Amperometry

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Using Single-Cell Amperometry To Reveal How Cisplatin Treatment Modulates the Release of Catecholamine Transmitters during Exocytosis

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Abstract: *The pretreatment of cultured pheochromocytoma (PC12) cells with cis-diamminedichloroplatinum (cisplatin), an anti-cancer drug, influences the exocytotic ability of the cells in a dose-dependent manner. Low concentrations of cisplatin stimulate catecholamine release whereas high concentrations inhibit it. Single-cell amperometry reflects that 2 μM cisplatin treatment increases the frequency of exocytotic events and reduces their duration, whereas 100 μM cisplatin treatment decreases the frequency of exocytotic events and increases their duration. Furthermore, the stability of the initial fusion pore that is formed in the lipid membrane during exocytosis is also regulated differentially by different cisplatin concentrations. This study thus suggests that cisplatin influences exocytosis by multiple mechanisms.*

Chemotherapy with cisplatin (*cis*-diamminedichloroplatinum, CDDP) induces mild cognitive impairment ("chemo brain"), including memory loss, difficulties with multitasking and focusing, and confusion.[1] High doses of cisplatin can also cause peripheral neuropathy.[2] Thus far, the reasons for the mild cognitive impairment or peripheral neuropathy induced by chemotherapy are unknown. We were thus interested in investigating the influence of cisplatin on the nervous system.

Single-cell amperometry, as a real-time, quantitative chemical method, can be used to analyze neurotransmitter release during exocytosis, the main process for neuronal and hormonal communication.[3] By analyzing the characteristics of the exocytotic peaks, certain information about the geometry and kinetics of the fusion pore and release process can be obtained. This method has contributed to our understanding of the fundamental mechanisms of exocytosis and the effects of pharmacology and changes in physicochemical conditions on exocytosis.[3c,4]

It is of great interest to examine the influence of cisplatin on the exocytotic release of dopamine from pheochromocytoma (PC12) cells. For our studies, PC12 cells were pretreated with different concentrations of cisplatin for specific periods of time. Single-cell amperometry was subsequently used to monitor exocytosis at the top of the cells. Interestingly, it appears that cisplatin treatment can modulate exocytosis in completely different directions depending on the drug concentration. Analysis of the peaks revealed that cisplatin appears to regulate the fusion pore geometry, the duration of its opening, and its closure.

The following discussion shows that cisplatin treatment influences the exocytosis ability of a cell in a dose-dependent manner. A carbon fiber microelectrode held at 700 mV was placed on top of a PC12 cell (Figure 1 A). Exocytosis was triggered by stimulating the cell with a solution containing a high concentration of K^+ ions, eventually leading to a train of peaks in the amperometric recording. The PC12 cells were incubated for three hours in RPMI 1640 media alone or with different concentrations of cisplatin. Figure 2 shows the percentage of cells generating at least five exocytotic peaks over the number of cells tested as a function of the cisplatin concentration. At lower concentrations (i.e., below 5 μM), cisplatin treatment increases the probability that a cell shows exocytotic capability. However, a clear inhibitory effect was observed in the presence of high cisplatin concentrations (10–100 μM), as fewer cells responded to chemical stimulation.

