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Effect of betulinic acid on intracellular-free Ca^{2+} levels in Madin Darby canine kidney cells

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Abstract

The effect of betulinic acid, an anti-tumor and apoptosis-inducing natural product, on intracellular-free levels of Ca^{2+} ($[Ca^{2+}]_i$) in Madin Darby canine kidney (MDCK) cells was examined by using fura-2 as a Ca^{2+} dye. Betulinic acid caused significant increases in $[Ca^{2+}]_i$ concentration dependently between 25 and 500 nM with an EC_{50} of 100 nM. The $[Ca^{2+}]_i$ signal was composed of an initial gradual rise and a plateau. The response was decreased by removal of extracellular Ca^{2+} by $45 \pm 10\%$. In Ca^{2+}-free medium, pretreatment with 1 μM thapsigargin (an endoplasmic reticulum Ca^{2+} pump inhibitor) abolished 250 μM betulinic acid-induced $[Ca^{2+}]_i$ increases. Conversely, pretreatment with betulinic acid only partly inhibited thapsigargin-induced $[Ca^{2+}]_i$ increases. Addition of 3 mM Ca^{2+} induced a $[Ca^{2+}]_i$ increase after pretreatment with 250 nM betulinic acid in Ca^{2+}-free medium for 5 min. This $[Ca^{2+}]_i$ increase was not altered by the addition of 20 μM SKF96365 and 10 μM econazole. Inhibiting inositol 1,4,5-trisphosphate formation with the phospholipase C inhibitor U73122 (2 μM) abolished 250 nM betulinic acid-induced Ca^{2+} release. Pretreatment with 10 μM La^{3+} inhibited 250 nM betulinic acid-induced $[Ca^{2+}]_i$ increases by $85 \pm 3\%$; whereas 10 μM of verapamil, nifedipine and diltiazem had no effect. In Ca^{2+} medium, pretreatment with 2.5 nM betulinic aid for 260 s potentiated 10 μM ATP and 1 μM thapsigargin-induced $[Ca^{2+}]_i$ increases by $33 \pm 3\%$ and $45 \pm 3\%$, respectively. Trypan blue exclusion revealed that acute exposure of 250 nM betulinic acid for 2–30 min decreased cell viability by $6 \pm 2\%$, which could be prevented by pretreatment with 2 μM U731222. Together, the results suggest that betulinic acid induced significant $[Ca^{2+}]_i$ increases in MDCK cells in a concentration-dependent manner, and also induced mild cell death. The $[Ca^{2+}]_i$ signal was contributed by an inositol 1,4,5-trisphosphate-dependent release of intracellular Ca^{2+} from thapsigargin-sensitive stores, and by inducing Ca^{2+} entry from extracellular medium in a La^{3+}-sensitive manner.

Keywords: Betulinic acid; Ca^{2+}; Ca^{2+} store; Fura-2; MDCK cell; Thapsigargin

1. Introduction

Betulinic acid (3 beta-hydroxylup-20(29)-en-28-oic acid) is a pentacyclic triterpene prepared from betulin of white-barked birch trees (Pisha et al., 1995; Nick et al., 1995; Schuhly et al., 1999). Betulinic acid has a direct effect on mitochondria, resulting in the release of soluble apoptogenic factors such as cytochrome *c* or apoptosis-inducing factor into the cytosol where they activate caspases and endonucleases (Fulda et al., 1998). Additionally, betulinic acid was shown to be a potent apoptosis inducer, and has been used experimentally to treat malignant brain-tumor cells and neuroectodermal tumors (Fulda et al., 1999a,b). Recently, betulinic acid was thought to inhibit human immunodeficiency virus type 1 (HIV-1) via interfering with HIV-1 entry into cells at a postbinding step (Soler et al., 1996; Evers et al., 1996).

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Among the various messengers involved in the initiation and modulation of cellular functions, an increase in intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) is a key signal (Berridge, 1993, 1997; Bootman et al., 1993; Clapman, 1995). A $[Ca^{2+}]_i$ increase may occur as a result of release of Ca^{2+} from intracellular stores and/or an entry of Ca^{2+} from extracellular space. The inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store is an important intracellular Ca^{2+} pool, which actively discharges Ca^{2+} into cytosol when the inositol 1,4,5-trisphosphate receptors on these stores bind cytosolic inositol 1,4,5-trisphosphate (Berridge, 1993, 1997). In many cell types, this Ca^{2+} mobilization may cause Ca^{2+} influx across plasma membrane via the process of capacitative Ca^{2+} entry (Putney 1986). Prolonged elevations in $[Ca^{2+}]_i$ or abnormal regulations of $[Ca^{2+}]_i$ are known to lead to cell injury and apoptosis (Bootman et al., 1993; Clapman, 1995). The effect of betulinic acid on Ca^{2+} signaling has not been examined previously in any cell type. The present study was aimed to explore the effect of betulinic acid on $[Ca^{2+}]_i$ in Madin Darby canine kidney (MDCK) cells. Previous results showed that many agents can cause significant increases in $[Ca^{2+}]_i$ in this renal tubular cell. These agents include inositol 1,4,5-trisphosphate-dependent ATP and bradykinin (Jan et al., 1998a,b), the endoplasmic reticulum Ca^{2+} pump inhibitors thapsigargin and 2,5-di-*tert*-butylhydroquinone (Jan et al., 1999a,b), and other agents such as Zn^{2+}, fendiline, chloroform, sevoflurane, etc. (Jan et al., 1999c, 2000a,b; Jan and Chen, 2000).

