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Nitric Oxide Modulates a Late Step of Exocytosis*[S]

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José D. Machado‡, Fernando Segura, Miguel A. Brioso, and Ricardo Borges§

From the Unidad de Farmacología, Facultad de Medicina, Universidad de La Laguna, 38071 La Laguna, Tenerife, Spain

The effects of nitric oxide (NO) on the late phase of exocytosis have been studied, by amperometry, on Ba^{2+}-stimulated chromaffin cells. Acute incubation with NO or NO donors (sodium nitroprusside, spermine-NO, *S*-nitrosoglutathione) produced a drastic slowdown of the granule emptying. Conversely, cell treatment with N^{ω}-nitro-L-arginine methyl ester (a NO synthase inhibitor) or with NO scavengers (methylene blue, 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide potassium) accelerated the extrusion of catecholamines from chromaffin granules, suggesting the presence of a NO modulatory tone. The incubation with phosphodiesterase inhibitors (3-isobutyl-1-methylxanthine or zaprinast) or with the cell-permeant cGMP analog 8-bromo-cGMP, mimicked the effects of NO, suggesting the involvement of the guanylate cyclase cascade. NO effects were not related to changes in intracellular Ba^{2+}. NO did not modify the duration of feet. Effects were evident even on pre-fusioned granules, observed under hypertonic conditions, suggesting that the fusion pore is not the target for NO, which probably acts by modifying the affinity of catecholamines for the intragranular matrix. NO could modify the synaptic transmitter efficacy through a novel mechanism, which involves the regulation of the emptying of secretory vesicles.

NO is a short-lived, highly reactive radical involved in several physiological functions such as vasodilatation, macrophage mobility, cytotoxicity, or gene transcription (see Ref. 1 for a review). In addition, NO is a modulator of neurotransmitter-mediated responses in the central nervous system (2).

In the adrenal gland, NO could be secreted from the chromaffin cell itself (3), or paracrine, being secreted from contiguous endothelium (4). In addition, NO could also be released by afferent nerves (5, 6). To date, many *in vitro* studies have been carried out to elucidate the role of NO/cGMP on the secretory processes of chromaffin cells. Results are still controversial; O'Sullivan and Burgoyne (7) reported a potentiation of CA[1] release induced by various NO-releasing agents, whereas others have found a dose-dependent inhibition of secretion (8, 9), or no changes at all (10, 11). NO is also reported to increase basal secretion (3, 9). NO induces CA synthesis through tyrosine hydroxylase activation (11). The present view is that the main role of NO is the control of adrenal blood flow, whereas its modulation on the bulk of CA release seems to be small (12).

Catecholamines and other soluble components are stored within chromaffin granules at very high concentrations: 0.5–1 M (13–16), thus creating a high intragranular osmotic pressure. Complexation of intragranular substances will reduce the osmotic forces, thereby preventing granule lysis (15–18). No mechanisms are known at present that regulate this intragranular matrix complex.

Amperometric techniques allow the direct observation of time-course kinetics of single secretory events and have been successfully used to study the late phase of exocytosis (19–22).

Here, we show conclusively that NO, acting on the guanylate cyclase cascade, produces dramatic changes on quantal release of CA by single chromaffin cells, probably acting on the intragranular matrix. To our knowledge, this is the first experimental report suggesting that the kinetic of vesicular release can be modulated by drugs or second messengers. In addition, we have found evidence indicating that the interaction of intragranular components can be modulated under physiological conditions. If this effect of NO were extended to dense core vesicles of sympathetic neurons, it would result in significant changes on synaptic efficacy, even releasing the same amount of noradrenaline quanta.

EXPERIMENTAL PROCEDURES

Materials—Noradrenaline, SNP, IBMX, zaprinast, methylene blue, 8-Br-cGMP, cultured media, sera, and collagenase type IA were purchased from Sigma-Aldrich (Madrid, Spain). Fura-2/AM, Pluronic acid, and *S*-nitrosoglutathione were obtained from Molecular Probes (Eugene, OR). Spermine-NO, L-NAME, and C-PTIO were purchased from RBI (Natick, MA). NO gas (N30) was purchased from Air Liquide (Tenerife, Spain). Urografin® was obtained from Schering España (Madrid, Spain). Culture plates were from Corning (Cambridge, MA). All salts used for buffer preparation were reagent grade.