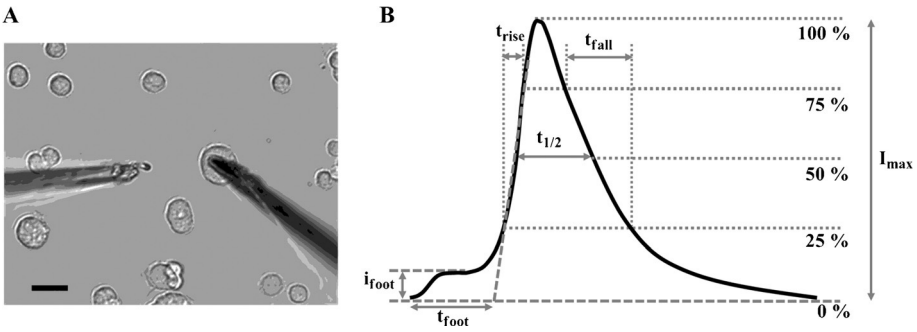

Figure 1. Experimental setup and peak analysis. A) Optical micrograph showing the experimental setup. Scale bar: 20 μm. B) Scheme showing the different parameters used for the peak analysis in this work. I_{max} = peak current, t_{rise} = rise time, $t_{1/2}$ = half peak width, t_{fall} = fall time,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

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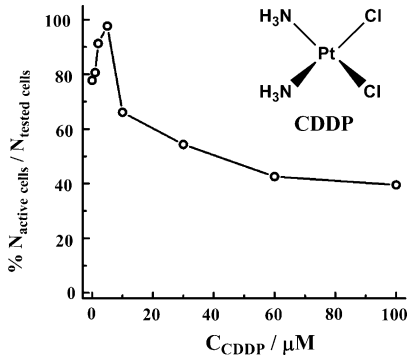

Figure 2. Cisplatin treatment modulates the exocytotic ability of a cell. The normalized fraction of cells generating at least five exocytotic peaks (active) over the number of cells tested is plotted as a function of the cisplatin concentration. In the control case, where no cisplatin was added, 78% of the cells were active.

Furthermore, the average number of events generated per cell and the average number of cumulative molecules released per cell upon chemical stimulation were studied as a function of the cisplatin concentration. Figure 3 shows that the number of events and the number of released molecules per cell increased after treatment with cisplatin at concentrations of 1–5 μM, but decreased at higher concentrations (10–100 μM). As an anticancer drug, cisplatin possibly decreases the cell viability, which consequently influences the exocytotic ability; therefore, MTT cell viability assays were carried out to examine how cisplatin decreases the PC12 cell viability over this concentration range. Under the conditions used in this study, the cell viability did not decrease upon cisplatin treatment (see the Supporting Information, Figure S1). This result suggests that cisplatin regulates exocytosis, which is in agreement with previous work with cell populations,[5] and that cisplatin is involved in promoting exocytosis at low concentrations and in inhibiting exocytosis at high concentrations, but not by influencing the cell viability.

Single-cell amperometry was then used to show that cisplatin treatment modulates individual exocytotic events. Exocytotic events from PC12 cells pre-incubated with 2 μM and 100 μM cisplatin were selected for peak analysis. The typical amperometric traces for each case are shown in Figure 4, left. Several parameters can be obtained from each

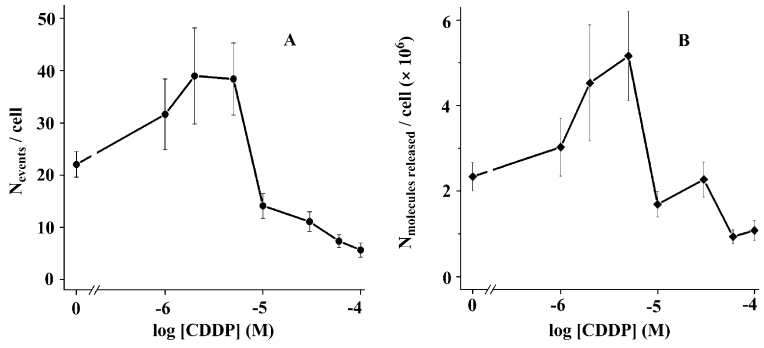

Figure 3. Average number of events recorded per cell (A) and average number of cumulative molecules released per cell (B) upon treatment with different concentrations of cisplatin in RPMI 1640 cell media for 3 h.

