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## **Somatostatin analogue pasireotide (SOM230) inhibits catecholamine secretion in human pheochromocytoma cells**

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**ABSTRACT**

Increasingly common, neuroendocrine tumors (NETs) are regarded nowadays as neoplasms potentially causing debilitating symptoms and life-threatening medical conditions. Pheochromocytoma is a NET that develops from chromaffin cells of the adrenal medulla, and is responsible for an excessive secretion of catecholamines. Consequently, patients have an increased risk for clinical symptoms such as hypertension, elevated stroke risk and various cardiovascular complications. Somatostatin analogues are among the main anti-secretory medical drugs used in current clinical practice in patient with NETs. However, their impact on pheochromocytoma-associated catecholamine hypersecretion remains incompletely explored. This study investigated the potential efficacy of octreotide and pasireotide (SOM230) on human tumor cells directly cultured from freshly resected pheochromocytomas using an implemented catecholamine secretion measurement by carbon fiber amperometry. SOM230 treatment efficiently inhibited nicotine-induced catecholamine secretion both in bovine chromaffin cells and in human tumor cells whereas octreotide had no effect. Moreover, SOM230 specifically decreased the number of exocytic events by impairing the stimulation-evoked calcium influx as well as the nicotinic receptor-activated inward current in human pheochromocytoma cells. Altogether, our findings indicate that SOM230 acts as an inhibitor of catecholamine secretion through a mechanism involving the nicotinic receptor and might be considered as a potential anti-secretory treatment for patients with pheochromocytoma.

**Key words:** somatostatin analogues, pasireotide (SOM230), octreotide, neuroendocrine tumor, pheochromocytoma, exocytosis, secretion

## 1. INTRODUCTION

Neuroendocrine tumors (NETs) are a heterogeneous group of neoplasms arising from hormone, amine and peptide secreting cells that are spread all over the body, and its incidence has constantly increased during the last decades. NETs are often associated with a deregulation of hormone secretion which can induce clinical complications [1-6].

Pheochromocytoma (Pheo) is a NET arising from chromaffin cells of the adrenal medulla, which store and then secrete catecholamines in the blood stream. While Pheos are rare, most of them are responsible for catecholamine hypersecretion that may lead to severe and potentially life-threatening clinical complications. Clinical symptoms in patients with Pheos are mainly related to catecholamine hypersecretion and include the classic triad of headaches, palpitations, and profuse sweating but also permanent or paroxysmal hypertension [7-9]. It has been also reported that Pheo can lead to severe acute cardiovascular complications including myocarditis, Takotsubo syndrome, as well as various forms of cardiomyopathies [10, 11]. Accordingly, a retrospective study has shown that patients with Pheo have a 14-fold higher rate of cardiovascular events than patients with essential hypertension [12]. Finally, it is interesting to note that approximately 1 Pheo is found every 2000 autopsies (0.05%), a proportion significantly higher than the estimated prevalence of the general population suggesting a premature mortality caused by Pheo [13, 14].

Although currently controversial, the symptoms of patients with Pheo can be treated by preoperative antihypertensive medications such as  $\alpha$ - and  $\beta$ -blockers or calcium channel blockers to counteract the negative effect of excessive catecholamine secretion during surgical resection [15]. In France, the most common antihypertensive treatments currently use are calcium channel blockers such as nifedipine [16, 17]. However, to this date, no drug has been shown to directly and specifically prevent hypersecretion of tumor chromaffin cells. Interestingly, somatostatin analogues are currently among the most widely used drugs to treat the symptoms of different NETs, including excessive hormone secretion [18]. Octreotide and lanreotide were the two first-generation analogues tested and are still currently used in the treatment of gastroenteropancreatic NETs and acromegaly caused by a pituitary adenoma [19]. These analogues were reported to have a dual effect by decreasing both tumor cell proliferation

and tumor hypersecretion [18, 20, 21]. In the early 2000s, a new generation of somatostatin analogues has emerged with the development of pasireotide (SOM230). While octreotide binds preferentially the somatostatin receptor SSR2, SOM230 is a multireceptor-targeted analogue with a 40-, 30- and 5-fold higher affinity than octreotide for somatostatin receptors SSR5, SSR1 and SSR3, respectively [22, 23]. For instance, SOM230 has been authorized for Cushing disease and acromegaly treatments [24, 25]. *In vitro* and *in vivo* data have also shown that this analog is able to effectively inhibit gastroenteropancreatic NETs secretion and improve symptoms in patients refractory to octreotide treatment [20, 26].

As somatostatin receptors are also expressed by Pheo [27, 28], the aim of the present work was to investigate whether somatostatin analogues impact catecholamine secretion of adrenal chromaffin cells *in vitro* and thus determine its potential to prevent catecholamine hypersecretion. Using carbon fiber amperometry, we have tested the effect of octreotide and SOM230 treatments on primary cultures of either bovine chromaffin cells or tumor cells directly cultured from freshly resected human Pheos and found that SOM230 has an effective anti-secretory effect.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Octreotide and pasireotide (SOM230) were obtained from Novartis, Basel, Switzerland. Analogues were dissolved in dimethyl sulfoxide (DMSO), and stock solutions at  $10^{-2}$  or  $10^{-3}$  M were stored at  $-20^{\circ}\text{C}$  and protected from light exposure.

### 2.2 Patient population

The medical files of patients with Pheo in 2 French centers between 2017 and 2021 were retrospectively reviewed. We collected the following data: initial diagnosis, including a clinical examination looking for hormonal-related symptoms and biological analysis. As recommended by the Endocrine Society clinical practice guideline published in 2014 [29], Pheo genetic testing was proposed to identify germline mutations in the major susceptibility genes (SDHB, SDHC, SDHD, VHL, NF1, RET, TMEM127, MAX) using Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA). Then, as recommended in the consensus statement published in 2017 [30], next-generation-sequencing (NGS)-based diagnostic was carried out for more recent patients. Biological analysis comprised the measurement of metanephrine (MN) and normetanephrine (NMN) levels (in urine and/or plasma). When available, chromogranin A (CGA) measurements were also registered. Levels of free MN, NMN and CGA in plasma, as well as urinary levels of MN and NMN are presented as ratios normalized by the normal upper limits. Urinary or plasma MN and NMN levels reaching two-fold the upper limit of the normal range and/or CGA exceeding the upper limit of the normal range was defined as the threshold of abnormal hormonal secretion [7]. Catecholamine-producing phenotype of Pheo were categorized as previously described [31]: adrenergic (AD) phenotype, when MN content exceeded 10% of the combined MN and NMN contents, or noradrenergic (NAD) phenotype when MN content remained below 10% of the combined MN and NMN contents. Pathological evaluation was reviewed, including tumor size, Ki-67 result and the PASS (Pheochromocytoma of the Adrenal Gland Scaled Score) as previously described [32].

### 2.3 Primary culture of chromaffin cells

Bovine chromaffin cells were cultured as previously described [33]. Human tumor cells were cultured from freshly resected Pheos following surgery [34]. In the operating room and

immediately after the resection, the adrenal gland was cut longitudinally in two parts. Roughly a 1 cm<sup>3</sup> piece of tumor tissue was dissected and immediately plunged into ice cold transport medium (Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free Hank's Balanced Salt Solution (CMF HBSS, Sigma) supplemented with 0.2% Fetal Bovine Serum (FBS, Gibco) and 1% penicillin/streptomycin (Sigma) or MACS Medium Tissue Storage solution (Miltenyi Biotec). Up to 3 hours after resection, the tumor sample was minced into 1 mm<sup>3</sup> pieces in a dish containing CMF HBSS. Chunks were collected, centrifuged at 250 g for 5 min at room temperature and the pellet resuspended in 15 mL of complete medium (RPMI 1640 GlutaMAX<sup>TM</sup> (Gibco), 15% FBS, 1% penicillin/streptomycin). Red blood cells, debris and fat were separated from minced tissue by sedimentation for 15 min at room temperature. The supernatant was removed and 15 mL of complete medium were added to the pellet before centrifugation at 250 g for 5 min. Tumor pieces were resuspended in HBSS (in 10 times the tissue volume), containing 1.5 mg/mL of collagenase B (Roche) and 1 mg/mL of the protease dispase II (Gibco) and gently rocked for 45 min at 37°C. 5 min before the end of protease digestion, 0.1 mg/mL DNase I (Roche) was added to remove potential DNA clumps. Samples were left for a few minutes to sediment at room temperature and supernatant recovered (fraction 1). The pellet was resuspended in 5 mL of CMF HBSS and triturated for a couple of minutes to dislodge tumor cells from chunks. The remaining pieces were left few minutes to sediment and the supernatant recovered (fraction 2). Both fractions were centrifuged at 800 g for 5 min at room temperature. Cell pellets were resuspended in 2 mL of CMF HBSS. 4 mL of Red Blood Cell Lysis Buffer (Roche) were added before being gently rocked for 10 min at room temperature. The fractions were centrifuged at 500 g for 5 min and resuspended into complete medium. Cell viability and density were estimated under a microscope and 300 µL of cell suspension were seeded into type I collagen (Corning)-coated 35 mm dishes (MatTek) for amperometry or polylysine (Sigma)-coated glass coverslips for calcium imaging and electrophysiology experiments. Cells were left to adhere overnight at 37°C in an incubator with water-saturated and 5% CO<sub>2</sub> atmosphere. 2 mL of complete RPMI were added the following day and cells were used within two days.

#### ***2.4 Catecholamine secretion assay***

Assays were performed as previously described [33]. Briefly, bovine chromaffin cells were seeded in 96 well plates (Thermo Fisher Scientific) at a density of 250,000 cells per well and were maintained in culture for 48 to 72 hours before experiments. Cells were washed 3 times for 7 min with 200 µL of Locke's solution (140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 11 mM glucose, 0.01 mM EDTA, 0.56 mM ascorbic acid and

15 mM HEPES, pH 7.5) at 37°C and stimulated for 10 min with 50 µL of an high K<sup>+</sup> solution (Locke's solution containing 59 mM KCl, pH 7.2) or 10 µM nicotine (Sigma) with or without SOM230 or octreotide. Basal secretion was obtained by maintaining cells in Locke's solution for 10 min. The secretion was stopped by placing cells at 4°C and supernatants were immediately collected. Cells were lysed with 200 µL of Locke's solution containing 1% Triton X-100 (Sigma) for 3 min at 37°C. The plate was then centrifuged at 3,000 g for 10 min at room temperature. 20 µL of each sample were transferred to a black 96-well plate (Thermo Fisher Scientific). 150 µL of CH<sub>3</sub>COOH (1M, pH 6) and 15 µL of K<sub>3</sub>Fe(CN)<sub>6</sub> (0.25%) were added to oxidize the noradrenaline and adrenaline to noradrenochrome and adrenochrome, respectively. Addition of 50 µL of NaOH (5M) containing ascorbic acid (0.3 mg/mL) transformed aminochromes to fluorescent noradrenolutine and adrenolutine. Fluorescence was measured ( $\lambda_{\text{excitation}}$ : 430 nm,  $\lambda_{\text{emission}}$ : 520 nm) with a spectrofluorometer (Mithras LB 940, Berthold). Amounts of secreted catecholamines are expressed as percent of total catecholamines. Data are representative of at least 3 independent cultures and each measurement was realized in triplicate. The sigmoid curve in Figure 1B have been fitted using the 4-parameter logistic regression curve method (Sigma Plot).

