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Increased Catecholamine Secretion from Single Adrenal Chromaffin Cells in DOCA-Salt Hypertension Is Associated with Potassium Channel Dysfunction

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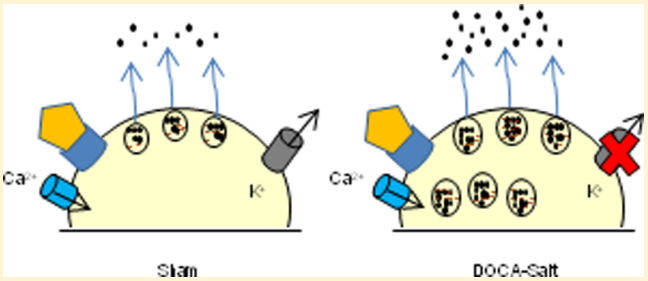
ABSTRACT: The mechanism of catecholamine release from single adrenal chromaffin cells isolated from normotensive and DOCA-salt hypertensive rats was investigated. These cells were used as a model for sympathetic nerves to better understand how exocytotic release of catecholamines is altered in this model of hypertension. Catecholamine secretion was evoked by local application of acetylcholine (1 mM) or high K^+ (70 mM), and continuous amperometry was used to monitor catecholamine secretion as an oxidative current. The total number of catecholamine molecules secreted from a vesicle, the total number of vesicles fusing and secreting, and the duration of secretion in response to a stimulus were all significantly greater for chromaffin cells from hypertensive rats as compared to normotensive controls. The greater catecholamine secretion from DOCA-salt cells results, at least in part, from functionally impaired large conductance, Ca^{2+}-activated (BK) and ATP-sensitive K^+ channels. This work reveals that there is altered vesicular release of catecholamines from these cells (and possibly from perivascular sympathetic nerves) and this may contribute to increased vasomotor tone in DOCA-salt hypertension.



KEYWORDS: *chromaffin cells, catecholamines, exocytosis, continuous amperometry, potassium channel function, DOCA-salt hypertension*

Increased sympathetic drive contributes to hypertension in humans[1−3] and in several animal models of the disease.[4−6] Evidence for heightened drive includes increased plasma levels of norepinephrine (NE) and hyperactive sympathetic nerve activity.[1,7,8] Our laboratory is investigating how the sympathetic neural control of vasomotor tone becomes altered in salt-sensitive hypertension. Vasomotor control mechanisms maintain the tone or diameter of blood vessels and, therefore, are an important regulator of blood pressure. To date, we have found that there is differential neurogenic control of arteries and veins in rats[9,10] and that two prejunctional control mechanisms become dysfunctional in the DOCA-salt model of hypertension:[10−12] (i) the α_2-adrenergic autoreceptor and (ii) the norepinephrine transporter (NET).

As a consequence of the altered prejunctional control, there is increased NE availability at sympathetic neuroeffector junctions and this contributes to increased vascular contractility, hence increased blood pressure. In vitro electrochemical measurements made at the adventitial surface of blood vessels enable real time monitoring of NE near its sites of release and action.[13−15] NE is a vasoconstricting neurotransmitter released from sympathetic nerves supplying arteries and veins. The action of NE is mediated by the postjunctional α_1-adrenergic receptor expressed by smooth muscle cells. Activation of α_1-adrenoreceptors causes muscle contraction and elevated blood pressure.

Amperometry can also be used to monitor catecholamine release from single adrenal chromaffin cells.[16−20] Sympathetic neurons communicate with smooth muscle cells by Ca^{2+}-dependent exocytosis of vesicular stores of NE and other vasoconstrictor transmitters.[21] This leads to the expulsion of vesicular content into the extracellular space.[21−23] Electrochemical measurements at single secretory cells have been used over the years to investigate the individual stages of exocytosis with high spatial and temporal resolution.[16,18,24,25] Measurements using single cells aid in the understanding of exocytosis, more so than can be accomplished through studies of intact tissues. This is because of the small size of the neuroeffector junction (~100 nm) that prohibits placement of a recording electrode in a single junction. Additionally, the coupled actions of the prejunctional α_2-autoreceptor and NET that serve to regulate of extracellular levels of NE at perivascular sympathetic nerve fibers, make it difficult to independently investigate exocytotic release of neurotransmitters.

Adrenal chromaffin cells secrete NE and epinephrine (EPI), and increased secretion from adrenal glands has been found in both hypertensive humans[1,7,26] and animals models.[27,28] Circulating catecholamine levels are elevated in DOCA-salt

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Table 1. Catecholamine and Metabolite Levels in Adrenal Medulla Isolated from Sham and DOCA-Salt Hypertensive Rats (n = number of rats, two adrenal medulla were used per rat)[a]

	NE	EPI	DA	NMN	MN	DHPG
Sham (n = 5)	168 ± 17	958 ± 72	6.1 ± 0.5	46 ± 5.0	52 ± 3.7	26 ± 11
DOCA-salt (n = 5)	257 ± 34[b]	1302 ± 49[b]	6.7 ± 0.6	79 ± 13	71 ± 7.4	2.0 ± 0.4

[a]NE, norepinephrine; EPI, epinephrine; DA, dopamine; NMN, normetanephrine; MN, metanephrine; DHPG, 3,4-dihydroxyphenylglycol. Data are mean ± SEM in units of μg/g tissue wet weight. [b]$P < 0.05$ vs corresponding Sham value, Student's t test.

hypertension, undoubtedly contributed to by chromaffin cells.[27,28] Chromaffin cells are a viable model for studies of the mechanisms and dynamics of exocytosis.[16,17,24] Since chromaffin cells do not express the α_2-adrenergic autoreceptor or NET, as do perivascular sympathetic nerves, they can be used in conjunction with electrochemical methods to study catecholamine secretion independent of autoreceptor inhibition or transporter-mediated reuptake. Understanding how and why there is increased quantal release would lead to a better understanding of DOCA-salt hypertension as a multifactoral disease. In addition to affecting catecholamine homeostasis, dysfunction in the adrenal gland has been linked to other pathways affecting blood pressure. For example, recent work has linked aldosteronism in BKβ1-KO mice to elevated blood pressure due to increased water retention and poor electrolyte handling.[29] The investigators in this work found that, in the adrenal gland, the BKβ1 channel expression is restricted to the medulla, suggestive of a link between adrenal chromaffin cells and elevated blood pressure in aldosterone-mediated hypertension. These findings are further supported by Sausbier et al. who determined that small arteries in BKα-KO mice had increased vascular tone, decreased vasodialation and the animals exhibited higher levels of aldosterone, further linking vascular tone to adrenal gland function.[30]