By using fura-2 as a fluorescent Ca^{2+} probe, this study shows that betulinic acid induced a significant increase in $[Ca^{2+}]_i$ and mild death in MDCK cells. The concentration–response relationship was established, and the underlying mechanism of the $[Ca^{2+}]_i$ increase was evaluated.

2. Methods

2.1. Cell culture

MDCK cells obtained from American Type Culture Collection 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. The cells were kept at 37°C in 5% CO_2-containing humidified air.

2.2. Solutions

Ca^{2+} medium (pH 7.4) contained (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 2; Hepes 10; glucose 5. Ca^{2+}-free medium contained no Ca^{2+} plus 1 mM EGTA.

2.3. Optical measurements of $[Ca^{2+}]_i$

Trypsinized cells (10^6/ml) were allowed to recover in Dulbecco's modified Eagle medium for 1 h before being loaded with 2 μM 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*,*N*-tetraacetic acid pentaacetoxymethyl ester (fura-2/AM) for 30 min at 25°C in the same medium. The cells were washed and resuspended in Ca^{2+} 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 spectrofluorophotometer (Kyoto, Japan) by continuously 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 20 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as described previously (Grynkiewicz et al., 1985).

2.4. Cell viability assay

Cell viability was determined by Trypan blue exclusion. Fifty microliters of cell suspension was mixed with 50 μl of Trypan blue isotonic solution (0.2%; w/v) in the absence (control) or presence of 250 nM betulinic acid for 0–30 min. Then cell viability was determined on a hemocytometer under a microscope.

2.5. Chemical reagents

The reagents for cell culture were from Gibco (Grand Island, NY, USA). Fura-2/AM was from Molecular Probes (Eugene, OR, USA). Betulinic acid, U73122, and U73343 were from Biomol (Plymouth Meeting, PA, USA). The other reagents were from Sigma (St. Louis, MO, USA).

2.6. Statistical analyses

The traces are typical of four to six similar responses. All values were reported as means ± SEM of four to six experiments. Because the data from each experiment were the average of responses from 0.5 million cells, the variation among experiments was small. This suggests that the mean ± SEM of four to six experiments can reveal significant results. Statistical comparisons were determined by using Student's *t*-test, and significance was accepted when $P < 0.05$.

3. Results

3.1. Effect of betulinic acid on $[Ca^{2+}]_i$

Betulinic acid at concentrations between 25 and 500 nM increased $[Ca^{2+}]_i$ in a concentration-dependent manner in the presence of extracellular Ca^{2+}. Fig. 1A shows typical records of the $[Ca^{2+}]_i$ increases induced by 250 and 25 nM betulinic acid (traces a, b). At a concentration of 5 μM,

betulinic acid had little effect (trace c). The basal $[Ca^{2+}]_i$ was 51 ± 3 nM ($n = 6$). Over a time period of 350 s, the $[Ca^{2+}]_i$ signal induced by 250 nM betulinic acid comprised an initial rise, which reached a maximum value of 155 ± 8 nM ($n = 6$), followed by a plateau. The Ca^{2+} signal saturated at 250 nM betulinic acid because the responses induced by 250 and 500 nM of the drug were similar. Fig. 1C (filled circles) shows the concentration–response curve of the betulinic acid response. The curve suggests an EC_{50} value of about 100 nM by fitting to the Hill equation.

