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Effect of BayK 8644 on $[Ca^{2+}]_i$ and Viability in PC3 Human Prostate Cancer Cells

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Abstract

The effect of BayK 8644 on cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) and viability in PC3 human prostate cancer cells was explored. Fura-2 was applied to measure $[Ca^{2+}]_i$. BayK 8644 at 1-50 μM induced a $[Ca^{2+}]_i$ rise concentration-dependently. The response was reduced by removing extracellular Ca^{2+}. BayK 8644-evoked Ca^{2+} entry was inhibited by nifedipine, econazole, SK&F96365, and protein kinase C modulators. In Ca^{2+}-free medium, incubation with the endoplasmic reticulum Ca^{2+} pump inhibitor 2,5-di-tert-butylhydroquinone (BHQ) abolished BayK 8644-induced $[Ca^{2+}]_i$ rise. Inhibition of phospholipase C did not alter BayK 8644-induced $[Ca^{2+}]_i$ rise. BayK 8644 killed cells in a concentration-dependent manner, which was not reversed by chelating cytosolic Ca^{2+} with 1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid/acetoxy methyl (BAPTA/AM). Collectively, in PC3 human prostate cancer cells, BayK 8644 induced a $[Ca^{2+}]_i$ rise by evoking phospholipase C-independent Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} entry *via* protein kinase C-sensitive store-operated Ca^{2+} channels (and/or T-type Ca^{2+} channels). At high concentrations, BayK 8644 caused cell death.

Key Words: BayK 8644, Ca^{2+}, PC3

Introduction

In 1986, Fredholm *et al*. (9) reported that the dihydropyridine Ca^{2+}-channel agonist BayK 8644 inhibited the presynaptic effects of R-phenylisopropyl adenosine in the rat hippocampus. Subsequently, BayK 8644 has been used as a presumed L-type Ca^{2+} channel agonist in various models (5, 29, 31). However, evidence accumulates that BayK 8644 may act *via* Ca^{2+}-independent mechanisms. For example, Noh *et al*. (22) show that BayK 8644 inhibits RANKL-induced osteoclast formation *via* NFATc1 down-regulation. Marom *et al*. (20) show that conformational changes induced in voltage-gated Ca^{2+} channel by BayK 8644 modify the kinetics of secretion independently of Ca^{2+} influx. The possibility that BayK 8644 may cause a rise in cytosolic free Ca^{2+} level ($[Ca^{2+}]_i$) independently of activation of L-type Ca^{2+} channels is usually overlooked.

The aim of this study was to examine the effect

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of BayK 8644 on $[Ca^{2+}]_i$ in PC3 human prostate cancer cells. Ca^{2+} ions play a crucial role in different biological responses. A rise in $[Ca^{2+}]_i$ can initiate many pathophysiological cellular processes (1). However, a unregulated $[Ca^{2+}]_i$ rise may cause ion flux, dysfunction of proteins, apoptosis, and proliferation, *etc.*. Ca^{2+} signaling is shown to play a key role in development of prostate cancer (24). In general, non-excitable tissues, including the epithelium, do not express voltage gated Ca^{2+} channels. This is partly because the ranges of membrane potential changes in these cells are too small to activate these channels. However, recent studies show that T-type Ca^{2+} channels are expressed in cancerous cells such as prostate cancer cells (24). For instance, Diaz-Lezama *et al.* (6) show that ghrelin increased T-type Ca^{2+} channel expression and induced $[Ca^{2+}]_i$ rises in PC3 human prostate cancer cells. In this study, it was examined whether BayK 8644 could increase $[Ca^{2+}]_i$ and evoke death in PC3 cells. The PC3 cell is a useful system for prostate cancer cell research. It has been shown that in this cell, $[Ca^{2+}]_i$ can increase in response to the stimulation of various ligands such as MK-886 (14), desipramine (3) and safrole (2).

Fura-2 was used as a fluorescent Ca^{2+}-sensitive dye to measure $[Ca^{2+}]_i$ changes in the present study. BayK 8644-induced Ca^{2+} entry and Ca^{2+} release were explored. The $[Ca^{2+}]_i$ rises were characterized, the concentration-response plots were established, and the pathways underlying BayK 8644-evoked Ca^{2+} entry and Ca^{2+} release were explored. The effect of BayK 8644 on cell viability was examined.

