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SINGLE-VESICLE CATECHOLAMINE RELEASE HAS GREATER QUANTAL CONTENT AND FASTER KINETICS IN CHROMAFFIN CELLS FROM HYPERTENSIVE, AS COMPARED TO NORMOTENSIVE RATS

by

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Non-standard abbreviations: spontaneously hypertensive rats (SHR); Dulbecco´s modified Eagle´s medium (DMEM)

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Abstract

In a previous study performed in the intact adrenal gland (Lim et al., 2002) stimulation with acetylcholine (ACh) or high K^+ concentrations (K^+) produced greater catecholamine release in spontaneously hypertensive rats (SHR), as compared to normotensive animals. In this study the time course of secretion was in the range of minutes. Hence, we do not know whether enhanced release is due to greater quantal content and/or distinct kinetics in SHR and control animals. To get insight into the mechanism involved in such enhanced catecholamine secretory responses, we performed a single-vesicle release study in primary cultures of adrenal chromaffin cells, recorded with amperometry. Cells were stimulated with 2-s pulses of 1 mM ACh or 70 mM K^+. The secretory responses to ACh or K^+ pulses in SHR cells as compared to control cells had the following characteristics: (1) double number of secretory events; (2) 4-fold augmentation of total secretion; (3) cumulative secretion that saturated slowly; (4) 3-fold higher complex events with 2-4 superimposed spikes that may be explained by faster spike kinetics; (5) about 2-3 fold higher event frequency at earlier post stimulation periods; and (6) 2-5 fold higher quantal content of simple spikes. We conclude that SHR cells have faster and larger catecholamine release responses, explained by more vesicles ready to undergo exocytosis and greater quantal content of vesicles. This could have relevance to further understand the pathogenic mechanisms involved in the development of high blood pressure, as well as in the identification of new drug targets to treat hypertension.

Introduction

The differential release of the catecholamines norepinephrine and epinephrine from the adrenal medullary gland into the circulation, either in basal or stressful conditions, is tightly regulated by central (Folkow and Von Euler, 1954) and various peripheral splanchnic nerve stimulation patterns (Mirkin, 1961; Klevans and Gebber, 1970). Alteration of the activity of the sympatho-adrenal medullary axis and of the rate of catecholamine release, has been implicated in the pathogenesis of genetic essential hypertension, as proven by the classical use of drugs interfering with this axis to treat hypertensive patients i.e. blockers of α and β adrenergic receptors, reserpine, α-methyldopa, ganglionic blocking agents, guanethidine, angiotensin II receptor blockers, and so on (Westfall and Westfall, 2007). This is supported by the fact that the plasma levels of norepinephrine and epinephrine are augmented in spontaneously hypertensive rats (SHR) (Iriuchijima, 1973; Grobecker et al., 1975; Pak, 1981), as happens to be the case in humans suffering essential hypertension (Goldstein, 1983). This may be due to elevated sympathetic nerve activity, as revealed in hypertensive patients using microneurography (Anderson et al., 1989)

Numerous studies performed with different methodologies support that pre- and postsynaptic sympathetic dysfunctions are involved in the pathophysiology of primary hypertension in humans or laboratory animals (Tsuda and Masuyama, 1991; de Champlain et al., 1999). Studies on presynaptic mechanisms have been performed in SHR, a model of primary hypertension; they show that norepinephrine release is increased in different tissues rich in sympathetic nerve endings (Donohue et al., 1988). Although this increase of NA release from sympathetic nerve endings constitutes an important catecholaminergic dysfunction associated to primary hypertension, the precise mechanism involved in this dysfunction remains unknown.

A recent study on catecholamine release from intact perfused rat adrenal glands indicates that release stimulated by acetylcholine (ACh), the physiological neurotransmitter at the adrenal medulla splanchnic nerve-chromaffin cell synapse (Feldberg et al., 1934) or by high K^+ concentrations (K^+), is higher in adrenals from SHR, as compared to the responses obtained in control normotensive rats (Lim et al., 2002). However, in this study secretion was assessed upon collection of 4-min perfusate

samples, with a poor temporal resolution to study a fast secretory event such as exocytosis of chromaffin cells (Neher, 1998). This low-temporal resolution precluded the analysis in such study of the fast kinetics of secretion or quantal aspects of single-vesicle secretory events, upon stimulation of isolated individual chromaffin cells from control and SHR animals.

