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New insights into molecular mechanism(s) underlying the presynaptic action of nitric oxide on GABA release

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ABSTRACT

Background: Nitric oxide (NO) is an important presynaptic modulator of synaptic transmission. Here, we aimed to correlate the release of the major inhibitory neurotransmitter GABA with intracellular events occurring in rat brain axon terminals during their exposure to NO in the range of nanomolar–low micromolar concentrations.
Methods: Using [^{3}H]GABA and fluorescent dyes (Fluo 4-AM, acridine orange and rhodamine 6G), the following parameters were evaluated: vesicular and cytosolic GABA pools, intracellular calcium concentration, synaptic vesicle acidification, and mitochondrial membrane potential. Diethylamine NONOate (DEA/NO) and S-nitroso-N-acetylpenicillamine (SNAP) were used as NO donors.
Results: DEA/NO and SNAP (in the presence of dithiothreitol (DTT)) stimulated external Ca^{2+}-independent [^{3}H]GABA release, which was not attributed to a rise in intracellular calcium concentration. [^{3}H]GABA release coincided with increasing GABA level in cytosol and decreasing the vesicular GABA content available for exocytotic release. There was a strong temporal correlation between NO-induced increase in cytosolic [GABA] and dissipation of both synaptic vesicle proton gradient and mitochondrial membrane potential. Dissipation was reversible, and recovery of both parameters correlated in time with re-accumulation of [^{3}H]GABA into synaptic vesicles. The molar ratio of DTT to SNAP determined the rate and duration of the recovery processes.
Conclusions: We suggest that NO can stimulate GABA release via GABA transporter reversal resulting from increased GABA levels in cytosol. The latter is reversible and appears to be due to S-nitrosylation of key proteins, which affect the energy status of the pre-synapse.
General significance: Our findings provide new insight into molecular mechanism(s) underlying the presynaptic action of nitric oxide on inhibitory neurotransmission.

1. Introduction

Nitric oxide (NO) is a crucial signaling molecule involved in the regulation of cellular processes in CNS under a variety of physiological and pathological conditions [1–4]. Overproduction of NO during the course of neurological diseases or following acute brain injury causes oxidative stress and mitochondrial dysfunction and, as a consequence, often leads to the disturbances in synaptic transmission. At the same time, at low physiological concentrations, NO may serve as one of the key factors that regulate synaptic plasticity and neural communication [5–7]. It has been shown that nitric oxide acts directly in the presynaptic neuron to produce long-term potentiation in cultured hippocampal neurons [8]. There are some evidence for NO involvement in the modulation of acetylcholine [9], noradrenaline [10], serotonin [11], glutamate [12] and GABA secretion [13,14]. However, despite numerous investigations, data concerning the effect of nitric oxide on neurotransmission are controversial, showing either a significant stimulation [13,15] or, vise versa, an inhibition of neurotransmitter release [16,17]. Some authors [18] have reported that nitric oxide-related species inhibit evoked neurotransmission but enhance spontaneous miniature synaptic currents in central neuronal cultures.

Over the last decades, the regulatory role of NO in neurotransmission is intensively studied because it is important to understand the mechanism by which NO provides a retrograde control of synaptic transmission, and it opens up new perspectives for using NO donors as therapeutic agents. Two different mechanisms are proposed to underlie NO-induced modulation of neurotransmission: the activation of a soluble guanylyl cyclase, which potentiates a cGMP-protein kinase G pathway [19,20], and S-nitrosylation of key proteins that control important cellular functions of neurons [7,21–23]. Through S-nitrosylation nitric oxide may exert neurotoxic as well as neuroprotective effects. NO neurotoxicity in vivo is associated with the inhibition of respiration at the level of cytochrome c oxidase and mitochondrial complex I [24,25], whereas cyto- and neuroprotection is realized due to the inhibition of caspase activity [26] and feedback down-regulation of channel activity of NMDA receptors [27]. At low concentrations, nitric oxide, as a rule, reversibly nitrosates various neuronal proteins, thereby modulating their activity, sensitivity to inhibitors or activators, and interaction with downstream targets [28,29].

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Among all neurotransmitters, GABA is known to play a central role in controlling excitability, neuronal development and communications. In vivo, GABAergic neurotransmission can be modulated by NO released as a consequence of postsynaptic glutamate receptor activation or, for example, from NO-releasing drugs. Two distinct release mechanisms are thought to underlie nitric oxide-evoked GABA release. Some authors [30] have shown a direct stimulation of GABA exocytosis by NO, whereas other authors [31] have reported that GABA could also be released via reversal of the GABA transporter. At the same time, Sequeira et al. [32] suggested that NO differentially affects the exocytotic and carrier-mediated release of [^{3}H]GABA depending on NO concentrations.

With this in mind, in the present work we investigated the effect of nanomolar–low micromolar concentrations of nitric oxide on GABA release from rat brain nerve terminals and correlated this with intracellular events occurring in the pre-synapse. Our efforts were focused on studying NO-induced changes in synaptic vesicle proton gradient and plasma/mitochondrial membrane potential, given that these parameters directly determine the release, reuptake and accumulation of neurotransmitters within vesicles, and so regulate the efficiency of neurotransmission as a whole.

2. Materials and methods

2.1. Chemicals and solutions

The following chemicals were used: diethylamine NONOate (DEA/NO), S-nitroso-N-acetylpenicillamine (SNAP), dithiothreitol (DTT), rotenone, oligomycin, nipecotic acid, amino-oxyacetic acid, sodium pyruvate, D-glucose, sucrose, adenosine 5′-triphosphate magnesium salt (ATP), EDTA, EGTA, HEPES and dimethyl sulfoxide (DMSO) were purchased from Sigma (U.S.A.). Ficoll 400 was from Pharmacia LKB Biotechnology Inc. (Sweden); acridine orange, Rhodamine 6G and Fluo-4AM were from Invitrogen (U.S.A.); 4-aminopyridine (4-AP) was from RBI (U.S.A.); [^{3}H]GABA (94 Ci/@mmol) and Aqueous Counting Scintillant (ACS) were from Amersham (U.K.). Analytical grade salts were from Reachim (Ukraine).

Nipecotic acid and DTT were dissolved in bidistilled water to form stock solutions. Stock solution of SNAP (200 mM) was made with DMSO, aliquoted and frozen at −20 °C until analysis. DEA/NO was dissolved in NaOH (10 mM) and its concentration was determined spectrophotometrically at 250 nm using an extinction coefficient of 6500 M^{-1} cm^{-1} (Cayman chemical product information). Stock solution of DEA/NO was freshly prepared and kept on ice in the dark until use. Appropriate dilutions of DEA/NO and SNAP were made with bidistilled water just before application. Final concentration of DMSO in all experiments was ≤0.5%.

Experiments were performed in HEPES-buffered saline (HBS, pH 7.4) containing 126 mM NaCl, 5 mM KCl, 2.0 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 20 mM HEPES and 10 mM D-glucose. The Ca^{2+}-free medium was nominally Ca^{2+} free and contained 1 mM EGTA.

2.2. Isolation of rat brain synaptosomes

Experiments were carried out on male Wistar rats that were housed and handled in accordance with European Community Council Directive of 24 November 1986 (86/609/EEC). The animal (100–120 g body weight) was decapitated, and the brain was rapidly removed and placed in ice-cold solution containing 0.32 M sucrose, 0.2 mM EDTA and 5 mM HEPES (pH adjusted to 7.4 with NaOH). The tissue was minced and homogenized at the ratio of 10:1 (volume/tissue weight) using a glass homogenizer with a Teflon plunger (0.25 mm clearance). Isolated nerve terminals (synaptosomes) were prepared by differential and Ficoll-400 density gradient centrifugation of rat brain homogenate according to the method of Cotman [33] with slight modifications as described by Linetska et al. [34]. All manipulations were performed at 0–4 °C. Protein concentration was measured as described by Larson [35].