## 2.5 Mass Spectrometry

*Preparation and derivation:* bovine chromaffin cells were stimulated as described above in 2.4. The secretion medium was then recovered and 50 µL of H<sub>2</sub>O containing 0.1 mM ascorbic acid was added to the well containing chromaffin cells. Cells were detached mechanically and the cell lysates were sonicated (4 x 10 sec, 100W; Model 505 Sonic Dismembrator; Fisher Scientific). After centrifugation (20,000 g, 30 min, 4°C), supernatants were collected.

20 µL of the cell extract or 20 µL of the secretion medium were mixed with 30 µL of borate buffer, 10 µL AccQtag Ultra reagent (AccQ-Tag Ultra derivatization kit, Waters, Guyancourt) and 10 µL of internal standards containing 20 pmoles of D4-Dopamine, D6-Adrenaline and C6-Noradrenaline in 0.1 mM ascorbic acid. The mixture was incubated 10 min at 55°C under agitation. Then, 280 µL of acetonitrile was added in order to precipitate proteins, and samples were centrifuged at 20,000 g during 15 min at 4°C. The resulting supernatants were dried under vacuum and suspended in 20 µL of H<sub>2</sub>O containing 0.1% formic acid (v/v). Finally, 5 µL of the resulting extracts were analyzed by LC-MS/MS (see below). Samples were loaded into reverse phase Zorbax column (1SB-C18, #863600-902; 1 mm x 150 mm, 3.5 µm, Agilent Technologies).

*LC-MS/MS conditions:* a LC-MS/MS approach was used to quantify the presence of dopamine, adrenaline, and noradrenaline using the multiple reaction monitoring mode (MRM). Analyses were performed on a Dionex Ultimate 3000 HPLC system (Thermo Scientific) coupled with an Endura triple quadrupole mass spectrometer (Thermo Electron). The system was controlled by Xcalibur v. 2.0 software (Thermo Electron).

Elution of the compounds was performed at a flow rate of 90  $\mu\text{L}/\text{min}$ , at 40°C, according to the gradient detailed in Supplementary Table 1. Buffer A corresponded to H<sub>2</sub>O 98.9%/ACN 1%/formic acid 0.1% (v/v/v), whereas buffer B was ACN 99.9%/ formic acid 0.1% (v/v). The MRM mode was used to identify and quantify target molecules according to the instrument settings and target compounds molecular signatures detailed in Supplementary Table 2. The selection of the monitored transitions and the optimization of the collision energy (CE) were manually determined. The identification of the compounds was based on precursor ion, selective fragment ions and retention times obtained for dopamine, adrenaline, and noradrenaline and their corresponding internal standards. Amounts of neurotransmitters were quantified according to the isotopic dilution method [35].

## **2.6 Carbon fiber amperometry**

Chromaffin cells were washed with Locke's solution (without ascorbic acid) and processed for catecholamine release measurements by amperometry as previously described [36, 37]. A carbon fiber electrode of 5  $\mu\text{m}$  diameter (ALA Scientific Instruments) was held at a potential of +650 mV compared with the reference electrode (Ag/AgCl) and approached close to one cell. Secretion of catecholamines was induced by a 10 s pressure ejection of a 100  $\mu\text{M}$  nicotine (Sigma) solution from a micropipette positioned 10  $\mu\text{m}$  from the cell and recorded over 60 s. The somatostatin analogue (octreotide or SOM230) was added either in the stimulation pipette or directly in the incubation medium. The amperometric recordings were performed with an AMU130 amplifier (Radiometer Analytical), calibrated at 5 kHz, and digitally low-pass filtered at 1 kHz. Analysis of the amperometric recordings was performed as previously described with a macro (laboratory of Dr. R. Borges; <http://webpages.ull.es/users/rborges/>) written for Igor software (WaveMetrics), allowing automatic spike detection and extraction of spike parameters [38]. The spike parameters analysis was restricted to spikes with amplitudes higher than 5 pA, which were considered as exocytic events. All spikes identified by the program were visually inspected. Overlapping spikes and spikes with aberrant shapes were discarded for parameters analysis. Quantal size (spike charge, Q) of each individual spike was measured by calculating the spike area above the baseline. Spike area is defined as the time integral of each transient

current,  $I_{max}$  as the height of each spike, half-width as the width of each spike at half its height ( $T_{1/2}$ ) and  $T_{peak}$  as the spike rise time (Figure 2C).

### **2.7 Intracellular calcium imaging**

Changes in the intracellular  $Ca^{2+}$  concentration were detected by the ratiometric fluorescent probe Fura-2. Cells were loaded during a 1 hour incubation with 2  $\mu$ M of the cell-permeant precursor Fura-2 acetoxymethyl ester (Fura-2AM; Molecular Probes) and 0.001% (w/v) pluronic acid (Molecular Probes). The recording saline solution contained 135 mM NaCl, 5 mM KCl, 2 mM  $CaCl_2$ , 2 mM  $MgCl_2$ , 10 mM glucose and 10 mM HEPES, pH 7.4. Fluorescence measurements were performed on an inverted microscope (Axiovert 35; Zeiss) with an oil immersion 40 Nikon objective (Fluor 40, NA 1.30) and a cooled CCD camera (CoolSnap HQ; Photometrics). The Imaging Workbench 4.0 software (Axon Instruments) was used for image acquisition. Fluorescence was excited alternately at 350 and 380 nm with a Lambda-10 filter wheel (Sutter Instruments), and emitted light was collected above 520 nm. Pairs of images were acquired every 2 s. Intracellular calcium level is expressed throughout as the fluorescence ratio  $F_{350}/F_{380}$ , calculated after background subtraction. Experiments were performed at 34°C. During calcium measurements, cells were continuously perfused with saline solution: the whole dish by a bath perfusion of control medium and the recorded field by a single-tip multichannel gravity-fed system, allowing switching between various solutions. Analysis was performed using Clampfit 10.2 (MDS Analytical Technologies). Responses induced by 15  $\mu$ M nicotine in presence of SOM230 were normalized with respect to the average of the previous and subsequent responses induced by 15  $\mu$ M nicotine alone.

### **2.8 Electrophysiological recordings**

Whole-cell patch-clamp recordings were performed with an Axopatch 200A amplifier (Axon Instruments) and low resistance (3- 4  $M\Omega$ ) electrodes. The final series resistance during electrophysiological recordings was between 5 and 15  $M\Omega$ . Membrane currents were recorded under voltage-clamp at a steady holding potential of -60 mV. The recording saline solution was the same as for calcium imaging experiments (see above). The culture dish was continuously perfused with extracellular solution at a rate of 3  $mL \cdot min^{-1}$ . Recording electrodes were filled with: 140 mM KCl, 2 mM  $MgCl_2$ , 10 mM HEPES, 2 mM MgATP, pH 7.3. 1 mM QX314 (Alomone Labs) was added to the intrapipette solution to prevent spiking of the recorded cell. Experiments were performed at room temperature (20- 22°C). The different substances were applied or coapplied locally by means of a U- tube allowing a relatively fast solution exchange

around the cell (< 50 ms). Signals were low-pass filtered at 5 kHz, sampled at 20 kHz, digitized using a BNC-2110 data acquisition card (National Instruments) and acquired with the Strathclyde electrophysiology software (WinWCP, John Dempster, University of Strathclyde). Analysis was performed using Clampfit 10.2 (MDS Analytical Technologies). Responses induced by 15  $\mu$ M nicotine in presence of SOM230 were normalized with respect to the average of the previous and subsequent responses induced by 15  $\mu$ M nicotine alone.

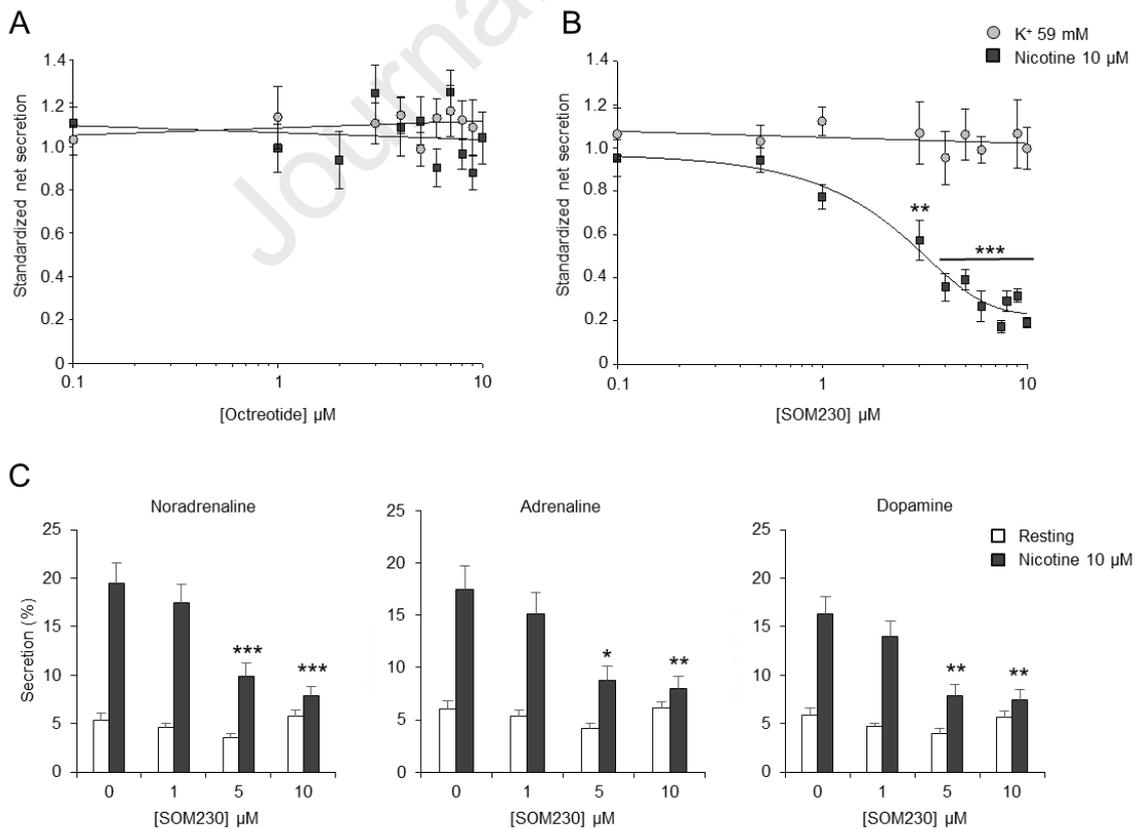
### ***2.9 Statistical analysis***

Statistical analyses were performed using SigmaPlot 13.0 (Ritme). One way ANOVA followed by Dunnett test were performed to assess the difference between multiple groups when data followed normal distribution and equal variance. If normality or equal variance failed, significance was estimated by non-parametric tests. Wilcoxon Rank Sum test or Mann-Whitney Rank Sum test was used for comparison between two groups and multiple comparisons. Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn's test were performed to assess the difference between multiple groups. A p value < 0.05 was considered to be significant for all tests.

