The firing frequency of spontaneous action potentials and their corresponding evoked exocytosis are increased in chromaffin cells of CCl₄-induced cirrhotic rats with respect to control rats

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Abstract

High catecolamine plasma levels because of sympathetic nervous system over-activity contribute to cirrhosis progression. The aim of this study was to investigate whether chromaffin cells of the adrenal gland might potentiate the deleterious effect exerted by this over-activity. Electrophysiological patch-clamp and amperometric experiments with carbon-fibre electrodes were conducted in single chromaffin cells of control and CCl₄-induced cirrhotic rats. The spontaneous action potential firing frequency was increased in chromaffin cells of cirrhotic rats with respect to control rats. The exocytosis evoked by that firing was also increased. However, exocytosis elicited by ACh did not vary between control and cirrhotic rats. Exocytosis triggered by depolarizing pulses was also unchanged. Amperometric recordings confirmed the lack of increased catecholamine release. The overall Ca²⁺ entry through voltage-dependent Ca²⁺ channels (VDCC), or in particular through Cav1 channels, did not vary between chromaffin cells of control and cirrhotic rats. The inhibition of VDCC by methionine-enkephaline or ATP was not either altered, but it was increased by adrenaline in cells of cirrhotic rats. When a cocktail composed by the three neurotransmitters was tested in order to approach a situation closer to the physiological condition, the inhibition of VDCC was similar between both types of cells. In summary, chromaffin cells of the adrenal gland might contribute to exacerbate the sympathetic nervous system over-activity in cirrhosis because of an increased exocytosis elicited by an enhanced spontaneous electrical activity.

Keywords: amperometry, chromaffin cell, cirrhosis, exocytosis, patch-clamp, spontaneous action potentials.

J. Neurochem. (2019) 148, 359–372.

Received August 15, 2018; revised manuscript received October 9, 2018; accepted October 18, 2018.
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Abbreviations used: CC, current clamp; Cₘ, plasma membrane capacitance; HSCs, hepatic stellate cells; RRID, research resource identifier; sAP, spontaneous action potentials; SHR, spontaneously hypertensive rats; SNS, sympathetic nervous system; TTX, tetrodotoxin; VC, voltage clamp; VDCC, voltage-dependent calcium channel; Vₛ, holding potential.

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Cirrhosis is a pathologically defined entity associated to a spectrum of characteristics clinical manifestations, which should be viewed as a common pathway of many types of chronic liver injury. Regardless of the cause of cirrhosis, the pathologic features consist of the development of fibrosis and architectural distortion with the formation of regenerative nodules. Eventually, cirrhosis progresses to hepatic insufficiency, complications of portal hypertension and death.

Several lines of evidence indicate that sympathetic nervous system (SNS) over-activity develops in the advanced stages of cirrhosis and contributes to disease progression. Activation of the SNS with increased plasma levels of noradrenaline and adrenaline into the circulation under in vivo sources that might provide noradrenaline for HSC function in vivo is as yet unclear. Thus, the relevance of SNS over-activity in cirrhosis has fostered the interest to identify whether other sources of catecholamines, such as the chromaffin cells of the adrenal gland, may potentiate the SNS deleterious effect in this disease. We aimed at investigating in chromaffin cells of the adrenal gland of rats with CCl4-induced cirrhosis whether exocytosis elicited at basal conditions because of the spontaneous firing of action potentials, or exocytosis triggered by different stimuli (ACh or depolarizing pulses), might be altered in cirrhosis. We also investigated whether Ca^{2+} currents, flowing through voltage-dependent Ca^{2+} channels (VDCC) or their modulation by neurotransmitters, were modulated in chromaffin cells of the adrenal gland of cirrhotic rats in comparison to control rats.

**Material and methods**

**Animals**

Male Wistar Hanover rats (RccHan:WIST) (Envigo, Horst, The Netherlands, cat. #168) of 20 to 31 weeks old were used for all the experiments (Fig. 1). They had an average weight of 385 g. Animals were fed with a standard laboratory diet with water and food provided ad libitum. The study was not pre-registered. Experiments were approved by and performed in accordance with the Ethic Committee of the Universidad Autónoma de Madrid and Universidad de Alcalá regulations and conducted according to the European Directive 2010/63/EU and Royal Decree 53/2013 from Spain. No randomization method was employed to allocate animals to different experimental groups.

**Induction of cirrhosis**

Cirrhosis was induced by CCl4 (Sigma Aldrich, Madrid, Spain, cat. #289116), feeding by gavage on a weekly basis, along with phenobarbital (Química Farmacéutica Bayer, Barcelona, Spain) bought in a local pharmacy, added to the drinking water (0.35 g/L). The initial 20 μL dose of CCl4 was subsequently increased, depending on the animal weekly change in body weight until ascites formation (Runyon et al. 1991). Experiments were performed 7 days after the last CCl4 dose (Fig. 1). Cirrhosis was confirmed by trichrome staining of livers. Only rats with ascites were included in the study (n = 18). Two rats with cirrhosis but without ascites were excluded from the study. Phenobarbital-treated age- and sex-matched rats were used as the control group (n = 16). No statistical methods were performed to determine the sample size. Barbiturates have been reported to inhibit nicotinic acetylcholine receptors in a reversible manner (Watanabe et al. 1999), and therefore, phenobarbital should not affect the response to ACh in these experiments.

