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Characterization of cocaine-elicited cell vacuolation: the involvement of calcium/calmodulin in organelle deregulation

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Abstract

The sizes of organelles are tightly regulated in the cells. However, little is known on how cells maintain the homeostasis of these intracellular compartments. Using cocaine as a model compound, we have characterized the mechanism of deregulated vacuolation in cultured rat liver epithelial Clone 9 cells. The vacuoles were observed as early as 10 min following cocaine treatment. Removal of cocaine led to vacuole degeneration, indicating vacuolation is a reversible process. The vacuoles could devour intracellular materials and the vacuoles originated from late endosome/lysosome as indicated by immunofluorescence studies. Instant calcium influx and calmodulin were required for the initiation of vacuole formation. The unique properties of these late endosome/lysosome-derived vacuoles were further discussed. In summary, cocaine elicited a new type of deregulated vacuole and the involvement of calcium/calmodulin in vacuolation could shed light on prevention or treatment of cocaine-induced cytotoxicity.

Introduction

The size distribution of an organelle is extremely tight and its size control is fundamentally important in maintaining normal cellular homeostasis [1]. This phenomenon has been studied with either abnormal organelles of mutant cells or chemicals/toxins that can disturb the function of organelles [2–4]. The process of cytoplasmic vacuolation has been observed with a variety of toxins. For example, treatment of cells with *Helicobacter pylori*-produced cytotoxin, VacA, can disturb endocytic membrane flow by abnormally moving Rab7, a membrane-fusion mediator [5], to endosomes, leading to the generation of large vacuoles [6]. Proaerolysin-induced vacuolation has been well acknowledged as a process derived from fragmentized endoplasmic reticulum (ER) [7]. In addition, alkyl amines have been shown to cause vacuolation through lysosomal swelling [8]. Antibiotic X537A induces selective Golgi apparatus vacuolation in vascular and intestinal smooth muscle cells [9]. Thus, different toxins may induce vacuoles that originate from various deregulated organelles such as ER, Golgi, lysosomes or endosomes.

Cocaine, a major alkaloid isolated from the coca leaf, is one of the most abused drugs in the world. Cocaine has been shown to cause cardiovascular, central nervous and neuromuscular system toxicity, complications of renal and pulmonary injury, hapatotoxicity as well as genotoxicity [10–12]. In addition to its systematic toxicity, cocaine has also been known to affect morphology or functions of intracellular apparatus, including disturbance in spindle formation [13], perturbation of neurite

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extension [14], disruption of function and morphology of mitochondria [15], induction of ER dilation [16] and impairment of lysosomal proteolysis [17]. One of the most intriguing morphological alterations upon cocaine exposure is vacuole formation in the cytoplasm. Cocaine-induced cell vacuolation has been reported in animals [18], isolated organs [19] and cultured cell systems [19, 20]. It has been proposed that cellular vacuolation is associated with cell injury or cytotoxicity [21, 22]. However, the exact mechanism of cocaine-induced vacuolation has not been elucidated.

In this study, we therefore used cocaine as a model compound to characterize the behavior of the deregulated vacuoles. By exploring the mechanism of cocaine-induced vacuolation, we hope to provide a clue to prevent or treat cocaine-induced cell injury or cytotoxicity.

Materials and methods

Chemicals and media

Cocaine–HCl was obtained from the official provider of narcotics for medical and scientific purposes, the National Bureau of Controlled Drugs, Taiwan. The cocaine–HCl was screened from a batch of active pharmaceutical ingredient (API)-grade raw material that met the requirements of British Pharmacopoeia 1993. The cocaine–HCl was further analyzed by gas chromatography/mass spectrometry to be 100.00% (as cocaine base) with negligible amounts of impurities, and then aliquoted for experiments. Since the hydrochloride salt of cocaine could readily dissolve in water, distilled water was used as the solvent for all experiments. Bafilomycin A1, verapamil, BPATA-AM (1,2-bis(2-aminophenoxy)ethane-*n*,*n*,*n*′,*n*′-tetraacetic acid acetoxymethyl ester), trifluoperazine (TFP), W7 (*n*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide) and cytochalasin B were purchased from Sigma Chemical Co. (St. Louis, MO). Calcein-AM, Fluo 3-AM and DilC_{18}(5) oil (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate) were purchased from Molecular Probes, Inc. (Eugene, OR). All media, including F-12, Ham's F12K, DMEM and McCoy's 5A, were purchased from Gibco BRL Co. (Grand Island, NY).

Cell culture

All cell lines tested in this study were purchased from American Type Culture Collection (Rockville, MD). The rat liver epithelial cell line, Clone 9, was grown in F-12 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah), 1% L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The Clone 9 cells were cultured at 37°C in a humidified incubator containing 5% CO_2 and 95% air. Other cell lines, including PC12, HeLa, and CHO-K1, were grown in Ham's F12K medium, DMEM and McCoy's 5A, respectively.

Examination of cell vacuolation

The Clone 9 cells were seeded on coverslips, and cultured overnight. After exposure to cocaine, the cells were washed twice with phosphate buffered saline (PBS), and then examined for vacuolation and photographed under the optical microscope. Bafilomycin A1, verapamil, BAPTA-AM, TFP and W7 were co-treated with cocaine for 2 h, respectively.