### 3. RESULTS

#### 3.1 Effect of octreotide and pasireotide on bovine chromaffin cells secretion

We first analyzed the potential effect of the first-generation somatostatin analogue octreotide and the next-generation somatostatin analogue pasireotide (SOM230) on catecholamine secretion from primary culture of bovine chromaffin cells. To analyze the global catecholamine release, we used an assay based on the oxidation of catecholamines into fluorescent aminolutin [33]. Secretion was evoked either by 10  $\mu\text{M}$  nicotine or by a high  $\text{K}^+$  depolarizing solution (59 mM). As shown in Figure 1A, increasing concentration of octreotide did not affect catecholamine secretion regardless of the stimulus type. On contrary, SOM230 treatment triggered a significant dose-dependent inhibition of the nicotine-induced catecholamine secretion ( $\text{IC}_{50}=2.64 \mu\text{M}$  and maximum efficacy=77% inhibition), whereas no effect was observed on  $\text{K}^+$ -evoked secretion (Figure 1B). At higher concentration, SOM230 inhibited catecholamine release up to  $81.0 \% \pm 2.4 \%$  (Figure 1B). In agreement with previous studies [39, 40], we also observed in our experimental conditions that somatostatin (SS14) inhibited catecholamine secretion in a concentration range similar to SOM230 (not shown).



**Figure 1. The somatostatin analogue pasireotide (SOM230) specifically inhibits catecholamine secretion from bovine chromaffin cells.**

Bovine chromaffin cells were stimulated with 10  $\mu$ M nicotine or with a high  $K^+$  depolarizing solution (59 mM) for 10 min in the presence of the indicated concentrations of octreotide (a) or SOM230 (b). Catecholamine release was quantified using an adrenolutine fluorescent assay (a, b) or mass spectrometry (c). For adrenolutine assay, basal release was subtracted to obtain the net catecholamine secretion and for each concentration of analogues, secretion was normalized to control secretion (untreated cells) which was fixed to 1. The proportion of catecholamines secreted in resting and stimulated conditions are indicated in Supplementary Figures 1A and B. Data are given as the mean values  $\pm$  SEM obtained from different cell cultures ( $n \geq 3$ ).

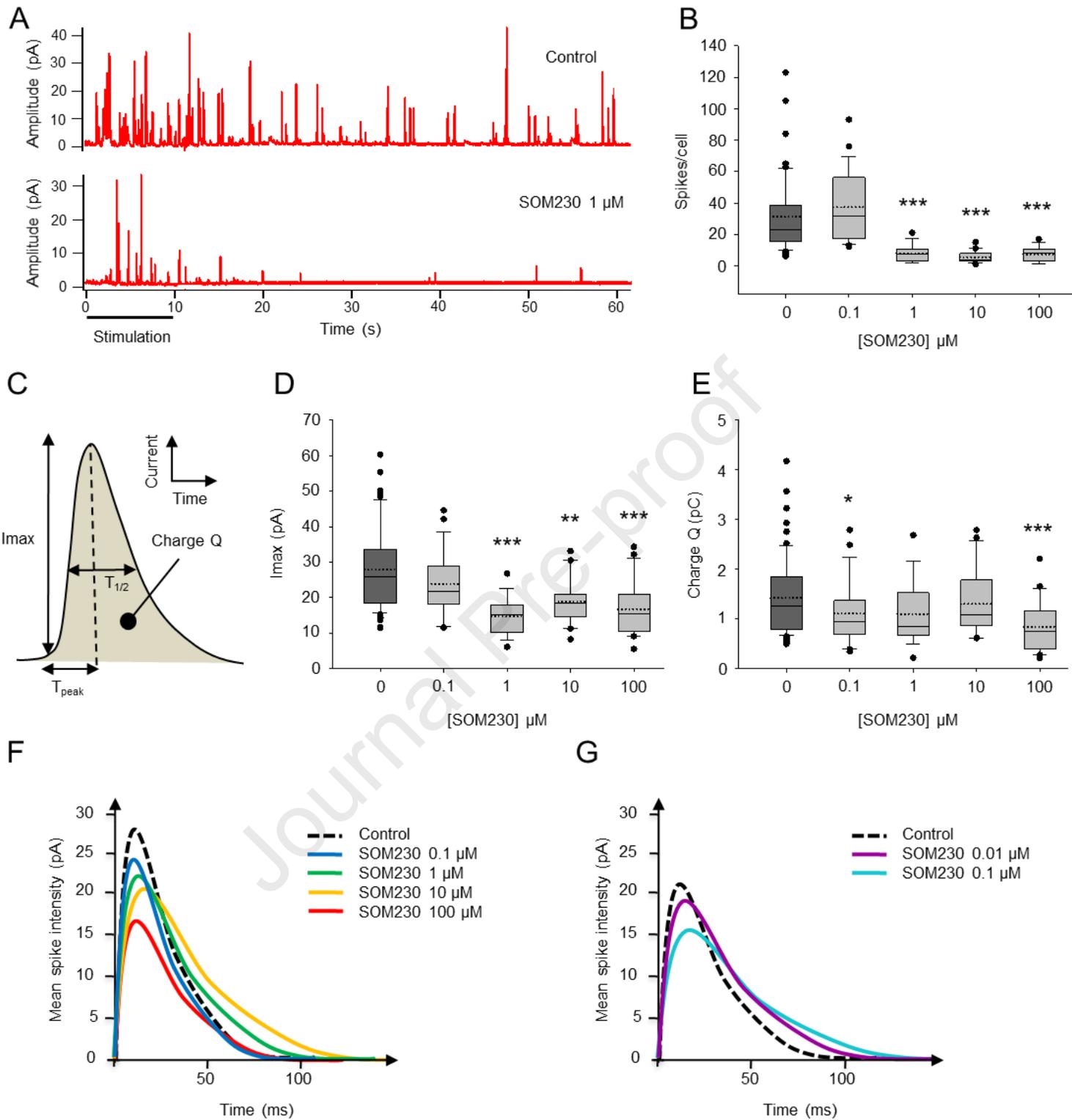
\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to control; Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn's test or One Way ANOVA followed by Dunnett's test.

To confirm that SOM230 inhibits secretagogue-evoked catecholamine release and to check whether the secretion of different catecholamines is equally affected, we quantified noradrenaline, adrenaline and dopamine release using mass spectrometry. As shown in Figure 1C, SOM230 similarly inhibited noradrenaline, adrenaline and dopamine secretion. In control experiments, we have shown that 10  $\mu$ M octreotide does not modify the secretion of any of the catecholamines (Supplementary Figure 1C).

To better investigate the effect of SOM230 on secretion, we next recorded catecholamine release by carbon fiber amperometry (Figure 2). More sensitive than the adrenolutine assay, carbon fiber amperometry is an electrochemical method that allows to calculate frequency, quantal size and kinetics of individual exocytic events from single cells [41, 42]. Cultured bovine chromaffin cells were stimulated by a puff application using a stimulation pipette containing 100  $\mu$ M nicotine, with or without the indicated concentration of SOM230. As illustrated by the representative amperometric traces in Figure 2A, the most striking effect of SOM230 treatment is a reduction in the number of amperometric spikes. Indeed, increasing concentrations of SOM230 reduced substantially the spike number per cell (reduction from 76 to 82%) indicating a strong decrease of exocytic events (Figure 2B and Table 1). These results clearly demonstrated that SOM230 treatment drastically inhibits exocytosis leading to a large reduction of catecholamine release.

Each individual spike represents a single granule fusion event and is composed of a rapid rise corresponding to the quick release of a high concentration of catecholamine through a fusion pore as it dilates, followed by a slower decay representing the subsequent diffusion of molecules from the release site to the electrode surface. Therefore, analyzing the individual amperometric spike provided valuable dynamic information on the remaining exocytic process. The surface area or quantal size (Q) is proportional to the amount of catecholamines released per event, the

spike amplitude value ( $I_{max}$ ) reflects the maximal flux of catecholamines, whereas the half-width ( $T_{1/2}$ ) and the time to peak ( $T_{peak}$ ) reflect the duration of exocytotic events and the kinetics of the fusion pore expansion, respectively (Figure 2C). The spike parameters means for each SOM230 concentration are listed in Table 1. From 1  $\mu$ M concentration in the stimulation pipette, SOM230 reduced the spike amplitude with or without reducing the quantal size (Figure 2D, E, Table 1). This effect on quantal size may depend on the capacity of SOM230 to change the spike kinetics and duration as a concomitant increase of  $T_{1/2}$  and  $T_{peak}$  has been observed only at the concentration of 1 and 10  $\mu$ M (Table 1). Accordingly, superimposing the average shape of spikes for each SOM230 treatment conditions revealed that the spike height is progressively decreasing upon increasing concentration of SOM230 with a mild shift of spike kinetics for 1 and 10  $\mu$ M SOM230 treatment (Figure 2F). Altogether, the amperometric spike analyses suggest that SOM230 treatment might also affect the fusion pore formation/expansion/closure of the residual exocytotic events or the granule catecholamine content.



**Figure 2. SOM230 affects different parameters of individual exocytotic events from single adrenal bovine chromaffin cells.**

Single bovine chromaffin cells were stimulated with a local application of 100  $\mu\text{M}$  nicotine for 10 s and catecholamine secretion was monitored using carbon fiber amperometry. (a) Example of a typical amperometric recording obtained from an untreated cell (control) or a cell treated with 1  $\mu\text{M}$  SOM230. (b) Box-and-whisker diagrams illustrating the number of amperometric spikes per cell. Each plot represents the 1st quartile (bottom line), the median (line in the box), the mean (dotted line in the box) and the third quartile (upper line). Whiskers correspond to the 5th (bottom) and the 95th (top) percentile

and black dots represent outlier observations; \*\*\*  $p < 0.001$  compared to control; Mann Whitney Rank Sum test. (c) Scheme of a spike representing a single granule fusion event and parameters that can be evaluated from each individual spike. (d, e) Increasing concentrations of SOM230 significantly affects the spike amplitude ( $I_{max}$ ; (d)) and the quantal size (Charge Q; (e)); \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared to control; Mann Whitney Rank Sum test. (f) The superimposition of average spikes for each condition illustrates the effect on the spike shape of SOM230 when applied through the stimulation pipette. All the average amperometric data are detailed in Table 1.

Puff application of SOM230 close to single recorded cells is likely to misjudge the effective inhibiting dose of SOM230 because of the rapid dilution of the SOM230 solution in the cell incubation medium. Therefore, we also tested different SOM230 concentrations directly in the bath (Supplementary Figure 2). Under those conditions, treatment of cells with 0.01 and 0.1  $\mu\text{M}$  SOM230 significantly inhibits the number of exocytic events by around 20 and 60%, respectively. However, the residual spike amplitude was inhibited by around 30% only at 0.1  $\mu\text{M}$ , whereas quantal size was not significantly affected most likely due to an increase of  $T_{1/2}$  and  $T_{peak}$ . These results indicate that SOM230 starts to significantly affect the exocytic process from 0.1  $\mu\text{M}$  and above.