Materials and Methods

Chemicals

The reagents for cell culture were from Gibco (Gaithersburg, MD, USA). Fura-2/AM and BAPTA/AM were from Molecular Probes (Eugene, OR, USA). BayK 8644 and other reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Cell Culture

PC3 human prostate cancer cells obtained from Bioresource Collection and Research Center (Taiwan) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Solutions Used in $[Ca^{2+}]_i$ Measurements

Ca^{2+}-containing medium (pH 7.4) had 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, and 5 mM glucose. Ca^{2+}-free medium contained similar chemicals as Ca^{2+}-containing medium except that $CaCl_2$ was replaced with 0.3 mM EGTA and 2 mM $MgCl_2$. BayK 8644 was dissolved in dimethyl sulfoxide as a 1 M stock solution. The other chemicals were dissolved in water, ethanol or dimethyl sulfoxide. The concentration of organic solvents in the experimental solutions did not exceed 0.1%, and did not affect viability or basal $[Ca^{2+}]_i$.

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

Confluent cells grown on 6 cm dishes were trypsinized and made into a suspension in culture medium at a density of 10^6/ml. Cell viability was determined by trypan blue exclusion (adding 0.2% trypan blue to 0.1 ml cell suspension). The viability was greater than 95% after the treatment. Cells were subsequently loaded with 2 mM fura-2/AM for 30 min at 25°C in the same medium. After loading, cells were washed with Ca^{2+}-containing medium twice and was made into a suspension in Ca^{2+}-containing medium at a density of 10^7/ml. 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 immediately after 0.1 ml cell suspension was added to 0.9 ml Ca^{2+}-containing or Ca^{2+}-free medium, by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-sec intervals. During the recording, reagents were added to the cuvette by pausing the recording for 2 sec to open and close the cuvette-containing chamber. For calibration of $[Ca^{2+}]_i$, after completion of the experiments, the detergent Triton X-100 (0.1%) and $CaCl_2$ (5 mM) were added to the cuvette to obtain the maximal fura-2 fluorescence. Then the Ca^{2+} chelator EGTA (10 mM) was added to chelate Ca^{2+} in the cuvette to obtain the minimal fura-2 fluorescence. Control experiments showed that cells bathed in a cuvette had a viability of 95% after 20 min of fluorescence measurements. $[Ca^{2+}]_i$ was calculated as previously described (11-13). Mn^{2+} quenching of fura-2 fluorescence was performed in Ca^{2+}-containing medium containing 50 μM $MnCl_2$. $MnCl_2$ was added to cell suspension in the cuvette 30 sec before the fluorescence recoding was started. Data were recorded at excitation signal at 360 nm (Ca^{2+}-insensitive) and emission signal at 510 nm at 1-sec intervals as described previously (21).

