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Abstract—The effects of the antihypertensive agent hydralazine (1 to 100 nmol/L) on the exocytotic process of single adrenal chromaffin cells have been studied using amperometry. Hydralazine does not reduce the frequency of exocytotic spikes but rapidly slows the rate of catecholamine release from individual exocytotic events by reducing the quantal size of catecholamine exocytosis. Confocal and standard epifluorescence microscopy studies show that hydralazine rapidly accumulates within secretory vesicles. The blockade of the vesicular H^+ pump with bafilomycin A_1 inhibits hydralazine uptake. Experiments with permeabilized cells show that hydralazine displaces catecholamines from secretory vesicles. The drug also displaces vesicular Ca^{2+}, as shown by fura-2 microfluorimetry. These data suggest that hydralazine acts, at least partially, by interfering with the storage of catecholamines. These effects of hydralazine occurred within seconds, and at the tissue concentrations presumably reached in antihypertensive therapy; these concentrations are a thousand times lower than those described for relaxing vascular tissues in vitro. We proposed that these novel effects could explain many of the therapeutic and side effects of this drug that are likely exerted in sympathetic nerve terminals. **(*Circ Res*. 2002;91:830-836.)**

Key Words: amperometry ■ bafilomycin A_1 ■ catecholamines ■ chromaffin granules ■ intragranular pH

Hydralazine (HYD) was a widely used agent in antihypertensive therapy. It has recently received considerable interest because it decreases the blood flow of tumoral masses,[1] reduces nitrite tolerance,[2] and is useful in the treatment of certain forms of drug-resistant hypertension. However, its mechanism of action remains far from understood.

The elusive nature of the site of action of HYD has interested researchers for decades; hence, many actions of the drug in vascular and nonvascular tissues have been reported.[3] However, to evaluate the relevance of a proposed mechanism only those effects produced at concentrations reached with clinical doses should be taken into account. Therapeutic plasma levels of HYD have been calculated to be around 100 ng/mL; a distribution volume of 1.5 L/kg[4] results in an extracellular concentration of about 500 nmol/L. Although these calculations cannot be immediately transformed into effective concentrations reaching the cells, they contrast with that described in most pharmacological studies performed in vitro. Many authors find the vasorelaxing effect of HYD only at concentrations three orders of magnitude higher, 10 to 1000 μmol/L. For instance, hydralazine inhibits the release of Ca^{2+} from the sarcoplasmic reticulum with an IC_{50} of 17 μmol/L and causes the maximal relaxation of rabbit aorta at 100 μmol/L.[5] Furthermore, HYD inhibits $^{45}Ca^{2+}$ uptake,[6] or increases cGMP[7] and cAMP levels[8] in the mmol/L range. It is therefore important that any proposed target for HYD must satisfy the concentration criteria before a relationship with its antihypertensive effect can be established.

It is important to make a distinction between effects on secretion, ie, the total amount of neurotransmitter released after a stimulus, and effects on the exocytotic event, ie, kinetics at the single event level. Both are closely related processes involved in the release of neurotransmitters and other substances, but the kinetics of exocytosis can be drastically modified even when the total amount of product secreted remains unchanged.[9] In addition, exocytosis under hypertonic conditions[10] or of empty granules[11] can also occur without being accompanied by secretion. For these reasons, most of the results from the present study are referred to as the kinetics of exocytosis at the single event level.

In adrenal chromaffin tissues, several drugs can alter the kinetics of exocytosis at the single event level as well as their quantal size.[9,12–14] The aim of the present study has been to test whether the mechanism of action of HYD can be explained by an interference with the processes of catecholamine storage or exocytosis. Our results demonstrate that, even at nanomolar concentrations, hydralazine accumulates rapidly into secretory vesicles, thus promoting dramatic changes in the kinetics of exocytosis at the single event level

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and reducing the quantal size of the secretory events. To our knowledge, this is the first time that this mechanism has been proposed to explain most of the clinical and side effects of HYD. This makes sense if we consider that HYD acts predominantly on the more densely sympathetically innervated vascular tissue like arterioles.[15,16]

Materials and Methods

Materials

Fura-2/AM was obtained from Molecular Probes. Urografin was obtained from Schering España. Culture plates were from Corning. Kits for cGMP and cAMP were from Calbiochem Novabiochem Corporation. All other drugs, culture media, and sera were from Sigma-Aldrich.

