigargin-induced Ca^{2+} release. Inhibition of phospholipase C activity with 2 μM U73122 did not alter 50 μM fendiline-induced Ca^{2+} release. Incubation with 50 μM fendiline for 10-30 min decreased cell viability by 10-20%. Together, the findings indicate that in smooth muscle cells fendiline induced $[Ca^{2+}]_i$ increases. Fendiline acted by activating Ca^{2+} influx via L-type Ca^{2+} channels, and by releasing internal Ca^{2+} in a phospholipase C-independent manner. Prolonged exposure of cells to fendiline induced cell death.

Key Word: A10 cells, fendiline, fura-2, Ca^{2+} signaling, smooth muscle

Introduction

A rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is a key messenger for many physiological and pathological events in essentially all types of cells (1-3); however, prolonged elevations in $[Ca^{2+}]_i$ may lead to apoptosis (4).

Fendiline is an anti-anginal drug for treatment of coronary heart diseases (5). In vitro, fendiline was found to inhibit L-type Ca^{2+} channels (6, 7) and calmodulin (8). Notably, fendiline was recently shown to act as a Ca^{2+} mobilizer in renal tubular cells, by releasing stored Ca^{2+} and activating external Ca^{2+} influx (9). The present study examined whether

Corresponding author: Dr. Chung-Ren Jan, Department of Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung 813, Taiwan, ROC. Tel: 886-7-3422121-1509; Fax: 886-7-3468056; E-mail: crjan@isca.vghks.gov.tw
Received December 11, 2000; Revised January 16, 2001; Accepted January 18, 2001.

fendiline could increase $[Ca^{2+}]_i$ in A10 smooth muscle cells. A10 cells have been shown to posses L-type Ca^{2+} channels (10, 11), and internal Ca^{2+} stores that could be released by thapsigargin (12), an endoplasmic reticulum Ca^{2+} pump inhibitor (13).

In this study, by using fura-2 as a Ca^{2+} probe it was shown that fendiline induced significant increases in $[Ca^{2+}]_i$ in A10 cells. The concentration-response relationship was established, and the underlying mechanism of the fendiline response was evaluated. The effect of fendiline on cell viability was examined by trypan blue exclusion.

Materials and Methods

Cell Culture

A10 smooth muscle cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 g/ml streptomycin. Cells were kept at 37C in 5% CO_2-containing humidified air.

Solutions

Physiological buffer solution (pH 7.4) contained (in mM): NCl 150, KCl 5, Hepes 5, glucose 5. Ca^{2+}-containing medium (pH 7.4) contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8, Hepes 10, glucose 5. Ca^{2+}-free medium contained no added Ca^{2+} plus 1 mM EGTA. The experimental solution contained less than 0.1% of solvent (dimethyl sulfoxide or ethanol) which did not affect $[Ca^{2+}]_i$ (n=3).

Optical Measurements of $[Ca^{2+}]_i$

After removal of medium, cells were rinsed twice with physiological buffer solution. After aspirating physiological buffer solution, the cells were bathed in a solution containing 0.05% (w/v) trypsin and 0.53 mM EDTA for 30 s. After cells were detached from the flask, they were suspended in Ca^{2+} medium (10^6/ml) and was loaded with the ester form of fura-2, fura-2/AM (2 μM) for 30 min at 25°C. Cells were washed and resuspended in Ca^{2+} medium before use. 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 RF1503PC spectrofluorophotometer (Shimadzu Incorp., Kyoto, Japan) by recording excitation signals at 340 and 380 nm and emission signal at 510 nm at 1-s intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 (+ 10 mM $CaCl_2$) and 20 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as described previously (14-16).

Chemical Reagents

The reagents for cell culture were from Gibco (Grand Islands, NY, USA). Fura-2/AM was from Molecular Probes (Eugene, OR, USA). All the other reagents were from Sigma (St. Louis, MO, USA).

Cell Viability

Assay Cell viability was determined by trypan blue exclusion. Fifty l of cell suspension was mixed with 50 l of trypan blue isotonic solution (0.2%; w/v) in the absence (control) or presence of 50 μM fendiline. Fendiline was added for 0-30 min before cell viability was determined on a hemocytometer under a microscope.

Statistical Analyses

The traces were typical of 4-6 experiments. All values were reported as the means±S.E.M (n=4-6). Statistical comparisons were determined by using the Student's t test, and significance was accepted when $P<0.05$.