Culture Chromaffin Cells—Bovine adrenal chromaffin cells, enriched in adrenaline, were prepared as described elsewhere (23). Cells were planted on 12-mm diameter glass coverslips at an approximate density of 5×10^5 cells/coverslip. Cells were maintained at 37 °C in a 5% CO_2 environment and used at room temperature between 1 and 4 days of culture.

Amperometric Detection of Exocytosis—Carbon fiber microelectrodes were prepared as described (24). Carbon fibers (5 μm radius; Thornel P-55, Amoco Corp., Greenville, SC) were the kind gift of Prof. R. M. Wightman (University of North Carolina at Chapel Hill, NC). Electrochemical recordings were performed using an Axopatch 200B (Axon Instruments, Foster City, CA). A fixed potential of +650 mV was maintained between the carbon fiber electrode *versus* an Ag/AgCl pellet reference electrode. Electrodes were backfilled with 3 M KCl to connect to the headstage. Electrodes were tested with a flow-injection system with noradrenaline standard solutions using an EI-400 potentiostat (Ensman Inst. Bloomington, IN) (24).

Glass coverslips with adhering adrenal cells were washed in Krebs-HEPES buffer solution containing (in mM): NaCl (140), KCl (5), $MgCl_2$

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[S] The on-line version of this article (available at http://www.jbc.org) contains Fig. 1S and legend.

‡ Recipient of a scholarship of Instituto Tecnológico de Canarias.

§ To whom correspondence should be addressed. Tel.: 34-922-319346; Fax: 34-922-655995; E-mail: rborges@ull.es.

[1] The abbreviations used are: CA, catecholamine; $[Ba^{2+}]_c$, cytosolic barium concentration; 8-Br-cGMP, 8-bromo-cGMP; $[Ca^{2+}]_c$, cytosolic calcium concentration; C-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium; CGA, chromogranin A; IBMX, 3-isobutyl-1-methylxanthine; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NOS, nitric-oxide synthase; PKG, cGMP-dependent protein kinase; SNP, sodium nitroprusside.

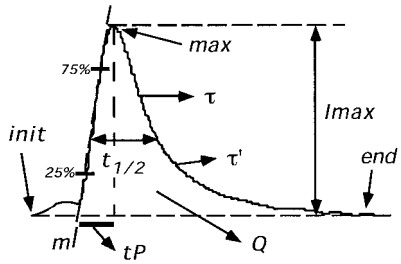

FIG. 1. **Parameters used for secretory spike kinetics characterization.** For explanation, see "Experimental Procedures."

(1.2), $CaCl_2$ (2), glucose (11), and HEPES (10), brought to pH 7.35 with NaOH. Cells were placed in a perfusion chamber positioned on the stage of an inverted microscope (Leica DM-IRB, Wetzlar, Germany). Amperometric measurements were performed with the carbon fiber microelectrode gently touching the cell membrane. Cell release was stimulated by 5-s pressure ejection of 5 mM Ba^{2+} from a micropipette placed 40 μm away from the cell. Ba^{2+} was used as a secretagogue because it does not require receptor activation or membrane depolarization and because it produces a low frequency of secretory event, so that during spike analysis the initial and final points of each wave can be easily distinguished.

Experiments using hypertonic solutions were performed as described previously (22). Briefly, cells were incubated in hypertonic Krebs (750 mosM, obtained by adding NaCl) solution for 5 min, in the presence or in the absence of 10 μM SNP. Under these hypertonic conditions, secretion was elicited by pulse injection of isotonic Krebs solution.

NO Solutions—In order to reduce NO degradation, free O_2 was reduced from the stock solution. Krebs solution was bubbled with pure N_2 into a sealed bottle for about 60 min. Five milliliters of the above solution were transferred into 7-ml sealed vials and bubbled again for another 10 min, keeping a pure N_2 atmosphere in the empty space. This degassing procedure reduced the pO_2 to 15–20 mmHg (ABL-2, Radiometer, Copenhagen, Denmark), equivalent to 20–40 μM free O_2.

