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Single cell amperometry reveals curcuminoids modulate the release of neurotransmitters during exocytosis from PC12 cells

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Abstract

We used single cell amperometry to examine whether curcumin and bisdemethoxycurcumin (BDMC), substances that are suggested to affect learning and memory, can modulate monoamine release from PC12 cells. Our results indicate both curcumin and BDMC need long-term treatment (72 h in this study) to influence exocytosis effectively. By analyzing the parameters calculated from single exocytosis events, it can be concluded that curcumin and BDMC affect exocytosis through different mechanisms. Curcumin accelerates the event dynamics with no significant change of the monoamine amount released from single exocytotic events, whereas BDMC attenuates the amount from single exocytotic event with no significant change of the event dynamics. This comparison of the effect of curcumin and BDMC on exocytosis at the single cell level brings insight into their different mechanisms, which might lead to different biological actions. The effect of curcumin and BDMC on the opening and closing of the exocytotic fusion pore were also investigated. These results might be helpful for understanding the improvement of learning and memory and the anti-depression properties of curcuminoids.

Keywords

Single cell amperometry; Exocytosis; Curcumin; Bisdemethoxycurcumin; PC12 cells; Model cells

1. Introduction

Curcuminoids are the primary polyphenolic components extracted from dietary spice turmeric. Curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC) are the major constituents of curcuminoids. Commercial curcumin is principally a mixture of these curcuminoids with approximately 77% curcumin, 18% DMC, and 5% BDMC [1]. Curcumin possesses effective antioxidant, anti-inflammatory, and anti-cancer properties. Moreover, curcumin has the ability to affect a variety of molecular objects such as growth factors, transcription compounds and enzymes to regulate significant signaling pathways. Curcumin has been explored as a potent neuroprotective compound and it has a critical

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capability to cross the blood brain barrier. Recent studies have shown curcuminoids affect learning and memory in aged animals or animals with Alzheimer's disease. It has been suggested that curcumin might be useful for the treatment of neurodegenerative disorders such as Alzheimer's and Parkinson's disease [2].

Pheochromocytoma (PC12) cells provide an excellent model for the study of different aspects of neuronal physiology and biochemistry and, thus, these have been used for investigating beneficial activities and the mechanism of curcumin. For example, curcumin appears to increase the viability of cultured PC12 cells in the presence of aluminum chloride used as an oxidative stress agent inducing Alzheimer's disease [3]. In addition, curcumin seems to inhibit the oxidative and toxicity damage of PC12 cells caused by beta-amyloid-induced toxicity [4]. Curcuminoids such as curcumin, DMC, and BDMC, differentially stimulate PC12 neurite outgrowth and differentiation ensued by expression of neuronal differentiation biomarkers [5]. Previous findings have demonstrated that a diet enriched with curcumin diminishes fear memory consolidation and reconsolidation processes. These properties may be helpful for the treatment of complaints such as posttraumatic stress disorder (PTSD) that are identified by abnormally robust reactivated fear memories [6].

Curcumin has been shown to benefit the process of learning and memory. In an Alzheimer's-disease-induced animal model, curcumin significantly improved learning and memory [3]. It also prevents apoptosis and impairs the neuropathological changes in the hippocampus, a region of the brain playing an important role in memory formation. It has been shown that curcumin reduces D-galactose-induced cognitive and memory deficiency [7]. Curcumin demonstrates anti-depression effects and can enhance learning and memory and improve cognition deficiency related to depression [8]. Although the mechanism of the anti-depression effects of curcumin is unclear, there is some evidence of biochemical and neurochemical changes in the brain such as modulating the level and distribution of serotonin and dopamine [9].

Single cell amperometry is a real time chemical method that can be used to quantitatively analyze electroactive neurotransmitter released from single cell during exocytosis, the main process for neuronal and hormonal communication [10,11]. In this method, a disk microelectrode is placed on the cell membrane to oxidize released hormone during individual exocytotic events. This has widely been used to monitor the release of substances such as epinephrine, norepinephrine, dopamine, insulin, and serotonin from chromaffin cells, PC12 cells, β -cells, and platelets. The transmitter is released through a fusion pore between the vesicle and the cell, diffuses away from the cell, and is oxidized at the electrode resulting in an amperometric peak. By analyzing the characteristics of the amperometric peak, certain parameters related to the geometry and kinetics of the fusion pore and release process can be obtained [12–14]. This method has been widely used to study the fundamental process of exocytosis and the effects of pharmacology and physicochemical change on neuronal communication [15–17].

