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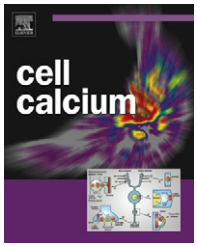

UVA-induced calcium oscillations in rat mast cells

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Mast cell;
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NAD(P)H oxidase

Summary UVA is a major bio-active component in solar irradiation, and is shown to have immunomodulatory and anti-inflammatory effects. The detailed molecular mechanism of UVA action in regard to calcium signaling in mast cells, however, is not fully understood. In this study, it was found that UVA induced ROS formation and cytosolic calcium oscillations in individual rat mast cells. *Exogenously added H_2O_2 and hypoxanthine/xanthine oxidase (HX/XOD) mimicked UVA effects on cytosolic calcium increases*. Regular calcium oscillation induced by UVA irradiation was inhibited completely by the phosphatidylinositol-specific phospholipase C inhibitor U73122, but U73343 was without effect. Tetrandrine, a calcium entry blocker, or calcium-free buffer abolished UVA-induced calcium oscillations. L-type calcium channel blocker nifedipine and stores-operated calcium channel blocker SK&F96365 had no such inhibitory effect. ROS induction by UVA was abolished after pre-incubation with anti-oxidant NAC or with *NAD(P)H* oxidase inhibitor DPI; such treatment also made UVA-induced calcium oscillation to disappear. UVA irradiation did not increase mast cell diameter, but it made mast cell structure more granular. Spectral confocal imaging revealed that the emission spectrum of the endogenous fluorophore in single mast cell contained a sizable peak which corresponded to that of *NAD(P)H*. Taken together, these data suggest that UVA in rat mast cells could activate *NAD(P)H* oxidase, to produce ROS, which in turn activates phospholipase C signaling, to trigger regular cytosolic calcium oscillation.

Introduction

Mast cells are specialized secretory cells that synthesize and store neurotransmitters (histamine, serotonin, substance P), cytokines (IL3-6, TNF), proteases (chymase, tryptase), and others [1]. Although mast cell activation has been extensively studied [2–4], it is not completely understood as to how those different mediators might be specifically or differentially released [1,5,6]. A role for calcium has long been recognized in exocytosis [7–9], but a role for calcium oscillations in mast cell secretion is not fully understood, especially in regard to specific modulation of mediator release [10–14]. Mast cell calcium oscillation has not been extensively studied other than in the mast cell/basophil cell line RBL-2H3 [15], partly due to the fact that some early works found that mast cell cytosolic calcium concentration tended not to oscillate after anti-IgE stimulation [16].

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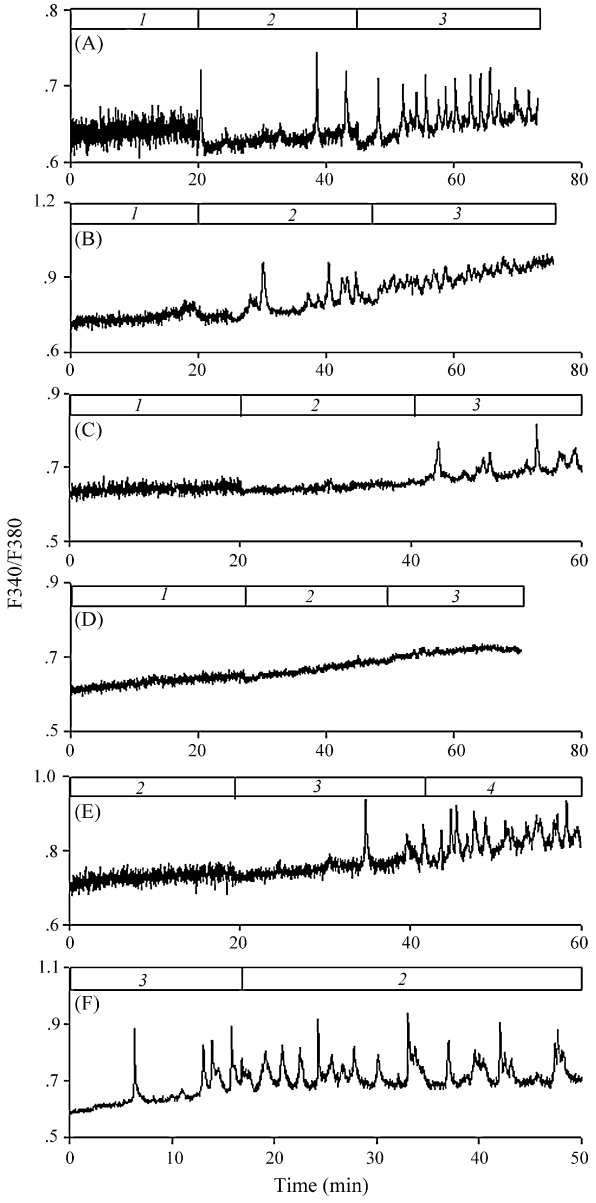

Figure 1 Light intensity-dependent induction of cytosolic calcium oscillations in isolated rat peritoneal mast cells. Fura-2-loaded-mast cells attached to Sykes-Moore chamber were perfused, and illuminated with monochromatic light from the DeltaRam V monochromater alternately at 340 nm/380 nm, the same as for normal calcium measurement. Both the in and out slits for the monochromater were initially set at a band width of 1 nm, and the resting calcium concentration remained stable at basal level. The slit width was subsequently increased as indicated (by the horizontal bars) and cytosolic calcium concentration increased in an oscillatory fashion. Traces A—D represent different response patterns observed in different mast cells. In a total of 45 experiments (only one cell could be examined in each experiment) with sequential incremental band widths from 1 to 2 to 3 nm, different response patterns were found as indicated: A: $N = 7/45$; B: $N = 15/45$; C: $N = 19/45$; D: $N = 4/45$. With sequential incremental band widths from 2 to 3 to 4 nm, all cells showed response at 4 nm (E: $N = 44/44$). With sequential band widths from 3 to 2 nm, the regular calcium oscillations tended to last with a stable baseline (F: $N = 11$).