Neutral red staining and quantitation of cell vacuolation

After incubation, cells were stained with 100 μM neutral red for 15 min at room temperature. Excessive neutral red was removed by washing twice with PBS. Intracellular neutral red was then extracted with 70% ethanol containing 0.37% HCl and quantitated spectrophotometrically. The absorbency was measured by a Packard Argus 400 microplate reader at 540 nm with subtraction of absorbency at 405 nm [6]

Preparations of cells for transmission electron microscopy (TEM)

The Clone 9 cells were seeded in 35-mm dishes overnight. After treatments, cells were washed with PBS twice and trypsinized by incubating with 1 ml of trypsin solution (0.05% trypsin, 0.53 mM EDTA.4Na) at 37°C for 1 min. The cells were then shaken off and fresh F12 medium was added for further centrifuge. The supernatant was discarded and cell pellets were fixed with 2% glutaldehyde in 0.1 M cacodylate buffer (pH 7.2) in dark at room

temperature for 1 h. Then, the cell pellets were washed three times with cacodylate buffer, 10 min for each time. The cells were incubated with 1% osmium tetraoxide in 0.1 M cacodylate buffer in dark at room temperature for 1 h. After being washed with water, the cells were dehydrated with 30%, 50%, 70%, 80%, 95% and 100% ethanol sequentially. Then, the cells were rinsed with 25%, 50% and 75% spurr (Electron Microscopy Sciences, Fort Washington, PA) in ethanol, and kept at room temperature overnight. Finally, pure spurr was applied at room temperature for 2 h and the preparations were transferred to 70°C for 24 h. Thin section was stained with lead citrate and uranyl acetate, and observed with a JEOL JEM 1200 electron microscope.

Immunofluorescence

The cells were rinsed with PBS and then fixed with 4% formaldehyde (pH 7.4 in PBS) for 15 min, followed by permeabilization with 0.1% SDS in PBS for 4 min. Immunofluorescent labeling was performed as follows. All primary antibodies were incubated at room temperature for 60 min and secondary antibodies were incubated at 37°C for 30 min. The ER network was visualized with 1:50 dilution of mouse anti-Bip monoclonal antibody (Transduction Laboratories, Lexington, KY), followed by treatment with 1:100 of FITC-conjugated rat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO). Golgi was labeled with 1:100 dilution of mouse anti-TGN38 monoclonal antibody (Transduction Laboratories, Lexington, KY), followed by incubation with 1:100 of FITC-conjugated rat anti-mouse IgG. Endosomes were marked with either an early endosome marker, mouse anti-Rab4 monoclonal antibody (1:100 dilution, Transduction Laboratories, Lexington, KY) or a late endosome marker, rabbit anti-Rab7 antiserum (1:200 dilution, Cytosignal Research Products, Irvine, CA), followed by incubation with 1:100 or 1:4,000 dilution of FITC-conjugated rat anti-mouse or rabbit IgG (Transduction Laboratories, Lexington, KY). Lysosomal labeling was performed with 1:100 dilution of Lamp1 monoclonal antibody (Calbiochem Com., San Diego, CA), followed by 1:4,000 dilution with fluorescein-conjugated rat anti-sheep IgG (ICN Pharmaceuticals, Inc., Aurora, Ohio). After antibody binding reaction, cells were mounted with 90% glycerol containing 1 mg/ml of 1,4-phenylendiamine and were observed with FITC filter microscopically.

Measurement of Ca^{2+} concentration change within cells

Relative concentration of intracellular calcium was determined according to Ronde's method with some modifications [23]. Cells were seeded in 60 mm dish overnight. After preloading with 10 μM of the fluorescent Ca^{2+} indicator Fluo-3 for 30 min, cells were washed with PBS twice and challenged with cocaine or cocaine plus verapamil (0.1, 0.2, 0.3 mM), BAPTA-AM (5, 10, 20 μM), W7 (5, 10, 20 μg/ml), or TFP (5, 10, 20 μM), respectively, in Hepes buffer. Images were acquired at 1 min intervals with a Bio-Rad model MRC-600 argon/krypton ion laser-scanning confocal imaging system attached to a Zeiss Axiovert 135 M inverted microscope (63X water immersion epifluorescent objective), and measured fluorescence emitted in response to 480 nm excitation. The tested compounds were rapidly applied after recording the first image (as basal line and defined as 1.0). All quantitation of fluorescence intensities associated with 10–15 cells, as recorded in digitized images, was performed by using OPTIMAS image analysis software (Optimas Co., Seattle, WA). For averaged data, all cells within the microscopic field that responded to test compounds were included in the quantitation.

Statistical methods

For quantitation of cocaine-induced vacuolation by neutral red staining, each experiment was repeated three times and the results were represented as the mean ± S.D. of three independent experiments. The results were then compared using One-Way ANOVA. A P-value of <0.05 was considered significant.