### ***3.2 Effect of SOM230 treatment on catecholamine secretion of human pheochromocytoma cells***

The ability of SOM230 to also inhibit catecholamine secretion of human pheochromocytoma (Pheo) cells was the next important point we decided to address. Ten patients (6 females and 4 males, mean age of  $62 \pm 10$  years) with histologically confirmed Pheo were included in this study (Table 2). Seven patients (70%) were diagnosed with hormonal-related symptoms whereas all of them presented abnormal hormonal secretion (100%). All were considered to have an adrenergic phenotype although three had excess normetanephrines. Two out of 10 patients (20%) were diagnosed with a neurofibromatosis type 1 and one had a confirmed sporadic disease while the genotype of the 7 other patients was not known yet. The mean tumor size was 4.3 cm (range 1.8-7.0 cm). Other pathological characteristics are detailed in Table 2.

Catecholamine secretion analysis by carbon fiber amperometry was performed on tumor cells directly cultured from 8 of these freshly resected human Pheos. Four patient's cultures have been treated with SOM230 present in the stimulation pipette (patients 1 to 4), whereas the 4 others cultures have been treated with SOM230 directly added to the cell culture medium with lower SOM230 concentrations (patients 5 to 8). One to three different concentrations have been

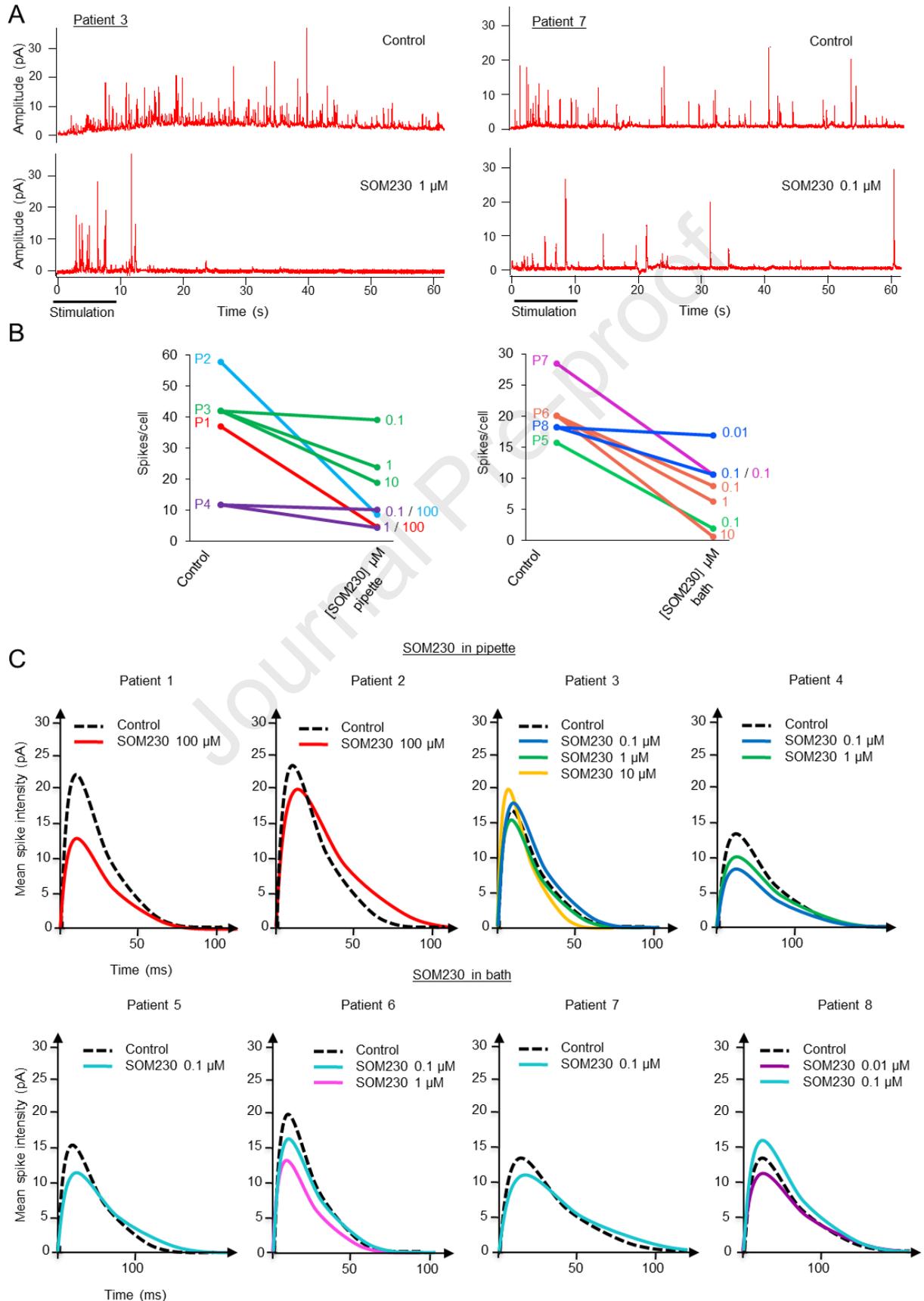
tested per patient depending on the culture viability over time and on the amount of material. All the amperometric parameters measured on human Pheo cells are summarized in Table 3.

Figure 3A shows representative amperometric traces recorded from untreated human Pheo cells (control) or from Pheo cells treated with 1  $\mu\text{M}$  SOM230 in stimulation pipette (left) or with 0.1  $\mu\text{M}$  SOM230 in cell culture medium (right). From these recordings it is noticed that, as observed in bovine chromaffin cells, SOM230 treatment drastically reduced the number of spikes. Figure 3B illustrates the variation of the total amount of spikes per cell for each patient. Importantly, all patients were sensitive to SOM230 showing a significant decrease of exocytic events. Furthermore, the inhibitory effect of SOM230 on catecholamine release was dose-dependent in all the cases, when different concentrations of SOM230 were tested on cells from the same patient. Of note, the inhibitory effect of SOM230 on catecholamine secretion appears more potent when it is present in the cell medium than when it is added to the pipette, because of the rapid dilution of SOM230 resulting from this type of application. Indeed, when present in the cell medium, SOM230 inhibits catecholamine secretion from 0.1  $\mu\text{M}$  (patients 5, 6, 7 and 8 with inhibition by around 88%, 56%, 63% and 42%, respectively), a concentration that corresponds more or less to a puff of 1  $\mu\text{M}$  through the stimulation pipette. Accordingly, 1  $\mu\text{M}$  SOM230 treatment of Pheo cells through the stimulation pipette led to a significant inhibition of the number of spikes by around 43% (patient 3) and 61% (patient 4).

Next we analyzed the dynamic parameters of the residual spikes upon treatment with different concentrations of SOM230. Figure 3C shows the average shape of spikes for each patient. Spike amplitude was reduced in 80% of patients' Pheo cells (ranging from 15 to 40% inhibition depending on SOM230 concentration) either alone (patients 4, 6 and 8) or associated with a decrease of the quantal size (patients 1 and 5) or with an increase of the kinetic and the duration of the exocytic events (patients 2 and 7). No significant changes have been observed for patient 3. These data tend to indicate that the residual exocytic events might be differentially affected from one patient to another.

Note that when octreotide treatment was tested on tumor cells from patient 2 at the highest dose of SOM230 (100  $\mu\text{M}$ ; Supplementary Figure 3), only a modest decrease of the spike amplitude (around 15%) was observed, whereas the number of exocytic events and the spike parameters

remained unchanged. These data support a lack of effect of octreotide on catecholamine secretion from human Pheo cells and validate the specificity of the effect of SOM230.



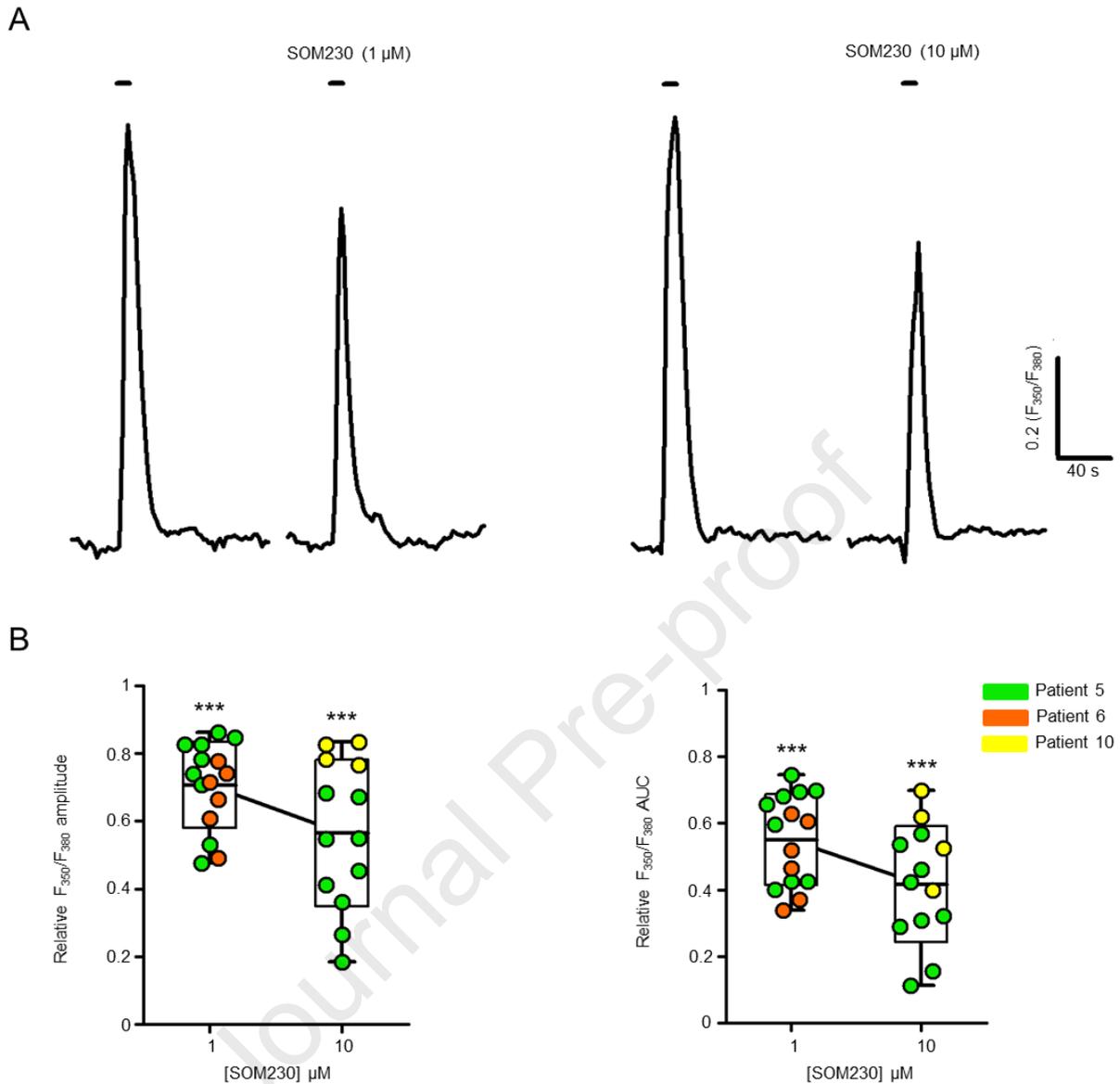
**Figure 3. SOM230 inhibits the secretion of human pheochromocytoma cells.**

Single human pheochromocytoma cells were stimulated with a local application of 100  $\mu\text{M}$  nicotine for 10 s in the absence or the presence of SOM230 and catecholamine secretion was monitored using carbon fiber amperometry. (a) Example of a typical amperometric recording obtained from untreated cells (controls, top) or a cell treated with 1  $\mu\text{M}$  SOM230 in pipette (patient 3, bottom left), or with 0.1  $\mu\text{M}$  SOM230 in the cell incubation bath (patient 7, bottom right). (b) Representation of the mean number of spikes per cell in response to SOM230. Patients (P) are color-coded. SOM230 was present in the stimulation pipette (left) or added directly to the incubation bath (right) and the concentration is indicated. (c) Superimposition of average spike obtained for cells of each patient according to treatment conditions. SOM230 was applied either through the stimulation pipette (top) or in the incubation bath (bottom). All the average amperometric data are detailed in Table 3.