In the present study, PC12 cells were treated with curcumin, BDMC, or vehicle for specific intervals. Single cell amperometry on top of the cells was then used to measure exocytotic

release. Interestingly, it appears that curcuminoids not only influence the dynamics of exocytosis but also the amount of the transmitter released during single exocytotic process.

2. Material and methods

2.1. Chemicals and solutions

Analytical grade chemicals were obtained from Sigma-Aldrich (unless stated otherwise) and used as received. The HEPES physiological saline contains 150 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 5 mM glucose, 10 mM HEPES, and 2 mM CaCl₂. The high K⁺ stimulating solution consists of 55 mM NaCl, 100 mM KCl, 1.2 mM MgCl₂, 5 mM glucose, 10 mM HEPES, and 2 mM CaCl₂. All solutions were made with 18 MΩ cm water from a Purelab Classic purification system (ELGA, Sweden), and the solution pH was adjusted to 7.4 with concentrated NaOH (3.0 M).

2.2. Fabrication of disk carbon fiber microelectrodes (CFMEs)

The disk CFMEs were fabricated as previously described [18]. Briefly, a 5-μm diameter carbon fiber was aspirated into a borosilicate capillary (1.2 mm O.D., 0.69 mm I.D., Sutter Instrument Co., Novato, CA, U.S.A.).

The capillaries were subsequently pulled with a micropipette puller (Model P-1000, Sutter Instruments Co., Novato, CA, U.S.A.). The electrode was sealed by dipping the pulled tip in a solution of epoxy (Epoxy Technology, Billerica, MA, U.S.A.). The glued electrodes were cured at 100 °C overnight and subsequently cut at the glass junction and beveled at 45° angle (EG-400, Narishige Inc., London, UK). Prior to the experiments, the electrode response was tested with cyclic volt-ammetry (−0.2 to 0.8 V vs. Ag/AgCl, 100 mV/s) in 100 μM dopamine in PBS (pH 7.4). Only electrodes showing good reaction kinetics and stable steady-state currents, which were in agreement with theoretically calculated values for 5-μm disk electrodes, were used for experiments.

2.3. Cell culture and treatment

PC12 cells, a gift from Lloyd Greene (Columbia University), were maintained in RPMI-1640 medium (PAA Laboratories, Inc. Australia) supplemented with 10% donor equine serum (PAA Laboratories) and 5% fetal bovine serum (PAA Laboratories) in a 7% CO₂, 100% humidity atmosphere at 37 °C. The cells were grown on mouse collagen-coated cell culture flasks (collagen type IV, BD Biosciences, Bedford, MA) and were sub-cultured every 7–9 days. The medium was replaced every 2 days throughout the lifetime of all cultures. For single cell exocytotic experiments, PC12 cells were sub-seeded sparsely on mouse collagen coated culture dishes (type IV, BD Biosciences, Bedford, MA) in RPMI-1640 medium supplemented with 10% donor equine serum and 5% fetal bovine serum 4–5 days before the amperometric recording. Cells were treated with vehicle (0.1% DMSO), 20 μM curcumin or 20 μM BDMC in low serum (1% horse serum and 0.5% FBS) medium in a humidified incubator for 3 h or 72 h immediately followed by amperometric recording.