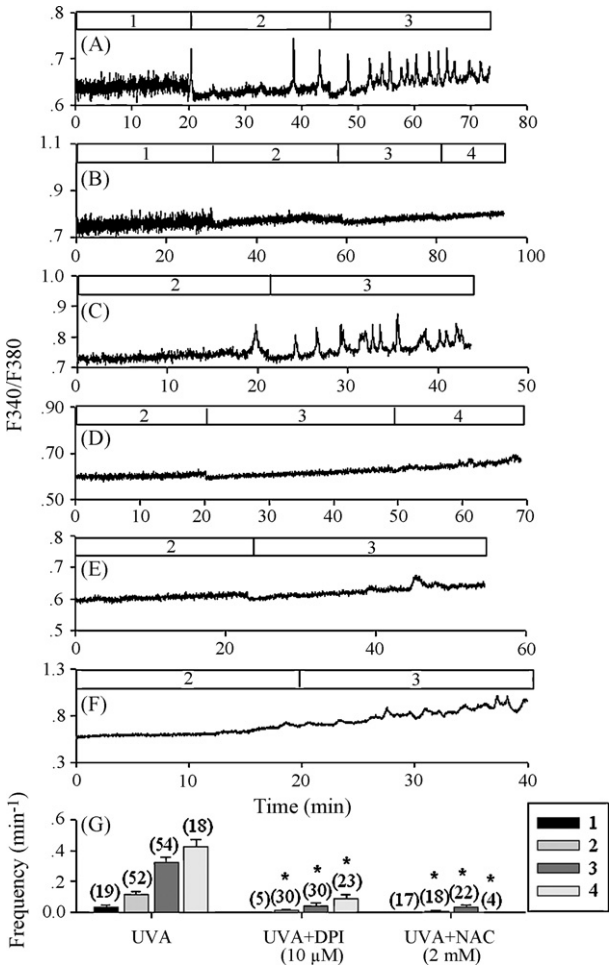

Figure 2 Antioxidant NAC and *NAD(P)H* oxidase inhibitor DPI inhibited cytosolic calcium oscillations induced by monochromatic UVA light (340 nm/380 nm). Sequential band widths from 1 to 2 to 3 nm, and from 2 to 3 nm induced regular calcium oscillations (A = 22, C = 31). In the presence of NAC 2 mM (B and E) or DPI 10 μM (D and F), such calcium oscillations were either completely absent even when the slit width was increased further to 4 nm (B: $N = 20$, D: $N = 22$), or only sparse fluctuations remained with an elevated baseline (E: $N = 10$; F: $N = 5$). The average oscillation frequency with UVA, UVA + NAC, and UVA + DPI at different slit width (1, 2, 3, 4 nm) was calculated and plotted in panel G. With UVA alone, the average calcium oscillation frequency (min^{-1}) at slit width of 1, 2, 3 and 4 nm was 0.033 ± 0.018 ($N = 19$), 0.118 ± 0.020 ($N = 52$), 0.326 ± 0.028 ($N = 54$) and 0.424 ± 0.049 ($N = 18$), respectively. With UVA + DPI, the average calcium oscillation frequency (min^{-1}) at slit width of 1, 2, 3 and 4 nm was 0 ($N = 5$), $0.015 \pm 7.910 \times 10^{-3}$ ($N = 30$), 0.041 ± 0.019 ($N = 30$) and 0.087 ± 0.029 ($N = 23$), respectively. With UVA + NAC, the average calcium oscillation frequency (min^{-1}) at 1, 2, 3 and 4 nm was 0 ($N = 17$), $5.848 \times 10^{-3} \pm 5.848 \times 10^{-3}$ ($N = 18$), 0.033 ± 0.017 ($N = 22$) and 0 ($N = 4$), respectively. Asterisk (*) indicates statistical significance with $P < 0.01$ when compared with UVA alone.

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Oscillatory increases in intracellular calcium concentration ($[Ca^{2+}]_c$) is commonly found in *several* cell types [17], which regulate diverse cellular functions [17,18]. Oscillatory calcium concentration increases do so by frequency modulation (FM), amplitude modulation (AM), and spike shape modulation (SM), therefore encode multiple and specific signals. Lower oscillatory frequency is known to activate pro-inflammatory transcription factors NF-κB, whereas higher frequency oscillations stimulate all three factors NF-AT, Oct/OAP and NF-κB [18,19]. Calcium oscillations are frequently induced by endogenous physiological factors such as hormones and neurotransmitters at low, physiological concentration [18]. In the present work, we found that UVA at low doses could also trigger calcium oscillations in freshly isolated rat mast cells.

UVA is a major component of solar radiation that reaches the earth surface and is therefore a major biologically relevant UV band [20—22]. We in this work investigated the detailed mechanisms of low dose UVA-triggered cytosolic calcium oscillations. It was found that UVA (alternately at 340 and 380 nm) from the DeltaRam V monochromator of the PTI calcium measurement system readily increased intracellular ROS via activating *NAD(P)H* oxidase, which in turn induced cytosolic calcium oscillation by activating the PLC pathway. Our work is the first such report on UVA-induced *NAD(P)H* oxidase activation and ROS generation leading to calcium oscillations in mast cell, confirming UVA-induced calcium increase in other cell types such as skin keratinocytes [23].

Material and methods

Materials

SK&F96365 and tetrandrine were bought from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). U73122, U73343, nifedipine, diphenylene iodonium (DPI) and *N*-acetyl-cysteine (NAC), hypoxanthine, xanthine oxidase were from Sigma—Aldrich (St. Louis, MO, USA). Fura-2 AM, and 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) were purchased from Molecular Probes (Eugene, OR, USA). Carboxyamido-triazole (CAI) was a gift from Dr. Elise Kohn, ICR, NIH (Bethesda, MD, USA). H_2O_2 was from Beijing Chemical Engineering Factory (Beijing, China).

Isolation of rat peritoneal mast cells

Rat peritoneal mast cells were obtained as reported previously [24,25] with slight modifications. Rat of the Sprague—Dawley strain (200—400 g) was killed by cervical dislocation. Standard buffer was injected into the abdomen of the rat with a syringe. The standard buffer had the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.13, NaH_2PO_4 1, glucose 5.6, HEPES 10, glutamine 2.0, bovine serum albumin 0.2%, amino acid mixture (Gibco BRL) (50×), pH was adjusted to 7.4 with NaOH 4 M. Buffer was oxygenated with O_2 100% for 30 min before use. The rat abdomen was massaged for 5 min and then the abdominal cavity was opened with scissors. The ascites was centrifuged ($100 \times g$) for 2×5 min period. Mast cells could be readily identified by their large size and were re-suspended in standard buffer before use.

