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NPC-15199, A Novel Anti-Inflammatory Agent, Mobilizes Intracellular Ca^{2+} in Bladder Female Transitional Carcinoma (BFTC) Cells

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Abstract

This report demonstrates that NPC-15199 [(N-(9-fluorenylmethoxycarbonyl)L-leucine)], a novel anti-inflammatory agent, increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in human bladder female transitional cancer (BFTC) cells. Using fura-2 as a Ca^{2+} probe, NPC-15199 (0.1-2 mM) was found to increase $[Ca^{2+}]_i$ concentration-dependently. The response saturated at 2-5 mM NPC-15199. The $[Ca^{2+}]_i$ increase comprised an initial rise, a slow decay, and a plateau. Ca^{2+} removal partly inhibited the Ca^{2+} signals. In Ca^{2+}-free medium, pretreatment with 1 mM NPC-15199 abolished the $[Ca^{2+}]_i$ increase induced by 1 μM thapsigargin (an endoplasmic reticulum Ca^{2+} pump inhibitor); and after pretreatment with thapsigargin, NPC-15199-induced Ca^{2+} release was dramatically inhibited. This indicates that NPC-15199 released internal Ca^{2+} mostly from the endoplasmic reticulum. Adding 3 mM Ca^{2+} increased $[Ca^{2+}]_i$ in cells pretreated with 1 mM NPC-15199 in Ca^{2+}-free medium. Together, the findings suggest that in BFTC bladder cancer cells, NPC-15199 induced Ca^{2+} release from the endoplasmic reticulum and activating Ca^{2+} entry.

Key Word: BFTC cells, bladder cell carcinoma, NPC-15199, fura-2, Ca^{2+} signaling

Introduction

Anti-inflammatory properties have been ascribed to a series of N-(fluorenyl-9-methoxy-carbonyl) amino acids that inhibit the activity of granulocytes and T-lymphocytes. The compounds were active against oxazolone dermatitis in mice and adjuvant arthritis in rats, models in which activated T lymphocytes are implicated (4). Among these compounds, NPC-15199 was studied in more detail. NPC-15199 was found to inhibit ileitis in guinea pigs (8) and block recruitment of neutrophils into the inflammatory site (4). NPC-15199 was effective in blocking antigen arthritis in rabbits and was effective in a therapeutic protocol, reversing oxazolone edema (4). However, the mechanisms of NPC-15199's action are largely unknown.

In an attempt to serch for new chemotherapeutic drugs, in the present study the effect of NPC-15199 on Ca^{2+} signaling in human baldder female transitional cancer (BFTC) cells was examined. 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 (3); however, prolonged elevations in $[Ca^{2+}]_i$ may lead to apoptosis (3).

In non-excitable cells, activation of receptors coupled to phospholipase C often results in an increase in $[Ca^{2+}]_i$ (2). The $[Ca^{2+}]_i$ signal is usually caused by internal Ca^{2+} release and/or external Ca^{2+} influx.

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Inositol 1,4,5-trisphosphate (IP_3) is an important messenger for Ca^{2+} release (1). By mechanisms yet to be defined, Ca^{2+} store depletion results in external Ca^{2+} influx via a process termed "capacitative Ca^{2+} entry"(9).

BFTC cells have been used as an *in vitro* model for investigation of molecular biology of bladder cell carcinoma (5). Ca^{2+} signaling in this epithelial-like cell line has not been examined. In the present study it was found that NPC-15199 increased $[Ca^{2+}]_i$ in BFTC cells, by using fura-2 as a Ca^{2+} probe. The concentration-response relationship has been established, and the underlying mechanisms of the NPC-15199 response determined.

Materials and Methods

Cell Culture

BFTC 905 human bladder female transitional cancer cells were cultured in Macoy's 5a medium supplemented with 5-10% 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+} 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 $[Ca^{2+}]_i$

Trypsinized cells (10^6/ml) were loaded with the ester form of fura-2, fura-2/AM (2 μM) for 30 min at 25°C in Ca^{2+} medium. 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 of cells. Fluorescence was monitored with a Shimadzu RF1503PC spectrofluorophotometer by recording excitation signals at 340 and 380 nm and emission signal at 510 nm at 1-sec intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 and 20 mM EGTA sequentially at the end of an experiment. $[Ca^{2+}]_i$ was calculated as described previously (6).

Chemical Reagents

The reagents for cell culture were from Gibco. Fura-2/AM was from Molecular Probes. NPC-15199 was from TOCRIS. All other reagents were from Sigma.

Statistical Analyses

The traces are typical of 4-5 experiments. All values were reported as the means±S.E. (n=4-5). Statistical comparisons were determined by using the Student's paired t test, and significance was accepted when $p<0.05$.