Culture of Chromaffin Cells

Bovine adrenal chromaffin cells were obtained as described previously[17] and plated on 12-mm–diameter glass coverslips at an approximate density of 5×10^5 cells/well.

Amperometric Detection of Exocytosis

Carbon fiber electrodes were prepared and tested as described.[18] Electrochemical recordings were performed using an Axopatch 200B (Axon Instruments).[9]

Glass coverslips with adhering cells were placed in a perfusion chamber mounted on the stage of an inverted microscope and bathed in a Krebs-HEPES solution containing (in mmol/L) NaCl 140, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 2, HEPES 10, and Glucose 11; pH 7.35 (NaOH). Carbon electrodes were gently placed onto the cell membrane. Secretion was elicited by a 5-second pulse of a secretagogue from a glass micropipette located 40 μm from the cell.

Data Analysis

Amperometric signals were low-pass filtered at 1 KHz and sampled at 4 KHz as described.[9] For data analysis, we used locally written macros for IGOR (Wavemetrics, Lake Oswego, Oregon). Several parameters were extracted from each spike.[19] Macros can be freely downloaded from the Research Team in Neurosecretion Web site.[20]

To avoid day-to-day variations in electrode sensitivity and cell responsiveness, control experiments and drug tests were done within the same day using the same electrode. Amperometric spike data were averaged by cell to avoid misinterpretation due to the different number of spikes recorded from each cell.[14] Statistical analysis was performed by the nonparametric Mann-Whitney *U* test. Changes observed in the kinetics of exocytosis at the single event level cannot be attributed to a modification in the electrode responsiveness because HYD does not affect the electrode responses to standard solutions of noradrenaline (50 μmol/L) in a flow-stream calibration[18] (data not shown). Although HYD was oxidized at +650 mV, thus increasing the background current, this effect is only evident at micromolar concentrations and not at the nanomolar concentrations used in this study.

Measurement of Cytosolic Ca^{2+}

Fura-2 measurements were performed as described.[9] Data were collected at 1 Hz and expressed as fluorescence ratios $(F_{340})/(F_{380})$.

Accumulation of HYD

Cells were placed in a perfusion chamber of an epifluorescence microscopy and excited at λ_{485} for 10 ms at 1 Hz. Excitations with λ_{800} were used as blank. Emission was recorded at λ_{510}. Intracellular distribution of HYD was monitored on a confocal microscope (BioRad MRC1024), coupled with an argon laser using a standard FITC method. Hydralazine was added either directly to the bath or by means of a 5-μm–tip glass micropipette placed 40 μm away from the cell. Statistical analysis was performed by Student's *t*-test.

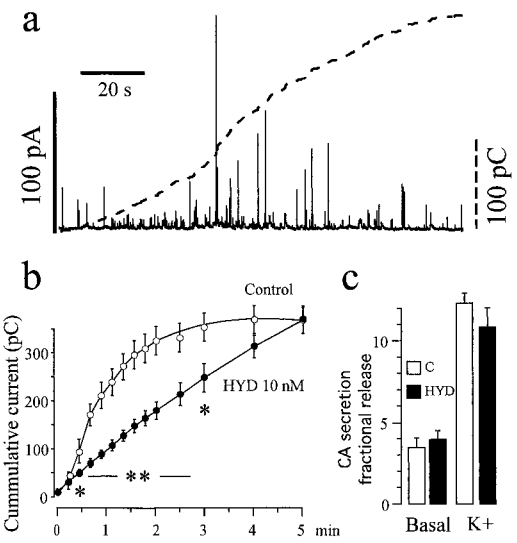


Figure 1. Effects of hydralazine (HYD) on catecholamine secretion. Chromaffin cells were stimulated with 5 mmol/L $BaCl_2$ for 5 seconds in the presence and in the absence of 10 nmol/L HYD (which is present 10 minutes before and during the stimulus). a, Typical trace showing the amperometric spikes. Panel also shows the cumulative secretion curve obtained by integration of the trace. Calibration bars are pA for direct record (solid trace) and pC for the cumulative secretion (dashed trace). b, Mean±SEM from the measurements done at the end of the integration intervals were pooled and expressed in pC. $^{**}P<0.01$, $^{*}P<0.05$ (Student's *t* test). c, Total catecholamines released measured by fluorimetry. Cells are stimulated with 70 mmol/L K^+ for 5 minutes in the absence (c) and in the presence of 10 nmol/L of HYD.

