Effect of NPC-15199 on Ca^{2+} Levels in Renal Tubular Cells

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Abstract

In Madin-Darby canine kidney (MDCK) cells, effect of NPC-15199 on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was investigated by using fura-2. NPC-15199 (100-1000 μM) caused a rapid and sustained increase of $[Ca^{2+}]_i$ in a concentration-dependent manner (EC_{50}=500 μM). NPC-15199-induced $[Ca^{2+}]_i$ rise was prevented by 70% by removal of extracellular Ca^{2+}, but was not changed by dihydropyridines, verapamil and diltiazem. In Ca^{2+}-free medium, carbonylcyanide m-chlorophenylhydrazone (CCCP; 2 μM), a mitochondrial uncoupler, and thapsigargin (1 μM), an inhibitor of the endoplasmic reticulum (ER) Ca^{2+}-ATPase, caused a monophasic $[Ca^{2+}]_i$ rise, respectively, after which the increasing effect of NPC-15199 (1 mM) on $[Ca^{2+}]_i$ was substantially attenuated; also, pretreatment with NPC-15199 abolished CCCP- and thapsigargin-induced $[Ca^{2+}]_i$ rises. U73122, an inhibitor of phoispholipase C, abolished 10 μM ATP (but not 1 mM NPC-15199)-induced $[Ca^{2+}]_i$ rise. These results suggest that NPC-15199 rapidly increases $[Ca^{2+}]_i$ by stimulating both extracellular Ca^{2+} influx and intracellular Ca^{2+} release via as yet unidentified mechanism(s).

Key Words: Ca^{2+}, Ca^{2+} stores, fura-2, MDCK, NPC-15199, renal tubular cells

Introduction

Anti-inflammatory properties have been ascribed to a series of N-(fluorenyl-9-methoxycarbonyl) amino acids called leumedins that inhibit the activity of granulocytes and T-lymphocytes (4). Among these leumedins, NPC-15199 (N-(9-fluorenylmethoxycarbonyl-L-leucine) has been shown to prevent mucosal injury and dysfunction in a guinea pig model of intestinal inflammation (19). It was found that inhibition of granulocyte infiltration was not essential for the beneficial effects of NPC-15199 and

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Received: June 19, 2002; Revised: August 2, 2002; Accepted: August 12, 2002.

alternative actions of NPC-15199 might be involved. It has been recently shown that NPC-15199 and another similar drug NPC-14686 caused significant $[Ca^{2+}]_i$ rises in bladder cells and renal tubular cells, respectively (9, 14, 15). Since NPC-15199 and its metabolites are most likely excreted via urine, the present study was aimed to explore the effect of NPC-15199 on Ca^{2+} movement in renal tubular cells.

An increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) plays a crucial triggering role in diverse physio-pathological processes in all cell types (1, 2). However, many cytotoxical phenomena are linked to abnormal Ca^{2+} movement (3). $[Ca^{2+}]_i$ is controlled by an intricate interplay of many events. In non-excitable cells, activation of receptors coupled to phospholipase C results in a $[Ca^{2+}]_i$ increase (2). The Ca^{2+} signal is caused by store Ca^{2+} release and/or extracellular Ca^{2+} influx. One major Ca^{2+} store is depleted by an increase in cytosolic levels of inositol 1,4,5-trisphosphate, a second messenger formed by phospholipase C (1). Mobilization of store Ca^{2+} may result in Ca^{2+} influx via the process of store-operated Ca^{2+} entry (20). Other unknown Ca^{2+} entry pathways may exist (7).

The Madin-Darby canine kidney (MDCK) cell line is a useful model for renal research. It has been shown that in this cell, $[Ca^{2+}]_i$ can increase in response to the stimulation of various endogenous and exogenous compounds, such as ATP (11), bifonazole (5), and bradykinin (10), etc.. Using fura-2 as a Ca^{2+}-sensitive dye, here we show that NPC-15199 induces concentration-dependent $[Ca^{2+}]_i$ rises both in the presence and absence of extracellular Ca^{2+} in MDCK cells. The Ca^{2+} responses are characterized, the concentration-response relationships in the presence and absence of extracellular Ca^{2+} are established, and the pathways underlying NPC-15199-induced Ca^{2+} influx and Ca^{2+} release are evaluated.