Cell Viability Assays

The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by de-

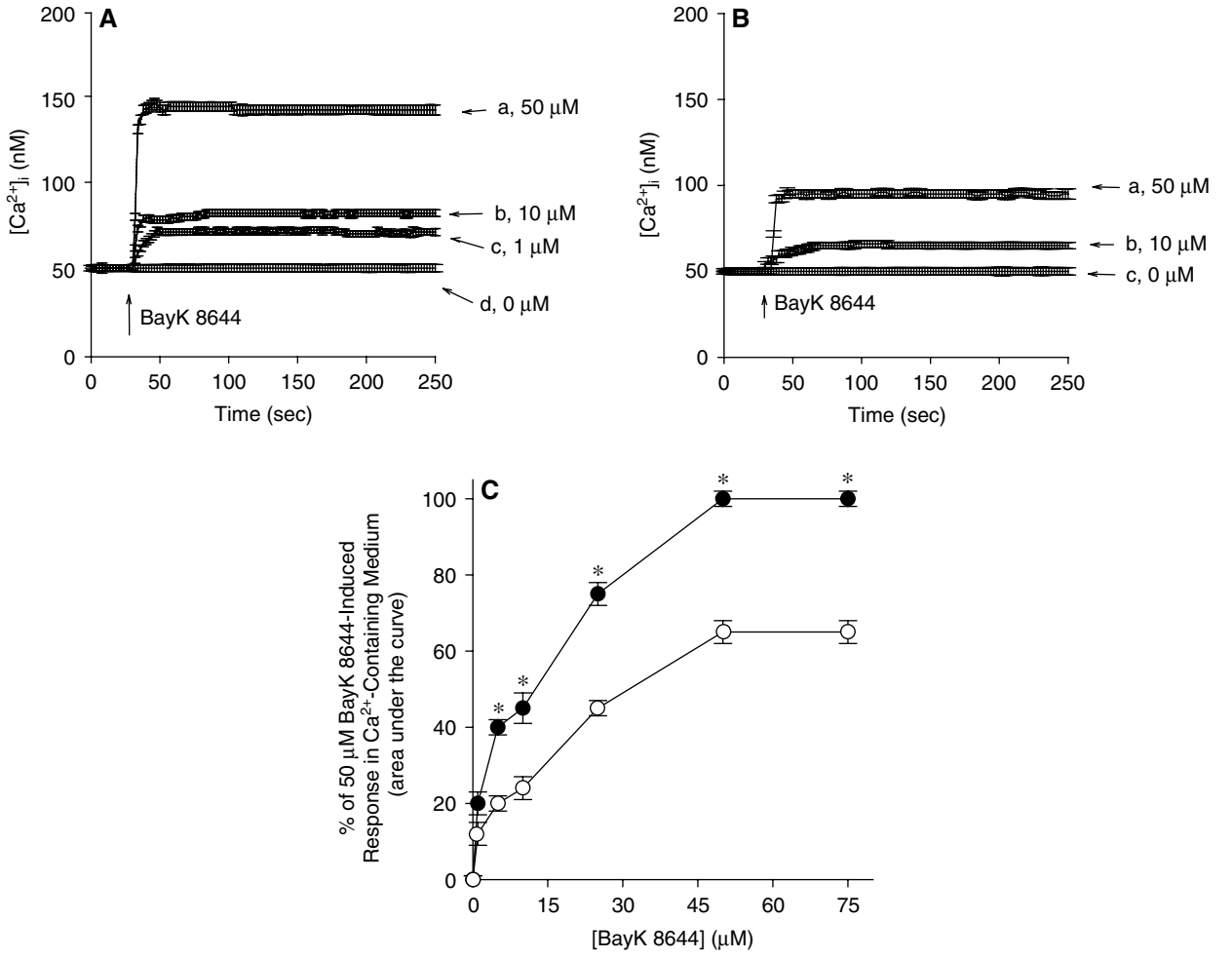

Fig. 1. (A) Effect of BayK 8644 on $[Ca^{2+}]_i$ in fura-2-loaded PC3 cells. BayK 8644 was added at 25 sec. The concentration of BayK 8644 was indicated. The experiments were performed in Ca^{2+}-containing medium. (B) Effect of BayK 8644 on $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. BayK 8644 (10 or 50 μM) was added at 25 sec in Ca^{2+}-free medium. (C) Concentration-response plots of BayK 8644-induced $[Ca^{2+}]_i$ rises in the presence or absence of extracellular Ca^{2+}. Y axis is the percentage of the net (baseline subtracted) area under the curve (25-250 sec) of the $[Ca^{2+}]_i$ rise induced by 50 μM BayK 8644 in Ca^{2+}-containing medium. Data are means ± S.E.M. of three separate experiments. $*P < 0.05$ compared to open circles.

hydrogenases. Increase in the amount of developed color directly correlated with the number of live cells. Assays were performed according to manufacturer's instructions designed for this assay (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at a density of 10,000 cells/well in culture medium for 24 h in the presence of BayK 8644. The cell viability detecting reagent 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1, 3-benzene disulfonate] (WST-1; 10 μl pure solution) was added to samples after BayK 8644 treatment, and cells were incubated for 30 min in a humidified atmosphere. In experiments using BAPTA/AM to chelate cytosolic Ca^{2+}, cells were treated with 10 μM BAPTA/AM for 1 h prior to incubation with BayK 8644. The cells were washed once with Ca^{2+}-containing medium and incubated with/without BayK 8644 for 24 h. The absorbance of samples (A_{450}) was determined using an enzyme-linked immunosorbent assay (ELISA) reader. Absolute optical density was normalized to the absorbance of unstimulated cells in each plate and expressed as a percentage of the control value.

Statistical Analysis

Data are reported as means ± S.E.M. of three separate experiments. Data were analyzed by one-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS®, SAS Institute Inc., Cary, NC, USA). Multiple comparisons between group means were performed by *post-hoc* analysis using the Tukey's HSD (honestly significantly difference) procedure. A *P*-value less than 0.05 were considered significant.

