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RESEARCH PAPER

The quantal secretion of catecholamines is impaired by the accumulation of β-adrenoceptor antagonists into chromaffin cell vesicles

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Background and purpose: The delayed onset of certain effects of antagonists of β-adrenoceptors (β-blockers), such as lowering arterial blood pressure (several days), cannot be explained solely by their effects on β-adrenoceptors, an action that occurs within minutes. Although several mechanisms have been proposed, none of them explain this temporal delay. This work aimed at providing a new explanation based on the interference of these drugs with the functional accumulation of catecholamines within neurosecretory vesicles.
Experimental approach: We used the simultaneous on-line monitoring of catecholamine and labetalol release from bovine isolated chromaffin cells and from rat perfused adrenal glands, as well as single cell amperometry, intracellular electrochemistry, patch amperometry and HPLC.
Key results: Using amperometry, three β-blockers, labetalol, atenolol and propranolol, reduced the quantal size of secretory events in chromaffin cells, accompanied by a slowing down of exocytosis. By patch amperometry, we found that treatment with β-blockers also increases the chromaffin vesicle volume, thereby creating a functional dilution of catecholamines. Experiments with intracellular electrochemistry show that vesicles cannot uptake new catecholamines. There was progressive accumulation of labetalol in secretory vesicles of bovine adrenal chromaffin cells, and this β-blocker was co-released with catecholamines from rat and bovine chromaffin tissues.
Conclusions and implications: We propose that β-blockers are progressively concentrated into sympathetic secretory vesicles, and interfere with the storage of catecholamines and are co-released with the natural transmitters, resulting in a decrease in the sympathetic tone. This could explain the delayed onset of the hypotensive effects of β-blockers.
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Keywords: amperometry; chromaffin cells; patch amperometry; secretion; catecholamines; β-adrenoceptor; labetalol; propranolol; atenolol

Abbreviations: β-blocker, β-adrenoceptor antagonist; CgB, chromogranin B; DMPP, dimethylphenyl piperazinium; I_{max}, maximal intensity of amperometrical spikes; *m*, ascending slope of spikes; *Q*, spike charge; $t_{1/2}$, spike width at a half height

Introduction

For more than 40 years, β-adrenoceptor antagonists (β-blockers) have received considerable attention in medicine because of their proven efficacy in the treatment of hypertension. Propranolol does not immediately reduce blood pressure in hypertensive patients; however, after a few days of treatment, vascular resistance falls, causing its hypotensive effect (Man in't Veld *et al.*, 1988). Even though short-term effects can be fully explained based solely on their ability to interact with β-receptors, adequate explanation of the effects of long-term use of β-blockers is still lacking. Several hypotheses have been proposed in an effort to explain how these drugs work: reduction in plasma renin activity, blockade of presynaptic β-adrenoceptors, reduction in cardiac output or CNS effects. However, there are exceptions to all of these explanations, and none of them can account for their delayed onset (Goodman *et al.*, 2006). Moreover, there is no plausible explanation for the differences observed between plasma half-life

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and their therapeutic doses, especially for the β-blockers with high lipophilicity such as propranolol.

Chromaffin cells have been widely used as a model of sympathetic peripheral neurons to study stimulus-secretion coupling (Garcia *et al.*, 2006). Tissue accumulation and the release of β-blockers have been demonstrated in chromaffin cells (Kent *et al.*, 1981; Boksa, 1986), PC12 (Bright *et al.*, 1985; Webb *et al.*, 1988; Bagwell *et al.*, 1989), cortical synaptosomes (Bright *et al.*, 1985) and in peripheral sympathetic nerves (Bright *et al.*, 1985), thus prolonging their biological half-life. However, β-blockers do not modulate the secretion of catecholamines in chromaffin cells when they are applied acutely (Wakade, 1981; Orts *et al.*, 1987; Wan *et al.*, 1988), and their precise effects on quantal transmission were not studied due to technical limitations.

β-Blockers are sequestered by protonation into acidic organelles such as lysosomes (Henneberry *et al.*, 1986) or chromaffin granules (Kent *et al.*, 1981). The consequences of the vesicular accumulation of β-blockers for lowering blood pressure have not received much attention since they were described in the 1980s, even though propranolol and catecholamines are released after nerve stimulation (Russell *et al.*, 1983; Daniell *et al.*, 1988) or during exercise (Hurwitz *et al.*, 1983). However, any substance that is accumulated in a secretory vesicle in neurons might displace the natural neurotransmitter, thus reducing the final concentration of that transmitter in synaptic clefts. Furthermore, when the concentrated substance is pharmacologically active, its release in the synaptic cleft will also modify the transynaptic response considerably.

Here, we directly demonstrate the uptake and release of labetalol by chromaffin secretory vesicles, and how this agent and also atenolol and propranolol influenced the quantal release of catecholamines. We have also found that the accumulation of these β-blockers inside secretory vesicles is time and concentration dependent, and that this mechanism could, at least partially, explain the delayed onset in lowering arterial pressure in persons with elevated sympathetic tone, as observed in hypertensive patients.

Methods

For a more detailed description, please see the Supporting Information.

Culture of chromaffin cells

Bovine cells were prepared as described elsewhere (Moro *et al.*, 1990). Cells were plated in 35 mm Ø Petri dishes at 3×10^6 cells per well for on-line secretion analysis. Rat cells were cultured according to Gandia *et al.* (1995). Cells were plated on round glass coverslips placed in 24-well culture plates at an approximate density of 5×10^4 cells per well for amperometry and patch amperometry studies. Cells were used after 1–5 days in culture, at room temperature.