Measurement of cytosolic calcium concentration

Isolated mast cells were incubated with Fura-2 AM (final concentration 10 μM) for 40 min at 37 °C in a shaking water bath (50 cycle min^{-1}), with oxygenation every 20 min. Fura-2 loaded-mast cells were attached to Cell-Tak coated coverslip in Sykes-Moore perfusion chamber. Attachment was allowed for 20 or 30 min before perfusion with standard buffer (without BSA and amino acid mixture), and stimulus was added by changing perfusion buffer containing relevant chemical reagents.

Sykes-Moore chamber was placed on the platform of an inverted fluorescence microscope (Olympus IX70), which was coupled to a PTI (Photon Technology International Inc., Lawrenceville, NJ, USA) microfluorometric calcium measurement system (DeltaRam V), with a photon-counting PMT (Hamamatsu PMT R1527P, i.e., PTI PMT81) as detector. Calcium concentration was indicated as fluorescence ratios F340/F380 (Figs. 1, 2 and 4—6).

A calcium imaging system (EasyRatioPro, PTI Inc., Lawrenceville, NJ, USA) was used in the detection of exogenous ROS-induced calcium increases (Fig. 3). In this imaging system, the monochromater used was DeltaRam X, inverted fluorescence microscope was Nikon TE2000U with objective Super Fluor 40 X, CCD was QuantEM 512 CS (Roper Scientific, Koto-Ku, Tokyo, Japan). Note that the fluorescence ratio (F340/F380) was higher than that obtained with the DeltaRam V (Figs. 1, 2 and 4—6).

Calcium oscillation induced by UVA irradiation

The monochromater of the PTI DeltaRam V monochromator was used as the UVA irradiation source. *UV light intensity was adjusted by changing width of the slits (both in and out) in the monochromater from 1 to 4 nm.* Basal calcium level was measured in Fura-2-loaded-mast cells with the slit-width set at 1 nm, with excitation alternating between 340 nm/380 nm and emission at >510 nm. After determining the condition of the mast cells of the day, regular calcium oscillation was induced in subsequent cells with increased slit width, which was then changed back to a smaller slit width during the measurement. For instance, if calcium oscillation was inducible at 3 nm, the silt with was set at 2 nm during measurement. Other chemicals were introduced by a change of the perfusion buffer containing the relevant chemicals. The UVA irradiance at each slit width was measured by an IL 1700 research radiometer (International Light Inc., Newburyport, MA01950, USA), and shown in Table 1.

Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS production was measured with ROS indicator CM-H_2DCFDA. In order to avoid artifacts, the loading of CM-H_2DCFDA was performed after UVA irradiation, as

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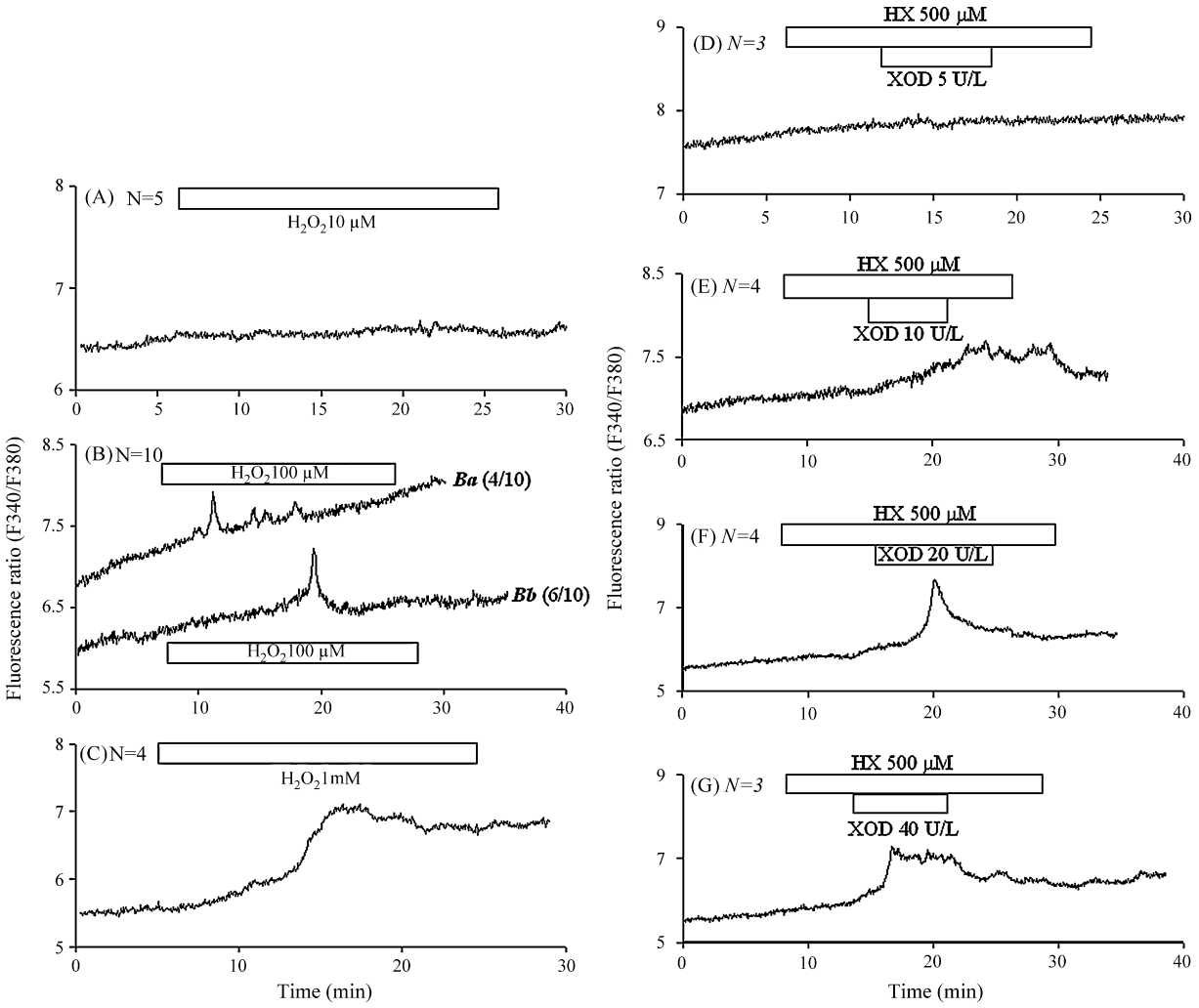