Results

Effect of BayK 8644 on $[Ca^{2+}]_i$

The effect of BayK 8644 on basal $[Ca^{2+}]_i$ was examined. Fig. 1A shows that the basal $[Ca^{2+}]_i$ level was 51 ± 2 nM. At concentrations between 1 and 50 μM, BayK 8644 induced a $[Ca^{2+}]_i$ rise in a concentration-dependent manner in Ca^{2+}-containing medium. At a concentration of 10 μM, BayK 8644 evoked a $[Ca^{2+}]_i$ rise that attained to a net increase of 27 ± 2 nM (n = 3) followed by a sustained phase. The Ca^{2+} response saturated at 50 μM BayK 8644 because at a concentration of 75 μM, BayK 8644 evoked a similar response as that induced by 50 μM. Fig. 1B shows that in the absence of extracellular Ca^{2+}, 10 μM BayK 8644 induced a $[Ca^{2+}]_i$ rise of 15 ± 2 nM; and at a concentration of 50 μM, BayK 8644 induced a $[Ca^{2+}]_i$ rise of 50 ± 2 nM. Fig. 1C shows the concentration-response plots of BayK 8644-induced responses. The EC_{50} value was 13 ± 2 μM in Ca^{2+}-containing or Ca^{2+}-free medium by fitting to a Hill equation.

BayK 8644-Induced Mn^{2+} Influx

Experiments were performed to confirm that BayK 8644-evoked $[Ca^{2+}]_i$ rise involved Ca^{2+} influx. Mn^{2+} enters cells through similar mechanisms as Ca^{2+} but quenches fura-2 fluorescence at all excitation wavelengths. Therefore, quenching of fura-2 fluorescence excited at the Ca^{2+}-insensitive excitation wavelength of 360 nm by Mn^{2+} implicates Ca^{2+} influx. Fig. 2A shows that 50 μM BayK 8644 evoked an instant decrease in the 360 nm excitation signal by 130 ± 2 (n = 3) arbitrary units. This suggests that Ca^{2+} influx was involved in BayK 8644-evoked $[Ca^{2+}]_i$ rise.

Pathways of BayK 8644-Induced Ca^{2+} Entry

Experiments were conducted to explore the Ca^{2+} entry pathway of the BayK 8644-induced $[Ca^{2+}]_i$ rise. Three Ca^{2+} entry inhibitors: nifedipine (1 μM), econazole (0.5 μM) and SK&F96365 (5 μM); phorbol 12-myristate 13 acetate (PMA; 1 nM; a protein kinase C activator); and GF109203X (2 μM; a protein kinase C inhibitor) were applied 1 min before 50 μM BayK 8644. These agents all significantly inhibited BayK 8644-induced $[Ca^{2+}]_i$ rise (Fig. 2B).

Internal Stores of BayK 8644-Induced $[Ca^{2+}]_i$ Rise

Efforts were made to explore the Ca^{2+} store involved in BayK 8644-induced $[Ca^{2+}]_i$ rise. Previous studies have shown that the endoplasmic reticulum is the major Ca^{2+} store in PC3 cells (14). Fig. 3A shows that in Ca^{2+}-free medium, addition of 50 μM 2,5-di-tert-butylhydroquinone (BHQ), an endoplasmic reticu-