Thus, we felt that a study to analyze the kinetics of single-vesicle secretory events, using a carbon fibre electrode and amperometry, was timely. This high-resolution technique provides insight not only on quantitative, but also on qualitative kinetic aspects of the last fusion steps of exocytosis in isolated chromaffin cells in the millisecond time range (Wightman et al., 1991; Borges et al., 2005). We found that short pulses (2 s) of ACh or K^+, elicited a more sustained production of spike secretory events, and a drastic augmentation of the quantal catecholamine content of individual secretory vesicles, that had a faster fusion kinetics in SHR, as compared to normotensive rats.

Materials and methods

Control and spontaneously hypertensive rats

Animals were manipulated according to the guidance of the Ethics Committee for Handling Research Animals, of our Medical School, Universidad Autónoma de Madrid. Male 16-week-old Sprague-Dawley (SD) and SHR (Spontaneously Hypertensive Rats) weighing around 300 g were housed at $24 \pm 2°C$ with $60 \pm 20\%$ relative humidity, on a 12 h light/12 h dark cycle. Animals were fed a standard diet and water ad libitum and periodically weighed. Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (bpm) were evaluated with a pressure meter (LETICA 2006, Cibertec, Madrid, Spain), by cannulating the femoral artery. As in our present study, several other studies on hypertensive rats used SD rats as control normotensive rats (Wexler, 1980; Chao and Chao, 1988; Jandeleit-Dahm et al., 1997; Shimosawa et al., 2004).

Isolation and culture of rat adrenal medulla chromaffin cells from control and hypertensive rats

To prepare each cell batch we used 1-2 adult rats that were killed by cervical dislocation. The abdomen was opened, the adrenal glands exposed, and quickly removed and decapsulated and both adrenal medullae isolated under a stereoscope. They were placed in Ca^{2+}- and Mg^{2+}- free Locke buffer of the following composition (in mM): NaCl 154, KCl 3.6, $NaHCO_3$ 5.6, glucose 5.6, and Hepes 10 (pH 7.2) at room temperature. Tissues were collected under sterile conditions. Medullae digestion was achieved by incubating the pieces in 6 ml of Ca^{2+}/Mg^{2+}-free locke buffer containing 6 mg collagenase, 12 mg bovine serum albumin, for 20 min at 37ºC; gentle agitation was applied at 5-10 min intervals by using a plastic Pasteur pipette. The collagenase was washed out of the cells with large volumes of Ca^{2+}/Mg^{2+}-free Locke buffer. The cell suspension was centrifuged at 120xg for 10 min. After washing 2 times, the cells were resuspended in 1 ml of Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 5% foetal calf serum containing 50 IU ml^{-1} penicillin and 50 µg ml^{-1} streptomycin. Cells were plated on circular glass coverslips, previously treated with 0.1 mg/ml of poly-d-lysine during 30 min, followed by a thorough washout with water. After 30 min, 1 ml DMEM was added to each well. Cells were then incubated at 37 ºC in a water saturated, 5% CO_2 atmosphere; they were used within 1-2 days after plating

Amperometric monitoring of catecholamine release with a carbon fibre microelectrode.

Carbon fibre microelectrodes were prepared by cannulating a 7-µm-diameter carbon fibre in polyethylene tubing. The carbon fibre tip was glued into a glass capillary for mounting on a patch-clamp headstage and backfilled with 3 M KCl to connect to the Ag/AgCl wire, which was kept at +700 mV. The electrode was positioned at the middle right side of a spherical cell, gently touching the cell. Amperometric currents were recorded using an EPC-9 amplifier and PULSE software running on an Apple Macintosh computer. Sampling was performed at 14.5 kHz and samples were digitally filtered at 2 kHz. The sensitivity of the electrodes was routinely monitored before and after the experiments, using 50 µM epinephrine as standard solution. Only fibres that

rendered 200-300 pA of current increment after a 50 µM epinephrine pulse, were used for the experiments. Cell secretion was stimulated by pulses of 70 mM K$^+$ or 1 mM ACh during 2 s, delivered from a micropipette located 40 µm away from the cell right side of the cell being explored; solutions bathed the cells by gravity, upon opening of computer-driven valves.

Spike analysis and statistics

Spike analysis was performed using the Pulse Program (Heka) and Igor Pro Software (Max Planck Institute), which includes the Ricardo Borges´s macro package that allows the analysis of single events (Segura et al., 2000). A threshold of 4.5 times the first derivative of the noise standard deviation was calculated to clearly detect amperometric events.