On-line analysis of catecholamines and labetalol released by cultured cells

The procedure for cell perfusion was similar to that previously reported (Kumakura *et al.*, 1986). Cells were plated in 35 mm diameter Petri dishes at 3×10^6 cells per well for on-line secretion analysis. Cells were scraped with a rubber policeman, gently centrifuged (500× *g*) and resuspended in 100 μL of Krebs–HEPES (in mmol·L^{-1}) NaCl (140), KCl (5.9), $MgCl_2$ (1.2), $CaCl_2$ (2), HEPES (10) and glucose (11), pH 7.35 (NaOH). Cells were then placed on a nitrocellulose filter with 3 μm pores (Millipore Iberica, Madrid, Spain, REF# SCWPO2500) in a plastic cell chamber. A roller pump delivered Krebs–HEPES solution with a flow rate of 1 mL·min^{-1}. The solution emanating from the cells was successively passed through a fluorimetric detector (Waters 470, Waters, Milford, MA, USA) to monitor the release of labetalol ($Ex_{\lambda 335\ nm}/Em_{\lambda 420\ nm}$), and through an electrochemical detector (CANSTAT-4, University of La Laguna, Tenerife, Spain) set to an oxidative potential of +650 mV for the analysis of catecholamines, as detailed in Supporting Information Figure S1. The fluorescence characteristics of labetalol are pH dependent; for this reason, it was important to adapt the detection conditions for each environmental pH (Supporting Information Figure S3).

Secretion could not be normalized as a percentage of the catecholamine content, because the accumulation of labetalol modified the amount of catecholamines present in cells. On the other hand, attempts to normalize secretion to the amount of protein present in the filters failed due to their interference with either the bicinchoninic acid or Bradford tests. To avoid the problems derived from the different amount of cells retained in the filters and the different responsiveness to dimethylphenyl piperazinium (DMPP) from one batch of cells to another, data are expressed as the fluorescence/amperometry ratios.

On-line analysis of catecholamines and labetalol released by rat adrenal glands

All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by The Ethical Committee of the University of La Laguna. Thirty adult male rats were distributed in three groups, given tap water (control), 40 mg·kg^{-1}·day^{-1} of labetalol or 4 mg·kg^{-1}·day^{-1} of (*S*)·(-)-propranolol dissolved in tap water, for a week. In anaesthetized rats (pentobarbital 50 mg·kg^{-1}), the left adrenal glands were retrogradely perfused through the adrenal vein with Krebs–bicarbonate solution (in mmol·L^{-1}): NaCl (119), KCl (5.6), Mg_2SO_4 (1.2), K_2HPO_4 (1.2), $CaCl_2$ (2.5), NaH_2CO_3 (25) and glucose (11) as described (Borges, 1993). The glands were placed into sealed plastic chambers, and the perfusate was analysed on-line as described for cultured cells (Supporting Information Figure S1).

Amperometry

Carbon fibre microelectrodes were prepared and calibrated as described (Kawagoe *et al.*, 1993; Machado *et al.*, 2008). Measurements were taken in a perfusion chamber positioned on the stage of an inverted microscope using an Axopatch 200B (Molecular Devices, Sunnyvale, CA, USA) with the microelectrode gently touching the cell membrane. Unless otherwise mentioned, cell release was stimulated by 10 s pressure ejection of 5 mmol·L^{-1} Ba^{2+} from a micropipette placed 40 μm away from the cell. Barium was used as the secretagogue for

single-cell measurements because it produces low-frequency secretory events, so that the initial and final points of each spike can be easily distinguished during spike analysis.

Patch amperometry

The procedure for electrode and pipette construction precisely followed the procedure described elsewhere (Dernick *et al.*, 2005) adapted to an Axopatch 200B (for capacitance and fusion pore conductance measurements) and to a VA-10 (NPI Electronics, Tamm, Germany; for amperometric measurements; see also Supporting Information Figure S5).

Data analysis

Data are shown as means ± SEM. Significant differences between means were assessed by the Mann–Whitney test, as the data were not normally distributed. For comparison of more than two means, we used ANOVA, as indicated in the figure legends.

Materials

Labetalol, atenolol, propranolol and pargyline were all obtained from Sigma-Aldrich, Madrid, Spain. The anaesthetic pentobarbital was from Vetoquinol, Buckingham. UK.

Results

Chromaffin granules accumulate labetalol concentration dependently

Bovine cells were incubated for 48 h with labetalol at 0.1, 1 or 10 μmol·L^{-1}. The drug was removed from the cultures by three consecutive washes, and the granules were purified. The granule contents of labetalol, analysed by HPLC, were (means ± SEM) 0.13 ± 0.002, 1.94 ± 0.37 and 11.2 ± 2.30 nmol·μg^{-1} protein for 0.1, 1 or 10 μmol·L^{-1} respectively. The absence of chromogranin B in the cell supernatant also indicated that labetalol did not affect the integrity of the granules (not shown).