**Isolation and culture of rat chromaffin cells**

Animals were killed inside a gas chamber and were opened along the peritoneal cavity. To obtain the glands, tweezers of sharp tip and scissors were employed. Once removed, they were placed on a 35 mm diameter Petri dish with Locke solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO3, 5 mM HEPES and 5.6 mM glucose,) maintained on ice. Under the microscope, fat tissue and cortex were taken off by means of tweezers and scalpel on the Petri dish with ice. The medullae were introduced in Locke solution, and afterwards they were incubated during 60 min in control rats, or...
50 min in cirrhotic rats, in a solution with trypsin (2.5%) (GIBCO, Invitrogen Life Technologies, Massachusetts, USA, cat. #15090-046) and collagenase (1%) (Sigma-Aldrich, cat. #C0130) at 37°C. After this time they were washed with Locke, and later on with Dulbecco’s Modified Eagle’s Medium (Sigma-Aldrich, cat. #D6546) that was supplemented with 1% penicillin-streptomycin (Sigma-Aldrich, cat. #P4333), 1% GlutaMAX (GIBCO, cat. #35050038) and 5% foetal bovine serum (Labelcins, cat. #S181B). Mechanic digestion was performed by passing the medullae through a pipette tip until obtaining an homogeneous suspension. The cells were plated on glass coverslips previously treated with polylysine (0.1 mg/mL) (Sigma-Aldrich, cat. #P2636). Finally they were introduced in an incubator with saturated atmosphere of water vapour, 95% of O₂ and 5% of CO₂. Experiments were performed 1–4 days after plating on matched time periods.

Electrophysiological recordings and analysis of data

For the conventional whole-cell recordings of Ca²⁺ currents, the external solution was (in mM): 10 BaCl₂, 100 NaCl, 45 TEACl, 10 HEPES, 5.5 KCl, 0.002 TTX and 10 glucose (pH 7.4). The intracellular solution composition was (in mM): 10 NaCl, 100 CsCl, 10 TEACL, 5 MgATP, 0.3 NaGTP, 14 EGTA and 20 HEPES (pH 7.2).

For the perforated-patch whole-cell recordings of Ca²⁺ currents, the external solution was (in mM): 5 CaCl₂, 100 NaCl, 45 TEACl, 5.5 KCl, 1 MgCl₂, 0.2 d-tubocurarine, 0.002 TTX, 0.0002 apamin, 10 HEPES and 10 glucose (pH 7.4). The intracellular solution composition was (in mM): 145 Cs-glutamate, 8 NaCl, 1 MgCl₂, 0.5 amphotericin B and the pH was adjusted to 7.2 with CsOH. To record action potentials in the current-clamp perforated-patch configuration, the external solution was (in mM): 2 CaCl₂, 145 NaCl, 5 KCl, 1 MgCl₂, 10 HEPES and 10 glucose (pH 7.4). The intracellular solution composition was (in mM): 145 K-glutamate, 8 NaCl, 1 MgCl₂, 10 HEPES and 0.5 amphotericin B (pH 7.2).

An amphotericin B (Sigma-Aldrich, cat. #A4888) stock solution was prepared every day at a concentration of 50 mg/mL in dimethyl sulphoxide and kept protected from light. The final concentration of amphotericin B was prepared by ultrasonicating 10 μL of stock amphotericin B in 1 mL of Cs-glutamate internal solution in the dark. Pipettes were tip-dipped in amphotericin-free solution for several seconds and back-filled with freshly mixed intracellular amphotericin solution.

Chemical compounds were purchased from Sigma-Aldrich. Experiments were performed at room temperature (22–24°C). There were no sample size differences between the beginning and end of the experiments. No blinding procedures were performed.

Drugs were applied by means of a multi barrelled pipette that was constructed using polyethylene tubing with an inner diameter of 0.4 mm. These tubes coalesced to a single outlet tube with a 0.28 mm inner diameter. Drugs were delivered by gravity and were controlled by a valve controller triggered by the amplifier. A Picospritzer III (General Valve Corp., Fairfield, NJ, USA) was used to apply ACh to the cell by means of pressure ejection (15 psi) through a glass capillary tube (World Precision Instruments, Sarasota, FL, USA; cat. 1B200F-4) that was pulled to obtain an opening of 1–2 μm in diameter. For a rapid wash-out of ACh, a pipette with a polyethylene tube with an inner diameter of 0.58 mm was used. The outlet of this tube was placed just behind, close to the glass capillary tube of the Picospritzer.

