Communication

Newly Synthesized Phosphatidylinositol Phosphates Are Required for Synaptic Norepinephrine but Not Glutamate or \(\gamma\)-Aminobutyric Acid (GABA) Release*

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Newly synthesized phosphatidylinositol phosphates have been implicated in many membrane-trafficking reactions. They are essential for exocytosis of norepinephrine in PC12 cells and chromaffin cells, suggesting a function in membrane fusion. We have now studied the role of phosphatidylinositol phosphates in synaptic vesicle exocytosis using synaptosomes. Under conditions where phosphorylation of phosphatidylinositols is blocked, norepinephrine secretion was nearly abolished whereas glutamate and GABA release was still elicited. Thus phosphatidylinositides are essential only for some membrane fusion reactions, and exocytotic release mechanisms differ between neurotransmitters.

At synapses, similar mechanisms of synaptic vesicle exocytosis are thought to effect the secretion of different neurotransmitters (e.g. glutamate, GABA, \(^1\) and norepinephrine; reviewed in Refs. 1–4). The same vesicle proteins are present in presynaptic nerve terminals independent of neurotransmitter type (e.g. see Refs. 5–8). In norepinephrine-secreting chromaffin cells and in PC12 cells, phosphorylation of phosphatidylinositols by PI 4-kinase is required for Ca\(^{2+}\)-triggered exocytosis (9–12). In these cells, PI 4-kinase can be potently inhibited by phenylarsine oxide (PAO), resulting in a block of exocytosis (11, 12). Several proteins involved in synaptic vesicle exocytosis bind to the products of PI 4-kinase activity, PIP and PIP\(_2\) (13–17). Furthermore, phosphoinositides have also been implicated in a number of other membrane trafficking reactions (reviewed in Refs. 18–20). Together these findings suggested that phosphorylation of PI is essential for exocytosis and membrane fusion. To explore the role of PI phosphorylation in neurotransmitter release, we have now studied neurotransmitter release from synaptosomes. We demonstrate that inhibition of PIP and PIP\(_2\) synthesis results in dramatically different effects for different neurotransmitters and that PIP and PIP\(_2\) synthesis is not a requirement for most synaptic exocytosis.

EXPERIMENTAL PROCEDURES

Treatments and Phospholipid Analysis of Synaptosomes—Synaptosomes were prepared as described (21) and resuspended in aerated (95% O\(_2\), 5% CO\(_2\)) ice-cold Krebs-bicarbonate buffer, pH 7.4 (composition in mM: NaCl 118, KCl 1.25, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 25, glucose 11.5, and HEPES-NaOH 5) or phosphate-free Krebs-bicarbonate buffer (for \(^32\)P-labeling experiments). Synaptosomes were \(^32\)P-labeled for 1.5 h at 35 °C in a 95% O\(_2\), 5% CO\(_2\) atmosphere with \(^32\)P(orthophosphate (1 mM)). After labeling, PAO (from Aldrich) freshly made in Me\(_2\)SO or Me\(_2\)SO alone (<1% of total volume) was added, and synaptosomes were incubated for the indicated times in triplicate. Reactions were stopped on ice. Lipids were extracted with 3.75 volumes of chloroform:methanol:concentrated HCl (100:200:1). After 10 min on ice, 10 μg of phosphatidylethanolamine (Sigma) was added as a carrier, and phase partitioning was induced by with 1.25 volumes of chloroform and of 0.1 N HCl. The chloroform phase with phospholipids was washed twice with cold methanol:0.1 N HCl (1:1). Equal volumes of the extracts were loaded on TLC plates with phospholipid standards (10–20 μg each). TLC plates were developed in 1-propanol:HO\(_2\):concentrated NH\(_4\)OH (65:20:15) and analyzed by autoradiography; phospholipid standards were identified with iodine vapors. \(^32\)P incorporation was quantified with a PhosphoImager (Molecular Dynamics, CA).

Measurements of \(^3H\)-Glutamate, \(^3H\)-GABA, and \(^3H\)-Norepinephrine Release—Synaptosomes were incubated for 5 min with 140 nM \(^3H\)-glutamate (specific activity, 15 Ci/mmol), 130 nM \(^3H\)-norepinephrine (90 Ci/mmol), or 66 nM \(^3H\)-GABA (90 Ci/mmol). Buffers for the norepinephrine experiments and experiments in which glutamate and norepinephrine release were measured in the same synaptosomes also contained 0.4 mM ascorbic acid, 30 μM EDTA, and 10 μM pargyline. \(^3H\)-Loaded synaptosomes (0.1 ml) were trapped on glass fiber filters (GF/B, Whatman), overlaid with 50 μl of a 50% Sephadex G-25 slurry, and superfused at 33 °C with Krebs-bicarbonate buffer (flow rate, 0.8 ml/min) under continuous aeration with 95% O\(_2\), 5% CO\(_2\). For sucrose-triggered neurotransmitter release experiments, synaptosomes were superfused with Ca\(^{2+}\)-free Krebs-bicarbonate buffer containing 0.1 mM EGTA. After 12 min of washing, two 1-min fractions were collected to determine base-line release. We then evoked release from synaptosomes by the following agents: 1) 25 mM KCl for 30 s; 2) 5 mM ionomycin for 30 s; 3) 0.5–3 mM α-latrotoxin for 1 min; 4) 0.5 mM succrose for 30 s in Ca\(^{2+}\)-free Krebs-bicarbonate buffer. All stimuli were applied by rapid switching of the superfusion lines between regular and stimulation buffers. The amounts of \(^3H\)-glutamate, \(^3H\)-norepinephrine, or \(^3H\)-GABA released into the superfusate and remaining in the synaptosomes at the end of the experiment were quantified by liquid scintillation counting. Fractional neurotransmitter release was calculated by dividing the amount of neurotransmitter released during a time interval by the amount of transmitter remaining in the synaptosomes at that time. To obtain the total GABA, glutamate, and norepinephrine release induced by a given stimulus, the evoked release above baseline was integrated over the time of the experiment. To test the effect of PAO on release, synaptosomes were treated with PAO in Me\(_2\)SO, Me\(_2\)SO alone (control; <1% of total volume), or vanadyl hydroperoxide (VOOH; prepared as a complex with 1,10-phenanthroline as in Ref. 22) for 20 min at 35 °C in Ca\(^{2+}\)-containing aerated (95% O\(_2\), 5% CO\(_2\)) Krebs-bicarbonate buffer. During the last 5 min, \(^3H\)-labeled neurotransmitters were added for loading the synaptosomes, which were then used for release measurements as described above. PAO treatment partly inhibited \(^3H\)-norepinephrine and \(^3H\)-GABA but not \(^3H\)-glutamate uptake. To control for this and other possible indirect effects, we performed experiments in which synaptosomes were first loaded with \(^3H\)-labeled neurotransmitters and then treated with PAO, with identical results to those shown here. For each experiment, results are expressed in percent release compared with control conditions.

