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Effect of oleamide on Ca^{2+} signaling in human bladder cancer cells☆

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Abstract

The effect of oleamide, a sleep-inducing endogenous lipid in animal models, on intracellular free levels of Ca^{2+} ($[Ca^{2+}]_i$) in non-excitable and excitable cells was examined by using fura-2 as a fluorescent dye. $[Ca^{2+}]_i$ in pheochromocytoma cells, renal tubular cells, osteoblast-like cells, and bladder cancer cells were increased on stimulation of 50 μM oleamide. The response in human bladder cancer cells (T24) was the greatest and was further explored. Oleamide (10–100 μM) increased $[Ca^{2+}]_i$ in a concentration-dependent fashion with an EC_{50} of 50 μM. The $[Ca^{2+}]_i$ signal comprised an initial rise and a sustained plateau and was reduced by removing extracellular Ca^{2+} by 85 ± 5%. After pre-treatment with 10–100 μM oleamide in Ca^{2+}-free medium, addition of 3 mM Ca^{2+} increased $[Ca^{2+}]_i$ in a manner dependent on the concentration of oleamide. The $[Ca^{2+}]_i$ increase induced by 50 μM oleamide was reduced by 100 μM La^{3+} by 40%, but was not altered by 10 μM nifedipine, 10 μM verapamil, and 50 μM Ni^{2+}. In Ca^{2+}-free medium, pre-treatment with thapsigargin (1 μM), an endoplasmic reticulum Ca^{2+} pump inhibitor, abolished 50 μM oleamide-induced $[Ca^{2+}]_i$ increases; conversely, pretreatment with 50 μM oleamide reduced 1 μM thapsigargin-induced $[Ca^{2+}]_i$ increases by 50 ± 3%. Suppression of the activity of phospholipase C with 2 μM U73122 failed to alter 50 μM oleamide-induced Ca^{2+} release. Linoleamide (10–100 μM), another sleep-inducing lipid with a structure similar to that of oleamide, also induced an increase in $[Ca^{2+}]_i$. Together, it was shown that oleamide induced significant $[Ca^{2+}]_i$ increases in cells by a phospholipase C-independent release of Ca^{2+} from thapsigargin-sensitive stores and by inducing Ca^{2+} entry.

Keywords: Ca^{2+}; Ca^{2+} stores; Fura-2; Oleamide; Thapsigargin; T24 bladder cells

1. Introduction

It was shown that cis-9,10-octadecenoamide (oleamide), isolated from the cerebrospinal fluid of sleep-deprived cats, is a natural constituent of the cerebrospinal fluid of cats, rats, and human, and synthetic oleamide induced sleep when

Abbreviations: ATP, adenosine 5′-triphosphate; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; fura-2/AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N,N-tetraacetic acid pentaacetoxymethyl ester; SKF96365, 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride; U73122, 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione; U73343, and 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione.

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injected into rats [1–3]. Oleamide has been considered as a new class of biological signaling molecules [1] because of its role in events related to cancer, inflammation, and other disorders [4]. However, the mechanism underlying oleamide's action is unclear.

Oleamide has been shown to modulate multiple *in vitro* responses. Oleamide activated serotonin 5-HT_7 neurons in mouse thalamus and hypothalamus [5] and modulated 5-HT_2 receptor-mediated behavior in the rat [6]. Oleamide modulated $GABA_A$ receptors, inhibitory synaptic currents [7,8], and voltage-gated Na^+ channels [9]. Furthermore, oleamide has been used as a gap junction inhibitor [10,11].

An increase in intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) is a key signal for diverse cellular processes [15–18]. A $[Ca^{2+}]_i$ increase can occur by Ca^{2+} release and/or Ca^{2+} entry. The inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store is an important intracellular Ca^{2+} pool that actively discharges Ca^{2+} into cytosol when the inositol 1,4,5-trisphosphate receptors on these stores bind cytosolic inositol 1,4,5-trisphosphate [15,16]. This Ca^{2+} release may cause Ca^{2+} influx across plasma membranes via the process of capacitative Ca^{2+} entry [19]. The effect of oleamide on Ca^{2+} signaling and the underlying mechanism are unclear. We have recently found that linoleamide, another sleep-inducing lipid with a structure similar to that of oleamide, increased $[Ca^{2+}]_i$ in renal tubular cells, and we evaluated the underlying mechanisms [20]. The present study explored the effect of oleamide on $[Ca^{2+}]_i$ in several epithelial cells including human bladder cancer cells (T24, BFTC), human osteoblast-like cancer cells (MG63), canine renal tubular cells (MDCK), and PC12 rat pheochromocytoma cells. By using fura-2 as a fluorescent Ca^{2+} probe, this study shows that oleamide induced a significant $[Ca^{2+}]_i$ increase in all five cell types. The concentration-response relationship was established, and the underlying mechanisms of the $[Ca^{2+}]_i$ increase was evaluated for T24 cells.