Altered catecholamine secretion has been previously found in spontaneously hypertensive rats (SHR). We believe that these changes are a consequence of hypertension and similar alterations may be present in the DOCA-salt model of hypertension. In the present work, we report on the use of single adrenal chromaffin cells, as a model for sympathetic nerve endings, to investigate the mechanisms of catecholamine release. Our goal was to verify if similar alterations in catecholamine secretion were evident in the DOCA-salt model of hypertension and investigate possible mechanisms that would cause any alterations found. We tested two hypotheses: (i) that catecholamine secretion from single adrenal chromaffin cells is increased in DOCA-salt hypertension and (ii) that impaired K^+ channel function contributes to the increased release.

RESULTS AND DISCUSSION

Neuroendocrine chromaffin cells are part of the adrenal gland, which is innervated by the sympathetic division of the autonomic nervous system. Consequently, these cells essentially function as modified postganglionic sympathetic neurons. In our prior work studying sympathetic neuroeffector transmission to arteries and veins, we used electrochemical methods to measure NE concentrations at the adventitial surface of the blood vessel.[9,10] In these measurements, an oxidation current is measured that reflects the extracellular NE concentration in the vicinity of the recording electrode. This concentration is contributed to by release from multiple nearby sympathetic varicosities. In such measurements, it is impossible to decouple the neurotransmitter release step (vesicle extrusion) from prejunctional autoinhibition, reuptake, and diffusion. Chromaffin cells serve as a viable model for sympathetic neurons, and their use in these studies allowed us to investigate disease-induced alterations in catecholamine release.

In related work, Miranda-Ferreira et al.[16] monitored chromaffin cell secretion using continuous amperometry and found that, for cells isolated from spontaneously hypertensive rats (SHR), a greater number of catecholamine molecules are released per stimulus from diseased cells as compared to healthy controls. Their work also indicated that catecholamine secretion is altered in chromaffin cells isolated from SHR.[16] Our results in the DOCA-salt model of salt-sensitive hypertension corroborate their findings and provide new mechanistic insights as to a cause for the altered release; impaired K^+ channel function.

Steady-State Catecholamine and Metabolite Levels in the Adrenal Medulla. To determine if alterations in catecholamine metabolism contrinute to the impaired catecholamine secretion from DOCA-salt chromaffin cells, HPLC was used to measure whole-tissue levels of catecholamines and metabolites in the adrenal medulla from normotensive and hypertensive rats. Whole medulla NE and EPI levels were ∼1.4-fold greater in DOCA-salt compared to Sham rats (Table 1). In contrast, dopamine (DA), normetanephrine (NMN), metanephrine (MN), and 3,4-dihydroxyphenylgycol (DHPG) levels were all statistically similar in the two tissues. NMN and MN metabolite levels normalized to their precursor levels (NMN/NE and MN/EPI) were used as a measure of catecholamine metabolism. The NMN/NE ratio was 0.27 ± 0.01 in adrenal medulla from Sham rats and 0.29 ± 0.03 ($P > 0.05$) in glands from DOCA-salt rats. The MN/EPI ratio was 0.05 ± 0.01 in both Sham and DOCA-salt adrenal medulla ($P > 0.05$).

We found that whole tissue levels of NE and EPI are elevated in the adrenal medulla isolated from hypertensive rats as compared to normotensive controls, consistent with previous reports for SHR [31] and DOCA-salt rats.[32] There were, however, no statistically different differences in levels of NMN, MN, and DHPG metabolites between the two groups. These results indicate that the metabolic degradation pathways for NE and EPI in the medulla are not altered in DOCA-salt hypertension. Therefore, a change in metabolism is not the cause for the increased number catecholamine molecules detected from DOCA-salt cells.[33] However, there is a clear buildup of catecholamines in the adrenal medulla. It is believed that the majority of catecholamine metabolism occurs in the cytoplasm after leakage from vesicles or extracellular reuptake.[34] This would suggest that the increased catecholamine content must be building up somewhere inside the cell other than the cytoplasm, which would most likely be inside the storage vesicles (i.e., increased packaging).

Increased Catecholamine Release from DOCA-Salt Chromaffin Cells. Continuous amperometric recordings of catecholamine release from single chromaffin cells isolated in culture. Release was elicited using an application of ACh (1

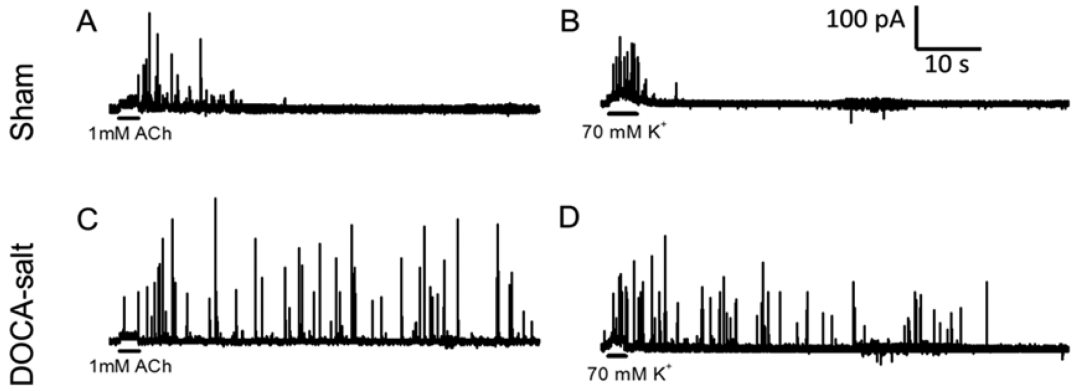

Figure 1. Continuous amperometric recordings from single Sham (A, B) and DOCA-salt (C, D) adrenal chromaffin cells. Current spikes arise from the electrochemical oxidation of catecholamines released from a cell with each spike representing a release event. Secretion evoked by ACh (1 mM) (A, C) and K^+ (70 mM) (B, D) is increased in DOCA-salt chromaffin cells compared to controls.