Results

Cocaine-induced cell vacuolation is a rapid and reversible process

Cocaine induced vacuole formation in all cells tested, including HeLa, PC12, CHO K1 (data not shown) and Clone 9 (Supplemental figure 1). The

Clone 9 cell line was chosen in this study because of its convenience for morphology examination. To quantitate the extent of cell vacuolation, cocaine-exposed cells were stained with neutral red, a basic dye that has been used to label and measure the acidic vacuole triggered by bacterial toxin VacA-induced acidic vacuoles [24]. Our results showed that neutral red labeled the cocaine-elicited vacuoles promptly (Figure 1A), implicating the acidic interior of the vacuoles. As shown in Figure 1B, uptake of neutral red was increased as the doses of cocaine were increased. The uptake of neutral red was also increased in a time-dependent manner (Figure 1C). After cocaine was removed, vacuoles disappeared within 1–2 h (Figure 1D), indicating that the process of vacuolation was reversible. These results showed that vacuolation was a dynamic process upon cocaine exposure.

Cocaine-induced vacuoles can engulf cytoplasmic materials

The cocaine-induced vacuoles could be labeled with a membrane probe $DiIC_{18}(5)$ oil, indicating the vacuoles possess a membrane-enclosed property

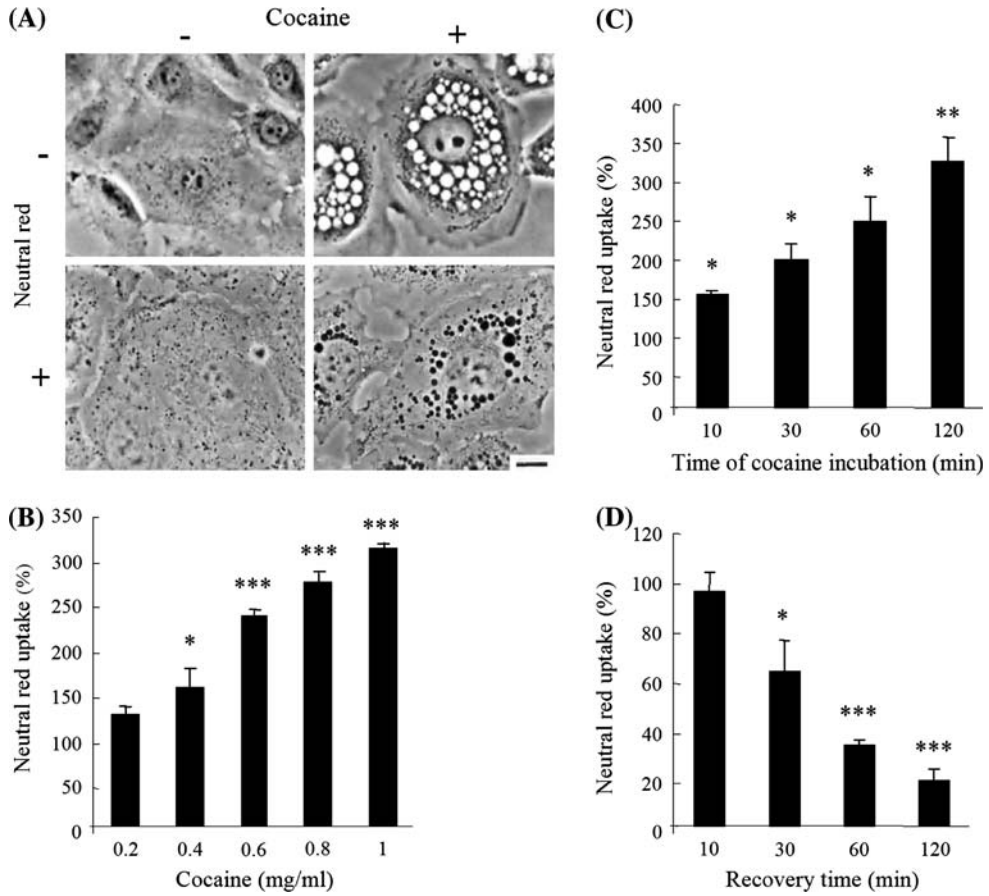


Figure 1. Dose and time dependency of cocaine-induced cell vacuolation. Untreated cells or cocaine (1 mg/ml)-treated cells were stained with 100 μM neutral red or vehicle for 15 min at room temperature, followed by microscopic photography (A), or extraction and quantitation spectrophotometrically (B–D). (A) Phase contrast photos of untreated cells showed little neutral red uptake, however, cocaine-provoked vacuoles were heavily labeled with the basic dye. (B) Uptake of neutral red in cells treated with various doses of cocaine for 2 h. (C) Uptake of neutral red in cells treated with 1 mg/ml of cocaine added for 0–120 min. (D) After treatment with 1 mg/ml of cocaine for 2 h, the cells were cultured in cocaine-free medium for 0–120 min followed by staining and quantitation with neutral red. Neutral red uptake of untreated cells was defined as 100% in (B) and (C). The neutral red uptake by the cells challenged with cocaine without recovery was defined as 100% and that of untreated cells was 0% in (D). Results are represented as the mean ± S.D. of three independent experiments. Significant difference between untreated and cocaine-treated cells is given by $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