Results

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

In the presence of external Ca^{2+}, NPC-15199 (0.1-2 mM) increased $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 1A). The response saturated at 2 mM NPC-15199 because the responses caused by 2 and 5 mM NPC-15199 were similar. Over a time period of 5 min the $[Ca^{2+}]_i$ signal comprised a slow initial rise, a slow decay and a sustained phase. For example, at a concentration of 1 mM, NPC-15199 induced a $[Ca^{2+}]_i$ increase which reached a maximum 48±3 s (n=4, $p<0.05$) later at a net value of 189±12 nM (n=4, $p<0.05$), followed by a sustained phase. The rise of the Ca^{2+} signal was slower in response to lower concentrations of NPC-15199. Figure 1B shows that NPC-15199 (1 mM) induced an increase in the 340 nm excitation signal accompanied by a corresponding decrease in the 380 nm excitation signal. This suggests that the rises in fura-2 340/380 ratio signals induced by NPC-15199 most likely reported increases in $[Ca^{2+}]_i$.

Effect of External Ca^{2+} Removal on the NPC-15199 Response

Experiments were performed to explore the effect of external Ca^{2+} removal on NPC-15199-induced $[Ca^{2+}]_i$ increase. Shown in Figure 1C was the effect of Ca^{2+} removal on 1 mM NPC-15199-induced response. The maximum value of this response was 60±5 nM (n=4, $p<0.05$). The concentration-response relationships of NPC-15199-induced $[Ca^{2+}]_i$ increase in the presence of external Ca^{2+} are illustrated in Figure 1D.

The Internal Source of the NPC-15199 Response

Figure 2A shows that in Ca^{2+}-free medium, adding 1 μM thapsigargin, an endoplasmic reticulum Ca^{2+} pump inhibitor (10), induced a significant $[Ca^{2+}]_i$ increase with a net maximum value of 51±4 nM (n=4; $p<0.05$). This suggests that thapsigargin