In the experiments with [^{3}H]GABA, synaptosomal suspensions were used during 2–4 h after isolation. To prolong the state of metabolic competence of synaptosomes in the long-term fluorescence experiments, sodium pyruvate (4 mM) was added to the incubation medium [36].

2.3. Isolation of synaptic vesicles

Synaptic vesicles were obtained according to the procedures of De Lorenzo and Freedman [37] with slight modifications [38]. In briefly, crude synaptosomes (P2 fraction) were lysed by rapid resuspension in 1 mM EGTA, 10 mM Tris–HCl, pH 8.1 (3 mL/g of brain tissue) and incubated at 4 °C for 60 min. The preparation was centrifuged at 20,000 *g* for 30 min, and then the supernatant was centrifuged at 55,000 *g* for 60 min. After that, the supernatant of the last centrifugation was centrifuged again at 130,000 *g* for 60 min, and the pellet of synaptic vesicles was resuspended in HEPES buffer (10 mM HEPES–Tris (pH 7.4), 120 mM sucrose, 140 mM K-gluconate, 4 mM NaCl and 2 mM $MgCl_2$) to a protein concentration of 2 mg/mL. This preparation is referred to as crude synaptic vesicles.

2.4. GABA release experiments

Synaptosomes were diluted with HBS to 2 mg protein/mL and, after pre-incubation at 37 °C for 5 min, were loaded with [^{3}H]GABA (50 nM, 4.7 μM/mL) for 10 min. After loading, the suspension was washed with ice-cold oxygenated HBS (1:10) by centrifugation at 4000 *g* for 10 min, and then the pellet was resuspended in the same solution to a protein concentration of 1 mg/mL. Amino-oxyacetic acid, a GABA transaminase inhibitor, was present at 100 μM throughout all experiments involving [^{3}H]GABA loading and release. To estimate the release of [^{3}H]GABA from synaptosomes, the following procedure was carried out: samples were pre-incubated with or without 1 mM dithiothreitol (DTT) in the medium containing 4 mM pyruvate for 10 min at 37 °C, and then nitric oxide donors were applied. At selected time points, nipecotic acid or 4-AP were added to the various experimental tubes, in which synaptosomes were exposed to NO donors for different time periods (5–10–15 min). In each case, the action of nipecotic acid/4-AP was restricted for 5 min, after which 120 μL sample aliquots were taken out and rapidly sedimented in a microcentrifuge for 15 s at 10,000 *g*. [^{3}H]GABA content was measured in aliquots of supernatants (90 μL) by liquid scintillation counting with Aqueous Counting Scintillant (1.5 mL) as scintillation cocktail and was expressed as a percentage of the total amount of [^{3}H]GABA loaded into the synaptosomes.

Ca^{2+}-dependent exocytotic [^{3}H]GABA release was triggered by 2 mM 4-aminopyridine (4-AP). Ca^{2+}-independent transporter-mediated [^{3}H]GABA release was stimulated by 100 μM nipecotic acid. Neurotransmitter release from synaptosomes incubated without any stimulating agents in Ca^{2+}-supplemented or Ca^{2+}-free medium was defined as basal release. Stimulated neurotransmitter release was calculated by subtracting the basal release value from the value obtained in the presence of stimulants.

2.5. Measurement of the relative level of intrasynaptosomal calcium

The relative changes in intrasynaptosomal calcium were measured using an acetoxymethyl ester derivative of Fluo-4. Synaptosomes were incubated with Fluo-4AM (3 μM final concentration) for 40 min in a shaking waterbath (30 °C), and then were washed with HBS (1:10) by centrifugation at 4000 *g* for 10 min. The pellet was resuspended in ice-cold nominally Ca^{2+}-free HBS to a final protein concentration of 1.5 mg/mL and kept in the refrigerator until using. For each assay, 0.5 mL of Fluo-loaded synaptosomes was rapidly centrifuged (30 s, 12,000 *g*) in a microcentrifuge (Eppendorf® MiniSpin®), and the resulting pellet was resuspended in HBS, placed in a quartz cuvette

and left to equilibrate for 15 min at 35 °C. Fluorescence kinetics was monitored using a QuantaMaster 40 spectrofluorometer (PTI, FelixGX-4.1.0 software) at excitation wavelength of 496 nm and emission wavelength of 518 nm. Results are presented as normalized responses (F/F_0), where F_0 is the fluorescence intensity at the time of NO donor addition. Responses to elevated extracellular K^+ as well as to calcium ionophore A 23187 and SDS were used as the criteria for synaptosomal functionality.

2.6. Measurement of membrane potential

Membrane potential measurements were performed with the potentiometric optical dye rhodamine 6G using a Hitachi MPF-4 (Japan) spectrofluorometer with a home-made system of computer data acquisition. Excitation and emission wavelengths were 528 and 551 nm, respectively (slit width, 5 nm each). Rhodamine 6G (final concentration 0.5 μM) was added to synaptosomes (0.2 mg protein/mL) pre-incubated for 10 min in a stirred cuvette thermostated at 35 °C. Dye uptake was recorded for 2–3 min until the steady state level of rhodamine 6G fluorescence had been reached, and then NO donors were applied. Fluorescence intensity was normalized to the time point just before NO donor addition.

To single out the contribution of mitochondria to overall fluorescence changes, experiments were carried out under non-respiring conditions, when mitochondrial membrane potential ($\Delta\psi_m$) was collapsed by 4 μM rotenone plus 4 μg/mL oligomycin. In this case fluorescence intensities were normalized to the time point = 0, and the ratio (F) was estimated:

$$F = F_t / F_0$$

where F_t and F_0 are fluorescence intensities of rhodamine 6G in the absence and presence of synaptosomes respectively. F_0 was calculated by extrapolation of exponential decay function to $t = 0$. F_t was corresponded to the dye fluorescence at a certain time interval.

2.7. Measurement of synaptic vesicle acidification

To monitor the changes in synaptic vesicle acidification, we used acridine orange (AO), a pH-sensitive fluorescent dye, which was selectively accumulated by synaptic vesicles according to pH gradient [39]. The particular advantage of this methodological approach is a possibility to monitor not only vesicle acidification (i.e. ability of synaptic vesicles to keep protons), but also exo/endocytosis that allow to elucidate which processes occur during NO exposure. Fluorescence measurements were carried out on a Hitachi MPF-4 spectrofluorometer at excitation and emission wavelengths of 490 and 530 nm, respectively (slit width, 5 nm each). The reaction was started by the addition of AO (final concentration 5 μM) to synaptosomes (0.3 mg protein/mL) pre-incubated for 10 min in a stirred cuvette thermostated at 35 °C. The equilibrium level of dye fluorescence was achieved for 15 min, and then NO donors were applied. Fluorescence intensity was normalized to the time point just before NO donor addition.

In the experiments with isolated synaptic vesicles, the vesicles (50 μg protein/mL) pre-incubated for 10 min at 35 °C with AO (final concentration 2 μM) were able to accumulate the dye just after the addition of ATP (0.5 mM). The equilibrium level of dye fluorescence was achieved for 3 min, and then NO donors were applied.

2.8. Nitric oxide detection

The concentration of nitric oxide released from NO donors spontaneously or in the presence of DTT was assessed using the Griess reagent that provides a simple and well characterized colorimetric assay for nitrite detection [40]. The absorbance was measured at 543 nm using UV–VIS spectrometer Specord M-40 (Carl Zeiss JENA, Germany). For calibration, sodium nitrite solutions with concentrations between 0.01 and 10 μm were prepared in deionized water.

2.9. Statistics

Statistical analyses were carried out using 'Origin Pro 8.6.0, b70' (OriginLab Corporation, Northampton, MA, USA). Statistical differences among more than two groups were tested using one-way ANOVA followed by Bonferroni multiple comparison test. All data are presented as the mean ± standard error of the mean (SEM). Results were considered statistically significant at p-values ≤ 0.05.