mM) to a single cell, which evoked a burst of oxidation currents (Figure 1). ACh elicits catecholamine secretion through activation of the nicotinic-acetylcholine receptor (nAChR), which increases intercellular Ca^{2+}, leading to exocytosis.[35,36] Secretion was blocked with (i) hexamethonium (100 μM), an nAChR antagonist, and (ii) in Ca^{2+}-free extracellular medium (data not shown). Catecholamine release from cells isolated from Sham normotensive rats occurred as a burst of secretion events lasting 10−15 s (Figure 1A). ACh also evoked bursts of oxidation spikes from DOCA-salt chromaffin cells (Figure 1C). These bursts were, however, longer in duration (30−45 s), the number of release events was 2.5-fold greater, and the total charge detected over the course of a recording was 3-fold greater for DOCA-salt compared to Sham cells.

Analyzing the data by either grouping the data by the total number of cells investigated (Table 2) or the number of animals investigated (data not shown) for Sham and DOCA-salt animals, respectively, revealed the same statistically significant differences. The total number of release events (spikes) per stimulus was 2× greater for DOCA-salt cells, the total oxidation charge measured per stimulus was 2−3× greater for DOCA-salt cells, and the total number of catecholamine molecules detected (oxidized) was 2−3× greater for DOCA-salt cells as compared to Sham controls. These trends were found whether using ACh of high K^+ stimulation. The increased number of spikes per stimulus seen for the DOCA-salt cells is consistent with more vesicles undergoing fusion and releasing greater amounts of catecholamines.

Table 2. Analysis of ACh (1 mM) and K^+ (70 mM) Evoked Catecholamine Secretion from Single Adrenal Chromaffin Cells Maintained in Primary Culture[a]

	total events per stimulus	total charge per stimulus (pC)	total molecules oxidized per stimulus ($\times 10^8$)
Sham			
ACh (n = 19)	22 ± 2	15 ± 2	0.5 ± 0.1
K^+ (n = 11)	25 ± 5	18 ± 5	0.6 ± 0.1
DOCA-salt			
ACh (n = 13)	49 ± 5[b]	42 ± 4[b]	1.3 ± 0.2[b]
K^+ (n = 14)	57 ± 12[b]	47 ± 9[b]	1.6 ± 0.3[b]

[a]Data are grouped by animal type. Each cell was used for a single amperometric recording. In the ACh experiments, 19 and 13 cells were used from 6 Sham and 5 DOCA rats, respectively. For the K^+ recordings, 11 and 14 cells were used from 4 Sham and 4 DOCA rats respectively. Data are mean + SEM. n is the number of cells investigated. [b]$P \leq 0.05$ vs corresponding Sham values, Student's t test.

One possible explanation for the increased number of vesicles undergoing fusion and secretion (individual spikes) from the DOCA-salt cells could be an up-regulation in nAChR expression. To test this possibility, high extracellular K^+ (70 mM) was also used to evoke release. Application of high K^+ evokes catecholamine secretion by directly depolarizing the cell membrane causing activation of voltage-gated Ca^{2+} channels. The data revealed that high K^+, like ACh, evoked increased catecholamine secretion from DOCA-salt cells as compared to Sham controls (Figure 1B and D). These results suggest that the primary cause of increased secretion from DOCA-salt chromaffin cells is not due to increased nAChR expression. It has been shown that exocytosis occurs in multiple steps (complete or partial release) and can be modulated by various stimuli.[37−40] Due to the high concentrations of our chemical stimuli, the release events being detected are most likely complete vesicular release.

ACh release from preganglionic sympathetic nerves, acting at nAChRs, is the endogenous stimulant of catecholamine release from chromaffin cells.[35,41] When cells were stimulated with ACh in vitro, the charge per spike, the frequency of individual oxidation current spikes, the total number of spikes or release events per stimulus, and the total number of catecholamine molecules released were significantly greater from DOCA-salt as compared to Sham cells. High K^+ depolarizes secretory cells, independent of nAChR function, by activating L-type Ca^+ channels.[42] The fact that catecholamine release was not different between high K^+ and ACh stimulation confirms that the increased secretion is not due to altered pathways associated with nAChR activation or increased expression of this receptor.

Release Kinetics. It is proposed that the individual spikes correspond to single vesicular exocytotic release events.[20,22,24,25,43] In other words, we suppose that each current spike corresponds to the electro-oxidation of catecholamines released from an individual vesicle. This supposition is supported by the fact that the charge per spike (Table 3, ∼0.8−1.1 pC) is similar to values reported previously for single vesicular release events (∼1 pC per spike).[22,24,43] For each spike, the integral of the current over time yields the charge. The charge can be converted to the number of molecules secreted and oxidatively detected using Faraday's law ($Q = nFN$), where Q is charge, n is the number of electrons

Table 3. Summary of the spike charge, Rise Time, and Full Width at Half Maximum (half width) for Secretory Events from Sham and DOCA-Salt Chromaffin Cells[a]

	avg charge/spike (pC)	rise slope (pA/ms)	10−90% rise time (ms)	half width (ms)
Sham				
ACh (1 mM) (n = 365)	0.79 ± 0.03	51 ± 2.4	5.1 ± 0.2	13.1 ± 0.3
K^+ (70 mM) (n = 246)	0.80 ± 0.04	57.2 ± 1.7	5.3 ± 0.2	11.6 ± 0.3
DOCA-salt				
ACh (1 mM) (n = 494)	1.12 ± 0.03[b]	66.2 ± 2.0[b]	4.4 ± 0.1[b]	10.7 ± 0.2[b]
K^+ (70 mM) (n = 669)	0.98 ± 0.03[b]	76.6 ± 1.3[b]	5.3 ± 0.1	11.7 ± 0.1

[a]Data are mean + SEM. n is the number of single event amperometric spikes used in the data analysis. The number of animals and cells used in these experiments is the same as in Table 2. [b]$P < 0.05$ vs corresponding Sham values, Student's t test.