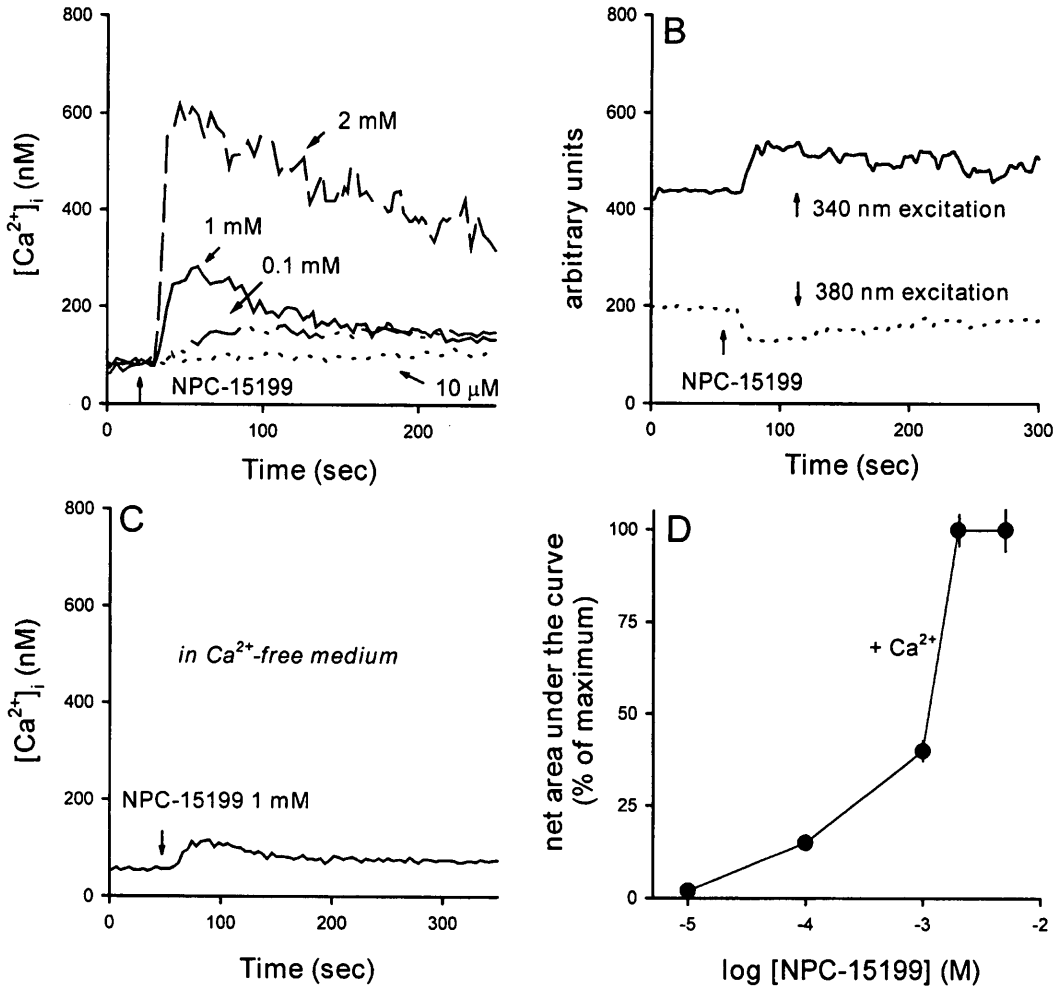

Fig. 1. Effects of NPC-15199 on $[Ca^{2+}]_i$ in fura-2-loaded BFTC cells. *A*, NPC-15199-induced $[Ca^{2+}]_i$ increases in Ca^{2+} medium. Concentrations of NPC-15199 were 2 mM, 1 mM, 0.1 mM and 10 μM, respectively. The experiments were performed in Ca^{2+} medium. *B*, NPC-15199 (1 mM)-induced changes in the 340 nm and 380 nm excitation wavelength signals (emission wavelength was 510 nm). *C*, Effect of external Ca^{2+} removal on NPC-15199-induced $[Ca^{2+}]_i$ increase. NPC-15199 (1 mM) was added at 30 s in Ca^{2+}-free medium. *D*, A concentration-response plot of NPC-15199-induced Ca^{2+} signal in Ca^{2+} medium. The y axis is the percentage of maximum response in the net area under the curve (30-250 s).

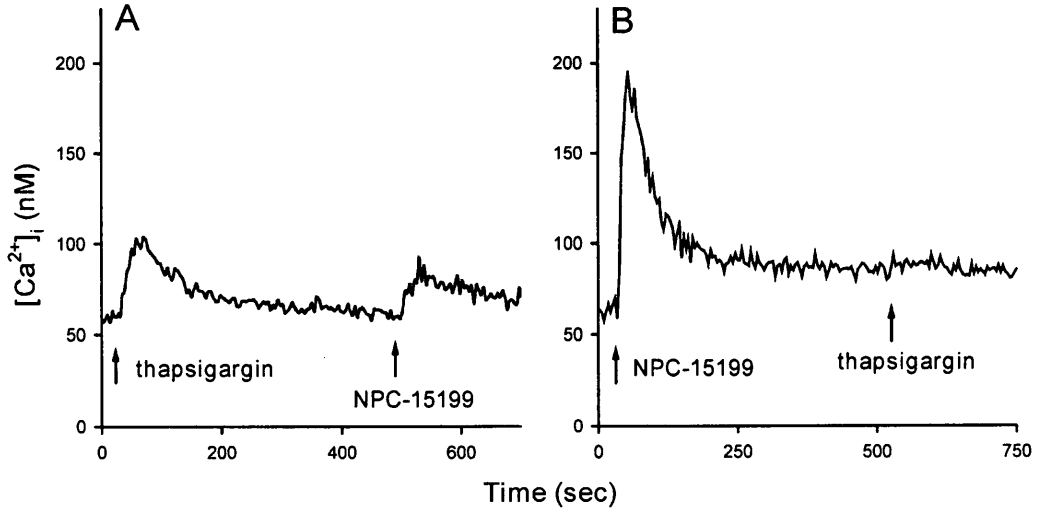

Fig. 2. Internal Ca^{2+} stores of NPC-15199-induced Ca^{2+} release. *A*, In Ca^{2+}-free medium, 1 μM thapsigargin and 1 mM NPC-15199 were added as shown. *B*, In Ca^{2+}-free medium, 1 mM NPC-15199 and 1 μM thapsigargin were added as shown.

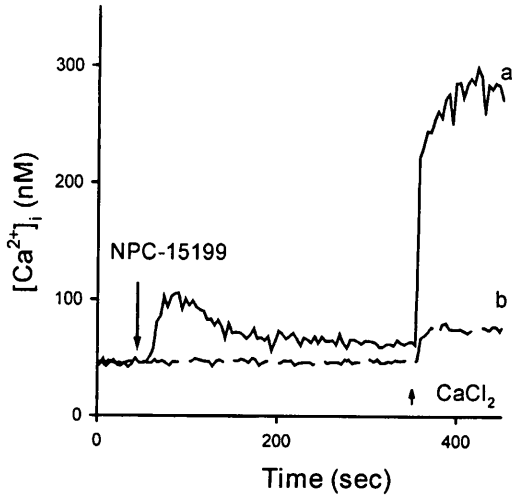

Fig. 3. NPC-15199-induced capacitative Ca^{2+} entry. Capacitative Ca^{2+} entry was induced by depleting Ca^{2+} stores in Ca^{2+}-free medium followed by adding 3 mM $CaCl_2$. *Trace a*: NPC-15199 (1 mM) was added at 30 s followed by $CaCl_2$ at 350 s. *Trace b* (control): $CaCl_2$ was added without NPC-15199 preincubation.