3. Results

3.1. The choice of nitric oxide donors

As a source of NO, we have used two nitric oxide donors, diethylamine NONOate (DEA/NO) and S-nitroso-N-acetylpenicillamine (SNAP), which are distinct in their pathways of NO liberation. In physiological buffer, DEA/NO generates NO by the dissociation to free amine and NO, whereas SNAP liberates NO spontaneously or undergoes rapid decomposition to NO, thiols and disulfides in the presence of reducing agents [41]. As a relatively stable NO donor, SNAP can serve as a model of endogenous S-nitrosothiols, which mediate S-nitrosylation of protein thiols in vivo during nitric oxide signaling [42]. To estimate the concentrations of NO released from SNAP, the production of nitric oxide metabolites (NO^x) was measured by using the Griess reagent [40]. It was revealed that SNAP, at 0.5 mM concentration, released 61.14 ± 1.36 nM of free NO^x (data not shown) that correlated with the data of Matthews et al. [43], who reported that the concentration of free NO^x released from SNAP was 3–4 log units lower than SNAP concentration. In the presence of 1 mM dithiothreitol (DTT), the amount of measured NO^x, was significantly increased (up to 168.47 ± 1.67 nM in 10 min after the reaction start). The interaction of thiols with S-nitrosothiols is shown to enhance their decomposition [44,45] and leads to the formation of active species [10]. As for DEA/NO, it, according to chemical product information, released 1.5 mol of NO per mole of parent compound, however different research groups have recorded the lowest values of NO liberated from DEA/NO. For instance, Ridnour et al. have shown that peak rates of $^{\bullet}NO$ released from 100 μM DEA/NO occurred in the first 2 to 5 min and reached approximately 10 nmol/min [46]. Chen and Wang assessed the concentration of NO immediately after the addition of DEA/NO to the culture medium (at 37 °C), and obtained the value of 1.8 μM for 125 μM DEA/NO [47]. Taking into account all above mentioned, in our study we applied NO donors at concentrations, which released NO in the range of nanomolar to low micromolar concentrations.

3.2. Nitric oxide donors cause a reversible redistribution of [^{3}H]GABA between the vesicular and cytosolic pools

In the first series of experiments, we studied the effect of both NO donors on the release of GABA from synaptosomes preloaded with [^{3}H]GABA. Measuring the extracellular [^{3}H]GABA concentrations at 2, 5, 10 and 15 min after NO donor application has shown that DEA-NO (50 μM) as well as SNAP (100 μM) in the presence of DTT (1 mM) stimulated the initial increase in the extracellular [^{3}H]GABA concentration, which was followed by subsequent decrease in extracellular [^{3}H]GABA concentration (Fig. 1A and B). It was of interest that the kinetics of NO-induced [^{3}H]GABA release was not similar to that we previously observed during depolarization-evoked [^{3}H]GABA release by exocytosis. In contrast to the latter, NO-induced [^{3}H]GABA release was gradual, but not instantaneous, and required more time to reach the maximum level (5 and 10 min for DEA-NO and SNAP (plus DTT), respectively). These data as well as the insensitivity of the process to the omission of extracellular Ca^{2+} (Fig. 1C) suggest that external Ca^{2+}-activated

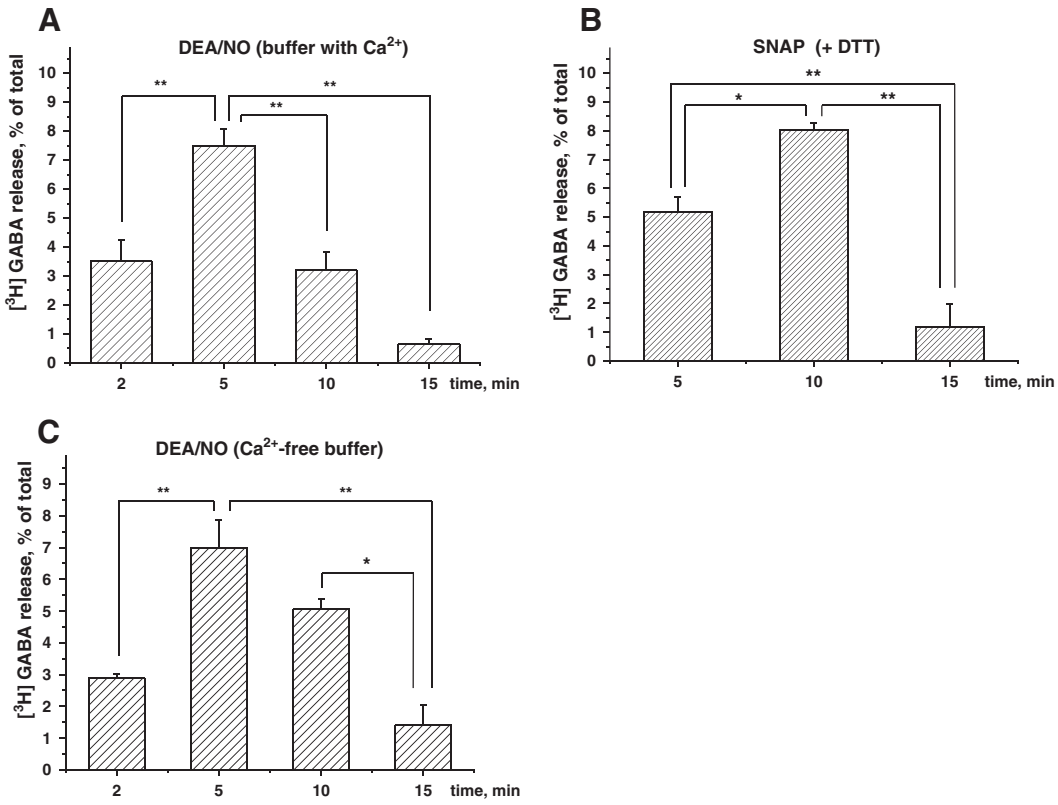

Fig. 1. The kinetics of NO donor-induced [^{3}H]GABA release from rat brain synaptosomes. [^{3}H]GABA release was measured at certain time points after application of DEA/NO (50 μM) or SNAP (0.1 mM) (A and B, respectively) to synaptosomes pre-loaded with [^{3}H]GABA in Ca^{2+}-containing or Ca^{2+}-free (C) medium. In the experiments with SNAP (B), 1 mM DTT was added 5 min before SNAP application. Basal release of [^{3}H]GABA was subtracted from NO-induced [^{3}H]GABA release. Data are presented as mean ± SEM of three–five independent experiments. * $p < 0.05$, ** $p < 0.01$, one-way ANOVA followed by Bonferroni's post-hoc test.

exocytosis seems not to be responsible for GABA release from synaptosomes exposed to NO.

To clarify the pathway by which GABA was released, the vesicular and cytosolic [^{3}H]GABA pools were assessed at a certain time period. The experiments were performed with 4-AP, a K^+-channel blocker, which is known to specifically stimulate Ca^{2+}-induced exocytotic GABA release, and nipecotic acid (NA), a transportable inhibitor of GABA re-uptake. When nipecotic acid is extracellularly applied to nerve terminals, transporter operates as an exchanger coupling an influx of nipecotic acid to an efflux (by hetero-exchange) of cytosolic GABA [48].