Materials and Methods

Cell Culture

MDCK cells obtained from American Type Culture Collection (CRL-6253) were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were kept at 37°C in 5% CO^{2+}-containing humidified air.

Solutions

Ca^{2+}-containing medium (pH 7.4) had (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 2; Hepes 10; glucose 5. Ca^{2+}-free medium contained similar components as Ca^{2+}-containing medium except that $CaCl_2$ was substituted with 1 mM EGTA. Chemicals were dissolved in water, ethanol or dimethyl superoxide as stock solutions. Final concentrations of organic solvents in the $[Ca^{2+}]_i$ measurements were less than 0.1% and did not alter basal $[Ca^{2+}]_i$ (n=3; not shown).

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

Trypsinized cells (10^6/ml) were allowed to recover in culture medium for 1 hour before loading with 2 μM fura-2/acetoxy methyl for 30 min at 25°C. The cells were washed and re-suspended in Ca^{2+}-containing medium. 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 spectrofluorophotometry (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 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 (8).

Mn^{2+} quench of fura-2 fluorescence was performed in Ca^{2+}-containing medium containing 50 μM $MnCl_2$, by recording the excitation signal at 360 nm and emission signal at 510 nm at 1-s intervals.

Chemicals

The reagents for cell culture were from Gibco (Gaithersburg, MD, USA). Fura-2/acetoxy methyl was from Molecular Probes (Eugene, OR, USA). U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) and U73343 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione) were from Biomol (Plymouth Meeting, PA, USA). NPC-15199 (N-(9-fluorenylmethoxycarbonyl-L-leucine) was from Tocris (Bristol, UK). The other reagents were from Sigma (St. Louis, MO, USA).

Statistics

Data are reported as means±S.E.M. of 3-5 replicates. Statistical comparisons were determined by using Student's *t* test, and significance was accepted when $P<0.05$.

Results

Effect of NPC-15199 on $[Ca^{2+}]_i$

In Ca^{2+}-containing medium, NPC-15199 (≥100 μM) caused an immediate increase in $[Ca^{2+}]_i$, which