Chromaffin cells release labetalol together with catecholamines from bovine and rat chromaffin cells

Figure 1 shows the on-line monitoring of secretion measured by simultaneous electrochemical and fluorescence detection. We chose labetalol for these experiments after analyzing the fluorescent properties of 12 different β-blockers (Supporting Information Table S1). Although almost all β-blockers were fluorescent, they were not suitable for these purposes because their excitation spectra were in the far UV range where many released substances were fluorescent. However, even under the more restricted fluorescence conditions set for labetalol ($Ex_{\lambda 335\ nm}/Em_{\lambda 420\ nm}$), for pH 7.35 (Supporting Information Figure S2), depolarizing stimuli such as DMPP caused small fluorescent signals (3–5 f.a.u.), even from untreated cells, caused by native fluorescent product(s) (Figure 1A). Conversely, the fluorimetric signals were consistently bigger in the perfusate from bovine cells treated with labetalol (26–63 f.a.u.) after

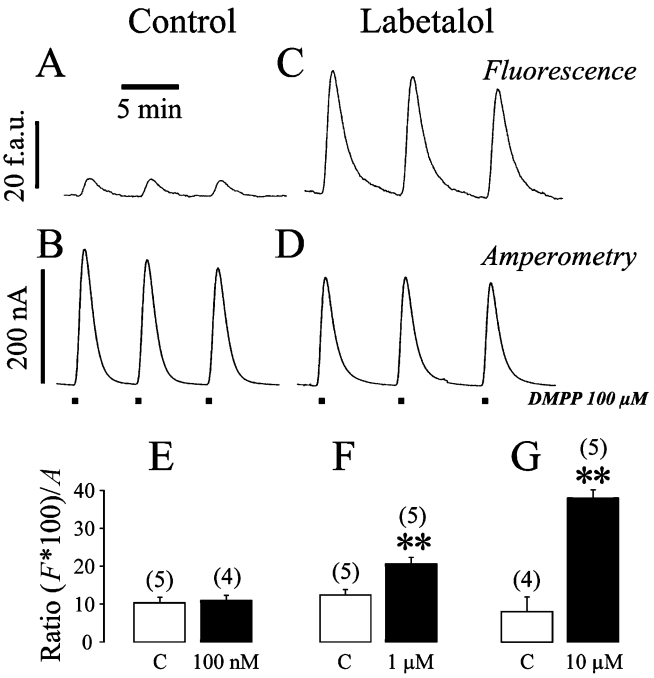

Figure 1 Labetalol is released together with catecholamines in superperfused bovine chromaffin cells. The experimental system is described in Supporting Information Figure S1. (A–D) The on-line recordings of the release of a fluorescent (f.a.u.) substance(s) and catecholamines (expressed in nA of oxidation current) in response to 10 s pulses of 100 μmol·L^{-1} of the nicotinic agonist DMPP applied every 5 min. (A) and (B) Representative traces obtained from untreated cells. (C) and (D) Obtained from cultured cells incubated for 24 h with 10 μmol·L^{-1} of labetalol. (E–G) The pooled data of fluorescence/amperometry ratios obtained by integration of the traces (areas under the curves) from cells treated for 24 h at the concentrations mentioned. Numbers in brackets indicate the number of different experiments carried out in the absence (C, control) and in the presence of labetalol. $^{**}P < 0.01$ (Mann–Whitney rank sum test).

triggering the release with DMPP. This effect was accompanied by a reduction in the amperometric signal indicating a lower catecholamine release per stimuli (Figure 1D). Therefore, the fluorescence/amperometry ratio directly increased with the concentration of labetalol. Fluorescence signals increased with the concentration and the time of incubation with labetalol. The differences in the total amount of cells trapped in the filters and in their responsiveness to DMPP caused large differences in the secretory responses from different batches of cells, and this obliged us to use fluorescence/amperometry ratios (Figures 1E–G and 2A).

Depletion of extracellular Ca^{2+} (replaced with Mg^{2+} plus EGTA 200 μmol·L^{-1}) eliminated both the fluorimetric and the amperometric responses, thus confirming the exocytotic nature of both signals. These experiments were done on two different cell batches (not shown).

The uptake and release of labetalol were also studied in intact adrenals using a similar set-up to that used for bovine chromaffin cells (Supporting Information Figure S1). We compared the secretion of catecholamines and β-blockers from adrenal glands of rats treated with labetalol with the release from adrenals of the control animals. The secretion of substances monitored with the fluorescence detector, set for labetalol, increased after stimulation with DMPP. Conversely, the

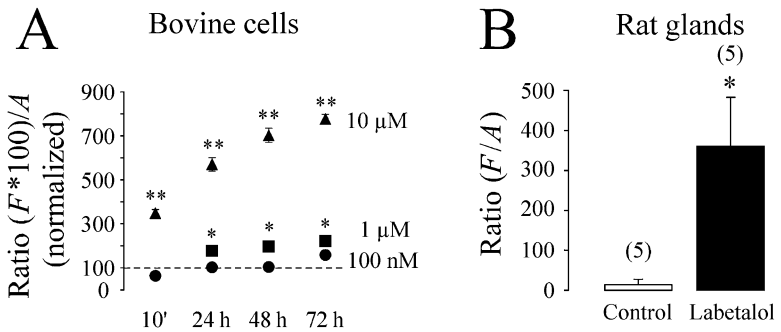

Figure 2 Time and concentration dependence of the release of labetalol from bovine chromaffin cells and rat adrenal glands. Experiments were conducted as in Figure 1. Secretion was elicited by 100 µmol·L^{-1} of DMPP applied for 10 s. To reduce the influence of culture conditions, the results, as the ratios of fluorescence/amperometry (*100), were normalized to their own control cells. (A) Release from four to eight different experiments (means ± SEM) conducted with bovine cells incubated with labetalol at the time and concentrations indicated. Error bars are not shown when error bars are smaller than symbols. Comparisons were made with their own control untreated cells, *$P < 0.05$; **$P < 0.01$ (ANOVA). (B) The set-up was modified for the retrograde perfusion of rat isolated adrenal glands, as described in Supporting Information Figure S1. Glands were stimulated with 100 µM of DMPP for 10 s. The labetalol group was taken from rats that orally received labetalol 40 mg·kg^{-1}·day^{-1} for 7 days; control rats received the same volume of water. The ratios of the areas under curves from both fluorescence and electrochemical detection traces were calculated. *$P < 0.05$ (Mann–Whitney rank sum test).

