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ROLE OF THE ENDOPLASMIC RETICULUM AND MITOCHONDRIA ON QUANTAL CATECHOLAMINE RELEASE FROM CHROMAFFIN CELLS OF CONTROL AND HYPERTENSIVE RATS

by

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Running title: Quantal secretion from chromaffin cells of hypertensive rats

Non-standard abbreviations: DMEM, Dulbecco´s modified Eagle´s medium; CRT, mixture of caffeine, ryanodine and thapsigargin; CCCP, carbonylcyanide chloromethoxy phenylhydrazone; FCCP, carbonylcyanide P-(trifluoromethoxy) phenylhydrazone; SERCA, sarcoendoplasmic reticulum calcium ATPase.

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Abstract

Here we present the first study on the effects of compounds that interfere with calcium (Ca^{2+}) handling by the endoplasmic reticulum (ER) and mitochondria, on amperometrically measured quantal catecholamine release from single adrenal chromaffin cells of control and spontaneously hypertensive rats (SHRs). Acetylcholine (ACh) or K^+ pulses triggered spike bursts of secretion by Ca^{2+} entry through Ca^{2+} channels. ER Ca^{2+} release triggered by CRT (a mixture of caffeine, ryanodine, and thapsigargin) or FCCP (a mitochondrial protonophore) also caused bursts of secretory spikes. The spike bursts generated by ACh, K^+, CRT and FCCP were 3-4 times longer in SHRs, as compared with control cells; furthermore, the individual spikes were faster and had 3-fold greater quantal size. In additional experiments, a 90-s treatment was made with CRT or FCCP to block Ca^{2+} handling by the ER and mitochondria. In these conditions, the integrated spike burst responses elicited by ACh and K^+ were potentiated 2-3-fold in control and SHR cells. This suggests that variations in Ca^{2+} entry and its subsequent redistribution into the ER and mitochondria are not responsible for the greater secretion seen in SHR, as compared with control cells; rather such differences seem to be due to greater quantal content of spike bursts and to greater quantal size of individual amperometric events.

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Introduction

The differential release into the circulation of the catecholamines norepinephrine and epinephrine from the adrenal medullary gland, either in basal or stressful conditions, is tightly regulated by various central (Folkow and Von Euler, 1954) and peripheral splanchnic nerve stimulation patterns (Mirkin, 1961; Klevans and Gebber, 1970). Alteration of the activity of the sympathoadrenal medullary axis and of the rate of catecholamine release has been implicated in the pathogenesis of essential hypertension, as proven by three facts: (1) the classical effects of drugs interfering with this axis, used to treat hypertensive patients such as blockers of α and β adrenergic receptors, reserpine, α-methyldopa, ganglionic blocking agents, guanethidine or angiotensin II receptor blockers (Westfall and Westfall, 2007); (2) 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); and (3) hypertensive patients have elevated sympathetic nerve activity, as revealed with microneurography (Anderson *et al.*, 1989).

Numerous studies support the notion that pre- and postsynaptic sympathetic dysfunctions are involved in the pathophysiology of human or animal primary hypertension (Tsuda and Masuyama, 1991; de Champlain et al., 1999). Studies on presynaptic mechanisms have been performed in SHRs, showing that norepinephrine release is increased in different tissues rich in sympathetic nerve endings (Donohue *et al.*, 1988). Although this increase of norepinephrine release constitutes an important catecholaminergic dysfunction associated to primary hypertension, the precise mechanism involved remains unknown.

A study on catecholamine secretion from intact perfused rat adrenal glands indicated that release stimulated by acetylcholine (ACh), or by potassium (K^+), was higher in adrenals from SHRs, as compared with control normotensive rats (Lim *et al.*, 2002b). We recently performed the first study on the kinetics of single-vesicle secretory events, using a carbon fiber electrode and amperometry in isolated single chromaffin cells from control and SHRs (Miranda-Ferreira *et al.*, 2008). This high-resolution technique provides insight on quantitative and qualitative kinetic aspects of the last fusion steps of exocytosis in isolated single chromaffin cells in the millisecond time range (Wightman et al., 1991; Borges et al., 2005). We found that SHR chromaffin

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cells, stimulated with short pulses of ACh or K^+, had a more sustained production of spike secretory events and a drastic augmentation of the quantal catecholamine content of individual secretory vesicles with faster fusion kinetics, compared with normotensive rats (Miranda-Ferreira *et al.*, 2008).

Differences in the nicotinic receptors and/or voltage-dependent Ca^{2+} channels in chromaffin cells (Garcia *et al.*, 2006) could explain the different secretion patterns seen in SHRs, as compared with control rats. Alterations of intracellular Ca^{2+} homeostatic mechanisms could also account for the differences. In fact, a large Ca^{2+} influx and a high cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) has been seen even during rest in conduit arteries i.e., aorta, carotid and femoral arteries from SHRs as compared with normotensive rats (Jelicks and Gupta, 1990; Asano et al., 1996); this has also been found in small mesenteric arteries from SHRs, where the sarcoplasmic reticulum has a larger capacity for Ca^{2+} storage (Nomura and Asano, 2002).

In trying to clarify the mechanisms involved in the kinetic differences of single-vesicle quantal release of catecholamine between SHR and normotensive rats found in our recent study (Miranda-Ferreira *et al.*, 2008), we felt of interest to study whether alterations of Ca^{2+} handling by the endoplasmic reticulum and mitochondria could explain those differences in the exocytotic responses. Here we present such study in which we have compared the kinetics of quantal catecholamine release elicited by the following stimuli applied to single chromaffin cells from normotensive and hypertensive rats: (1) pulses of ACh or K^+, to favour Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Douglas and Poisner, 1961); (2) acute application of a mixture of caffeine, ryanodine and thapsigargin (CRT), to quickly release the Ca^{2+} stored in the ER (Cuchillo-Ibáñez et al., 2002); (3) acute application of the protonophore FCCP, to cause release into the cytosol of Ca^{2+} accumulated into mitochondria during previous cell stimulations with ACh or K^+ pulses (Montero *et al.*, 2000); and (4) ACh or K^+ pulses given after cell treatments with CRT or FCCP. We demonstrate that (i) quantal secretory responses elicited by Ca^{2+} entry or intracellular Ca^{2+} release were much longer in SHRs, as compared with controls; (ii) the responses to ACh and K^+ were markedly enhanced in cells previously treated with CRT or FCCP; (iii) the individual single-vesicle secretory spikes were faster and had a greater quantal size in SHRs, as compared with control rats.

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Methods

Animals

Animals were manipulated according to the guidance of the Ethic´s Committee for Handling Research Animals, of our Medical School, Universidad Autónoma de Madrid. Male 16-week-old Sprague-Dawley (SD) weighing around 300 g were housed at 24 ± 2°C with 60 ± 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.

Isolation and culture of rat adrenal medulla chromaffin cells

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 and 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^{-1} 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.

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Amperometric monitoring of catecholamine release with a carbon fiber 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 fibers that rendered 200-300 pA of current increment after a 50 µM epinephrine pulse were used for the experiments.

Experimental protocols

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 right side of the cell being explored; solutions bathed the cells by gravity from left to right, upon opening of computer-driven valves. After the stimuli with ACh and/or K^+, cells were treated with a cocktail containing caffeine (20 mM), ryanodine (10 µM) and thapsigargin (1 µM), to study the role of endoplasmic reticulum Ca^{2+} on quantal catecholamine release. This cocktail will be referred to as CRT throughout. To study the role of mitochondria, cells were perifused with FCCP (1 µM). Both combined treatments were also used in order to study the participation of the two organelles on the secretory response. After 30 s with this treatment, the same cells were challenged with ACh or K^+ during 2 s and then these cells were bathed with Tyrode solution during 4 min to be challenged once more with ACh and K^+ pulses.