2.4. Single cell experiments

Electrochemical recordings of single PC12 cells were performed on an inverted microscope (IX81, Olympus), in a Faraday cage. The working electrode was held at +700 mV versus a Ag/AgCl reference electrode (Scanbur, Sweden) using an Axon 200B potentiostat (Axopatch 200B, Molecular Devices, Sunnyvale, CA). The recorded signal was filtered at 2 kHz with a 4-pole Bessel filter and digitized at 10 kHz with a Digidata model 1440 A and Axoscope 10.4 software (Axon Instruments Inc., Sunnyvale, CA, U.S.A). Before experiments, the cells were rinsed three times with HEPES physiological saline at 37 °C and were maintained under these conditions throughout the experiment. A glass micropipette containing high K⁺ stimulating solution was positioned 30 µm from the cell. Each cell was then stimulated once with a single 5-s K⁺ injection pulse (20 psi) through the micropipette coupled to a microinjection system (Picospritzer II, General Valve Corporation, Fairfield, NJ). Optical images of experimental setup were obtained with a 40× objective (0.65 n.a.) in an inverted microscope (IX81, Olympus). All cell experiments were performed at 37 ± 1 °C.

2.5. Data processing and statistics

The amperometric traces were processed using an IgorPro 6.22 routine originating from David Sulzer's group [19]. The filter for the current was 1 kHz (binomial smoothing filter). The threshold for peak detection was five times the standard deviation of the noise ($I_{\max} \sim 2$ pA). The traces were carefully inspected after peak detection and false positives were manually rejected. All the peaks larger than 2 pA were collected and analyzed.

The parameters obtained from the peaks were $I_{\max}$ (the peak current), t_{rise} (the rise time, defined as the time separating 25% of the maximum from 75% of the maximum on the ascending part of the spike), $t_{1/2}$ (the half peak width, defined as the width of the exocytotic at half of its magnitude), t_{fall} (the fall time, defined as the time that takes for the current to drop from 75% to 25% of $I_{\max}$), and N (the number of molecules released). The number of moles, N , was evaluated with Faraday's law ($N = Q/nF$), where Q is the area under the peak, n is the number of electrons in the oxidation reaction (2 for monoamines), and F is Faraday's constant. For the pre-spike feet, the parameters obtained were I_{foot} (the foot current, defined as the average of the measured current over the duration of the foot or the plateau current when a steady-state is reached), t_{foot} (the foot duration), and N_{foot} (the number of molecules observed to be released in the foot, evaluated with Faraday's law ($N = Q/nF$) as explained above).

For comparison, the mean of the median of all the parameters calculated from single cells was used to minimize the impact of the cell-to-cell variations. Pairs of data sets were compared with a Wilcoxon–Mann–Whitney test. For Tau analysis, all the spikes recorded from all the cells were treated equally and the median was used for comparison since it is less sensitive to extremes compared to mean.

The exponential decays were fitted with double exponential with IgorPro and the parameters (T_1 and T_2) were obtained. The normalized distance, d , was calculated as previously reported [20,21].

3. Results and discussion

As stated above, curcumin, DMC, and BDMC are the main components of curcuminoids in turmeric. They have similar chemical structures (Fig. 1) with unsaturated carbons that are thought to be the main reason for their antioxidant properties. In the present study, curcumin and BDMC were chosen as representatives for single cell exocytosis study since curcumin is the major constituent (77%) of the commercial curcumin product and BDMC has a more distinct chemical structure from curcumin when compared with DMC. Furthermore, it has been reported that curcumin and DMC, but not BDMC, involve the same series of signaling pathways for their neuroprotective function. The concentration of curcumin and BDMC used in this study (20 μ M) is in the range of curcumin usually used in the literature [5,8,22–28].

Cells were pretreated with a control solution of 0.1% DMSO (used to dissolve the drugs) or drugs for specific intervals and exocytosis was monitored by placing a 5- μ m disk carbon fiber microelectrode on top of the cell following stimulation with high K^+ (Fig. 2A). The chemical messengers (mostly dopamine for PC12 cells) released during exocytosis were oxidized at the electrode, producing an exocytotic transient. Several parameters can be obtained from each individual exocytotic transient and these are presented in Fig. 2B.