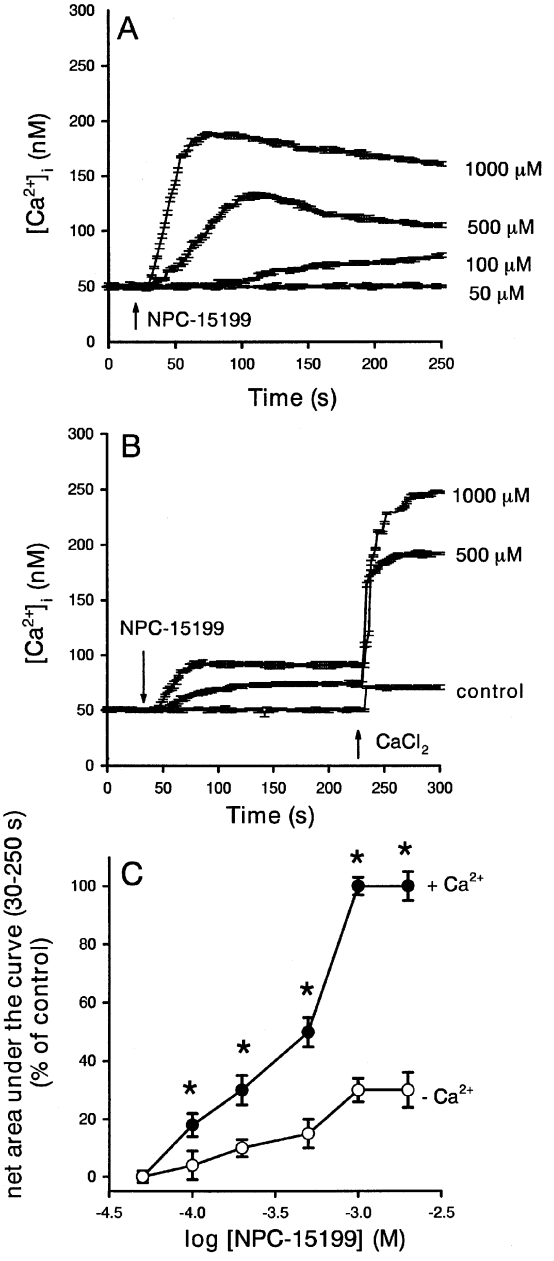

Fig. 1. NPC-15199-induced concentration-dependent $[Ca^{2+}]_i$ rises in MDCK cells: effect of extracellular Ca^{2+} removal. In Ca^{2+}-containing (A) or Ca^{2+}-free (B) medium, NPC-15199 was added at time points indicated by arrows. Fig. 1A: The concentration of NPC-15199 was indicated besides the trace. Fifty μM NPC-15199 failed to increase $[Ca^{2+}]_i$. (B) NPC-15199 (500 or 1000 μM) was added at 30 s followed by 3 mM Ca^{2+} added at 230 s. Control: Ca^{2+} was added without NPC-15199 treatment. (C) A concentration-response curve of NPC-15199 for $[Ca^{2+}]_i$ rises measured in Ca^{2+}-containing (●) or Ca^{2+}-free (○) medium. A value of 100% represents the $[Ca^{2+}]_i$ rise (baseline subtracted) obtained in Ca^{2+}-containing medium with 1000 μM NPC-15199. Data are means±S.E.M. (n=3-5). *P<0.05 compared with Ca^{2+}-free medium.

lasted for, at least, 220 s after the addition of NPC-15199 (Fig. 1A); e.g. NPC-15199 (1000 μM)-induced $[Ca^{2+}]_i$ rise attained to 195±2 nM (n=5) over baseline (50±1 nM; n=5). The increasing effect of NPC-15199 was concentration-dependent with an EC_{50} of 500 μM (Figs. 1A and 1C).

Effect of Extracellular Ca^{2+} Removal on NPC-15199-Induced $[Ca^{2+}]_i$ Rise

To examine whether/how influx of extracellular Ca^{2+} and/or mobilization of Ca^{2+} from the intracellular store site(s) may contribute to the NPC-15199-induced $[Ca^{2+}]_i$ rise, effect of NPC-15199 on $[Ca^{2+}]_i$ was measured in the absence of extracellular Ca^{2+} (Fig. 1B). In Ca^{2+}-free medium, the $[Ca^{2+}]_i$ rises caused by 500 and 1000 μM NPC-15199 were attenuated, with no change in the basal $[Ca^{2+}]_i$ (50±3 nM; n=5). NPC-15199 (1000 μM) increased $[Ca^{2+}]_i$ by 45±2 nM (n=5) over baseline and the signal last for at least 200 s without decay. The data further show that addition of 3 mM Ca^{2+} afterwards induced an immediate $[Ca^{2+}]_i$ rise of 162±2 nM, which was 7.7±0.2-fold ($P<0.05$) over control (21±2 nM; n=4; no NPC-15199 pre-treatment). Fig. 1C shows that removal of extracellular Ca^{2+} inhibited NPC-15199 (100-1500 μM)-induced $[Ca^{2+}]_i$ rise by 72±2%, without changing the EC_{50} of 500 μM.

Effect of NPC-15199 on Mn^{2+}-Induced Quench of Fura-2 Fluorescence

Experiments were performed to confirm that the reduced NPC-15199-induced $[Ca^{2+}]_i$ rise by removal of extracellular Ca^{2+} was not due to EGTA-caused depletion of store Ca^{2+}. Mn^{2+} enters cells through similar pathways as Ca^{2+} but quenches fura-2 fluorescence at all excitation wavelengths (18). Thus, quench of fura-2 fluorescence excited at the Ca^{2+}-insensitive excitation wavelength of 360 nm by Mn^{2+} indicates Ca^{2+} influx. Fig. 2 shows that 1 mM NPC-15199 induced an immediate decrease in the 360 nm excitation signal, and the decrease attained to 51±3 (n=4) arbitrary units within 70 s, which was significantly lower than control (no NPC-15199 was added) ($P<0.05$). Subsequently, NPC-15199-induced Mn^{2+} influx gradually attenuated.