Electrophysiological measurements were made using an EPC-10 amplifier and PULSE software (HEKA Elektronik, Lambrecht, Germany) running on a PC computer. Glass electrodes were pulled from borosilicate glass capillaries (Kimbal Chase, cat. #3400-99) using a P97 pipette puller (Sutter Instruments, Novato CA, USA). These electrodes had resistances between 1.5 and 3 MΩ when they were filled with the internal electrode solutions. Borosilicate glass capillary tubes were partially coated with wax and fire polished. Only recordings in which the leak current and access resistance were lower than 20 pA and 25 MΩ in the perforated-patch configuration.
respectively, were accepted. We noticed more difficulty in perforating the chromaffin cell membrane of the 5–7 months old rats of this study with respect to younger rats. Cell membrane capacitance (Cm) changes as an index of exocytosis were estimated by the Lindau-Neher technique implemented in the ‘Sine-DC’ feature of the ‘PULSE’ lock-in software. A 1 kHz, 70 mV peak-to-peak amplitude sinewave was applied at a holding potential (Vh) of −80 mV. The signals were sampled at 10 kHz and filtered at 1 kHz through a Bessel filter. Cells were clamped at a Vh of −80 mV with the exception of those experiments performed using ACh as a stimulus and the “triple-step” protocol to measure exocytosis (Pérez-Alvarez and Albillos 2007), which were carried out at the resting membrane potential of each individual cell. Chromaffin cells of control or cirrhotic rats exhibited the same specific capacitance, 7.8 ± 0.4 mF (n = 70) and 7.1 ± 0.3 mF (n = 77), respectively, reflecting a similar size.

Analysis of electrophysiological data was conducted using IGOR Pro software (RRID: SCR_000325, WaveMetrics, Lake Oswego, Oregon). The ROUT test of GraphPad Prism was used to identify outliers. In the analysis of the inhibition by ATP of the Ca2+ current, 1 of 9 control cells and 1 cell of 10 cirrhotic cells, respectively, were excluded from the analysis (they potentiated the current by 2.2% and 22.1% respectively). In the case of the inhibition of VDCC by adrenaline, 1 of 13 control cells was excluded from the analysis (it potentiated the current by 84%). In addition, 1 of 17 control cells and 1 of 12 cirrhotic cells showed potentiation in the perforated-patch clamp configuration after perfusion with a cocktail of methionine-enkephalin, ATP and adrenaline (4.3% and 9.5%, from control and cirrhotic rats respectively). These cells were not either included in the analysis of the inhibition of VDCC by the cocktail of compounds.

In the triple step protocol, the Cm increment was calculated from the baseline in Step 1 to the initial value of Cm recorded in Step 3, once conductances activated in Step 2 returned to the zero value. In the case of Cm increments because of depolarizing pulses, they were calculated from the baseline Cm recorded before the depolarizing pulse to the maximum value obtained after the pulse, once activated conductances during the depolarization returned to zero. Inhibition of Ca2+ currents were calculated as the decrease between the peak control current value and the corresponding current value in the presence of the drug under study. The nonspecific background current and Cm recorded under 200 µM CdCl2 were subtracted off-line from Ca2+ current and Cm traces. Protocols were repeated every 5 min in order to obtain an average value of two or three measurements of exocytosis.

Amperometric recordings and analysis of data
Carbon fibre electrodes were prepared by cannulating a 10 µm-diameter carbon fibre in polyethylene tubing (diameter: outer, 1 mm; inner, 0.5 mm). The carbon fibre tip was glued into a glass capillary for mounting on a patch-clamp headstage, and back filled with 3 M KCl to connect to the Ag/AgCl wire, which was kept at +700 mV. The carbon fibre electrode was gently placed on top of the cell under study. Amperometric currents were recorded using an EPC-10 amplifier and PULSE software running on a PC computer. The sampling rate was 14.5 kHz. Samples were digitally filtered at 0.1 kHz. The sensitivity of the electrodes was routinely monitored before and after the experiments using 50 µM of adrenaline as standard solution. Only fibres that rendered 200–300 pA of current increment after 50 µM of adrenaline pulse were used for the experiments. The tip of the fibre was recut for each experiment and calibrated again.

Chromaffin cells were perfused with a solution containing (in mM): 145 NaCl, 1 MgCl2, 10 HEPES, 5.5 KCl, 2 CdCl2 and 10 glucose (pH 7.4). Cells were stimulated with 300 µM ACh or 100 mM K+. There were no sample size differences between the beginning and end of the experiments. No blinding procedures were performed.

Spike analysis was performed using IGOR Pro software and macros that allowed the analysis of single events and the rejection of overlapping spikes (Mosharov and Sulzer 2005). A threshold of 4.5 times the first derivative of the noise standard deviation was calculated to clearly detect amperometric events. Then, among the events whose first derivative was above this threshold, only those showing one peak and one rising and falling phase, were considered as single spikes.

Statistical analysis of data
Statistical analysis was performed using SPSS 24.0 (RRID: SCR_002865, Armonk, NY, USA). Data were given as the mean ± SEM for the number (n) of cells. The normality test Kolmogorov–Smirnov was first performed. Data were then compared using the unpaired Student’s t-test or Mann-Whitney U test. Data were found statistically significant when *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001.