As Fig. 2A shows, nipecotic acid (0.1 mM) added to synaptosomes at 5 min after DEA/NO (50 μM) application induced a more than twofold increase in [^{3}H]GABA efflux from cytosol compared with control (without DEA/NO) experiments (25.78 ± 3.49% versus 10.1 ± 2.02%). At the same time point, 5 min, 4-AP-stimulated exocytotic [^{3}H]GABA release was significantly attenuated (5.02 ± 0.4% versus 10.76 ± 1.15% in the control) (Fig. 2B). These data indicate that DEA/NO appears to cause the redistribution of endogenous GABA between synaptic vesicles and cytosol in favor of the latter, and suggest that the maximal increase in the extracellular [^{3}H]GABA concentration observed at 5 min after DEA/NO application (see Fig. 1A) is mainly attributed to carrier-mediated release of [^{3}H]GABA from cytosol. It was interesting to observe the opposite effects at 15 min after DEA-NO application, i.e. NA-induced [^{3}H]GABA release from cytosol was reduced (Fig. 2A), whereas 4-AP-triggered exocytotic release was essentially increased up to initial level (Fig. 2B). Given that the half-life of DEA/NO is 2 min, these findings illustrate that synaptic vesicles were able to fully restore their vesicular GABA pool after the end of NO action. It should be noted that at 10 μM concentrations, the kinetics of DEA/NO-stimulated [^{3}H]GABA release was analogous to that observed at 50 μM, but the relatively small magnitude of the responses did not allow us to assess vesicular and cytosolic [^{3}H]GABA pools correctly.

Similar results were obtained with SNAP (100 μM), when it was added to synaptosomes pre-incubated for 5 min with DTT (1 mM). At 10 min after SNAP application, NA-induced [^{3}H]GABA release from cytosol was essentially increased in comparison with control (Fig. 2C), whereas 4-AP-evoked [^{3}H]GABA release from synaptic vesicles was drastically decreased (Fig. 2D). The subsequent attenuation of NA-induced [^{3}H]GABA release at 15 min of SNAP action was accompanied with the reuptake of [^{3}H]GABA into synaptosomes, its reaccumulation into synaptic vesicles and, as a consequence, with the massive 4-AP-evoked [^{3}H]GABA release from vesicles (Fig. 2C and D). It should be noted that at 1 mM, DTT alone had no stimulating effect on [^{3}H]GABA release and, moreover, it even slightly decreased the basal release of [^{3}H]GABA, thereby showing a stabilizing effect on synaptosomes.

Thus, our results show that at nanomolar–low micromolar concentrations nitric oxide stimulates external Ca^{2+}-independent GABA release, which appears to have a non-vesicular origin. Nevertheless, to be certain that observed process was not attributed to spontaneous exocytosis triggered by calcium released from intracellular stores, we next studied the changes in cytosolic calcium level ($[Ca^{2+}]_i$) in nerve terminals exposed to NO donors.

3.3. Effect of NO donors on intrasynaptosomal calcium level

The addition of 10 μM DEA/NO to Fluo-4-loaded synaptosomes in Ca^{2+}-free/EGTA solution did not cause any detectable changes in $[Ca2+]_i$, whereas the subsequent application of calcium ionophore A23187 (5 μM) was accompanied with a rapid and significant increase in Fluo-4 fluorescence due to the release of calcium from intracellular stores (Fig. 3A). Some elevation of $[Ca2+]_i$ was detected at higher

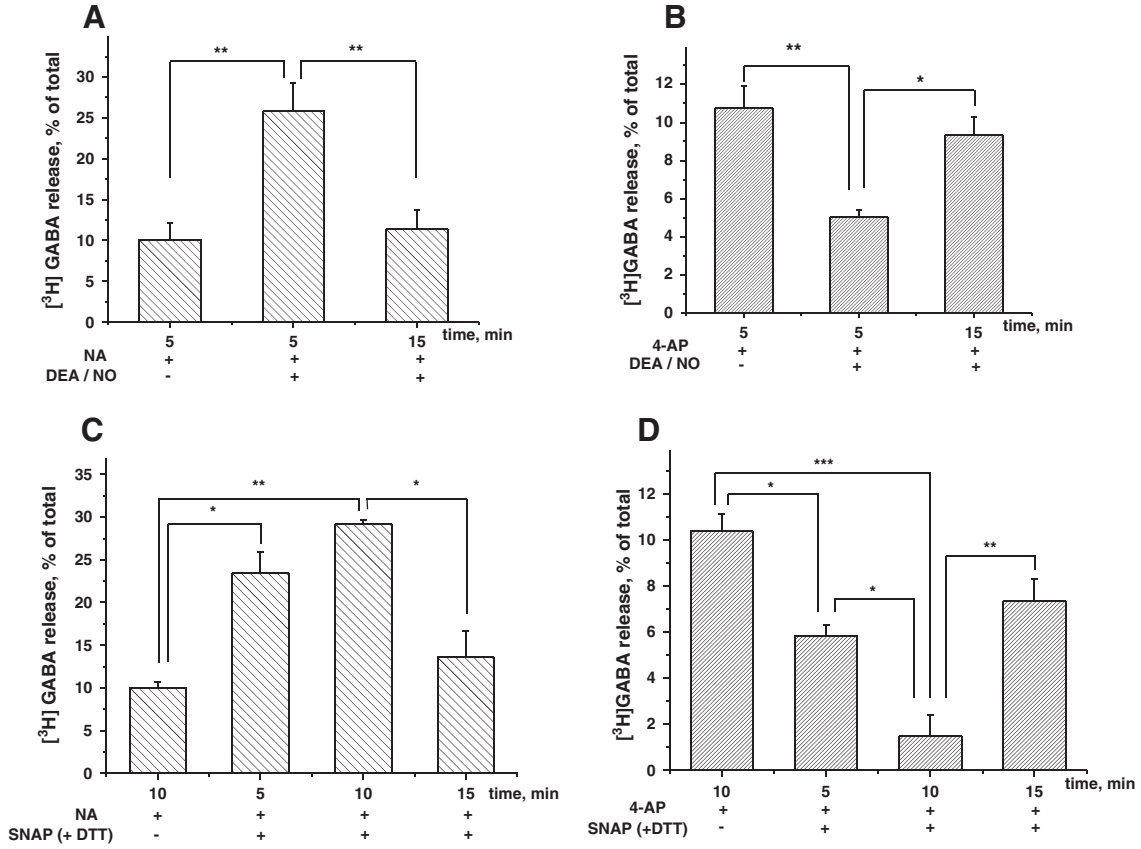

Fig. 2. Temporal differences in NA- and 4-AP-evoked release of [^{3}H]GABA from synaptosomes exposed to NO donors. NA (0.1 mM) or 4-AP (2 mM) were added to [^{3}H]GABA-loaded synaptosomes at 5-th and 15-th min after DEA/NO (50 μM) application (A and B), and at 5-th, 10-th and 15-th min after SNAP (0.1 mM) application (C and D). In the experiments with SNAP, 1 mM DTT was applied 5 min before SNAP addition. [^{3}H]GABA content was measured in sample aliquots taken out and centrifuged 5 min after NA or 4-AP addition. NA-stimulated [^{3}H]GABA release was estimated by subtracting the basal release value from the value obtained in the presence of stimulants (NA alone or together with NO donors). For correct estimation of exocytotic GABA release during NO donor action, 4-AP-stimulated [^{3}H]GABA release was expressed as a value from which [^{3}H]GABA release evoked by NO donors alone was subtracted. Data are presented as mean ± SEM of three–four independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA followed by Bonferroni's post-hoc test.

concentration of DEA/NO (50 μM), however as can be seen in Fig. 3A, such an elevation was delayed over 5 min, and so could not be a trigger of massive [^{3}H]GABA release observed in previous section.

Cytosolic [Ca^{2+}] was also not enhanced when DEA/NO at concentrations of 10 μM and lower was applied to synaptosomes pre-incubated in standard HBS containing 2 mM Ca^{2+} (Fig. 3B). At the same time, the subsequent addition of high K^+ (30 mM final concentration) induced the fluorescence spike as a fast response to depolarization-evoked calcium influx. Similar to DEA/NO, SNAP at concentrations up to 100 μM also had no effect on cytosolic [Ca^{2+}] (data not shown).

