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Noradrenaline inhibition of Ca^{2+} channels and secretion in single patch-clamped insulinoma cells

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Abstract Noradrenaline effects on voltage-operated calcium channels and exocytosis were studied, for the first time, in single patch-clamped RINm5F insulin-secreting cells. Noradrenaline, despite small and variable inhibition of calcium currents, strongly inhibited the increase in membrane capacitance (a measure of exocytosis) stimulated by both step depolarizations and the calcium ionophore, ionomycin. Noradrenaline similarly inhibited KCl- and ionomycin-induced [^{3}H]serotonin release from RINm5F cell populations. Noradrenaline effects were mediated by PTX-sensitive G proteins. Noradrenaline inhibitory effects on secretion are, therefore, mainly exerted downstream from Ca^{2+} influx.

Key words: Calcium channel; Exocytosis; Serotonin release; Noradrenaline

1. Introduction

Many hormones and neurotransmitters inhibit release from nerve terminals and endocrine cells. Their mechanisms of action include K^+ channel activation, Ca^{2+} channel inhibition, as well as effects on the secretory apparatus downstream from Ca^{2+} elevations [1-6].

In relation to the adrenergic inhibition of insulin secretion from pancreatic beta cells, both inhibition of voltage-operated calcium channels (VOCCs) [7,8] and direct effects on the secretory apparatus have been demonstrated [9-12]. However, while the modulation of calcium channels is mainly studied at the single cell level, insulin release is usually studied from cell populations, under experimental conditions that differ dramatically in terms of membrane potential, ionic composition of the buffers and time course.

Recently, patch-clamp techniques have been described which allow the simultaneous measurement of both Ca^{2+} currents (I_{Ca}) through VOCCs, and the associated changes in cell membrane capacitance (ΔC_m) due to exocytosis [13-19]. This technique has been applied to many cell types including pancreatic beta cells [20-22] and, recently, the insulinoma cell line RINm5F [23].

Here we report the first simultaneous characterization of adrenergic modulation of VOCCs and exocytosis at the single cell level in insulin-secreting RINm5F cells. Our results demonstrate that single RINm5F cells are heterogeneous in their responses to the inhibitory neurotransmitter, and that inhibition of exocytosis at a point downstream from Ca^{2+} influx plays a crucial role in the modulation of secretion from these cells. We have also confirmed that the noradrenaline-induced inhibition of secretion is mediated by a pertussis toxin (PTX)-sensitive G protein. Corroborative evidence that the inhibition of ΔC_m represented a reduction in release was obtained by measuring [^{3}H]serotonin ([^{3}H]5HT) release from RINm5F cell populations.

2. Materials and methods

2.1. Cell culture

The rat insulinoma RINm5F cell line provided by Dr. C.B. Wollheim (University of Geneva, Switzerland) was grown in Roosevelt Park Memorial Institute (RPMI)-1640 medium, supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 mg/ml streptomycin. Cells, incubated at 37°C and at a 5% CO_2 concentration in air, were trypsinized weekly and replated in either 35 mm tissue culture Petri dishes for electrophysiology or in 24 multiwell plates for [^{3}H]5HT release experiments. Cells were used 4–7 days after plating.

2.2. Electrophysiological recordings

I_{Ca} and C_m were measured in the whole-cell patch-clamp configuration [24], as recently described for RINm5F cells [23]. Briefly, data were acquired with an EPC9 amplifier (Instrutech, NY, USA) using HEKA software run on a Macintosh Centris 650. Isolated RINm5F cells were held at −90 mV and corrected for leak currents by subtracting four scaled hyperpolarizing pulses to zero-current command potentials. Series resistance (R_s) was compensated by at least 50% and signals were filtered at 2.9 Hz.

C_m and R_s were measured automatically by the EPC9 amplifier. This time-domain method of capacitance measurement has been shown to produce equivalent values of C_m to those obtained with the frequency-domain method [16,18]. Cells in which changes in C_m were accompanied by either parallel or anti-parallel changes in R_s were discarded. Similarly, recordings in which the holding current was greater than −100 pA were also removed from the data set. In all experiments the reported changes in C_m were determined by averaging the 4 C_m values before each voltage command and subtracting it from the average of the 4 C_m values after the command pulse. The temporal resolution of C_m points was ~300 ms, providing 3 data points per second. The rate of ionomycin-induced increase in C_m was measured in individual cells by linear fit ($y=mx+b$) of the relevant segment of the C_m trace using Igor Pro (Wavemetrics, OR, USA). Data were acquired at room temperature (~26°C). Perfusion of the cells was achieved by gravity flow using a multi-barrelled pipette system with a flow rate of 0.1 ml/min producing a local exchange at the cell within 5 s.