Results

Effect of Fendiline on $[Ca^{2+}]_i$

In Ca^{2+}-containing medium, fendiline at concentrations between 10-50 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner. Figure 1A shows typical records of the $[Ca^{2+}]_i$ increase induced by 50 μM (trace a), 20 μM (trace b), and 10 μM (trace c) fendiline. At a concentration of 1 μM fendiline did not have an effect (trace d). Over a time period of 250 s the $[Ca^{2+}]_i$ signal comprised an initial rise and a sustained phase. The basal $[Ca^{2+}]_i$ was 45±2 nM (n=6). The Ca^{2+} signal-induced by 50 μM fendiline had a net (baseline subtracted) maximum value of 3565 nM (n=6). Fendiline-induced $[Ca^{2+}]_i$ increases saturated at 50 μM because 75 μM fendiline did not induce a greater response. Figure 1C (solid circles) shows the concentration-response curve of the fendiline response. The curve suggests an EC50 value of 20 μM by fitting to the Hill equation.

Effect of External Ca^{2+} Removal on the Fendiline Response

Figure 1B shows that in Ca^{2+}-free medium, 20 μM (trace b) and 50 μM (trace a) fendiline induced concentration-dependent $[Ca^{2+}]_i$ increases (time points

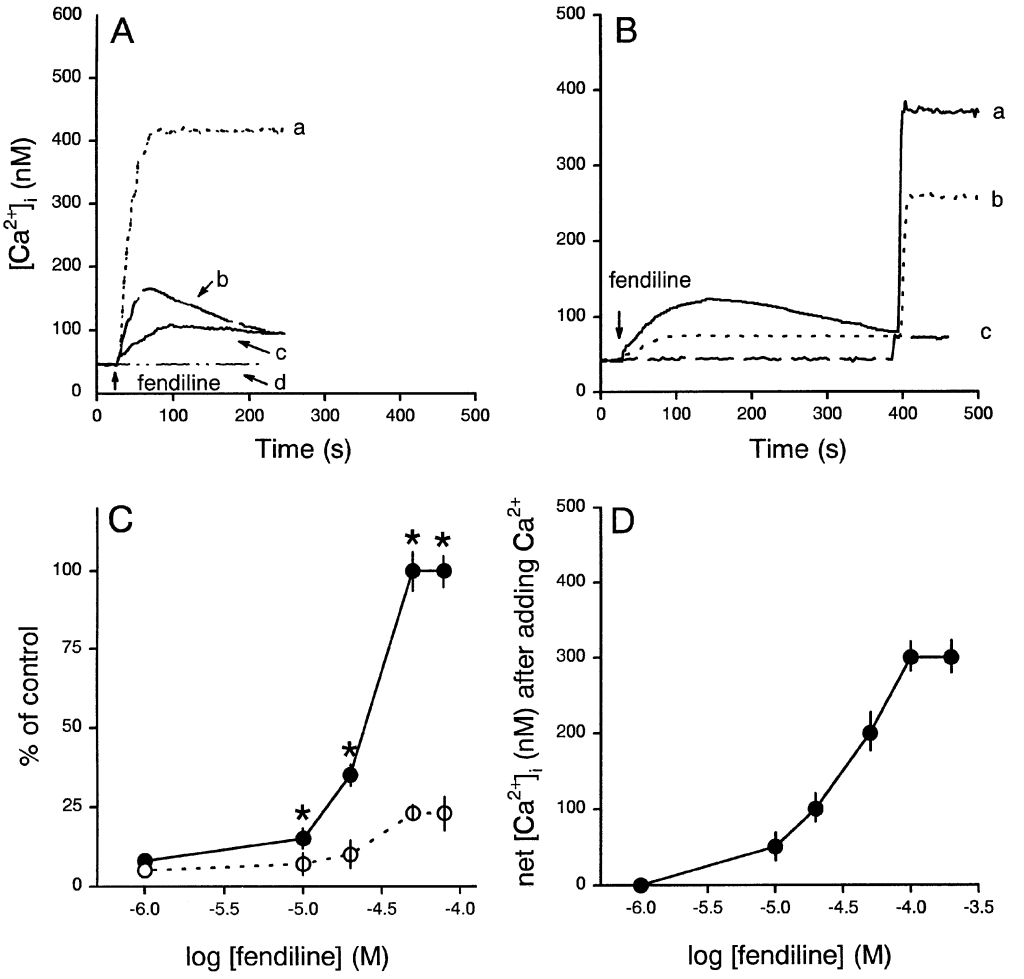

Fig. 1. Effect of fendiline on $[Ca^{2+}]_i$ in fura-2-loaded A10 cells. **A**, typical signals induced by fendiline in Ca^{2+}+-containing medium. Concentrations of fendiline were 50 (trace a), 20 (trace b), 10 (trace c), and 1 (trace e) μM, respectively. **B**, effect of external Ca^{2+} removal on fendiline-induced $[Ca^{2+}]_i$ increases and the effect of readmission of Ca^{2+}. Trace a: fendiline (50 μM) was added at 30 s in Ca^{2+}-free medium followed by addition of 3 mM $CaCl_2$ at 400 s. Trace b: similar to trace a except that 20 μM fendiline was used. Trace c: Ca^{2+} was added without pretreatment with fendiline. **C**, Concentration-response plots of fendiline-induced Ca^{2+} signals in the presence and absence of Ca^{2+}. The y axis is the percentage of control. Control was the net (baseline subtracted) maximum value of the $[Ca^{2+}]_i$ increase induced by 50 μM fendiline in Ca^{2+}-containing medium. Data were mean±SEM. of 4-6 experiments. *P<0.05. **D**, Concentration-response plots of reintroduction of Ca^{2+} based on the data from **B**. The y axis is the net $[Ca^{2+}]_i$ (nM) after adding Ca^{2+}. The x scale is the concentration of fendiline. Data were mean±SEM. of 4-6 experiments. *P<0.05. Traces were typical of 4-6 experiments.