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Spike analysis and statistics

Total cumulative secretion elicited by ACh and K^+ pulses, or the transient initial secretion elicited by CRT, FCCP or CRT/FCCP treatments were calculated by successive additions of individual spike areas (Q), and expressed in pC. Baseline elevation during a given stimulus is due to spike overlapping caused by faster secretion (Miranda-Ferreira et al., 2008). Note that the number of spikes shown in the histograms in several figures may be underestimated because baseline elevation in some traces can not be resolved in separate spikes. Thus, in some cases a correlation between cumulative secretion and spike number elicited by a given treatment or stimulation, was not obvious (see further comments in Results).

Kinetic analysis of individual spikes 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.

We studied the integral charge, the individual charge, the number of spikes, and the kinetic parameters i.e. time to peak (t_{max}), half-time ($t_{1/2}$) and amplitude (I_{max}) before and after the ER and mitochondrial treatments.

Differences between means of group data fitting a normal distribution were assessed by using Student's t test and ANOVA (difference between treatments). A p value equal or smaller than 0.05 was taken as the limit of significance.

Materials and solutions

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

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Results

The quantal catecholamine release responses triggered by ACh or K^+ pulses in chromaffin cells from normotensive and hypertensive rats.

All experiments began with an initial 5-min perifusion resting period, to adapt the targeted cell to its environment. No spontaneous secretory amperometric spikes were usually seen during this period. Thus, the low-frequency spontaneous action potentials reported to occur in primary cultures of rat chromaffin cells (Kidokoro and Ritchie, 1980),were not apparently capable of eliciting exocytosis under our present experimental conditions.

To stimulate secretion, the basal Krebs-Hepes solution containing 2 mM Ca^{2+} was quickly switched to another containing 1 mM ACh that bathed the cell during 2 s (the duration of the ACh pulses). Fig. 1A shows the spike burst produced by ACh in an example control cell. Usually, a given cell was stimulated five times with ACh at regular 4-min intervals; during those intervals, the cell remained silent. The responses to the second pulse decayed by 30% with respect to pulse 1; the following responses underwent little decay (not shown). Thus, when used, pharmacological treatments (i.e. CRT of FCCP) were given between ACh pulses 2 and 3.

Fig. 1B shows a spike trace from an example SHR cell. Note the initial spike burst, with some baseline elevation due to fast simultaneous secretory events. Also note the long-lasting duration of spike activity for near 20 s, in spite of the fact that the ACh pulse duration lasted for only 2 s. This drastic difference of burst duration was better seen when the cumulative secretion versus time was plotted (Fig. 1C). The response of the control cell saturated after about 6 s while that of SHR cells began to saturate after 18 s.

The integrated secretion (area in pC of all spikes secreted by each ACh pulse) is plotted in Fig. 1D; this secretion was about 2.6-fold higher in SHR, as compared to control cells. Since the spike number in an ACh pulse response was also in this range (about 2.8-fold higher; Fig. 1E) it seemed logical to conclude that the mean quantal size of single spike events should be similar; however, it was near 3-fold higher in SHR, as compared with control cells (Fig. 1F). This may be explained by spike overlapping due

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to a faster secretion in SHR cells; in fact, complex spikes having 3-4 "fused" spikes are 3.5-fold more frequent in SHR, as compared with control cells (Miranda-Ferreira *et al.*, 2008).

A picture similar to ACh was found in cells stimulated with high K^+ pulses (70 K^+ , 2 s). In the example control cell shown in Fig. 2A, the K^+ pulse elicited a spike burst with a delay of only 0.2 s. The secretory response lasted for 4-5 s. Again, the SHR cell responded with a secretory activity (Fig. 2C) that did not reach saturation even after 20 s of the K^+ pulse (see the cumulative secretion plot in Fig. 2B). The total secretion (Fig. 2D) and spike number (Fig. 2E) were about 2.5-fold higher in SHR cells. Once more, the quantal size of single vesicle events was 3-fold higher (Fig. 2F), indicating a faster secretion and vesicle overlapping in SHR, with respect to control cells.

The quantal catecholamine release responses triggered by CRT or FCCP in chromaffin cells from normotensive and hypertensive rats.

We have previously used CRT, a mixture of caffeine (20 mM), ryanodine (10 μM), and thapsigargin (1 μM) to cause a rapid release of ER Ca^{2+} into the cytosol and a subsequent irreversible ER Ca^{2+} depletion in bovine chromaffin cells (Cuchillo-Ibáñez *et al.*, 2002). Here we have used CRT acutely, to see whether the sudden release of Ca^{2+} into the cytosol evoked by such drastic treatment, could elicit similar or different secretory responses in control and SHR cells. We first applied two ACh or K^+ pulses; these pulses elicited secretory responses similar to those of Figs. 1 and 2 and hence they are not shown here. In the example control cell of Fig. 3A, CRT caused spike activity after a delay of 2 s. Such activity ceased after about 7 s in spite of the continued presence of CRT. The secretion pattern in the SHR cell evoked by CRT (Fig. 3B) lasted for about 30 s (Fig. 3C). Total secretion in control cells was around 140 pC, and 2.2-fold higher in SHR cells (Fig. 3D). The spike number was about 2.4-fold higher in SHR (Fig. 3E) and the single-vesicle quantal size was 3-fold higher (Fig. 3F). Thus, the differences in secretory patterns between control and SHR cells were similar with CRT (ER Ca^{2+} release) and ACh or K^+ (Ca^{2+} entry from the extracellular milieu). This is clearly seen upon comparison of figures 1C, 2C, and 3C.

The mitochondrial protonophores CCCP and FCCP have previously been shown to augment the secretory responses evoked by ACh or K^+ pulses in populations of

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perifused bovine adrenal medulla chromaffin cells (Montero et al., 2000; Cuchillo-Ibáñez et al., 2002; Villalobos et al., 2002b; Cuchillo-Ibanez et al., 2003; Cuchillo-Ibanez et al., 2004) as well as in single voltage-clamped bovine chromaffin cells stimulated with depolarizing pulses or action potential waveforms (Giovannucci *et al.*, 1999). Thus, in the present experiments we used FCCP to study its effects on secretion.

Fig. 4A shows that in a control cell, FCCP (1 μM) produced a large burst of secretory spikes that lasted for about 8 s; the cell became silent thereafter, in spite of the fact that FCCP was present for 30 s. After a 1 s delay, FCCP also caused a fast secretory activity in the example SHR cell shown in Fig. 4B. The duration of such response lasted for about 30 s, as shown in Fig. 4C. The total quantal secretion elicited by FCCP in control cells was 200 pC, more than double of the secretion achieved by ACh and K^+, and 50% higher than the response to CRT. Total secretion in SHR cells reached 350 pC, about 20% above the responses elicited by the other three secretagogues. Spike number followed a similar pattern, 100 spikes in control cells and 255 in SHR cells (Fig. 4E). The quantal size of the individual spikes triggered by FCCP in control cells was 1.7 pC and in SHR cells reached as much as 3.5 pC. In conclusion, with some minor differences, the pattern of the secretion responses triggered by FCCP was similar to that of the other three secretagogues.