3.1. Long-term treatment with curcumin alters the dynamics of the exocytotic response

Fig. 3 displays typical amperometric traces of exocytosis from vehicle or drug-treated PC12 cells for different intervals. For a 3-h treatment with low content of serum (1% horse serum and 0.5% FBS), the number of exocytotic events per cell for curcumin-treated cells (23 ± 4) is the same as that for vehicle-treated cells (23 ± 3). When the treatment time is increased to 72 h, the number of exocytotic events per cell for both curcumin-treated cells (48 ± 10) and the vehicle-treated cells (37 ± 5) is increased. This appears to result, at least in part, from the change of membrane permeability following long-term treatment with DMSO despite the very low concentration (0.1%) [29]. There is a trend towards an increase in event number for the curcumin-treated cells (72 h) when compared with the corresponding control even though not statistically significant.

The peak parameters obtained from the control and drug treatments are summarized in Table 1. The distribution of the exocytotic parameters for each cell is asymmetric and strongly deviates from normality (data not shown), motivating the use of the median instead of the mean for comparison. As shown in Table 1, the peak parameters for curcumin-treated cells were not changed significantly after a 3-h treatment. Taken together with the lack of change for the frequency of events at short incubation time, it appears curcumin does not influence exocytosis over a time of even a few hours. For the 72-h treatment with curcumin, $t_{1/2}$ and t_{fall} decreased by 17% and 24% compared to the control (vehicle, 72 h), respectively. Thus, the exocytosis events are faster for the curcumin-treated cells, possibly indicating the effects of curcumin are centered more in the regulation of the event duration and not in event frequency or amount released.

3.2. Long-term treatment with BDMC alters the amount of molecules released in exocytosis

Similarly to curcumin, a 3-h treatment with BDMC has no significant influence on the frequency of exocytotic events per cell (21 ± 3) and peak parameters, as shown in Table 1. Increasing the treatment time to 72 h, the frequency of exocytotic events per cell for BDMC-treated cells increases to 40 ± 6 , a value that is not significantly different from the vehicle-treated and curcumin-treated cells. When cells are treated with BDMC for 72 h, the amperometry parameters measured related to exocytotic dynamics are not changed significantly (Fig. 4). However, the value of $I_{\max}$ and the amount of molecules released are decreased significantly (38%, $p = 0.0007$ and 28%, $p = 0.0018$, respectively). The decrease in the amount of molecules released might result from the depletion of vesicular monoamine content or a decrease in the fraction released during exocytosis [30]. The changes observed following BDMC treatment are different from those for curcumin, suggesting curcumin and BDMC influence exocytosis through different mechanisms.

The mechanism of curcuminoids promoting neurite outgrowth in PC12 cells has been explored with the same conditions used in the present study. It has been reported that extracellular signal-regulated protein kinase 1/2 (ERK1/2)- and protein kinase C-dependent pathways are involved in the changes observed following curcumin, DMC and BDMC treatment. However, they concluded that the cAMP response element-binding protein coupling with cAMP response element-dependent gene transcription are only involved for curcumin and DMC, suggesting BDMC has a different cellular target or interaction pathway from curcumin and DMC [5]. This certainly might be related to the different effects observed on exocytosis reported here.

3.3. Effect of curcuminoids on the opening and late kinetic characteristics of the pore

Pre-spike amperometric feet were observed in some exocytotic events. As reported before, these are a critical indicator of the formation of fusion pore generated during exocytosis [19,31,32]. To further study the effect of curcuminoids on exocytosis and their mechanism, pre-spike feet were analyzed. Only peaks with a foot current higher than 2 pA were used for analysis in order to minimize the effect of noise. The parameters for pre-spike feet were analyzed according to the electrochemical parameters shown in Fig. 1B and the results are summarized in Table 2.

The probability to observe a peak with a pre-spike foot is slightly higher for cells treated with curcumin than the control cells. However, there is no significant change for I_{foot} , t_{foot} and N_{foot} . For cells treated with BDMC for 72 h, the probability to observe a peak with a pre-spike foot is lower with no significant change for the foot parameters. In amperometric detection of exocytosis, t_{foot} is thought to represent the dynamic opening of the fusion pore, whereas I_{foot} is related to the fusion pore geometry with a larger diameter pore giving a larger current. With the experimental approaches used here, the foot analysis suggests curcuminoids do not significantly affect the opening of fusion pore. Hence, we searched for another mechanism by which these compounds might affect exocytosis.