Thus, nitric oxide, at used concentrations, does not induce any changes in [Ca2 +]$_i$, confirming that NO-stimulated GABA release occurs by non-exocytotic mechanism, and so is likely to be a consequence of the increase in GABA concentration in the cytosol. To clarify the possible

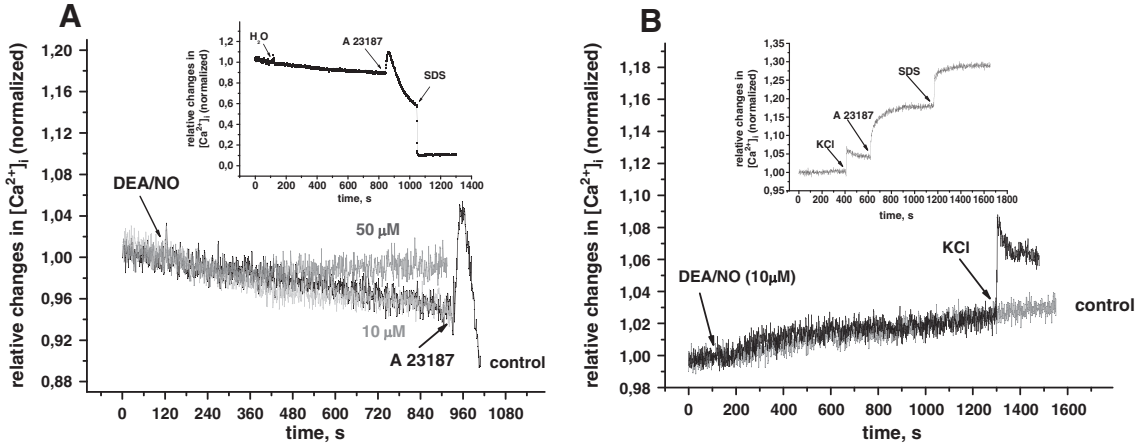

Fig. 3. The relative changes in intrasynaptosomal [Ca^{2+}]$_i$ during the exposure of synaptosomes to DEA/NO in Ca^{2+}-free/EGTA- (A) and Ca^{2+}-containing (B) medium. Responses to high K^+ (30 mM final concentrations) as well as to A23187 (5 μM) and SDS (10%) shown in the inset serve as the criteria for synaptosomal functionality. All fluorescence signals were corrected for dilution factor. Traces are representative of four–five independent experiments.

causes of such an increase, in the next series of experiment we studied the influence of NO donors on the synaptic vesicle proton gradient, taking into account that the accumulation of neurotransmitters within vesicles is tightly coupled with synaptic vesicle electrochemical proton gradient ($\Delta\mu H^+$) generation.

3.4. Reversible dissipation of the synaptic vesicle proton gradient of synaptosomes exposed to nanomolar–low micromolar NO concentrations

Given the high sensitivity of vesicular GABA uptake to ΔpH component of $\Delta\mu H^+$, we measured the changes in synaptic vesicle acidification using pH-sensitive fluorescent dye AO. As Fig. 4A shows, the influx of the dye into synaptic vesicles was accompanied by a partial quenching of the fluorescence intensity, the steady-state level of which was achieved after the 15th min. The subsequent application of DEA/NO induced the kinetic changes in the fluorescence signal characterized by a gradual increase in fluorescence, peaking at 5–7 min after DEA/NO addition and then declining to a baseline level. As can be seen, the effect of DEA/NO was dose-dependent, and the height of fluorescence curve was directly proportional to DEA/NO concentration. The enhancement of AO fluorescence reflects the release of the protons from synaptic vesicles, and so it could be a consequence of either the dissipation of synaptic vesicle proton gradient or the release of the vesicular content during exocytotic process. As Fig. 4B shows, DEA/NO-induced process was insensitive to the omission of calcium from the external medium. A selective inhibitor of NO-sensitive guanylyl cyclase, 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ, 10 μM), also had no effect on DEA/NO-evoked response (data not shown), indicating the non-involvement of cGMP-protein kinase G pathway in this process. The lack of any effects of external Ca^{2+} and cGMP allows suggesting that the dissipation of synaptic vesicle proton gradient rather than exocytosis appears to underlie the effect of NO at nanomolar–low micromolar concentrations. An additional argument in favor of this assumption is that the kinetics of the response to DEA/NO was distinctive from that observed in the case of high K^+ application (Fig. 4B). High K^+-induced fluorescence spike, according to Zoccarato et al. [39], is a marker of depolarization-evoked exo-endocytosis and, in contrast to NO-induced response, as expected, it was not observed in Ca^{2+}-free medium (Fig. 4B).

Similar results were obtained with other NO donor, SNAP, but only in the presence of DTT. As Fig. 4C shows, at 100 μM concentration, SNAP alone did not cause any changes in AO fluorescence, indicating its inability to affect vesicular proton gradient at this concentration. However, the further application of DTT (0.5 mM) triggered a gradual increase in the fluorescence signal analogous to that observed for DEA/NO. The same enhancement of the fluorescence was also seen when SNAP was added to synaptosomes pre-incubated with DTT, and the subsequent decrease in fluorescence intensity was depended on the concentration of SH-reducing agent, being more pronounced at the higher DTT concentrations (Fig. 4D). As can be seen, the molar ratio of DTT to SNAP, denoted as R, determines the rate and duration of fluorescence signal recovery showing that the higher R, the faster the recovery of the proton gradient. The further application to nerve terminals of 30 mM KCl (at 20th min after SNAP addition) triggered fluorescence spike, the magnitude of which was directly proportional to R (Fig. 4D inset). So, the higher the reducing potential nerve terminals have, the more quickly synaptic vesicles restore their proton gradient and capacity of the readily releasable pool. The high sensitivity of NO-induced processes to SH-reducing agent suggests that nitric oxide,