The quantal catecholamine release responses to ACh and K^+ pulses, in control and SHR cells that had been pretreated with CRT or FCCP

As described above CRT and FCCP caused by themselves a burst of quantal secretory spikes. Such secretory activity lasted 4-5 s in control cells and 20-30 s in SHR cells. This transient response could be due to cell damage. Thus, we decided to treat the cells for a 90-s period with CRT or FCCP for 90 s and at the end of this period, when basal secretory activity was absent, an ACh or K^+ pulse was applied. With this protocol we seeked two pieces of information: (1) to know whether after these strong drug pretreatments the secretory machine was still viable; and (2) to determine how the suppression of the ability of ER and mitochondria to take up Ca^{2+} from the cytosol and to release Ca^{2+} back into the cytosol, could affect the responses to ACh and K^+. It is firmly established that CRT suppresses the capacity of ER to handle Ca^{2+} and FCCP suppresses the ability of mitochondria to handle such cation (Alonso et al., 1999;

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Montero et al., 2000; Cuchillo-Ibáñez et al., 2002; Villalobos et al., 2002b; Cuchillo-Ibanez et al., 2004).

With this protocol we observed that CRT pretreatment did not deteriorate the secretory responses to ACh or K^+; on the contrary, such responses were more than doubled after CRT treatment of both control and SHR cells (Fig. 5). FCCP was even more efficacious than CRT in causing an augmentation of ACh- and K^+- elicited responses. For instance, in control cells FCCP augmented 4.5-fold the ACh response in control cells (Fig. 6A) and near 3-fold in SHR cells (Fig. 6B). The K^+ responses were augmented 3-fold in control cells (Fig. 6C) and 2.3-fold in SHR cells (Fig. 6D)

In a few control and SHR cells we performed experiments with pretreatment with the combined CRT and FCCP. We did not observe a further enhancement of secretion. In fact, the cells responded to ACh and K^+ with poorer responses, indicating that this drastic treatment could damage the cells.

Kinetics of individual quantal spike events released by ACh, K^+, CRT, or FCCP

As shown in Figs. 1-4, the quantal size of individual secretory events released from control cells is about 1 pC, and that for SHR cells is 3-fold higher (Figs. 1-4). We wanted to know more about the kinetic features of control and SHR single spikes and analyzed other parameters such as (1) $t_{1/2}$, an indication of spike width; (2) t_{max}, the time to peak, an indication of the spike activation rate; and (3) I_{max}, the spike amplitude.

Table 1 shows that the ACh-evoked secretory events in control cells had a $t_{1/2}$ of 8.9 ms, a t_{max} of 12.5 ms, and an I_{max} of 155 pA. In SHR cells, these parameters were as follows: $t_{1/2}$ 7.5 ms, 20% lower; t_{max} 8.5 ms, 30% lower; and I_{max} 283 pA, 45% higher. These changes indicate that the spikes of SHR cells were narrower, faster, and of higher amplitude, with respect to those of control cells. Their 3-fold greater quantal content of catecholamines, expressed by spike area, gave rise to larger and sharper spikes.

The K^+-elicited spikes were in the range of those evoked by ACh, the SHR cells having a $t_{1/2}$ and t_{max} 20-25% lower, and I_{max} 50% higher than control cells. Similar results were obtained for the kinetics of spikes generated by cell stimulation with CRT or FCCP. Thus, it seems that these kinetic differences are due to variations in the intrinsic properties of control and SHR cells (i.e. greater quantal size and more vesicles available for release) rather than to the type of secretagogue used to stimulate secretion

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by Ca^{2+} entry (ACh or K^{+}) or by Ca^{2+} release and blockade of Ca^{2+} fluxes at intracellular organelles (CRT or FCCP).

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Discussion

We have found in this study drastic differences in the secretion kinetics of adrenal medulla chromaffin cells from control normotensive rats and those isolated from hypertensive animals. Such differences of SHRs with respect to control cells can be summarized as follows: (1) a longer lasting burst of secretory spikes; (2) a greater cumulative burst secretion per stimulus; (3) a 3-fold higher quantal content of individual secretory spikes; (4) a faster secretory response of individual vesicles. Overall, this pattern suggests that in SHRs the adrenal gland could respond with a sharper and prolonged release of catecholamines into the circulation upon stressful conflicts.

In a recent study we compared the patterns of secretory responses elicited by ACh and K^+ and concluded that "*the much greater catecholamine release responses in SHRs are explained by faster exocytosis of more vesicles, with greater quantal catecholamine content, during a much longer secretory period*". The present study corroborates this conclusion and adds novel elements on the implication of an alteration of catecholamine secretory mechanisms to the understanding of the pathogenesis of hypertension. Thus, an alteration of the Ca^{2+} handling by the ER and mitochondria could also alter the secretory responses and explain their differences between control and SHR cells. In fact, alterations of intracellular Ca^{2+} homeostatic mechanisms occur in vascular smooth muscle cells in SHRs. For instance, a large Ca^{2+} influx and a high basal $[Ca^{2+}]_c$ have been seen during rest in conduit arteries (i.e. the aorta, carotid and femoral arteries) from SHRs as compared with normotensive rats (Jelicks and Gupta, 1990; Asano et al., 1996). This has also been found in small mesenteric arteries from SHRs, where the sarcoplasmic reticulum has a larger capacity for Ca^{2+} storage (Nomura and Asano, 2002).

Donohue *et al*. (1988) found an enhanced norepinephrine release from different tissues with rich sympathetic innervation in SHR rats, with respect to normotensive rats. More recently, enhanced catecholamine release has also been shown in perfused adrenal glands (Lim *et al.*, 2002a) or single adrenal chromaffin cells of SHRs, compared with control animals (Miranda-Ferreira *et al.*, 2008). Such enhanced release could be due to different Ca^{2+} entry pathways or to differences in the Ca^{2+} redistribution into, and Ca^{2+} release from the ER and mitochondria. In fact, a functional triad controlling Ca^{2+} and exocytotic signals is present in adrenal chromaffin cells, as revealed through the use of ER-targeted aequorin (Alonso *et al.*, 1999) or mitochondria-targeted aequorin (Montero

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et al., 2000; Villalobos *et al.*, 2002a). Such triad is formed by high-voltage activated Ca^{2+} channels, the ER and mitochondria located nearby the plasmalemma. The triad will control Ca^{2+} entry and its subsequent intracellular redistribution, shaping in this manner the local $[Ca^{2+}]_c$ transients, vesicle traffic and exocytotic responses generated by cell activation with depolarizing pulses of ACh or high K^+ (Garcia *et al.*, 2006). Mitochondria also play a relevant role in the clearance of the $[Ca^{2+}]_c$ elevations elicited by stimulation of rat chromaffin cells (Herrington et al., 1996; Park et al., 1996; Babcock et al., 1997). On the other hand, ER Ca^{2+} release by histamine or muscarine causes the release of catecholamines (Wakade, 1981) suggesting that as in bovine chromaffin cells, rat chromaffin cells could also have a functional triad to control $[Ca^{2+}]_c$ and exocytotic signals.