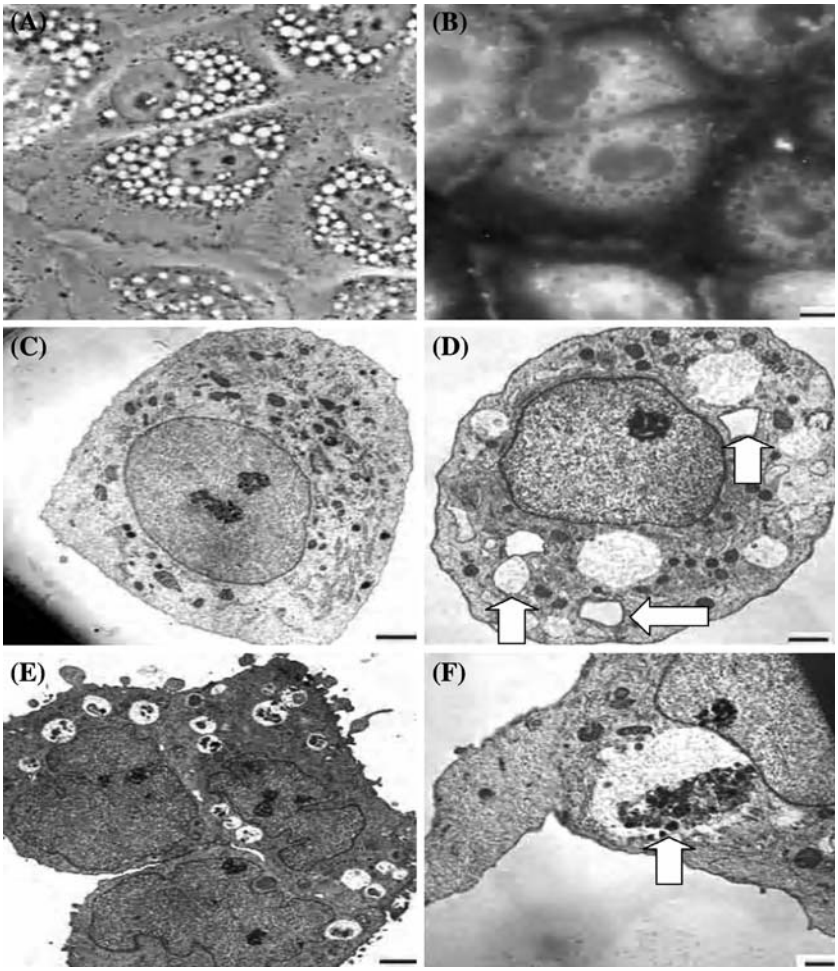


Figure 2. Properties of cocaine-induced vacuoles. The cocaine-induced vacuoles were membrane-enclosed and frequently contain cytoplasmic subtances. (A, B) Labeling of vacuolar membrane with $DiIC_{18}(5)$ oil. Cocaine (1 mg/ml)-treated cells were incubated with 100 μM $DiIC_{18}(5)$ oil for 30 min. Subsequently, the treated cells were washed with PBS and examined by phase contrast (A) or fluorescent microscope equipped with rhodamine filter (B). Untreated (C) and vacuolated cells elicited by cocaine (D–F) were examined with TEM. Arrows in D indicate vacuoles with enclosed membrane and the arrow in F represents organelle like particle in a vacuole. The bars in (B), (C–E) and (F) represent 10, 2 and 1 μm, respectively.

(Figure 2A, B). As shown by TEM, there was no significant vacuolation observed in the untreated cells (Figure 2C). However, vacuoles with varied sizes were formed upon cocaine exposure and some of the vacuoles apparently were enclosed by membrane (indicated by *arrows* in Figure 2D). Interestingly, electron-dense particles were found in part of the cocaine-treated cells (Figure 2E). Many of such electron-dense materials were organelles-like (Figure 2F). These results suggested that the vacuoles were likely to engulf intracellular materials. Indeed, the cocaine-induced vacuoles were shown to uptake cytoplasmic fluorescent dye calcein (Supplementary figure 1), implicating the devouring property of these inducible organelles.

The vacuoles may originate from late endosome/lysosome compartments

To explore the origins of the vacuoles, immunofluorescent staining assays were performed with antibodies against early endosomal marker Rab4 [25], late endosomal marker Rab7 [26] or lysosomal marker Lamp1 [27] in cocaine-treated cells. The results showed that Rab4 signals were not

co-distributed with the vacuoles upon cocaine treatment, but signals of Rab7 and Lamp1 were localized at the margins of the vacuoles (Figure 3). Further examination on the Rab7/Lamp1 distribution in control and cocaine-treated cells, we found that the amounts of Rab7/Lamp1 protein were not significantly raised (data not shown), while the two proteins became intensively localized to the vacuolated cells upon cocaine treatments. In contrast, the Golgi or ER marker, Bip or TGN38, was not associated with the vacuoles, excluding the involvement of these organelles in forming cocaine-induced vacuoles (data not shown).