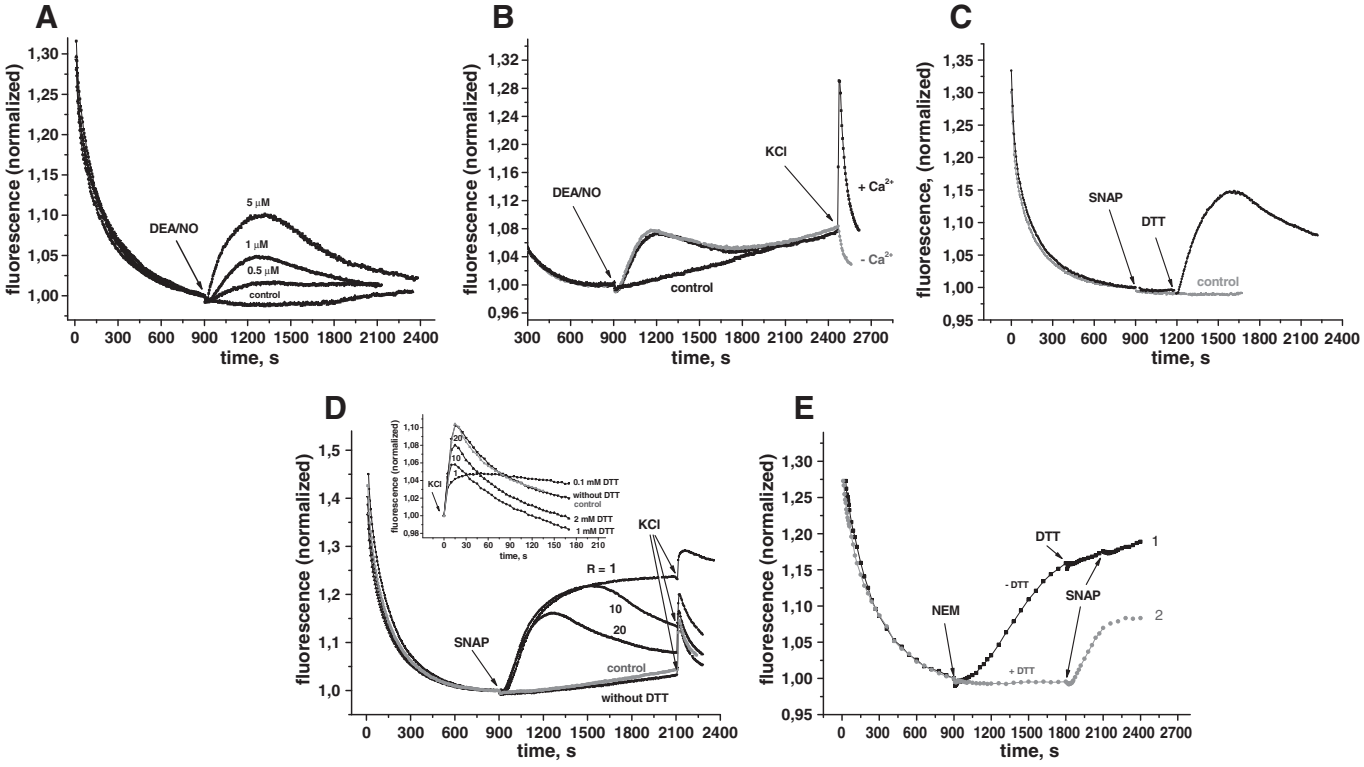

Fig. 4. The kinetics of NO donor-induced changes in the acidification of SVs, which are inside the synaptosomes. (A) Dose-dependent effect of DEA/NO; (B) The effect of the omission of external calcium on 1 μM DEA/NO-induced changes in SV acidification and subsequent exocytosis evoked by high K^+ (30 mM KCl); (C) SNAP (0.1 mM) affects SV acidification only after the addition of DTT (0.5 mM); (D) The effect of different DTT concentrations on SNAP-induced changes in SV acidification and subsequent high K^+-induced response. Synaptosomes were pre-incubated with 0.05–2.5 mM DTT, equilibrated with AO, and then SNAP (50 μM) and KCl (30 mM) were added as indicated by arrows; (D, inset) The time course of the high K^+-induced exocytotic spikes; (E) The effect of NEM on SV acidification and SNAP-induced action. NEM (50 μM) was added to AO-loaded synaptosomes pre-incubated without (curve 1) or with (curve 2) DTT (1 mM). The following addition of SNAP (0.1 mM) and DTT (1 mM) is indicated by arrows. Traces are representative of three–five independent experiments.

either alone or in the form of S-nitrosothiols, affects synaptic vesicle acidification via S-nitrosylation of the proteins, which influence the generation of vesicular proton gradient.

To confirm this assumption, we used N-ethylmaleimide (NEM), which is known to irreversibly alkylate protein SH-groups. Fig. 4E (curve 1) shows that NEM (50 μM) caused a gradual irreversible dissipation of H^+-gradient, which was not influenced by the subsequent application of DTT (1 mM) and SNAP (0.1 mM). At the same time, NEM failed to induce any changes in the vesicular proton gradient, when it was added to synaptosomes pre-incubated with DTT (Fig. 4E, curve 2). In this case, DTT added in excess to NEM inactivated the latter, but stimulated SNAP action. These data indicate that the modification of protein sulfhydryl groups appears to underlie the NO-induced changes in synaptic vesicle acidification.

The next question was whether NO-induced dissipation of the vesicular proton gradient was a consequence of the direct action of NO on V-ATP-ase or/and the decrease in the ATP level in nerve terminals. To elucidate this question, we studied the effect of NO donors on the acidification of synaptic vesicles (SVs) isolated from synaptosomes. As can be seen in Fig. 5A, neither SNAP nor DEA/NO, applied to SVs at much more higher concentrations (0.3 mM), induced the drastic changes in AO fluorescence similar to that observed when SVs were within the synaptosomes. In contrast to NO donors, concanamycin A, a well-known inhibitor of V-ATP-ase, completely dissipated the proton gradient of the isolated SVs (Fig. 5A) as well as SVs within the synaptosomes (Fig. 5B). It should be noted that DTT (1 mM) applied to SVs before or after SNAP addition did not influence the effect of SNAP on the acidification of isolated SVs (data not shown).

The results with isolated SVs suggest that the direct action of NO on V-ATP-ase seems not to be a major cause of NO-induced dissipation of the vesicular proton gradient. Given that V-ATP-ase activity is extremely sensitive to the ATP level in nerve terminals, in the next series of experiments we studied the effect of NO donors on the mitochondrial membrane potential (ΔΨm), which is known to be a driving force for ATP synthesis by mitochondria.

3.5. Reversible dissipation of the mitochondrial membrane potential of synaptosomes exposed to nanomolar–low micromolar NO concentrations

Under normal conditions, potentiometric dye rhodamine 6G (Rh 6G), being both lipophilic and cationic, easily penetrates into synaptosomes and bounds with plasma and mitochondrial membranes, yielding potential-dependent distribution. This process, as can be seen in Fig. 6A, is accompanied by the quenching of Rh 6G fluorescence, the steady-state level of which is achieved after 2 min. The subsequent application of DEA/NO led to a dose-dependent increase in the fluorescence intensity, which was followed by a gradual decrease in fluorescence up to basal level (Fig. 6A). These data indicate that NO released from DEA/NO causes the membrane depolarization of synaptosomes, which is fully reversible after the end of NO action.

To estimate the contribution of mitochondria to overall fluorescence changes evoked by DEA/NO, $\Delta\Psi_m$ was collapsed by rotenone and oligomycin as inhibitors of the respiratory chain and ATP synthase, respectively. Fig. 6B illustrates that under non-respiring conditions synaptosomes accumulated dye significantly less effectively since only the plasma membrane bonded it, and even at 200 μM concentrations, DEA/NO failed to alter the fluorescence signal. At the same time, as can be seen in Fig. 6B, KCl-induced depolarization of the plasma membrane led to the same increase in fluorescence irrespectively of the presence of rotenone + oligomycin in the extracellular medium. Thus, nitric oxide in the concentration range of nanomolar–low micromolar reversibly depolarizes the mitochondrial membrane and does not cause any appreciable changes in the plasma membrane potential.

Similar results were obtained with SNAP (50 μM), when it was added to synaptosomes pre-incubated with DTT (Fig. 6C). As in the case of the experiments with AO, the molar ratio of DTT to SNAP determined the rate and duration of the membrane potential recovery, i.e. the higher the reducing potential nerve terminals had, the more quickly the membrane potential was restored to the resting level. It should be noted that, as our preliminary findings showed, DTT alone did not influence synaptosomal membrane potential in the range of tested concentrations (data not shown). Under non-respiring conditions, SNAP as well as DEA/NO failed to alter Rh 6-G fluorescence confirming the high vulnerability of the mitochondrial membrane and the insensitivity of the plasma membrane to low NO concentrations (Fig. 6D). A specific thiol-alkylating agent NEM caused irreversible membrane depolarization of synaptosomes (Fig. 6E, curve 1), which was completely prevented by the preliminary addition of DTT at excessive concentrations (Fig. 6E, curve 2). It was important to note that SNAP + DTT-induced depolarizing effect (curve 2) was the same magnitude as that observed in the case of the sequential addition of NEM and SNAP (+DTT) (curve 1). The additivity of the effects of both SH-modifying agents, NEM and SNAP (+DTT), suggests that NO-induced depolarization of mitochondria is a consequence of the changes in the redox state of proteins responsible for maintaining the proper mitochondrial membrane potential.