Results
Exocytosis elicited by the firing of spontaneous action potentials
The exocytosis or fusion process of chromaffin vesicles with the plasma membrane was determined by recording the increments in plasma membrane capacitance (Cm) (Lindau and Neher 1988; Albillos et al. 1997). We first investigated whether exocytosis because of the firing of spontaneous action potentials (sAP) at rest could be responsible for the increased basal catecholamine plasma levels previously reported in cirrhosis. Chromaffin cells fire sAP (Biales et al. 1976; Marcantoni et al. 2007, 2009, 2010; Pérez-Alvarez et al. 2011; Hernández-Vivanco et al. 2017), but it has not been probed so far that these sAP can evoke the fusion of secretory vesicles with the plasma membrane, although catecholamine release has been determined (Vandael et al. 2015a). Here we measured for the first time the exocytosis because of the firing of sAP using the ‘triple-step’ protocol previously performed in our laboratory (Pérez-Alvarez and Albillos 2007). It consists in the successive switch from the voltage-clamp configuration to determine basal Cm (Step 1), to the current-clamp configuration to allow sAP firing (Step 2), and then again to the voltage-clamp configuration to measure the increased Cm because of the previous firing of sAP (Step 3). A scheme of the protocol is displayed in Fig. 2a. This protocol was repeated every 5 min in order to
Fig. 2 Exocytosis elicited by the firing of spontaneous action potentials (sAP). (a) Scheme of the 'Triple-step' protocol used to record the exocytosis elicited by sAP, which consisted in the switch from the voltage-clamp configuration (VC) in the Step 1 to measure basal exocytosis, to the current-clamp configuration (Step 2), and then switch again to the VC configuration (Step 3) to record the increment in $C_m$ elicited by the sAP recorded in Step 2. $V_h$ was the resting membrane potential of each particular cell. This protocol was performed in control $n = 5$ cells (panel b) and cirrhotic $n = 7$ cells (panel c) from three independent cultures. It was repeated two-three times every 5 min. The time course of firing is showed in the separate panels corresponding to the grey windows above.

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obtain an average value of two or three measurements of exocytosis. The \( V_h \) was the resting membrane potential of each cell, which was similar between chromaffin cells of control and cirrhotic rats. The resting membrane potential was obtained 5 min after establishing the current-clamp configuration. Values were \(-52.5 \pm 2.6 \text{ mV} \) in control rats \((n = 8)\) and \(-54.6 \pm 3.5 \text{ mV} \) in cirrhotic rats \((n = 13)\). The sAP firing frequency was \(1.4 \pm 0.4 \text{ Hz} \) \((n = 5)\) and \(2.6 \pm 0.3 \text{ Hz} \) \((n = 7)\) \((p \leq 0.05)\), and the exocytosis achieved was \(46.8 \pm 13 \text{ fF} \) \((n = 5)\) and \(160.3 \pm 31.1 \text{ fF} \) \((n = 7)\) in chromaffin cells of control and cirrhotic rats respectively \((**p \leq 0.01)\). Representative recordings of this type of experiment are shown in Fig. 2b and c for control and cirrhotic rats respectively. The time course of firing is showed into more detail in the grey separate panels on an expanded time scale. The half-width \((\text{duration of the sAP at 50\% of its peak amplitude})\) and amplitude \((\text{maximal potential achieved from the basal potential})\) of the sAP were unchanged between control and cirrhotic rats. Values amounted to \(6 \pm 0.7 \text{ ms} \) and \(5.6 \pm 0.3 \text{ ms} \) for the half-width and \(50.6 \pm 2.6 \text{ mV} \) and \(46.4 \pm 3.7 \text{ mV} \) for the amplitude of the sAP in control \((n = 5)\) and cirrhotic rats \((n = 7)\) respectively.

**Exocytosis evoked by short pulses of ACh or depolarizing pulses**

Plausible changes in relation to the stimulated exocytosis were also evaluated by applying two types of stimuli in control rats in comparison to cirrhotic rats: ACh, the physiological neurotransmitter at the synapse chromaffin cell-splanchnic nerve, and depolarizing stimuli. In the first case, 10 ms pulses of 300 \(\mu\text{M} \) concentration ACh (11 pulses) at 0.2 Hz were applied at the resting membrane potential of each cell. That concentration of ACh was chosen for two reasons. First, in a post-synaptic area of 1 mm\(^2\) with a 50-nm-wide synaptic cleft, the peak ACh concentration is \(0.3 \text{ mM} \) (Kuffler and Yoshikami 1975; Scimemi and Beato 2009). Second, 300 \(\mu\text{M} \) is the concentration of ACh that elicits larger nicotinic currents with the minimum open-channel block effect in human chromaffin cells (Pérez-Alvarez and Albillos 2007; Pérez-Alvarez et al. 2012a,b; Hone et al. 2015, 2017), and also in rat chromaffin cells (Hone et al., unpublished data). We tested a very short duration of ACh pulses (10 ms) applied at the resting membrane potential of the cell and at the basal firing frequency of the splanchnic nerve, to mimick conditions closer to the physiological situation. The overall exocytosis evoked by ACh was also determined by using the ‘triple-step’ protocol (increment between the \( C_{in} \) of Step 3 and 1) (Fig. 3a). Changes in the membrane potential because of the application of 10 ms ACh pulses were recorded in the current-clamp configuration (Step 2). ACh evoked ‘Spike-like’ changes in the membrane potential. The triple-step protocol was repeated every 5 min to obtain an average value of exocytosis. The exocytosis triggered by the ACh pulses was \(278.1 \pm 76 \text{ fF} \) \((n = 8)\) and \(272.6 \pm 86 \text{ fF} \) \((n = 6)\) in control and cirrhotic rats respectively. Representative recordings of this protocol are shown in Fig. 3b and c for control and cirrhotic rats respectively.