Ca^{2+}/Calmodulin is involved in the cocaine-provoked cell vacuolation

Several studies have reported that cocaine induces Ca^{2+} influx in many cell types, including liver cells [28–30]. The role of Ca^{2+} in the process of cell vacuolation was therefore investigated. To monitor the rise of Ca^{2+} concentration in the cytoplasm, the relative changes of Ca^{2+} binding fluorescence intensity in the untreated and cocaine-treated cells were detected. The rise of intracellular Ca^{2+} concentration was observed 2 min after cocaine treatment, and the intracellular Ca^{2+} concentration rapidly reached plateau (Figure 4A). Co-incubation of verapamil, calcium channel blocker,

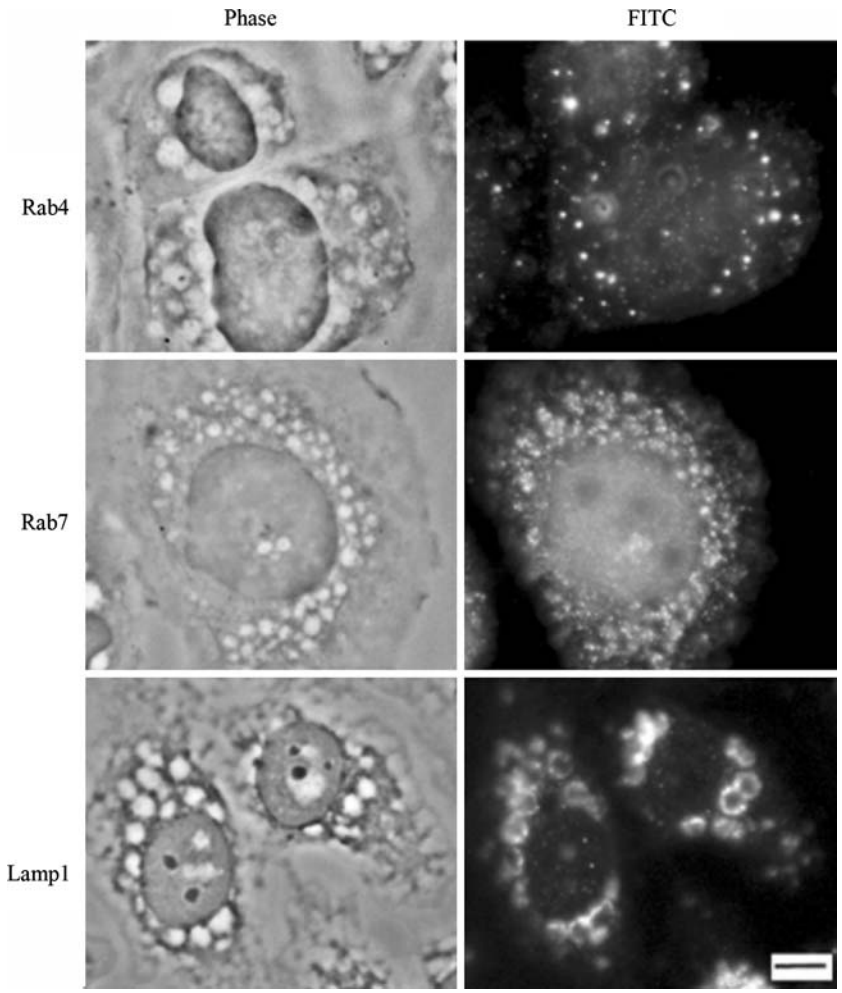
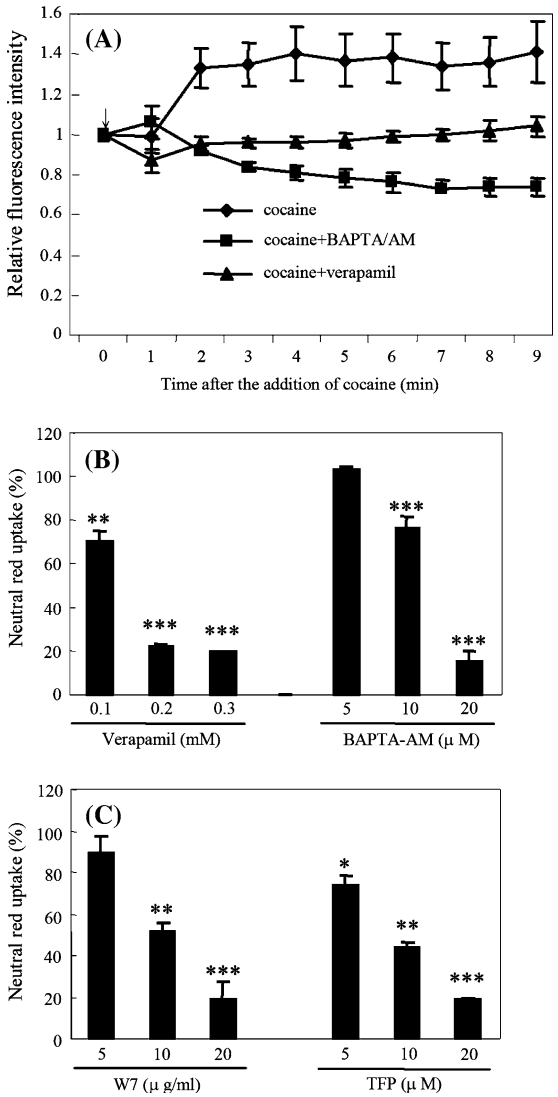


Figure 3. Origin of cocaine-induced vacuoles from late endosomes/lysosomes. Cells were fixed with 4% formaldehyde followed by permeabilization with 0.1% SDS. Subsequently, antibodies against Rab 4 (early endosomal marker), Rab 7 (late endosomal marker) or Lamp 1 (lysosomal marker) were incubated with cells. These cells were examined and photographed with fluorescent microscope.