Differences between means of group data fitting a normal distribution, were assessed by using Student's t test. A P value equal or smaller than 0.05 was taken as the limit of significance. The cumulative secretion (Figs. 1C and 2C) was analyzed by repeated measures with ANOVA followed by the Bonferroni test.

Materials and solutions

The following materials were used: collagenase type I and ACh from Sigma (Madrid, Spain); Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin fraction V, fetal calf serum, and antibiotics were from Gibco (Madrid, Spain). All other chemicals used were reagent grade from Merck and Panreac Química (Madrid, Spain)

Results

Body weight, adrenal weight, and hemodynamic parameters of control and SHR rats

Sixteen week-old rats were used. Control rats weighed 328.2 g and SHR rats 318.9 g (table 1). The dry weights of adrenal glands were 22.3 mg for control and 22.7 mg for SHR. The hemodynamic parameters were significantly higher in SHR rats as shown in table 1. Thus, the systolic blood pressure, the diastolic blood pressure, and the mean arterial pressure were 55.2%, 59.3%, and 56.4% higher than the values obtained in control animals. The basal heart rate of control rats was 337 bpm, and that of SHR animals 371 bpm, 10.2% higher.

The amperometric catecholamine secretory responses elicited by ACh

Occasionally, rat adrenal chromaffin cells maintained in primary cultures fire low-frequency spontaneous action potentials (Kidokoro et al., 1982). This predicts that cells might spontaneously produce some secretory spikes. However, in our experiments this spontaneous activity was rare and when produced, the cell was discarded.
Usually, an experiment began with about 2-5 min of cell perifusion with the basal Krebs-Hepes solution, to allow its equilibration. When possible, two or more cells were explored with the carbon fibre microelectrode in the same coverslip, going from right (the position of the microelectrode) to left (the position of the local superfusion pipette). In this manner we did not expose to ACh or K^+ stimuli, the second or third cell explored in the same coverslip.

Fig. 1A shows a prototype secretory response elicited by an ACh pulse (1 mM, given focally for 2 s), in a control cell. After a delay of about 600 ms, a burst of secretory spikes appeared. The duration of the burst overlasted the ACh application by about 2.5 s. The spike frequency was initially higher to decline after 2 s or so. Note that the rest of the trace was silent and no spikes clearly different from noise were produced.

The behaviour of the SHR cell shown in the trace of Fig. 1B was quite different. Firstly, the delay between the ACh pulse and the appearance of the first spike was considerably shortened to about 150 ms. Secondly, the baseline secretion was markedly

enhanced likely as a result of overlapping secretory spikes. Thirdly, the amplitudes of some spikes seemed to be larger; and fourthly, the spike response overlasted the ACh pulse duration during a substantially longer period, about 15 s.

Fig. 1C shows a plot of the mean cumulative secretion from various cells explored following the protocol of Fig. 1A and 1B. The secretion evoked by the ACh pulse in each individual cell was calculated at 2-s intervals, as the area of spikes present within such 2-s period that was expressed in picoculombs (pC) of catecholamine released and cumulatively added to obtain the curves of Fig. 1C. Note that in control cells, secretion during the first 6 s after the ACh pulse saturated at about 120 pC, and stopped after 14 s. In contrast, in SHR cells secretion continued rising to about a 600 pC peak at the 35th second.

The amperometric catecholamine secretory responses elicited by K^+

Saline solutions containing high K^+ concentrations cause chromaffin cell depolarization, enhanced Ca^{2+} entry, and catecholamine release. This stimulus directly recruits voltage-dependent Ca^{2+} channels, thus bypassing the nicotinic receptors stimulated by ACh (Garcia et al., 2006). Hence, to define whether ACh evoked greater responses in SHR cells just through a nAChR-delimited effect, secretion was also studied in cells stimulated with K^+-depolarizing saline solutions.

In the experiments shown in Fig.2A, a protocol similar to that of Fig.1 was followed, except for the fact that here, a control cell was stimulated for 2 s with a solution containing 70 mM K^+ (isoosmolar reduction of Na^+). Note that the secretory spikes appeared soon after K^+ application, with a delay of only 200 ms; the secretory activity over lasted the K^+ pulse and kept going for an additional 4-s period.

The trace of Fig. 2B was obtained from a SHR cell. Here, a pronounced elevation of baseline secretion, indicating the superposition of many catecholamine release quanta was seen. Furthermore, the secretory response continued for about 20-30 s after the K^+ pulse. Fig. 2C shows that in control cells, cumulative secretion gradually rose to reach a plateau at the 10th second, at about 150 pC; the secretory activity stopped after 10 s of the K^+ pulse. In contrast, secretion in SHR cells lasted much longer and rose to a plateau of near 1000 pC after 40 s of the K^+ pulse.