In a different set of experiments, depolarizing pulses of 200 ms to the peak current voltage were applied every 5 min. In order to know the voltage at which Ca\(^{2+}\) current was maximal, a ramp test of 200 ms from -100 mV to 100 mV was applied at the beginning of the experiment. The increment achieved in \( C_{in} \) was \(71 \pm 12.4 \text{ fF} \) \((n = 13)\) and \(95.8 \pm 20 \text{ fF} \) \((n = 11)\), in control and cirrhotic rats respectively. No significant statistical differences were found between both groups of rats. Endocytosis was also similar between cells of control and cirrhotic rats, amounting to \(71.7 \pm 21.3 \text{ fF} \) \((n = 13)\) and \(79.1 \pm 23.8 \text{ fF} \) \((n = 11)\) respectively. Some cells did not exhibit endocytosis, whereas other cells showed a variable degree of compensatory endocytosis in both groups of cells. Representative \( C_{in} \) recordings are displayed in Fig. 3d and e for control and cirrhotic rats respectively.

**Release of catecholamines in cells stimulated with ACh or K\(^+\)**

In order to confirm the data obtained by \( C_{in} \) measurements in relation to the trigger of the exocytosis by ACh or depolarization, the release of catecholamines elicited by activation of nAChRs using ACh as stimulus, or by depolarization of the plasma membrane using 100 mM K\(^+\), was now recorded using a carbon fibre electrode. The transient current of oxidation because of the catecholamines oxidised at the surface of the electrode, at +700 mV potential, was recorded and compared in chromaffin cells of control and cirrhotic rats. Representative recordings of catecholamine release of single chromaffin cells stimulated with 2 s pulses of 300 \(\mu\text{M} \) ACh are displayed from control and cirrhotic rats (Fig. 4a and b respectively) or 100 mM K\(^+\) (Fig. 4c and d respectively). The charge corresponding to the overall catecholamines released was similar for both stimuli between control and cirrhotic rat chromaffin cells. The values achieved were \(25.3 \pm 5 \text{ pC} \) \((n = 13)\) and \(39 \pm 10 \text{ pC} \) \((n = 13)\) for control and cirrhotic ACh-treated cells, respectively, and \(37.4 \pm 6.3 \text{ pC} \) \((n = 17)\) and \(46.3 \pm 8 \text{ pC} \) \((n = 14)\) for control and cirrhotic K\(^+\)-treated cells, showing no significant statistical differences.

A detailed analysis of the amperometric spikes obtained was performed. The following parameters were determined: \( I_{\text{max}} \): peak amplitude; \( Q \): charge; \( m \): ascending slope; rise 25-75, time calculated from the linear portion of the ascending trace between 25\% and 75\% of the \( I_{\text{max}} \); fall 75-25, time calculated from the linear portion of the descending trace between 75\% and 25\% of the \( I_{\text{max}} \): base, time between the spike beginning and end respectively; and \( t_{1/2} \), half-width or duration of the amperometric signal at 50\% of its peak amplitude. A scheme of these parameters is shown in
Fig. 4e. Values were shown in bar diagrams for control (black) and cirrhotic (grey) rats in Fig. 4f and g, for ACh and K+ respectively. It was found that the amperometric spikes evoked by ACh or K+ in cirrhotic rats exhibited larger $I_{\text{max}}$ and $m$ values, and smaller rise 25-75, fall 75-25, base or $t_{1/2}$ values (Table 1). This reflects that catecholamines were released faster in chromaffin cells of cirrhotic rats, although the total charge remained unchanged.

$Ca^{2+}$ entry charge flowing through VDCC and their modulation by opioids, ATP and adrenaline

Depolarizing pulses of 50 ms were applied at the voltage at which $Ca^{2+}$ current was maximal in the perforated-patch configuration. To know the value of this voltage, a ramp test of 200 ms from $-100$ mV to 100 mV was applied at the beginning of each experiment. The total $Ca^{2+}$ charge recruited by these depolarizing pulses was $11.1 \pm 1 \mu C$ ($n = 16$) and $10.7 \pm 1.5 \mu C$ ($n = 11$) in control and cirrhotic rats respectively. No significant statistical differences were found. Therefore, it was not expected that Cav1 channel expression would have been modified, as reported in activated HSCs in comparison with quiescent HSCs (Bataller et al. 2001).

