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Reduced calcium current density in female versus male mouse adrenal chromaffin cells *in situ*

Shyue-An Chan, Jacqueline Hill, and Corey Smith

Department of Physiology and Biophysics Case Western Reserve University Cleveland, Ohio, USA

SUMMARY

Neuroendocrine adrenal medullary chromaffin cells are a main output of the sympathetic nervous system. Acute stress activates the sympatho-adrenal stress reflex, excites adrenal chromaffin cells, and elicits catecholamine secretion into the circulation. Previous studies have demonstrated that stress-evoked serum catecholamine levels are greater in males. We investigated potential mechanistic bases for this gender dimorphism at the level of the adrenal medulla. We utilized *in situ* single-cell perforated patch voltage clamp to measure basic electrophysiological parameters that affect cell excitability. We found that chromaffin cells from male and female mice exhibit statistically identical depolarization-evoked calcium currents. However, the resting capacitance, an index of cell surface area, was significantly higher in cells from female mice. Thus the current density in female cells was significantly lower. We found that inhibition of Protein kinase C, an enzyme shown to regulate both exocytosis and endocytosis, eliminates the cell surface area gender dimorphism. Finally, we performed kinetic simulations of the secretion process and report a predicted elevated secretory capacity in male cells. Thus, regulation of cell size may act to decrease cell excitability in female cells and may in-part represent the mechanistic basis for increased stress-evoked catecholamine secretion described in males.

Keywords

Gender; Exocytosis; Adrenal; Stress; Capacitance; Cell Volume

INTRODUCTION

The sympathetic nervous system sets the overall metabolic status of an organism and coordinates the appropriate physiological response to stress. Under basal conditions, the sympathetic nervous system fires at a modest rate, setting the sympathetic tone. Under these conditions, sympathetic nerve activity excites neuroendocrine adrenal medullary chromaffin

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Please address correspondence to: Corey Smith, Ph.D., Department of Physiology and Biophysics, Case Western Reserve University, 2109 Adelbert Road, Cleveland, Ohio, 44106 USA, Tel: +1-216-368-3497 Fax: +1-216-368-5586, Corey.Smith@Case.Edu.

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cells to release modest amounts of catecholamine into the circulation. Basal catecholamine help regulate physiological parameters including shunting of blood from the periphery to viscera, increasing enteric activity and maintaining basal heart rate. Emotional or psychological stress, injury or environmental insult initiates the sympatho-adrenal ‘fight or flight’ stress response and drives adrenal chromaffin cells to greatly increase catecholamine release [1, 2]. Specific physiological consequences of stress-evoked adrenal catecholamine release include increased glucagon secretion and decreased insulin release from the pancreas, as well as increased hepatic blood flow, which all combine to elevate blood glucose levels. Additionally, catecholamines elevate blood pressure, cardiac output and pulmonary function to increase oxygen delivery to the musculature. Misregulation of serum catecholamine levels results in pathologies including hypertension, cardiac myopathy and diabetes mellitus [3–6].

Studies have shown a gender dimorphism in the physiological response to stress in human and in animal models. In humans, males express a greater increase in serum catecholamine in response to stressors [7–9]. The mechanistic basis for this gender bias is difficult to ascertain in human subjects due to multiple uncontrolled variables including body mass index, physical fitness and other risk factors [10]. However, direct evidence has recently been obtained where gender-specific genetic factors including transcription of neuropeptides has been reported in neuroendocrine cells [11]. Studies in animal model systems have begun to provide potential mechanistic insight, indicating that the gender bias may at least in part be due to a gender-specific regulation of excitatory and inhibitory neuronal sympatho-adrenal input [12], activity-evoked electrical coupling between the secretory adrenal chromaffin cells themselves [13, 14] and differential levels of transmitter molecules [15]. Given the relationship between stress-evoked adrenal catecholamine release, and gender-specific predisposition for hypertension [10], we set out to test if adrenal chromaffin cells themselves express gender-specific intrinsic differences in excitability. We employ single cell electrophysiological recordings to compare the stimulus-evoked influx of Ca^{2+} in male and female mice, and we report a significantly decreased current density in female chromaffin cells. Inhibition of protein kinase C, an enzyme shown to regulate multiple aspects of membrane trafficking [16–18] and thus steady state membrane surface area, reverses the current density gender dimorphism. Lastly, we employ an established quantitative kinetic model of chromaffin cell exocytosis [19, 20] to simulate the impact of the differential current density on catecholamine release from male versus female cells. The data from the simulation recapitulate key aspects of the observed stress/gender dimorphism reported in humans and in rodent model systems and identify regulation of cell surface area as a key target for further study of gender-specific catecholamine release in response to stress.