released Ca^{2+} from the endoplasmic reticulum Ca^{2+} store. After the thapsigargin response had subsided, 1 mM NPC-15199 only induced a transient increase in $[Ca^{2+}]_i$ with a net maximum of 24±3 nM (n=4, $p< 0.05$). Figure 2B shows that in Ca^{2+}-free medium, pretreatment with 1 mM NPC-15199 for ~ 500 s abolished the $[Ca^{2+}]_i$ increase induced by thapsigargin (n=5).

The Mechanism of NPC-15199-Induced Ca^{2+} Influx

Depletion of internal Ca^{2+} pools often triggers Ca^{2+} influx via capacitative Ca^{2+} entry (9). However, whether capacitative Ca^{2+} entry exists in bladder tumor cells is completely unknown. Figure 3A shows that in Ca^{2+}-free medium, after pretreating with NPC-15199 for 310 s, adding 3 mM $CaCl_2$ evoked an increase in $[Ca^{2+}]_i$ with a net maximum of 250±5 nM (*trace a*; n=4; $p<0.05$). Adding $CaCl_2$ alone only induced a small $[Ca^{2+}]_i$ increase with a net maximum of 24±2 nM (trace b; n=4; $p<0.05$).

Discussion

To our knowledge, this study is the first to report that the novel anti-inflammatory agent, NPC-15199, can cause significant increases in $[Ca^{2+}]_i$ in a human bladder tumor cell line, and to dissect the underlying mechanisms and regulation of this $[Ca^{2+}]_i$ signal.

We found that NPC-15199 increased $[Ca^{2+}]_i$ at concentrations between 0.1-2 mM, and the response saturated at 2-5 mM of the drug. In inflammatory events, it was shown that NPC-15199 blocks neutrophil recruitment via inhibiting leukocyte adhesion to endothelial cells with an IC_{50} of tens of μM (4). Further, in animal experiments, NPC-15199 was administered daily (10 or 100 mg/kg, s.c.) in guinea pig and was found to exhibit potent inhibition of ileitis (8). Thus, the concentrations (0.1-1 mM) at which NPC-15199 increases $[Ca^{2+}]_i$ in bladder cancer cells may be physiologically revelant.

The Ca^{2+} signal induced by NPC-15199 results from external Ca^{2+} influx and internal Ca^{2+} release, because external Ca^{2+} removal partly inhibited the signal. The Ca^{2+} store of 1 mM NPC-15199-induced Ca^{2+} release appears to mainly consist of the thapsigargin-sensitive endoplasmic reticulum store, because in Ca^{2+}-free medium, pretreatment with 1 mM NPC-15199 completely depleted the Ca^{2+} stores sensitive to 1 μM thapsigargin, and pretreatment with thapsigargin for 500 s inhibited ~75% of the NPC-15199-induced $[Ca^{2+}]_i$ response. NPC-15199 (1 mM) essentually failed to release $Ca^{2+}]_i$ after thapsigargin pretreatment for 1000 s (not shown).

Another question was how NPC-15199 induces Ca^{2+} influx. It was found that after 1 mM NPC-15199 depleted Ca^{2+} stores for 400-500 s in Ca^{2+}-free medium, addition of Ca^{2+} induced an increase in $[Ca^{2+}]_i$ which was 10-fold greater than control in maximum value. This suggests that NPC-15199-induced Ca^{2+} influx was via capacitative Ca^{2+} entry which was triggered by Ca^{2+} store depletion, or via other pathways such as receptor-operated Ca^{2+} channels or second messenger-operated channels.

Together, this study investigated the effect of NPC-151999, a novel anti-inflammatory drug, on $[Ca^{2+}]_i$ in BFTC human bladder tumor cells, and examined the underlying mechanisms. This is the first study to show that NPC-15199 is capable of inducing prolonged increases in $[Ca^{2+}]_i$ in a tumor cell. Since prolonged elevations in $[Ca^{2+}]_i$ will lead to apoptosis, the potential of this drug as an anti-tumor agent deserves further investigation.

Acknowledgments

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