Anyway, the contribution of Cav1 channels to the overall $Ca^{2+}$ current was evaluated using 3 $\mu$M nifedipine to isolate Cav1 channels by subtracting the current in the presence of the drug from the control current, as performed in previous studies.
Ca2+ currents were elicited by 100 ms depolarizing pulses. Nifedipine block of the Ca2+ charge was similar in control and cirrhotic rats, amounting to 44.4 ± 6% (n = 9) and 42.8 ± 3.6% (n = 14) respectively. Representative recordings of Ba2+ current traces before and after perfusion with nifedipine are shown in Fig. 5a and b, respectively, for control and cirrhotic rats.

However, modulation of VDCC by the neurotransmitters released by chromaffin cells might be impaired in cirrhotic rats, modifying the amount of Ca2+ entry to the cytosol. This would lead to impaired exocytosis and catecholamine release. The block of methionine-enkephalin, ATP and adrenaline was analyzed separately using the conventional whole-cell configuration of the patch-clamp technique and 10 mM Ba2+ as cation charger in control and cirrhotic rats. This configuration of patch-clamp was employed in order to introduce GTP inside of the cell, avoiding a decrease in the modulatory effect by the neurotransmitters because of GTP consumption along the experiment. The protocol consisted of depolarizing pulses of 50 ms from the Vh to 0 or +10 mV, applied every 15 s. The three neurotransmitters were used at 10 μM concentration (Albillos et al. 1996a and b; Gandía et al. 1993). Methionine-enkephalin inhibited by 21.5 ± 5% (n = 12) and 15.8 ± 4.1% (n = 9) the Ba2+ charge in control and cirrhotic rats respectively (Fig. 5c and d). ATP inhibited by 24.6 ± 9% (n = 8) and 27.4 ± 10.4% (n = 9) the Ba2+ charge in control and cirrhotic rats respectively. Representative current recordings before and after perfusion ATP are displayed in Fig. 5e and f, for control and cirrhotic rats respectively. Finally, adrenaline inhibited by 14 ± 3.6% (n = 12) and 35 ± 6% (n = 6) the Ba2+ charge in control and cirrhotic rats respectively (**p ≤ 0.01). Representative current recordings before and after perfusion adrenaline are shown in Fig. 5g and h, respectively, for control and cirrhotic rats.

In order to know whether differences might exist when neurotransmitters were applied together, a condition closer to the physiological situation, the effect of a cocktail of...
methionine-enkephalin, ATP and adrenaline was investigated on VDCC in the conventional whole-cell configuration using 10 mM Ba$^{2+}$ as charge carrier in control and cirrhotic rats. The block of Ca$^{2+}$ charge achieved by the cocktail of neurotransmitters was $37.2 \pm 5\%$ ($n = 18$) and $50.5 \pm 7\%$ ($n = 12$) in control and cirrhotic rats respectively. No significant statistical differences were obtained. Representative recordings before and after perfusion the cocktail of compounds are shown in Fig. 5i and j respectively. We wanted to confirm the results obtained in the conventional whole-cell configuration using the whole-cell perforated-patch configuration (in 5 mM Ca$^{2+}$ as charge carrier) which avoids dilution of intracellular factors. Using this condition, the cocktail of compounds inhibited by $42.1 \pm 8.1\%$ ($n = 16$) and $39.4 \pm 6\%$ ($n = 11$) the Ca$^{2+}$ charge in control and cirrhotic rats. No significant statistical differences were obtained. Representative recordings before and after perfusion the cocktail of compounds are displayed in Fig. 5k and l, respectively, for control and cirrhotic rats.

**Discussion**

Cirrhosis is characterized by a SNS over-activity with elevated noradrenaline and adrenaline plasma levels that contribute to the progression of the disease. It has been postulated that other sources of catecholamines different to sympathetic nerve fibres, such as chromaffin cells of the adrenal gland medulla, may also regulate HSCs (Oben and Diehl 2004). Elevated catecholamines plasma levels have been reported in cirrhotic patients (Henriksen et al. 1984, 1985, 1987, 1988, 1998; Nicholls et al. 1985; Pozzi et al. 1994; Pozzi et al. 2001). In addition, also patients suffering essential hypertension (Goldstein et al. 1983) or spontaneously hypertensive rats (SHR) (Iriuchijima 1973; Grobecker et al. 1975) exhibit high catecholamine plasma levels. In this latter case, greater catecholamine release was observed, as compared with normotensive rats (Lim et al. 2002). Indeed, SHR chromaffin cells showed faster and larger catecholamine responses, explained by more vesicles ready to undergo exocytosis and greater quantal content of vesicles (Miranda-Ferreira et al. 2008) rather than altered Ca$^{2+}$ entry and subsequent redistribution into the endoplasmic reticulum or mitochondria (Miranda-Ferreira et al. 2009). Based on these results, our hypothesis posed that impaired Ca$^{2+}$ entry to the cytosol through VDCC or altered spontaneous actions potentials (sAP) in chromaffin cells might lead to a larger exocytosis and catecholamine release, that would contribute to exacerbate the negative effects of the SNS over-activity reported in liver cirrhosis. Similarly to what it was obtained in SHR chromaffin cells, amperometric spikes recorded using ACh or high concentration of K$^{+}$ showed faster catecholamine responses in cirrhotic with respect to control chromaffin cells. However, the quantal content of the vesicles remained unchanged.