Catecholamine Release and Cyclic Nucleotide Measurements

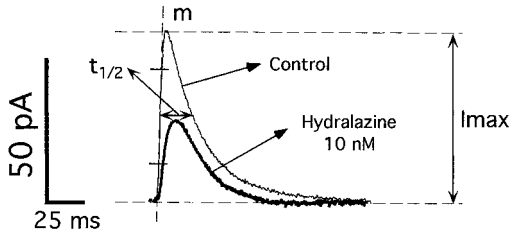
Cells were cultured on 24-well plates at 5×10^5/well for 48 hours. Cells were washed twice in Krebs-HEPES. Catecholamine measurement was done either by HPLC-ED[21] or fluorimetric assay[22] and expressed as fractional release from the total cell content.

Cyclic nucleotide (cAMP and cGMP) measurements were performed as described.[9,12] Data are calculated in fmol/μg total protein content, measured by the bicinchoninic acid method as described by Sigma. Statistical analysis was performed by the Tukey test.

Results

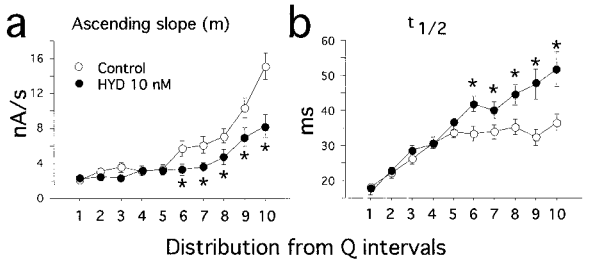
Hydralazine Modifies the Kinetics but Not the Amount of Catecholamine Released by Chromaffin Cells Over a 5-Minute Period

Experiments with single cells show that HYD did not reduce the number of exocytotic events. Thus, from 10 control cells, the averaged number (±SEM) of secretory spikes counted during the first 5 minutes after Ba^{2+} stimulation was 186±16; in 12 cells treated with 10 nmol/L HYD, the number of spikes was 195±17. In the absence of HYD, cumulative release is rapid in the first minute after stimulation, then tapered off to approach a maximum by 3 minutes (Figure 1a). In the presence of HYD, cumulative release was much less in the first minute, but continued linearly for 5 minutes, at which time it reached the same level as the control cells (Figure 1b). This equality was confirmed by quantifying the total amount of catecholamine secreted during this 5-minute period using a fluorimetric method[22] (Figure 1c). In addition, HPLC analysis



	Imax (pA)	Q (pC)	$t_{1/2}$ (ms)	m (nA/s)	n spikes	n cells
Control	22.9±2.3	0.9±0.05	31.5±1.9	5.7±1.6	783	11
HYD 10 nM	13.9±1.9	0.6±0.06	31.1±0.7	3.6±0.7	832	14

Figure 2. Effects of hydralazine on kinetics of exocytosis at the single event level. Representative spike traces were built using the kinetic parameters from the inserted table. Hydralazine was applied as a brief 5 second pulse together with $BaCl_2$. Vertical and horizontal bars are the calibration for the oxidation current and time. Note the differences in the ascending slope (m) and in the net charge (total area under each trace, Q). Accompanying table shows the effects of HYD incubation compared with their own control cells. Data are averaged values and are expressed in the units indicated in brackets. Normalized data are shown in a separate Table.



Q intervals	1	2	3	4	5	6	7	8	9	10
From (pC)	0.06	0.19	0.27	0.34	0.41	0.49	0.61	0.75	1.01	1.55
To	0.19	0.27	0.34	0.41	0.49	0.61	0.75	1.01	1.55	6.71

Figure 3. Hydralazine preferentially affects the larger secretory vesicles. Secretory spikes from both control (n=1512) and 10 nmol/L HYD-treated cells for 10 minutes (n=1109) were pooled, sorted by their quantal size, and distributed into boxes with the same number of events. Each interval contains spikes with a variable range of Q size but with a fixed number of events. Ranges of values of Q are shown in the accompanied table. Each box contains 200 events (control+HYD, in a distribution that does not exceed 65% of either group). Average of m (a) and $t_{1/2}$ (b) from each experimental condition is plotted ±SEM. This box analysis shows that HYD treatment affects preferentially the biggest vesicles, those whose charges are larger than 0.5 pC in our experimental conditions *$P<0.05$ Student's *t* test.

of the secreted products shows no changes in the adrenaline/noradrenaline ratio (not shown).