Figure 3 H_2O_2 and HX/XOD mimicked UVA effects on cytosolic calcium concentration. Freshly isolated rat peritoneal mast cells were stimulated with H_2O_2 or HX/XOD as indicated in A—G. A—C: Perfused Fura-2-loaded peritoneal mast cells were stimulated with H_2O_2 at 10 (A), 100 (B) and 1000 μM (C). D—G: Mast cells were perfused with hypoxanthine (HX) 500 μM, together with xanthine oxidase (XOD) of 5 (D), 10 (E), 20 (F), 40 (G) $U\,L^{-1}$. Drugs were added as indicated by the horizontal bars. The N numbers mean each calcium trace is representative of N separate experiments: A, $N = 5$; B, $N = 10$ (Ba, $N = 4$; Bb, $N = 6$); C, $N = 4$; D, $N = 3$; E, $N = 4$; F, $N = 4$; G, $N = 3$.

Table 1 UVA irradiance from PTI DeltaRam V at alternating 340 nm/380 nm with different slit width ($\times 10^{-7}$ W cm^{-2})

Slit width (nm)	Irradiance ($\times 10^{-7}$ W cm^{-2})
1	3.02 ± 0.11
2	12.56 ± 0.36
3	28.92 ± 0.81
4	51.91 ± 1.17
5	79.45 ± 1.79
6	111.74 ± 2.32
8	193.98 ± 4.33

The sensor of an IL 1700 research radiometer was placed on the IX70 Olympus microscope platform at a position similar to mast cells in the Sykes-Moore chamber, a stable reading was recorded on 10 separate occasions at each slit width from 1 to 8 nm. Data are presented as mean ± S.E.M.

cautioned by others [26]. Cells were attached to Cell-Tak treated cover-slip in the Sykes-Moore perfusion chamber and treated as described above to obtain regular calcium oscillation. Mast cells were incubated with CM-H_2DCFDA (4 μM) for 30 min immediately after UVA treatment. CM-H_2DCFDA fluoresces after oxidization in the cytoplasm. CM-H_2DCFDA-loaded cells were perfused, fluorescence (Fs) in a single mast cell was read at λ_{ex} 490 nm and λ_{em} 525 nm for 30 s continuously. Background fluorescence (Fb) was read under the same condition. Relative ROS level in single mast cell was expressed as Fs/Fb. The fluorescence in this series of experiments was measured in the PTI DeltaRam V system.

Confocal imaging and emission spectrum of single mast cells

The emission spectrum of the endogenous fluorophore in single mast cells was determined in a spectral confocal

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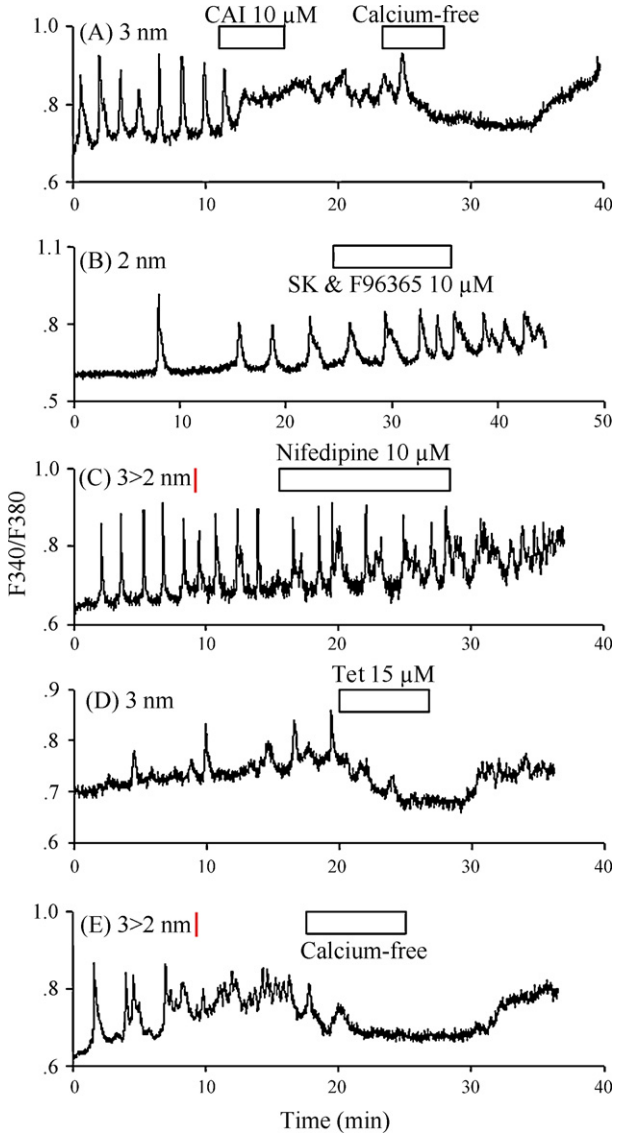

Figure 4 Effects of modulating calcium entry on UVA-induced calcium oscillations in mast cells. Regular calcium oscillations were induced individually for each mast cell. Effects of CAI 10 μM, SK&F96365 (10 μM), nifedipine 10 μM, tetrandrine 15 μM and calcium-free buffer on UVA-induced calcium oscillations in mast cells were shown. Each trace in panels A—E is representative of *N* independent experiments: A, *N* = 3; B, *N* = 8; C, *N* = 9; D, *N* = 10; E, *N* = 3. All drugs were added as indicated by the horizontal bars. The slit width was 3 nm throughout the experiment in panels A and D, 2 nm for panel B; the slit width was changed from 3 to 2 nm at time indicated by the vertical line in panels C and E.