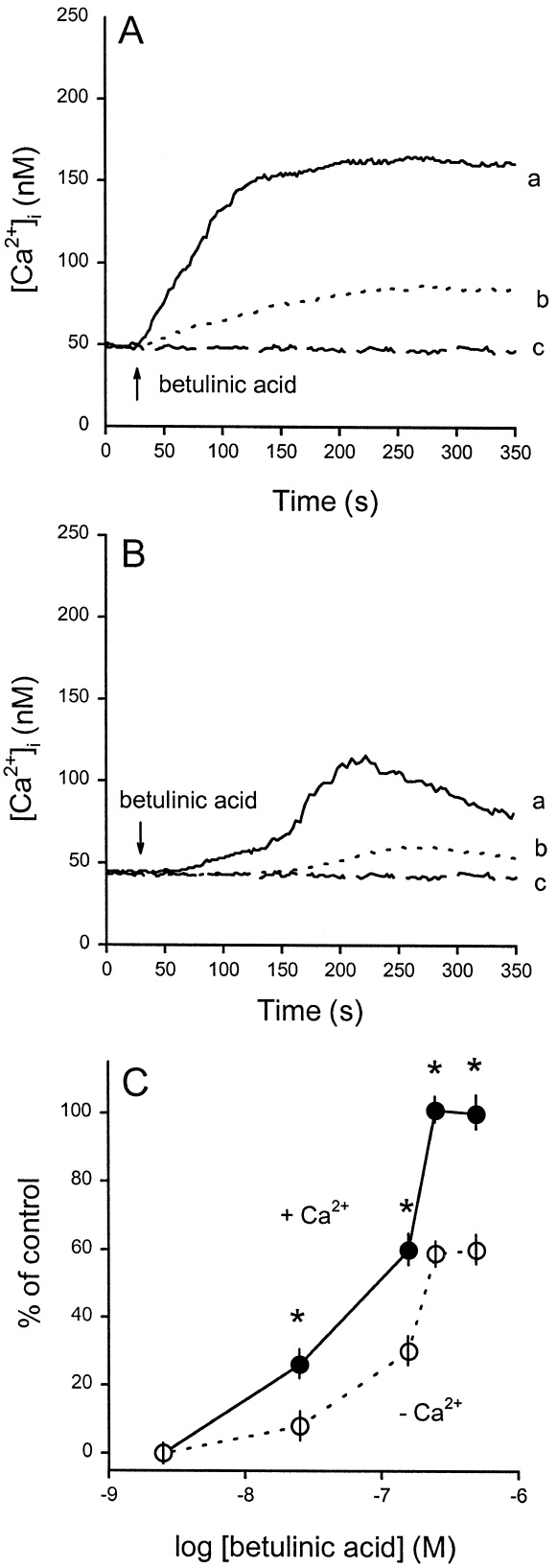

Fig. 1. Effect of betulinic acid on $[Ca^{2+}]_i$ in fura-2-loaded MDCK cells. (A) Concentration-dependent effects of betulinic acid. The concentration of betulinic acid was 250 nM in trace a, 25 nM in trace b, and 5 nM in trace c. Experiments were performed in Ca^{2+} medium. (B) Similar to (A) except that experiments were performed in Ca^{2+}-free medium. Traces in (A) and (B) were typical of four to six experiments. (C) Concentration–response plots of betulinic acid-induced responses in the presence (open circles) or absence (filled circles) of extracellular Ca^{2+}. The *y*-axis is percentage of control, which is the net maximum $[Ca^{2+}]_i$ value of the $[Ca^{2+}]_i$ increase induced by 250 μM betulinic acid in Ca^{2+} medium. The data are means ± SEM of four to six similar experiments. $^{*}P < 0.05$ between filled circles and open circles.

3.2. Effect of extracellular Ca^{2+} removal on the betulinic acid response

Experiments were performed to evaluate the relative contribution of intracellular Ca^{2+} release and extracellular Ca^{2+} entry in the betulinic acid response. Fig. 1B shows that in Ca^{2+}-free medium (no added Ca^{2+} plus 1 mM EGTA to chelate residual Ca^{2+}), 250 (trace a) and 25 (trace b) nM betulinic acid induced an increase in $[Ca^{2+}]_i$ in a concentration-dependent manner. The basal $[Ca^{2+}]_i$ in Ca^{2+}-free medium was 48 ± 2 nM ($n = 6$). The $[Ca^{2+}]_i$ increase induced by 250 nM betulinic acid reached a maximum value of 102 ± 4 nM ($n = 5$). The $[Ca^{2+}]_i$ signal was followed by a gradual decay. The concentration–response curve of betulinic acid-induced $[Ca^{2+}]_i$ increases in Ca^{2+}-free medium is shown in Fig. 1C (open circles). The data suggest that Ca^{2+} removal inhibited $45 \pm 10\%$ of the $[Ca^{2+}]_i$ increases induced by 25–500 nM betulinic acid.