2. Materials and methods

2.1. Cell culture

T24 and BFTC human bladder cancer cells were cultured in Macoy's 5a medium and RPMI-1640 medium, respectively. MDCK (Madin-Darby canine kidney) renal cells and MG63 human osteoblast-like cancer cells were cultured in Dulbecco's modified Eagle medium. PC12 cells were cultured in RPMI-1640 medium. The media were 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.

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. The oleamide stock solution was made in 100% ethanol. The stock solutions for other agents were made in water, ethanol, or dimethyl sulfoxide. The concentration of the organic solvent in the final solution was less than 0.1%, which did not alter basal $[Ca^{2+}]_i$ (N = 4).

2.3. Fluorescence measurements

Trypsinized cells (10^6/mL) were allowed to recover in an appropriate medium for 1 hr before loading with 2 μM fura-2/AM for 30 min at 25°C in the same medium. Cells were washed and re-suspended 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 (Shimadzu Corporation, Kyoto, Japan) by continuously recording excitation signals at 340 nm and

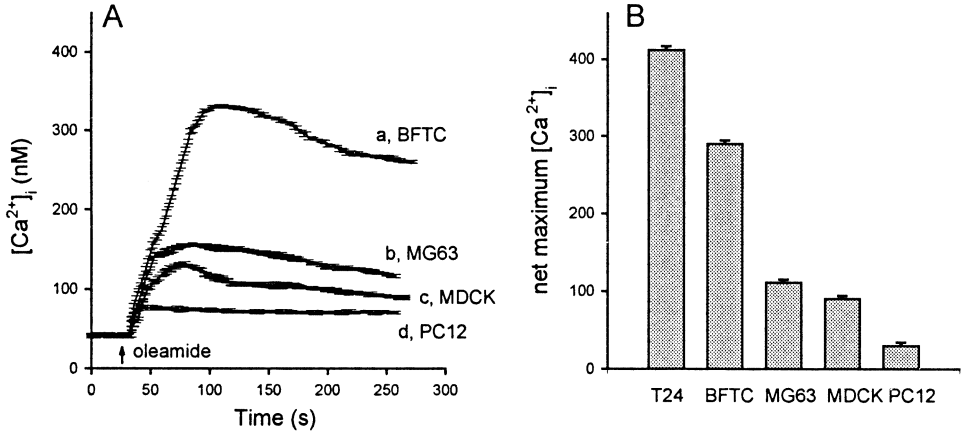

Fig. 1. The effect of oleamide on $[Ca^{2+}]_i$ in several cell types. (A) Oleamide (50 μM) was added at 30 sec in Ca^{2+} medium to BFTC (trace a), MG63 (trace b), MDCK (trace c), and PC12 (trace d) cells. (B) The net maximum (nM) of 50 μM oleamide-induced $[Ca^{2+}]_i$ increases in Ca^{2+} medium in the five cell types. Data are mean ± SEM of 4–6 replicates.

380 nm and emission signal at 510 nm at 1-sec intervals. Maximum and minimum fluorescence values were obtained by adding 10 μM digitonin (plus 10 mM $CaCl_2$) and 20 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as described previously assuming a K_d of 155 nM [21].

2.4. Materials

The reagents for cell culture were from Gibco (Grand Island, NY, USA). Fura-2/AM was from Molecular Probes (Eugene, OR, USA). Oleamide (9,10-octadecenoamide), linoleamide, U73122, and U73343 were from Biomol (Plymouth Meeting, PA, USA). The other reagents were from Sigma (St. Louis, MO, USA).

2.5. Statistics

The traces were the means ± SEM of 4–6 experiments. Because the data from each experiment were the average of responses from 0.5 million cells, the variation among experiments was small. Statistical comparisons were determined by using Student's *t* test, and significance was accepted when $P < 0.05$.