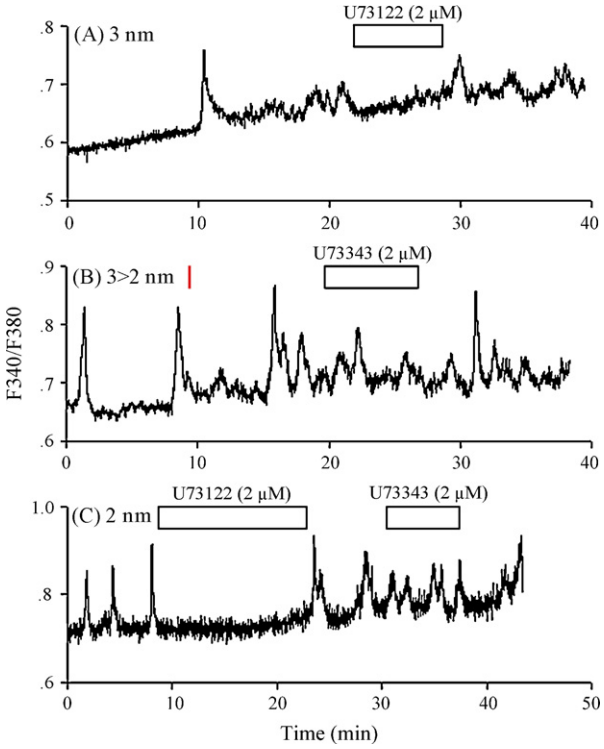

Figure 5 Role of phospholipase C in UVA-induced cytosolic calcium oscillations in rat mast cells. Regular calcium oscillations were induced individually for each mast cell. Effects of *phospholipase* C inhibitor U73122 (2 μM), and inactive analogue U73343 (2 μM) were investigated separately in different mast cells (A and B) or in the same cell (C), drug addition was as indicated by the horizontal bars. Each trace in panels A—C is representative of *N* similar experiments: A, *N* = 25; B, *N* = 15; C, *N* = 3. The slit width was 3 nm in panel A, and 2 nm in panel C; the slit width was changed from 3 to 2 nm at time indicated by the vertical line in panel B.

microscope (Leica TCS Sp2 plus Leica DMIR) with objective 40× (N.A. 1.25) equipped with a UV excitation lamp. Isolated mast cells adhered to Cell-Tak-coated cover-slip was excited at λ_{ex} 364 nm (band width 360—380 nm), and emission scanned from 400 to 600 nm. Subsequently the fluorescence images were taken at λ_{ex} 364 nm and λ_{em} 433 nm.

Statistical analysis

Each experiment reported in this work has been done at least three times on different days with different animals. Data are expressed as mean ± S.E.M. Figures were plotted by SigmaPlot. To examine the differences between different experimental groups, Student's *T* test was used, with $P < 0.05$ taken as statistically significant.

Results

UVA-induced regular calcium oscillations in rat mast cells

To determine UVA effects on cytosolic calcium concentration, Fura-2-loaded-mast cells attached to Cell-Tak-coated cover-slips were excited with alternating light of 340 and 380 nm. In most experiments excitation light was delivered from the DeltaRam V monochromator with both in and out slips set at 1 nm (i.e., band width of 1 nm). With this setting, basal calcium concentration remained stable. When the slit width was increased, regular calcium

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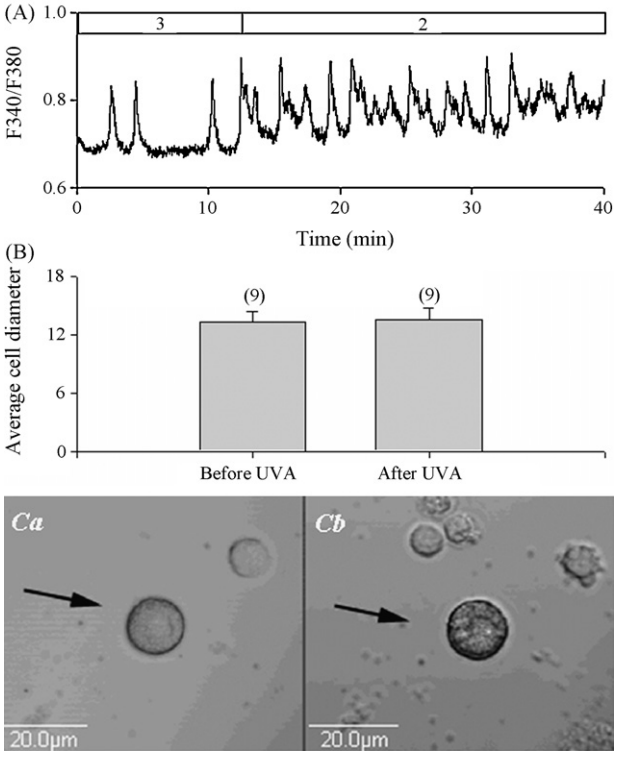

Figure 6 Morphological changes induced by UVA in rat mast cells. Regular calcium oscillations were induced in mast cells similar to Fig. 1F and as indicated in panel A, the slit width being indicated by the horizontal bars. Mast cell images were taken both before and after calcium measurement, and cell diameters (in μm) were compared. Mast cell diameter was measured with the ruler tool in Photoshop (B). Panel C shows mast cell morphological changes in a typical experiment. The arrows in Ca and Cb indicate the mast cell used for both calcium and diameter measurements. Panels A and C are representative of 9 independent experiments. *DIC image*s were taken in the scanning mode in an Olympus IX 71 microscope coupled to the FV300 laser scanning unit, with objective ×40 (N.A. 1.25).

oscillations appeared. Fig. 1A–D show 4 typical responses in cytosolic calcium concentration increases. The differences in responses in individual cells were obviously due to mast cell heterogeneity reported previously also by others [27]. Regular calcium oscillations were induced with the slit width at 2 nm (Fig. 1A and B), or 3 nm (Fig. 1C), with a minor number showing no oscillation even at 3 nm (Fig. 1D, 4/45). When the initial slit width was set at 2 nm, most cells started to show calcium oscillations when the slit width was increased to 3 nm. In Fig. 1D, a slow phasic increase in cytosolic calcium was found; this could be due to slow activation of a nonselective cationic current permeable to calcium as reported by others [28]. With a starting slit width of 3 nm, calcium oscillation was readily observed (Fig. 1E), and once calcium oscillation was induced, oscillation could be maintained at a lower slit width (Fig. 1F). It may be noted here that UVA irradiances at different slit width was measured and presented in Table 1. The average UVA dosage with alternating wavelength of 340 nm/380 nm used in this work was in the range of 3.02–51.91 $\times 10^{-7}$ W cm^{-2}, corresponding to slit width of 1–4 nm.