Effects of Ca^{2+} Channel Inhibitors on NPC-15199-Induced $[Ca^{2+}]_i$ Rise

In Ca^{2+}-containing medium, NPC-15199 (1 mM)-induced $[Ca^{2+}]_i$ rise was not altered by 10 μM nifedipine, nimodipine, verapamil or diltiazem (data not shown; n=3; $P>0.05$).

Stores for NPC-15199-Induced Ca^{2+} Release

Experiments were conducted to investigate the

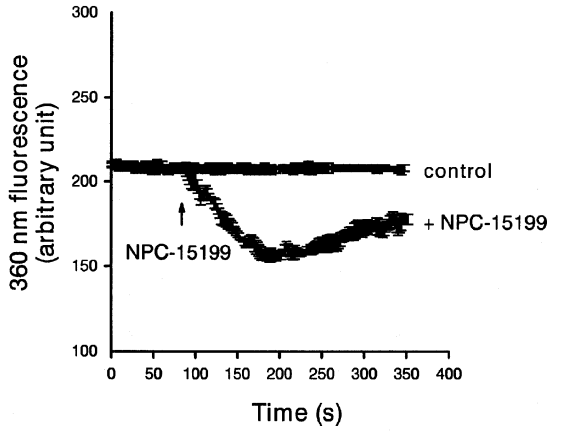

Fig. 2. Effect of NPC-15199 on Ca^{2+} influx by measuring Mn^{2+} quench of fura-2 fluorescence in Ca^{2+}-containing medium. $MnCl_2$ (50 μM) was added to cells before fluorescence measurements. Control: in the absence of NPC-15199. NPC-15199 (1 mM) was added at 70 s. Data are means±S.E.M. of five replicates.

role of the Ca^{2+} stored in the endoplasmic reticulum (ER) and mitochondria, the major Ca^{2+} stores in MDCK cells (12, 13, 16), in NPC-15199-induced Ca^{2+} release. Fig. 3A shows that in Ca^{2+}-free medium, addition of 1 mM NPC-14686 prevented carbonylcyanide m-chlorophenylhydrazone (2 μM; CCCP) and thapsigargin (1 μM; an ER ATPase inhibitor) (21) from releasing store Ca^{2+}; however, Fig. 3B shows that addition of CCCP or thapsiagrgin increased $[Ca^{2+}]_i$ by 23±2 nM or 80±3 nM, respectively (n=5). Subsequently applied NPC-15199 (1 mM) induced a small $[Ca^{2+}]_i$ rise of 152 nM which was 70% ($P<0.05$) smaller than control shown in Fig. 3A. CCCP did not potentiate the inhibiting effect of thapsigargin on NPC-15199-induced $[Ca^{2+}]_i$ rise (data not shown).

No Involvement of Phospholipase C in NPC-15199-Induced Ca^{2+} Release

We examined whether phospholipase C (PLC)-inositol 1,4,5-trisphosphate (IP3) pathway may be involved in NPC-15199-induced Ca^{2+} mobilization from the ER. Fig. 4A shows that 10 μM ATP, an agonist for P2Y type ATP receptors that mobilizes intracellular Ca^{2+} from the ER via increasing IP_3 (11), caused an instantaneous monophasic $[Ca^{2+}]_i$ rise (181±3 nM; n=3) in Ca^{2+}-free medium. Fig. 4B, however, shows that pretreatment with 2 μM U73122, an inhibitor of PLC (22), abolished ATP-induced $[Ca^{2+}]_i$ rise; in contrast, 10 μM U73343, a biologically inactive analogue of U73122 (22), failed to to so (data not shown, n=3). Even in the presence of 2 μM U73122, 1 mM NPC-15199 caused a $[Ca^{2+}]_i$ rise indistinguishable from control (Fig. 3A; $P>0.05$).