### *3.3 Effect of SOM230 on intracellular calcium levels and membrane currents evoked by nicotine in human pheochromocytoma cells*

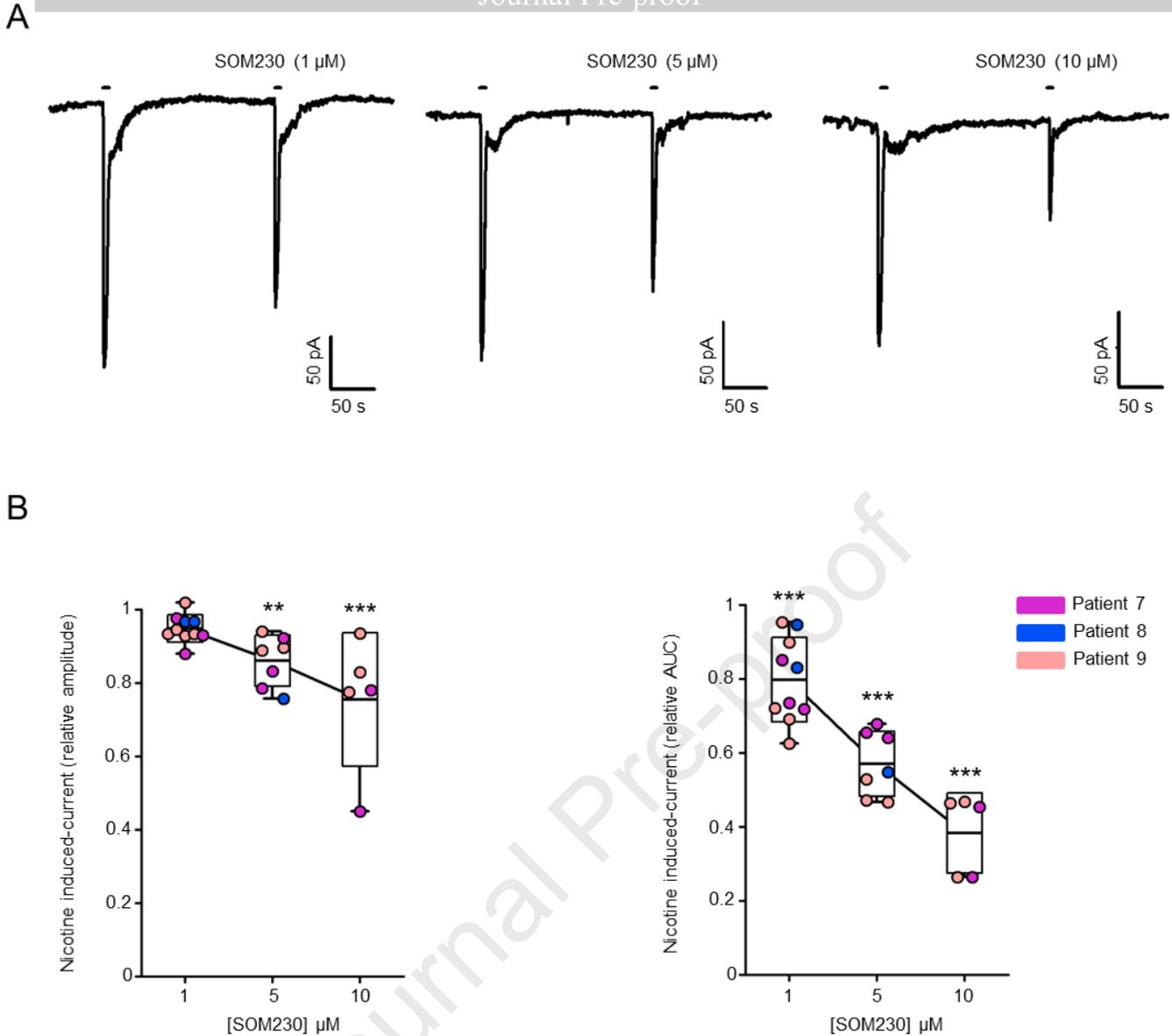
Nicotine-induced stimulation of exocytosis in chromaffin cells triggers an elevation of intracellular calcium concentration ( $[\text{Ca}^{2+}]_i$ ) [43]. We therefore examined whether the rise in  $[\text{Ca}^{2+}]_i$ -induced by nicotine was inhibited by SOM230. To this end, we performed  $[\text{Ca}^{2+}]_i$  imaging at single-cell resolution in tumor cells from 3 different human Pheos (patients 5, 6 and 10; Figure 4). Stimulation of chromaffin cells with 15  $\mu\text{M}$  nicotine generated a rapid increase in  $[\text{Ca}^{2+}]_i$  (Figure 4A). Co-application of nicotine with 1 or 10  $\mu\text{M}$  SOM230 reduced the amplitude of  $[\text{Ca}^{2+}]_i$  by 29% and 42% respectively and the total integrated signal (area under the curve) by 45% and 58 % respectively (Figure 4B).

Since SOM230 reduced  $[\text{Ca}^{2+}]_i$  responses to nicotine, we examined whether it affected the membrane currents evoked by nicotine. To this end, we performed whole-cell patch-clamp recordings in the voltage-clamp mode on human tumor cells cultured from 3 different Pheos (patients 7, 8 and 9). Co-application of 15  $\mu\text{M}$  nicotine with SOM230 (1, 5 or 10  $\mu\text{M}$ ) significantly reduced the amplitude and the area under the curve of the current evoked by nicotine in a concentration-dependent manner (Figure 5). On average, 1, 5 and 10  $\mu\text{M}$  SOM230 respectively reduced the amplitude of nicotine-induced current by 5%, 14% and 25% and the area under the curve by 20%, 43% and 62%. These data indicate that SOM230 has a fast and concentration-dependent action on the function of nicotinic receptors.



**Figure 4. Dose-dependent inhibition by SOM230 of calcium transients evoked by brief nicotine application on tumor cells from human pheochromocytoma.**

(a) Example of typical traces of calcium transients, represented as ratiometric changes in fluorescence emission during 10 s applications of nicotine 15 μM or during co-applications of nicotine with SOM230 1 or 10 μM on human pheochromocytoma (data from Patient 5). Nicotine application times are indicated by the horizontal lines. (b) Average amplitude (left) and area under the curve (AUC, right) of the ratiometric changes induced by 15 μM nicotine co-applied with SOM230 1 and 10 μM normalized to the ratiometric changes induced by 15 μM nicotine alone. \*\*\* $p < 0.001$  compared to control; Wilcoxon Rank Sum test.



**Figure 5. Dose-dependent inhibition by SOM230 of currents evoked by brief nicotine application on tumor cells from human pheochromocytoma.**

(a) Example of currents induced by 3 s applications of nicotine 15  $\mu\text{M}$  recorded in a cell incubated with 1, 5 or 10  $\mu\text{M}$  SOM230 (data from Patient 7). When SOM230 is co-applied with nicotine, the amplitude of the current is reduced. Nicotine application times are indicated by the horizontal lines. (b) Average amplitude (left) and area under the curve (AUC, right) of the current induced by nicotine coapplied with SOM230 1, 5 and 10  $\mu\text{M}$  normalized to the current induced by 15  $\mu\text{M}$  nicotine alone. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  compared to control; Wilcoxon Rank Sum test.

#### 4. DISCUSSION

Historically misunderstood as relatively rare and harmless, current understanding shows that NETs are increasingly common and are now regarded as neoplasms that can cause debilitating symptoms and are life-threatening for patients. Indeed, loss of control of hormone secretion by NETs can result in a broad spectrum of symptoms and clinical syndromes ranging from feeling ill up to severe complications such as hypertension, hypoglycemia, carcinoid syndrome, cardiopathy and stroke. Moreover, NETs with initial low secretory activity can transform into high secreting lesions with negative impact on prognosis just by progressively becoming hormonally active [44-47]. Today, an unmet need is to identify drugs targeting the secretory activity of the tumor.

The main anti-secretory therapies currently used in clinic on patients with certain NETs and endocrine syndromes are the somatostatin analogues [48, 49]. However, the impact of somatostatin analogues on pheochromocytoma-associated catecholamine hypersecretion has been poorly explored *in vitro*. First, we tested the effect of octreotide and pasireotide (SOM230), two different-generation somatostatin analogues, on animal model (bovine chromaffin cells) by measuring catecholamine release either at the whole population level by the adrenolutine assay and mass spectrometry or at the single cell level by carbon fiber amperometry. Using these 3 methods, we have shown that the somatostatin analogue SOM230 efficiently inhibits the nicotine-evoked catecholamine secretion, whereas octreotide has no significant effect. In agreement with our observations, Ribeiro et al. reported that octreotide had no effect on 1-methyl-4-phenylpyridinium secretion from the bovine chromaffin cells [50]. Moreover, the potential use of octreotide for Pheo treatment has been already investigated in clinic. Except for very few cases [51, 52], octreotide treatment did not significantly affect catecholamine secretion. For example, octreotide treatment did not significantly change the level of plasma and urinary catecholamine or metanephrine compared to placebo treatment in several patients with benign or malignant Pheo [53, 54]. Likewise, urinary catecholamine levels remained unchanged after octreotide treatment of patients with secreting head and neck paraganglioma [55].