Decay of secretory responses upon repeated pulsing with ACh or K$^+$

In a few SHR and in most control cells, repeated pulsing with ACh or K$^+$ produced secretory responses that decayed with time. For instance, in 4 SHR cells we could obtain two responses evoked by ACh pulses separated by a 5-min interval. Fig. 3A shows that the second secretory responses decayed to about 50% in control cells and by 40% in SHR cells. However, the response to both stimuli were significantly higher in SHR cells, about 2.5-fold above control cells. The number of spikes also decreased during the second ACh pulse in both cell types, but once more it was higher in SHR cells.

Secretory responses also decayed with successive high-K$^+$ pulses (Fig. 3C). For instance, in 3 SHR cells, the total secretion per pulse (Q) decreased from 220 pC (1st pulse), to about 100 pC (2nd pulse), and to around 60 pC (third pulse). A similar relative decline was seen in control cells, although such decline was smaller from the 2nd to the 3rd K$^+$ stimulus. Again, secretion was 3-4-fold higher in SHR cells, as compared to control cells during the 1st and the 2nd stimulus. A similar picture was observed on the total number of spikes per K$^+$ pulse; spike numbers were higher in SHR cells during the 1st and the 2nd K$^+$ stimulus while it was similar to control cells during the third stimulus. Since the decay of secretion to repeated pulsing was high, particularly in SHR cells, we performed the kinetic analysis of single-vesicle events by taking into account only the secretory responses elicited by each ACh or K$^+$ pulse in every individual cell.

Incidence of single and multiple spikes in the secretory responses elicited by ACH or K$^+$

To analyze some kinetic aspects of individual secretory spikes we investigated first the incidence of single separated spikes and that of secretory complexes consisting of multiple spikes that did not reach baseline secretion. Fig. 4 shows four examples of these spike types. It is known that in about 10% of the cases, partial vesicle fusion

proceeds to a full fusion event, resulting in a foot signal preceding an amperometric spike (Zhou et al., 1996). Most of the spikes obtained in this study had no foot, as the prototype shown in panel A. The spike shown in panel B has a foot that proceeds to a full fusion spike. Panel C shows a secretory event consisting of two partially superimposed spikes and panel D shows a complex secretory event with four spikes.

We counted the number of single or multiple spikes in all traces recorded from control and SHR cells stimulated with ACh or K^+ pulses. (Fig. 4E). In control cells stimulated with ACh, 10% of the 599 spikes analyzed had a complex profile. In SHR cells, 30% of spikes were complex, out of the 834 spikes analyzed. A similar picture emerged upon the analysis of K^+ elicited spikes: multiple spikes accounted for 9% in control cells and were 3.5-fold more frequent in SHR cells.

Frequency distribution of secretory events in the responses elicited by ACh or K^+ pulses

Another facet of interest was the analysis of spike frequency along the traces obtained in the example cells shown in Figs. 1 and 2. Such analysis was performed second by second and is presented in Fig. 5. Panel A shows the responses elicited by ACh. During the first 9 s of the trace, the spike frequency was in general higher in SHR, as compared to control cells. From this time to the 27th second, the control cell was silent while the SHR cell continued discharging spike events at about 5-7 Hz.
The responses to K^+ were considerably faster during the first 10 s, as indicated in Fig. 5B. For instance, during the first 5 seconds the control spikes reached frequencies between 7-17 Hz (compared to about 10 Hz with ACh); the spikes from SHR cells reached frequencies as high as 40 Hz.

Frequency distribution of secretory events as a function of spike amplitude

A significant higher spike number was counted along the secretory responses elicited by ACh or K^+ in SHR cells, with respect to control cells. The question is whether the distribution of spikes was uniform at different spike amplitudes; or rather, if they were randomly distributed along the trace recording obtained with each stimulus, independently of amplitudes.

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Fig. 6A shows the number of spikes generated by ACh pulsing as a function of amplitude ranges, grouped in 200 pA steps. In the lower amplitude range (0-199 pA), 39 spikes were found in control cells and 76 spikes in SHR cells, about 2-fold higher. In the range 200-399 pA these values decreased to 9 and 14 spikes respectively. The spikes with higher amplitudes were few and had no statistical differences between control and SHR rats.