In a fume hood, NO gas was on-line bubbled through a sealed bottle containing 5 N NaOH, to get rid of acid-generated material and then to an empty 7-ml vial for 1 min at 0.5 bar, maintaining the flow for 5 min. Two milliliters of the deoxygenated solution were injected into the vial containing pure NO using a gas-tight syringe through a rubber stopper. The vial was kept at 4 °C and used within 2 h. NO concentration in the solution, measured by Griess method, was 2.8 mM.

Data Analysis—Amperometric signals were low-pass filtered at 1 KHz and sampled at 4 KHz and collected using a locally written software (Labview for Macintosh, National Instruments, Austin, TX). Data analysis was carried out using locally written macros for IGOR (Wavemetrics, Lake Oswego, OR). These macros allow the automatic digital filtering, secretory spike identification, and build histograms for spike classification. All the above macros are free shareware.

Fig. 1 describes the parameters measured from each secretory spike. Once the beginning and the end points were found, the computer obtained the maximum amplitude of the oxidation current (I_{max}), which was expressed in pA. The ascending slope (m) was determined from the linear part of the trace located between 25% and 75% of the I_{max}; hence, this parameter is not affected by the presence of the pre-spike phenomenon (foot), m being expressed in nA/s. The time to peak (t_P) was determined between the point at which the back-extrapolation of the slope line crossed the base line and the point of I_{max}. This parameter partially shows the slow dissociation of adrenaline from intragranular proteic matrix. Total granule release (Q) was obtained by integration of the curve, which indicates the amount of oxidizing substances released and is expressed in pC. Q was normalized as the cubic root ($Q^{1/3}$) and two spike fade constants ($\tau = I_{max} - I_{max}/e$) and ($\tau' = I_{max}/e$) taken from the adjusted exponential decay.

Because of day-to-day variations in electrode sensitivity and cell responsiveness, significant differences were currently observed between untreated cells, used as controls, from different days. For this reason, effects of drugs on secretory spikes were compared with control experiments carried out under the same conditions. Statistical analysis was carried out by the non-parametric Mann-Whitney U test.

Measurement of Cytosolic $[Ba^{2+}]_c$—Glass coverslips with adhering adrenal cells were washed twice in Krebs buffer solution and incubated with 2 μM fura-2/AM (stock solution dissolved in 20% Pluronic F-127 in Me_2SO) and 0.1% fetal calf serum for 45 min. Cells were then washed twice to remove extracellular dye and placed in the perfusion chamber, as described above. Intracellular Ba^{2+} was measured using a computer-operated monochromator (TILL Photonics, Munich, Germany) controlled by Labview software. Fluorescence signals were low-pass filtered at 510 nm and detected by a photomultiplier mounted to a viewfinder (TILL Photonics) that defined the area of interest over which the fluorescence intensity was integrated.

Data of $[Ba^{2+}]_c$ time courses were collected at 10 Hz and expressed as fluorescence ratios (F_{360}) and (F_{380}).

RESULTS

NO Affected the Time Course of Secretory Spikes—The direct application of NO produced drastic effects on the time course of secretory spikes, which are summarized on Table I and II. These effects were reproduced with all of the NO donors tested (Table II). Spermine-NO was particularly potent promoting a fall in granules emptying kinetics. Incubation with *S*-nitrosoglutathione and direct NO application produced similar concentrationdependent changes, which relate closely to their described abilities for producing free NO (25). Spike decay was also affected; τ' increased with 200 nM NO from 12.9 ms to 20.3, whereas τ changed from 27.1 to 42.3 ms, indicating that NO strongly slowed down the last phase of exocytosis. The observed spike shape changes were not caused by a decrease in electrode sensitivity, as SNP did not modify the oxidation curves observed in the flow stream system used for electrode calibration (data not shown).