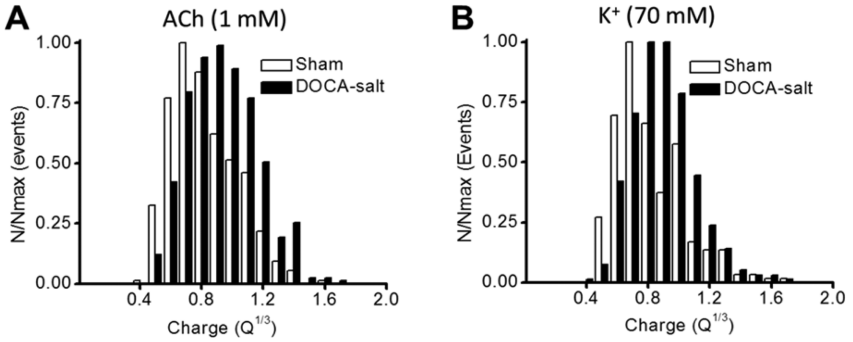

Figure 2. Distribution of the cubed root of the charge ($Q^{1/3}$) for individual release events from Sham and DOCA-salt cells evoked by ACh (A) and elevated K^+ (B) stimulation. Values were analyzed within 0.1 $pC^{1/3}$ bins and plotted against the maximum frequency observed among individual bins. A positive shift in charge values can be seen for events from DOCA-salt cells regardless of the stimulus used. Assuming an equal concentration distribution for the two cell types, the positive shift correlates to an increase in radius for vesicles from DOCA-salt cells as compared to normotensive cells. Cubed root means were compared between Sham and DOCA data using Student's t test and were statistically different at $P < 0.05$ (F-test was used to confirm no statistical differences between Sham and DOCA variances).

transferred per mole, F is Faraday's constant, and N is the number of moles of catecholamines oxidized. The number of moles can be converted to the number of molecules using Avogadro's number. Using the individual spike charge (see Table 3), a value of 2.5×10^6 molecules per vesicle is calculated for Sham secretory events evoked by both ACh and high K^+. This value is close to the 5.2×10^6 molecules per vesicle value reported previously for bovine adrenal chromaffin cells.[22,24,43]

Analyzing the data either by grouping the total number of current spikes recorded for each cell type (Table 3) or by the total number of cells studied from each animal type (Sham and DOCA-salt, data not shown) produced the same statistically different trends. Regardless of the stimulation, the average charge per spike is greater for DOCA-salt cells.

It has been reported that a relatively constant catecholamine concentration exists in each spherically shaped vesicle.[44] At high concentrations of chemical stimuli (1 mM ACh and 70 mM K^+) as used in this work, the likelihood of complete release of vesicular catecholamines is increased. Therefore, the number of catecholamine molecules reaching the electrode for detection is assumed to be independent of vesicular concentration and dependent on the vesicular volume, or radius ($Q = nFCV = nFCr^3$).[20,25] Figure 2 shows the distribution of the charge ($Q^{1/3}$), which is proportional to the vesicle radius, for single secretion events from Sham and DOCA-salt cells. Values were binned into 0.1 $pC^{1/3}$ intervals and then normalized to the highest number of events within a given bin. For both ACh and high K^+ stimuli, a right-ward shift in the distribution of charge per spike is seen for DOCA-salt cells. By taking the mean of all the cubed root values, average $Q^{1/3}$ values were obtained for ACh stimulation, which were 0.88 ± 0.01 and 0.99 ± 0.01 pC, respectively, for Sham and DOCA-salt cells. These values were similar to data for high K^+ stimulation. This increased $Q^{1/3}$ value for the DOCA-salt cells is significant and consistent with an increase in the average vesicle radius.

The temporal profiles of the current spikes were analyzed to assess the kinetics of release. As mentioned below in the Methods section, spikes were only analyzed if (i) they were monophasic, (ii) the peak amplitude was at least twice magnitude of the background current, (iii) the current was deflected positive, and (iv) the current transient returned to the baseline. Representative recordings of oxidation currents from Sham and DOCA-salt cells are presented in Figure 3A. When analyzing the data in Table 3, it can be seen that there are no differences in either the rise slope of the current spike or the 10−90% rise time for Sham or DOCA-salt cells, regardless of the stimulation. There is, however, a statistically significant

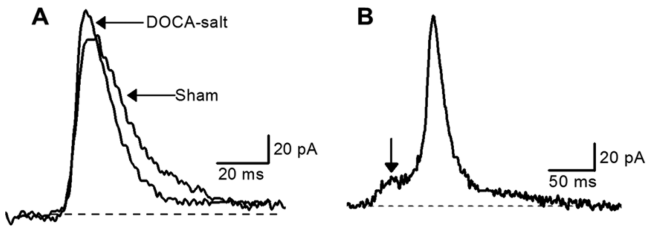

Figure 3. Single release events recorded from cultured adrenal chromaffin cells isolated from Sham and DOCA-salt rats (A). Release events from DOCA-salt cells occur faster than release events from Sham cells. An example of a spike event with prefoot event is shown (B). Approximately 10% of all spikes recorded from both Sham and DOCA-salt chromaffin cells exhibited this prefoot event.