As we observed an important inhibition of bovine chromaffin cell secretion by SOM230, we next tested this analogue on human tumor cells directly cultured from freshly resected Pheo. We show here that SOM230 inhibits catecholamine secretion of tumor cells with a significant

effect starting from 0.1  $\mu\text{M}$ . To our knowledge, this is the first evidence of an inhibitory effect of SOM230 on catecholamine secretion from human Pheo cells. Nevertheless, in agreement with our observations, the effect of SOM230 on hormone secretion has been tested on various types of other tumor cells *in vitro*. For example, SOM230 efficiently inhibits prolactin, growth hormone and adrenocorticotrophic hormone secretion from primary cultures of human secreting pituitary adenomas [56, 57]. It also significantly decreases secretion of chromogranin A in cells cultured from tumor of human gastroenteropancreatic NETs [20]. Moreover, Quinn et al. demonstrated *in vivo*, in a mouse model with insulinoma, that SOM230 injection inhibits hypersecretion of insulin thus preventing hypoglycemia [58].

Using carbon fiber amperometry, we have further dissected the anti-secretory effect of SOM230 on the exocytic pathway. The main effect observed after the application of 0.1  $\mu\text{M}$  SOM230 was an important inhibition of the number of exocytic events on both bovine chromaffin cells (decreased by around 60%) and human tumor cells (decrease ranging from 42 to 88%; patients 5 to 8). Note that, due to the dilution factor, the effect seen with 0.1  $\mu\text{M}$  in bath of SOM230 corresponds more or less to the one seen with 1  $\mu\text{M}$  co-incubated with nicotine in the stimulation pipette. Accordingly, under the latter experimental condition, the number of exocytic events was reduced by SOM230 with a comparable efficiency (by 43 and 61% in patients 3 and 4, respectively and by 75% in average for bovine model). These results support the notion that SOM230 affects early steps in the exocytotic process, such as the recruitment of the secretory granules or their docking at the plasma membrane. Moreover, the analysis of the amperometric parameters revealed that the amplitude of the remaining exocytic events tends to be lower upon SOM230 treatment, an effect sometimes accompanied by a decrease of the kinetics. This suggests that, even when secretory granule fusion is engaged, the release might be disturbed, being incomplete and/or slowed down. To our knowledge, only a single study investigated the *in vitro* effect of SOM230 on tumor cells cultured from human Pheos. Hence, Pasquali et al. showed that a long-lasting (24 h) treatment of 0.1 and 1  $\mu\text{M}$  SOM230 significantly decreased the intracellular content of dopamine and noradrenaline [28]. However, in contrast to our study, they didn't measure the secretory activity of the tumor cells. A reduction of cellular catecholamine levels can be the consequence of a decrease in the number of secretory granules and/or the level of catecholamine per granules. In our study, the total amount of catecholamines in cells remains unchanged which was expected because relatively short SOM230 treatments were performed. This indicates that the effect we observed on secretion is necessarily independent from its potential long-term effect on catecholamine metabolism. It is also of note that the inhibitory effect of SOM230 on catecholamine release

was seen only when the drug was present during the stimulation period and a pre-incubation period did not significantly further increase this inhibition of secretion (data not shown). These data show that the effect of SOM230 is rapid and that a long pre-incubation is not required.

In our experimental conditions, we found that SOM230 drastically inhibited catecholamine secretion of tumor cells at 100 nM but not at 10 nM. Accordingly, inhibitory effects with similar concentration have been previously observed in cells cultured from human cancer such as growth hormone-secreting pituitary adenoma and growth hormone-releasing hormone-producing bronchial carcinoid [59, 60]. However, this concentration is relatively high compared to the  $IC_{50}$  (ranging from 0.2 to 1 nM) reported after long lasting treatment of SOM230 on the secretory activity of rat pituitary cells, rat pancreatic islets, mouse corticotroph adenoma cells and human pituitary adenoma cells [56, 57, 61, 62], raising the question of the mechanisms by which SOM230 inhibits catecholamine secretion in human Pheo cells. As frequency and amplitude of exocytic events depend on the level of secretagogue-evoked intracellular calcium elevation [63, 64], it was tempting to imagine that SOM230 might affect secretory granule exocytosis upstream of calcium entry. Indeed, we showed that SOM230 significantly inhibited nicotine-evoked increase of intracellular calcium in human tumor cells. One other interesting aspect is that SOM230 acts instantly and exclusively on secretion induced by nicotine without affecting secretion induced by high  $K^+$  solution. In agreement with our results, several studies demonstrated that somatostatin itself was able to inhibit catecholamine release when chromaffin cells are stimulated with nicotine or acetylcholine but not upon membrane depolarization [39, 40, 65, 66]. In fact, we show here by whole cell recordings in the voltage-clamp mode that SOM230 is able to reduce the nicotinic current. Since membrane potential remains constant under these recording conditions, this effect most likely involves an effect on the function of nicotinic receptors and not on voltage-dependent  $Na^+$  and  $Ca^{2+}$  channels. Accordingly, co-application of somatostatin with nicotine reversibly inhibits the nicotine-induced inward current in guinea pig chromaffin cells [67]. Whether SOM230 indirectly affects the function of the nicotinic receptor via its own somatostatin receptor or via a direct action on the nicotinic receptor is currently unknown. Interestingly, several evidences indicated that somatostatin receptors are able to either homo-oligomerize or to hetero-oligomerize between different subtypes of somatostatin receptors or with other types of receptor like the dopamine receptor D2 or the  $\mu$ -opioid receptor MOR1 [68-72]. Potential oligomerization between somatostatin and nicotinic receptors has never been reported and will require further investigations.

This pre-clinical study allowed us to uncover a novel anti-secretory role of SOM230 on tumor adrenal medulla cells from patients with functional Pheo and abnormal secretion. If SOM230 treatment could actually improve the management of patients remains a key question that will require further clinical studies. As SOM230 efficiently inhibits catecholamine secretion by directly acting on the tumor secretion, it might be considered for preoperative medical preparation or intraoperative management of highly secreting Pheo. SOM230 might also be useful to treat some metastatic Pheos resistant to conventional antihypertensive medications, especially as high hormonal secretion impacts overall survival in patient with metastatic Pheo [73]. Moreover, as long lasting effect of SOM230 is known to reduce catecholamine synthesis in Pheo cells [28], treatment of patients may lead to a combination of reduced catecholamine synthesis and secretion which is in fact likely to boost the effect of SOM230 on lowering catecholamine levels in patient with Pheo.

In conclusion, a long-acting release formulation of SOM230 might be a potential lead to consider a therapy aimed at treating the symptoms induced by excessive catecholamine secretion of Pheo. This new data may be used to better control catecholamine hypersecretion when needed and should be evaluated in clinical practice.

## **5. Ethics approval and consent to participate**

The present study used the data and the human biological material of the biological collection “Approche moléculaire des tumeurs corticosurréaliennes” which was agreed by the “Comité de Protection des Personnes Est III” ethical advisory committee, and was conducted according to currently accepted ethical guidelines, including informed written consent approval signed by all patients prior to inclusion.

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## **7. Conflict of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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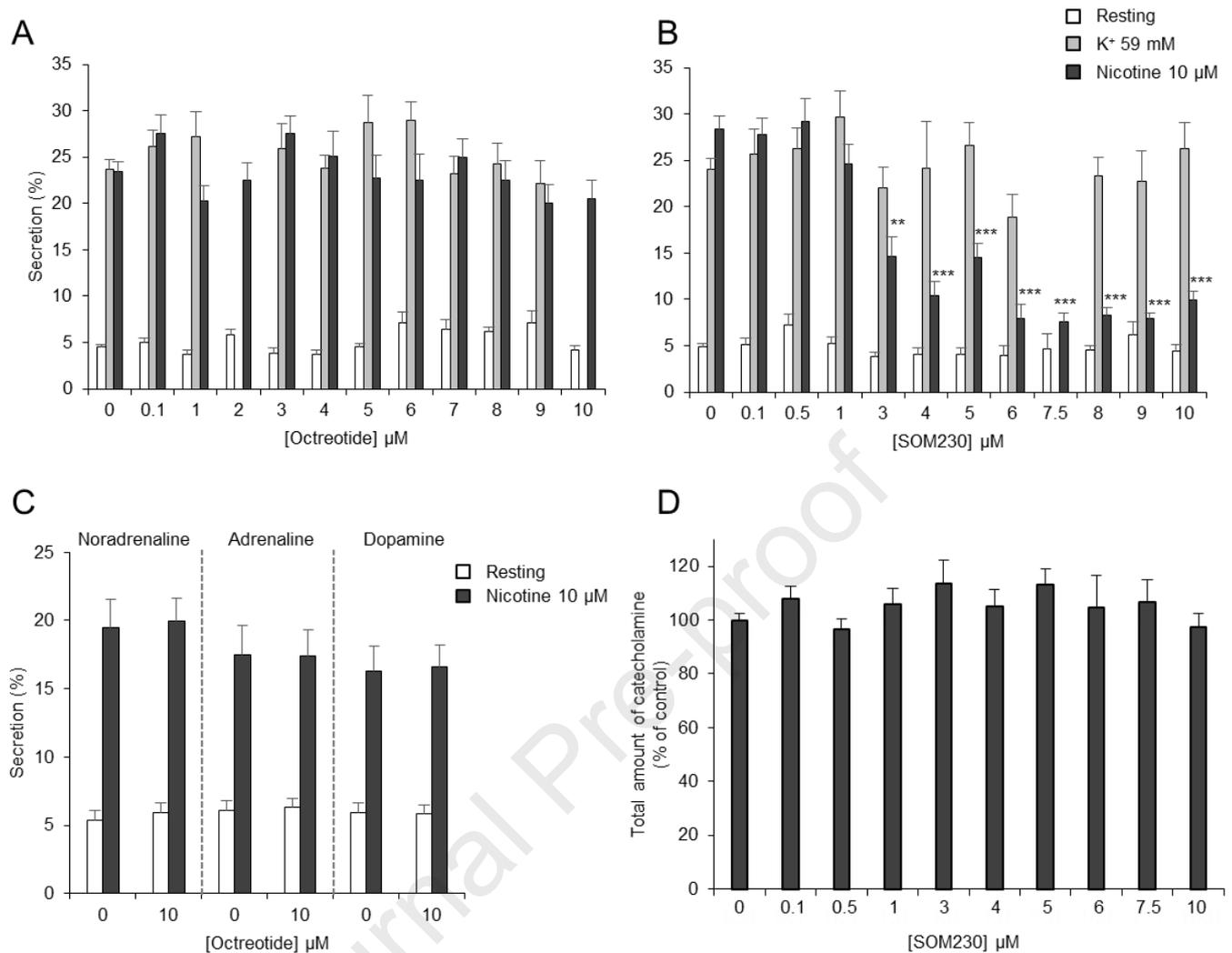
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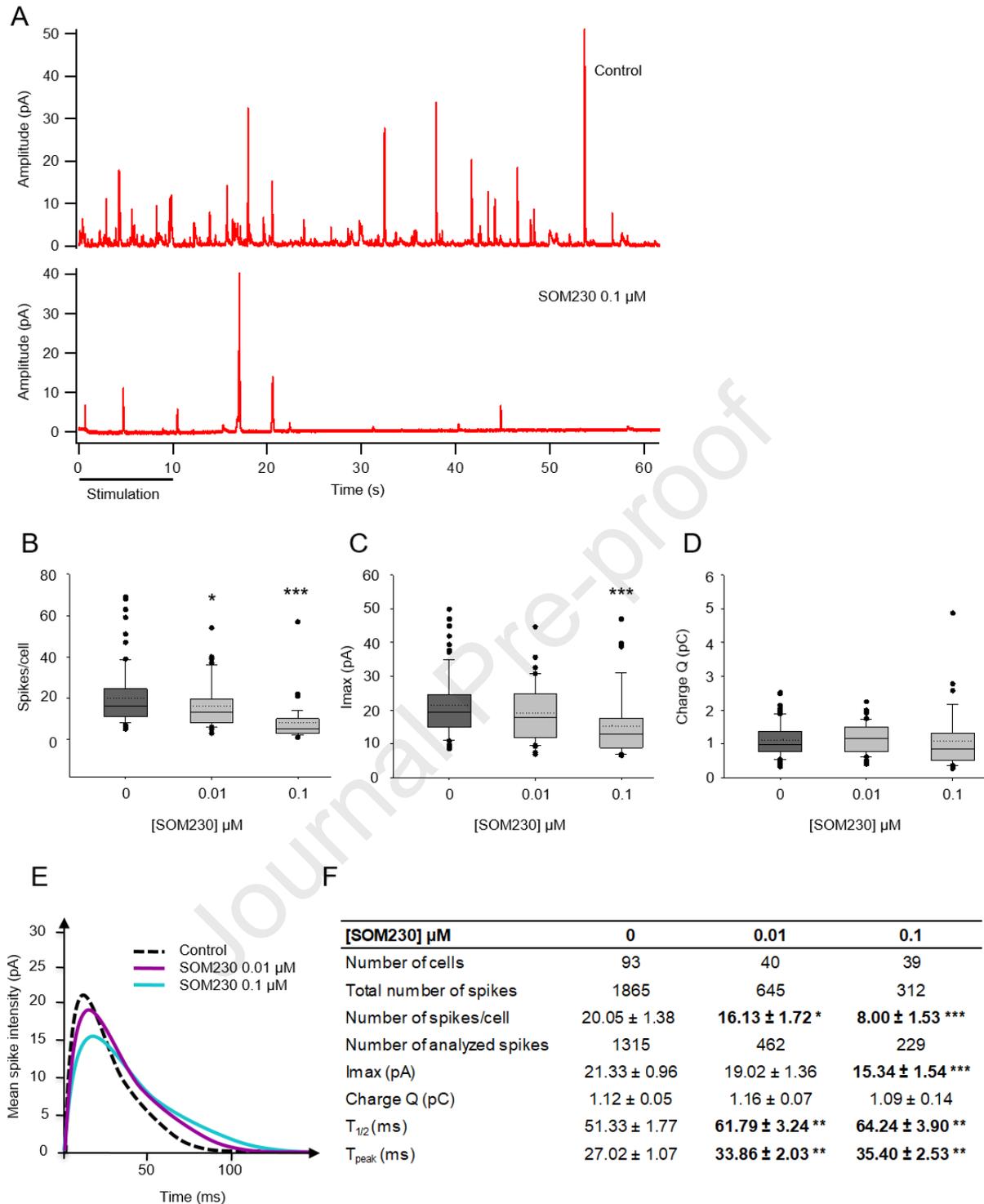
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**Supplementary Figure 1. Experimental details on the effect of octreotide and SOM230 on catecholamine secretion in bovine chromaffin cells.**