Fig. 2 shows histograms from secretory spikes obtained in the absence or in the presence of 10 μM of the NO donor SNP incubated for 10–20 min. SNP caused a dramatic reduction in the spike I_{max}, averaging a fall to a 36% of control that was accompanied by a $t_{1/2}$ average increase of 161% (Table II). Virtually, no spikes over 60 pA were found upon SNP treatment. Conversely, the number of events with a $t_{1/2}$ of over 40 ms was greatly increased. The releasing speed decayed as the ascending slopes of spikes were drastically reduced. The histogram in Fig. 2 shows that the number of secretory events with a t_P over 10 ms in duration was largely increased. NO effects were even more pronounced with 100 μM SNP, but a dramatic reduction in the number of spikes prevented us from using these data. Total granule release remained unaltered at low concentration of the drug, whereas a reduction was observed when SNP was raised to 10 μM.

In order to rule out SNP effects caused by NO metabolites accumulated along drug incubation, 10 μM SNP was also applied for 10 s in the vicinity of a cell. The effects of this brief application, although less pronounced, were qualitatively similar (Table II); I_{max} dropped from 45 to 34 pA, and $t_{1/2}$ rose 36%.

Fig. 3 describes how NO affected the time course of spikes. Incubation with 100 μM spermine-NO for 10 min produced a drastic change in spike shape, which included a reduction in the I_{max} and in the m (ascending slope), accompanied with an increase in the t_P, τ, τ', and $t_{1/2}$. The effect of NO on exocytotic kinetics occurs in few seconds (Fig. 4).

Due to the large differences within control data from one day to another, each treatment was compared with its own untreated control cells, from the culture of the same day using the same electrode. Table II shows data normalized with their own control. Although the effects of NO on total CA released by Ba^{2+} were not analyzed in detail, a discrete reduction in spike firing, of about 15%, was observed. In addition, the average spike charge observed was reduced by 20–40%.

cGMP Mimicked the Effects of NO—The guanylate cyclase PKG is known to be the main cellular transduction system for NO. In order to test if cGMP could mimic the NO effects, cells were treated with 10 μM cGMP-permeable analog 8-Br-cGMP. Results are summarized on Table II. Incubation for 20–30 min caused changes of spike shape qualitatively similar to those found with NO and NO donors. The secretory speed was profoundly slowed, and spikes were indistinguishable from NO-

TABLE I

The effects of NO on secretory spike parameters

The effects of NO incubation are shown together with their own control cells (see "Results"). Data are expressed in the units indicated. See Fig. 1 for explanation of each parameter.

	I_{max}	Q	$t_{½}$	m	t_P	n	n
	pA	*pC*	*ms*	*nA/s*	*ms*	*spikes*	*cells*
Control	41.9 ± 1.8	1.4 ± 0.06	24.8 ± 0.8	11.6 ± 0.6	20 ± 1	1761	7
NO (20 nM)	20.1 ± 0.9	1.0 ± 0.05	33.1 ± 1.1	4.2 ± 0.2	33 ± 2	716	8
NO (200 nM)	19.0 ± 1.1	1.1 ± 0.07	41.0 ± 1.6	2.7 ± 0.2	51 ± 4	332	6

TABLE II

The effects of various NO/guanylate cyclase activators and blockers on secretory spike parameters (normalized data)

Data were normalized as percentages of their own control group. Statistical analysis (Mann-Whitney U test) was performed on original data. Significant differences, $p < 0.01$ (**) or $p < 0.001$ (*). SNP was incubated at 10 μM.

	I_{max}	Q	$t_{½}$	m	t_P	ns^a	nc^a
NO (20 nM)	48*	71*	133*	36*	165*	716	8
NO (200 nM)	45*	79	165*	23*	255*	332	6
Puffed SNP (10 μM)	74*	100	136*	61*	155*	1059	14
SNP 1 μM	64*	94	154*	33*	247*	1310	11
SNP 10 μM	36*	62*	161*	24*	371*	871	21
SPER-NO 100 μM	27*	55*	177*	18*	150*	523	13
GSNO 20 μM	54*	73*	140*	31*	360*	1446	7
8-Br-cGMP	36*	63*	137*	34*	110	319	11
IBMX 5 mM	73*	104	128*	54*	135*	1286	15
IBMX + SNP	64*	88	125*	50*	115*	946	15
Zaprinast 10 μM	63*	110	166*	37*	195*	572	12
Zaprinast + SNP	60*	124*	217*	26*	248*	523	11
L-NAME 10 μM	98**	87	85*	117*	63*	1536	13
L-NAME 100 μM	78*	56*	69*	96	63*	837	13
C-PTIO 10 nM	223*	111	64*	331*	104	785	10
Methylene blue	142*	62*	52*	290*	58*	364	13

[a] *ns*, number of spikes; nc, number of cells.