In our present investigation we have seen that the secretory responses to ACh and K^+ were similar, indicating that differences in Ca^{2+} entry during nicotinic receptor activation or direct cell depolarization did not account for the greater responses of SHR cells. Furthermore, CRT and FCCP caused secretory responses that were qualitatively and quantitatively similar, also suggesting that differences in intracellular ER Ca^{2+} release from the ER or mitochondria did not explain the much greater responses in SHRs, as compared with control cells. Additionally, control and SHR cells with their ER depleted by CRT pretreatment, or with blockade of mitochondrial Ca^{2+} uptake through their uniporter elicited by FCCP pretreatment, responded with similar enhanced responses to ACh and K^+. This indicates that differences in Ca^{2+} redistribution into the ER and mitochondria, and/or its release from these organelles do not explain the much greater secretory responses seen in SHRs with respect to control cells, in the different experimental conditions studied. However, ER Ca^{2+} release is known to be taken up by mitochondria in chromaffin cells (Montero et al., 2000); thus, the possibility exists that this "Ca^{2+} shuttle" is altered in SHR, as compared to control cells. So, pending of a more direct study on $[Ca^{2+}]_c$ transients and their clearance, as that performed in Bertill Hille laboratory in normotensive rats (Herrington et al., 1996; Park et al., 1996; Babcock et al., 1997), our quantal secretion experiments indicate that the functional triad that control Ca^{2+} homeostasis in chromaffin cells (Garcia *et al.*, 2006) is not altered in SHRs chromaffin cells.

Rather, differences in the size of the docked vesicle pool underneath the plasmalemma (Neher, 1998) as well as in the kinetics of single-vesicle exocytosis could

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explain the drastic differences in the secretory responses obtained in control and SHR cells. First of all, the quantal size of individual vesicles was as much as 3-fold higher in SHRs, as compared with control cells; this was true for all protocols used to stimulate secretion i.e. ACh, K^+, CRT, FCCP, or ACh and K^+ after cell pretreatment with CRT or FCCP. The obvious conclusion from these data is that at least 3-fold greater catecholamine secretion will be seen in SHR cells. Secondly, the quantal content of integrated burst spikes was also 2-4-fold greater in SHR cells. And thirdly, the individual amperometric events had a faster kinetics. Overall, these properties suggest that upon a given stimulus, the adrenal medulla in the intact animal will respond much more quickly and with a more prolonged response in SHRs than in normotensive rats. This seems to derive from differences in the structure and function of the secretory machinery, rather than to differences in Ca^{2+} handling by intracellular organelles. It will be interesting to perform an electron microscopic study to find out whether the number of chromaffin vesicles and their disposition at subplasmalemmal sites are different in control and SHR chromaffin cells. This type of morphological studies may provide information on the size of different vesicle pools nearby the secretory machinery (Vitale et al., 1995). Another interesting question is related to the possibility that the enhanced norepinephrine release from sympathetic nerve terminals in SHR, as compared to normotensive rats (Donohue et al., 1988) could also underly a mechanism similar to that described here for chromaffin cells.

An additional relevant issue in this study concerns the controversy on the role of the ER Ca^{2+} store in controlling the release of catecholamines. Concerning the effect of ER Ca^{2+} depletion on depolarization-evoked secretion, three studies are available in bovine chromaffin cells. Mollard et al. (1995) showed no effect, Pan and Fox (2000) showed potentiation followed by depression, and Cuchillo-Ibáñez et al. (2002) showed a pronounced depression of ACh responses but no effect on K^+-evoked secretory responses. Here, we have shown that under ER Ca^{2+} depletion, the ACh and K^+ responses were drastically augmented in both control and SHR cells. This may be explained by an augmentation of vesicle traffic due to Ca^{2+} release from the ER during the CRT treatment period preceding the ACh or K^+ pulses; this will lead to accumulation of more vesicles underneath the plasma membrane, as suggested by von Ruden and Neher (1993) to be the case upon stimulation of ER Ca^{2+} release with histamine.

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In conclusion, we have found that the quantal secretion of catecholamines from chromaffin cells is higher and of longer duration in SHRs, as compared with control rats. This was true for the release triggered by Ca^{2+} entry (ACh or K^+ stimulation), or by Ca^{2+} release from the ER (CRT) or the mitochondrion (FCCP). Furthemore, the individual amperometric events are faster and have a 3-fold higher quantal size in SHRs, as compared with control rats. We believe that these data are valuable for the understanding of the pathogenesis of hypertension. Additionally, they might provide clues to identify new targets for the development of novel antihypertensive compounds acting on the exocytotic machinery.

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Footnotes

Footnote to title: Financial support

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LEGENDS FOR FIGURES

Fig.1 ACh pulses produced longer lasting quantal catecholamine secretory responses in SHR chromaffin cells, compared with control cells. **A**, example record obtained from a control cell stimulated with an ACh pulse (1 mM, 2 s) as indicated by the dot at the bottom. **B**, record obtained from an example SHR cell, similarly stimulated with an ACh pulse. **C**, cumulative secretion, calculated at 2-s intervals in traces similar to those shown in A and B; the area of spikes is expressed and represented in pC (ordinate) as a function of time (abscissa) **D**, secretion per pulse (integrated area of all spikes generated by the ACh pulse) in pC, ordinate. **E**, total number of spikes secreted per each ACh pulse (ordinate). **F**, quantal content of individual secretory events, expressed in pC (ordinate). Data in D and E are means±S.E. of the number of cells shown in parentheses on top of each column, from at least 3 different cultures. Data in F are means±S.E. of the number of individual spikes shown in parentheses on top of each column; those spikes are from the experiments of panels D and E. ***$p<0.001$, with respect to control cells.

Fig.2 K^+ pulses produced a longer lasting burst of quantal secretory spikes in SHR chromaffin cells, compared with control cells. **A**, example record obtained from a control cell stimulated with a K^+ pulse (70 mM K^+, 2 s) as indicated by the dot at the bottom. **B**, record obtained from an example SHR cell, similarly stimulated with a K^+ pulse. **C**, cumulative secretion, calculated at 2-s intervals in traces similar to those shown in A and B; the integrated area of spikes found in 2-s periods was cumulatively added and represented in pC (ordinate) as a

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function of time (abscissa). **D**, total secretion per pulse (integrated area of all spikes generated by the K^+ pulse) in pC, ordinate. **E**, total number of spikes secreted per each K^+ pulse (ordinate). **F**, quantal content of individual secretory events, expressed in pC (ordinate). Data in D and E are means±S.E. of the number of cells shown in parentheses on top of each column, from at least 3 different cultures. Data in F are means±S.E. of the number of individual spikes shown in parentheses on top of each column; those spikes are from the experiments of panels D and E. ***p<0.001, with respect to control cells.

Fig. 3 CRT produced a longer lasting burst of quantal secretory spikes in SHR chromaffin cells, compared with control cells. **A**, example record obtained from a control cell stimulated with CRT (a mixture of 20 mM caffeine, 10 μM ryanodine, and 1 μM thapsigargin) as indicated by the horizontal bar at the bottom. **B**, record obtained from an example SHR cell, similarly stimulated with CRT. **C**, cumulative secretion, calculated at 2-s intervals in traces similar to those shown in A and B; the integrated area of spikes found in 2-s periods was cumulatively added and represented in pC (ordinate) as a function of time (abscissa) **D**, total secretion (integrated area of all spikes generated during CRT treatment) in pC, ordinate. **E**, total number of spikes secreted during CRT treatment (ordinate). **F**, quantal content of individual secretory events, expressed in pC (ordinate). Data in D and E are means±S.E. of the number of cells shown in parentheses on top of each column, from at least 3 different cultures. Data in F are means±S.E. of the number of individual spikes shown in parentheses on top of each column; those spikes are from the experiments of panels D and E. ***p<0.001, with respect to control cells.