A role for ROS in UVA-induced calcium oscillations in mast cells

UVA irradiation indeed induced regular calcium oscillations. To determine the possible mechanisms involved, Fura-2-loaded-mast cells were treated with standard buffer, with ROS scavengers NAC (2 mM), or with *NAD(P)H* oxidase inhibitor DPI (10 μM) for 1 h before UVA irradiation. As shown in Fig. 2A and C, mast cells without any pre-treatment showed regular calcium oscillations with UVA irradiation; whereas after incubation with NAC (Fig. 2B and E) or DPI (Fig. 2D and F), calcium oscillations completely disappeared when UVA irradiated. UVA alone induced regular calcium oscillations in 89% of the mast cells examined (41 of 46). In the presence of NAC and DPI, no oscillations were observed, but a slow increase was found in 14% (3 of 21), and 22% (6 of 27) cell examined, respectively (Fig. 2E and F). The average oscillatory frequency at 1–4 nm, under different conditions were analyzed and presented in Fig. 2G. It was obvious that both DPI and NAC markedly blocked UVA-induced calcium oscillations. From these data, it was obvious that intracellular ROS was essential for UVA induction of calcium oscillations.

To corroborate further a role for ROS in UVA-induced increases in cytosolic calcium concentration, effects of exogenous ROS was examined. H_2O_2 and hypoxanthine/xanthine oxidase (HX/XOD) system were used to mimick effects of superoxide anion. It was found that H_2O_2 induced dose-dependent increases in cytosolic calcium concentration. H_2O_2 at 10 μM was without any obvious effect, but at 100 μM induced sparse or recurrent calcium spikes, and at 1000 μM induced a plateau increase (Fig 3.1). Similarly, the HX/XOD system also induced readily cytosolic calcium concentration increases. In control experiments it was found that HX (500 μM) alone had no effect. HX (500 μM) plus XOD induced increases in cytosolic calcium concentration dependent on the concentration of XOD. HX plus XOD at 5 U L^{-1} induced no obvious increase, at 10 U L^{-1} induced fluctuations in cytosolic calcium concentration, at 20 and 40 U L^{-1} induced a phasic increase with a much broader peak at 40 than at 20 U L^{-1} (Fig. 3.2). Therefore both H_2O_2 and the HX/XOD system induced fluctuatory increases in cytosolic calcium concentration in peritoneal mast cells, mimicking the effects of UVA irradiation.

A role for calcium influx in UVA-induced calcium oscillations in mast cells

Other than intracellular ROS, it was found that calcium influx was needed for UVA-induced calcium oscillations to persist. In this series of experiments, regular calcium oscillations were induced with large slit width (2–3 nm); then oscillations were maintained at lower slit numbers. The calcium influx blocker carboxyamido-triazole [29] at 10 μM was able to disrupt regular calcium oscillations, subsequent removal of extracellular calcium completely reduced calcium concentration to the basal level (Fig. 4A). On the other hand, both SK&F96365 [30] (Fig. 4B) and nifedipine

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were without any effect on UVA-induced calcium oscillations (Fig. 4C). Tetrandrine 15 μM, an active ingredient from Chinese medicine and a calcium channel antagonist, effectively reduced UVA-induced oscillatory calcium increase to below the basal level (Fig. 4D). Removal of extracellular calcium effectively blocked UVA-induced oscillatory calcium increases (Fig. 4E). These data suggest that the presence of external calcium was needed for UVA-induced calcium oscillation to persist in mast cells.

Involvement of phospholipase C in UVA-induced calcium oscillations in mast cells

To assess the critical source of calcium oscillation for UVA irradiation-induced calcium oscillations, U73122, a phospholipase C inhibitor, was used. U73122 at 2 μM completely abolished UVA-induced calcium oscillations in mast cell (Fig. 5A and B), whereas the structural analogue U73343 at 2 μM was without any effect (Fig. 5B and C), both in different cells (Fig. 5A and B) and in the same mast cell (Fig. 5C). These data indicate that like *neurotransmitter-or hormone*-induced calcium oscillations in glandular and liver cells [31–33], UVA-induced calcium oscillation is also mediated by the PI-PLC signaling pathway. Further, UVA irradiation-induced PLC-activation was reversible.

To assess the extent of mast cell activation by UVA irradiation, mast cells were imaged by laser confocal microscopy both before and after UVA treatment and calcium measurement. Mast cell was first imaged before UVA irradiation (Fig. 6Ca), then calcium oscillations were induced with UVA (Fig. 6A); 40 min later, the same mast cell was imaged again (Fig. 6Cb). The average diameter of mast cells both before and after UVA irradiation did not show any significant change (Fig. 6B). Although the average cell diameter did not change, mast cells underwent a major structural change after UVA irradiation (cf. Fig. 6Ca and Cb); a granular texture was found in UVA-irradiated cells, although no obvious exosomes were detected.

UVA irradiation-induced intracellular ROS generation in single mast cells

It has been reported before that UV irradiation generates intracellular ROS in non-phagocytic cells such as human keratinocytes [23,34]. To confirm a role for ROS in UVA-induced calcium oscillations, the actual generation of ROS after UVA irradiation was measured with the ROS probe CM-H_2DCFDA. CM-H_2DCFDA when oxidized by ROS inside the cell to DCF, shows a significant increase in fluorescence. To avoid direct effects of UVA irradiation on CM-H_2DCFDA, in our experiments, mast cells were first UVA irradiated as above, then UVA-irradiated mast cells were loaded with the ROS probe CM-H_2DCFDA. The *difference* in DCF fluorescence was taken as an indicator of ROS generation. As shown in Fig. 7, the relative ROS in control mast cells not treated with UVA was about 1.232 ± 0.154, after UVA irradiation, the relative ROS increased to 3.092 ± 0.875. Notably, when mast cells were pre-treated with ROS scavenger NAC (2 mM), UVA-induced ROS was inhibited markedly (Fig. 7A and B).