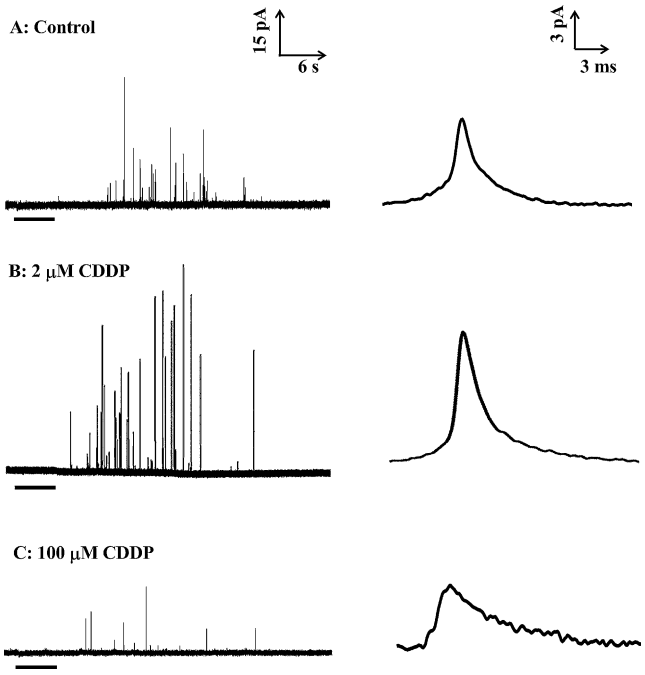

Figure 4. Left: Typical traces obtained from the control (top), 2 μM CDDP (middle), and 100 μM CDDP treated (bottom) PC12 cells. The black bar indicates the 5 s K^+ stimulation. Right: Average peaks obtained from the corresponding typical traces.

individual exocytotic event, as shown in Figure 1 B. The corresponding averaged peaks obtained from the typical traces for the control, 2 μM, and 100 μM cisplatin treatment are also shown in Figure 4 (right panels). Exposure to 2 μM cisplatin leads to sharper (higher and narrower) exocytosis events, whereas exposure to 100 μM cisplatin leads to less intense and broader events in comparison to the control cells.

The peak parameters obtained from the control treatment and the cells incubated with 2 μM and 100 μM cisplatin are summarized in Table 1. The distribution of the exocytotic parameters is unsymmetric and strongly deviates from normality (Figure S3), hence motivating the use of the median for statistical analysis, as it is less sensitive to extremes. These data reveal that treatment with 2 μM cisplatin leads to significant decreases in the characteristic peak times, $t_{1/2}$ and t_{fall}, and an increase in the peak current, I_{max}. Interestingly, these effects are opposite to those observed when the cisplatin concentration is increased to 100 μM. This result suggests that cisplatin has at least two different effects or mechanisms in the regulation of the lipid pore that is created during exocytosis. Furthermore, 2 μM cisplatin treatment does not alter the number of molecules released by each vesicle (N), but 100 μM cisplatin treatment increases N significantly. Intracellular vesicle electrochemical cytometry has been developed to quantify the neurotransmitter content in mammalian vesicles.[6] Here, we examined the change in the vesicular dopamine content in cisplatin-treated cells by this method. The result (Figure S4) indicates that the vesicular catecholamine content did not change significantly upon

Table 1: Experimental results obtained from K^+-stimulated PC12 cells, comparing control (43 cells, 1443 peaks) to CDDP-treated cells (2 μM: 18 cells, 892 peaks; 100 μM: 14 cells, 244 peaks).[a]

	I_{max} [pA]	$t_{1/2}$ [ms]	t_{rise} [ms]	t_{fall} [ms]	N [10^3 molec.]
control	6.02 ± 0.42	2.94 ± 0.15	0.90 ± 0.04	2.54 ± 0.14	81.5 ± 3.9
2 μM CDDP	8.22 ± 0.80	1.93 ± 0.11	0.74 ± 0.05	1.62 ± 0.12	79.7 ± 8.0
variation	+37%**	−34%***	−18%	−36%***	−2%
100 μM CDDP	4.27 ± 0.21	3.87 ± 0.23	1.03 ± 0.07	5.35 ± 0.37	105.3 ± 6.5
variation	−29%**	+32%***	+14%	+111%***	+29%**