MATERIALS AND METHODS

Adrenal slice preparation

Adult C57BL/6 mice (4 to 8 week old) from Jackson Laboratories (Bar Harbor, ME) were used in this study. Anesthesia and euthanasia protocols were approved by Case Western Reserve University's institutional animal care and use committee (IACUC), a federal

oversight body (Federal welfare assurance number A3145-01). Animals were deeply anesthetized by isoflurane (USP, Halocarbon Products Corp., River Ridge, NJ) inhalation and euthanized by decapitation. Adrenal glands were immediately excised and immersed in ice-cold low-calcium bicarbonate-buffered saline (BBS) containing (in mM): 140 NaCl, 2 KCl, 0.1 CaCl₂, 5 MgCl₂, 26 NaHCO₃, 10 glucose and bubbled with 95% O₂/5% CO₂. All chemicals were from Fisher Scientific, (Fair Lawn, NJ) except MgCl₂ (Sigma-Aldrich, St. Louis, MO). Osmolarity of the BBS solution was 320 mOsmol l⁻¹. Glands were trimmed of excess fat and connective tissue and embedded in 3% low melting temperature agarose (Lonza, Rockland, ME). Agarose was prepared beforehand by melting in low calcium BBS at 110° C followed by equilibration in a 35° C water bath. Immediately after embedding, glands and agarose were placed on ice to set. Gelled agarose was trimmed into 3–5 mm blocks, each containing a single adrenal gland. Agarose blocks were glued to a vibrotome sectioning stage (WPI, Sarasota, FL). The stage was placed in a slicing chamber filled with ice-cold low calcium BBS that was continuously bubbled with 95% O₂/5% CO₂. Adrenal glands were sectioned at 200 µm. Sections were collected, and placed in a holding chamber containing low calcium BBS bubbled with 95% O₂/5% CO₂ at 25°C. Experiments were carried out within 7 hrs of slice preparation.

Electrophysiology

Tissue slices were constantly superfused during recordings with HEPES-buffered Ringer solution containing (in mM): 150 NaCl, 10 HEPES-H, 10 Glucose, 2.8 CaCl₂, 2.8 KCl, and 2 MgCl₂. The Ringer solution was adjusted to pH 7.2 with NaOH and osmolarity was adjusted with mannitol to 320 mOsmol l⁻¹. Tissue slices were held in place in the recording chamber by placing silver wires over the agarose perimeter of the adrenal slice. Patch pipettes were pulled from borosilicate glass (~ 1 µm tip diameter; 4–5 MΩ resistance). Pipette tips were dipped in molten dental wax and fire polished with a microforge (Narishige, Tokyo, Japan). Data presented in this study were recorded from cells perforated to a series resistance of no more than 25 MΩ. Records with unstable series resistance were excluded from analysis.