◀*Figure 4*. Involvement of Ca^{2+} and calmodulin in cocaine-induced cell vacuolation. (A) Effects of verapamil or BAPTA-AM on the ratio of cocaine-induced calcium binding fluorescence intensity. Cells were preloaded with 10 μM of fluo-3 for 30 min. Then, cells were treated with cocaine alone, cocaine plus verapamil or cocaine plus BAPTA-AM simultaneously. Fluorescent intensities were quantitated with recorded digital images from 10 to 15 cells. Data were expressed as the ratio of fluorescent intensities of drug-exposed cells at 1–9 min over cells at 0 min. The ratio of untreated cells remained unchanged during the 10-min detection period (data not shown). Arrow indicates the time cocaine or cocaine plus verapamil/BAPTA-AM was added. Results are represented as the mean ± S.D. of three independent experiments. (B) Verapamil (0.1, 0.2, 0.3 mM), BAPTA-AM (5, 10, 20 μM) or (C) W7 (5, 10, 20 μg/ml), TFP (5, 10, 20 μM) were co-incubated with cocaine for 2 h. After the cells were washed with PBS twice, 100 μM of neutral red was incubated for 15 min at room temperature. Uptakes of neutral red were quantitated with microplate reader (see "Materials and methods"). Cocaine-treated cells were defined as 100% of neutral red uptake, and untreated control was expressed as 0% of uptake. Results are represented as the mean ± S.D. of three independent experiments. Asterisks denote significant difference between cocaine and cocaine/verapamil or BAPTA-AM or cocaine/W7 or TFP-treated cells. $^{**}P < 0.01$, $^{***}P < 0.005$.

blocked cocaine-elicited Ca^{2+} influx. In addition, co-treatment of BAPTA-AM, an intracellular calcium chelator, also inhibited cocaine-induced intracellular Ca^{2+} rise and even reduced Ca^{2+} to a concentration below basal level. More importantly, when cells were co-incubated with cocaine and verapamil or BAPTA-AM, cocaine-induced cell vacuolation was significantly inhibited (Figure 4B). These results indicated that Ca^{2+} played an important role in the formation of cocaine-elicited vacuoles.

Cocaine induced a slight increase of intracellular calcium concentration within 10 min while apparent vacuolation had been observed significantly (Figure 4A). This result suggests that ionic imbalance was unlikely to trigger cocaine-induced vacuolation. Alternatively, calcium influx was more likely to be involved in either signaling processes or enzymatic reactions. It is well acknowledged that calmodulin, a cellular regulator for Ca^{2+}, functions concurrently with Ca^{2+} in many cellular processes. TFP or W7, two antagonists of calmodulin, efficiently prevented cocaine-induced cell vacuolation (Figure 4C). These data indicated that Ca^{2+}/calmodulin were essential components in cocaine-induced cell vacuolation.

Discussion

In this study we tried to elucidate the mechanism of cocaine-induced vacuolation. The following results were obtained: (i) Cocaine-induced cell vacuolation was a rapid and reversible process. (ii) The vacuoles could engulf cytoplasmic materials including a few organelle-like structures, resembling autophagic process. (iii) The vacuoles were derived from late endosome/lysosome, based on the presence of Rab7 and Lamp1 proteins on vacuolar membrane. (iv) Calcium/calmodulin was involved in the cocaine-induced cell vacuolation.

The cocaine-induced vacuoles could originate from the late endosomes/lysosomes as shown in this study. However, it remains unclear why these