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**Table 1 Kinetics parameters of the amperometric spikes obtained by stimulation with 300 μM ACh or 100 mM K$^{+}$ in chromaf

| Stimulus     | Number of Cells | Number of Spikes | I$_{\text{max}}$ (pA) | t$_{1/2}$ (ms) | Base (ms) | Rise 25-75 (ms) | Fall 75-25 (ms) | m (pA/ms) | Q_{0} (pC) | Q_{0} (pC) |
|--------------|-----------------|------------------|------------------------|----------------|-----------|----------------|----------------|-----------|-----------|-----------|
| ACh Control  | 16,13           | 225              | 13                     | 2.04 ± 0.1      | 0.9 ± 0.06 | 3.4 ± 0.4     | 0.8 ± 0.04     | 13.0 ± 1.1 | 0.47 ± 0.09 | 0.47 ± 0.09 |
| ACh Cirrhosis| 16,13           | 249              | 17                     | 2.2 ± 0.1       | 0.9 ± 0.04 | 3.4 ± 0.4     | 0.8 ± 0.04     | 16.0 ± 1.7 | 0.58 ± 0.08 | 0.58 ± 0.08 |
| K$^{+}$ Control | 16,13          | 269              | 17                     | 6.0 ± 0.6       | 0.58 ± 0.04 | 3.4 ± 0.4     | 0.8 ± 0.04     | 23.0 ± 1.3 | 0.58 ± 0.08 | 0.58 ± 0.08 |
| K$^{+}$ Cirrhosis | 16,13       | 293              | 14                     | 4.0 ± 0.4       | 0.58 ± 0.04 | 3.4 ± 0.4     | 0.8 ± 0.04     | 28.0 ± 1.3 | 0.58 ± 0.08 | 0.58 ± 0.08 |

* p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001.
Chromafin cells of the adrenal gland are modified post-ganglionic sympathetic neurons innervated by the splanchnic nerve that mainly control the release of adrenaline to the bloodstream, to prepare muscle and cardiovascular systems to a situation of stress. In humans, most of chromafin cells possess an adrenergic phenotype (Pérez-Alvarez et al. 2008).

Chromaffin cells store a pleayde of compounds which include catecholamines at concentrations as high as 1 M, opioids, ATP, chromogranins (components of the vesicular matrix) or Ca\(^{2+}\) in chromaffin vesicles, which are dense core granules that fuse with the plasma membrane through a Ca\(^{2+}\)-dependent mechanism called exocytosis. This process is mediated through SNAREs (Soluble N-ethylmaleimidesensitive factor Attachment Protein Receptors) and Rab family proteins. During exocytosis, a narrow fusion pore is formed, allowing the release of free catecholamines, and afterwards, this pore expands, releasing the whole content of the vesicle (‘full fusion event’). Alternatively, the opening of the fusion pore can be transient, allowing only the release of catecholamines but not of the vesicular matrix proteins (‘kiss and run event’) (Albillos et al. 1997). The transport of secretory vesicles, the docking of these vesicles with the plasma membrane, as well as the regulation of the expansion of the fusion pore, are also Ca\(^{2+}\)-dependent mechanisms. Therefore, regulation of exocytosis and the release process can be performed through regulation of Ca\(^{2+}\) entry, which would include, among other targets, VDCC.

We evaluated the exocytosis yielded by the firing of sAP in these cells by measuring the \(C_{Ca}\) increments owing to that firing. In mouse chromaffin cells, the catecholamine released by the firing of sAP was previously recorded using the carbon fibre amperometry in combination with current-clamp recordings (Vandael et al. 2015a). We achieved that measurement by using the ‘triple-step’ protocol previously
described in our laboratory that allows to determine $C_m$ increments because of the changes in membrane potential (Pérez-Alvarez and Albillos 2007). We obtained that the frequency of sAP firing in chromaffin cells of cirrhotic rats was increased with respect to control rats, and this elicited a higher exocytotic response. It has been shown that the firing mode can influence secretion in chromaffin cells (Duan et al. 2003; Vandael et al. 2015a; Guarina et al. 2017). However, in this study, the firing frequency of action potentials and the corresponding exocytosis increased without changing the firing mode.

Many ion channel types regulate chromaffin cell excitability, and therefore, might be responsible for the increased firing frequency. In chromaffin cells, Cav1 channels fire spontaneous action potentials (Marcantoni et al. 2007, 2010; Pérez-Alvarez et al. 2011). They express two Ca$^{2+}$ channel subtypes, Cav1.2 and Cav1.3, which open at relatively low membrane potentials and allow Ca$^{2+}$ to enter the cells near resting potentials (Marcantoni et al. 2010; Pérez-Alvarez et al. 2011). In this way, these channels shape the action potential waveform and pacemaker activity. Besides that, Cav1.3 supports most of the pacemaking current that sustains action potential firings and part of the catecholamine secretion (Vandael et al. 2015b). In cirrhotic rats a higher expression of Cav1 channels in HSCs has been shown (Bataller et al. 2001), which would allow larger Ca$^{2+}$ entry to the cytosol and therefore, Ca$^{2+}$ dependent-exocytosis and neurotransmitter or collagen release. Therefore, a larger expression of Cav1 channels, in particular of Cav1.3, might explain the increased frequency of firing in chromaffin cells of cirrhotic rats reported in this study. However, nifedipine block was similar in both types of rats, which indicates that a similar amount of Cav1 channels are expressed. Anyway, we can not exclude that Cav1.3 channels are more expressed in cirrhotic rats, as the dihydropyridine blocks both channel subtypes.