3.3. Intracellular sources of betulinic acid-induced $[Ca^{2+}]_i$ increases

Experiments were performed to explore whether betulinic acid releases Ca^{2+} from the endoplasmic reticulum, an important Ca^{2+} stores in MDCK cells (Jan et al., 1998a,b). Fig. 2A shows that in Ca^{2+}-free medium, adding 1 μM thapsigargin, an endoplasmic reticulum Ca^{2+} pump inhibitor (Thastrup et al., 1990), induced a significant $[Ca^{2+}]_i$ increase with a net maximum of 134 ± 11 nM ($n = 6$). After this Ca^{2+} store was depleted by thapsigargin for 600 s, the addition of 250 nM betulinic acid failed to induce significant increases in $[Ca^{2+}]_i$ ($n = 6$). Con-

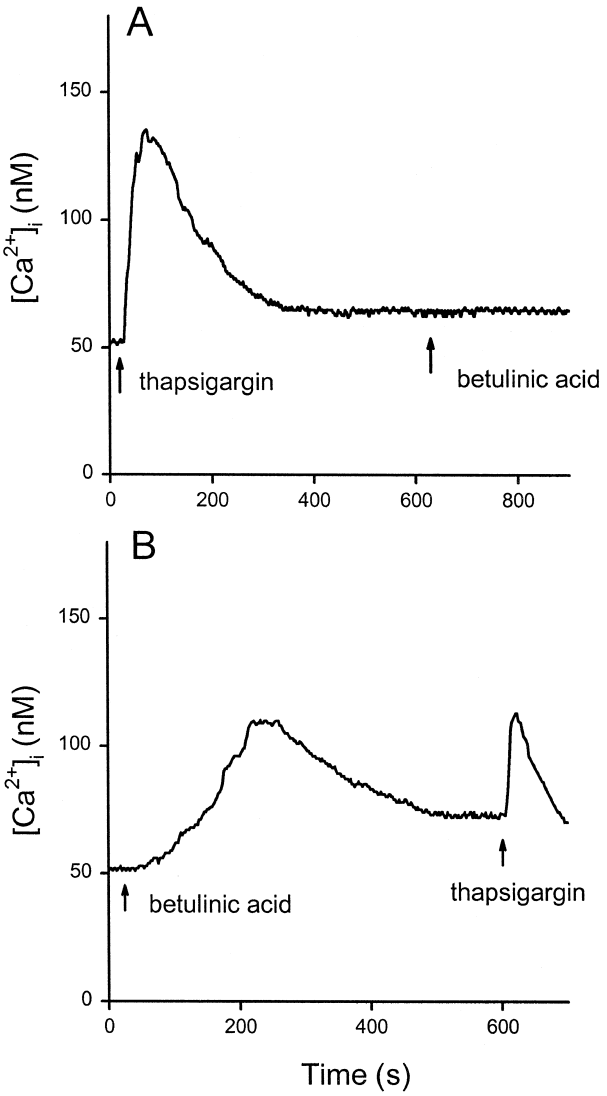

Fig. 2. Intracellular sources of betulinic acid-induced $[Ca^{2+}]_i$ increases. (A)–(B) All experiments were performed in Ca^{2+}-free medium. Drugs were applied at the time indicated by arrows. The concentrations of drug were thapsigargin (1 μM) and betulinic acid (250 nM). Traces were typical of four to six experiments.

versely, Fig. 2B shows that after pretreatment with 250 nM betulinic acid for 600 s, 1 μM thapsigargin still induced an increase in $[Ca^{2+}]_i$ with a net peak value of 41 ± 3 nM ($n = 5$), which is $31 \pm 3\%$ of the control shown in Fig. 2A.

3.4. Effect of inhibiting inositol 1,4,5-trisphosphate formation on betulinic acid-induced intracellular Ca^{2+} release

The phospholipase C inhibitor U73122 (Thompson et al., 1991) was applied to suppress inositol 1,4,5-trisphosphate formation to see whether inositol 1,4,5-trisphosphate is responsible for betulinic acid-induced Ca^{2+} release. Fig. 3A shows that in Ca^{2+}-free medium, the well-known inositol 1,4,5-trisphosphate-dependent intracellular Ca^{2+} releaser ATP (10 μM) induced a $[Ca^{2+}]_i$ increase with a peak value of 321 ± 10 nM ($n = 5$). However, pretreatment with 2 μM U73122 for 200 s prevented 10 μM ATP from increasing $[Ca^{2+}]_i$ (Fig. 3B). U73343 (2 μM), an inactive U73122 analogue, had no effect on basal or ATP-induced $[Ca^{2+}]_i$ increases (data not shown; $n = 4$). These results suggest that U73122 effectively inhibited inositol 1,4,5-trisphosphate formation as shown previously (Jan et al., 1998c). Fig. 3B further shows that after incubation with 2 μM U73122 for 5 min, addition of 250 nM betulinic acid hardly caused an increase in $[Ca^{2+}]_i$ as compared to the control betulinic acid response (Fig. 1B, trace a).