catecholamine released per stimuli fell. The responses were normalized as the fluorescence/amperometry ratios as explained in Methods (Figure 2B). The presence of labetalol in the effluent buffer was confirmed by HPLC.

β-Blockers modify the quantal characteristics of catecholamine exocytosis in bovine and rat chromaffin cells

We incubated chromaffin cells with three β-blockers for different concentrations of drugs and periods of time to check whether the accumulation of labetalol and other β-blockers was also accompanied by changes in the quantal characteristics of the release.

Figure 3 shows the kinetic parameters measured from secretory spikes obtained with conventional amperometry in bovine chromaffin cells. The three β-blockers tested (atenolol, labetalol and propranolol) produced a reduction in the I_{max} and Q. These effects on exocytosis which were caused by all the drugs exhibited time dependence. Atenolol was the most potent agent tested in slowing down exocytosis, and reduced the quantal size (Q) as its action on the I_{max} was observed at 100 nmol·L^{-1} (Figure 3B and E). Conversely, labetalol required 10 µmol·L^{-1} and 48 h of incubation to produce a significant reduction of I_{max} and Q (Figure 3A and D). We did not carry out further experiments using atenolol because its effects in exocytosis were similar to labetalol, and atenolol does not have fluorescent properties as suited as those of labetalol to the fluorescence assay.

The concentration dependence was only tested with propranolol. Cell incubation with 1 µmol·L^{-1} required 72 h of incubation to affect both the I_{max} and Q (Figure 3C and F). The effects of the incubation with propranolol 10 µmol·L^{-1} on I_{max} were evident after 3 h, and reduced the Q progressively at 24 and 48 h (Figure 3C and F). However, drug incubation for 72 h eliminated exocytosis (10 cells from two different cultures; not shown). The less active isomer (*R*)-(+)-propranolol (10 µM) was also tested, and a non-significant reduction (≈12%) in the quantal size was observed after 24 h of treatment (not shown). In our experience, the best amperometric recordings are usually obtained during the first 3 days after culture, which means that longer incubations with β-blockers will not produce optimal recordings. To prolong the period of study, we tested the effects of β-blockers on cultured chromaffin cells from rats that received labetalol or propranolol for 7 days. Neither labetalol nor propranolol modifed the spike firing profile (Supporting Information Figure S3). Figure 4A shows spikes reconstructed with the absolute data of I_{max}, Q, t_p and $t_{1/2}$. These parameters are described in Figure 4B. Labetalol at 40 mg·kg^{-1}·day^{-1} started to cause changes in the kinetic parameters after 7 days of treatment, but these changes were not statistically significant. Conversely, propranolol slowed down the exocytosis without altering the vesicular content of the catecholamines (Figure 4C). No further concentrations of drugs or treatment times were tested on rats. Because amperometrical measurements were performed on cultured cells 1 day after isolation, it is possible that these effects were underestimated as β-blockers could leak out during the 24 h in culture in absence of any drugs in the culture medium.

Treatment with labetalol inhibited the uptake of newly synthesized catecholamines in bovine cells

Figure 5A shows the effects of labetalol incubation on total secretion measured with single-cell amperometry. We incubated cells with 10 µM of labetalol for 24 h, a concentration that only caused small alterations in the kinetics of single-event exocytosis (Figure 3A and D). Chromaffin vesicles from untreated bovine cells were able to take up catecholamines as was confirmed by the increase of spike charge (Figure 5B) and in the I_{max} (Figure 5C). Note that I_{max} increased more than the spike charge. This is usually attributed to the relatively larger abundance of catecholamines in the free portion of the vesicles. Conversely, chromaffin vesicles from cells treated with labetalol could not take up more catecholamines, which means that neither the total secretion nor the quantum sizes of vesicles could have increased.