Low Concentration of HYD Causes a Slow-Down of the Kinetics of Exocytosis at the Single Event Level

We examined the difference in early secretion ±HYD by looking at catecholamines secretion at the single event level. Figure 2 shows that acute application of HYD 10 nmol/L caused a drastic change in the kinetics of exocytosis at the single event level. There was a decrease of the maximum concentration that reaches the electrode (I_{max}) and a decrease in the ascending slope (m) of amperometric spikes, which indicates that the exocytosis becomes slower. These effects were accompanied by a reduction in the apparent granule content of catecholamines (Q).

The effects of HYD were observed within seconds after coapplication for 5 seconds of 10 nmol/L HYD plus Ba^{2+} (Figure 2). Moreover, there were few differences between the short application and long incubation (2 hours) with HYD (Table). We have used Ba^{2+} as a secretagogue through this work because it causes a sustained secretion with very few overlapped spikes that are ideal for individual characterization (Figure 1). However, K^+-evoked secretion produced similar effects (Table), although with many superimposed spikes. Because of this reason, only a fraction of the total secretory events could be reliably analyzed. Hydralazine 10 nmol/L did not modify the time course or the total amount of catecholamines evoked by K^+ stimulation. The effects of HYD were observed along the entire recording period (2 to 5 minutes).

In order to know whether HYD affects evenly all of the granules, or rather a subpopulation of them, we performed an interval distribution or "box analysis." Each box contained spikes sorted by their catecholamine content, regardless of the treatment received. Once the distribution in boxes was performed, the two kinetic parameters m and $t_{1/2}$ were averaged separately from control and HYD-treated cells. These parameters (m and $t_{1/2}$) were extracted from the spikes of each interval and plotted. Figure 3 shows that HYD preferentially slows the kinetics of exocytosis (m and $t_{1/2}$) of

Effects of HYD on Secretory Spike Parameters (Normalized Data)

Drug Treatment	I_{max}	Q	$t_{1/2}$	m	Spikes, n	Cells, n
HYD, 1 nmol/L	80*	93	90*	64*	1449	6
HYD, 10 nmol/L	60*	66*	99	99	1109	14
HYD, 100 nmol/L	48**	70*	89*	26*	806	8
HYD, 10 nmol/L puffed	61*	67*	99	63*	832	14
K^++HYD, 10 nmol/L	47*	88	151*	27*	616	6
HYD, 10 nmol/L (120 minutes)	60*	63*	91	56*	486	10

Each parameter was normalized to their own control group of cells (100%). No. of control spikes computed ranged from 287 to 1480 from 9 to 15 cells.

*$P<0.05$ and **$P<0.01$ (Mann-Whitney *U*). Significant differences were performed comparing mean values of cells before the normalization of data. K^+ was applied for 5 seconds at 70 mmol/L; all other experiments were performed with 5 mmol/L $BaCl_2$ applied for 5 seconds.

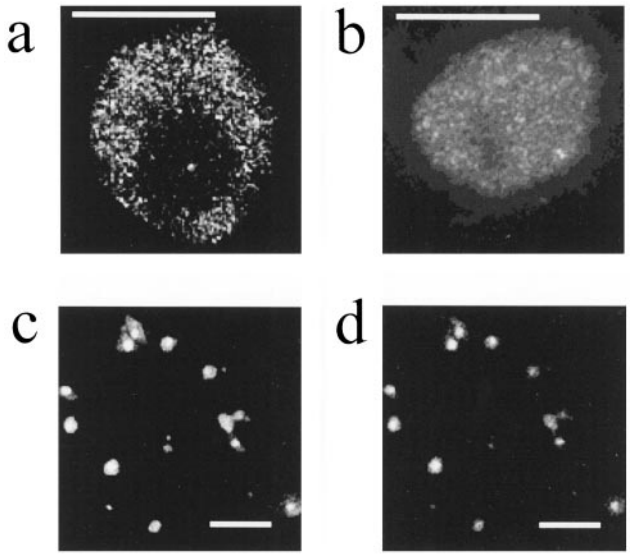


Figure 4. Hydralazine is rapidly taken up into chromaffin granules. a and b, Confocal images obtained taking advantage of HYD autofluorescence. Pictures were taken 10 minutes after the addition of 10 μmol/L (a) and a few seconds after the addition of 10 mmol/L HYD (b). These high concentrations are necessary due to the weak fluorescence potency of HYD. c and d, Uptake of 10 nmol/L of acridine orange (AO) in the absence or after the incubation with 1 nmol/L of bafilomycin. Calibration bars: a and b=10 μm; c and d=50 μm.