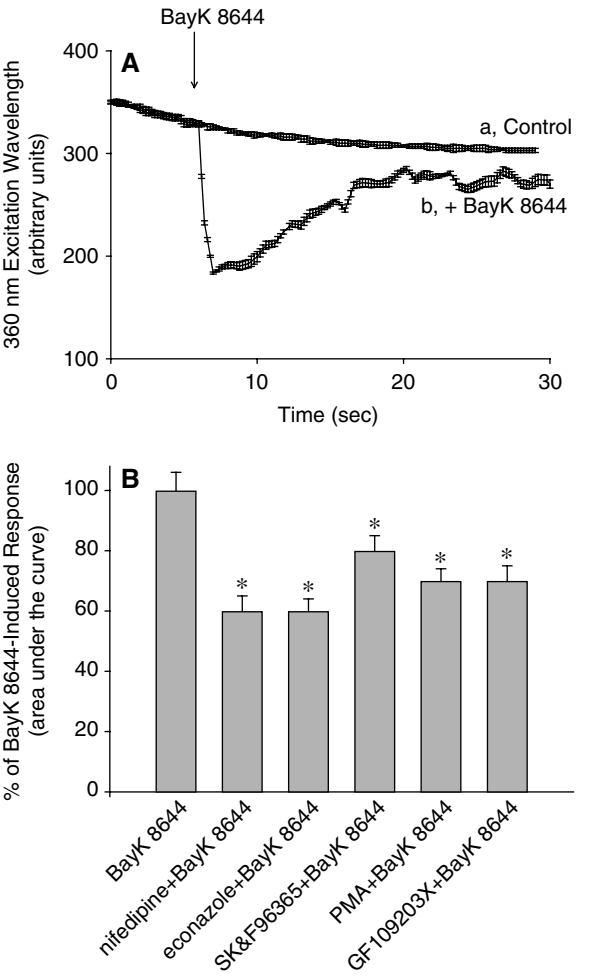

Fig. 2. (A) Effect of BayK 8644 on Ca^{2+} influx by measuring Mn^{2+} quenching of fura-2 fluorescence. Experiments were performed in Ca^{2+}-containing medium. $MnCl_2$ (50 μM) was added to cells 1 min before fluorescence measurements. The y axis is fluorescence intensity (in arbitrary units) measured at the Ca^{2+}-insensitive excitation wavelength of 360 nm and the emission wavelength of 510 nm. Trace a: control, without BayK 8644. Trace b: BayK 8644 (50 μM) was added as indicated. Data are means ± S.E.M. of three separate experiments. (B) Effect of Ca^{2+} channel blockers or modulators on BayK 8644-induced $[Ca^{2+}]_i$ rise. A. In blocker- or modulator-treated groups, the reagent was added 1 min before BayK 8644 (50 μM). The concentration was 1 μM for nifedipine, 0.5 μM for econazole, 5 μM for SK&F96365, 10 nM for phorbol 12-myristate 13-acetate (PMA), and 2 μM for GF109203X, Data are expressed as the percentage of control (1st column) that is the area under the curve (25-200 sec) of 50 μM BayK 8644-induced $[Ca^{2+}]_i$ rise, and are mean ± S.E.M. of three separate experiments. *$P < 0.05$ compared to the 1st column.

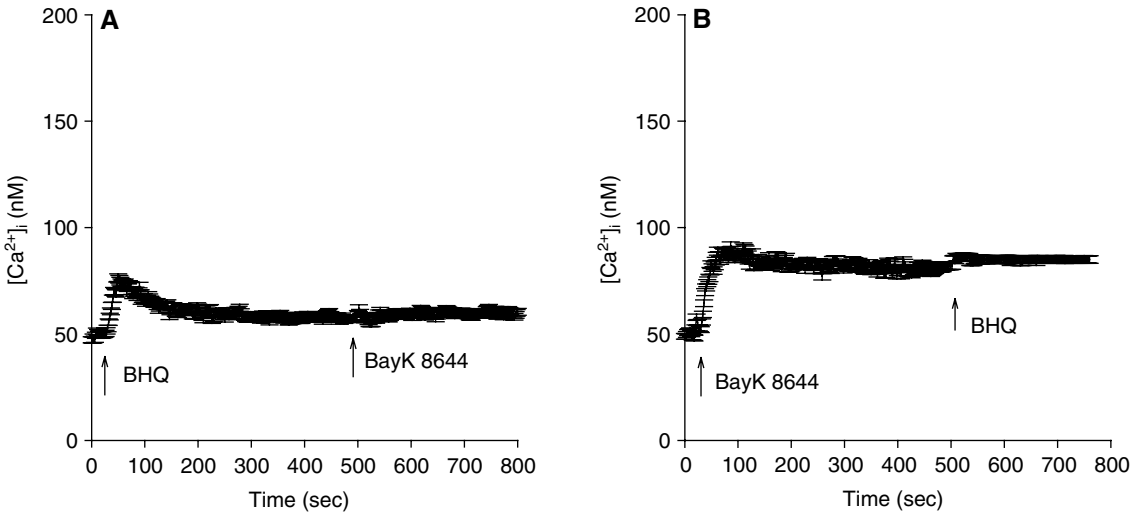

Fig. 3. Intracellular Ca^{2+} stores of BayK 8644-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. BayK 8644 (50 μM) and BHQ (50 μM) were added at time points indicated. Data are means ± S.E.M. of three separate experiments.