For voltage clamp experiments, pipettes were filled with an internal patch solution containing (in mM): 145 Cs-Glutamate, 10 HEPES-H, 8 NaCl, and 0.5 TEA-Cl. Internal solution had a pH of 7.2 and osmolarity of 310 mOsmol l⁻¹. Amphotericin B (Fisher Scientific) was prepared in DMSO (Acros Organics, NJ) as a 100x stock solution daily and diluted into internal solution to a final concentration of 0.53 mM. The liquid junction potential between HEPES Ringer solution and the internal solution was measured to be 17.8 mV through an agar bridge. Junction potentials and series resistance compensation were adjusted in all records accordingly. For whole-cell current clamp experiments, pipettes were filled with an internal solution containing (in mM) 145 K-Glutamate, 10 HEPES-H, 8 NaCl, 1 MgCl₂, 2 MgATP, 0.002 NaGTP and 0.001 EGTA; pH was adjusted to 7.2 and osmolarity was adjusted to 310 mOsmol l⁻¹.

Electrophysiological records were filtered at 10 kHz and digitized at 20 kHz using an EPC-9 amplifier under the control of “Pulse” software (v. 8.8; HEKA Elektronik, Bellmore, NY). All electrophysiological data were analyzed using Igor Pro software (WaveMetrics Inc).

Lake Oswego, OR). The action potential voltage template was obtained from a cell held in whole-cell current clamp and stimulated by current injection to fire. Two hundred records were averaged with respect to current injection to obtain the voltage template. This template was then fed into the voltage-clamp of the EPC-9 and used to stimulate cells. The protocol used to construct and collect current-voltage (IV) plots was as follows: cells were held at -80mV and delivered 30 depolarizing steps from -100mV to $+45\text{mV}$ in 5mV increments, with each potential pulse lasting 50ms and an inter-sweep interval of 15s .

Computer simulation of evoked secretion

We utilized a computer simulation of secretory granule fusion based on the “Two Step Model of Secretion Control” [19] with adaptation for more detailed Ca^{2+} driving functions and time-resolved dynamic secretory behavior as previously described [20]. A brief summary of the model and alterations from published parameters is supplied here. The Heinemann “Two-step” scheme for secretion describes the transition of vesicles between 3 separate states. Pool A represents a reserve pool of vesicles that mature, in a Ca^{2+} -dependent manner, into the release-ready vesicles of pool B (RRP). Vesicles in B pool either regress and rejoin pool A, or undergo evoked Ca^{2+} -dependent fusion with the cell membrane. The fused state is defined by pool C.

For the simulations, we used measured, independent input parameters wherever possible (cell size, equivalent circuit parameters, evoked calcium influx etc.). Other parameters were either taken from the literature (basal Ca^{2+} concentration, cytosolic Ca^{2+} dynamics [18]) or fitted by within the constraining measured parameters. Pool A was defined as 5pF (approximately 2900 granules, [19–21]). The forward rate constant k_1 between pools A and B is dependent upon $[\text{Ca}^{2+}]_i$, and is described by the Michaelis-Menten relationship in eq. (2);

$$k_1 = \frac{a_1 [\text{Ca}^{2+}]_i}{b_1 + [\text{Ca}^{2+}]_i} \quad (2)$$

where a_1 is the upper limit value of k_1 and b_1 represents a dissociation constant of the Ca^{2+} -mediated step. In the simulations the stimulus-evoked $[\text{Ca}^{2+}]_i$ transient was based on measured current values, measured cell surface area, and defined accessible cytosolic volume and endogenous Ca^{2+} buffering [22]. From these measured parameters, we calculated expected cytosolic Ca^{2+} in a dynamic manner. Following influx, the driving Ca^{2+} wave followed a mono-exponential decay back toward baseline with a time constant of 5.5s between pulses [18]. At the end of the train the $[\text{Ca}^{2+}]_i$ decay was described by the measured time constants $\text{Ca}\tau_4$ and $\text{Ca}\tau_5$ (see table 1 and legend to figure 3 and ref [20] for description). The backward rate constant k_{-1} represents the spontaneous loss of vesicles from pool B to pool A and is derived from fitting the steady state size of pool B and the refilling

kinetics of pool B following depletion. The rate of vesicle exocytosis (population in pool C) is represented by the rate constant k_2 and is defined by eq. (3);

$$k_2 = a_3 \left[Ca^{2+} \right]_i^3 \quad (3)$$

where a_3 is calculated from the data of Heinemann *et al.* [23], according to the secretion study of Klingauf and Neher [24] to be equal to $0.035 \mu M^{-3} s^{-1}$ (see [18] for a more detailed description of a_3 calculation).