vesicles with a content of catecholamines larger than 0.5 pC. This strategy increases the resolution of histograms or descriptive statistics and permits us to make a relation between two parameters.[23] Other details are described in the legend of Figure 3.

Hydralazine Rapidly Accumulates Within Chromaffin Granules

Hydralazine is a weakly fluorescent compound that emits light at 504 nm when excited with a 483 nm monochromatic light. This spectrum is roughly similar to fluorescein (485/510) and permits use of cube filters designed for FITC.

Confocal microscopy reveals that HYD rapidly accumulates into vesicular structures. These vesicles are presumably chromaffin granules because of their distribution, abundance, and the large the fraction of subcellular volume occupied by chromaffin granules (≈17%). Images were usually obtained immediately after drug addition (Figure 4a). Large HYD concentrations (10 mmol/L) or prolonged incubation times (over 15 to 20 minutes) also stain the cell nucleus (Figure 4b).

The pattern of HYD distribution within the cell was similar to the dye acridine orange (AO), whose accumulation is highly pH-dependent. Figures 4b and 4c show that the accumulation of AO was impaired by incubation with 10 nmol/L of the vesicular H^+ pump inhibitor bafilomycin. However, because HYD is a weaker fluorescent compound than AO (≈1000 times less potent), it was necessary to increase HYD concentrations to 10 μmol/L to observe its accumulation in secretory vesicles. Hydralazine and AO seemed to accumulate in the same vesicular structure as HYD, drastically reducing the AO accumulation (online Figure, available at http://www.circresaha.org in the data supplement). Their different pKa (7.3 and 10.45, respectively) ensured that AO was sequestered stronger. Note the different scale in Figure 5.

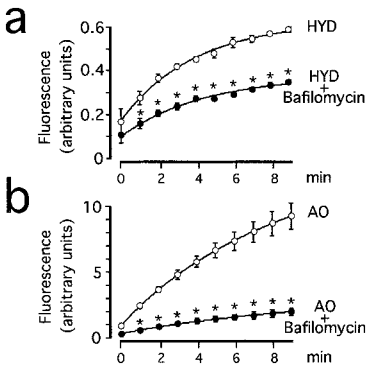


Figure 5. Time course of hydralazine and acridine orange accumulation in single bovine adrenal chromaffin cells. a, Hydralazine was applied for 10 seconds by pressure at 10 μmol/L. Data also show the effects of incubation with 100 nmol/L bafilomycin. b, Acridine orange (AO) was applied for 10 seconds at 10 nmol/L in the presence or in the absence of 10 nmol/L bafilomycin. Note the different fluorescence scale. Cell fluorescence was recorded as described in Materials and Methods.

The HYD fluorescence was rapidly photo bleached, even during the time required to obtain the confocal image, thus reducing the apparent light intensity. However, a few tenths of a second is enough for the fluorescent image to recover, suggesting that new HYD is continuously accumulating inside the granules.

The time course of HYD accumulation can be followed in a standard epifluorescence microscope, as shown in Figure 5. This accumulation can be prevented by 5-minute preincubation with 100 nmol/L of bafilomycin. These data suggest that HYD is mostly compartmentalized by protonation within chromaffin granules, although accumulation in other acidic organelles like lysosomes could also occur.

The accumulation curve of HYD recorded from Figure 5a follows the double exponential probably caused by the delay of HYD for crossing the plasma membrane:

$$\text{Fluorescence} = K_o + K_1 e^{-\alpha t} + K_2 e^{-\beta t}, \quad (1)$$

where K_o is the apparent saturation level (0.61), $K_1 = -0.51$, $K_2 = -0.13$, $\alpha = 0.007$, and $\beta = 0.17$.