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Fig. 4 FCCP produced a longer lasting burst of quantal secretory spikes in SHR chromaffin cells, compared with control cells. **A**, example record obtained from a control cell stimulated with FCCP (1 μM) as indicated by the horizontal bar at the bottom. **B**, record obtained from an example SHR cell, similarly stimulated with FCCP. **C**, cumulative secretion, calculated at 2-s intervals in traces similar to those shown in A and B; the integrated area of spikes found in 2-s periods was cumulatively added and represented in pC (ordinate) as a function of time (abscissa). **D**, total secretion (integrated area of all spikes generated during FCCP treatment) in pC, ordinate. **E**, total number of spikes secreted during FCCP treatment (ordinate). **F**, quantal content of individual secretory events, expressed in pC (ordinate). Data in D and E are means±S.E. of the number of cells shown in parentheses on top of each column, from at least 3 different cultures. Data in F are means±S.E. of the number of individual spikes shown in parentheses on top of each column; those spikes are from the experiments of panels D and E. ***$p<0.001$, with respect to control cells.

Fig. 5 Quantal catecholamine release responses evoked by ACh or K^+ pulses in control and SHR cells pretreated with CRT. Cells were initially stimulated with an ACh (1 mM, 2 s) or K^+ pulse (70 mM, 2 s). Cells were then perifused with basal Krebs-Hepes solution for 3.5 min and subsequently with CRT, a mixture of 20 mM caffeine, 10 μM ryanodine and 1 μM thapsigargin to cause ER Ca^{2+} depletion and to irreversibly block ER Ca^{2+} fluxes, for 1.5 min. Once silent, the cell was challenged with a second ACh or K^+ pulse. Total secretion per pulse (integrated area of all spikes generated by each one of the two ACh or K^+ pulses) is shown in the ordinates. A, B, control and SHR cells stimulated with ACh, before and after CRT treatment; C, D, control and SHR cells stimulated

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with K^+. Data are means ± S.E. of the number of cells shown in patentheses on top each column, from at least 3 different cultures. *** $p< 0.001$ with respect to the response obtained before CRT treatment.

Fig. 6 Quantal catecholamine release responses evoked by ACh or K^+ pulses in control and SHR cells pretreated with FCCP. Cells were initially stimulated with an ACh (1 mM, 2 s) or K^+ pulse (70 mM, 2 s). Cells were then perifused with basal Krebs-Hepes solution for 3.5 min and subsequently with FCCP (1 μM) to block mitochondrial Ca^{2+} fluxes, for 1.5 min. Once silent, the cell was challenged with a second ACh or K^+ pulse. Total secretion per pulse (integrated area of all spikes generated by each one of the two ACh or K^+ pulses) is shown in the ordinates. A, B, control and SHR cells stimulated with ACh, before and after FCCP treatment; C, D, control and SHR cells stimulated with K^+. Data are means ± S.E. of the number of cells shown in patentheses on top each column, from at least 3 different cultures. ** $p< 0.01$, *** $p< 0.001$ with respect to the response obtained before FCCP treatment.

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Table 1. Kinetic parameters of individual secretory events released from control and SHR cells, from the experiments shown in Figs. 1 to 4.

stimulus	Type of chromaffin cell	$t_{1/2\ (ms)}$	$t_{max\ (ms)}$	$I_{max\ (pA)}$	n
ACh	Control	8.9 ± 0.3	12.5 ± 0.5	155± 7.6	307
	SHR	7.5 ± 0.4*	8.5 ± 0.8***	283 ± 9.1***	517
K^+	Control	7.2 ± 0.3	11.3 ± 0.7	130 ± 6.3	330
	SHR	6.3 ± 0.2*	9.2 ± 0.5*	260 ± 7.3***	689
CRT	Control	7.8 ± 0.4	8.5 ± 0.3	105 ± 6	450
	SHR	6.0 ± 0.3***	6.8 ± 0.4***	161 ± 9***	749
FCCP	Control	7.1 ± 0.4	8.2 ± 0.2	137 ± 8	660
	SHR	5.4 ± 0.3***	6.5 ± 0.4***	171 ± 10***	823

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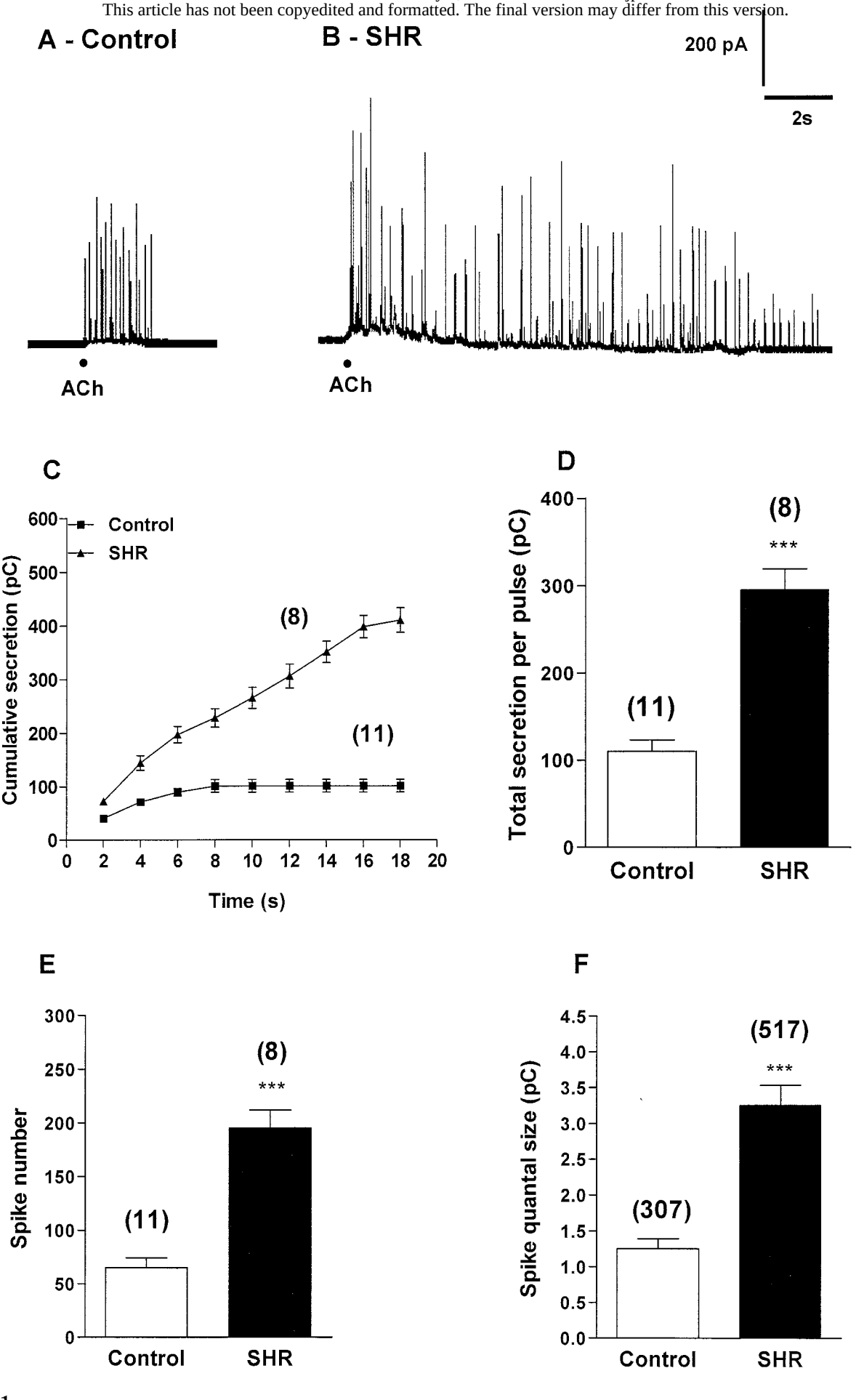