The $[Ca^{2+}]_i$ -dependent steady state size of pool B is described by eq. (4);

$$B_{\infty} = \frac{Ak_1}{(k_{-1} + k_2)} \quad (4)$$

where A is the size of pool A in Farads.

In order to estimate the post-stimulus recovery of pool B, the differential equations derived from the model were solved using the Runge-Kutta integration method. The parameter set used in the simulation is summarized in Table 1. For further description of the limitations and assumptions of the model, see [18–20, 23].

Statistical analysis

The specific analysis used for quantification of the data in each figure is included in the text and/or figure legends. Student's t-test was utilized to assess significance in data sets where control and treatment conditions were from the same cell. Statistical barriers for either condition are indicated in legends.

RESULTS

The stress-evoked surge in serum catecholamines, epinephrine and norepinephrine, in response to stress has been shown to be greater in males [7, 10, 25]. The adrenal medulla is a major source for circulating norepinephrine and the sole source for peripheral epinephrine [5, 26]. Thus, a secretory dimorphism between male and female adrenal medullae is predicted. We designed a study to determine if the gender-specific secretory dimorphism is present at the level of the single neurosecretory adrenal chromaffin cell. We investigated the possibility that male and female mouse chromaffin cells express different evoked calcium current magnitudes in response to electrical excitation, and thus a differential response to sympathetic activation. We utilized an *in situ* mouse adrenal slice preparation in combination with perforated patch voltage clamp recordings to measure the physiological response to stimulation. We conducted perforated patch recordings from single chromaffin cells in tissue slices cut from age-matched male and female mice as described in Methods.

Female mice exhibit a decreased evoked Ca^{2+} current density that is sensitive to PKC inhibition

We initiated our study by investigating Ca^{2+} influx as a parameter that could be responsible for gender-specific catecholamine release. In order to investigate influx in response to a physiologically meaningful stimulus, we measured Ca^{2+} influx in voltage-clamped cells stimulated with an action potential voltage command template (Fig. 1A). For the voltage clamp recordings, we utilized a Cs^+ -containing internal solution to block K^+ conductance in order to reveal Na^+ and Ca^{2+} currents. Example recordings from cells obtained from male and female adrenal slices are provided in Fig. 1Bi, showing an initial inward current previously shown to be carried by a rapidly inactivating Na^+ current [27], followed by an inward, temporally isolated Ca^{2+} current (grey box). No significant differences between male and female current records were noted. However, we did note that female cells expressed a consistently higher basal capacitance compared to cells from male slices (10.57 ± 0.15 pF versus 7.99 ± 0.29 pF, respectively; $p < 0.0001$). Indeed, when the same example currents presented in Fig. 1Bi are plotted as current density, the female record exhibits a much lower current density (Fig. 1Bii). We considered potential reasons for why female cells might be larger than male cells, including potential differences in cell actin cortex or differences in membrane trafficking. Previous work in adrenal chromaffin cells has shown both exocytic membrane fusion with the cell surface as well as endocytic membrane fission from the surface to be modulated by the activity of protein kinase C (PKC; [16, 18, 28]). We pretreated tissue slices with the potent general PKC inhibitor, Gö6983 and repeated the AP-stimulus protocol (Gö6983 pretreatment was with 100 nM bath incubation for 5 minutes prior to, and maintained throughout the recording). Pooled data for control and Gö6983-treated cells are provided in Fig. 1Ci and show that PKC-inhibition did not significantly alter measured peak Ca^{2+} influx. However, Gö6983 treatment did reconcile the basal capacitance values (Fig. 1Cii). While the capacitance measured from male cells was statistically unchanged, Gö6983 treatment did decrease the capacitance measured in female cells to match that in male cells (8.57 ± 0.17 pF and 8.65 ± 0.14 pF, respectively). Thus, female cells exhibit a larger basal membrane capacitance that is dependent on a tonic gender-specific PKC activity. Moreover, when the raw recorded currents presented in Fig. 1Ci are converted into current densities, and their magnitudes compared, pretreatment with Gö6983 eliminates the Ca^{2+} current density gender dimorphism observed in untreated cells.