Hydralazine Displaces Catecholamines From Secretory Vesicles

The reduction of the granule catecholamine content and the vesicular accumulation of HYD suggest a displacement of catecholamines from secretory vesicles to the cytosol. Experiments from Figure 6 were conducted to test this hypothesis. Cells were incubated in Krebs-HEPES buffer lacking Ca^{2+} (200 μmol/L EGTA) and permeabilized with a 10-second pulse of 20 μmol/L digitonin.[24] Under these conditions digitonin application did not evoke secretory spikes; however, a small elevation of the basal release occurred due to the leak of free catecholamines from the cytosol. When digitonin was applied on a HYD-treated cell, this elevation was significantly increased (Figure 6).

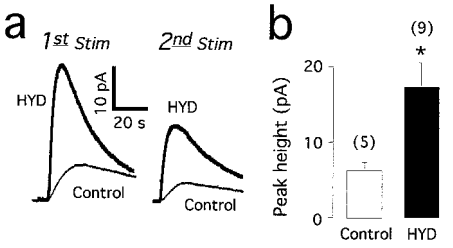


Figure 6. Hydralazine displaces catecholamines from the vesicular to the cytosolic compartment. Cells were bathed in a Ca^{2+}-free medium (200 μmol/L EGTA) and permeabilized by a short (10 second) pulse of 20 μmol/L digitonin in the absence and in the presence of 10 nmol/L HYD. Digitonin pulses were repeated 2 minutes later. a, Typical traces obtained by a carbon fiber electrode placed onto the cell surface. b, Average values (±SEM) obtained by measuring the maximum amplitude of the traces. *$P<0.05$ Student's t test.

Hydralazine Displaces Ca^{2+} From Intracellular Stores

A brief HYD application increases the intracellular free Ca^{2+} levels. Cells were bathed in a medium nominally lacking Ca^{2+} (200 μmol/L EGTA added) for the 5 minutes before the application of HYD. The time course of Ca^{2+} elevation did not follow a uniform pattern like that usually observed on the application of other types of stimuli like caffeine or histamine (Figure 7). This erratic pattern suggests that Ca^{2+} release is not mediated by an intracellular signal like that mediated by a drug receptor coupled with a specific internal store (like IP_3 production); rather, cytosolic Ca^{2+} rise might be due to its displacement from internal stores. Furthermore, HYD promoted the Ca^{2+} free elevation even after depletion of endoplasmic reticulum with three consecutive stimuli of 10 mmol/L caffeine, suggesting that HYD is acting on a different intracellular store (Figure 7, bottom trace).

Hydralazine Does Not Modify Intracellular Nucleotide Levels

We have shown that NO acting on a soluble guanylate cyclase promotes the slowing of exocytosis.[9] It is hence an attractive hypothesis that HYD could modulate this step by increasing the levels of cGMP. However, hydralazine 1 to 10 000 nmol/L did not promote any significant change in chromaffin cells. Hence, cGMP from control cells and from cells treated for 15 minutes with HYD 100 nmol/L were 12.4±1.8 and 11.1±3.2 fmol/L per μg protein, respectively. In addition, hydralazine did not modify the increase in cGMP elicited by sodium nitroprusside (data not shown).

No changes in the cAMP levels (4.2±0.3 pmol/L per μg protein) were found after 15 minutes treatment with 10 nmol/L or 100 nmol/L HYD (3.9±0.5 and 4.1±0.4 pmol/L per μg protein, respectively).

Discussion

Chromaffin cells share many important features with sympathetic neurons, and chromaffin granules are similar to the large dense cored vesicles found in sympathetic nerve terminals.[25] It is hence likely that both cells share common releasing mechanisms.