Summarizing the results presented in this study, it is important to emphasize that isolated nerve terminals exposed to nitric oxide in the range of nanomolar–low micromolar concentrations were able, without any washing procedures, to almost completely recover their key

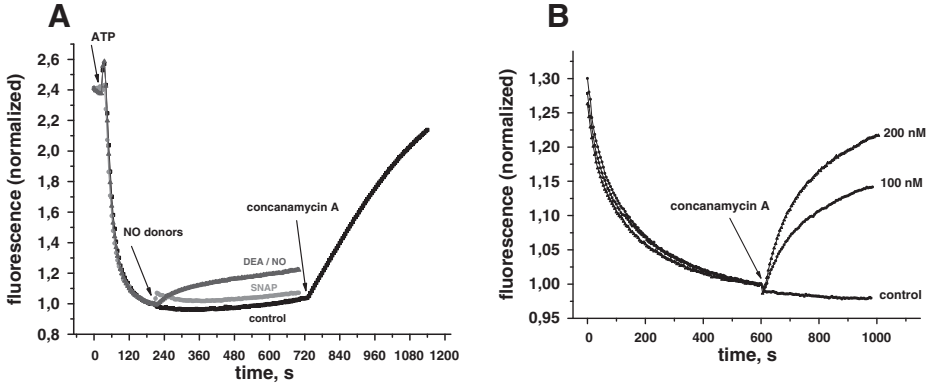

Fig. 5. The effect of NO donors and concanamycin A on the acidification of SVs isolated from synaptosomes. (A) The loading of isolated SVs with AO (2 μM) was triggered by ATP (0.5 mM), and then DEA/NO (0.3 mM), SNAP (0.3 mM) or concanamycin A (200 nM) was added as indicated by arrows; (B) Dose-dependent effect of concanamycin A on the acidification of SVs within the synaptosomes. Concanamycin A was added to synaptosomes pre-loaded with AO (5 μM). Traces are representative of at least three independent experiments.

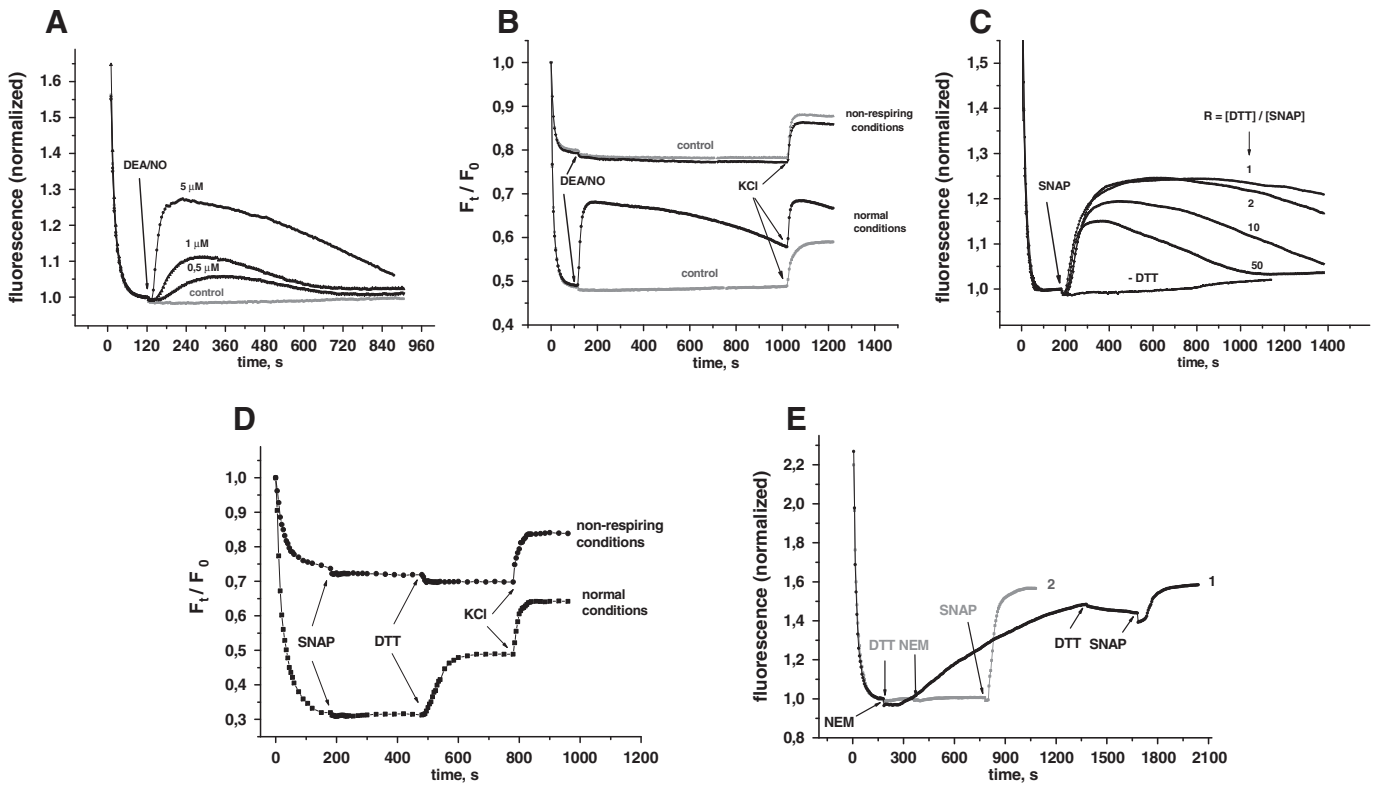

Fig. 6. The time course of the changes in the membrane potential of synaptosomes during their exposure to NO donors. (A) Dose-dependent effect of DEA/NO; (B) The effects of DEA/NO (200 μM) and high K^+ (30 mM KCl) under normal and non-respiring (in the presence of oligomycin (4 μg/mL) and rotenone (4 μM)) conditions; (C) The effect of different DTT concentrations on SNAP-induced changes in the membrane potential of synaptosomes. SNAP (50 μM) was added to synaptosomes pre-incubated with 0.05–2.5 mM DTT; (D) The effects of SNAP (0.1 mM), DTT (1 mM) and high K^+ (30 mM KCl) under normal and non-respiring conditions; (E) The additivity of the effects of NEM and SNAP + DTT. NEM (50 μM) was added before (curve 1) or after (curve 2) the application of DTT (1 mM) and then SNAP (0.5 mM) was applied. Traces are representative of three–five independent experiments.

functional parameters, such as the mitochondrial membrane potential, vesicular proton gradient and ability to reuptake neurotransmitter with its subsequent accumulation into synaptic vesicles.

4. Discussion

The main goal of this study was to improve our knowledge of the molecular mechanism(s) underlying the presynaptic action of nitric oxide on the neurosecretory system. In our work, we aimed to correlate the release of the major inhibitory neurotransmitter GABA with intracellular events occurring in the axon terminals during their exposure to NO in the range of nanomolar–low micromolar concentrations. Our findings support the assumption that nitric oxide, alone or in the form of S-nitrosothiols, stimulates GABA release, which appears to be attributed to the reversal of GABA transporters as a direct consequence of an increased cytosolic GABA concentration. The shift in the distribution of the endogenous GABA is reversible and seems to be due to S-nitrosylation of key proteins, which control the functions of synaptic vesicles and mitochondria. What is the basis for such an assumption?

Among all triggers of neurotransmitter release, NO is a unique, since it can stimulate this process independently of extra- and intracellular calcium [49–51]. According to our results with [^{3}H]GABA, nitric oxide induced external Ca^{2+}-independent GABA release, which seemed to be a consequence of non-vesicular neurotransmitter release via reversal of the GABA transporter. Non-vesicular GABAergic neurotransmission regulates the level of tonic inhibition and is thought to play an important role in the control of brain excitability [52]. Reverse transport occurs when dynamic equilibrium of the transporter is disrupted [48], and one of the possible reasons that may lead to such a transport during NO exposure is an increased concentration of GABA in the cytosol. Indeed, as our data show, the maximum concentration of ambient GABA recorded at 5 and 10 min after the application, respectively, of DEA/NO and SNAP (plus DTT) was attributed to GABA released from cytosol (Fig. 2A and C). At these time points, vesicular GABA content was minimal as evidenced by a significant decrease in exocytotic GABA release triggered by 4-AP (Fig. 2B and D).