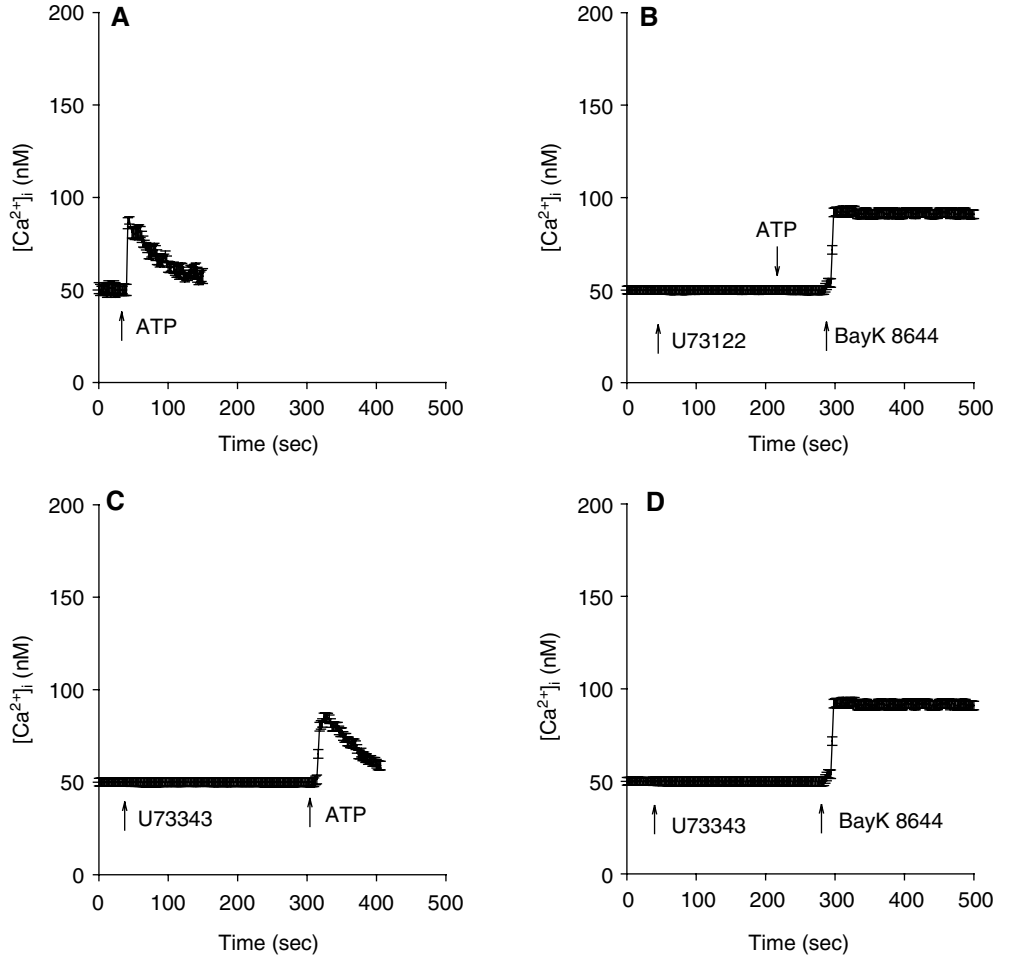

Fig. 4. Lack of an effect of U73122 on BayK 8644-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. (A) ATP (10 μM) was added as indicated. (B) U73122 (2 μM), ATP (10 μM), and BayK 8644 (50 μM) were added as indicated. Data are means ± S.E.M. of three separate experiments. (C) U73343 (2 μM) and ATP (10 μM), were added as indicated. Data are means ± S.E.M. of three separate experiments. (D) U73122 (2 μM) and BayK 8644 (50 μM) were added as indicated. Data are means ± S.E.M. of three separate experiments.

lum Ca^{2+} pump inhibitor (30), induced a $[Ca^{2+}]_i$ rise of 27 ± 2 nM. Subsequently added BayK 8644 (50 μM) at 500 sec failed to induce a $[Ca^{2+}]_i$ rise. Fig. 3B shows that after BayK 8644 pretreatment, BHQ (50 μM) added at 500 sec also failed to induce a $[Ca^{2+}]_i$ rise.

Lack of a Role of Phospholipase C in BayK 8644-Induced $[Ca^{2+}]_i$ Rise

Phospholipase C-dependent production of inositol 1,4,5-trisphosphate is a key process for releasing Ca^{2+} from the endoplasmic reticulum. Because BayK 8644 released Ca^{2+} from the endoplasmic reticulum, the role of phospholipase C in this event was examined. U73122 (27), a phospholipase C inhibitor, was used to see whether the activation of this enzyme was required for BayK 8644-induced Ca^{2+} release. Fig. 4A shows that ATP (10 μM) induced a $[Ca^{2+}]_i$ rise of 41 ± 2 nM. ATP is a phospholipase C-dependent agonist of $[Ca^{2+}]_i$ rise in most cell types (8). Fig. 4B shows that incubation with 2 μM U73122 did not change basal $[Ca^{2+}]_i$ but abolished ATP-induced $[Ca^{2+}]_i$ rise. This suggests that U73122 effectively suppressed phospholipase C activity. Fig. 4B also shows that incubation with 2 μM U73122 and ATP did not alter BayK 8644-induced $[Ca^{2+}]_i$ rise. U73343 (2 μM), a U73122 analogue, failed to have an inhibition (Fig. 4C). Fig. 4D shows that incubation with 2 μM U73122 did not alter BayK 8644-induced $[Ca^{2+}]_i$ rise.