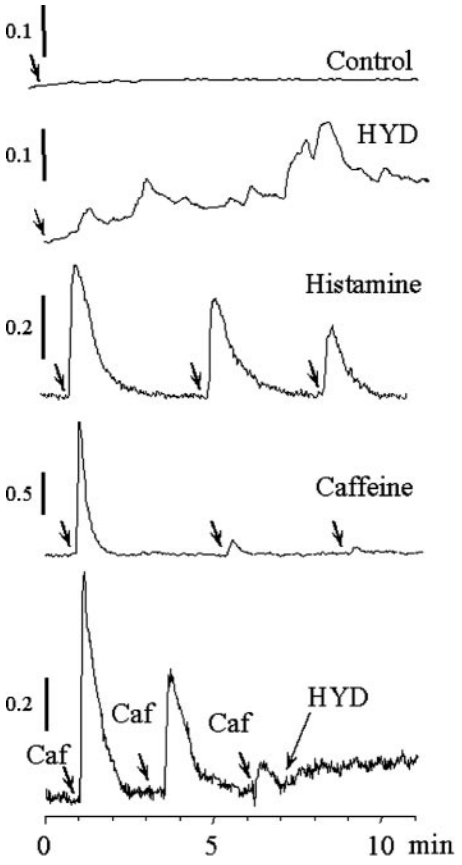


Figure 7. Hydralazine rapidly releases intravesicular Ca^{2+}. Cells loaded with fura-2AM were bathed in a Ca^{2+}-free medium (200 μmol/L EGTA). Figure shows several time courses of intracellular free Ca^{2+} elicited by a 20 second pulse of Ca^{2+} free buffer (Control) or 10 nmol/L HYD. Figure also shows the effects of 5-second stimulation with 30 μmol/L histamine or 10 mmol/L caffeine in the same conditions. Bottom trace, HYD still elevating Ca^{2+} even when the intracellular stores has been emptied with caffeine. Drugs were applied from a pipette placed at 40 μm from the cell at the time indicated by arrows. $[Ca^{2+}]_c$ was continuously monitored. Figures show typical traces from 4 to 6 experiments. Note the different size of the calibration bar, which indicates the fluorescence ratio obtained at 340 and 380 nm of excitation light.

Amperometry is a potent tool for the study of the kinetics of exocytosis at the single event level and to monitor apparent quantal size of secretory spikes, measured as the parameter Q.[26] When an electrode is gently touching the cell membrane, only a thin water layer is between the cell and the electrode surface. This distance can approximately be considered as the width of a synaptic junction.[27] The electrode is thus "seeing" the cell surface and the exocytotic phenomenon like a postsynaptic cell. Therefore, changes of I_{max} will reflect changes in the concentration of the neurotransmitter reaching its target receptors.[9,12]

Hydralazine is a potent vasodilator drug whose mechanism(s) of action remains unclear even after 50 years of clinical use. Although a series of putative cellular targets for this drug have been proposed,[3,28,29] most of these mechanisms occur only when high HYD concentrations are used. Because hydralazine produces higher relaxing effects on those vascular tissues that are profusely innervated by sympathetic nerve

terminals,[15] it points toward the presynaptic nerve terminal as the target for HYD.

It has been shown that HYD reduces the amount of catecholamine released from chromaffin cells but only with high concentrations of the drug.[30] Conversely, Chevillard et al[31] report that HYD, in a range of concentrations that are similar to those used in the present study, reduces ^{3}[H]-noradrenaline output from sympathetic nerve terminals.

Amperometry experiments show that HYD does not produce significant changes in the frequency of spike firing. Although it reduces the amount of catecholamine secretion during the first minutes HYD does not change the total secretion is not altered after 5 minutes as measured by HPLC and fluorimetry (Figures 1b and 1c).

Nanomolar concentrations of HYD rapidly produce a slower rate of transmitter release from single events of exocytosis and a decrease in the apparent quantal size (Figure 2 and Table), suggesting the involvement of HYD in the storage mechanisms of catecholamine. It cannot be explained in terms of a reduction in catecholamine synthesis or vesicular monoamine transporter (VMAT) inhibition because it occurs within 30 to 40 seconds. The fragile equilibrium of the intravesicular components seems to be easier to alter than the total number of vesicles that undergo exocytosis.

A possible explanation for the lack of effect of HYD on the total amount of catecholamines released after 5 minutes of recording, in spite of the smaller amount of amine secreted by each exocytotic event, might be the nonexocytotic release. Hydralazine displaces the vesicular catecholamines, as shown in Figure 6, and it could be possible that they leak out the cell by a nonquantal mechanism. This effect would be similar to that recently described for other weak bases like tyramine or amphetamine in chromaffin cells.[32] The nonquantal release of catecholamines from sympathetic nerve terminals is a well-documented phenomenon. However, to our knowledge, weak bases are poor secretagogues in adrenomedullary tissues and their nonquantal release of catecholamines has not been so far demonstrated in cultured chromaffin cells. Although an increase in the basal levels of catecholamines is frequently observed on HYD application, we cannot ensure that this small amount can account for the different time courses of cumulative secretion shown in Figure 1.