[a] The data are presented as mean of the median ± SEM. The pairs of data sets were compared using a two-tailed Wilcoxon–Mann–Whitney rank-sum test, and the result is indicated next to the variation. ***: $p < 0.001$; **: $p < 0.01$.

cisplatin treatment. Therefore, cisplatin likely influences exocytosis by altering the fusion pore dynamics rather than the vesicle properties. Evidence from electrochemical cytometry,[6,7] post-spike feet analysis,[8] and several models of the amperometric event[9] has shown that during exocytosis, the vesicle expands beyond the initial fusion pore or kiss-and-run state, but does not normally open all the way. This open and closed exocytosis and the closing of the lipid pore again occur before complete expulsion of the vesicular contents, thus leaving the vesicle to be used again. Furthermore, partial expulsion makes it possible to regulate the amount of transmitter released without changing the vesicle or its resting content. In the case of cisplatin, the higher N value at high drug concentrations appears likely to result from a longer lifetime of the open pore, causing larger depletion of the vesicle content during exocytotic release.

Cisplatin can react with various types of proteins outside and inside of cells by coordination to cysteine, methionine, histidine, and tyrosine residues.[10] For instance, cisplatin can bind extensively to serum proteins, transport proteins, copper-transporting ATPases, insulin, metallothioneins, cytochrome c, superoxide dismutase, and ubiquitin, for example.[11] To date, only few proteins involved in exocytosis have been reported to react with cisplatin, which might be the reason for why the influence of cisplatin on exocytosis has not received much attention. Considering that the cells were incubated with a low concentration of cisplatin for only three hours, a clear influence on protein expression by modulating DNA expression is highly unlikely. Therefore, we propose that the influence of cisplatin on exocytosis results from its direct interaction with the proteins associated with exocytosis.

The SNARE (soluble NSF attachment protein receptor) proteins include synaptobrevin, which is located in the vesicle membrane, and syntaxin and SNAP-25, which are located in the plasma membrane. The formation of a complex of syntaxin, SNAP-25, and synaptobrevin is essential for triggering exocytosis.[5b,12] Synaptobrevin knockout leads to a lower event frequency without changing the peak shape except for the pre-spike foot.[13] Selective cleavage of syntaxin and SNAP-25 with Botulinum neurotoxin[14] or RNA interference[15] reduces the occurrence of the release events without any changes to the release dynamics. Furthermore, cells expressing a SNAP-25 mutant that lacks the C terminus have been shown to display broader peaks with a lower amperometric current, but the number of molecules released was not significantly changed.[16] The differences between these previous reports and our data (Table 1) suggest that the modulatory effect of cisplatin on exocytosis does not mainly occur through its interaction with SNARE proteins, but other proteins are also implicated in regulating open and closed exocytosis.

Dynamin and actin have been found to be involved in exocytosis.[17] Rapid inhibition of the GTPase activity of dynamin with dynasore results in peaks of shorter duration, with a decrease in $t_{1/2}$ and t_{fall} that is similar (ca. 30%) to that observed upon 2 μM cisplatin treatment. Furthermore, inhibition of dynamin with anti-dynamin antibodies inhibits rapid endocytosis, which is thought to be the last step in kiss-and-run exocytosis.[17c,d] Fewer release events coupled with an increased number of released molecules are consistent with our data for 100 μM cisplatin treatment. The actin cytoskeleton has been shown to be disrupted in cells upon cisplatin incubation.[18] Furthermore, actin takes part in regulating exocytosis. The inhibition of actin polymerization with latrunculin A leads to peaks of longer duration with more molecules released.[17b] Protein kinase C (PKC) is also a regulator of exocytosis. Activation of PKC with the phorbol ester PMA stimulates exocytosis by increasing the release frequency with shorter peak durations.[14] The similarity of these reports with our data led us to speculate that the observed effect of cisplatin might be due to the influence of cisplatin on these proteins, and this might explain the differential effect at low and high concentrations where the effect on one protein is more pronounced at low concentration, and the effect on other proteins is more dominant at high cisplatin levels.