Effect of BayK 8644 on Cell Viability

Given that acute incubation with BayK 8644 induced a substantial and lasting $[Ca^{2+}]_i$ rise, and that unregulated $[Ca^{2+}]_i$ rise often alters cell viability (1), experiments were performed to examine the effect of BayK 8644 on cell viability. Cells were treated with 0-200 μM BayK 8644 for 24 h, and the tetrazolium assay was performed. In the presence of 50 μM BayK 8644, cell viability was not altered. In the presence of 100, 150 and 200 μM BayK 8644, cell viability was decreased in a concentration-dependent manner (Fig. 5).

Lack of a Relationship between BayK 8644-Induced $[Ca^{2+}]_i$ Rise and Cell Death

The next question was whether the BayK 8644-induced cell death was caused by a preceding $[Ca^{2+}]_i$ rise. The intracellular Ca^{2+} chelator BAPTA/AM (28) was used to prevent a $[Ca^{2+}]_i$ rise during BayK 8644 treatment. Fig. 5 also shows that 10 μM BAPTA/AM loading did not alter the control value of cell viability. BayK 8644 (50 μM) did not induce a $[Ca^{2+}]_i$ rise in BAPTA/AM-treated cells (not shown). In the presence of 100-200 μM BayK 8644, BAPTA/AM loading did not reverse BayK 8644-induced cell death.

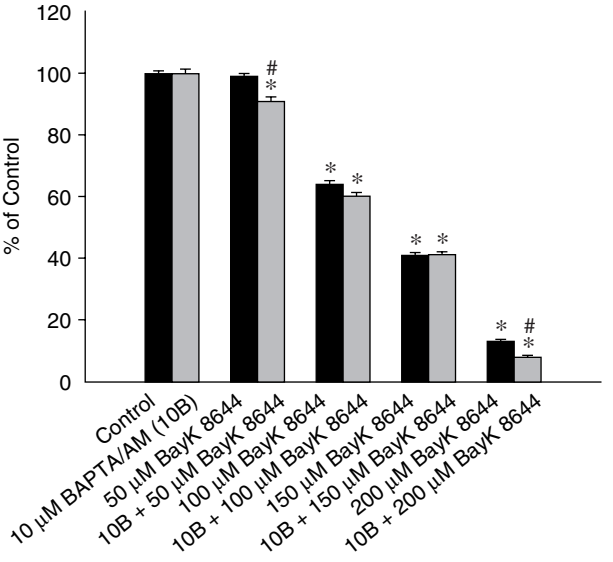

Fig. 5. Effect of BayK 8644 on viability of cells. Cells were treated with 0-200 μM BayK 8644 for 24 h, and the cell viability assay was performed. Data are means ± S.E.M. of three separate experiments. Each treatment had six replicates (wells). Data are expressed as the percentage of control that is the increase in cell numbers in BayK 8644-free groups. Control had 10,556 ± 71 cells/well before experiments, and had 13,555 ± 711 cells/well after incubation for 24 h. $^*P < 0.05$ compared to control. In each group, the Ca^{2+} chelator BAPTA/AM (10 μM) was added to cells followed by treatment with BayK 8644 in Ca^{2+}-containing medium. Cell viability assay was subsequently performed. $^{\#}P < 0.05$ compared to the pairing group.

Discussion

Our study shows that BayK 8644 induced a $[Ca^{2+}]_i$ rise in PC3 human prostate cancer cells. The results show that BayK 8644 induced a concentration-dependent $[Ca^{2+}]_i$ rise. BayK 8644 increased $[Ca^{2+}]_i$ by depleting Ca^{2+} stores and causing Ca^{2+} entry. Removal of Ca^{2+} reduced BayK 8644-induced $[Ca^{2+}]_i$ rise throughout the measurement, suggesting that Ca^{2+} entry occurred during the whole interval. This Ca^{2+} entry was confirmed by BayK 8644-induced Mn^{2+} entry that led to quenching of fura-2 fluorescence.