3.5. Mechanism of betulinic acid-induced extracellular Ca^{2+} entry

Release of intracellularly stored Ca^{2+} into the cytosol may trigger Ca^{2+} influx via capacitative Ca^{2+} entry in MDCK cells (Jan et al., 1998a,b). In this study, capacitative Ca^{2+} entry was evaluated by reintroduction of Ca^{2+} to cells depleted of intracellular Ca^{2+} by betulinic acid in Ca^{2+}-free medium for several minutes. Fig. 3C (trace a) shows that in Ca^{2+}-free medium, after pretreatment with 250 nM betulinic acid for about 350 s, addition of 3 mM $CaCl_2$ induced a rapid $[Ca^{2+}]_i$ increase with a net maximum of 158 ± 10 nM (baseline subtracted; $n = 5$). The $[Ca^{2+}]_i$ increase stayed stable without decay for 4 min. The $[Ca^{2+}]_i$ increase was not altered by adding the following Ca^{2+} entry blockers 30 s before 3 mM Ca^{2+}: 20 μM SKF96365 and 10 μM econazole ($n = 4$; data not shown). Adding $CaCl_2$ alone without betulinic acid pretreatment only induced a small $[Ca^{2+}]_i$ increase with a net maximum of 28 ± 2 nM (trace b; $n = 4$).

3.6. Effect of Ca^{2+} entry blockers on betulinic acid-induced $[Ca^{2+}]_i$ increases

These experiments were aimed to further explore the pathway underlying the betulinic acid-induced Ca^{2+} entry. Fig. 4A shows that in Ca^{2+} medium, the $[Ca^{2+}]_i$ increase induced by 250 nM betulinic acid was significantly inhibited by pretreatment with 10 μM La^{3+} (trace b vs. trace a). Fig. 4B shows that the betulinic acid-induced $[Ca^{2+}]_i$ increase at the time point of 200 s was inhibited by La^{3+} by $85 \pm 3\%$ ($n = 5$; $P < 0.05$). Nifedipine (10 μM), verapamil (10 μM), and diltiazem (10 μM) had no effect ($n = 4$; data not shown).

3.7. Effect of a low concentration of betulinic acid on ATP and thapsigargin-induced $[Ca^{2+}]_i$ increases

Experiments were performed to examine the effect of a low concentration of betulinic acid (that does not increase $[Ca^{2+}]_i$) on the Ca^{2+} signals induced by the inositol

1,4,5-trisphosphate-dependent Ca^{2+} mobilizer ATP and inositol 1,4,5-trisphosphate-independent Ca^{2+} mobilizer thapsigargin. Fig. 5A shows that in Ca^{2+} medium, incubation with 2.5 nM betulinic acid for 260 s did not increase $[Ca^{2+}]_i$. Subsequently added 10 μM ATP induced a $[Ca^{2+}]_i$ increase with a net (basal $[Ca^{2+}]_i$ subtracted) peak value of 241 ± 10 nM, which was $33 \pm 3\%$ greater than the control ATP response (180 ± 8 nM; $n = 5$; $P < 0.05$) shown in Fig. 5B. The kinetics of the ATP-induced Ca^{2+} signal was