of 30-400 s). The 50 μM fendiline-induced $[Ca^{2+}]_i$ increases had a net maximum value of 78±2 nM (n=5). The concentration-response curve of fendiline-induced $[Ca^{2+}]_i$ increases in Ca^{2+}-free medium is shown in Figure 1C (open circles). The data suggest that Ca^{2+} removal inhibited 10-75 μM fendiline-induced $[Ca^{2+}]_i$ increases by 75±10% in the net maximum value (n=4-6).

Mechanism of Fendiline-Induced Ca^{2+} Influx

In most cells, depletion of internal Ca^{2+} pools usually leads to Ca^{2+} influx via store-operated Ca^{2+} influx (17). Store-operated Ca^{2+} influx was evaluated by reintroduction of Ca^{2+} to cells depleted of Ca^{2+} by fendiline in Ca^{2+}-free medium. Figure 1B (time points of 400-450 s) shows that in Ca^{2+}-free medium, after pretreatment with 20 μM (trace b) or 50 μM (trace a) fendiline for 370 s, addition of 3 mM $CaCl_2$ induced an $[Ca^{2+}]_i$ increase with a net maximum value of 199±2 and 310±5 nM, respectively (n=6). Addition of $CaCl_2$ alone without fendiline pretreatment only induced a small $[Ca^{2+}]_i$ increase with a net maximum of 28±2 nM (trace c; n=4).

Effects of Ca^{2+} Entry Blockers on Fendiline-Induced $[Ca^{2+}]_i$ Increases

Figure 2 shows that in Ca^{2+}-containing medium, 50 μM fendiline-induced $[Ca^{2+}]_i$ increases were

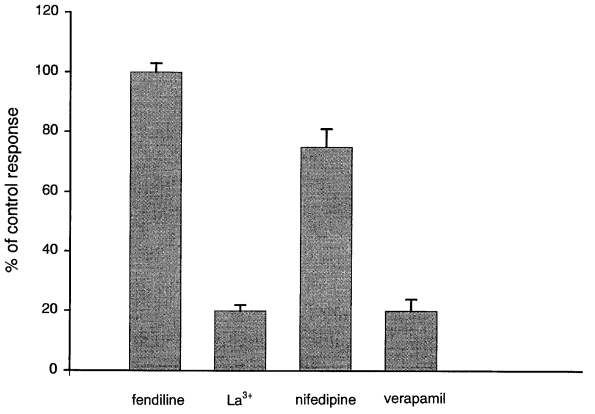

Fig. 2. Effect of Ca^{2+} entry blockers on fendiline-induced $[Ca^{2+}]_i$ increases. In Ca^{2+}-containing medium, 10 μM of La^{3+}, nifedipine or verapamil was added 30 s prior to fendiline. Data were the percentage of control response. Control was the net maximum value of 50 μM fendiline-induced $[Ca^{2+}]_i$ increases. Traces were typical of 4-6 experiments.

markedly inhibited by La^{3+} (10 μM) or verapamil (10 μM), and slightly reduced by 10 μM nifedipine (n=5-6).