In the case of spikes elicited by K^+, a similar pattern emerged. So, in the lower range (0-199 pA), control cells exhibited 54 spikes while SHR cells had 103 spikes, about 2-fold higher. In the range 200-399 pA, 9 spikes were in control cells and 19 spikes in SHR cells. Again, at higher amplitudes fewer spikes were seen.

Catecholamine quantal content and kinetics of single amperometric spikes

We also analyzed the quantal catecholamine content of individual spikes (Q, in pC). Partially superimposed spikes that begun above baseline, or that did not reach baseline when decaying, were hard to accurately estimate their area and hence their quantal catecholamine contents; therefore, we did not include them in this analysis. The area of these single spikes reflects the release of the total vesicular content of catecholamines (Zhou et al., 1996).

Spikes were distributed in groups according to their amplitudes, in 200 pA steps. The maximal numbers of spikes were found in the range 0-199 pA amplitude, both for ACh and K^+ pulses, in control as well as SHR cells (Fig. 7A,B). In this range, the quantal content of spikes from control cells stimulated with ACh approached 1 pC, in agreement with Q values of 0.8 pC of mouse chromaffin cells (Arroyo et al., 2006) and 1.3 pC in bovine chromaffin cells (Ardiles et al., 2007). A surprising finding was the much higher Q of SHR spikes, about 3-5 fold higher than the values of Q for control cells stimulated with ACh. The spikes triggered by K^+ exhibited a pattern similar to ACh (Fig. 7B); their quantal content was about 2-4 fold higher in SHR, compared with control cells.

We also analyzed the kinetics of individual spikes. Table 2 shows that the time to peak (t_{max}) was around 16 ms in spike events elicited by ACh or K^+ stimulation of control cells; t_{max} was reduced by about 40% in SHR spikes. This indicated faster spike

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ascension (m) that was 35-50% higher in SHR cells, compared with control cells. The spike half-width ($t_{1/2}$) was reduced by 21% in SHR cells.

Discussion

We have found that the stimulation of single rat adrenal chromaffin cells with short ACh or K^+ pulses causes catecholamine secretory responses that are substantially higher and longer lasting in cells obtained from SHR rats, as compared with responses generated in cells from control normotensive animals (Figs. 1, 2 and 3). This corroborates the observation of Lim et al. (2002) who in the perfused intact adrenal gland observed that stimulation with ACh or K^+ enhanced more the catecholamine present in 4-min perfusate samples of SHR, as compared with control rats. However, in such study using the intact gland, the slow perfusion rate and the low-sensitivity of the catecholamine fluorescence assay permitted only to analyze global catecholamine release responses in the minute range, precluding to get insight into the kinetic mechanisms involved in such enhanced secretion. In our study we measured the amperometric detection of single-vesicle exocytotic events in cultured single chromaffin cells, allowing us a high temporal resolution (millisecond to second) analysis of secretory responses. We found that the integrated total catecholamine release elicited by an ACh pulse was 4-fold higher in SHR rats, as compared to control rats (Fig. 1C). However, the number of secretion spikes generated by an ACh pulse was only two-fold higher in SHR cells (Fig. 1D). In cells stimulated with K^+ pulses, a similar outcome was seen (Fig. 2C, D). Thus, augmented secretion in SHR versus control cells may have at least two components: (i) a higher number of secretory events; and (ii) a higher quantal content of individual events, as shown in Fig. 7.

Another interesting feature was related to the number of events having single or multiple spikes. It is generally accepted that a separate well identified spike corresponds to the quantal total release of the catecholamine stored in a single secretory vesicle, through a fusion pore formed by the fusion of the vesicle membrane with the plasmalemma (Breckenridge and Almers, 1987); this pore usually expands to release

the total vesicular content expressed by the area of a single spike (Zhou et al., 1996). Multiple spike events indicate that various vesicles suffered almost simultaneous exocytosis; this was more pronounced with K^+ stimulation of SHR cells, that caused a clear elevation of baseline secretion (Fig. 2B). In SHR cells, about 30% of events had multiple spikes (2-4 spikes), while control cells had only 10% of events with multiple spikes; this was true with ACh or K^+ stimulation (Fig. 4E), suggesting that in SHR cells more vesicles were docked and primed at subplasmalemmal sites (Neher, 1998) to undergo exocytosis upon ACh or K^+ stimulation. Alternatively, it may also indicate that vesicle fusion with the plasmalemma and the ensuing quantal catecholamine release was faster (table 2), giving rise to more exocytotic sites available and to more spike overlapping.