3. Results

Effects of oleamide on $[Ca^{2+}]_i$ in several cell types were examined. Fig. 1A shows that 50 μM oleamide induced a $[Ca^{2+}]_i$ increase in human BFTC bladder cancer cells, human MG63 osteoblast-like cells, canine MDCK renal tubular cells, and rat PC12 pheochromocytoma cells (N = 4–6). Fig. 1B compares the net (baseline subtracted) maximum value of oleamide-induced $[Ca^{2+}]_i$ increases in these cell types. The magnitude of 50 μM oleamide-induced $[Ca^{2+}]_i$ increases in these cells had an order of T24 > BFTC > MG63 > MDCK > PC12.

Because oleamide induced greater responses in T24 cells than in the other four cell types, the effect of oleamide in this cell was further explored. In T24 cells, oleamide at concentrations between 10–100 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner in Ca^{2+} medium. Fig. 2A shows the $[Ca^{2+}]_i$ increases induced by 100 μM (trace a), 50 μM (trace b), 25 μM (trace c), and 10 μM (trace d). At a concentration of 1 μM, oleamide had no effect (trace e). Over a time period of 250 sec, the $[Ca^{2+}]_i$ signals induced by 50–100 μM oleamide were composed of an initial rise and a sustained phase. The Ca^{2+} signal induced by 50 μM oleamide (trace b) had a net (baseline subtracted) maximum of 412 ± 3 nM (N = 6). The signal gradually decayed and showed a net $[Ca^{2+}]_i$ of 330 ± 4 nM at the time point of 250 sec. At the concentrations of 100 μM and 150 μM oleamide induced similar effects. Fig. 2C (filled circles) shows the concentration-response relationship of the oleamide response. The data suggest an EC_{50} value of 50 μM.