We next turned to a more detailed protocol to resolve the complete current-voltage relationship for stimulus-evoked Ca^{2+} influx. We designed a current-voltage (I-V) stimulus template that held cells at a membrane potential of -80 mV and stepped them in 5 mV increments between -120 mV and $+50$ mV. Each test step was 50 ms in duration to allow for complete inactivation of inward Na^+ currents and reliable measure of Ca^{2+} currents at the end of the test step (example recording provided in Fig. 2A). Example I-V plots for both raw current values and current densities are provided in figure 2B and recapitulate the results in obtained from simple AP-template stimulation (figure 1); maximal inward calcium current magnitudes are equivalent between genders (Fig. 2Bi). However, due to an elevated basal capacitance in cells from female glands, the current densities in female cells are smaller (Fig. 2Bii). For comparison to the AP data in figure 1, we again repeated this protocol in cells from untreated male and female tissues slices (Fig. 2Ci) and from

equivalent slices bathed in 100 nM G6983 (Fig. 2Cii). Again, pretreatment with the PKC inhibitor decreased basal capacitance in the cells recorded from female slices, resulting in an equilibration of the current densities.

Simulations recapitulate increased stimulus-secretion coupling in male adrenal chromaffin cells

We considered the possibility that the relatively greater Ca^{2+} current density measured in chromaffin cells of male adrenal slices may be at least partially responsible for the elevated serum and urinary catecholamine levels observed in stressed males [7, 8]. We initially considered conventional membrane capacitance or electrochemical amperometric assays to measure secretory function. However, we decided that the temporal overlap of exocytosis and endocytosis during repetitive stimulation, as expected under physiological stress activation, would make interpretation of time-resolved capacitance measures for specific exocytic activity difficult. Moreover, measuring spike activity through the tissue bed and subsequent equivocal assignment of measured current to a single cell made amperometry-based secretion analysis an equally difficult approach to adopt in the tissue slice context. Instead, we turned to simulation of the secretory process to predict the specific functional impact of the current density gender dimorphism established in figures 1 and 2. This approach allows us to isolate and focus on the specific impact cell volume regulation is expected to have on Ca^{2+} dynamics, stimulus-secretion coupling and granule fusion by implementing a limited and defined parameter set.

The adrenal chromaffin cell has served as a well-described model system for the stimulus-secretion process. A series of highly-controlled and quantitative studies of Ca^{2+} -dependent secretory granule fusion is present in the literature [19, 23, 24, 29–31] and has provided an accurate kinetic model for chromaffin cell exocytosis. More recent adaptations incorporated Ca^{2+} influx through channels in response to repetitive stimulation [20], to reflect persistent sympathetic drive, as subsequent secretory function [18, 20] (see Methods and Table 1 for details of the simulation). Thus, while not an experimental measure of secretory activity, the vetted kinetic simulations do provide a reduced test bed to isolate and investigate individual parameters contributing to the secretory process from other dimorphic parameters. Primary components of the simulations and their output are provided in figure 3. Independent variables measured from experimental conditions were basal cell capacitance and maximal evoked Ca^{2+} influx in response to a 50 ms depolarization to -20 mV as in figure 2. We simulated a train of 20 depolarizations delivered to the cell at 5 s intervals and utilized a Ca^{2+} influx that included experimentally-determined run-down of Ca^{2+} influx during subsequent stimuli. We approximated expected intracellular Ca^{2+} from the cell volume predicted by the measured cell capacitance, and the experimentally determined accessible volume and endogenous Ca^{2+} buffer capacity of chromaffin cells [22]. We were limited to the assumption of a spherical cell, which is not necessarily the case in the tissue slice preparation. Thus, this simplified morphology must be considered a limitation to the overall quantitative accuracy of the simulations, but is not likely to limit the ability to compare predicted stimulus-secretion coupling and evoked secretory behavior between conditions. As demonstrated in figure 3, left column, decreased current density (and increased cell diameter and cytosolic volume, Fig. 3A) in female mouse chromaffin cells results in a decreased