To further investigate the influence of cisplatin on the mechanism of exocytosis and the initial fusion pore formed in this process, the pre-spike feet, thought to be caused by the initial formation and stabilization of the fusion pore, [4a,17a,19] were examined. To prevent issues with poor signal-to-noise ratios, only peaks with a foot current greater than 2 pA were used for analysis. The parameters for the pre-spike feet were analyzed according to the procedure presented in Figure 1B, and the results are summarized in Table 2.

The probability to observe a peak with a pre-spike foot is higher for cells treated with 2 μM cisplatin than for the control cells. Upon 2 μM cisplatin treatment, i_{foot} increased while t_{foot} decreased, which is similar to the overall trend for exocytosis events presented above. As t_{foot} is related to the dynamics of the fusion pore whereas i_{foot} is related to the fusion pore geometry, the foot analysis suggests that 2 μM cisplatin treatment induces a less stable fusion pore that is prone to dilate and close faster than in the control case. This is in agreement with the influence of dynamin inhibition by dynasore on the foot, namely that when the pore is not framed by the dynamin coil, the pore does not open as widely as in the control case and tends to collapse more rapidly, suggesting that the action of dynamin on the pore opening might be blocked by the 2 μM cisplatin treatment.

Table 2: Foot parameters obtained from K^+-stimulated PC12 cells ($i_{foot} > 2$ pA), comparing control (43 cells, 54 peaks with foot) to CDDP-treated cells (2 μM: 18 cells, 91 peaks with foot; 100 μM: 14 cells, 2 peaks with foot).[a]

	i_{foot} [pA]	t_{foot} [ms]	N_{foot} [10^3 molec.]	Peaks with a foot
control	2.4 (2.2–3.0)	3.6 (2.4–5.6)	37 (22–57)	3.7%
2 μM CDDP	3.0 (2.4–4.0)	2.9 (1.6–5.5)	33 (16–67)	10%
variation	+25%**	−19%*	−11%	
100 μM CDDP	2.2	7.2	54	0.8%
variation	−8.3%	+100%	−46%	

[a] The data are presented as the median (1st quartile–3rd quartile). The pairs of data sets were compared using a two-tailed Wilcoxon–Mann–Whitney rank-sum test, and the result is indicated next to the variation. **: $p < 0.01$, *: $p < 0.05$.

For cells treated with 100 μM cisplatin, fewer peaks with feet were recorded, so that there were not enough peaks with feet for a valid statistical analysis of the parameters. However, as actin is thought to aid in closing the pore, we speculate that this observation might support a mechanism whereby depletion of the actin cytoskeleton at high cisplatin concentrations acts to make the fusion pore expand more easily, hence providing less chance to observe the pre-spike feet.[17b]

In summary, we have analyzed the effects of cisplatin on the neurotransmitters released during exocytosis by single-cell amperometry. Cisplatin differentially regulates exocytosis at low and high concentrations, apparently by two different mechanisms. The stability of the fusion pore and the late kinetic characteristics of the fusion pore during exocytosis are affected in a manner that suggests that cisplatin interacts with two proteins recently shown to be associated with the regulation of open and closed exocytosis, actin and dynamin, although other possibilities cannot be ruled out. For example, cisplatin has been shown to affect membrane tension and the K^+ mechanosensitive channel TREK-1, which might then influence release.[20] These data might help to understand the neurological side effects of cisplatin, such as mild cognitive impairment and peripheral neuropathy, at the single-cell level.

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