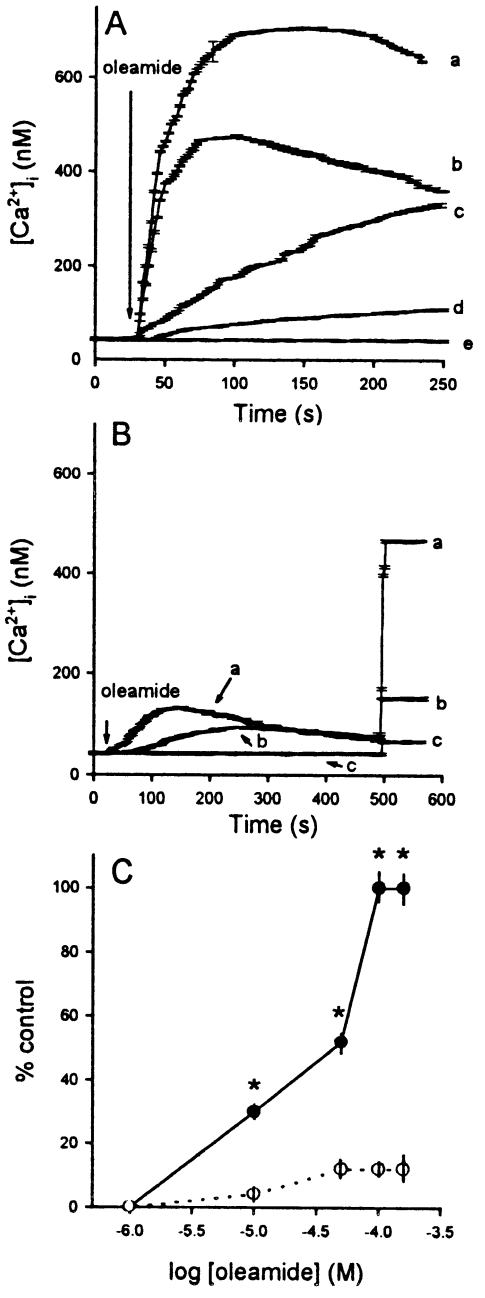

Fig. 2. Effect of oleamide on $[Ca^{2+}]_i$ in T24 human bladder cancer cells. (A) Concentration-dependent effects of oleamide. The concentration of oleamide was 100 μM (trace a), 50 μM (trace b), 25 μM (trace c), 10 μM (trace d), and 1 μM (trace e). Experiments were performed in Ca^{2+} medium. (B) Effect of oleamide on $[Ca^{2+}]_i$ in Ca^{2+}-free medium and the effect of reintroduction of Ca^{2+}. In Ca^{2+}-free medium, oleamide was added at 20 sec followed by adding 3 mM $CaCl_2$ at 500 sec. The concentration of oleamide was 100 μM (trace a), 50 μM (trace b), and zero (trace c). (C) Concentration-response plots of oleamide-induced responses in the presence (filled circles) or absence (open circles) of extracellular Ca^{2+}. Y-axis is the percentage of control. Control was the net (baseline subtracted) maximum $[Ca^{2+}]_i$ value of 100 μM oleamide-induced $[Ca^{2+}]_i$ increases in Ca^{2+} medium. Data are means ± SEM of 4–6 replicates. *$P < 0.05$ compared between filled and open circles.

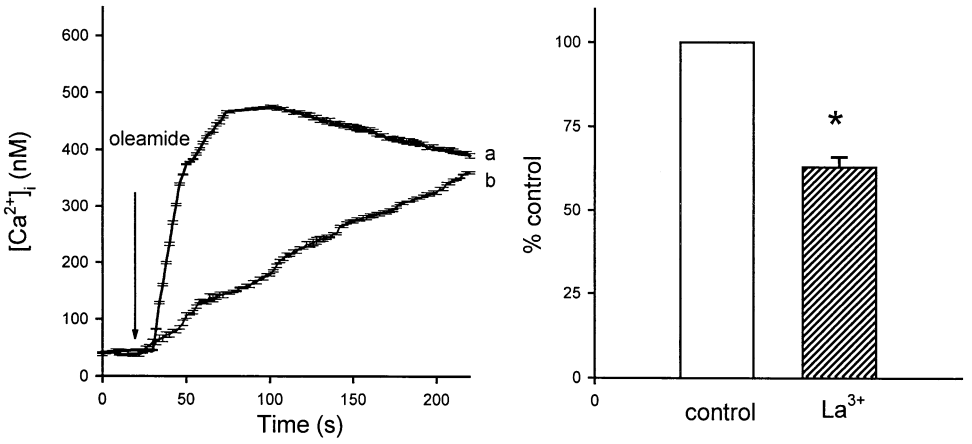

Fig. 3. Effect of La^{3+} on oleamide-induced $[Ca^{2+}]_i$ increases. (A) In Ca^{2+} medium, 50 μM oleamide was added at 30 sec in the absence (trace a) and presence (trace b) of 100 μM La^{3+}. La^{3+} was added 20 sec prior to oleamide. (B) The inhibitory effect of La^{3+} pretreatment on oleamide-induced $[Ca^{2+}]_i$ increases. The data are presented as percentage of control. Control was the net area under the curve between time points of 30–230 sec in trace a in (A). The area was calculated by a program installed in the Sigmaplot software. Data are mean ± SEM of 4–6 replicates. $*P < 0.05$ compared with control.

The data between time points of 0–500 sec in Fig. 2B shows that in Ca^{2+}-free medium (Ca^{2+} was substituted with 1 mM EGTA), 100 μM (trace a) and 50 μM (trace b) oleamide induced a $[Ca^{2+}]_i$ increase in a concentration-dependent manner in T24 cells. The $[Ca^{2+}]_i$ increase induced by 50 μM oleamide reached a net maximum of 33 ± 2 nM (N = 5). The Ca^{2+} signal was followed by a slow decay and showed a net value of 21 ± 2 nM at the time point of 500 sec. The concentration-response relationship of oleamide-induced $[Ca^{2+}]_i$ increases in Ca^{2+}-free medium is shown in Fig. 2C (open circles). The data suggest that Ca^{2+} removal decreased 85 ± 5% of the $[Ca^{2+}]_i$ increases induced by 10–100 μM oleamide (N = 5–6; $P < 0.05$).