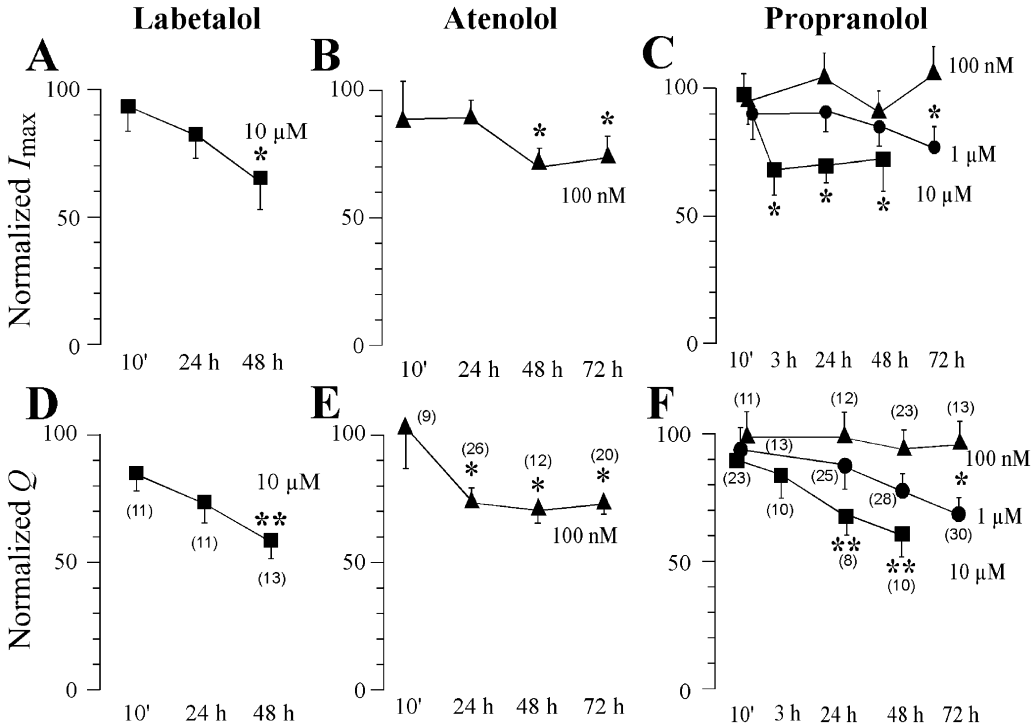

Figure 3 Effects of incubation with β-blockers on the exocytosis in single bovine chromaffin cells measured with amperometry. Cells were incubated with the drugs for the time and the concentrations indicated. Secretion was elicited with a 10 s pulse of 5 mmol·L^{-1} $BaCl_2$ from a pipette situated 40 µm from the cell. Secretory spikes were detected by single-cell amperometry. Experiments were carried out by alternating treated with untreated cells (10–18 cells for each point) using the same electrode. Each data set was normalized and compared to its own control cells. (A–C) The effects on the spike height (I_{max}); (D–F) The effect on the apparent quantum size (Q). Incubation with propranolol for 72 h abolished secretion. The number of cells assayed is indicated in brackets. *P < 0.05, **P < 0.01 (Mann–Whitney rank sum test).

Labetalol accumulated in secretory vesicles dilute the vesicular catecholamines

The experiments summarized in Figure 6 were conducted, using patch amperometry in cell-attached patch amperometry configuration, to study the effects caused by the accumulation of β-blockers on the vesicle size and in the vesicular concentration of catecholamines. Patch amperometry permits the simultaneous recording of single-vesicle capacitance and the electrochemical detection of the catecholamines released from a vesicle, thus allowing the calculation of inner concentration of catecholamines. In addition, it is also possible to study the characteristics of the fusion pore in some recordings (Supporting Information Figure S5).

Patch amperometry measurements showed that labetalol treatment slightly, but not significantly, reduced the spike charge (Figure 6A), significantly decreased the time (delay) between the granule fusion and the release of amines (Figure 6C) and significantly increased the size of vesicles. It is likely that the increase observed in the size was caused by the swelling of the vesicles due to the accumulation of more soluble material. The effects on spike charge were similar to those observed with amperometry (Figure 3D) and similar to that described for L-DOPA accumulation in secretory vesicles of PC12 (Colliver *et al.*, 2000), and later in chromaffin cells (Gong *et al.*, 2003). The combination of an increased granule size (Figure 6B) and a similar amount of catecholamines (Figure 6A) resulted in the reduction of the concentration of catecholamines (Figure 6D). The conductance and the dilatation kinetics of the fusion pores did not seem to be affected (data not shown).

Incubation with labetalol increased the cytosolic free catecholamines of bovine chromaffin cells

We used patch amperometry to measure the concentrations of free amines in the cytosol in the configuration of the whole cell so that we could perform the electrochemical analysis of the electrochemically active substances inside the cell. This approach did not discriminate between the catecholamines, L-DOPA or other substances. However, amperometric measurements are not affected by labetalol.

The membrane was broken by suction after the electric seal between the pipette and the cell was made. The amperometric trace showed a rapid increase that was followed by an exponential fall. The presence of secretory spikes (Figure 7A) is frequently observed, which is probably caused by the fusion of vesicles with the broken cell membrane or as a result of the impact and fission of granules on the carbon fibre surface. We subtracted the area of these spikes for the analysis of the free cytosolic amines; data are summarized in Figure 7B. All experiments were carried out in the presence on the MAO inhibitor pargyline (10 µM) to reduce the enzymatic degradation of the amines and to rule out the effects of labetalol on MAO activity.