stimulus-evoked cytosolic Ca^{2+} level (Fig. 3B, top plot) and thus a decreased stimulus-secretion function (middle plot) and a reduced overall fusion of secretory granules (bottom plot). Treatment with the PKC inhibitor Gö6983, eliminates the gender-specific volume dimorphism (Fig. 3A, right column), predicting an equivalent cytosolic Ca^{2+} profile in response to stimulation (Fig. 3B, right column, top plot), stimulus-secretion function (middle plot) and overall secretory behavior (bottom plot) for both genders.

DISCUSSION

The adrenal medulla is a primary output of the sympathetic nervous system, receiving synaptic input through the splanchnic nerve and releasing epinephrine and norepinephrine into the circulation. Normal sympatho-adrenal signaling is a vital component of the native physiological stress response to environmental danger, physical exertion or injury [4, 32] and regulates the ability of an organism to mount an appropriate response to specific stressors. For example, disruption of splanchnic-adrenal synaptic communication results in the fatal inability of mice to respond to insulin shock [33]. Inappropriate adrenal catecholamine secretion, in response to intermittent hypoxic events as experienced under sleep apnea, leads to hypertension [3, 34, 35]. Misregulation of blood glucose levels due to improper adrenal epinephrine secretion can lead to diabetes mellitus and other symptoms of the metabolic syndrome [6]. Lastly, sexual dimorphism of both the stress response and resultant serum catecholamines underlies the gender-bias for multiple pathologies including heart disease and hypertension [7, 8, 10, 11]. Yet, despite this clear physiological importance, remarkably little is known regarding the gender-specific basis for sympatho-adrenal catecholamine release.

In this study, we report a gender dimorphism in the adrenal medulla. Previous studies have identified gender dimorphisms in other secretion-associated parameters including differential expression of neuropeptide Y (a potent neuro-regulatory peptide) [15], tyrosine hydroxylase [36] and transcription factors regulating chromogranin expression [11]. The later is of particular interest in that there is a link between chromogranin expression and granule size [37], indicating the potential that there is also a dimorphism in granule morphology. We provide primary data showing that neurosecretory adrenal chromaffin cells, a major source for circulating norepinephrine and the sole source for serum epinephrine [5, 26, 32], show a gender-specific difference in resting surface area. This difference decreases the stimulus-evoked elevation in cytosolic Ca^{2+} in female mice relative to male mice and thus, decreases the predicted stimulus-secretion function. Moreover, we show that this difference in cell secretory capacity is due to a tonic activity of the key regulatory enzyme, protein kinase C (PKC). PKC has been the focus of study in adrenal medulla secretory behavior for quite some time, and has been shown to be responsible for multiple aspects of adrenal catecholamine secretion. A key study by Gillis *et al.* [28] showed that PKC activity increases the secretory output from adrenal chromaffin cells by increasing the number of secretory granules that comprise the readily-releasable pool (RRP, pool B in the Two-step model). Still other work has shown that PKC activation can occur in a Ca^{2+} -mediated manner in response to repetitive cell stimulation [20]. Subsequent reports have shown that the PKC-sensitive step in RRP augmentation is likely the phosphorylation of the SNARE protein SNAP-25 [38], and that PKC-mediated augmentation is a key component of