It is unlikely that differences in the nicotinic receptors and/or voltage-dependent Ca^{2+} channels present in rat chromaffin cells (Garcia et al., 2006) could explain the different secretion pattern seen in SHR, as compared to control cells. This conclusion is supported by the quite similar responses generated by ACh, that indirectly depolarizes the chromaffin cells (Douglas et al., 1967) and activates exocytosis by enhancing Ca^{2+} entry through Ca^{2+} channels (Douglas and Poisner, 1961), or by high K^+ concentrations that causes direct cell depolarization (Douglas et al., 1967) and recruitment of voltage-dependent Ca^{2+} channels, in both control and SHR cells. However, it will be interesting to study the inward currents through nicotinic receptors generated by ACh, as well as the inward Ca^{2+} currents generated by square depolarizing pulses or by action potentials in both, normotensive and hypertensive rats.

Another possibility rests in a different Ca^{2+} homeostatic mechanism in SHR as compared to control cells. We have seen different patterns of exocytosis in bovine and mouse chromaffin cells, likely related to different Ca^{2+} handling by mitochondria, upon cell depolarization with K^+ or electrically-evoked depolarizing pulses (Ales et al., 2005). Also, we found differences in ACh- or K^+-evoked secretory responses in bovine chromaffin cell populations when the endoplasmic reticulum Ca^{2+} movements were disturbed (Cuchillo-Ibanez et al., 2002). A functional triad controlling $[Ca^{2+}]_c$ and exocytotic signals has been described to be present in bovine chromaffin cells using endoplasmic reticulum- or mitochondria-targeted aequorins, and measurements of inward Ca^{2+} channel currents (Alonso et al., 1999; Montero et al., 2000; Garcia et al., 2006). It will be interesting to test whether such functional triad is present in chromaffin

cells of control normotensive rats, and if it is altered at some points in hypertensive rats. Alterations of these Ca^{2+} homeostatic mechanisms occur in vascular smoth muscle cells. In fact, a large Ca^{2+} influx and a high $[Ca^{2+}]_c$ are reported even during rest in conduit arteries (e.g., the aorta and the carotid and femoral arteries) from SHR as compared with normotensive rats (Jelicks and Gupta, 1990; Asano et al., 1996); this has also been found in small mesenteric arteries from SHR, where the sarcoplasmic reticulum has a larger capacity for Ca^{2+} storage (Nomura and Asano, 2002).

In conclusion, we have shown that short ACh or K^+ pulses cause catecholamine secretory responses that drastically differ in chromaffin cells of normotensive and hypertensive rats. The much greater catecholamine release responses in SHR rats are explained by the faster exocytosis of more vesicles with greater quantal catecholamine content, during a much longer secretory period. This drastic different behaviour of the chromaffin cell secretory responses may contribute to the understanding of the pathogenic mechanism of hypertension development in humans, and facilitate the identification of novel therapeutic targets to control high blood pressure.

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Footnotes

LEGENDS FOR FIGURES

Fig.1   ACh pulses produced longer lasting catecholamine secretory responses in SHR chromaffin cells, as compared to control cells. Panel A shows an example record obtained from a control cell stimulated with an ACh pulse (1 mM ACh, 2 s), as indicated by the horizontal bar at the bottom. Panel B displays a record obtained from an example SHR cell, similarly stimulated with an ACh pulse. Note the prolonged duration of the period showing post-stimulus amperometric spikes. Panel C shows the cumulative secretion, calculated at 2-s intervals in traces similar to those shown in panels A and B; the area of spikes is expressed and represented in pC (ordinate) as a function of time after the ACh pulse (abscissa). Data are means $\pm$ s.e. of the number of cells shown in parentheses. Statistical difference between control and SHR cells was at *** $p < 0.001$, using Bonferroni test after analysis by repeated measures with ANOVA.

Fig.2 High-K^+ pulses produced longer lasting catecholamine secretory responses in SHR chromaffin cells, as compared to control cells. Panel A shows an example record obtained from a control cell stimulated with a high-K^+ pulse (70 mM K^+, 2 s), as indicated by the horizontal bar at the bottom. Panel B displays a record obtained from an example SHR cell, similarly stimulated with K^+. Note the striking post-stimulus secretory spike activity in this SHR cell. Panel C shows the cumulative secretion, calculated at 2-s intervals from traces similar to those of panels A and B, and represented in pC (ordinate) as a function of time. Data are means $\pm$ s.e. *** $p < 0.001$, ANOVA.