endocytic compartments could be larger than 3–5 μm. The typical size of endosomes was far below 1 μm in diameter [31]. To interpret the deregulated growth of the vacuoles, we propose that smaller compartments could fuse with each other, leading to a rapid enlargement of the vacuoles. Several lines of observations support this concept. First, Rab7, reported to promote inter-endosomal fusion [32], was detected to localize on the vacuolar membrane (Figure 3). It has been shown that there are a number of small vesicles scattered throughout the cytoplasm of a Rab7 dominant negative mutant. Overexpression of Rab7 with an active mutant form leads to the appearance of atypically large endocytic structures [33]. These findings clearly indicate that Rab7 plays a crucial role in the membrane fusion. Further studies show that the process of endosomal fusion can be divided into four stages: priming, tethering, ducking and fusion. In yeast, the homologue of Rab7 (Ypt7p) is believed to take part in the tethering stage [5]. In this study, we found that Rab7 was intensively localized around the vacuoles elicited by cocaine. Hence, it is likely that extensive fusion between endosomes could proceed to promote cell vacuolation upon cocaine exposure. Second, endocytic fusion within cells depends on F actin [34]. Disruption of F actin decreased the size of vacuoles (Supplementary table 1), indicating that enlargement of vacuoles is F actin-dependent. Therefore, the interruption of F actin could block intervacuolar fusion. Third, Ca^{2+} and calmodulin are involved in cell vacuolation elicited by cocaine. It has been documented that Ca^{2+}/calmodulin is involved in the last step of membrane fusion [35]. Collectively, all these evidences strongly suggest that the larger vacuoles are produced through the fusion of smaller late endosome/lysosome compartments. On the other hand, the vacuoles could reach extraordinary size, implying huge amount of membrane components is required for vacuole formation, and therefore arguing organelles other than endocytic compartment are possibly involved to serve as membrane sources. We investigated the possibility by examining the membrane marker of ER or Golgi, i.e. Bip [36] or TGN38 [37], and treating with colcemid or brefeldin A able to disturb ER [38] or Golgi [39], respectively. The vacuoles were not labeled with Bip or TGN38, and colcemid or brefeldin A could not interfere with cocaine-induced vacuolation (data not shown), excluding the involvement of ER or Golgi in the development of such vacuoles.

The vacuoles were formed at the first 10 min of cocaine exposure, and degenerated largely within 1–2 h after cocaine removal (Figure 1). Hence, it is plausible to conceive that the underlying mechanism for such vacuolation involves an effective and responsive machinery. Cocaine caused a rapid calcium influx that reached its plateau within 2 min of cocaine exposure (Figure 4A). Therefore, Ca^{2+} influx, with the help of calmodulin, could serve as a signal to trigger cocaine-induced cell vacuolation. Moreover, we found that Na^+/K^+-ATPase inhibitor, ouabain [40], did not block cocaine-induced cell vacuolation (data not shown), excluding the involvement of Na^+/K^+ in the process of cocaine-elicited vacuole formation. These data indicate that a specific signal, Ca^{2+} along with calmodulin, rather than global ionic imbalance, is employed to trigger vacuolation.

Vacuolation has been shown with several model systems. Bacterial toxin aerolysin or VacA has been reported to cause cell vacuolation via receptor binding in mammalian cells [6, 7]. Ionic imbalance has also been suggested to result in α-adrenergic receptor-mediated vacuolation [41]. Alkyl amines, after entering lysosomes, are trapped in their protonated forms, which finally lead to lysosomal swelling [8]. Hyperoxic exposure-induced vacuolation is possibly based on oxidative stress [42]. In this study we propose a novel mechanism for cell vacuolation by which calcium/calmodulin is involved.

There are other organelles with structure or behavior similar to the vacuole triggered by cocaine. For example, alkyl amines such as NH_4Cl, is trapped and accumulated in acidic lysosome, leading to hypertonic interior of the organelle and subsequent swelling. The lysosomal swelling brings about the generation of huge vacuoles in cytoplasm [8]. Cocaine is also a basic tertiary amine in structure, raising a possibility that cocaine might cause vacuole formation through a similar mechanism. Moreover, autophagosome is a typical organelle with the ability to engulf cellular materials, which represents a non-selective mechanism for the bulk degradation of cytoplasm [43]. The vacuoles elicited by cocaine share a structural character of lysosome and display autophagy-like behavior. Thus, it would be of interest to distinguish the three different cellular processes. As shown in Table 1,

Table 1. Comparison of drug response and structural characteristics among cocaine-induced vacuolation, NH_4CI-triggered lysosomal swelling, and autophagy.

Compared items	Cocaine-induced vacuolafion	NH_4CI-triggered lysosomal swelling	Autophage	References[b]
Drug responses[a]				
1. 3-Methyl adenine	+	–	+	[46]
2. Cytochalasin B	+	–	+	[47]
3. Sodium azide	+	ND	+	[48]
4.GTPγs	–	ND	+	
5.Wortmannin	–	ND	+	[50]
6.Okadaic acid	–	ND	+	[51]
7.Bafilomycin Al	+	+	+	[52]
8.Verapamil	+	–	+	[53]
9.BAPTA-AM	+	–	+	[54]
10. TFP	+	–	?	NF
11.W7	+	–	?	NF
Structural/behavioral aspects				
12. Multiple membrane	No	ND	Yes	[45]
13. Calcein-AM inclusion	Yes	No	?	NF
14. Lucifer Yellow uptake	Yes	No	?	NF

ND, not detected; NF, references not found; +, drug with inhibitory effect on vacuole formation; –, no effect on vacuole formation; ?, not known yet.

[a]Cells were treated with cocaine (1 mg/ml) and various chemicals simultaneously for 1 hour, including 3-methyl adenine (20 mM), cytochalarin B (5 μg/ml), sodium azide (0.2 mM), α-toxin (1.76 μg/ml) ± GTP-γs (500 μM), wortmannin (0.1 μM), okadaic acid (1 μM), bafilomycin A1 (3.2 nM), verapamil (300 μM), BAPTA/AM (20 μM), TFP (15 μM), W7 (20 μg/ml). On the other hand, NH_4Cl (50 mM) was added to cells for 1 hour.