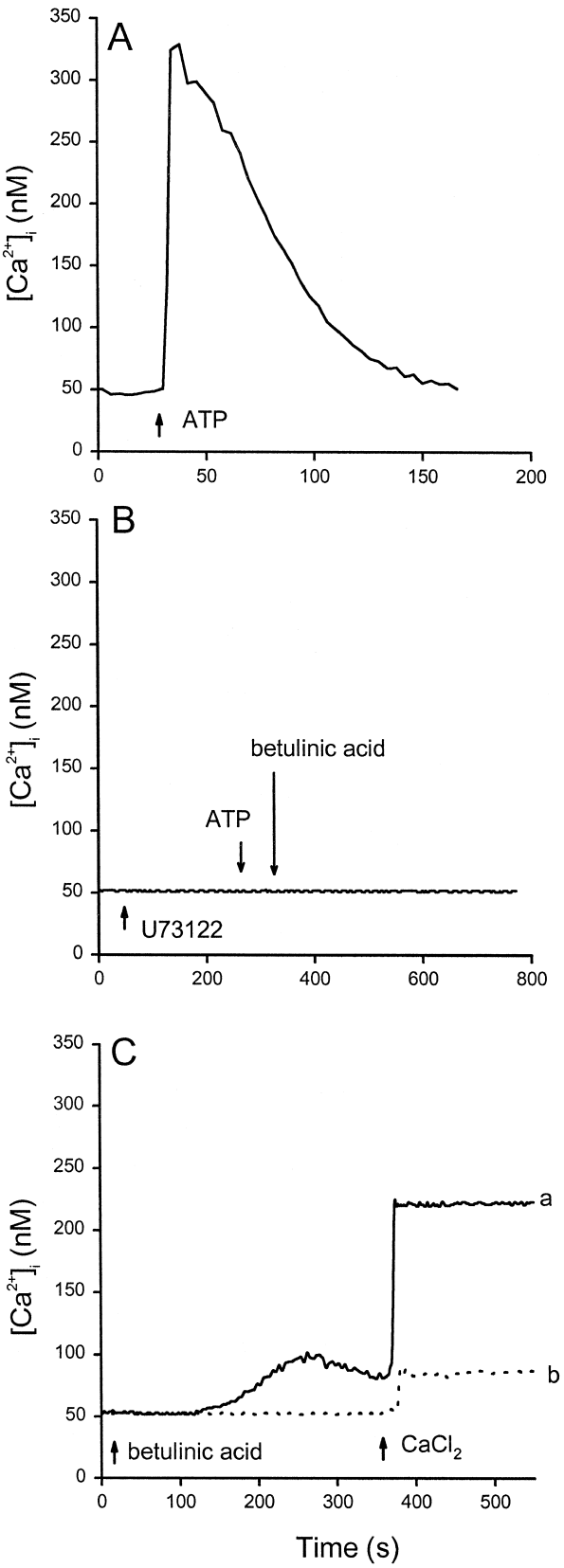

Fig. 3. Mechanisms of betulinic acid-induced extracellular Ca^{2+} influx and intracellular Ca^{2+} release. All experiments were performed in Ca^{2+}-free medium. (A) Effect of ATP on $[Ca^{2+}]_i$. ATP (10 μM) was added at 30 s. (B) Effect of inhibiting inositol 1,4,5-trisphosphate formation on betulinic acid-induced Ca^{2+} release. In Ca^{2+}-free medium, 2 μM U73122 was added at 30 s followed by 10 μM ATP at 240 s and 25 nM betulinic acid at 350 s, respectively. (C) Effect of reintroduction of Ca^{2+} on betulinic acid-induced $[Ca^{2+}]_i$ increase. Trace a: betulinic acid (250 nM) was added at 30 s followed by $CaCl_2$ at 380 s. Trace b: $CaCl_2$ was added at 380 s without betulinic acid pretreatment. Traces were typical of four to six experiments.

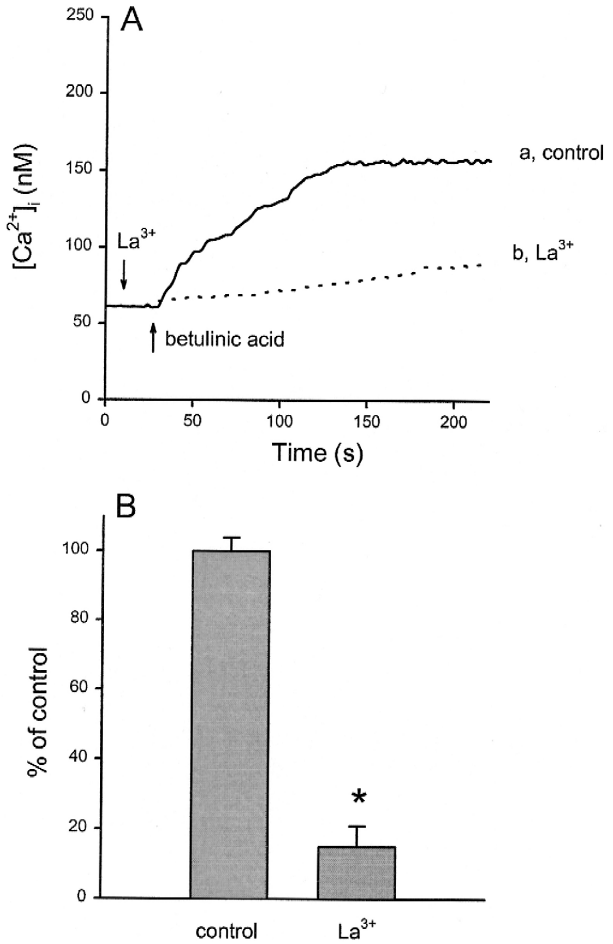

Fig. 4. Effects of La^{3+} on betulinic acid-evoked $[Ca^{2+}]$ increases. (A) Trace a: in Ca^{2+} medium, 250 nM betulinic acid was added at 30 s. Trace b: $LaCl_3$ (10 μM) was added 20 s before 250 nM betulinic acid was added. (B) A bar graph showing the inhibitory effect of La^{3+} on betulinic acid-evoked $[Ca^{2+}]_i$ increases. *Y*-axis is the percentage of control, which is the net $[Ca^{2+}]_i$ increase (baseline subtracted) induced by 250 nM betulinic acid at the time point of 200 s. $^{*}P < 0.05$. Traces were typical of four to six experiments.