The extracellular solution contained (in mM): NaCl 125, $CaCl_2$ 10, $MgCl_2$ 1, 4-aminopyridine (4-AP) 3, tetrodotoxin (TTX) 0.0005, pH 7.3 with NaOH. Patch pipettes, coated with dental wax to reduce stray capacitance, were filled with an intracellular solution containing (in mM): Cs-glutamate 125, HEPES 30, tetraethylammonium bromide (TEA-Br) 20, NaCl 10, MgATP 3, $MgCl_2$ 1, GTP 0.3, cAMP 0.1, EGTA 0.05, pH 7.3 with CsOH.

Data are expressed as mean ± SEM and statistical significance was calculated using Student's *t*-test.

2.3. [^{3}H]5HT release

[^{3}H]5HT release was assayed as recently described [25]. Briefly, RINm5F cells were washed three times in phosphate buffered saline (PBS). They were then loaded with [^{3}H]5HT creatinine sulfate (100

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nM) in a Krebs-Ringer-HEPES (KRH) solution containing (in mM): NaCl 150, KCl 5, $MgSO_4$ and KH_2PO_4 1.2, $CaCl_2$ 2, glucose 6, and 25 HEPES-NaOH 25 (pH 7.4) and equal amounts of ascorbic acid and the monoamine oxidase inhibitor pargyline (0.1 mg/ml). After 1 h at 37°C, the cells were washed three times with PBS and returned to KRH containing 1 μM of the 5HT reuptake inhibitor Cl-imipramine. [^{3}H]5HT release was stimulated with either 50 mM KCl or 1 μM ionomycin for 10 min at 37°C. Cells were then washed three times in ice-cold PBS, dissolved in 0.6 ml of 1 M NaOH and transferred to plastic vials containing 5 ml of Ultima Gold (Camberra Packard) in order to determine the amount of radioactivity remaining associated with the cells. Radioactivity was counted in a scintillation beta counter (TriCarb 2100 TR, Camberra Packard) with an efficiency of 65%. The amount of [^{3}H]5HT release is expressed as percent increase over basal. Basal release was measured in parallel 10 min incubations with control KRH alone.

2.4. Materials

Culture media and supplements were obtained from Seromed (Biochrom KG, Berlin, Germany) and plastic sterile dishes and multiwell plates from Corning (New York, NY, USA). Pargyline, ionomycin, ascorbic acid, 4-AP, EGTA, noradrenaline and PTX were purchased from Sigma (Milan, Italy). Cl-imipramine was from RBI (Natick, MA, USA), [^{3}H]5HT creatinine sulfate (specific activity 15.1 Ci/mmol) from Amersham (Buckinghamshire, UK), TEA-Br from Aldrich (Milan, Italy), CsOH from Alfa (Wardhill, MA, USA) and TTX from Calbiochem (San Diego, CA, USA). All other salts and reagents were purchased from either Merck (Darmstadt, Germany) or Sigma.

3. Results

Repetitive 200 ms step depolarizations to +10 mV delivered every 10 s from a holding potential (V_h) of −90 mV in the presence of 10 mM external Ca^{2+}, produced large ΔC_m in control RINm5F cells (Fig. 1, Ai). The responses of RINm5F cells are voltage-dependent and are abolished in the presence of external Cd^{2+}, and therefore represent true Ca^{2+}-dependent exocytosis [23]. The large capacitance steps seen in RINm5F cells are probably due to several factors, foremost being that RINm5F cell size is variable and the cells used in the present study were frequently larger than normal beta cells. Secondly, the Ca^{2+} concentration of the bath solution was raised to 10