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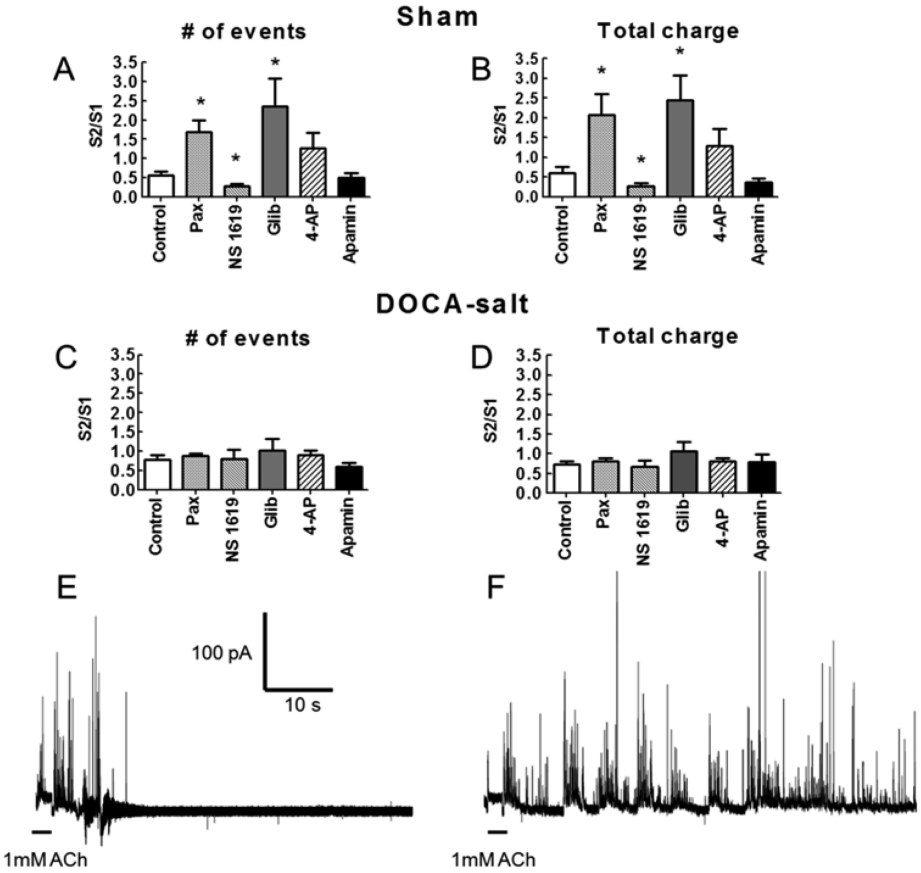

Figure 4. K^+ channel blockers alter catecholamine release kinetics in chromaffin cells from Sham but not DOCA-salt rats. Cells were stimulated with 1 mM ACh, and then after a 20 min interval they were stimulated a second time with ACh. Data are the ratio of the second (S2) stimulation to the first (S1). The control bars were obtained from cells that were not treated with a K^+ channel blocker showing baseline depletion of catecholamine content. Paxilline (BK channel blocker) and glibenclamide (ATP-sensitive channel blocker) caused an increase in the number of release events and total charge detected compared to the control (A, B) ($P < 0.05$, one-way ANOVA). To check if the BK channel function properly in Sham cells, the agonist NS 1619 was also applied to cells and caused a decrease in secretion from Sham cells compared to the control (A, B) ($P < 0.05$, one-way ANOVA). None of the drugs used affected catecholamine release from chromaffin cells from DOCA-salt rats (C, D) ($P > 0.05$). (*n* values for each antagonist represent the number of cells investigated. Sham data *n*: control, 7; paxilline, 10; NS 1619, 14; glibenclamide, 10; 4-aminopyridine, 10; apamin, 10. DOCA data *n*: control, 8; paxilline, 10; NS 1619, 11; glibenclamide, 12; 4-aminopyridine, 11; apamin, 12.). Amperometric recordings elicited from 1 mM ACh before (E) and after (F) application of the BK channel antagonist paxilline. Recording are obtained from a chromaffin cell isolated from a Sham rat. The BK channel antagonist increases the number of release events and the duration of secretion in a similar manner to recording obtained from DOCA-salt chromaffin cells (Figure 2C).

decrease in the half width for the DOCA-salt cells when using ACh stimulation. The rise time (10−90%) of a spike was used as a measure of the rate of catecholamine release. The $t_{1/2}$ was used as a measure of the rate of extrusion of chemical messengers after vesicle fusion.[19] The results suggest that the time course of catecholamine secretion is shorter for DOCA-salt release events as compared to Sham release events for ACh stimulation. However, only the rise slope was altered for DOCA-salt release events when stimulated with potassium. The rate-limiting step in exocytosis is thought to be rate of fusion pore expansion following vesicle-cell fusion.[17,24,25,45] The lack of a K^+ effect on the $t_{1/2}$ of individual events may be due to how this stimulation recruits Ca^{2+} in the cell. It is known that the SNARE proteins are responsible for tethering the vesicle to the cell membrane and that complete exocytosis versus partial secretion is calcium dependent.[46] The differences in calcium handling could provide an explanation as to the difference between the $t_{1/2}$ values for ACh and K^+ stimulation.

A characteristic of some of the oxidation current transients is the presence of a prefoot event. The prefoot event originates from the fusion of a vesicle membrane with the cell membrane and partial opening of the fusion pore. This leads to the partial release of the vesicular content.[17,24,25] An example of a prefoot event is shown in Figure 3B. There was no difference in the frequency of occurrence of the prefoot events for Sham and DOCA-salt cells with approximately 10% of all current spikes exhibiting a prefoot. Amatore et al. have shown that the prefoot event can be further classified by the limiting current, time length and quantal charge of the foot.[17] Our analysis of the secretory event "prefoot" was limited to simply noting the existence of a "foot" within an amperometric spike. Since no significant differences were found in their occurrence in Sham versus DOCA-salt cells, we did not investigate their features in any detail.