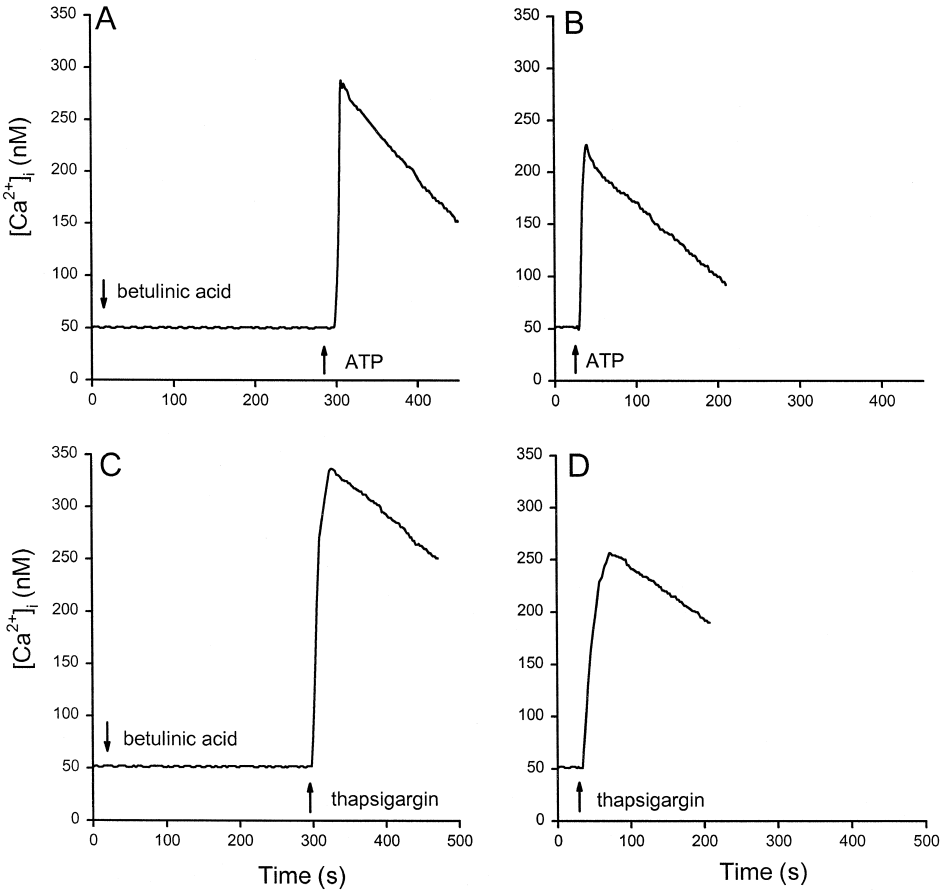

Fig. 5. Effect of a low concentration of betulinic acid on ATP and thapsigargin-induced $[Ca^{2+}]_i$ increases. The experiments were performed in Ca^{2+} medium. (A) Betulinic acid (2.5 nM) was added at 30 s followed by 10 μM ATP added at 280 s. (B) ATP (10 μM) was added at 30 s. (C) Betulinic acid was added at 30 s followed by 1 μM thapsigargin added at 290 s. (D) Thapsigargin (1 μM) was added at 30 s. Traces were typical of four to six experiments.

not significantly altered by betulinic acid treatment. Fig. 5C shows that after incubation with 2.5 nM betulinic acid for 260 s, 1 μM thapsigargin induced a $[Ca^{2+}]_i$ increase with a net peak value of 291 ± 11 nM, which was 45 ± 3% greater than the control thapsigargin response (200 ± 6 nM; $n = 5$; $P < 0.05$) shown in Fig. 5D. The kinetic of the thapsigargin response was not affected by betulinic acid pretreatment.

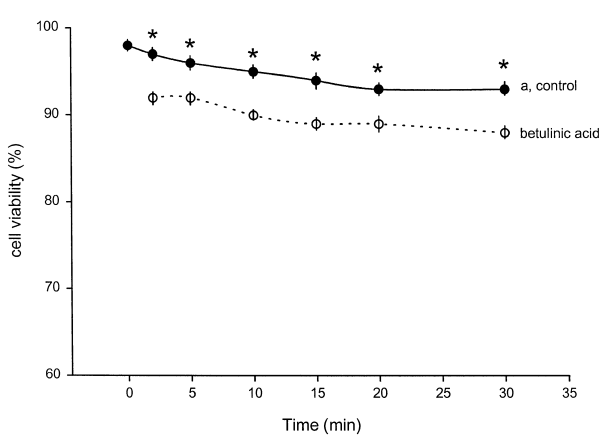

Fig. 6. Effect of betulinic acid on cell viability. Cell viability was assayed by Trypan blue exclusion. Trace a: control viability in the absence of betulinic acid. Trace b: cell viability in the presence of 250 nM betulinic acid. *Y*-axis is cell viability in percentage. *X*-axis is the incubation time. Data were means ± SEM of four to six experiments. * $P < 0.05$.