The incubation of cells with L-DOPA notably increased the amount of cytosolic amines. Labetalol caused a net increase of

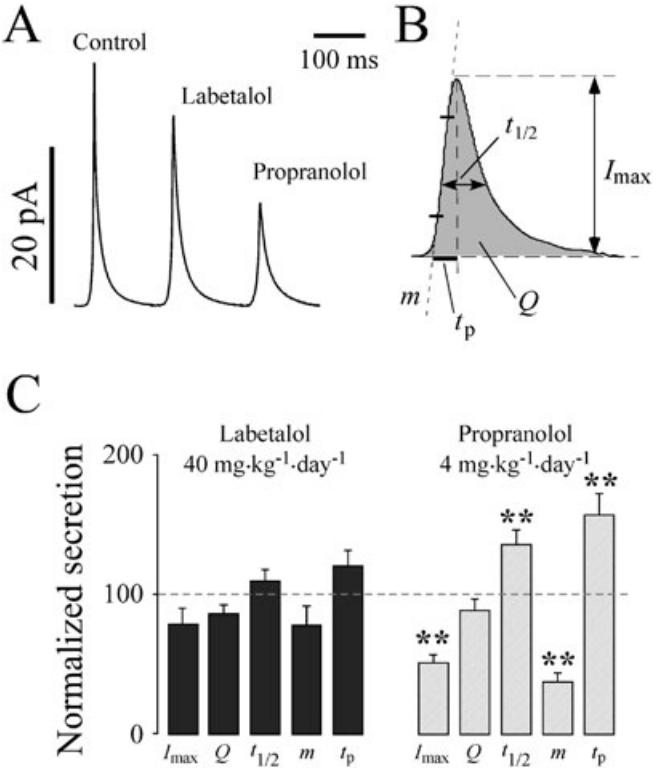

Figure 4 Effects of subchronic treatment of rats with propranolol and labetalol on the kinetics of exocytosis measured with single-cell amperometry. Rats were given labetalol (40 mg·kg^{-1}) or propranolol (4 mg·kg^{-1}) by mouth, for 7 days. Chromaffin cells from these animals were isolated and cultured, and their exocytotic characteristics studied by single-cell amperometry. Secretion was elicited with a 10 s pulse of 5 mmol·L^{-1} $BaCl_2$. (A) Averaged secretory spikes from control rats or from rats treated with labetalol or propranolol. These representative spikes were plotted using the data obtained under each condition. (B) The analysis of individual exocytotic events included the measurement of the following parameters: I_{max}, maximum oxidation current, expressed in pA; $t_{1/2}$, spike width at half height, expressed in ms; *Q*, spike net charge, expressed in pC; *m*, ascending slope of spike, expressed in nA·s^{-1}; see Methods in the Supporting Information Appendix S1 or Machado *et al.* (2000) and Segura *et al.* (2000) for further details. (C) Effects of treatment *in vivo* with β-blockers, on exocytosis. The kinetic parameters were extracted from amperometric spikes. Twenty-seven cultured cells from four control rats, 26 cells from four rats treated with labetalol and 28 cells from four rats treated with propranolol were used. Comparisons were made with their own control cells from untreated rats, *$P < 0.05$; **$P < 0.01$, Mann–Whitney rank sum test.

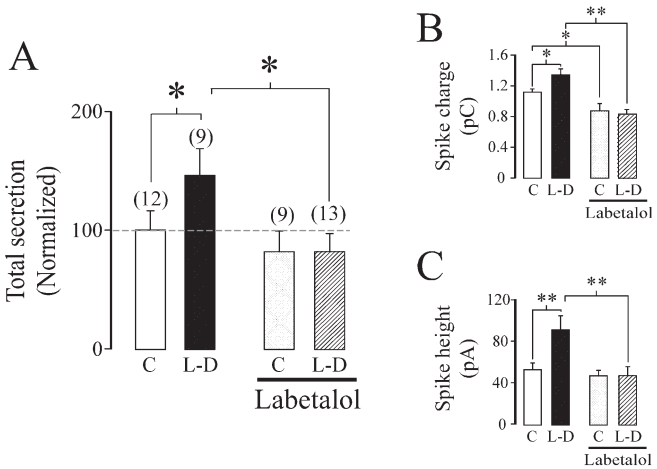

Figure 5 Incubation with labetalol impaired the ability of bovine chromaffin granules to take up newly synthesized amines. Cells were incubated in the absence or in the presence of labetalol (10 μmol·L^{-1}) for 24 h, and the secretory responses were measured with amperometry. A group of cells were incubated with 100 μmol·L^{-1} of the catecholamine precursor L-DOPA (L-D) for 90 min. (A) Cumulative secretion normalized to their own control cells. To measure the total catecholamines secreted, the secretory spikes were integrated for 2 min after a 5 mmol·L^{-1} Ba^{2+} stimulation for 10 s. (B) Effects of L-DOPA incubation on the spike charge (*Q*, see Figure 4B) of exocytosis from control and from cells treated with labetalol. (C) As in (B), but measuring the spike height (I_{max}). The number of cells assayed is indicated in brackets. *$P < 0.05$; **$P < 0.01$, ANOVA test.

the free amines in both basal conditions and after L-DOPA incubation.

Discussion and conclusions

We have considered the hypothesis of whether the slow accumulation of β-blockers inside secretory vesicles could underlie their hypotensive effects and could explain the discrepancies between the short half-life of some β-blockers (especially the hydrophobic drugs) and their clinical effects. The persistence of β-blockers in sympathetic synapses may account for their long-lasting effects.

We have studied the effects of three β-blockers on quantal neurosecretion to show that some of their effects seem to be unrelated to their β-adrenoceptor antagonist properties. We chose propranolol, a lipid soluble β-blocker with high pKa, because of its wide clinical use. Labetalol was selected because of its unique fluorescent profile that made the direct on-line detection by fluorimetry possible (Supporting Information Table S1). Although labetalol is also an antagonist at α_1-adrenoceptors, these receptors do not affect the chromaffin cell (Powis and Baker, 1986). Atenolol is a selective β_1-adrenoceptor antagonist with low lipid solubility, which probably uses the vesicular amine carrier VMAT2 to enter the vesicles (Kent *et al.*, 1981; Webb *et al.*, 1988; Bagwell *et al.*, 1989), and it efficiently binds chromogranins, with a similar affinity to that of catecholamines (Videen *et al.*, 1992).