Additional evidence confirming that NO-stimulated [^{3}H]GABA release mentioned above occurs by non-exocytotic mechanism comes from our experiments with Fluo 4AM showing that nitric oxide, at the used concentrations, does not induce any changes in intracellular calcium concentrations. Some elevation of $[Ca2+]_i$ evoked by 50 μM DEA/NO in Ca^{2+}-free/EGTA medium, as can be seen in Fig. 3A, was delayed over 5 min, and so could not be a trigger of massive [^{3}H]GABA release by exocytosis. However, we could not exclude a possible involvement of exocytosis in NO-induced GABA release at much higher NO concentrations, since a much more rapid and significant rise in $[Ca2+]_i$ was detected by us (data not shown) and other [53] at higher NO concentrations.

We suggest that our results may reconcile previously reported contradictory findings showing that NO can either stimulate [13,15], or inhibit [16,17], or have a dual effect, enhancing the spontaneous and inhibiting the evoked neurotransmitter release [18]. Spontaneous neurotransmitter release is usually referred to as external Ca^{2+}-independent spontaneous exocytosis, which is triggered by calcium released from intracellular Ca^{2+} stores [54]. But, there is evidence that some drugs, such as vigabatrin and gabapentin, induce spontaneous GABA efflux, which has a non-vesicular origin [55]. These anticonvulsants irreversibly block GABA transaminase that leads to an increase in neuronal cytosolic [GABA] and to the shift of the setpoint for GABA transporter to a higher ambient [GABA]. We hypothesize that NO-evoked GABA release described above is also a result of the increased cytosolic GABA concentration, but due to the redistribution of the endogenous GABA from synaptic vesicles to the cytosol. This process is accompanied by the decrease in vesicular GABA content and, as a result, in

the decrease of depolarization-evoked exocytotic release. Different actions of NO on the exocytotic and carrier-mediated GABA release have been shown by Sequeira et al. [32], who have proposed that NO inhibits the exocytotic and stimulates the carrier-mediated release. We also tend to consider the observed attenuation of exocytotic GABA release as a consequence of partial depletion of the vesicles, rather than the inhibition of the exocytotic machinery.

What might be the cause of such vesicle depletion? The driving force for neurotransmitter accumulation into synaptic vesicles is known to be an electrochemical proton gradient ($\Delta\mu H^+$), which influences vesicular neurotransmitter filling and thereby the efficacy of neurotransmission. Given the high sensitivity of GABA uptake to ΔpH component of $\Delta\mu H^+$ [56,57], it becomes evident that any changes in the vesicle acidification would influence vesicle filling with GABA. Indeed, as our results with AO show, nitric oxide causes reversible dissipation of synaptic vesicle proton gradient, and this process is closely correlated in time with NO-induced changes in [^{3}H]GABA release mentioned above. At the time point when the proton gradient was dissipated maximally (Fig. 4A and D), the [^{3}H]GABA level was minimal in the vesicles and maximal in the cytosol (Fig. 2). On the contrary, the re-acidification of synaptic vesicles in 15–20 min after NO donor application (Fig. 4A and D) was paralleled with the increase in the vesicular GABA content (Fig. 2B and D). So, our findings allow us to suggest that nitric oxide can modulate synaptic efficacy through a presynaptic mechanism that influences the acidification and, consequently, filling of synaptic vesicles with neurotransmitters. The tight correlation between the efficacy of inhibitory transmission and vesicle acidification has been recently reported by Riazanski et al. [58], who have shown that defective vesicular acidification in *Clcn3*$^{-/-}$ mice results in the reduction in loading of GABA into synaptic vesicles and, therefore, the decrease in the evoked GABAergic synaptic transmission.

The next question we asked was what underlies NO-induced changes in vesicle acidification. Proton transport is known to be provided by vacuolar-type H^+-ATPase (V-ATPase) and, consequently, NO can affect proton pumping activity of V-ATPase either via direct action on critical groups in the enzyme or via ATP depletion. Direct action of NO on V-ATPase was previously reported by Wolosker et al. [59] and Forgac [60], who revealed that NO inhibits both pumping and ATP-ase activity of the enzyme through S-nitrosylation of its critical sulfhydryl groups, thereby down-regulating the vesicular neurotransmitter pool. According to our results, NO donors dissipate the proton gradient of isolated synaptic vesicles; however, in contrast to experiments with intact nerve terminals, this dissipation was irreversible throughout the experimental time period and induced by much higher NO concentrations (Fig. 5A). This suggests that another reason is mainly responsible for NO-induced reversible H^+-gradient dissipation of synaptic vesicles, which are inside the nerve terminals. We assume that ATP depletion seems to be such a reason. Indirect evidence in favor of this assumption comes from our data showing that a gradual dissipation of the vesicular proton gradient is correlated in time with the depolarization of the mitochondrial membrane potential (Fig. 4), which is known to influence the energy status of the cell. It was previously reported that at low concentrations nitric oxide potently deenergizes isolated brain mitochondria [61], and a progressive concentration-dependent depletion of cellular ATP is a result of a rapid NO-induced depolarization of mitochondrial membrane [62]. The more direct evidence for the role of ATP in neurotransmitter release was recently obtained by Rudkouskaya et al. [22], who have measured ATP levels in synaptosomal preparations and established that NO donors suppress vesicular neurotransmitter release via inhibition of energetic metabolism due to S-nitrosylation of intrasynaptosomal proteins.

Nitric oxide impairs mitochondrial function, affecting the electron-transport chain at multiple sites, the most sensitive of which are cytochrome *c* oxidase [24] and mitochondrial respiratory complex I [25]. The inhibition of complex I activity is thought to be a result of S-nitrosylation of the critical thiol residues in complex I, and this inhibition can be readily reversed by light or reduced thiols, such as glutathione and dithiothreitol [63]. Our experiments with thiol-modifying agents support the assumption that the observed effects of NO are mainly attributed to its reaction with SH-groups of proteins responsible for the maintenance of proper mitochondrial respiratory activity. Irreversible alkylation of thiol groups with NEM completely abolished the dissipating effects of NO on both synaptic vesicle and mitochondrial proton gradients (Figs. 4E and 6E), indicating that thiol-groups are a common target for these agents.

As for dithiothreitol, it had a dual effect: at first DTT stimulated SNAP-induced dissipation of both SV proton gradient and mitochondrial potential, but later it dose-dependently facilitated reverse processes. We suggest that DTT promotes SNAP action, not so much due to the increase in NO concentration (≈three-fold, according to our data), but rather to the appearance of highly active species (trans-nitrosating intermediates) capable of readily transnitrosating critical thiols in mitochondrial proteins. Since the redox-sensitive processes are known to be strongly influenced by the molar ratio of reductant to oxidant, the observed acceleration of the recovery process at higher molar ratio of DTT to SNAP becomes clear. We have first shown that the higher the reducing potential nerve terminals have, the more quickly they are able to restore their key functional parameters such as mitochondrial membrane potential, synaptic vesicle proton gradient and capacity to accumulate neurotransmitter. The almost complete recovery of exocytotic neurotransmitter release in response to high K^+ (Fig. 4D, inset) indicates that this appears to be the case.

In conclusion, our study provides new insights into molecular mechanism(s) underlying the action of nanomolar-low micromolar concentrations of nitric oxide on GABA release. Recent studies increasingly indicate that S-nitrosylation, along with phosphorylation, represents an important pathway that influences most aspects of cellular physiology and pathophysiology [7,21,28,29]. Given the high oxidative potential of nitric oxide as well as its unique feature to stimulate neurotransmitter release by Ca^{2+}- and cGMP-independent mechanism, the reversible post-translational modification of mitochondrial and synaptic vesicle thiol proteins seems to be one of the crucial pathways underlying the presynaptic action of nitric oxide on inhibitory neurotransmission.

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