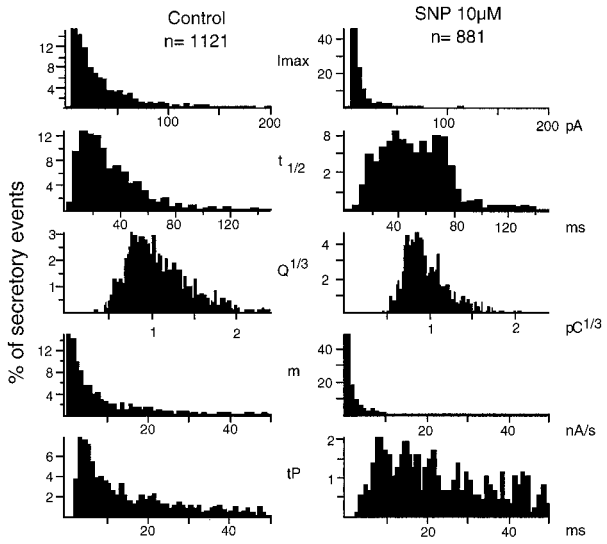

FIG. 2. **NO effects on spike shape.** Histograms from secretory spikes were obtained in the absence and in the presence of 10 μM SNP for 10–20 min. Data are from 18 and 23 different cells, respectively. *Columns* at the *right* of each graph indicate an increase in the number of higher (I_{max}), wider ($t_{1/2}$), larger ($Q^{1/3}$), sharper (m), or flatter top (t_P) spikes; $Q^{1/3}$ results from cubic root of Q data (see "Experimental Procedures"). Normalized data are summarized in Table II.

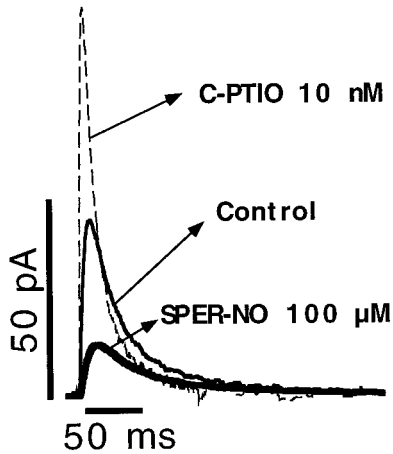

FIG. 3. **Representative traces with mean spike characteristics.** Spikes were plotted from the data sets of Table I. Control spike is indicated by the *solid thin line*, spermine-NO by a *solid thick line*, and C-PTIO by a *dashed line*.

treated cells. Similarly, τ values were affected to the same extend, τ' increased from 9.47 to 12.7 ms, whereas τ changed from 20.8 to 31.4 ms, indicating that cGMP affected as well the very last phase of exocytosis.

Endogenous cellular levels of cGMP can also be increased by inhibiting its degradation. Table II shows the effects of 20 min of incubation with two phosphodiesterase inhibitors, IBMX and the more specific inhibition of cGMP-phosphodiesterase, zaprinast. When applied alone, both substances produced net changes on spike shape similar to those observed with NO donors. In the presence of 10 μM SNP, slight additive effects were observed, suggesting that both agents act through the same mechanism. Zaprinast increased τ' from 13.4 to 22.5 ms and τ from 30 to 49.3 ms, whereas these values were increased by IBMX from 19 to 24.7 ms and 36.6 to 47.3 ms, respectively; the addition of SNP did not significantly modify the τ values obtained with IBMX.