The ability of certain drugs to accumulate in secretory vesicles and to be released as 'false neurotransmitters' is frequently ignored. We have reported that hydralazine, a drug classified as 'direct vasodilator', is rapidly sequestered inside chromaffin secretory granules (Machado *et al.*, 2002); the same mechanism could be imitated by other drugs like the β-blockers, which are concentrated in secretory vesicles, although at a much slower speed.

The vesicular accumulation of β-blockers and their co-secretion with catecholamines was demonstrated by the direct measurements by on-line detection of the secretory products from both superfused bovine chromaffin cells and retrogradely perfused rat adrenal glands (Figures 1 and 2). These data also showed that the labetalol accumulation was accompanied by a reduction in the amount of catecholamines released per depolarizing stimuli, suggesting that β-blockers

displaced the natural transmitter from the secretory vesicles. The electrochemical and fluorimetric signals were Ca^{2+} dependent, indicating that both products were co-released by exocytosis and not by diffusion from the cytosol.

The HPLC analysis of chromaffin granules from cells incubated with labetalol showed that its vesicular accumulation increased with the concentration of the drug. Conversely, the catecholamine content in granules decreased proportionally to the concentration of β-blockers used (Figure 3), suggesting that β-blockers compete with the endogenous catecholamines, thus displacing them from vesicular stores (Kent *et al.*, 1981; Boksa, 1986). This idea received additional support from the experiments with L-DOPA overload, which demonstrated the lack of ability of chromaffin vesicles to take up newly synthesized amines (Figure 5). The quantitative differences observed in the ability of vesicles to take up labetalol and the increase observed in the secretion ratios shown in Figure 1 could be explained by the asymmetrical distribution of labetalol within the vesicles (see below).

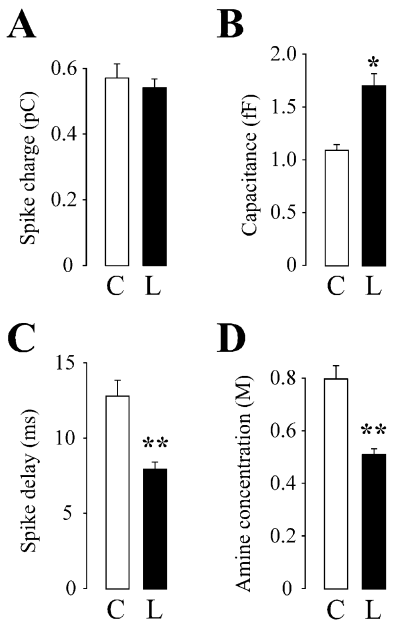

Figure 6 Effects of labetalol (L) incubation on the fusion of secretory vesicles analysed by patch amperometry in bovine chromaffin cells. (A) Spike charge obtained from the amperometric recording of 52 events from control-(C) and 136 from treated-cell spikes (L), expressed in pCoulombs. (B) Capacitance data from the same number of events, expressed in fFarads. (C) Delay (Δt in C) between the capacitance step and the beginning of the amperometric spike $n = 33$ control- and 79 treated-cells, expressed in ms. (D) estimated molar (M) concentration of vesicular amines obtained by the spike charge (amol) and vesicle size (aL). Cells were incubated with labetalol (10 μmol·L^{-1}) for 24 h. *$P < 0.05$; **$P < 0.001$ Mann–Whitney rank sum test.

Experiments from Figure 3 demonstrate that β-blockers in a time-dependent process slowed down the exocytosis and reduced the apparent content of catecholamines inside the vesicles. Cell incubations with large concentrations of propranolol (10 μmol·L^{-1}) for over 72 h eliminated the secretion, probably through an effect unrelated to β-adrenoceptors, but through the blockade of Na^+ channels (Orts *et al.*, 1987). The secretion evoked by Ba^{2+} was not dependent on Na^+ channel activity.

The estimated plasma concentrations of propranolol to reduce tachycardia caused by exercise by 50% are in the range of 15–90 ng·mL^{-1} (Riddell *et al.*, 1987), which roughly correspond to 60–350 nM, which are slightly below those used in this work. However, the lipid solubility of propranolol suggests that an important fraction of the drug may be accumulated in tissue. Although 10 μmol·L^{-1} can be considered as a high concentration of β-blockers when it is compared to the plasma concentration reached during a chronic therapy, we had to use this to force the experimental conditions in some experiments. It is important to stress that drug therapy requires at least 7–15 days of continuous treatment to be clinically relevant. For this reason and because it better resembles the conditions during drug therapy, we performed experiments on cultured cells obtained from rats treated for a week with the following two β-blockers: labetalol and