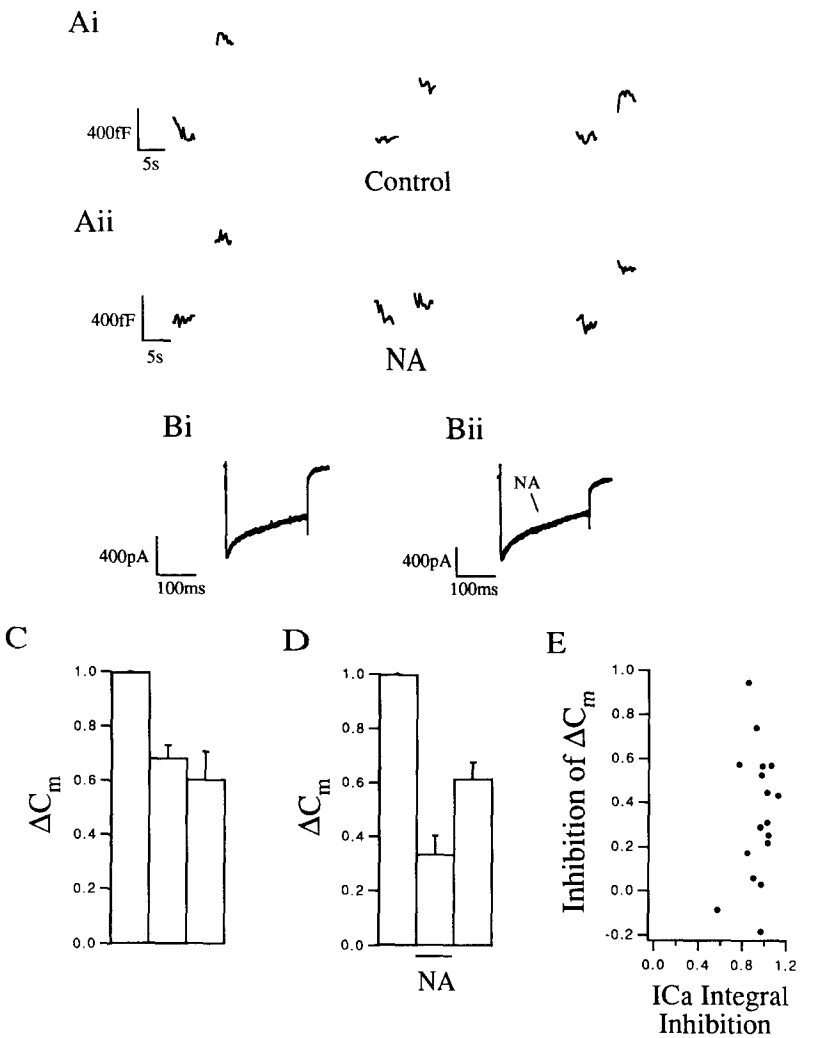

Fig. 1. Noradrenergic inhibition of I_{Ca} and C_m increases in single RINm5F cells. A: Examples of three consecutive C_m responses to 200 ms. depolarizations from a V_h of −90 mV to +10 mV. Responses were elicited every 10 s. The middle response in Ai was in control solution while Aii was in the presence of 20 μM noradrenaline (NA). The break in C_m sampling occurred during the depolarizing step command when I_{Ca} shown in Bi and Bii were acquired. Bi and Bii: Three superimposed I_{Ca} traces corresponding to the C_m data in A are shown. In this particular cell, I_{Ca} was almost unaffected by NA, while the C_m response was significantly inhibited. C and D: The average secretory response observed for three repetitive depolarizations in control solution (C, $n = 15$) and with exposure to 20 μM noradrenaline during the second step (D, $n = 17$) are shown. Data were normalized to the first C_m response for each cell. E: Scatter plot for the noradrenergic inhibition of ΔC_m versus the inhibition of I_{Ca} integrals. Both ΔC_m and the integral of I_{Ca} are normalized to the first response in each cell.

mM to ensure robust responses. For the same reason we also included 0.1 mM cAMP in the pipette solution. The calculated relationship between Ca^{2+} influx measured as the integral of the current versus the capacitance increase calculated for the first step response was 7.44 fF/pC. This value compares favorably with the value of 8.2 fF/pC reported for mouse beta cells [22].

Despite a relatively stable I_{Ca} (Fig. 1, Bi) there was a gradual depression of the secretory response in control cells, possibly due to depletion of readily releasable secretory granules [21,23,26]. The average values for three repetitive stimulations from 15 cells are shown in Fig. 1C.