NOS Inhibition Accelerated the Last Stage of Exocytosis—Cells were treated with L-NAME at 37 °C for 30 min and exocytotic spikes recorded in the presence of the drug. Low L-NAME concentrations (10 μM) promoted significant changes on the spike $t_{1/2}$, m, and t_P values (Table II). Although data obtained with 100 μM L-NAME were qualitatively similar, they should be interpreted with caution because of the total granule release reduction observed (44%). The effects of L-NAME per-

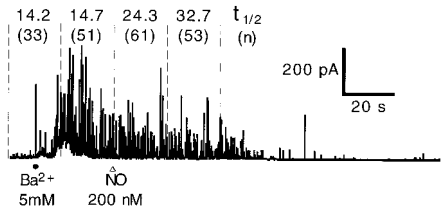

FIG. 4. **Time course of NO effects on quantal release.** Typical amperometric trace (from five) of a secretory response from a cell stimulated for 5 s with 5 mM Ba^{2+} (*dot*); 30 s later NO solution was added to the cell chamber to get an estimated NO concentration of 200 nM (*triangle*). *Vertical dashed lines* indicate periods of 20 s where $t_{1/2}$ values were measured. *Numbers* between *lines* show average values for $t_{1/2}$. The number of secretory spikes computed are in *parentheses*. *Calibration bars* are shown on the *right*.

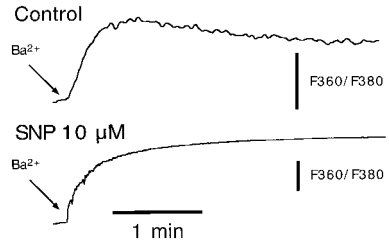

FIG. 5. **The effect of SNP on the time course of $[Ba^{2+}]_c$.** Cells were loaded with fura-2 and recorded as described under "Experimental Procedures." A pressure-injected pulse of 5 mM $BaCl_2$ was applied for 5 s as indicated by *arrows*. Traces show the time course in the absence (*upper trace*) and in the presence of 10 μM SNP (*lower trace*). Traces are representative of 10 untreated cells and 6 cells incubated with SNP.

sisted during incubation but rapidly disappeared upon drug removal, indicating a reversible NOS inhibition. High L-NAME concentrations (1 mM) resulted in a drastic reduction of the number of secretory spikes, probably due to a nonspecific or toxic effect (data not shown).

NO Reduction Promoted an Increase in the Number of Sharper Spikes—The presence of NOS within chromaffin cells suggested the existence of a NO basal tone which probably modulates continuously the kinetics of the exocytosis. This basal tone was revealed by NO sequestration using NO scavengers. Table II shows the effects of cell incubation with methylene blue and C-PTIO on Ba^{2+}-evoked secretory spikes. Neither methylene blue nor C-PTIO caused CA release. However, both agents induced a concentration-dependent reduction of the $t_{1/2}$, which was accompanied with an increase in m and a shortening of t_P. C-PTIO was more potent than methylene blue, probably because of its specificity and ability to serve as NO scavenger; the I_{max} was significantly increased to 223% after only 4–5 min of incubation, revealing the presence of a basal NO activity within cultured cells. An unexpected effect observed with NO scavengers was the changes found on spike charge. Table II shows that C-PTIO increased Q, whereas methylene blue induced a reduction. However, in all cases, an increase in the I_{max} together with a reduction in $t_{1/2}$ and τ was observed. Fig. 3 summarizes the effect of 10 min of incubation with a low concentration of C-PTIO (10 nM); spikes became taller and thinner, and the CA concentration reaching electrode was much bigger. Note that NO could account for 10-fold changes in the CA concentration reaching electrode (I_{max}).

NO Donors Did Not Reduce the $[Ba^{2+}]_c$—One possible target site of NO could be the interference with Ba^{2+} movements. A series of experiments was done measuring $[Ba^{2+}]_c$ in the absence and in the presence of 10 μM SNP. Fig. 5 shows representative traces of F_{360}/F_{380} ratios obtained with cells loaded with fura-2. Cells treated with SNP showed no changes on the ascending part of the traces. However, a significant increase on $[Ba^{2+}]_c$ of 18 ± 2% was observed on the plateau of $[Ba^{2+}]_c$ traces (6 cells of each group). In any case, the increased $[Ba^{2+}]_c$ levels were maintained in both groups of cells for 6–8 min after the stimulus, the time usually taken for amperometric recording.