Fig. 1

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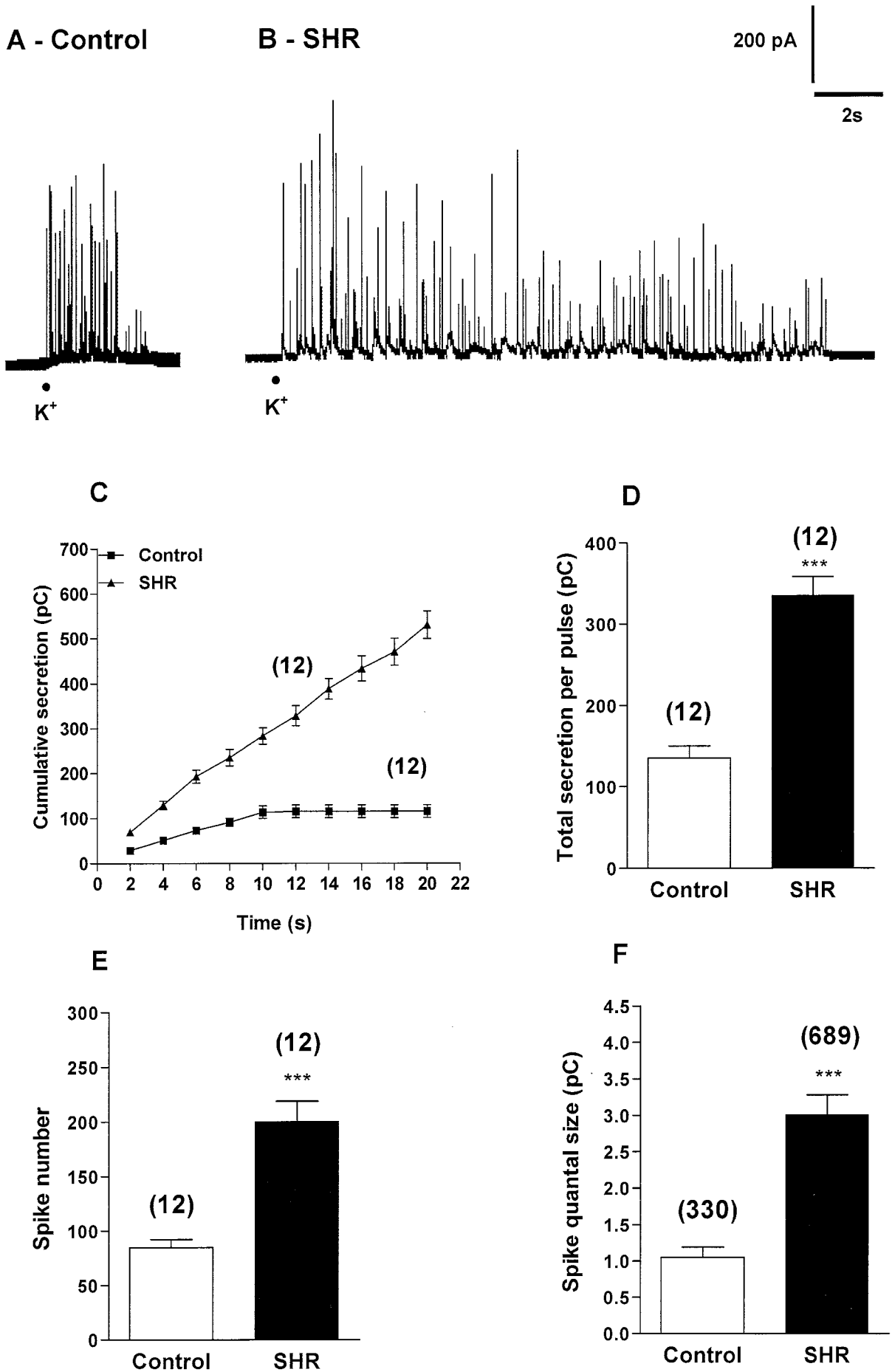

Fig. 2

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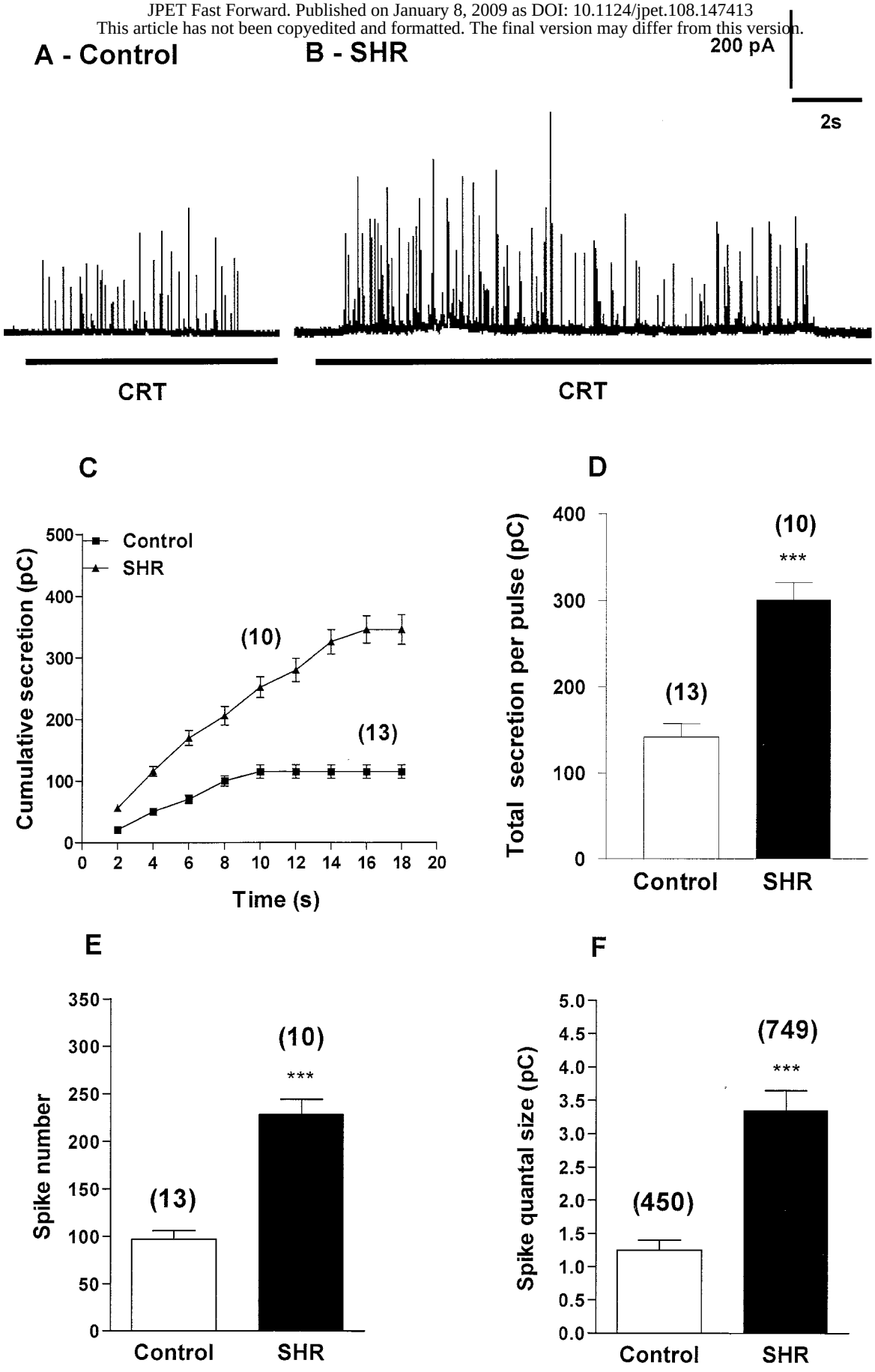