Our results suggest that catecholamine-containing vesicles in DOCA-salt chromaffin cells have a greater catecholamine molecule loading than do vesicles in Sham cells. It was also found that the rate of extrusion is more rapid from DOCA-salt cells compared to Sham cells, as evidenced by a decreased $t_{1/2}$ of the current spikes in the former for ACh stimulation.[19,20,22,24,25,43] Consistent with the greater loading is the increase in apparent vesicular radius based on the greater $Q^{1/3}$

Table 4. Summary of Potassium Channel Drug Effects on Secretion from Sham and DOCA-Salt Chromaffin Cells[a]

	control	paxilline	NS 1619	glibenclamide	4-AP	apamin
Sham release events	0.55 ± 0.11 (n = 9)	1.7 ± 0.3[b] (n = 12)	0.27 ± 0.07[b] (n = 13)	2.3 ± 0.7[b] (n = 12)	1.3 ± 0.4 (n = 12)	0.51 ± 0.11 (n = 11)
DOCA release events	0.79 ± 0.12 (n = 8)	0.89 ± 0.05 (n = 11)	0.81 ± 0.24 (n = 12)	±0.3 (n = 12)	0.91 ± 0.11 (n = 11)	0.60 ± 0.12 (n = 12)
Sham total charge detected	0.60 ± 0.18 (n = 9)	2.1 ± 0.5[b] (n = 12)	0.28 ± 0.08[b] (n = 14)	2.4 ± 0.6[b] (n = 12)	1.3 ± 0.6 (n = 12)	0.37 ± 0.09 (n = 11)
DOCA total charge detected	0.73 ± 0.07 (n = 8)	0.81 ± 0.08 (n = 10)	0.67 ± 0.16 (n = 12)	1.1 ± 0.2 (n = 12)	0.81 ± 0.10 (n = 11)	0.80 ± 0.19 (n = 12)

[a]Data are provided as a ratio of the response of the second ACh stimulation (S2) normalized to the response for the first stimulation (S1) (S2/S1). Statistical differences for drug effects are determined relative to control experiments where no drugs were applied. Data are mean + SEM. Data are grouped by the total number of cells (n) for each animal type. [b]$P \leq 0.05$ vs control values within a given an animal group, Student's t test.

value for the DOCA-salt cells. This makes sense as vesicles appear to possess greater catecholamine loading in the DOCA-salt cells and would therefore be expected to release more catecholamine molecules into the extracellular space as compared to Sham controls. According to published work that indicates that adrenal chromaffin cell vesicles maintain a constant catecholamine concentration with varying vesicle size,[37] an increase in the vesicle radius would be expected with greater catecholamine packaging (i.e., volume accompanying the increased loading). This was in fact observed. A limitation of our study, however, is that we have no microscopic evidence to confirm the suspected increase in vesicle radius.

In addition to NE and EPI, the vesicular matrix also contains chromogranin A, ATP (~120 mM), Ca^{2+} (17−30 mM), and ascorbate (~20 mM).[22,47] Vesicles of larger radii would have an increased membrane surface area. This could provide more points of contact with SNARE proteins located in the cellular membrane. SNARE proteins facilitate secretion by overcoming the natural repulsion energies of the vesicle and cell membrane, and promote docking and fusion.[48] It has also been shown that membrane tension and fusion pore length facilitate spontaneous fusion pore dilation.[49−51] More docking points would increase the probability of vesicle extrusion and this could be a cause for the greater number extruding vesicles (per stimulus) in DOCA-salt cells.

An additional cause for the increased secretion from DOCA-salt cells could be a greater number of vesicles available in the readily releasable pool as chromaffin cells maintain several storage pools.[25,52] Previously published work has shown that glucocorticoid treatment of adrenal chromaffin cells increases coupling of voltage-gated Ca^{2+} channels to the readily releasable pool of vesicles.[53] Our study was not focused on determining if the size of the vesicle pools is altered in DOCA-salt hypertension.

Impaired K^+ Channel Function in DOCA-Salt Chromaffin Cells. The literature reveals that in animal models of spontaneous and salt-sensitive hypertension, there is impaired K^+ channel function in nerves supplying blood vessels.[54,55] Our data show that the catecholamine release is significantly prolonged for DOCA-salt cells. In other words, there are a greater number of vesicles per stimulus releasing their contents from DOCA-salt cells. We hypothesized that this could be due to impaired K^+ channel function.[55−59] To test this, experiments were conducted in the absence and presence of different K^+ channel antagonists: paxilline (0.5 μM), 4-aminopyridine (1 mM), apamin (0.1 μM), and glibenclamide (50 μM). We also used the BK channel potassium agonist, NS 1619 (100 nM). Due to vesicle depletion, control experiments showed that there is approximately a 50% reduction in the charge evoked by the second (S2) of two ACh stimuli (S1 and S2) applied to a single cell. Therefore, experiments were performed in a paired manner with ACh-induced secretion from individual cells being recorded in the absence and presence of a specific K^+ channel antagonists with a 20 min delay between the first and second ACh stimulation. Paxilline (BK antagonist) and glibenclamide (ATP-sensitive) both increased the total number of release events as well as the total charge detected (total number of catecholamine molecules oxidized) from Sham cells but not from DOCA cells. 4-AP (voltage-gated K^+ channels) and apamin (SK) did not affect catecholamine release from either cell type. NS 1619 (BK agonist) effectively decreased secretion in Sham cells (Figure 4A−D); however, it had little effect on DOCA-salt secretion. Figure 4E and F also shows two amperometric recordings from a Sham cell before and after paxilline treatment. In the presence of paxilline, the number of release events and the duration of secretion are both increased, in a manner similar to that for DOCA-salt cells (Figure 1C). The results are summarized in Table 4.

We hypothesized that one or more K^+ channels become impaired in this model of hypertension and this contributes to increased catecholamine secretion due to the inability of the cell to timely repolarize. We found that BK and ATP-sensitive K^+ channel function is in fact impaired in chromaffin cells from DOCA-salt rats. However, to further verify that the receptors are functional in Sham cells, we used the BK channel agonist NS 1619 and found that activating BK channels caused a decrease in secretion from Sham cells, yet had no effect on DOCA-salt cells. This impairment leads to the prolonged release from DOCA-salt cells. However, the cause for the impaired channel function is still unknown.

Recent related work by Miranda-Ferreira et al. has shown that cytosolic and mitochondrial calcium handling is altered in SHR chromaffin cells and this leads to greater and more prolonged catecholamine release from stimulated chromaffin cells.[60] The authors found greater levels of calcium in chromaffin cell cytoplasm and in the mitochondria of chromaffin cells isolated from spontaneously hypertensive rats (SHR) as compared to normotensive controls. Increased calcium levels were also accompanied by a lower time-constant for calcium decay from SHR cells. A possible explanation for the slower clearance of calcium could be prolonged cell depolarization as a consequence of impaired potassium channels, as shown in our studies.