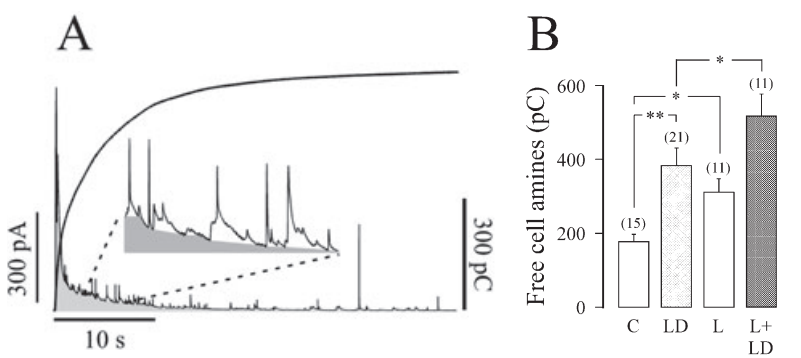

Figure 7 Impairment of vesicular uptake increased the concentration of free cytosolic catecholamines in bovine chromaffin cells. (A) Patch amperometry in the configuration of whole cell for the direct analysis of the free amines present in the cytosol. (B) Cytosolic oxidizable molecules were monitored by the microelectrode as a rapid increase in the amperometric current followed by a slow falling wave. Observe the presence of amperometric spikes in the enlarged section of the falling slope. After the subtraction of all spike areas, the total area of this oxidation current wave (grey) was used as the measure of the cumulative amount of cytosolic free catecholamines depicted as the smooth ascending line. Calibration bar on the left is for the amperometric trace (in pA), and the bar on the right for the cumulative trace (in pC). (C) Average values (mean ± SEM) from the integration of the curves from cells incubated with L-DOPA (100 μM) + pargyline (10 μM); (LD) treatment for 90 min in the absence (C) or presence of labetalol (L) (10 μmol·L^{-1} for 24 h). The net increase caused by L-DOPA in labetalol-treated cells was 1.6 times larger than in control cells. The numbers of cells assayed are shown in brackets. Comparisons were made with their own control untreated cells, *$P < 0.05$, **$P < 0.01$ ANOVA test.

propranolol. Both drugs reproduced the findings observed in acutely treated cultures of bovine chromaffin cells, although these effects were only significant in the propranolol group (Figure 4). As mentioned above, it is possible that the effects of β-blockers were underestimated in these experiments because part of the drug could leak out during the 24 h of cell culture after isolation in the absence of drugs. However, even considering this probable reduction in the amount of β-blockers present in vesicles, 1 week of exposure to propranolol produced profound effects on exocytosis. Labetalol seemed to require more treatment time to attain the same effects, but these experiments were not performed.

Soluble components of vesicles are stored in two dynamic forms. The major portion is associated with the vesicular matrix, which is thought to be composed of amines, ATP, H^+, Ca^{2+} and chromogranins; this accounts for 90–99% of the total content (Schroeder *et al*., 1996). The vesicular matrix is in slow equilibrium with the free fraction present in the clear halo that surrounds the matrix. The soluble components of the free fraction are rapidly exchanged with the cytosolic medium, and this partition largely depends on the pH gradient and on the free concentrations in the cytosol. The accumulation of β-blockers should initially occur in this compartment where they compete with catecholamines (Videen *et al*., 1992). Labetalol increased the size of secretory vesicles in a similar way to that described for L-DOPA (Pothos *et al*., 2000; Sulzer and Edwards, 2000; Gong *et al*., 2003). However, unlike L-DOPA, β-blockers are not converted into catecholamines, which means that a larger-sized vesicle caused a functional dilution of amines (Figure 6).

Theoretically, the reduction of vesicular cargo could be caused by an inhibition of cellular uptake. However, our data do not support this explanation. The uptake of catecholamines in chromaffin cells is very poor when compared with sympathetic nerve terminals, and the amount of free amines in the culture media is very low. In addition, the data from Figure 7B show significant large concentrations of cytosolic free catecholamines in the cells treated with labetalol. Another possible cause is inhibition of catecholamine synthesis; however, in the absence of L-DOPA, the free basal cytosolic levels of amines in the cells treated with labetalol were larger than in control cells. The L-DOPA experiments summarized in Figures 5 and 7 do not support this because the usual rate-limiting step of the catecholamine synthesis (tyrosine hydroxylase) was bypassed, and no effect of L-DOPA (in terms of newly synthesized catecholamines) on quantal size was observed. Other possibilities include the inhibition of vesicular uptake by labetalol, or competition for the same intravesicular matrix binding structures (Videen *et al*., 1992). Although either of these two mechanisms would be compatible with our results, we find the latter the most feasible.

If we extrapolate these effects to the sympathetic nerve terminals, β-blockers could act as false neurotransmitters, reducing the amount of amine released by each quantum (Hurwitz *et al*., 1983; Russell *et al*., 1983), therefore altering the synaptic performance in target tissues like arterial vasculature. Therefore, the hypotensive effects of β-blockers might be partially independent of their actions on adrenoceptors, although small effects on the kinetics of exocytosis were also observed with the less active isomer of propranolol, suggesting that this drug possesses a stereo-selective uptake. However, β-blockers are not inert compounds, and the release of these substances in locally high concentrations as vesicular contents could reduce the positive feedback of noradrenaline on presynaptic β-adrenoceptors. Taken together, our data provide a plausible explanation for the delayed onset and the paradoxical effects of β-blockers in lowering arterial blood pressure.

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Conflict of interest

The authors state no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Perfusion system used for the on-line monitoring of secretion of catecholamines and labetalol.
Figure S2 Parameters obtained from amperometric recordings.
Figure S3 Spectral characteristics of labetalol and its dependency on pH.
Figure S4 The treatment with β-Bs did not modify the spike firing characteristics.
Figure S5 Patch amperometry.
Table S1 Fluorimetric properties of several β-Bs.
Appendix S1 Materials and methods.

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