Fig.3 Decay of secretory responses upon repeated cell pulsing with ACh (1 mM, 2 s) or K$^+$ (70 mM, 2 s) is much more pronounced in SHR cells, as compared to control cells. Panel A shows the total secretion per pulse (integrated area of all spikes obtained in each stimulus, in pC, ordinate) in control and SHR cells stimulated twice with a 5-min interval. Panel B shows the total number of spikes secreted per each ACh pulse, in the same cells of panel A. Panels C and D show total secretion and spike number per pulse, respectively, in cells stimulated with 3 successive K$^+$ pulses given at 5-min intervals. Data are means $\pm$ s.e. of the number of cells given in parentheses. * p< 0.05, ** p< 0.01, *** p<0.001, with respect to control cells.(Student´s t test).

Fig.4 Incidence of single and multiple amperometric spike events in the secretory responses evoked by ACh (1 mM, 2 s) or high K$^+$ pulses (70 mM K$^+$, 2 s), in control and SHR cells. Panels A and B show examples of single spikes, with or without a foot, and panels C and D show samples of multiple spikes. To draw the histogram of panel E, single or multiple spikes were counted in the trace secretory responses elicited by ACh or K$^+$. The incidence of multiple spikes in each individual cell was expressed as % of total spikes, counting single or "multiple" spikes as "individual" events. Data are means $\pm$ s.e. *** p< 0.001 with respect to control. (Student´s t test).

Fig. 5 Histograms of frequency spike distribution, calculated from the traces shown in Figs. 1A,B and 2A,B, corresponding to the secretory responses elicited by ACh or K$^+$, in control and SHR cells. The number of spikes present in 1-s time periods were calculated and plotted as frequencies in Hz (ordinates). Data are

representative of cells stimulated with a 2-s ACh pulse (as indicated by the bottom

horizontal bar in panel A) or by a 2-s K^+ pulse (as shown by the horizontal bar in

panel B).

Fig. 6 Distribution of secretory spikes as a function of their amplitudes, in control and

SHR cells stimulated with ACh or K^+ pulses. Spikes from all traces obtained in the

experiments shown in Figs. 1 and 2 were grouped according to their amplitudes in

200 pA range (abscissae), counted and their numbers given in the ordinates. Panel

A shows the spike distribution in cells stimulated with ACh pulses, and panel B

shows that obtained from cells stimulated with K^+ pulses. Data are means $\pm$ s.e. *

$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, with respect to control cells. (Student´s t test).

Fig. 7 Analysis of the quantal content of individual secretory events obtained from

control and SHR cells (Figs. 1 and 2). Spike areas (quantal catecholamine content

of individual vesicles, expressed in pC, Q, in the ordinates) were individually

analyzed and distributed according to the amplitude range, in pA, shown in the

abscissae. Data are means $\pm$ s.e. of the number of spikes shown in parentheses on

top of each column. Panel A shows the analysis of spikes obtained with ACh

stimulation of control and SHR cells, and panel B shows that corresponding to K^+

stimulation. Data are means $\pm$ s.e. of the number of spikes shown in parentheses

on top of each column. ** $p < 0.01$, *** $p < 0.001$, with respect to control cells.

Table 1. Body weight, adrenal gland weight, and hemodynamic parameters of control and SHR rats. n, number of animals; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean blood pressure; bpm, beats per minute. Data are means $\pm$ s.e. of the number of animals shown in n. In parentheses, the % increases above control rats are given. *** $p < 0.001$ (Student´s t test).

	n	Body Weight (g)	Adrenal gland Weight (mg)	SBP	DBP	MAP	Heart rate (bpm)
CONTROL	20	328.2 ± 2.67	22.3 ± 0.51	125.7 ± 0.63	99.3 ± 0.68	109.1 ± 0.74	336.7 ± 1.31
SHR	20	318.9 ± 1.98	22.75 ± 0.53	195.1 ± 1.2 *** (155%)	158.2 ± 1.7 *** (159%)	170.6 ± 1,5 *** (156%)	371 ± 2.6 *** (110%)

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Table 2. Kinetic parameters of the spike secretory events in control and SHR cells. t_{max}, time to peak; m, rate of spike ascension; $t_{1/2}$, spike half width. Data are means $\pm$ s.e. of the number spikes, n. *** $p< 0.0001$, * $p< 0.01$, compared with their respective ACh or K^+ controls (Student´s t test).