Internal Sources of the Fendiline Response

Experiments were performed to explore whether fendiline releases internal Ca^{2+} from the the endoplasmic reticulum, the major Ca^{2+} stores in most cells. Figure 3A shows that in Ca^{2+}-free medium, addition of 1 μM thapsigargin induced a $[Ca^{2+}]_i$ increase with a net maximum of 15±2 nM (n=6). After the endoplasmic reticulum Ca^{2+} store was depleted by thapsigargin for 470 s, addition of 50 μM fendiline induced a $[Ca^{2+}]_i$ increase with a net maximum value of 513 nM which was 50% of the control fendiline response shown in Figure 3B (100±2 nM; n=6; $P<0.05$). Figure 3B shows that after pretreatment with 50 μM fendiline for 470 s, 1 μM thapsigargin failed to increase $[Ca^{2+}]_i$ (n=6).

Effect of Inhibiting Phospholipase C on Fendiline-Induced Ca^{2+} Release

Pretreatment with the phospholipase C inhibitor U73122 (18) in Ca^{2+}-free medium did not alter basal $[Ca^{2+}]_i$ or 50 μM fendiline-induced $[Ca^{2+}]_i$ increases (n=4; not shown).

Effect of Fendiline on Cell Viability

Figure 4 shows that cells were 964% viable (open circles; n=6). Cell viability was not altered by incubation with 50 μM fendiline for 2-5 min, but was reduced to 853% after incubation for 10 min, and was further reduced to 753% after incubation for 15-30 min (n=6).

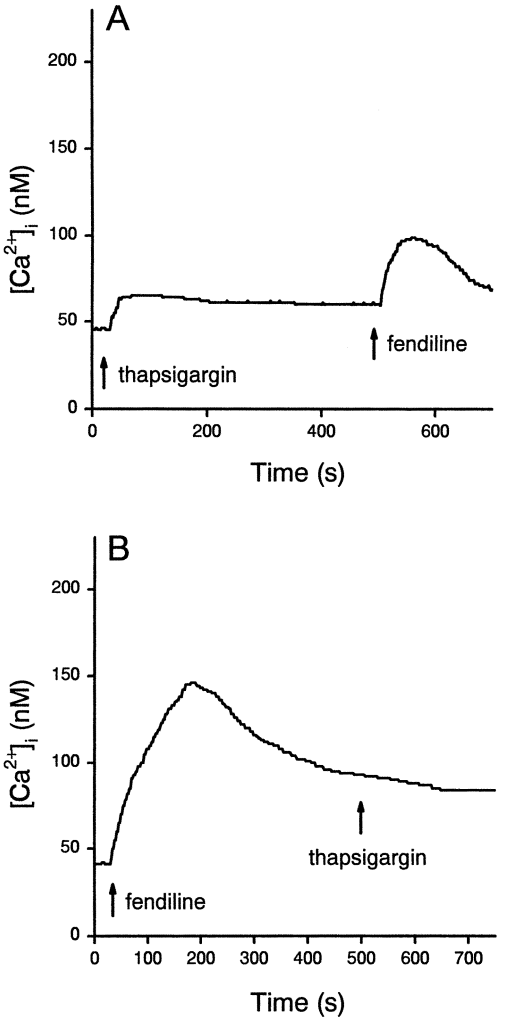

Fig. 3. Internal sources of the fendiline response. **A**, In Ca^{2+}-free medium, 1 μM thapsigargin was added at 30 s followed by 50 μM fendiline added at 500 s. **B**, similar to A except that the order of drug application was reversed. Traces were typical of six experiments.