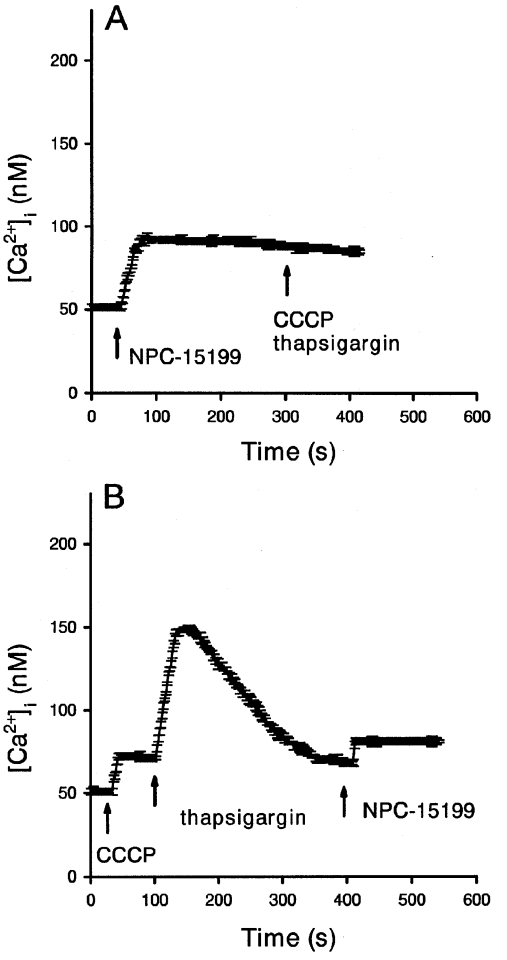

Fig. 3. Intracellular sources of NPC-15199-induced $[Ca^{2+}]_i$ rises. The experiments were performed in Ca^{2+}-free medium. (A) NPC-151199 (1 mM) was added at 30 s followed by CCCP (2 μM) and thapsigargin (1 μM) added together at 300 s. (B) CCCP was added at 30 s followed by thapsigargin and NPC-15199 added at 100 s and 400 s, respectively. Data are means±S.E.M. of five replicates.

Discussion

This study shows that NPC-15199 evoked a concentration-dependent $[Ca^{2+}]_i$ rise in renal tubular cells. This is consistent with our previous data that another leumedin (NPC-14686) exerted similar effects. A regulated rise in $[Ca^{2+}]_i$ has been shown to be a key message for normal renal physiology. The balance of a high extracellular osmolarity in the kidney medulla is regulated by osmolytes in the cells. The control of cell volume during hypotonic conditions results in a Ca^{2+}-dependent release of osmolytes (23). Many endogenous compounds, such as ATP, activate renal cells via causing a well-tuned $[Ca^{2+}]_i$ rise (11). However, an uncontrolled $[Ca^{2+}]_i$ rise may lead to tubular injury resulting in a fall in glomerular filtration rate, including increased tubuloglomerular feedback and distal tubular obstruction, in ischemic acute renal

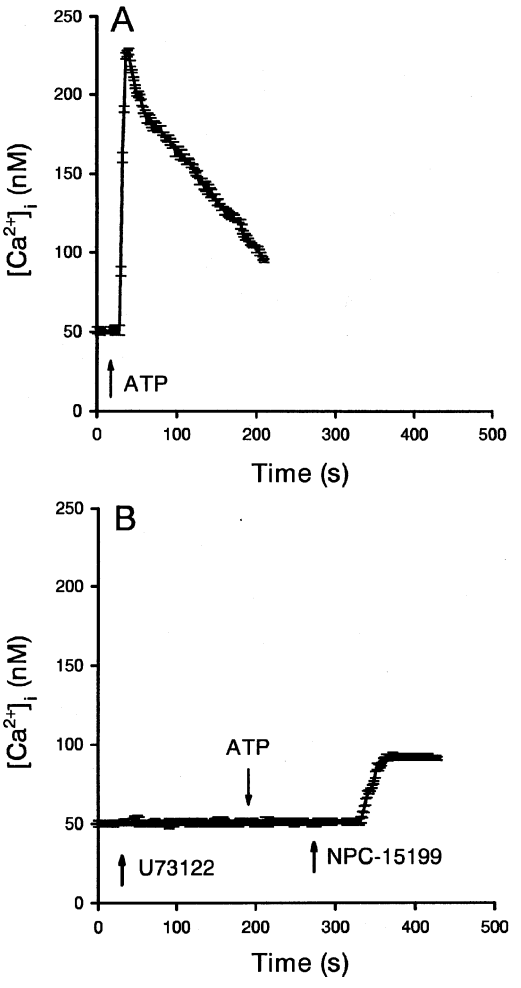

Fig. 4. Role of inositol 1,4,5-trisphosphate in NPC-15199-induced Ca^{2+} release. The experiments were performed in Ca^{2+}-free medium. (A) ATP (10 μM) was added at 20 s. (B) U73122 (2 μM), ATP (10 μM), and NPC-15199 (1 mM) were added at time points indicated by arrows. Data are means±S.E.M. of four replicates.