The Intragranular Matrix as the Probable Target of NO/Guanylate Cyclase—A series of experiments was carried out in order to elucidate the cellular target site for NO. Foot (prespike feature) duration indicates the elapsed time for formation of the fusion pore. If a given substance modifies the fusion pore machinery, the duration of the foot might be altered. However, no differences on foot duration were found between foot produced in control conditions and cells incubated with 10 μM SNP: 17.4 ± 1.1 ms (n = 99) *versus* 15.5 ± 1.2 ms (n = 84), or 8-Br-cGMP: 14.8 ± 0.8 ms (n = 87) *versus* 15.6 ± 1.3 ms (n = 64). In amperometric recordings, only the 35% of spikes exhibited foot (13, 19). In this study, measurements were only performed on spikes where the beginning and finishing points of foot were clearly distinguishable.

We have shown that cell stimulation under conditions of high tonicity (*i.e.* >700 mosM), promoted the partial exocytosis of chromaffin granules (22). Ba^{2+} application caused an increase in the $[Ba^{2+}]_c$, which was not accompanied by secretory spikes. However, exocytotic pore formation had already occurred, as demonstrated by the fact that brief pressure injection of isotonic saline caused many exocytotic events, which lasted throughout the time of application. These secretory spikes had lost 50% of their content, and they did not possess foot because they came from pre-fusioned granules that could only swell in response to isotonic media (Fig. 1[S]).

After Ba^{2+} stimulation under hypertonic conditions, granules were already opened; changes produced in spike shape must not be caused by an effect on the fusion pore but on another target, probably on the affinity of CA for the intragranular matrix. Data obtained from Fig. 6 (*table inset*) show significant changes on spike shape obtained from pre-fusioned granules, which mimicked those produced under normal conditions. Moreover, pre-fusioned granules progressively lost CA, indicated by the gradual fall in Q values along the time from Ba^{2+} stimulation. NO partially prevented this loss. As shown in Fig. 6, there were statistical differences between Q values obtained from control *a*nd SNP groups, indicating that NO increased the affinity of CA for its intragranular matrix.

DISCUSSION

The results of this study demonstrate that NO, even at low concentrations, produces profound effects on the kinetics of secretory spikes (Figs. 2 and 3). We also show that exocytosis is modulated by a basal NO tone present within the cultured cells. Our data suggest that most of the NO action is carried out through the guanylate cyclase PKG pathway, as incubation with cGMP analog 8-Br-cGMP and phosphodiesterase inhibitors mimicked NO effects.

Previous studies have reported changes on spike shape by altering temperature (21), ionic composition (26–28), or osmotic strength (22) of the extracellular media. Also, selective amino acid deletions on the granule fusion complex protein, SNAP-25, caused changes on the quantal release kinetics (29). However, these maneuvers are unlikely to occur under physiological conditions.

A possible target site for NO would be the interaction with Ca^{2+} homeostasis. Calcium participates in the fusion pore dynamics (28) and in the regulation of the "kiss and run" phenomenon (27). NO has been implicated in the modulation of Ca^{2+} channels (30), as well as in the control of membrane potential, through the activation of Ca^{2+}-activated K^+ channels (31). Brief Ba^{2+} application, in the absence of depolarizing

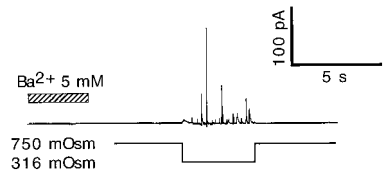

FIG. 6. **Effects of SNP on secretory spikes from pre-fusioned granules.** Cells were incubated in the absence and in the presence of 10 μM SNP in an hypertonic (750 mosM) Krebs solution. Under these conditions, Ba^{2+} stimulation did not evoke secretory spikes until a brief (5 s) pulse of isotonic saline was applied. The effects of SNP on pre-fusioned granules are summarized in the table (*inset*). Units are the same as in Table I. #, $p < 0.01$; and *, $p < 0.001$ by Mann-Whitney U test.

stimuli, resulted in a slow and long-lasting $[Ba^{2+}]_c$ time course due to its poor efflux from the cell (22, 32). Fig. 5 shows that SNP did not reduced the $[Ba^{2+}]_c$ responses but produced a slight increase. These results do not support the assumption that NO effects on exocytosis were caused by a reduction of $[Ba^{2+}]_c$.