Pre-treatment of mast cells with *NAD(P)H* oxidase inhibitor DPI for 1 h prior to UVA light, completely abolished UVA-induced ROS generation. The relative ROS level in UVA + DPI, and UVA + NAC groups was 1.834 ± 0.340, and 1.504 ± 0.316, respectively. These results suggest that *NAD(P)H* oxidase was involved in UVA-induced ROS generation and calcium oscillations.

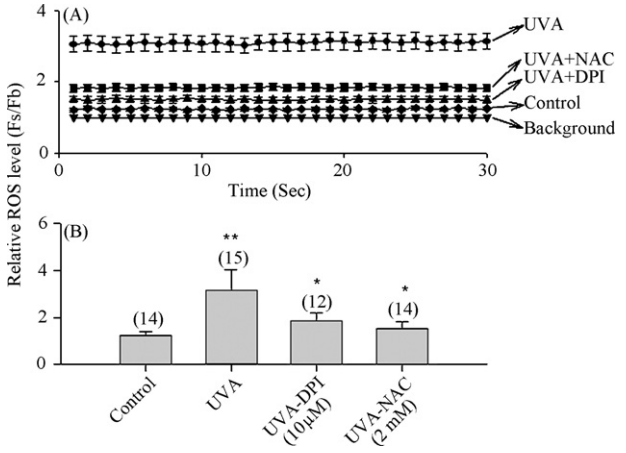

Figure 7 UVA-induced ROS generation and its inhibition by antioxidants NAC and *NAD(P)H* oxidase inhibitor DPI. In these experiments, mast cells were exposed to UVA as shown in Fig. 1A. After UVA irradiation mast cells were immediately incubated with CM-H_2DCFDA at 4 μM for 30 min at room temperature. Fluorescence was then measured, with the monochromater slit-width set at 1 nm. Control cells were not previously exposed to UVA. For NAC and DPI groups, NAC 2 mM and DPI 10 μM was added to adherent mast cells 1 h before UVA irradiation. DCFDA fluorescence was monitored for 30 s after such treatments, and expressed as fluorescence ratio Fs/Fb, Fs being mast cell fluorescence, Fb being background fluorescence of blank region of the same size. Panel A plots real time measurement, Panel B plots averaged ROS generation over 30 s in mean ± S.E.M. Control, $N = 14$; UVA, $N = 15$; UVA + DPI, $N = 12$; UVA + NAC, $N = 14$. The asterisks (**) denotes $P < 0.001$ compared with control, and (*) denotes $P < 0.01$, compared with UVA alone.

Subcellular localization and spectral features of the endogenous chromophore

To identify the potential molecular target for UVA action that led to cytosolic calcium oscillations, an emission spectrum of endogenous chromophores were obtained. Excitation at 360–380 nm induced ready fluorescence in mast cells (Fig. 8). With the Leica SP2, it was possible to determine the emission spectrum (Fig. 8A). A major emission peak was found at 434 nm, with 3 minor peaks at longer wavelengths. With λ_{ex} 364 nm and λ_{em} 434 nm, it was possible to image the endogenous chromophores. At the molecular level, various chromophores have been identified, and DNA remains the major chromophore. It was obvious that the major chromophores *here* were located in the cytosol instead of the nucleus (Fig. 8B). *Further*, the major chromophore responded to UVA in rat peritoneal mast cells is not DNA (Fig. 8). In the experiments measuring $[Ca^{2+}]_c$ oscillation and intracellular ROS, mast cells were irradiated by UVA of 340 and 380 nm wavelength. In a Leica confocal SP2, mast cell fluorescence image was taken with λ_{ex} from 360

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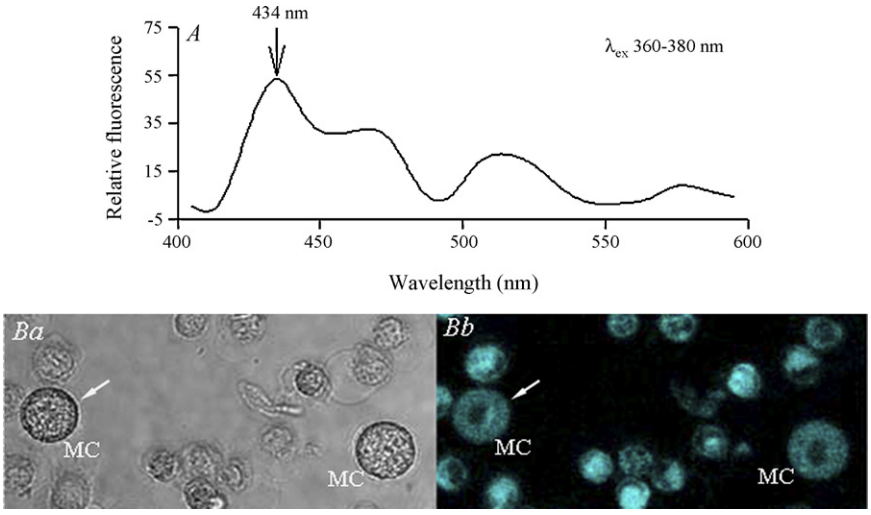

Figure 8 Subcellular localization and spectral features of the UVA responsive endogenous chromophore in rat mast cells. Freshly isolated rat mast cells were imaged in a Leica confocal microscope (TCS Sp2 plus DMIR) (ocular 10×, objective 40×, N.A. 1.25). A: Emission spectrum of a single mast cell (indicated by the arrow in Ba and Bb). Excitation light was through a band-pass filter (360—380 nm) with maximum transmission at 364 nm; emission was measured from 400 to 600 nm. Maximum emission was found to be at 434 nm. Ba and Bb are bright field and fluorescent images of examined mast cells (MC). Fluorescent image in Bb was taken with λ_{em} at 434 nm. Note the cytosolic localization of the endogenous chromophore.

to 380 nm. The image clearly indicated that the endogenous fluorophore was mainly cytosolic (Fig. 8B). So the possible target of UVA action in mast cell is unlikely to be genomic DNA. With λ_{ex} set at 360—380 nm, a λ_{em} maximum was found at 433 nm, with 3 smaller peaks at longer wavelengths (Fig. 8A). The implications of such data are discussed later in the 'Discussions'.

Discussions

Both ultraviolet A (320—400 nm) and ultraviolet B (290—320 nm) are major solar UV components that could reach the torrential surface, and therefore are the most biologically relevant. UVA has both immunomodulatory [35—37] and immunoprotective effects [38,39]. Such effects may be related to UVA modulation of mast cells.