Previous studies have shown that the decaying part of the exocytotic amperometric peak can be fit either with a single or a double exponential [19,33]. The two exponentials are thought

to reflect the opening and closing of the pore during exocytosis, but might also be the result of differential diffusion from the dense core and open fluid space in the vesicle [34]. It is possible that different populations of fusion pores appear to result from variations in the number of SNARE complexes, concentration of Ca^{2+} , synaptotagmin interactions, the effects of dynamin or actin on pore opening and closing [20,21,33,35]. A single exponential decay is explained as the diffusion of vesicular contents through the fusion pore according to numerical modeling. The closing of the fusion pore during the latter stage of the release event is consistent with the double exponential decay where the secondary exponential is probably related to the closing of the fusion pore combined with diffusion. Consequently, we consider two situations: a purely diffusive case, where the decay is controlled only by the diffusion of the neurotransmitters through the lipid nanotube (single exponential), and in the other case the efflux is also controlled by a closing pore (double exponential).

In the open and closed mechanism of exocytosis, only a fraction of neurotransmitters is released during normal exocytosis [30,36,37,38]. For some transmitters to remain in the vesicle after exocytosis, the rate of fusion pore constriction has to be faster than the diffusion rate of neurotransmitters coming out of the vesicle [20,21]. Thus T_1 is characteristic of the pore constriction while T_2 is that of the diffusion of neurotransmitters by defining $T_1 < T_2$.

If we plot T_2 versus T_1 we can then calculate a normalized distance, d , which is equal to $\sin\theta$, where θ is the angle between the point (T_1, T_2) and the line $(T_1 = T_2)$. This is shown in Fig. 5A and can be used to evaluate whether decay is better fit with a single or double exponential. The value of d is calculated as follows:

$$d = \sin\theta = \frac{T_2 - T_1}{\sqrt{2(T_1^2 + T_2^2)}} \quad (1)$$

When d is small, the peak is better fit with a single exponential. Fig. 5B shows the cumulative values of d obtained for control, 72-h treatment with curcumin, and BDMC. The y-axis displays the calculated d , and the x-axis displays the fraction of the peak population where the d value is below this value. The d values for curcumin-treated and BDMC-treated cells are slightly shifted to the left when $d < 0.4$. For example, about 47% of the peaks show a $d < 0.2$ for the control treatment, whereas 43% for curcumin- or BDMC- treated PC12 cells. This small change might indicate that treatment with the curcuminoids increases the prevalence of high d values, thus favoring the double exponential decays. As the second exponential fitting is considered to be evidence for more rapid pore constriction, this result suggests that curcuminoids slightly increase the probability of observing a spike generated by contraction of a fusion pore with a speed faster than the diffusion clearance of monoamine transmitter from the vesicle. This is a subtle effect, but subtle effects can have large consequences in biological systems.

4. Conclusions

In summary, single cell amperometry has been used to investigate the influence of curcumin and BDMC treatment on monoamine release during exocytosis in PC12 cells. The results

indicate that continuous treatment with curcumin or BDMC for 72 h can affect the dynamics and amount of exocytosis, but a 3-h treatment has no significant effect. Curcumin and BDMC were found, however, to have differing effects on exocytotic release at the longer time. Curcumin appears to accelerate the release dynamics, whereas BDMC appears to decrease the amount of monoamine released. This suggests that curcumin and BDMC influence monoamine release by different mechanisms. These electro-chemical data might help in the understanding of the function of curcuminoids on learning and memory and depression at the single-cell level.

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Fig. 1.
Chemical structures of curcumin and bisdemethoxycurcumin (BDMC).

Fig. 2.

Experimental setup and peak analysis for curcuminoids exocytosis experiments. (A) Optical micrograph showing the experimental setup. Scale bar, 20 μm . (B) Scheme showing the different parameters used for peak analysis in this work. I_{max} , peak current; t_{rise} , rise time; $t_{1/2}$, half peak width; t_{fall} , fall time; I_{foot} , foot current; t_{foot} , foot duration.

Fig. 3. Typical traces of K^+ -stimulated exocytosis from PC12 cells treated with vehicle (top), 20 μ M curcumin (middle), or 20 μ M BDMC (bottom) for 3 h (left panel) or 72 h (right panel).