The time course of exocytosis could have been altered, at least, through three mechanisms: changing the fusion pore expansion (27, 28, 33), altering the Cl^-/water flow into the granule (18, 34), or modifying the affinity of CA for intragranular matrix (35, 19).

Data presented here cannot conclude whether the target of NO/cGMP is the fusion pore expansion, the Cl^-/H_2O or the CA/CGA association. However, there are some arguments in favor of the later: (i) measurements done on feet duration did not support changes in fusion pore expansion after cell treatment with SNP or 8-Br-cGMP (Fig. 1[S]); (ii) NO effects were observed even on pre-fusioned granules obtained eliciting secretion under hypertonic conditions, and secretion was elicited only on return to isotonic conditions and (iii) in these pre-fusioned granules, NO prevented the CA leakage. Chromogranin A, ATP, and Ca^{2+} have been implicated in the intragranular Donnan complexation of CA (15–17). To date, this process has been considered to be only a passive mechanism for keeping the intragranular solutes isotonic with the cytoplasm (35). However, it is known that even little modifications on CGA conformation can account for large changes on its affinity for CA (19–22, 36).

The possible role of cGMP kinase on the intragranular matrix is difficult to explain since no cellular transduction routes have been described so far to explain how a second messenger could modify the kinetics of CA-CGA association. The only granule membrane protein, described so far, capable of interacting with CGA is the IP_3 receptor (38, 39). However, although it is known that PKG can phosphorylate IP_3 receptors (40), the real existence of an IP_3 receptor on granule surface has been questioned (41).

It is possible that NO can interact with the movements of Cl^-/water through the granule membrane; however, it is unlikely that this mechanism could operate under hypertonic conditions.

Fusion pore complex proteins posses several sites suitable for phosphorylation by PKG and other kinases (37). A delay in the fusion pore dilatation could produce a decrease in the speed of adrenaline. In addition, Criado *et al.* (29) reported changes in the spike kinetics parameters ($t_{1/2}$, m, t_P) of exocytosis in chromaffin cells with altered SNAP-25, one of the fusion pore complex proteins, although the changes caused by NO on spike shape were qualitatively different from those obtained in that study.

An effect of NO difficult to explain, however, is the change observed in Q. Assuming that this parameter reflects the total amount of adrenaline present within a granule, it is likely that this amount should be kept constant regardless of the kinetics of release. It is possible that NO can cause a true reduction in the quantal size of released CA. However, the more likely explanation may be the underestimation of spike charge resulting from the very slow release of CA upon granule fusion (22). The flattened end of the wider spikes implies very small concentrations of oxidative substances, which fall under electrode detection threshold, and as a result they are missed within base-line noise. Because the NO effect is observed a few seconds after its application (Fig. 4), it cannot be caused by an inhibition of CA synthesis (12) or by an inhibition of the monoamine carrier at the granule membrane.

Considering that dense core vesicles and chromaffin granules are similar organelles, NO could also modulate the speed of exocytosis in sympathetic nerve terminals. This will result in a decrease in their synaptic performance. In our study conditions, electrodes were touching the cell membrane; the distance between the electrode and the cell surface should be as short as an intervening water layer (≈20 nm), similar to the width of a synaptic cleft. In the example given in Fig. 3, NO accounts for a 10-fold change in the CA concentration reaching the electrode or cell surface. The concentration of CA released from a chromaffin granule, measured by cyclic voltammetry, was estimated to be around 34 mM (19). Assuming that, during resting conditions, a variable NO tone was present, NO levels could account for variations on CA concentration ranging from 8 to 76 mM, using the same granular content. Hence in sympathetic nerves, NO may potentiate its own vasodilatory effects by impairing the sympathetic compensatory activity through a reduction in synaptic efficacy.

In conclusion, our experiments suggest that NO, acting through the activation of the guanylate cyclase route, modifies the exocytotic kinetics of chromaffin granules. The cellular target for cGMP could be either the fusion pore dynamic or by altering the affinity of intragranular matrix for CA. To our knowledge, this is the first report indicating that, at its final stage, quantal secretion of a neurotransmitter could be physiologically modulated.

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