Discussion

This study showed that in A10 smooth muscle cells fendiline caused significant increases in $[Ca^{2+}]_i$. This is interesting because fendiline is conventionally thought to act as a blocker of L-type Ca^{2+} channels in smooth muscle cells at the concentrations used in the present study (5-8). Our data are supported by a recent study showing that fendiline induced $[Ca^{2+}]_i$ increases in epithelial cells (9). How fendiline acts as both a Ca^{2+} channel blocker in some conditions and as a Ca^{2+} mobilizer in others is unclear. Similarly, SKF96365 and econazole, two drugs commonly used to inhibit store-operated Ca^{2+} influx in various cell

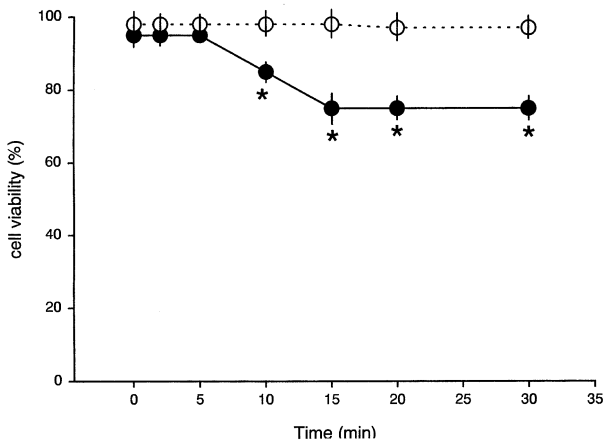

Fig. 4. Effect of fendiline on cell viability. Cell viability was assayed by trypan blue exclusion. The y axis is cell viability in percentage. The x axis is the incubation time of fendiline. Open circles: control. Filled circles: 50 μM fendiline was added. Data were mean±SEM of 4-6 experiments. *P<0.05.

types, were shown to cause $[Ca^{2+}]_i$ increases in renal cells (19, 20). Because our data showed that exposure of cells to fendiline for more than 15 min lead to decrease in cell viability, caution should be exercised in clinical and in vitro use of this drug.

The correlation between fendiline-induced $[Ca^{2+}]_i$ increases and its cytotoxic effect is unclear, but since prolonged elevations in $[Ca^{2+}]_i$ are closely linked to dysfunction and apoptosis in muscle cells (4), the effect of fendiline on $[Ca^{2+}]_i$ may lead to cytotoxicity.

The Ca^{2+} signal induced by fendiline resulted from both external Ca^{2+} influx and internal Ca^{2+} release because removal of external Ca^{2+} inhibited a large portion of the response. Fendiline released internal Ca^{2+} from stores involing the thapsigargin-sensitive endoplasmic reticulum pools bacause pretreatment with 1 μM thapsigargin in Ca^{2+}-free medium for about 9 min reduced 50 μM fendiline-induced $[Ca^{2+}]_i$ increases by half; and consistently, pretreatment with fendiline abolished thapsigargin-induced $[Ca^{2+}]_i$ increases. Our results suggest that fendiline-induced Ca^{2+} release appears to be uncoupled to phospholipase C activity because inhibiting phospholipase C with U73122 did not alter the fendiline response. How fendiline caused Ca^{2+} release from thapsigargin-sensitive stores is currently under investigation.

Fendiline may act Ca^{2+} influx via opening L-type Ca^{2+} channels because 50 μM fendiline-induced $[Ca^{2+}]_i$ increases in Ca^{2+}-containing medium were significantly reduced by L-type Ca^{2+} channel blockers verapamil and nifedipine. The possibility that fendiline may activate Ca^{2+} influx via store-operated Ca^{2+} influx pathway cannot be excluded because after depletion of Ca^{2+} stores with 50 μM fendiline for several min, addition of 3 mM Ca^{2+} induced a $[Ca^{2+}]_i$ increase 5-fold greater than control. Fendiline was recently reported to increase $[Ca^{2+}]_i$ in renal tubular cells by releasing internal Ca^{2+} and ativating external Ca^{2+} influx (9). The effect of fendiline found in the present study was similar to that found in renal cells except a major difference. In renal cells, 50 μM fendiline failed to release more Ca^{2+} after thapsigargin pretreatment, suggesting that fendiline exclusively released Ca^{2+} from thapsigargin-sensitive endoplasmic reticulum stores. This suggests that smooth muscle cells and renal tubular cells are different in the regulation of Ca^{2+} release in response to drug stimulation.

Acknowledgments

This work was supported by grants from National Science Council (NSC89-2320-B-075B-009), Veterans General Hospital-Kaohsiung (VGHKS90-07) and VTY Joint Research Program, Tsou's Foundation (VTY89-P3-21) to CRJ; and VGHKS89-110 to JKH.