When noradrenaline (20 μM) was perfused on the cell prior to the second depolarizing step, a strong inhibition of exocytosis was observed in most of the cells (Fig. 1, Aii), even though inhibition of I_{Ca} was small (Fig. 1, Bii) (see also [8]). The inhibition of exocytosis was rapidly reversed upon washout of noradrenaline, as seen from the recovery of ΔC_m by the third depolarization (Fig. 1, Aii). The average values from 17 noradrenaline-treated cells is shown in Fig. 1D. The average ΔC_m in response to the second step depolarization in the presence of noradrenaline was significantly smaller than the corresponding ΔC_m in control cells ($P = 0.0003$). Lack of a statistical difference between the average ΔC_m in response to the third step following noradrenaline washout (Fig. 1D), compared to the third response in control cells (Fig. 1C), confirmed that recovery of exocytosis was complete.

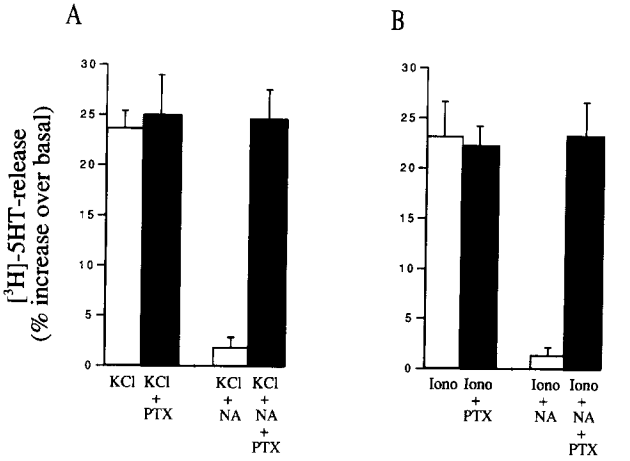

Fig. 3. Noradrenaline inhibits both KCl- and ionomycin-stimulated [^{3}H]5HT release from RINm5F cells. A: KCl (50 mM) induced [^{3}H]5HT release from RINm5F cells (10 min of incubation at 37°C, $n = 9$). NA (10 μM) inhibited KCl-induced secretion ($n = 9$). Pretreatment with PTX (200 ng/ml, 10 h, shaded bars) blocked the inhibitory action of NA ($n = 3$), without affecting KCl-induced [^{3}H]5HT release ($n = 3$). B: Ionomycin (1 μM)-induced [^{3}H]5HT release ($n = 5$) was also blocked by NA (10 μM, $n = 5$). While ionomycin-induced [^{3}H]5HT release was unaffected by PTX ($n = 3$), the NA inhibition was abolished by the PTX pretreatment ($n = 3$).