Experiments were performed to explore whether oleamide-induced Ca^{2+} influx in T24 cells involved capacitative Ca^{2+} entry, which is a Ca^{2+} refilling process triggered by depletion of stored Ca^{2+} [19]. Fig. 2B (time points between 500–580 sec) shows that in Ca^{2+}-free medium, after pre-treatment with 100 μM and 50 μM oleamide in Ca^{2+}-free medium, addition of 3 mM $CaCl_2$ induced a rapid $[Ca^{2+}]_i$ increase with a net maximum of 401 ± 5 nM and 98 ± 4 nM, respectively (traces a and b; N = 4–6). The $[Ca^{2+}]_i$ increase stayed stable without decay. Adding $CaCl_2$ without oleamide pre-treatment induced a small $[Ca^{2+}]_i$ increase with a net maximum of 25 ± 1 nM (trace c; N = 4). The effect of several Ca^{2+} entry blockers on oleamide-induced Ca^{2+} influx was examined. Fig. 3A shows that in Ca^{2+} medium, the $[Ca^{2+}]_i$ increase induced by 50 μM oleamide (trace a) was inhibited by pre-treatment with 100 μM La^{3+} (trace b). The initial rising phase of the oleamide response was removed by the blocker. Fig. 3B shows that La^{3+} reduced the net (baseline subtracted) area under the oleamide response between time points of 30–230 sec by 37 ± 3% (N = 4–6; $P < 0.05$). Nifedipine (10 μM), Ni^{2+} (50 μM), verapamil (10 μM), and diltiazem (10 μM) had no effect (N = 4; not shown).

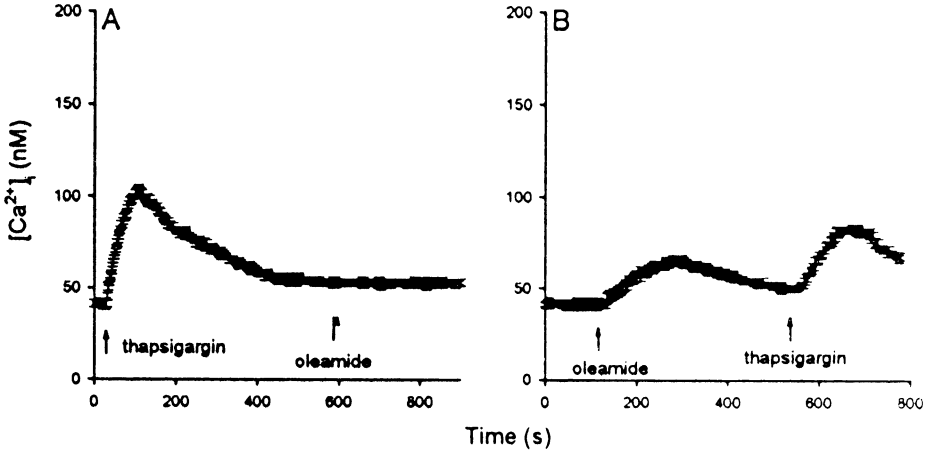

Fig. 4. Stores of oleamide-induced Ca^{2+} release. In Ca^{2+}-free medium, drugs were applied at the time indicated by arrows. The concentration was 1 μM for thapsigargin and 50 μM for oleamide. Traces are mean ± SEM of 4–6 replicates.

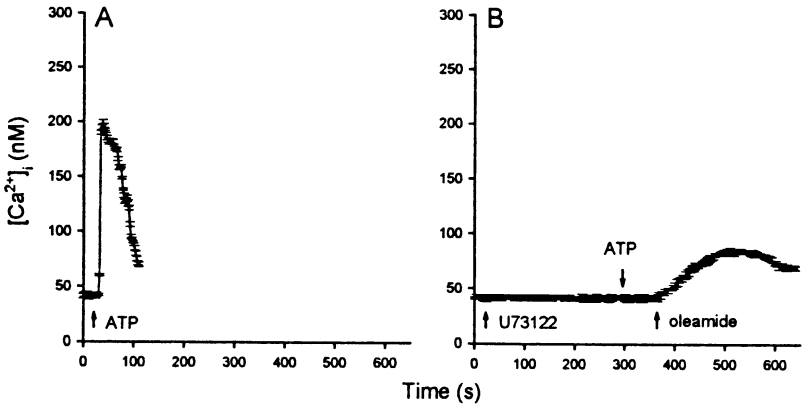

Fig. 5. Role of phospholipase C in oleamide-induced Ca^{2+} release. The experiments were performed in Ca^{2+}-free medium. (A) Effect of ATP on $[Ca^{2+}]_i$. ATP (10 μM) was added at 20 sec. (B) Effect of inhibiting phospholipase C activity on oleamide-induced Ca^{2+} release. U73122 (2 μM) was added at 30 sec followed by ATP (10 μM), and oleamide (50 μM) added at 300 sec and 360 sec, respectively. Traces were mean ± SEM of 4–6 replicates.