failure (6). Our data suggest that leumedins as a category may alter renal function via increasing $[Ca^{2+}]_i$; however, the effect of these amino acids in human has not been examined.

The data show that NPC-15199 increases $[Ca^{2+}]_i$ more by causing Ca^{2+} influx than by inducing store Ca^{2+} release because the response was reduced by 70% by removal of extracellular Ca^{2+}. This reduction in the response was not caused by EGTA-induced depletion of store Ca^{2+} because Mn^{2+} quench results support that Ca^{2+} influx occurred during NPC-15199 stimulation. NPC-15199 may induce Ca^{2+} influx via store-operated Ca^{2+} entry, a process triggered by store Ca^{2+} depletion (20) that has been previously shown to play a main role in Ca^{2+} influx in MDCK cells (9, 14, 15). This argument is supported by the findings that addition of Ca^{2+} after NPC-15199 had depleted store $^{2+}$ induced an immediate $[Ca^{2+}]_i$ rise, and that the Ca^{2+} influx was insensitive to voltage-gated Ca^{2+} entry blockers. However, NPC-151199 may also directly open some as yet unknown plasma membrane channels before inducing store Ca^{2+} release, judging from the dramatic effect of Ca^{2+} removal on the initial phase of NPC-15199-induced $[Ca^{2+}]_i$ rise (Fig. 1A vs. Fig. 1B). Recently, a Ca^{2+}-activated nonselective cation channel (TRPM4) has been cloned in excitable and non-excitable cells (17). TRPM4 is activated following receptor-mediated Ca^{2+} mobilization, representing a regulatory mechanism that controls the magnitude of Ca^{2+} influx by modulating the membrane potential and, with it, the driving force for Ca^{2+} entry through other Ca^{2+}-permeable pathways. Thus it remains possible that Ca^{2+} entry mechanisms other than depletion-activated channels may be important in agonist-evoked Ca^{2+} influx in non-excitable cells (17).

The thapsigargin-sensitive ER store (the dominant Ca^{2+} store in MDCK cells) (9, 14, 15), appears to play a major role in NPC-15199-induced Ca^{2+} release because NPC-15199 completely depleted the ER Ca^{2+} store, and thapsigargin treatment reduced a large part of the NPC-15199-induced $[Ca^{2+}]_i$ rise. Mitochondria did not play a significant role in NPC-15199-induced Ca^{2+} release because depletion of mitochondrial Ca^{2+} with CCCP did not potentiate thapsigargin-induced decrease in NPC-15199-induced Ca^{2+} release. The NPC-15199-induced Ca^{2+} release did not require a preceding elevation in cytosolic IP3 levels because it was not altered by suppression of phospholipase C. The IP_3-independent component of the Ca^{2+} releasing event is unknown, and may be related to inhibition of Ca^{2+} pump or permeabilization of the endoplasmic reticulum membranes.

Together, this study shows that NPC-15199 induced a $[Ca^{2+}]_i$ rise in MDCK cells by causing extracellular Ca^{2+} influx and intracellular Ca^{2+} release. Compared with bladder cancer cells (15), MDCK renal tubular cells respond similarly, in terms of Ca^{2+} signaling, to NPC-15199. The *in vivo* significance of NPC-15199-induced Ca^{2+} movement in the urinary system needs further investigation.

Acknowledgments

This work was supported by grants from National Science Council (NSC90-2320-B-075B-006), Veterans General Hospital-Kaohsiung (VGHKS91-17) to C.R.J.; VGHKS91-66 to Y.C.L.; VGHKS 91-21 to H.T.C.; VGHKS91-99 to B.P.J.; and VGHKS91-97 (NSC90-2320-B-075B-013) to J.K.H.

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