Nitric Oxide Modulates a Late Step of Exocytosis*

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The effects of nitric oxide (NO) on the late phase of exocytosis have been studied, by amperometry, on Ba²⁺-stimulated chromaffin cells. Acute incubation with NO or NO donors (sodium nitroprusside, spermine-NO, S-nitrosothioglutathione) produced a drastic slowdown of the granule emptying. Conversely, cell treatment with N⁶-nitro-L-arginine methyl ester (a NO synthase inhibitor) or with NO scavengers (methylene blue, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium) accelerated the extrusion of catecholamines from chromaffin granules, suggesting the presence of a NO modulatory tone. The incubation with phosphodies- terase 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 Ba²⁺. 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 CA¹ release induced by various NO-releasing agents, whereas oth-

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1 The abbreviations used are: CA, catecholamine; [Ba²⁺], cytosolic barium concentration; 8-Br-cGMP, 8-bromo-cGMP; [Ca²⁺], 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; t-NAMe, N⁶-nitro-L-arginine methyl ester; NOS, nitric-oxide synthase; PKG, cGMP-dependent protein kinase; SNP, sodium nitroprusside.
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-nitroso-glutathione and direct NO application produced similar concentration-dependent changes, which relate closely to their described abilities for producing free NO (25). Spike decay was also affected; \(\tau\) increased with 200 nM NO from 12.9 ms to 20.3 ms, whereas \(\tau\) changed from 27.1 ms 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 \(\mu\)M of the NO donor SNP incubated for 10–20 min. SNP caused a dramatic reduction in the spike \(t_{\text{max}}\) 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 \(t_{1/2}\) 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 \(t_{1/2}\) over 10 ms in duration was largely increased. NO effects were even more pronounced with 100 \(\mu\)M 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 \(\mu\)M.

In order to rule out SNP effects caused by NO metabolites accumulated along drug incubation, 10 \(\mu\)M 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); \(t_{\text{max}}\) 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 \(\mu\)M spermine-NO for 10 min produced a drastic change in spike shape, which included a reduction in the \(t_{\text{max}}\) and in the \(m\) (ascending slope), accompanied with an increase in the \(t_{1/2}\). 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 \(\mu\)M 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-
treated cells. Similarly, \( t \) values were affected to the same extend, \( t \) increased from 9.47 to 12.7 ms, whereas \( t \) 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 \( \mu \)M SNP, slight additive effects were observed, suggesting that both agents act through the same mechanism. Zaprinast increased \( t \) from 13.4 to 22.5 ms and \( t \) 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 \( t \) 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 \( \mu \)M) promoted significant changes on the spike \( t_{1/2} \), \( m \), and \( t_p \) values (Table II). Although data obtained with 100 \( \mu \)M 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-
The presence of NOS within chromaffin cells suggested the existence of a NO basal tone which probably modulates the number of secretory spikes, probably due to a nonspecific or nonsaturating 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 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 \(t_2\). C-PTIO was more potent than methylene blue, probably because of its specificity and ability to serve as NO scavenger; the \(m\) 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 \(m\) together with a reduction in \(t_1/2\) and \(\tau\) was observed. Fig. 3 summarizes the effect of 10 min of incubation with a low concentration of C-PTIO (10 \text{mM}); 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 (\(Q_{max}\)).

**NO Donors Did Not Reduce the \(I_{max}\)—**One possible target site of NO could be the interference with \(Ba^{2+}\) movements. A series of experiments was done measuring \(Ba^{2+}\) in the absence and in the presence of 10 \text{mM} SNP. Fig. 5 shows representative traces of \(P_{360}/P_{380}\) 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 (5 cells of each group). In any case, the increase \([Ba^{2+}]_c\) 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 (pre-pulse 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 \text{mM} 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 toxicity (\(i.e., >700 \text{mMNO}\)), 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 exocytic 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 and 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...
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stimuli, resulted in a slow and long-lasting [Ba\(^{2+}\)] response due to its poor efflux from the cell (22, 32). Fig. 5 shows that SNP did not reduce the [Ba\(^{2+}\)] response but produced a slight increase. These results do not support the assumption that NO effects on exocytosis were caused by a reduction of [Ba\(^{2+}\)].

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\(_2\)O 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[5]); (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 its real existence on chromaffin granule, measured by cyclic voltammetry, was estimated to be around 34 pmol (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 pmol, using the same granular content.

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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