increased adrenal output under native hypoxic stress in whole animals [3, 39]. Lastly, in a parallel signaling path, adrenal chromaffin cells have been shown to be highly sensitive to splanchnic release of the potent peptide secretagogue, PACAP [33, 40, 41]. Recent studies have demonstrated that PACAP-mediated adrenal excitation is sensitive to inhibition of PKC and that the PACAP/PKC signaling cascade culminates in an increased Ca^{2+} influx through the recruitment of a low voltage-activated T-type calcium conductance [42, 43]. Thus, PKC is emerging as a general excitation modulator of the adrenal medulla, acting at multiple sites including granule recruitment to the RRP, signaling excitation from splanchnic PACAP release and recruitment of low voltage-activated T-type calcium channels. This current study adds tuning of a sexual dimorphism in cell surface area, and thus in excitation, to the growing list.

The finding in this study clearly outlines several immediately testable hypotheses. First and foremost is to test the predictions forwarded by the simulations in figure 3: do female cells actually secrete less for a given Ca^{2+} influx? If so, is the decreased stimulus-secretion function due to tonic PKC activation. Chromaffin cells have served as a highly productive model system for secretion for several decades [44–48]. It would seem that if there were a gender-bias in the stimulus secretion function, it would have emerged by this point. However, the vast majority of studies have been conducted in isolated cells kept in culture for days up to a week or more. It is possible that the act of cell isolation and the period of cell quiescence in culture mitigates this mechanism. Many kinetic studies utilized UV flash-photolysis to elevate Ca^{2+} to levels saturating for the secretion process [23, 49, 50], which would bypass the influence of cell volume. Moreover, many of the studies conducted in isolated cells have historically been in bovine chromaffin cell cultures, where gender is likely not known. Only recently have mouse systems, providing access to gender identification, become more popular. Another factor is that the raw currents are identical between genders; only when the cell morphology is considered, is the impact of higher basal cell surface area brought to the forefront. These points will need to be experimentally tested. Lastly, a clear prediction is formulated from the primary data in figures 1 and 2 and predicted in figure 3 that describes an initial gender-bias in the sympatho-adrenal signaling reflex that should dissipate with persistent activity as activity-dependent PKC activation occurs in male cells to match the tonic activity in females. In this manner, it may be that the secretory deficit predicted for female cells in the current study may be transient and that male cells will match female cell excitability with activity and PKC activation. Further studies will need to be conducted to address this possibility.

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Figure 1. Females and males exhibit identical evoked calcium influx

(A) The voltage template of action potential stimulation is shown. Single cells in adrenal slices were held in the perforated patch voltage clamp configuration and stimulated with a voltage template generated by stimulating a chromaffin cell in current clamp (see Methods). (Bi) Evoked membrane currents were measured from male and female mouse chromaffin cells. The currents exhibit an initial inward Na^+ spike followed by a slower activating inward current carried by Ca^{2+} (grey box). (Bii) When the evoked currents are converted to current density as a function of cell capacitance (pA/pF), the evoked Ca^{2+} influx is lower in females than in males. (Ci) The peak inward Ca^{2+} current was measured and pooled for comparison between genders in control cells and cells pre-treated with Gö6983 (100 nm, 5 minute bath application prior to, and throughout the recording). Statistical significance of currents from female mouse cells was assessed versus male mouse cells by a two-tailed

Student's t-test. No statistical differences were found between genders in either condition. Sample sizes for the control group were 10 and 7 for males and females, respectively and 8 and 5 for the Gö6983 group, respectively. **(Cii)** Pooled, quantified data show that the peak calcium current density is significantly smaller in females compared to males and that this difference is eliminated in cells treated with Gö6983. Statistical significance is indicated by an asterisk and was determined by a Student's T-test at a barrier of $p < 0.02$.