Bovine chromaffin cells were stimulated with 10  $\mu\text{M}$  nicotine or with a high  $\text{K}^+$  depolarizing solution (59 mM) for 10 min in the presence of the indicated concentrations of octreotide or SOM230. Catecholamine amount was estimated using an adrenolutine fluorescent assay (a, b, d) or mass spectrometry (c). (a, b) Data describing the proportion of catecholamine secreted in resting and stimulated conditions upon treatment of octreotide and SOM230; \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to control; Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn's test or One Way ANOVA. Note that the effect of 2 and 10  $\mu\text{M}$  octreotide and 7.5  $\mu\text{M}$  SOM230 was not tested upon  $\text{K}^+$  stimulation. (c) Effect of 10  $\mu\text{M}$  octreotide on noradrenaline, adrenaline and dopamine release measured by mass spectrometry. (d) The total amount of catecholamine in bovine chromaffin cells was calculated from the adrenolutine secretion assay by adding secreted and cellular catecholamines. Cells were stimulated with 10  $\mu\text{M}$  nicotine for 10 min in the presence of the indicated concentrations of SOM230. Catecholamines were then measured both in the extracellular medium and in the cellular extract after stimulation. For each concentration of SOM230, the amounts of total catecholamine were normalized to control (untreated cells) which was fixed to 100%. Data are given as the mean values  $\pm$  SEM obtained from different cell cultures ( $n \geq 3$ ).



**Supplementary Figure 2. SOM230 in the culture medium affects different parameters of individual exocytotic events in single adrenal bovine chromaffin cells.**

Bovine chromaffin cells were stimulated with a local application of 100  $\mu\text{M}$  nicotine for 10 s and catecholamine secretion was monitored using carbon fiber amperometry. (a) Example of a typical amperometric recording obtained from an untreated cell (control) or a cell treated with 0.1  $\mu\text{M}$  SOM230 in bath solution. (b) Box-and-whisker diagram illustrating the number of amperometric spikes per cell. Each plot represents the 1st quartile (bottom line), the median (line in the box), the mean (dotted line in the box) and the third quartile (upper line). Whiskers correspond to the 5th (bottom) and the 95th (top)

percentile and black dots represent outlier observations; \* $p < 0.05$ , \*\*\* $p < 0.001$  compared to control; Mann Whitney Rank Sum test. (c, d) Increasing concentration of SOM230 significantly affects the spike amplitude ( $I_{max}$ ; (c)) but not the quantal size (Charge Q; (d)); \*\*\* $p < 0.001$  compared to control; Mann Whitney Rank Sum test. (e) The effect of SOM230 on spike shape is illustrated by superimposing average spikes according to cell treatment. (F) Table with all the average amperometric data; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to control; Mann Whitney Rank Sum test.

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**Supplementary Table. S1**

Elution gradient.

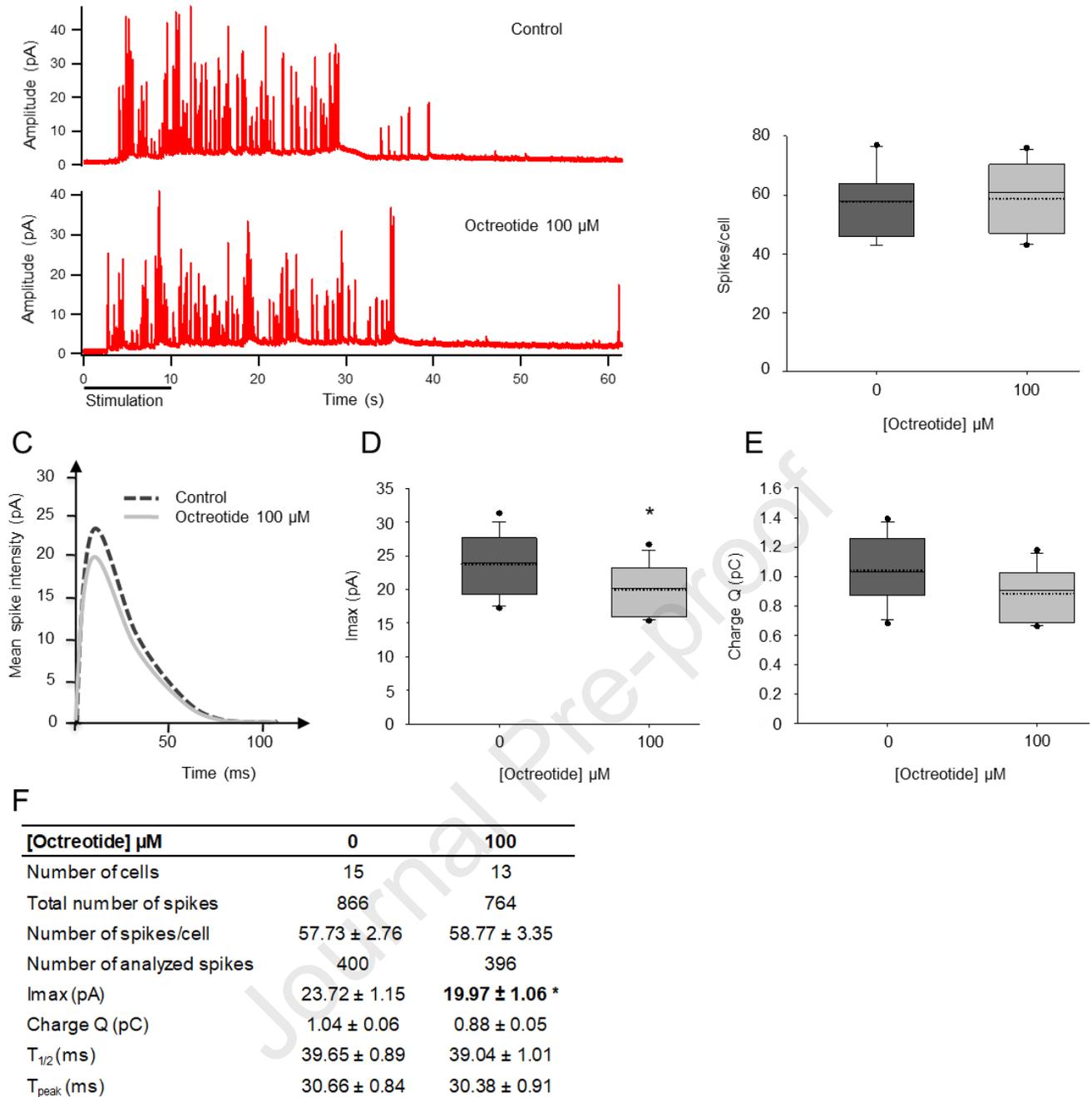
<b>Time (min)</b>	0	2.5	9	11	12	12.5	18
<b>% B mobile phase</b>	0	0	20	98	98	0	0

Detail of MS analysis conditions.