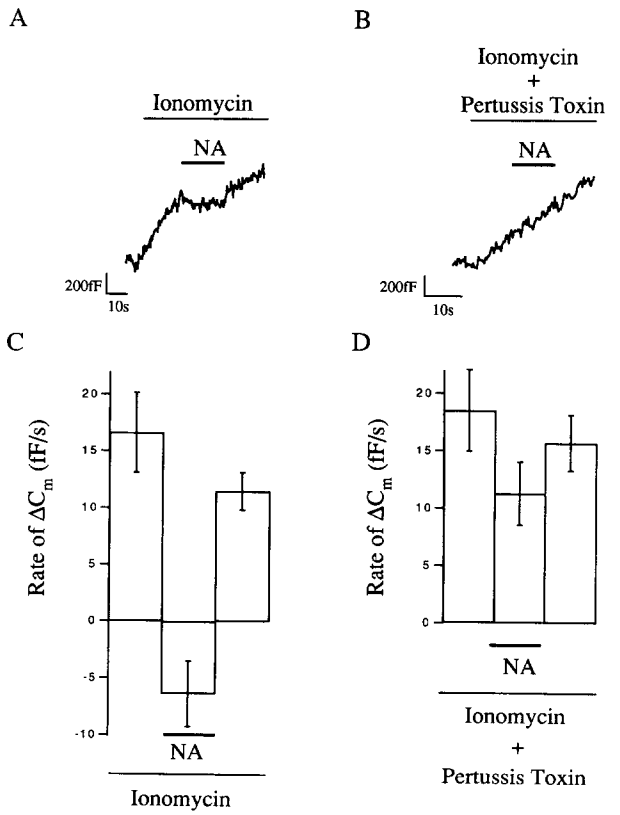

Fig. 2. Noradrenaline inhibits exocytosis by acting downstream from Ca^{2+} entry and in a PTX-sensitive manner. A: Example of the inhibition by 20 μM NA of the ΔC_m induced by 1 μM ionomycin in an individual RINm5F cell held at −90 mV. Drug applications are indicated by horizontal bars. B: Pretreatment of RINm5F cells with 200 ng/ml PTX for 10 h blocked the inhibitory action of NA. C and D: The average rate of the ΔC_m measured in individual cells by a linear fit to the C_m trace is plotted. In control cells, NA inhibited the increase in C_m induced by ionomycin (C, $n = 6$). In cells pretreated with PTX, NA had no significant effect on average rates of secretion (D, $n = 4$).

As shown in Fig. 1E, no correlation was found between the extent of I_{Ca} inhibition and the extent of ΔC_m inhibition; an almost complete block of exocytosis was observed in some cells with very little I_{Ca} inhibition. In a few cells neither parameter was affected by noradrenaline suggesting that in these cells the adrenergic receptors were absent, or their signalling deficient.

To further test the hypothesis that the inhibitory action of noradrenaline on exocytosis from RINm5F cells was predominantly mediated by a direct inhibition of the secretory apparatus, the Ca^{2+} ionophore ionomycin was used to stimulate secretion, thereby bypassing the contribution made by the adrenergic inhibition of the VOCCs [12]. Ionomycin (1 μM) produced a gradual increase in C_m which was completely abolished by co-perfusion with 20 μM noradrenaline (Fig. 2A). When noradrenaline was removed, the ionomycin-induced increase in C_m resumed and reached rates that were not significantly different from the rates measured prior to noradrenaline application. The average rate of ΔC_m due to ionomycin decreased significantly from 16.6 ± 3.5 fF/s to -6.4 ± 3.5 fF/s following noradrenaline application ($n = 6$, $P = 0.0005$, Fig. 2C). The average negative slope of ΔC_m in the presence of noradrenaline suggests that while exocytosis is strongly inhibited, endocytosis remains functional in the presence of the hormone.

Both noradrenaline-induced I_{Ca} inhibition [7,8] and the distal inhibition of insulin release from RINm5F cells [12] have been shown to be mediated by a PTX-sensitive G protein. To confirm this result at the single cell level, RINm5F cells were pretreated with 200 ng/ml PTX for 10 h. PTX pretreatment blocked the inhibitory action of 20 μM noradrenaline on ionomycin-stimulated exocytosis in all cells tested (Fig. 2B). The average rate of ΔC_m in PTX pretreated cells was not significantly altered during the noradrenaline application (Fig. 2D, $n = 4$).

To confirm that the observed reductions in ΔC_m caused by noradrenaline reflect inhibition of release (as opposed to a net increase in endocytosis), we examined the effects of noradrenaline on [^{3}H]5HT release from RINm5F cell populations [25]. Like glucose-induced [^{3}H]5HT release from mouse islets [27], KCl-evoked [^{3}H]5HT release from RINm5F cells was also found to be strongly inhibited (89.8 ± 1.2%, $n = 9$) by 10 μM noradrenaline (Fig. 3A). To verify that the inhibition of [^{3}H]5HT release is independent of the inhibition of the VOCCs, we also examined the effects of noradrenaline on ionomycin-induced [^{3}H]5HT release. Ionomycin-stimulated release was similarly reduced (92.2 ± 1.1%, $n = 5$) by 10 μM noradrenaline (Fig. 3B). The similarity in the extent to which KCl-evoked and ionomycin-induced [^{3}H]5HT release is inhibited by noradrenaline suggests that under these experimental conditions, VOCC inhibition plays a minor role in the actions of noradrenaline on hormone release from RINm5F cells.