References

1. Berridge, M.J. Elementary and global aspects of calcium signalling. *J. Physiol*. (Lond) 499: 291-306, 1997.
2. Berridge, M.J. Inositol trisphosphate and calcium signaling. *Nature* 361: 315-325, 1993.
3. Bootman, M.D., Berridge, M.J. and Lipp, P. Cooking with calcium: the recipes for composing global signals from elementary events. *Cell* 91: 367-373, 1993.
4. Bernardi, P. Mitochondria in muscle cell death. *Ital. J. Neurol. Sci.* 20: 395-400, 1999.
5. Bayer, R. and Mannhold, R. Fendiline: a review of its basic pharmacological and clinical properties. *Pharmatherapeutica* 5: 103-136, 1987.
6. Nawrath, H., Klein, G., Rupp, J. and Wegener, J.W. Shainberg A. Open state block by fendiline of L-type Ca^{2+} channels in ventricular myocytes from rat heart. *J. Pharmacol. Exp. Ther*. 285: 546-552, 1998.
7. Tripathi, O., Schreibmayer, W. and Tritthart, H.A. Fendiline inhibits L-type calcium channels in guinea-pig ventricular myocytes: a whole-cell patch-clamp study. *Br. J. Pharmacol*. 108: 865-869, 1993.
8. Orosz, F. and Christova, T.Y. Ovadi J Functional in vitro test of calmodulin antagonism: effect of drugs on interaction between calmodulin and glycolytic enzymes. *Mol. Pharmacol*. 33: 678-682, 1988.
9. Jan, C.R., Tseng, C.J. and Chen, W.C. Fendiline increases $[Ca^{2+}]_i$ in Madin Darby canine kidney (MDCK) cells by releasing internal Ca2+ followed by capacitative Ca^{2+} entry. *Life Sci.* 66: 1053-1062, 2000.
10. Roullet, J.B., Luft, U.C., Xue, H., Chapman, J., Bychkov, R., Roullet, C.M., Luft, F.C, Haller, H. and McCarron, D.A. Farnesol inhibits L-type Ca2+ channels in vascular smooth muscle cells. *J. Biol. Chem*. 272: 32240-32246, 1997.
11. Xuan, Y.T. and Glass, P.S. Propofol regulation of calcium entry pathways in cultured A10 and rat aortic smooth muscle cells. *Br. J. Pharmacol*. 117: 5-12, 1996.
12. Husain, M., Bein, K., Jiang, L., Alper, S.L., Simons, M., and

Rosenberg, R.D. c-Myb-dependent cell cycle progression and Ca^{2+} storage in cultured vascular smooth muscle cells. *Circ Res*. 80: 617-626, 1997.

13. Thastrup, O., Cullen, P.T., Drobak, B.K., Hanley, M.R. and Dawson, A.P. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci*. USA 87: 2466-2470, 1990.
14. Grynkiewicz, G., Poenie, M. and Tsien, R.Y. A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J. Biol. Chem*. 260: 3440-3450, 1985.
15. Jan, C.R., Yu, C.C. and Huang, J.K. NPC-15199, a novel anti-inflammatory agent, mobilizes intracellular Ca^{2+} in bladder female transitional carcinoma (BFTC) cells. *Chin. J. Physiol*. 43: 1-5, 2000.
16. Jan, C.R., Wu, S.N. and Tseng, C.J. A further investigation of ATP-induced calcium mobilization in MDCK cells. *Chin. J. Physiol*. 42: 33-39, 1999.
17. Putney, J.W. Jr. A model for receptor-regulated calcium entry. *Cell Calcium* 7: 1-12, 1986.
18. Thompson, A.K., Mostafapour, S.P., Denlinger, L.C., Bleasdale, J. E. and Fisher, S.K. The aminosteroid U73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH heuroblastoma cells. *J. Biol. Chem*. 266: 23856-23862, 1991.
19. Jan, C.R., Ho, C.M., Wu, S.N. and Tseng, C.J. Multiple effects of econazole on calcium signaling: depletion of thapsigargin-sensitive calcium store, activation of extracellular calcium influx, and inhibition of capacitative calcium entry. *Biochim. Biophys. Acta.* 1448: 533-542, 1999.
20. Jan, C.R., Ho, C.M., Wu, S.N. and Tseng, C.J. Multiple effects of 1-[b-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole (SKF 96365) on Ca^{2+} signaling in MDCK cells: depletion of thapsigargin-sensitive Ca^{2+} store followed by capacitaive Ca^{2+} entry, activation of a direct Ca^{2+} entry, and inhibition of thapsigargin-induced capacitative Ca^{2+} entry. *N-S Arch. Pharmacol.* 359: 92-101, 1999.