Another possible explanation for the altered potassium channel function could be oxidative stress. We have previously found that reactive oxygen species levels, mainly O_2^-, are elevated in perivascular sympathetic nerves in DOCA-salt hypertension and impaired prejunctional α2-AR function is

linked to the presence of O_2^{-}.[61] O_2^{-} may disrupt the secretory mechanism in the adrenal chromaffin cells from DOCA-salt rats. The work by Miranda-Ferreira et al.[60] suggests that investigating electron "leaking" from ATP production by the mitochondria may be a possible explanation for the altered secretion from SHR and DOCA-salt chromaffin cells, and could be caused by oxidative stress.

SUMMARY

The pathophysiology of salt-sensitive hypertension is multifactoral. This study provides direct evidence that there is increased catecholamine secretion from adrenal chromaffin cells in DOCA-salt hypertension. This contributes to the increased circulating catecholamine levels characteristic of this model of hypertension. Increased circulating catecholamines would be expected to contribute to increased vasoconstriction and blood pressure elevation. Additionally, these cells essentially function as modified postganglionic sympathetic neurons. The increased number of catecholamine molecules released per stimulus, the greater number of release events per stimulus, the more rapid rate of extrusion and the greater number of vesicles undergoing extrusion in DOCA-salt cells likely mimics what happens in perivascular sympathetic nerves supplying arteries and veins. Clearly, there is altered exocytotic release of catecholamines in this model of hypertension and this likely contributes to increased vasomotor tone. A cause for the greater number of vesicles undergoing extrusion and their prolonged release is impaired BK and ATP-sensitive K^+ channel function. Although measurements of release from individual sympathetic nerve varicosities are not possible, the adrenal chromaffin cell may be a suitable surrogate for further investigations of changes in catecholamine release in the sympathetic nervous system in hypertension.

METHODS

Sham and DOCA-Salt Rats. Experiments were performed using male Sprague−Dawley rats, 250−275 g (Charles River Laboratories, MI). All protocols were approved by the Michigan State University Committee on Animal Use and Care. Rats were housed 2−3 per cage in a temperature- and humidity-controlled room. They were exposed to a daily 12 h light/dark cycle and given free access to standard laboratory rat chow (8640 Rodent Diet; Harlan/Teklad) and water. Animals were euthanized with a lethal pentobarbital injection (100 mg/kg, i.p.). The adrenal glands were then removed through the abdomen via laparotomy. The surgical procedures and postoperative treatments have been described in detail elsewhere.[9−11] Briefly, the rats were anesthetized and a DOCA-salt steroid pellet was implanted subcutaneously producing a dose of 200 mg/kg (body weight). Animals were housed for an additional 4 weeks before being sacrificed for experiments to allow for full development of hypertension. Blood pressures were recorded the week the adrenal gland was harvested with nominal systolic blood pressure values of 137 ± 2 mmHg (n = 10) and 201 ± 5 mmHg (n = 9) for Sham and DOCA-salt rats, respectively.

Adrenal Medulla Catecholamine Levels. After removing both adrenal glands, the adrenal medulla was isolated and quickly frozen at −80 °C until time for the measurements (<2 weeks). The frozen medulla (2 medulla were used for each rat) was weighed (0.06 ± 0.02 g for Sham medulla and 0.04 ± 0.01 g for DOCA medulla, $p > 0.05$) and placed into 0.1 M perchloric acid at the time of analysis. The amount of acid used for the digestion was 4× the mass of the tissue sample. For both Sham and DOCA samples, the tissue was homogenized using an OMNI TH-01 homogenizer with a 5 mm blade. This was followed by centrifugation at 13 520g for 10 min at 4 °C. The resulting supernatant was then collected and passed through a 30 kD filter and recentrifuged. The centrifugate was analyzed immediately afterward by HPLC-EC for catecholamine and metabolite levels. A volume of 10 μL of the supernatant was directly injected onto a reversed-phase column for separation using an autosampler. The analysis was accomplished using a commercial HPLC system (ESA Biosciences, Chelmsford, MA) that consisted of a solvent delivery module (model 584), an autosampler (model 542) with sample cooling to 4 °C, and a coulometric detector (Coulochem III). The detector was equipped with a 5021A conditioning cell (electrode I) and a 5011A high-sensitivity analytical cell (electrode II and III). An HR-80 (C18, 3 μm particle size, 80 mm length × 4.6 mm ID) reversed-phase column (ESA Biosciences) was used for the separation. The mobile phase was a commercial Cat-A-Phase II (ESA Biosciences) that consisted of a proprietary mixture of acetonitrile, methanol, phosphate buffer, and an ion-pairing agent (pH 3.2). The separation was performed at 35 °C using a mobile phase flow rate of 1.1 mL/min. Quantification was achieved using external standards and creating a calibration curve for analytes of interest. No internal standard was used due to the fact that there was no extraction performed on the medulla tissue. HPLC data are presented as the mass of the molecule detected (μg) normalized to the mass of the frozen adrenal medulla (g).

Chromaffin Cell Isolation. Adrenal glands were removed and placed in cold Locke buffer (154 mM NaCl, 3.6 mM KCl, 5.6 mM $NaHCO_3$, 5.6 mM glucose, and 10 mM HEPES, pH 7.2). Adrenal medulla isolation was performed as described previously.[16] The adrenal medulla was then placed in a solution containing 12 mg of collagenase Type-I (Worthington Biochemical Corporation) and 24 mg of bovine serum albumin (Sigma-Aldrich) for 29 min with gentle agitation every 5−10 min. The tissue was then centrifuged for 5 min at 1200g. The collaganese solution was then replaced with warm, filtered Locke buffer, and the sample was recentrifuged. This process was repeated twice. The buffer was then replaced with 100−200 μL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum containing 50 IU/mL penicillin and 50 μg/mL streptomycin. Aliquots of 25−50 μL of the DMEM solution were then placed on glass coverslips coated with poly D-lysine (0.1 mg/mL, 45 min treatment). The coverslips were placed in Petri dishes, and after 30 min in an incubator (37 °C and 5% CO_2) 3 mL of DMEM was added to each dish. Cells were allowed to fully adhere to the coverslips for 2 h before beginning an experiment. A total of 2−4 coverslips were plated with chromaffin cells for each animal. For each coverslip, 1−2 cells were probed using continuous amperometry, allowing for investigation of multiple cells for each animal.