Fig. 4.

Summary of amperometric peak parameters including the N_{events} , I_{max} , $N_{\text{molecules}}$, $t_{1/2}$, t_{rise} , and t_{fall} for the control, 20 μ M curcumin-treated, and 20 μ M BDMC-treated cells.

Fig. 5.

(A) Scheme defining the normalized distance d ($\sin\Theta$) to the “ $T_1 = T_2$ ” line used in the analysis. (B) Single versus double exponential analysis showing the cumulative histograms of the normalized distance, d , for the vehicle, 20 μM curcumin, 20 μM BDMC treatment for 72 h. Inset: an expanded view of the area in the dotted rectangle.

Table 1

Experimental results for $I_{\max}$, $t_{1/2}$, t_{rise} , t_{fall} and N obtained from K^+ -stimulated PC12 cells of 3 h or 72 h treatment with vehicle (0.1% DMSO), 20 μM curcumin- or BDMC-treated cells.^a

Treatment	$I_{\max}/\text{pA}$	$t_{1/2}/\text{ms}$	$t_{\text{rise}}/\text{ms}$	$t_{\text{fall}}/\text{ms}$	$N/10^3$ molecules
Vehicle, 3 h (35 cells)	9.59 ± 1.52	2.29 ± 0.17	0.80 ± 0.06	1.71 ± 0.12	78.5 ± 5.9
20 μM curcumin, 3 h (34 cells)	7.53 ± 0.56	2.46 ± 0.18	0.74 ± 0.04	2.15 ± 0.15	88.2 ± 7.1
Variation	-21%	+7%	-8%	+26%	+12%
20 μM BDMC, 3 h (34 cells)	8.57 ± 0.83	2.10 ± 0.12	0.63 ± 0.03	1.80 ± 0.14	77.7 ± 6.8
Variation	-11%	-8%	-21%	+5%	-1%
Vehicle, 72 h (39 cells)	8.37 ± 1.27	2.29 ± 0.14	0.73 ± 0.04	1.96 ± 0.15	74.8 ± 4.9
20 μM curcumin, 72 h (37 cells)	8.50 ± 0.82	1.89 ± 0.11	0.67 ± 0.04	1.49 ± 0.10	73.4 ± 6.5
Variation	+2%	-17% [*]	-8%	-24% [*]	-2%
20 μM BDMC, 72 h (42 cells)	5.21 ± 0.30	2.19 ± 0.10	0.65 ± 0.03	2.07 ± 0.11	54.0 ± 3.7
Variation	-38% ^{***}	-4%	-10%	+6%	-28% ^{**}

^a The data are presented as mean of medians $\pm$ SEM. The pairs of data sets were compared using a Wilcoxon–Mann–Whitney test, and the result is indicated next to the variation.

p < 0.001.

**
p < 0.01.

*
p < 0.05.

Table 2

Foot parameters obtained from K^+ stimulated PC12 cells ($I_{\text{foot}} > 2$ pA) comparing control (75 peaks with foot from 1086 peaks) to curcumin-treated cells for 72 h (176 peaks with foot from 1789 peaks), BDMC-treated cells for 72 h (35 peaks with foot from 1677 peaks).^a

Treatment	$I_{\text{foot}}/\text{pA}$	$t_{\text{foot}}/\text{ms}$	$N_{\text{foot}}/10^3$ molecules	Peaks with a foot
Vehicle, 72 h	2.9 (2.3–3.7)	2.9 (1.6–4.8)	31 (18–61)	6.9%
20 μM curcumin, 72 h	3.0 (2.4–3.9)	3.0 (1.9–5.2)	33 (21–59)	9.8%
Variation	+3%	–3%	+6%	
20 μM BDMC, 72 h	2.6 (2.3–3.4)	3.4 (2.0–5.3)	35 (24–61)	2.1%
Variation	–10%	+17%	+13%	

^aThe data are presented as median (1st quartile–3rd quartile). The pairs of data sets were compared using a Wilcoxon–Mann–Whitney test, and no significant change was obtained.