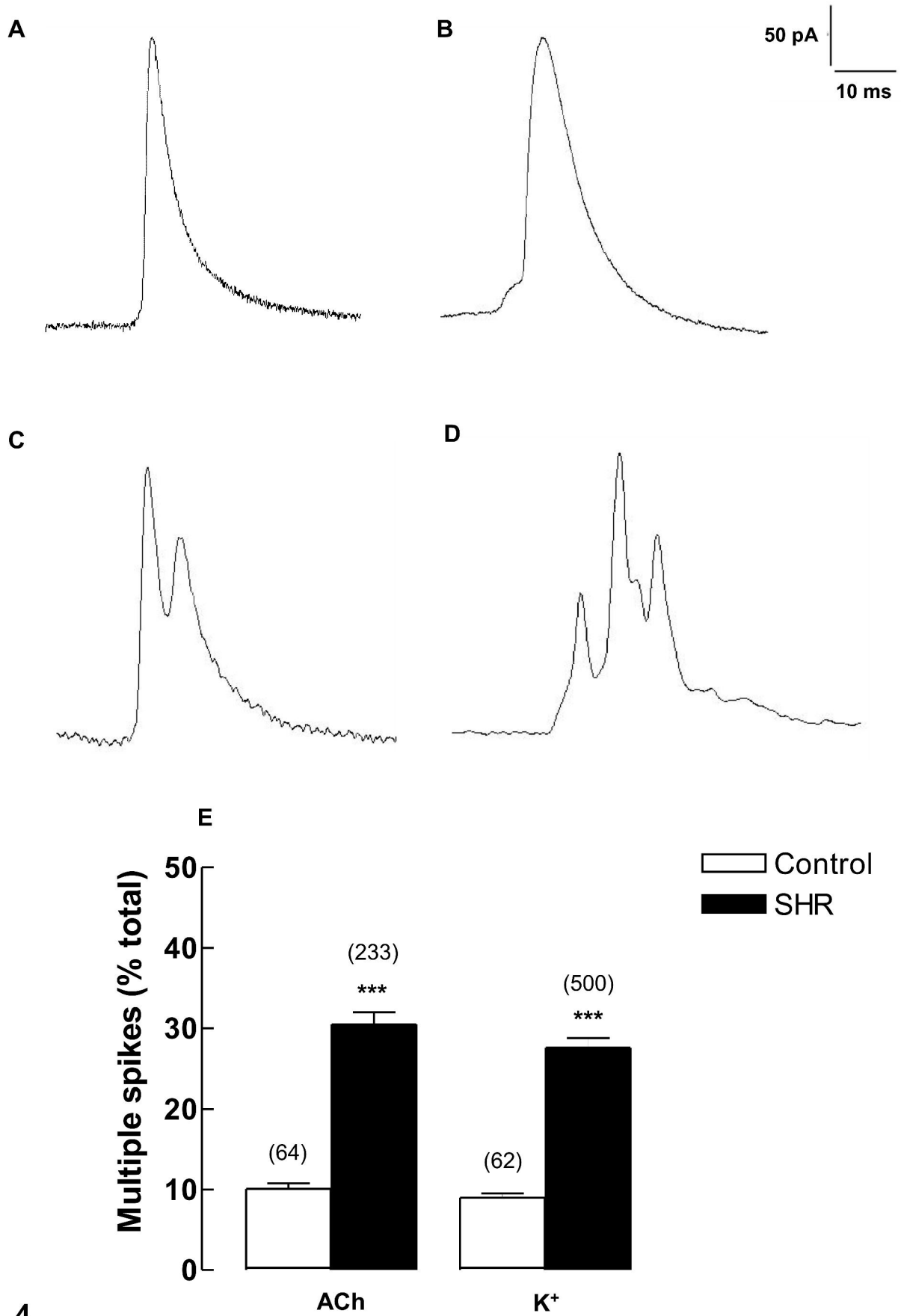|		t_{max} (ms)	m (pA/ms)	$t_{1/2}$ (ms)	n
Control	**ACh**	16.1 ± 0.83	31.9 ± 3.0	9.8 ± 0.2	584
	K^+	15.7 ± 2.2	55.3 ± 3.9	7.6 ± 0.2	678
SHR	**ACh**	9.4 ± 1.8 *** (58.4%)	43.3 ± 3.2 * (135.7%)	7.7 ± 0.3 *** (78.5%)	712
	K^+	10.4 ± 0.8 * (66.2%)	84.9 ± 3.2 *** (153.5%)	5.9 ± 0.1 *** (77.6%)	1638

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Fig. 1

Fig. 2

Fig. 3

A

B

50 pA

10 ms

C

D

E

Fig. 4

Fig. 5

Fig. 6

Fig. 7