<b>Mode</b>	positive
<b>Spray voltage</b>	3,500 V
<b>Nebulizer gas</b>	Nitrogen
<b>Desolvation (nitrogen) sheath gas</b>	18 Arb
<b>Aux gas</b>	7 Arb
<b>Ion transfer tube temperature</b>	297°C
<b>Vaporizer temperature</b>	131°C
<b>Q1 and Q2 resolutions</b>	0.7 FWHM,
<b>Collision gas (CID, argon) pressure</b>	2 mTorr

**Supplementary Table S2. Mass spectrometer ionization, selection, fragmentation, and identification parameters.**

Compound	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
<b>Dopamine</b>	positive	324.14	116.11	54.24	206
			145.11	33.36	
			171.05	23.90	
<b>D4-Dopamine</b>	positive	328.14	116.11	55.00	206
			128.05	48.67	
			171	20.76	
<b>Adrenaline</b>	positive	354.11	166.04	18.09	179
			171.06	27.69	
			184.04	14.60	
<b>D6-Adrenaline</b>	positive	360.17	171.06	27.19	184
			172.04	18.65	
			190.04	14.35	
<b>Noradrenaline</b>	positive	340.15	116.11	55.00	215
			145.11	36.19	
			171.06	24.61	
<b>C6-Noradrenaline</b>	positive	346.15	145.11	36.04	226
			158.11	18.34	
			171.06	25.62	



### Supplementary Figure 3. Octreotide does not affect catecholamine secretion of human pheochromocytoma cells.

Pheochromocytoma cells were stimulated with a local application of nicotine 100 μM for 10 s and catecholamine secretion was monitored using carbon fiber amperometry. (a) Example of a typical amperometric recording obtained from an untreated cell (control) or a cell treated with 100 μM octreotide in pipette (patient 2). (b) Box-and-whisker diagrams illustrate the number of amperometric spikes per cell. Each plot represents the 1st quartile (bottom line), the median (line in the box), the mean (dotted line in the box) and the third quartile (upper line). Whiskers correspond to the 5th (bottom) and the 95th (top) percentile and black dots represent outlier observations. (c) The effect of octreotide on spike shape is illustrated by superimposing average spikes according to cell treatment. (d, e) Octreotide significantly affects the spike amplitude (Imax; (d)) but not the quantal size (Charge Q; (e)); \*p < 0.05 compared to control; Mann Whitney Rank Sum test. (f) Table with all the average amperometric data; \*p < 0.05 compared to control; Mann Whitney Rank Sum test.

[SOM230] $\mu\text{M}$	0	0.1	1	10	100
Number of cells	69	28	16	20	21
Total number of spikes	2067	1043	125	110	155
Number of spikes/cell	31.32 $\pm$ 2.85	37.25 $\pm$ 4.24	<b>7.81 <math>\pm</math> 1.33</b> ***	<b>5.50 <math>\pm</math> 0.83</b> ***	<b>7.38 <math>\pm</math> 1.11</b> ***
Number of analyzed spikes	1463	735	57	53	72
I <sub>max</sub> (pA)	27.88 $\pm$ 1.39	23.84 $\pm$ 1.76	<b>14.70 <math>\pm</math> 1.31</b> ***	<b>18.87 <math>\pm</math> 1.41</b> **	<b>16.70 <math>\pm</math> 1.69</b> ***
Charge Q (pC)	1.42 $\pm$ 0.09	<b>1.11 <math>\pm</math> 0.12</b> *	1.10 $\pm$ 0.16	1.30 $\pm$ 0.14	<b>0.84 <math>\pm</math> 0.11</b> ***
T <sub>1/2</sub> (ms)	46.10 $\pm$ 1.86	41.94 $\pm$ 3.10	<b>65.49 <math>\pm</math> 6.62</b> **	<b>60.82 <math>\pm</math> 4.12</b> **	43.62 $\pm$ 2.17
T <sub>peak</sub> (ms)	22.76 $\pm$ 0.92	21.95 $\pm$ 2.06	<b>31.14 <math>\pm</math> 3.13</b> **	<b>29.75 <math>\pm</math> 2.40</b> **	22.00 $\pm$ 1.29

**Table 1: Characteristics of amperometric spikes from bovine chromaffin cells treated with puff application of SOM230 in pipette.** Amperometric recordings were performed on cultured bovine chromaffin cells. Cells were stimulated with 100  $\mu\text{M}$  of nicotine and the indicated SOM230 concentration for 10 s. The number of cells, amperometric spikes per cell, amperometric spikes, and single-event kinetic parameters are indicated. Results represent the mean  $\pm$  SEM. Bold values are considered significantly different from the control condition; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to control; Mann Whitney Rank Sum test.

#	Age	Gender	Symptoms	Urinary MN (ULN ratio)	Urinary NMN (ULN ratio)	Plasma free MN (ULN ratio)	Plasma free NMN (ULN ratio)	Plasma CGA (ULN ratio)	Size (cm)	Ki-67 (%)	PASS score	Genetics
1	47	F	Yes	4.7	23.4	-	-	1.2	7	1	0	Spor
2	58	F	Yes	-	-	1.9	2.5	-	2	1	1	NF1
3	58	F	Yes	2.8	10.4	4.4	7.4	-	3.5	1	4	-
4	56	F	No	-	-	4.9	8	4.9	4.9	1	1	-
5	72	M	Yes	10.3	9.6	-	-	-	6.5	1	2	-
6	70	F	Yes	1.4	5.6	-	-	-	3.5	2	2	NF1
7	52	F	No	13.3	0.8	21.9	2.0	-	5	1	2	-
8	61	M	No	1.2	1.3	0.9	2.3	0.8	1.8	4.8	1	-
9	77	M	Yes	10.6	4.6	-	-	-	4	5	2	-
10	72	M	Yes	3.8	2.1	3.4	1.45	2.2	2.8	1.5	1	-

**Table 2: Clinical, biochemical, and functional characteristics of the 10 patients with pheochromocytoma evaluated in this study.** Age at diagnosis, gender (F: female, M: male), **the presence (Yes) or absence (No) of hormonal** hypersecretion symptoms, biochemical and functional values are represented for each patient number (#).MN: metanephrine, NMN: normetanephrine, ULN: upper limit normal, CGA: chromogranin A, PASS: Pheochromocytoma of the Adrenal Gland Scaled Score, Spor: sporadic, NF1: neurofibromatosis type 1, - : not available.

Identification	Treatment	Number of cells	Total number of spikes	Number of spikes/cell	Number of analyzed spikes	I <sub>max</sub> (pA)	Charge Q (pC)	T <sub>1/2</sub> (ms)	T <sub>peak</sub> (ms)
Patient 1	Control	12	482	37.08 ± 5.96	334	21.58 ± 2.30	0.93 ± 0.14	37.39 ± 2.83	20.30 ± 3.34
	100 μM	13	61	<b>4.69 ± 1.87 ***</b>	46	<b>13.29 ± 1.86 *</b>	<b>0.50 ± 0.07 *</b>	33.05 ± 2.46	13.84 ± 1.40
Patient 2	Control	15	866	57.73 ± 2.76	400	23.72 ± 1.15	1.04 ± 0.06	39.65 ± 0.89	30.66 ± 0.84
	100 μM	22	191	<b>8.68 ± 0.77 ***</b>	70	<b>19.75 ± 1.58 *</b>	0.98 ± 0.11	<b>46.65 ± 2.81 *</b>	<b>35.79 ± 1.88 *</b>
Patient 3	Control	9	379	42.11 ± 5.25	276	15.84 ± 2.19	0.60 ± 0.10	33.53 ± 2.29	16.95 ± 1.58
	0.1 μM	4	156	39.00 ± 10.70	110	16.28 ± 3.38	0.72 ± 0.19	39.57 ± 2.33	20.41 ± 1.61
	1 μM	12	287	<b>23.92 ± 2.05 *</b>	170	14.99 ± 0.91	0.54 ± 0.04	33.06 ± 1.86	16.90 ± 1.23
	10 μM	7	132	<b>18.86 ± 4.58 *</b>	107	19.16 ± 4.80	0.56 ± 0.13	28.50 ± 1.42	13.30 ± 1.07
Patient 4	Control	12	187	11.69 ± 1.69	118	13.28 ± 1.23	1.33 ± 0.18	91.26 ± 6.40	47.43 ± 3.60
	0.1 μM	11	112	<b>10.18 ± 3.83 *</b>	76	<b>7.96 ± 0.73 **</b>	0.92 ± 0.08	105.13 ± 4.56	57.91 ± 3.91
	1 μM	14	63	<b>4.50 ± 1.39 ***</b>	44	9.89 ± 1.10	1.12 ± 0.18	101.16 ± 5.65	54.60 ± 3.49
Patient 5	Control	33	516	15.64 ± 1.85	436	15.21 ± 0.94	1.11 ± 0.06	71.74 ± 2.61	35.78 ± 1.40
	0.1 μM	24	46	<b>1.92 ± 0.48 ***</b>	40	<b>10.73 ± 2.86 ***</b>	<b>0.90 ± 0.15 *</b>	80.76 ± 5.36	40.89 ± 2.95
Patient 6	Control	33	662	20.06 ± 2.13	556	19.44 ± 2.15	0.72 ± 0.10	34.32 ± 2.07	16.46 ± 1.18
	0.1 μM	23	201	<b>8.74 ± 1.74 ***</b>	185	16.07 ± 2.79	0.66 ± 0.12	38.33 ± 1.87	19.55 ± 1.37
	1 μM	25	156	<b>6.24 ± 1.16 ***</b>	138	<b>13.09 ± 1.50 *</b>	0.49 ± 0.06	35.98 ± 1.87	17.82 ± 1.07
	10 μM	19	10	<b>0.53 ± 0.42 ***</b>	7	-	-	-	-
Patient 7	Control	22	627	28.50 ± 4.86	555	12.79 ± 0.83	0.68 ± 0.04	52.05 ± 2.27	25.85 ± 1.40
	0.1 μM	19	200	<b>10.53 ± 3.28 ***</b>	169	<b>10.28 ± 0.66 *</b>	0.64 ± 0.04	<b>59.30 ± 2.55 *</b>	<b>31.72 ± 1.69 *</b>
Patient 8	Control	29	547	18.23 ± 2.06	449	12.91 ± 0.88	0.82 ± 0.07	61.66 ± 3.54	34.18 ± 2.23
	0.01 μM	21	355	16.90 ± 3.43	314	<b>10.78 ± 0.74 *</b>	0.76 ± 0.05	65.91 ± 3.09	36.68 ± 2.15
	0.1 μM	24	254	<b>10.58 ± 2.13 **</b>	224	15.63 ± 1.67	1.00 ± 0.12	60.18 ± 2.74	32.39 ± 1.47

**Table 3: Characteristics of amperometric spikes from 8 pheochromocytomas treated with SOM230.** Amperometric recordings were performed on primary culture of human pheochromocytoma cells. Cells were stimulated with 100 μM of nicotine and the indicated SOM230 concentration for 10 s. For patients 1 to 4, SOM230 is in pipette stimulation and for patients 5 to 8, SOM230 is incubated in the cell medium. The number of cells, amperometric spikes per cell, amperometric spikes, and single-event kinetic parameters are indicated. Results represent the mean ± SEM. Bold values are considered significantly different from the control conditions; \*p< 0.05, \*\*p< 0.01; \*\*\*p< 0.001 compared to control; Mann Whitney Rank Sum test.

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## Highlights

- The somatostatin analogue Pasireotide (SOM230) inhibits catecholamine secretion in tumor cells cultured from freshly resected human pheochromocytoma.
- SOM230 inhibits catecholamine secretion by reducing the number of exocytic events per tumor cell.
- SOM230 inhibits nicotine-induced calcium influx and impact the function of the nicotinic receptor in pheochromocytoma cells.
- Treatment of pheochromocytoma cells with the somatostatin analogue octreotide has no effect on catecholamine secretion.

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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