A strong Cav1.3-BK channels coupling in wild-type mouse chromaffin cells as well as in rat chromaffin cells have been also reported, although rat chromaffin cells express higher densities of BK channels (Prakriya and Lingle 1999; Marcantoni et al. 2010). These channels are activated by both the action potential and Ca$^{2+}$ entering the cytoplasm during the interspike, which is mainly carried by Cav1.3 channels. BK currents sustain mainly the afterhyperpolarization of the short action potential and only partially the pacemaker current during the long interspike in mouse chromaffin cells (Marcantoni et al. 2010). In addition, a Cav1.3-driven SK channel activation regulates pacemaking and spike frequency adaptation in mouse chromaffin cells (Vandael et al. 2012). Other channels such as Na$^+$ (Nav1.3 and Nav1.7) or K$^+$ channels (Kv1-3, Kv4, Kv7, Kv11 and K2P), also contribute to regulate chromaffin cell excitability and impact on it under pathological circumstances (Lingle et al. 2018). Further research would be required to clarify the ion channel conductances altered in chromaffin cells in cirrhosis.

In relation to the regulation of VDCC, these can be modulated by the products released through a negative feedback mechanism in bovine chromaffin cells (Albillos et al. 1996a and b). In particular, ATP (Gándía et al. 1993), opioids (Albillos et al. 1996a) and adrenaline (Albillos et al. 1996b) inhibit the overall Ca$^{2+}$ current recruited by step-wise depolarizing pulses through a modulatory pathway mediated by G proteins. Here we obtained that this modulatory pathway was not impaired in chromaffin cells of the adrenal gland of rats with CLC-induced cirrhosis in the case of methionine-enkephaline or ATP, but it was increased in the case of adrenaline. This might indicate an adrenergic over-activity in chromaffin cells of cirrhotic rats, which would be in line with the SNS over-activity reported in cirrhosis. However, when methionine-enkephaline, ATP and adrenaline were perfused together, the inhibition was increased to values between 40-50%, and they were similar in both types of cells. This may be because of the fact that all these neurotransmitters act through the same G-protein-mediated pathway, which would be saturated, reaching maximal and identical values in both types of cells. These experiments were performed in the conventional whole-cell configuration of the patch-clamp technique in order to introduce GTP inside of the patch-pipette, to avoid a decrease modulatory effect of the tested neurotransmitters as a consequence of GTP consumption during the experiment. Later on, experiments were also conducted in the perforated-patch configuration, to record the modulation of VDCC by the cocktail of neurotransmitters under more physiological conditions, which might include a differential cytosolic concentration of GTP between control and cirrhotic rats that would lead to a different modulatory effect by neurotransmitters. Using both methods, we obtained that the cocktail of ATP, methionine-enkephaline and adrenaline did not exert a different inhibitory effect on Ca$^{2+}$ currents between control and cirrhotic rats.

In summary, our data reveal that in cirrhosis, adrenal gland chromaffin cells of the sympato-adrenal axis might contribute to potentiate the SNS over-activity that worsens this disease by increasing the exocytotic response triggered by an enhanced spontaneous electrical activity.

Acknowledgments and conflict of interest disclosure

This work was supported by the Spanish Ministerio de Ciencia, Innovación y Universidades [Grants BFU2012-30997 and BFU2015-69092 awarded to Almudena Albillos; SAF 2017-86343-R awarded to Agustín Albillos]; Spanish Ministerio de Sanidad, Consumo y Bienestar Social, Instituto de Salud Carlos III (Plan Estatal de I+D+i 2013-2016) awarded to Agustín Albillos [Grants PI14/00876 and PI051871, CIBERehd], cofinanced by the
European Development Regional Fund ‘A way to achieve Europe’. The authors have no conflict of interest to declare.

All experiments were conducted in compliance with the ARRIVE guidelines.

**Authorship contributions**

*Participated in research design: Almudena Albillos; Conducted experiments: Sara Sanz-Lázaro, Amanda Jiménez-Pompa, Beatriz Carmona-Hidalgo, María Ubeda, Leticia Muñoz, Jose Carlos Caba-González, Alicia Hernández-Vivanco, Sarai López-García; Performed data analysis: Sara Sanz-Lázaro, Amanda Jiménez-Pompa, Beatriz Carmona-Hidalgo; Wrote or contributed to the writing of the manuscript: Almudena Albillos, Agustín Albillos.*

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