Several weak bases like amphetamine or tyramine displace catecholamines from secretory vesicles, thus producing smaller secretory events.[32] The accumulation of these compounds, as well as catecholamines, is favored by lower intravesicular pH.[13,33,34] The driving force for catecholamine accumulation is the proton gradient created by the V-type ATP-dependent H^{+}-pump that maintains an intragranular pH around 5.5.[35] This pH coincides with the isoelectric point of chromogranin A,[36] the major granule matrix component, which has been considered to play an important physiological role in the intragranular complexation of soluble products.[10] The pH gradient is responsible for the accumulations of weak bases that ionize when transported across the vesicular membranes. This situation has been used to monitor the intragranular pH by the compartmentalization of AO. Hydralazine accumulation within secretory vesicles follows a similar pattern as AO and both are also reduced by bafilomycin (Figures 4 and 5). Both HYD and AO seem to compete for the same intracellular compartment (online Figure 1).

Lysosomes and early endosomes have an acidic inner media that favors the accumulation of weak bases like HYD. This pH gradient is also blocked by bafilomycin. It is likely that a small part of the HYD signal will come from these organelles. However, the number and the relative volume of chromaffin granules exceed any other organelle in chromaffin cell (≈17%). Data from measurements of free catecholamines in the cytosol also support the accumulation of HYD in granules.

Hydralazine is a weak base and its distribution follows the Henderson-Hasselbach equation:

$$(2)\qquad \frac{[C]_{vesicle}}{[C]_{extracell}} = \frac{(1+10^{pKa\text{-}pH\ vesicle})}{(1+10^{pKa\text{-}pH\ extracell})}$$

That means that at an intravesicular pH of 5.5, nearly 98% of HYD (pKa= 7.3) is ionized versus the extracellular pH 7.35 at which 50% of the HYD is ionized thus facilitating its vesicular accumulation.

Hydralazine could compete with catecholamines and displace them from the vesicular matrix (Figure 6); both substances possess an amine group, which can be implicated in the Donnan's association with chromogranin A. Alternatively, HYD could also displace catecholamines unbound to chromogranins[37] from intravesicular ATP, whose concentration is about 130 mmol/L.[37a] This latter hypothesis is supported by the fact that HYD affects preferentially vesicles with a larger catecholamines content (Figure 3) where the ratio of unbound catecholamines to catecholamines bound to the intragranular matrix is also larger. In any case, both mechanisms could explain the decrease observed in the quantal size (Figure 2 and Table) and the increase in the cytosolic free Ca^{2+} (Figure 7). Hydralazine still increases the cytosolic Ca^{2+} even after depletion of endoplasmic reticulum with repetitive stimuli with caffeine (Figure 7, bottom trace). Although Ca^{2+} could be stored in other organelles, the most plausible sources are the secretory vesicles.

The nonexocytotic catecholamine release could also explain why, under some conditions, there is not a decrease in the total catecholamine output in adrenomedullary tissues[30] and the paradoxical hypertensive response observed on HYD administration in clinical practice.

However, the efficiency of a given synapse depends on the size of the discrete packages of neurotransmitter released and of the postsynaptic responsiveness, but it does not depend of the nonexocytotic release caused by the slow leakage through the cytosol.

The activation of several second messenger cascades such as NO-guanylate cyclase–cGMP-dependent protein kinase (PKG)[9] or cAMP-dependent protein kinase (PKA)[12] decelerates kinetics of exocytosis at the single event level whereas activation of PKC[38,39] (R. Borges, unpublished observations, 1996) accelerates it. However, HYD does not change the intracellular levels of cAMP nor cGMP and, contrary to cAMP-mediated responses, HYD decreases the granule content. The erratic time course of Ca^{2+} released from intracellular stores (Figure 7), and the lack of effect on Ca^{2+} entry also supports this mechanism.

Our results suggest that the main effect of HYD is the displacement of the intravesicular catecholamines. The reduction of quantal size and the reduction of the kinetics of exocytosis at the single event level could explain most of the clinical and side effects of HYD. The physical-chemical nature of this new mechanism of action could explain the elusive nature of the putative HYD receptor, which has been sought for over five decades.

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