Continuous Amperometry. Carbon fiber microelectrodes were prepared according to a published procedure.[36] Coverslips with plated cells were placed in a flow cell containing HEPES buffer (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 12 mM glucose) flowing at 2−3 mL/min. The flow cell was positioned on the stage of an inverted microscope for monitoring the electrode positioning. Continuous amperometry was used to detect catecholamine release (both NE and EPI) as an oxidation current. A potential of +0.60 V vs Ag/AgCl (3 M KCl, Model EE009, Cypress System Inc.) was applied for detection using an Omni 90 analog potentiostat (Cypress Systems Inc.). At this potential, both NE and EPI were detected as an oxidation current. To perform an amperometric recording, the electrode was gently placed in contact with a chromaffin cell. Acetylcholine (ACh, 1 mM) or high K^+ (70 mM) was used to evoke catecholamine release and was applied using a pressurized burst of N_2 gas for 2 s (Picospritzer II, General Wave Corporation). Catecholamine secretion was recorded using a computer running Axoscope 8.1 and analyzed using Clampfit 10.0 (Axon Instruments). A Pt wire served as the counter electrode. In the ACh experiments, 19 cells from 6 Sham rats and 13 cells from 5 DOCA rats were analyzed. In the high K^+ experiments, 11 cells from 4 Sham rats and 14 cells from 4 DOCA rats were analyzed. Each cell was for only one measurement. For the K^+ channel studies, we used paxilline (BK channel blocker, 0.5 μM), 4-aminopyridine (voltage-gated K^+ channel blocker, 1 mM), apamin (SK channel blocker, 0.1 μM), glibenclamide (ATP-sensitive K^+ channel blocker, 50 μM), and NS 1619 (BK channel agonist, 100 nM). The effects of the K^+ channel agonist and antagonists were evaluated by measuring the S2/S1 ratio for which S2 was the oxidation current evoked by the second ACh application in the

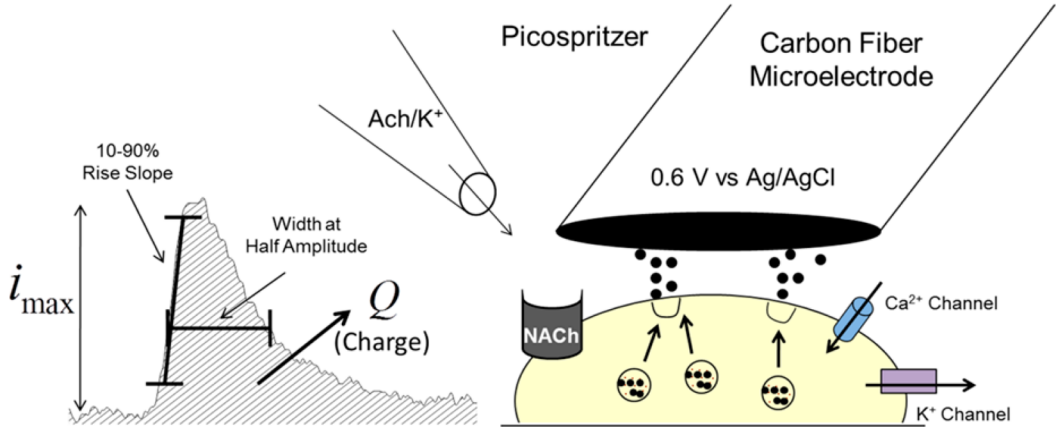

Figure 5. A carbon fiber microelectrode is positioned at the membrane surface of a chromaffin cell and used to detect catecholamine secretion as an oxidation current (A). A 2 s burst of ACh (1 mM) or K^+ (70 mM) was used to evoke catecholamine release. Secretion was monitored at 0.6 V vs a Ag/AgCl reference electrode. A representative release event is shown to the left. Current spikes were analyzed for their 10−90% rise time and slope, the full width at half-maximum (fwhm), the spike area, and the number of release events per stimulus.

presence of the drug relative to S1 which was the oxidation current measured initially in the absence of drug. In each experiment, after the initial recording (S1), a potassium channel drug would be applied through the flow bath for 20 min. Following the 20 min application, a second recording was performed to analyzed receptor function in the presence of the drug. An example of the experimental setup and a list of the kinetic parameters measured for amperometric events are shown in Figure 5 along with a diagram of the experimental setup.

Data Analysis. HPLC data are reported as a mean ± SEM with *n* values indicating the number of animals used for the study. Amperometric recordings were made at a sampling rate of 10 kHz and filtered using a low-pass Gaussian filter with a 1 kHz time constant. Individual oxidation currents (spikes) were analyzed using a threshold event detection routine. A threshold limit of twice the background current was used as an event discriminator. Spikes were not used in the analysis if there was any evidence of superimposed events, the current did not return to baseline, the signal was twice the background signal and only positive deflecting spikes were analyzed. The quantitative and kinetic parameters analyzed for each spike were the total charge (Q), the full width at half-maximum ($t_{1/2}$), the rise time (10−90%), and the number of exocytotic events. Data are reported as a mean ± SEM with *n*"indicating the number of cells or the number of individual release events from a cell. Statistical differences between groups were determined using a Student's *t* test for paired or unpaired data as appropriate. One-way analysis of variance (ANOVA) and Dunnett's multiple comparison test were used to analyze data from the K^+ channel antagonist studies. Differences were considered significant when $p < 0.05$.

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Author Contributions

All authors contributed fully to the experimental design, data analysis and interpretation, and the writing and editing of this manuscript.

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Notes

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ABBREVIATIONS

NE, norepinephrine; EPI, epinephrine; DOCA, deoxycorticosterone; ACh, acetylcholine

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