3.8. Effect of betulinic acid on cell viability

Trypan blue exclusion was performed to examine whether acute treatment with betulinic acid decreases cell viability. Fig. 6 shows that control viability was 98 ± 1% ($n = 4$) at the time point of 2 min, and was 96 ± 2% ($n = 4$) at the time point of 30 min (trace a). After exposure to 250 nM betulinic acid for 2 min, the cell viability was decreased by 6 ± 2% ($n = 5$). This decrease remained stable throughout the course of the measurement (30 min). This betulinic acid-induced mild cell death was prevented by adding 2 μM U73122 4 min before betulinic acid ($n = 5$; data not shown).

4. Discussion

Betulinic acid was shown to exhibit anti-tumor (Fulda et al., 1997, 1999a,b; Pisha et al., 1995; Selzer et al., 2000;

Yasukawa et al., 1991), anti-malarial (Bringmann et al., 1997), and anti-HIV (Evers et al., 1996; Kashiwada et al., 1996; Mayaux et al., 1994; Soler et al., 1996) action; however, it is not clear at all how this natural product exerts these effects except that this drug is a potent inducer of apoptosis (Schmidt et al., 1997; Wick et al., 1999). This report is the first to show that betulinic acid increased $[Ca^{2+}]_i$ in a renal tubular cell in a concentration-dependent manner between 25 and 500 nM with an EC_{50} of 100 nM. Betulinic acid increases $[Ca^{2+}]_i$ by causing both intracellular Ca^{2+} release and extracellular Ca^{2+} entry because the Ca^{2+} signals obtained in Ca^{2+} medium were partly inhibited by Ca^{2+} removal. The rising and sustained phases of the $[Ca^{2+}]_i$ increase were both reduced by Ca^{2+} removal, suggesting that the $[Ca^{2+}]_i$ increase involves Ca^{2+} entry and Ca^{2+} release throughout the 5-min measurement.

It appears that betulinic acid induces Ca^{2+} influx via a La^{3+}-sensitive pathway that was insensitive to voltage-gated Ca^{2+} channel blockers. The Ca^{2+} reintroduction experiments revealed that the betulinic acid-induced Ca^{2+} entry may be via capacitative Ca^{2+} entry, an intracellular Ca^{2+} refilling process, which is turned on by depleting stored Ca^{2+}. However, betulinic acid may also act by causing Ca^{2+} entry via directly opening a plasmalemmal Ca^{2+} channel in a manner dissociated from the extent of Ca^{2+} store depletion because the $[Ca^{2+}]_i$ increase was not inhibited by SKF96365 and econazole, two drugs that were shown to inhibit capacitative Ca^{2+} entry (Jan et al., 1999a,b).

The results indicate that betulinic acid evokes intracellular Ca^{2+} release mainly by discharging Ca^{2+} from the thapsigargin-sensitive stores, because in Ca^{2+}-free medium, pretreatment with 250 nM betulinic acid prevented 1 μM thapsigargin from releasing more Ca^{2+}. An important question is how betulinic acid causes Ca^{2+} release. Our findings suggest that inositol 1,4,5-trisphosphate may be involved in betulinic acid-induced Ca^{2+} release because the release was abolished by suppressing inositol 1,4,5-trisphosphate formation with the phospholipase C inhibitor U73122.

At a concentration that does not increase basal $[Ca^{2+}]_i$, betulinic acid potentiated ATP and thapsigargin-induced maximum $[Ca^{2+}]_i$ responses without altering the kinetics of the responses. Although the underlying mechanism is unclear, the data suggest a dual effect of betulinic acid: increasing basal $[Ca^{2+}]_i$ at higher concentrations and potentiating other Ca^{2+} mobilizers-induced Ca^{2+} signals at lower concentrations. Trypan blue exclusion revealed that acute exposure of cells to 250 nM betulinic acid for 2–30 min decreased cell viability by $6 \pm 2\%$ in a time-independent manner. This is consistent with previous findings that betulinic acid is cytotoxic (Fulda et al., 1997, 1999a,b; Pisha et al., 1995; Selzer et al., 2000). This mild effect of betulinic acid on viability is unlikely to be via apoptosis due to the rapidity of the effect. Our results suggest that the mild cytotoxic effect of betulinic acid was related to a prior $[Ca^{2+}]_i$ increase because the effect was abolished by inhibiting betulinic acid-induced $[Ca^{2+}]_i$ mobilization with U73122.

Together, this study shows that betulinic acid causes a significant increase in $[Ca^{2+}]_i$ and a mild decrease in viability in MDCK renal tubular cells, and has evaluated the underlying mechanisms of the $[Ca^{2+}]_i$ signal. The data suggest that betulinic acid increases $[Ca^{2+}]_i$ concentration dependently by releasing Ca^{2+} from the thapsigargin-sensitive stores in an inositol 1,4,5-trisphosphate-dependent manner, and also by inducing La^{3+}-sensitive extracellular Ca^{2+} entry. Due to the general importance of a $[Ca^{2+}]_i$ increase in cellular functions, these results may help to explain the diverse in vivo and in vitro effects of betulinic acid.

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