Experiments were performed to explore whether oleamide released Ca^{2+} from the endoplasmic reticulum in T24 cells. Fig. 4A shows that in Ca^{2+}-free medium, addition of 1 μM thapsigargin, an endoplasmic reticulum Ca^{2+} pump inhibitor [22], induced a $[Ca^{2+}]_i$ increase with a net maximum of 71 ± 3 nM (N = 6). After this Ca^{2+} store was depleted by thapsigargin, addition of 50 μM oleamide failed to induce a $[Ca^{2+}]_i$ increase (N = 6). Similar responses were found when 100 μM oleamide was used (not shown). Conversely, Fig. 3B shows that after pre-treatment with 50 μM oleamide, 1 μM thapsigargin still induced a $[Ca^{2+}]_i$ increase with a net maximum value of 32 ± 2 nM (N = 5), which is 45 ± 2% of the control shown in Fig. 4A.

Experiments were performed to examine whether oleamide-induced Ca^{2+} release was mediated by inositol 1,4,5-trisphosphate. U73122 is an inhibitor of phospholipase C, and has been used effectively in different cell types to block formation of inositol 1,4,5-trisphosphate [23]. Fig. 5A shows that in Ca^{2+}-free medium, the well-established inositol 1,4,5-trisphosphate-dependent Ca^{2+} releaser adenosine 5′ triphosphate (ATP); 10 μM induced a $[Ca^{2+}]_i$ increase with a net maximum value of 162 ± 3 nM (N = 5). This suggests that T24 bladder cells posses inositol 1,4,5-trisphosphate-sensitive Ca^{2+} mobilization machinery. Fig. 5B shows that in Ca^{2+}-free medium pretreatment with 2 μM U73122 abolished 10 μM ATP-induced $[Ca^{2+}]_i$ increases (N = 6). U73343 (10 μM), an inactive U73122 analog, had no effect on basal or ATP-induced $[Ca^{2+}]_i$ increases (not shown; N = 4). This suggests that U73122 effectively inhibited phospholipase C activity. The result further shows that subsequently added oleamide (50 μM) induced a $[Ca^{2+}]_i$ increase with a net maximum of 32 ± 3 nM (N = 6).

The effect of linoleamide, an unsaturated analog of oleamide that also induces sleep *in vivo* [20], on $[Ca^{2+}]_i$ in T24 cells was examined. Fig. 6A shows that linoleamide induced a $[Ca^{2+}]_i$ increase in a concentration-dependent manner between 10–50 μM. The $[Ca^{2+}]_i$ signal induced by 50 μM linoleamide had a net maximum value of 150 ± 2 nM (trace a; N = 6) and gradually decayed to a net $[Ca^{2+}]_i$ value of 91 ± 3 nM at the time point of 300 sec. Fig. 6B shows the

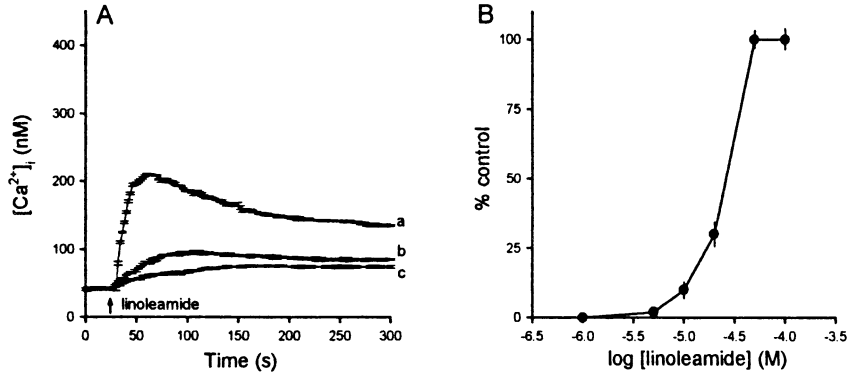

Fig. 6. Effect of linoleamide on $[Ca^{2+}]_i$. (A) Linoleamide was added at 30 sec in Ca^{2+} medium. The concentration of linoleamide was 100 μM (trace a), 50 μM (trace b), and 10 μM (trace c). (B) The concentration-response curve of linoleamide-induced $[Ca^{2+}]_i$ increases. Data are presented as percentage of control. Control was the net maximum $[Ca^{2+}]_i$ value induced by 100 μM linoleamide. Data are mean ± SEM of 4–6 replicates.

concentration-dependent relationship of the linoleamide response. The data suggest an EC_{50} value of 30 μM.