[b]References concerning autophase.

there are nine compounds that inhibit autophagosome formation according to literatures (Item 1–9). However, three of them, namely, GTPγs (nonhydrolyable GTP analog), wortmannin (PI3-kinase inhibitor) and okadaic acid (protein kinase inhibitor), all of which are known to inhibit autophagosome development at its early stage [44], failed to block cocaine-elicited vacuolation, implying different biogenetic mechanisms for these two cellular events. Furthermore, autophagosome is characterized as an inducible organelle possessing multiple membranes [45]. Careful examination of cocaine-provoked vacuole does not find this particular structure (Figure 2), implying these two types of vacuoles are formed through different mechanisms. On the other hand, most of the drugs capable of inhibiting cocaine-elicited cell vacuolation have no effect on NH_4Cl-induced lysosomal swelling (Items 1, 2, 8, 9, 11 and 12 of Table 1). It is noteworthy that the swollen lysosome could not engulf cytoplasmic fluorescent marker calcein (Table 1, Item 13), and failed to uptake Lucifer Yellow via endocytosis (Table 1, Item 14), implicating NH_4Cl-induced vacuoles are different from cocaine-elicited ones. Collectively, the cocaine-induced vacuole exhibits distinct characters and can be classified as a new type of autophagic vacuole.

Thus, the cocaine-initiated vacuolation triggers deregulation in endocytic flow. However, it remains to be determined if such vacuolation is associated with cell injury or cytotoxicity. Cocaine-elicited vacuolation is initially reversible, but it may cause principal damages in the end. First, bulky volumes of the vacuoles occupy huge space of the cytoplasm and numerous vacuoles maraud most of the membrane. The phenomenon is likely to cause global disturbances in cell physiology if vacuolation persists for a long time. Second, the late endosome/lysosome-derived vacuoles elicited by cocaine may perturb intracellular membrane trafficking. Endocytosis cycle may be hampered. Third, because of its devouring property, many cytoplasmic organelles or proteins may be recruited into the acidic vacuoles, leading to the morphological abnormality or probably functional impairment of the cells.

It has been reported that cocaine can induce cell death by either apoptotic or necrotic pathway [55–63]. Cocaine-elicited apoptosis has been observed in rat fetal locus coeruleus neurons, myocardial and vascular smooth muscle cells, mouse hepatocytes, and human epithelial cells of nasal mucosa [55–59]. For example, in fetal rat myocardial cells, p38alpha mitogen-activated protein kinase (MAPK) plays an important role in the cocaine-induced apoptosis by promoting cytochrome *c* release [58]. It would then be of interest to apply MAPK inhibitors to observe if cocaine-induced vacuolation could be blocked and apoptosis be inhibited. Since calcium influx was prompted by cocaine within 10 min to initiate a significant vacuolation, a signaling process-potentially through calmodulin- may be involved. Therefore, indicators of signal transduction leading to apoptosis, such as activation of tumor necrosis factor and Fas, or trigger of caspase cascade, could also be utilized for further approach. However, the reversibility of cocaine vacuolation argues its role to fit in the main theme of presumably irreversible programmed death. By contrast, the cocaine-induced necrosis is also reported from studies on hepatotoxicity in several mammalian species [10, 59–61]. This hepatotoxicity is mainly attributed to cocaine metabolites, which are catalyzed by cytochrome P450 (CYP) enzymes [10, 11, 62]. To make the issue even more complicated, higher (13-fold) concentrations of cocaine are needed for the hepatotoxicity in non-phenobarbital-induced hepatocytes than in phenobarbital-induced ones. Thus, two different mechanisms are proposed to explain the cocaine hepatotoxicity: while P450(CYP) catalysis is essential for the cytotoxicity of phenobarbital-induced hepatocytes, a non-transient rise in [Ca2+]i is evident for that of non-induced hepatocytes [63], which is in line with our observation that calcium inlflux plays an important role in the cocaine-induced vacuolation. If cocaine-induced vacuolation is associated with cytotoxicity, it also remains to be scrutinized whether the vacuolation proceeds through necrotic or apoptotic pathway.

Albeit cocaine concentrations used in this study seem very high, it is within the dose ranges that addicts usually take in their practice. Addicts may be able to tolerate up to 5 g a day without a manifestation of significant intoxication [64]. For an addict weighing 70 kg, he or she would need about 2.8 g to achieve the concentration of 0.2 mg/ml if cocaine is totally absorbed and distributed in the body. Active abusers may accumulate high concentrations either through drug tolerance, which may enormously increase their cocaine doses, or by binge behaviors, which may accumulate transient mega doses in a short time in some organs. For example, the lungs or nasal cavities of abusers may be locally exposed to high concentrations of cocaine through snorting crack cocaine. It would be of interest to know if some calcium channel blockers or calmodulin modulators would help reduce the acute toxicity caused by cocaine overdose. By scrutinizing the mechanism of cocaine-induced vacuolation, we hope at least parts of cocaine-initiated toxicological effects will be answered.

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Electronic supplementary material

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