The effect of BayK 8644 on $[Ca^{2+}]_i$ in PC3 cells does not appear to be mediated by activation of L-type Ca^{2+} channels because there is no evidence for the presence of these channels in this cell. Therefore, BayK 8644 may have additional molecular targets. Our results suggest that BayK 8644 might cause Ca^{2+} entry *via* store-operated Ca^{2+} entry which is induced by depletion of intracellular Ca^{2+} stores, based on the

inhibition of BayK 8644-induced $[Ca^{2+}]_i$ rise by nifedipine, econazole and SK&F96365. There is no evidence for the presence of receptor-operated Ca^{2+} channels (ROC) in PC3 cells. Therefore, a role of ROC in BayK8644-induced Ca^{2+} entry in PC3 cells could be ruled out. Although BTP-2 is a store-operated Ca^{2+} channels selective Ca^{2+} blocker, it is also a T-type Ca^{2+} channels blocker (19). If BTP-2 was used in our studies, it would not be clear whether BTP-2 inhibited BayK 8644-evoked Ca^{2+} entry through store-operated Ca^{2+} channels or T-type Ca^{2+} channels. Furthermore, nifedipine, econazole and SK&F96365 have often been applied as blockers of store-operated Ca^{2+} entry in different cell types (15, 16, 23, 25). Therefore, our data suggest that BayK 8644 might cause Ca^{2+} entry *via* store-operated Ca^{2+} entry. Because activation of phospholipase C produces IP_3 and diacylglycerol, which stimulates protein kinase C, the effect of regulation of protein kinase C activity on BayK 8644-induced $[Ca^{2+}]_i$ rise was explored. Both activation and inhibition of protein kinase C inhibited BayK 8644-induced $[Ca^{2+}]_i$ rise. This may suggest that a normally maintained protein kinase C activity was necessary for a full response of 50 μM BayK8644-induced $[Ca^{2+}]_i$ rise. Gao *et al*. (10) have shown that protein kinase C plays an important role in the activation of store-operated Ca^{2+} entry in airway smooth muscle cells. Protein kinase C-induced phosphorylation of Orai1 was shown to regulate $[Ca^{2+}]_i$ *via* the store-operated Ca^{2+} channel (17).

T-type Ca^{2+} channels appear to be present in PC3 cells. Diaz-Lezama *et al*. (6) showed that ghrelin activated T-type Ca^{2+} currents and increased $[Ca^{2+}]_i$ in PC3 cells. Our data suggest that nifedipine, econazole and SK&F96365 all significantly inhibited BayK 8644-induced $[Ca^{2+}]_i$ rise. Previous studies showed that nifedipine (18) and SK&F96365 (26) at μM concentrations both inhibited T-type Ca^{2+} channels. Therefore, it is possible that store-operated Ca^{2+} channels and T-type Ca^{2+} channels were both involved in BayK 8644-induced Ca^{2+} entry in PC3 cells.

Regarding the Ca^{2+} stores involved in BayK 8644-induced Ca^{2+} release, the endoplasmic reticulum stores might be the dominant one because BHQ pretreatment abolished BayK 8644-induced $[Ca^{2+}]_i$ rise; and conversely, BayK 8644 pretreatment abolished BHQ-induced Ca^{2+} release. Furthermore, it seems that phospholipase C-dependent pathways did not play a role in BayK 8644-induced Ca^{2+} release, since the response was not affected when phospholipase C activity was inhibited by U73122. Thus it appears that BayK 8644-induced Ca^{2+} release was caused by a phospholipase C-independent Ca^{2+} release from the endoplasmic reticulum.

Because BayK 8644 induced both $[Ca^{2+}]_i$ rises and cell death, it would be interesting to know whether the death occurred in a Ca^{2+}-dependent manner. Our data show that BayK 8644-induced cell death was not reversed when cytosolic Ca^{2+} was chelated by BAPTA/AM. This implies that in this case, BayK 8644-induced cell death was not triggered by a $[Ca^{2+}]_i$ rise. Emptying of intracellular Ca^{2+} stores and/or influx of extracellular Ca^{2+} can modulate cell viability in many cell types (1). However, Ca^{2+}-independent cell death could be found in some cell types such as hepatoma cells (4) and renal tubular cells (7) *etc*.. Note that BayK 8644 only induced cell death at concentrations higher than 50 μM after overnight treatment. Thus our data that 10-50 μM BayK 8644 induced a $[Ca^{2+}]_i$ rise was not complicated by a decrease in cell viability.

Together, the results show that BayK 8644 induced Ca^{2+} release from endoplasmic reticulum in a phospholipase C-independent manner and also caused Ca^{2+} influx *via* a protein kinase C-regulated, store-operated Ca^{2+} entry (and/or T-type Ca^{2+} channels) in PC3 human prostate cancer cells. BayK 8644 also induced Ca^{2+}-independent cell death.

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