4. Discussion

Because Ca^{2+} is a ubiquitous second messenger for most cellular events, alterations of $[Ca^{2+}]_i$ may lead to significant physio-pathological phenomena. The present study explored the effect of oleamide on $[Ca^{2+}]_i$ in epithelial cells including bladder cells, osteoblast-like cells, renal tubular cells, and in catecholamine-secreting cells. In T24 cells, it was found that oleamide increased $[Ca^{2+}]_i$ in a concentration-dependent manner between 10–100 μM. Oleamide at this concentration range has been routinely used by researchers to investigate its *in vitro* effect [5,7,9–11]. Because a $[Ca^{2+}]_i$ increase may affect diverse cellular events, caution should be exercised in interpreting the data derived from using oleamide as a gap junction inhibitor or for other use.

The oleamide-induced $[Ca^{2+}]_i$ increase in T24 cells was characterized by an initial rise and a lasting phase that did not decay or that decayed slowly, dependent on the concentration of oleamide. The Ca^{2+} signal was contributed mainly by Ca^{2+} influx because removal of extracellular Ca^{2+} reduced 85 $\pm$ 5% of the response. The results suggest that the oleamide-induced Ca^{2+} influx may involve capacitative Ca^{2+} entry because readding Ca^{2+} to cells depleted with stored Ca^{2+} by oleamide in Ca^{2+}-free medium increased $[Ca^{2+}]_i$ in a concentration-dependent manner. However, the data could also be interpreted as that oleamide induced Ca^{2+} influx by directly opening a Ca^{2+} channel independently of depleting stored Ca^{2+}. These two possibilities were difficult to distinguish because of the lack of specific blockers for capacitative Ca^{2+} entry. SKF96365 and econazole, commonly used as capacitative Ca^{2+} entry blockers in many studies [24,25], were of little use because it has been shown that these two drugs cause a $[Ca^{2+}]_i$ increase by releasing stored Ca^{2+} and causing Ca^{2+} influx in canine renal tubular cells [26,27] and human endothelial cells [28]. We found that these two drugs also increased $[Ca^{2+}]_i$ in T24 cells (not shown). A characteristic of the oleamide-induced Ca^{2+} influx is its partial sensitivity to La^{3+} and insensitivity to Ni^{2+}, verapamil, nifedipine, and diltiazem.

Oleamide appears to release stored Ca^{2+} in T24 cells solely from thapsigargin-sensitive endoplasmic reticulum stores because pre-treatment with thapsigargin abolished oleamide-induced Ca^{2+} release, and conversely, pre-treatment with oleamide partly inhibited thapsigargin-induced Ca^{2+} release. This suggests that oleamide-sensitive Ca^{2+} stores are included in thapsigargin-sensitive stores.

The data suggest that phospholipase C activation is not required for oleamide-induced Ca^{2+} release in T24 cells because the release was not changed when the activity of phospholipase C was suppressed. How exactly oleamide releases stored Ca^{2+} is unknown.

The data suggest that linoleamide, a structural analog of oleamide that was shown to induce sleep *in vivo*, also increased $[Ca^{2+}]_i$ in T24 cells with an EC_{50} slightly less than that of oleamide. We have recently characterized linoleamide-induced $[Ca^{2+}]_i$ increases in renal tubular cells [21]. The responses induced by linoleamide in T24 cells and MDCK cells were largely similar.

The most important finding in this study is that sleeping-inducing brain lipids such as oleamide and linoleamide induce significant increases in $[Ca^{2+}]_i$ in epithelial cells and endocrine cells. In T24 cells, oleamide appears to act by releasing Ca^{2+} from the endoplasmic reticulum in a phospholipase C-independent fashion, and also by causing Ca^{2+} entry. Because evidence shows that linoleamide and its metabolites are found in the urine of rats dosed with linoleamide [29], the findings that oleamide and linoleamide increased $[Ca^{2+}]_i$ in bladder cells and renal cells suggest that these endogenous lipids may alter the cellular function in the urinary system.

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