A bimodal effect of UVA on mast cells has been established early on: with exogenous photosensitizer present, low level UVA irradiation inhibited mast cell secretion, whereas high doses resulted in a stimulation of mast cell mediator release [40]. UVA irradiation alone triggers histamine release from mast cells, and leads to marked suppression of anti-IgE-induced histamine release [41]. *It may be noted here that low UV light has been shown to reduce angiotensin-induced cytosolic calcium increase in adrenal glomerulosa cells, although it had no effect on basal calcium levels* [42]. Those early studies notwithstanding, it is nevertheless not known in detail how UVA might affect the calcium signaling pathway in mast cells.

ROS are known to play an important role in mast cell activation and degranulation [34,43]. Stimulated rat mast cells release ROS [44,45]. Some authors argued that mast cell ROS production could be due to contaminating macrophages [46,47]. In the present study, ROS generation was directly measured in single rat mast cell microscopically, therefore directly confirming mast cell generation of ROS. *The fact that exogenous ROS (H_2O_2 and HX/XOD) mimicked UVA-induced calcium increases* (Fig. 3) *confirms a role for ROS in UVA activation of mast cells.*

Low level ($(12—51) \times 10^{-7}$ W cm^{-2}) UVA directly activated ROS production, which was nearly completely blocked by *NAD(P)H* oxidase inhibitor DPI (Fig. 7), implicating *NAD(P)H* oxidase as a source of ROS production. The ROS quencher NAC completely blocked CM-H_2DCFDA oxidation and associated fluorescence increase, further confirming the role for *NAD(P)H* oxidase. Most importantly, both NAC and DPI significantly blocked UVA-induced calcium oscillations (Fig. 2), suggesting an essential role for *NAD(P)H* oxidase-derived ROS in triggeration of mast cell cytosolic calcium oscillation. How cytosolic ROS might activate calcium oscillations is not known at the moment. But it is firmly established in the present work that PLC-activation is essential (Fig. 5).

Early work found that UVA irradiation led to the activation of a novel calcium-permeable cationic current in mast cells [28]. Calcium influx was also found to be important for the maintenance of UVA-induced calcium oscillations in this work (Fig. 4). But the calcium influx involved was not susceptible to known inhibitors of L-type voltage-dependent calcium channel inhibitor nifedipine or stores-operated calcium channel inhibitor SK&F 96365 (Fig. 4). Rather, the UVA-activated calcium influx was blocked by tetrandrine (Fig. 4D), an active component from the root of *Stephania tetrandra S Moore*. Tetrandrine as a wide-spectrum calcium antagonist effective towards both voltage-dependent and voltage-independent calcium channels has been widely recognized [48—51], although it may also have other effects, such as inhibition of IP_3 production and of other ion channels [52—54]. Tetrandrine in this work may have blocked UVA-induced calcium oscillation due to a combination of all these effects.

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UVA-induced calcium oscillations were not accompanied by obvious changes in mast cell size, although major structural changes were readily observed; the mast cell after UVA became more granular which suggested secretion (Fig. 6). It could be concluded that low level UVA in the range of $(1.2-5.1)\times10^{-6}\,W\,cm^{-2}$ was able to induce both calcium oscillation and possibly mast cell secretory response. The endogenous chromophore responsible was not identified at this moment, but it had a major emission peak at 434 nm and several smaller peaks at longer wavelengths. The chromophore was located mainly in cytosol (Fig. 8). The potential candidates at longer emission peaks may include NADH/*NAD(P)H* [55], and riboflavin [56–58], which would correspond to the smaller emission peaks at 470 and 520 nm, respectively (see Fig. 8A and [59,60]). Work by others has suggested that proteins and lipids near or in the plasma membrane may also be involved [61].

It may be noted here that it is not completely clear how phospholipase C was activated by UVA-induced ROS. But UVA irradiation has been shown to inactivate membrane-associated protein tyrosine phosphatase (PTP) by: (i) cysteine oxidation in the active center of PTP [62], or (ii) oxidation changing PTP conformation [63]. We have previously shown that singlet oxygen could modulate plasma membrane protein secondary structure [64]. ROS produced by *NAD(P)H* oxidase could therefore activate phospholipase C by direct oxidation of relevant amino acid residues at the reactive center, or by modulation of other residues which change the 3-D conformation of phospholipase C and leads to its activation or increased activity.

Fig. 6C as noted above suggests that UVA could possibly have induced mast cell secretion, the mast cell structure becoming more granular. This would possibly imply a physiological role for low dose UVA in mast cell biology. Mast cells after IgE stimulation are known previously to produce functionally significant amount of ROS but no nitric oxide [47]. *NAD(P)H* oxidase-mediated ROS production and subsequent induction of calcium oscillations reported in the present work may contribute to mast cell cytokine production, as reported before in other cell types [34]. A recent report found that high affinity IgE receptor activation induced ROS generation was completely abolished with 5-lipoxygenase inhibitors, but not with *NAD(P)H* oxidase inhibitor DPI. When IgE receptor-mediated ROS generation was blocked, mast cell degranulation and cytokine production remained un-affected in those human mast cells (huMC) and mouse bone marrow-derived mast cells (mBMMC), further implicating *NAD(P)H* oxidase-mediated ROS production in mast cell degranulation and cytokine production [65], i.e., different sources of ROS may produce different end effects. This is further confirmed in the present study in that exogenously added ROS by H_2O_2 and HX/XOD only MIMICKED UVA-induced calcium increase; regular calcium oscillations seen with UVA were not readily detected with exogenous ROS (Fig. 3). IgE and UVA induce different sources of ROS production and therefore differential release of mast cell mediators would make perfect sense.

In conclusion, the above data together suggest that UVA irradiation may sequentially activate *NAD(P)H* oxidase, to produce ROS, which in turn activate phospholipase C, to induce calcium oscillations in mast cells. The fact that UVA induces oscillatory increases in cytosolic calcium concentration suggests that UVA may encode multiple signals therefore be involved in the modulation of multiple and specific mast cell functions, or UVA may modulate the release of specific secretory mediators.

Conflicts of interest

We hereby declare that there is no conflict of interest in the work contained in the above MS.

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