Fig. 3

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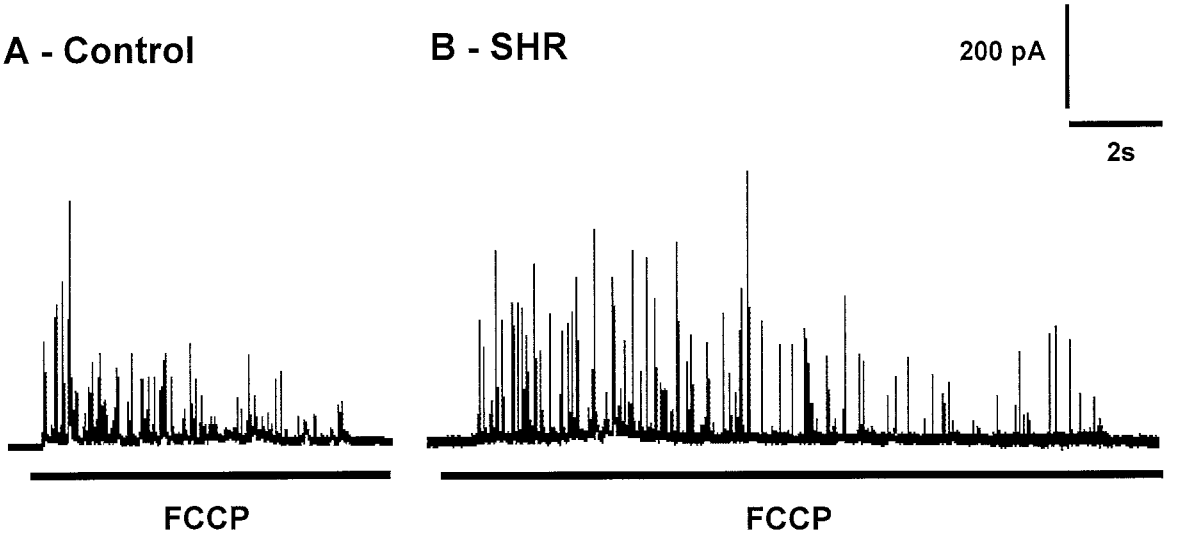