Figure 2. Current density is significantly lower in female cells

(A) A full current/membrane potential (I-V) analysis for Ca^{2+} influx was conducted. An example of evoked membrane currents is provided. (Bi) Representative current-voltage plots measured from chromaffin cells in male and female adrenal slices are provided. Currents were determined at the end of the 50 ms test pulse at each test potential and are plotted against membrane potential. (Bii) The same data presented in Bi are normalized for current density and demonstrate a gender dimorphism as in figure 1. (Ci) I-V data were collected and pooled for male, female, control and Gö6983-treated mice. Inward Ca^{2+} current was measured at the peak activation potential (-20 mV) for each condition and is provided in a category plot. Statistical significance of the female current density versus male was assessed by a Student's T-test with a barrier for significance of $p < 0.02$. Sample size was 10 and 7 for males and females, respectively and 8 and 5 for the Gö 6983 group, respectively. No statistical difference was found for any condition compared to control. (Cii) The maximal inward Ca^{2+} currents presented in panel Ci are re-plotted as a current density (pA/pF) and show the same behavior as in figure 1; females express a much lower density that is reversed by treatment with Gö6983. Statistical significance of data from female cells versus male cells and is indicated by an asterisk and was determined by a Student's T-test at a significance barrier of $p < 0.02$.

Figure 3. Kinetic simulation of evoked exocytosis predicts increased secretory response from male chromaffin cells

We utilized measured cell parameters in a kinetic simulation of cytosolic Ca^{2+} and evoked granule fusion to isolate the predicted impact of the altered current density. Further simulation parameters are provided in Table 1. (A) Cell capacitance and measured Ca^{2+} influx serve as input parameters for each of the four conditions, gender and Gö6983 treatment, are shown. The circles provide a to-scale representation of the differences in cell diameter. (B) The control condition is represented in the left column and the Gö6983-treated condition in the right column. The top plot in each represents the calculated global cytosolic free Ca^{2+} concentration for each condition. Initial Ca^{2+} influx was measured from cells for each condition and functions describing subsequent influx and rundown of Ca^{2+} currents during the train as well as post-train Ca^{2+} clearance were fitted from measurements as described in the text. The resultant Ca^{2+} wave served as the driving function in the kinetic

simulation. The middle plot provides the simulated stimulus-secretion function for each condition. The stimulus is represented by the calculated influx of Ca^{2+} in pC and the output is presented as granule fusion in fF. The bottom plot of each column represents the cumulative membrane addition to the cell surface in response to exocytic granule fusion.

Table 1

Summary of simulation parameters.

<i>Secretory capacity simulation parameter set.</i>	Time constant	Magnitude
Initial $[Ca^{2+}]_i$:	-----	153 nM
Cell capacitance at beginning of train:		Measured
Initial Ca^{2+} influx in repose to depolarization:		Measured
K_s , endogenous cytosolic Ca^{2+} buffer capacity		57
$Ca\tau_1$: Primary $[Ca^{2+}]_i$ decay following pulses within train:	5.5 s	-----
* $Ca\tau_2$: Fast Ca^{2+} current rundown within train:	8.8 s	137 pA
* $Ca\tau_3$: Slow Ca^{2+} current rundown within train:	116.9 s	253 pA
* $Ca\tau_4$: Fast $[Ca^{2+}]_i$ decay following train:	22 s	250 nM
* $Ca\tau_5$: Slow $[Ca^{2+}]_i$ decay following train:	77 s	250 nM
a_3 value:	-----	$0.035 \mu M^{-3} s^{-1}$
Pool A size:	-----	5 pF
a_1 value (control):	-----	$0.007 s^{-1}$
a_1 value (activated):	-----	$0.014 s^{-1}$
k_{-1} value:	-----	$0.031 s^{-1}$
b_1 value:	-----	1.9 μM

All values either obtained from the literature or measured experimentally in this study. * See legend to figure 3 for description.