4. Discussion

Noradrenaline, acting at α_2-adrenergic receptors, is a potent inhibitor of insulin release in vivo and in vitro [28]. Multiple targets of noradrenaline have been implicated in this inhibitory action, including VOCC inhibition [7,8] and direct inhibitory effects on the secretory apparatus [9-12]. We have now combined voltage clamp and membrane capacitance detection techniques to simultaneously monitor the relative contribution of I_{Ca} inhibition, and direct effects on the secretory apparatus, to the noradrenergic inhibition of exocytosis in single RINm5F cells.

Our results demonstrate that at the individual cell level, noradrenaline inhibits both VOCCs and secretion. This confirms and extends previous observations on VOCC modulation [7,8] and on inhibition of insulin release made at the cell population level [9-12]. However, the extent to which exocytosis from individual RINm5F cells was inhibited by noradrenaline was found to be quite variable, and this variability did not correlate with the extent of I_{Ca} inhibition. A dissociation between adrenergic effects on secretion and either $^{45}Ca^{2+}$ uptake from rat islets [9] or Quin2-detected Ca^{2+} signals in RINm5F cells [10] has been previously reported. Still, these data were obtained on cell populations and VOCC modulation was not directly assayed.

In RINm5F cells noradrenaline targets N-type VOCCs in a voltage-dependent manner and L-type VOCCs in a voltage-independent manner [8]. The remaining VOCCs (Q-like, [29]) are less sensitive to noradrenaline [8]. While the role of the Q-like VOCCs in the control of secretion from RINm5F cells is still unknown, both N- and L-type VOCCs have been shown to be involved in this process, the L-type being the more relevant [30,31]. The finding that noradrenaline has more potent actions on exocytosis than on I_{Ca} could be due to the fact that noradrenaline targets the few VOCCs 'crucially' involved in secretion, a role apparently played by L-type VOCC in both adrenal chromaffin [32] and pancreatic beta [33] cells.

Although we cannot completely exclude this hypothesis, two lines of evidence suggest that this explanation is unlikely. First, if the inhibition of I_{Ca} was causing the block of secretion, the extent to which I_{Ca} and ΔC_m were inhibited would be expected to be correlated, and this was not the case. Second, noradrenaline was potent in inhibiting both the exocytosis stimulated by Ca^{2+} influx through the VOCCs and that stimulated by ionomycin. From the above evidence we conclude that the direct inhibitory modulation of the secretory apparatus plays a crucial role in noradrenaline inhibition of exocytosis from single RINm5F cells, in agreement with previous data obtained studying insulin release from permeabilized rat islets [11], permeabilized RINm5F cells [12], and the data presented here on [^{3}H]5HT release from RINm5F cell populations. Based on these data we cannot exclude the possibility that noradrenaline also targets the Ca^{2+} handling capability of RINm5F cells; however, in the presence of ionomycin, which permits high sustained Ca^{2+} levels to be reached in the cell cytoplasm, secretion is clearly inhibited by noradrenaline (see also [12]).

These distal effects of noradrenaline are mediated by a PTX-sensitive G protein ([12] and this paper), but several PTX-sensitive G proteins are present in RINm5F cells [7]. Recent evidence obtained by Lang et al. [34] suggests that both G_i and G_o can participate in these distal adrenergic effects on secretion in normal rat beta and HIT-T15 insulinoma cells. However, a G_i-mediated inhibition of adenylate cyclase seems not to be the mechanism by which noradrenaline inhibits secretion 'distally' from RINm5F cell populations [12].

The molecular targets of the 'distal', G protein-mediated effects of noradrenaline are still uncertain. A direct inhibitory action on the secretory apparatus, however, is emerging as a general mechanism by which hormones and neurotransmitters inhibit release from both nerve terminals [1,2,4-6] and neuroendocrine cells [3,9-12]. Furthermore, there is evidence in both nerve terminals [2] and RINm5F cells [35] that not only the hormonal inhibition of release, but also its stimulation, could be achieved by the modulation of distal steps of the secretory process, downstream from second messenger production. We have recently found that while both exocytosis and endocytosis occur following Ca^{2+} influx in RINm5F cells, substitution with Ba^{2+} preferentially supports exocytosis [23], suggesting that the divalent ion sensitivities of the proteins involved in these two processes are different. Likewise, the observation at the single cell level that the adrenergic inhibition of exocytosis is not accompanied by an inhibition of endocytosis suggests that the proteins targeted by noradrenaline are specific to the exocytotic pathway.

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