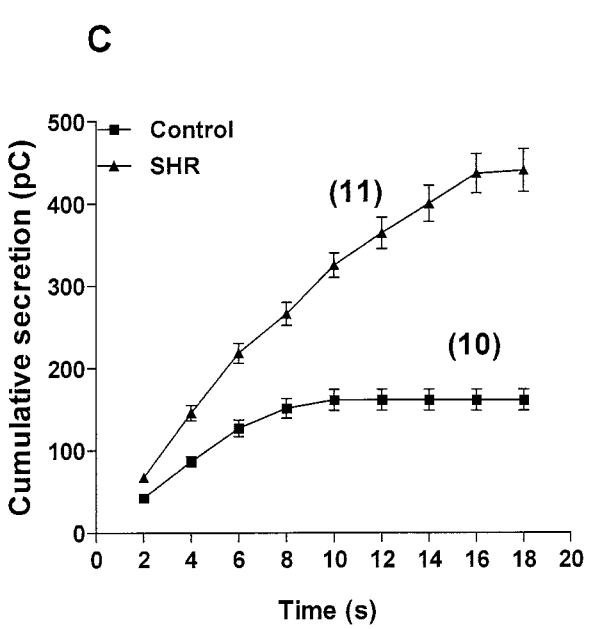

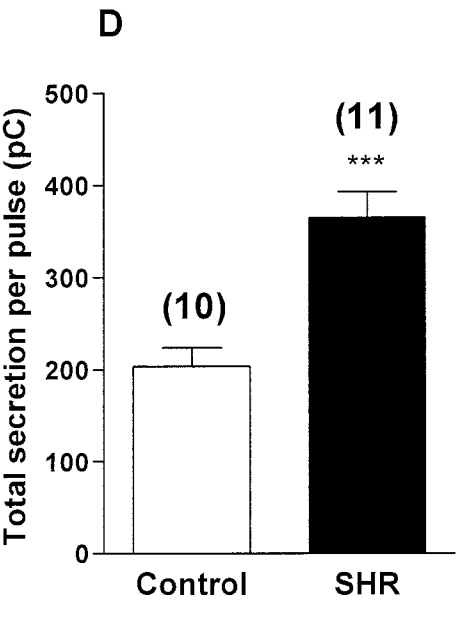

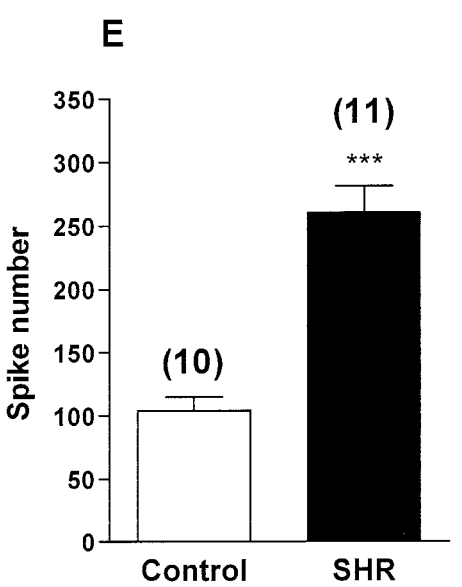

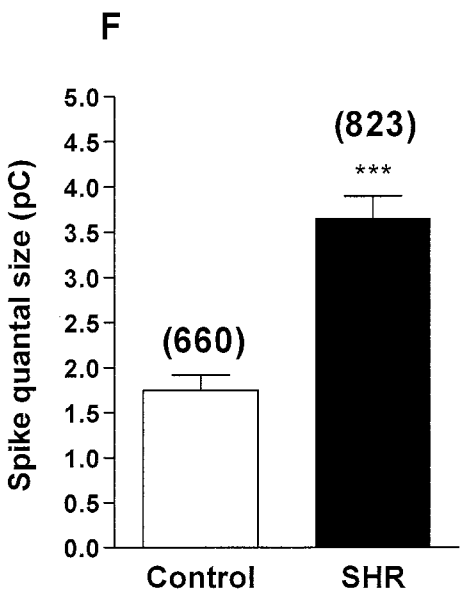

Fig. 4

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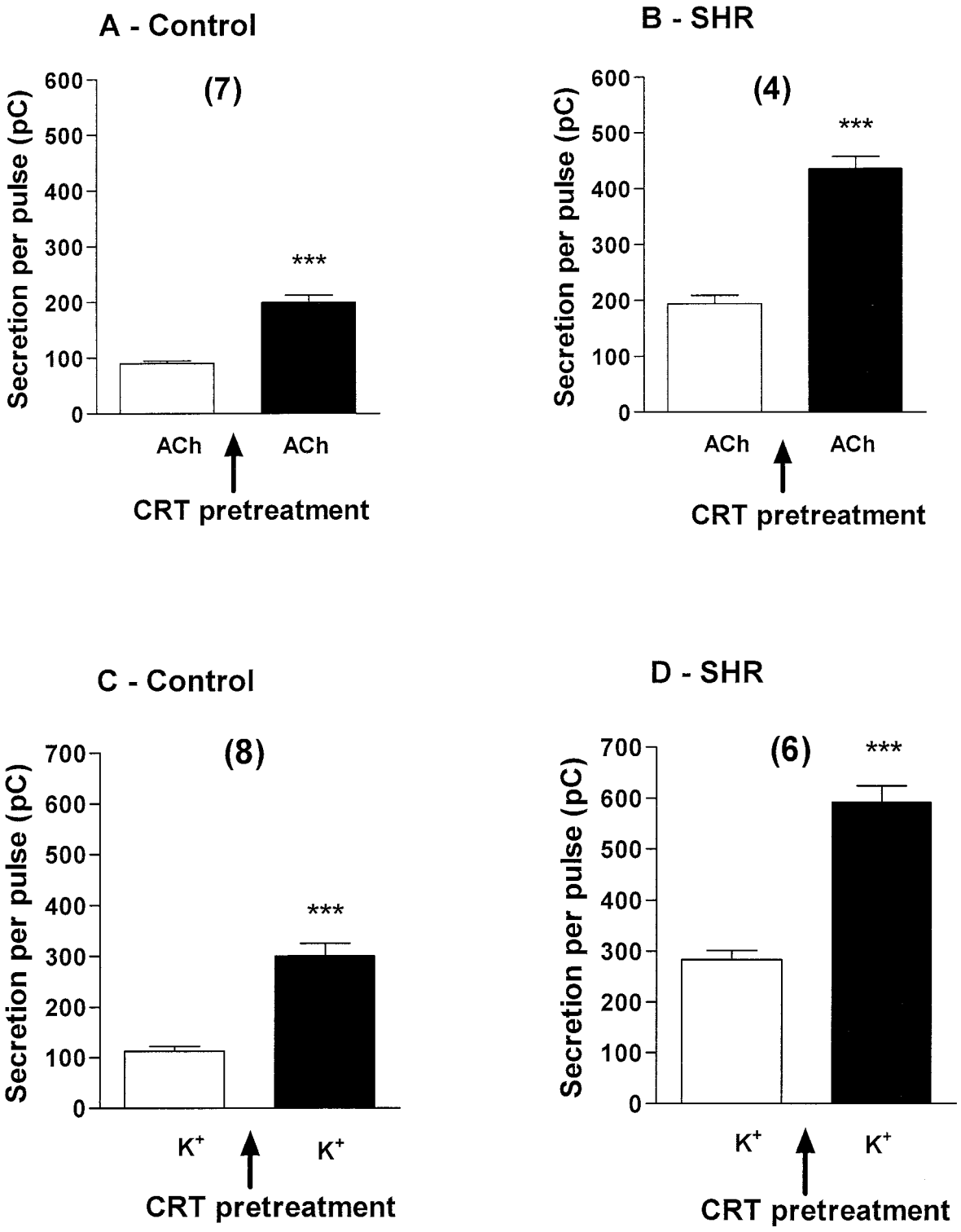

Fig. 5

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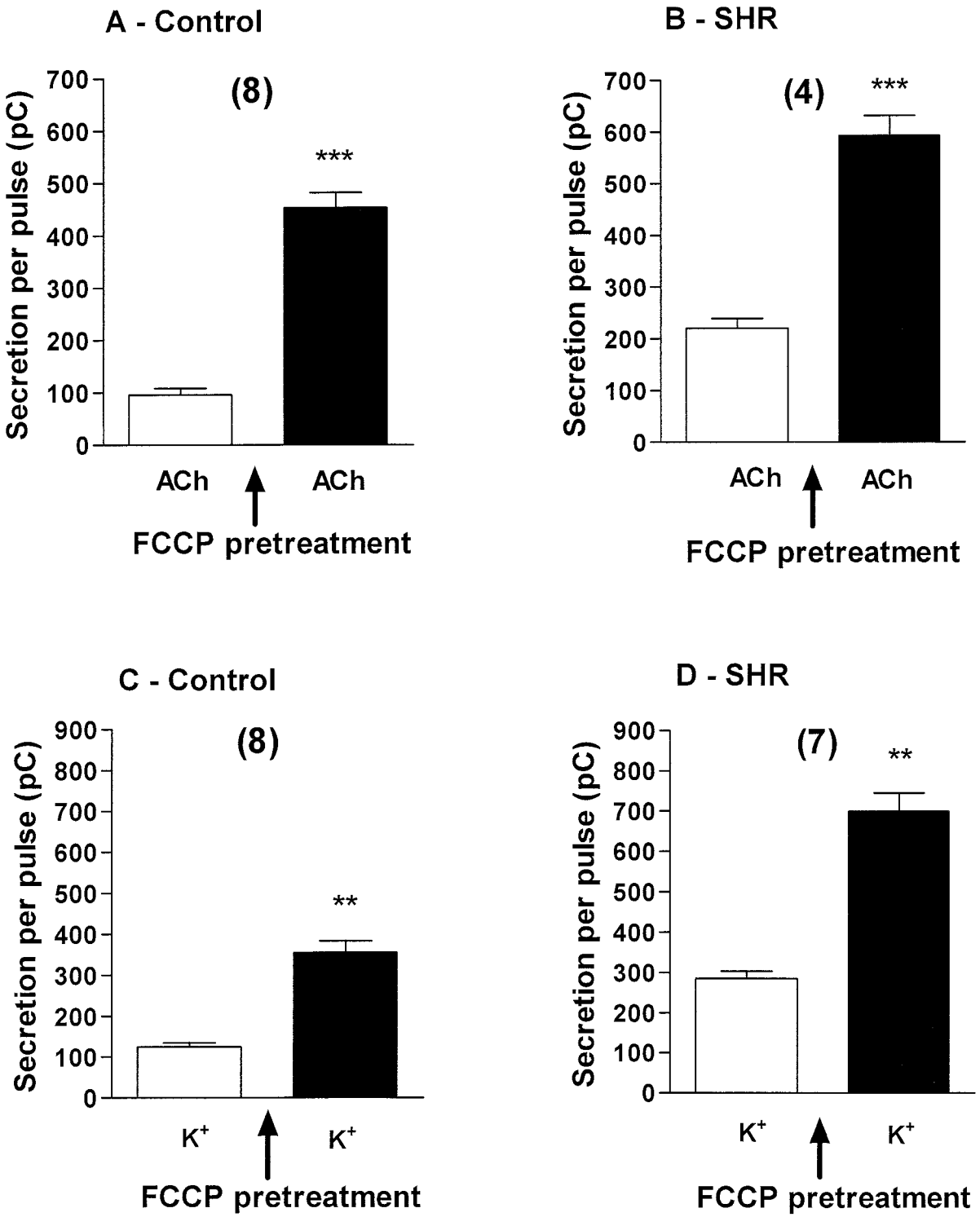

Fig. 6

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