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1 The abbreviations used are: GABA, \(\gamma\)-aminobutyric acid; PI, phosphatidylinositol; PAO, phenylarsine oxide; PIP, phosphatidylinositol phosphate; PIP\(_2\), phosphatidylinositol bisphosphate.
RESULTS

PAO Blocks PIP and PIP₂ Synthesis in Synaptosomes—We labeled synaptosomes with ³²P, and treated them with PAO or control buffer. The synaptosomes were then incubated under various control and stimulation conditions, and their phospholipids were analyzed by TLC. Three major ³²P-labeled phospholipids were observed and identified with unlabeled phospholipid standards as PIP, PIP₂, and closely co-migrating PI and phosphatidic acid (data not shown). The preferential labeling of PI, PIP, and PIP₂ in synaptosomes agrees well with the high turnover rate of PIPs (23–25). We then tested the effect of PAO on PIP and PIP₂ synthesis in synaptosomes. PAO caused a major inhibition of ³²P-labeling of PIP and PIP₂ but induced only marginal changes in PI and phosphatidic acid (Fig. 1 and data not shown). Stimulation of synaptosomes by α-latrotoxin or KCl depolarization elicited moderate decreases in the levels of ³²P-labeled PIP and PIP₂ in the absence of PAO but had no effect on the inhibition of PI and PIP phosphorylation by PAO (data not shown). We did not determine at which positions the phosphatidylinositol rings are phosphorylated in these experiments. However, similar studies in chromaffin cells showed that the majority of PIP and PIP₂ is phosphorylated at the 4- and 5-positions (11, 12). These results argue that, as in chromaffin cells, PAO inhibits phosphorylation of PIP and PIP₂ at the 4- and 5-positions of the inositol ring in synaptosomes (11, 12).

To optimize the PAO treatment conditions, we measured the time and dose dependence of the effect of PAO (Fig. 1). Synaptosomes were incubated with various concentrations of PAO for 20 min, and the levels of PIP and PIP₂ were quantified. Data are means ± S.E. from a single experiment repeated multiple times with similar results.

Fig. 1. PAO inhibits PI and PIP phosphorylation in synaptosomes. A, time course of PAO action. ³²P-labeled synaptosomes were treated with 30 μM PAO for the indicated times. ³²P-labeled PIP and PIP₂ were analyzed by TLC and quantified by PhosphorImager measurements. B, PAO concentration dependence. Synaptosomes were incubated with various concentrations of PAO for 20 min, and the levels of PIP and PIP₂ were quantified. Data are means ± S.E. from a single experiment repeated multiple times with similar results.

Ca²⁺-dependent and Ca²⁺-independent Release of Norepinephrine, Glutamate, and GABA from Synaptosomes—To study release, we loaded synaptosomes with [³H]norepinephrine (top), [³H]glutamate (middle), or [³H]GABA (bottom) were superfused with aerated Krebs-bicarbonate buffer. Released neurotransmitters were measured in the efflux and are plotted as fractional release (see “Experimental Procedures”). In the experiments on the left, release was stimulated by brief pulses of 25 mM KCl (K) and 5 μM ionomycin (I) in regular or Ca²⁺-free buffer. In the experiments on the right, secretion was evoked by application of 25 mM KCl (K) and 3 mM α-latrotoxin (L) in Krebs-bicarbonate buffer containing or lacking 50 μM La³⁺. Note the scale differences between graphs.

Fig. 2. Characterization of norepinephrine, glutamate, and GABA release from synaptosomes. Synaptosomes preloaded with [³H]norepinephrine (top), [³H]glutamate (middle), or [³H]GABA (bottom) were superfused with aerated Krebs-bicarbonate buffer. Released neurotransmitters were measured in the efflux and are plotted as fractional release (see “Experimental Procedures”). In the experiments on the left, release was stimulated by brief pulses of 25 mM KCl (K) and 5 μM ionomycin (I) in regular or Ca²⁺-free buffer. In the experiments on the right, secretion was evoked by application of 25 mM KCl (K) and 3 mM α-latrotoxin (L) in Krebs-bicarbonate buffer containing or lacking 50 μM La³⁺. Note the scale differences between graphs.
intracellular Ca\textsuperscript{2+} that results in the continuous recruitment of vesicles for exocytosis. α-Latrotoxin triggered neurotransmitter release by a Ca\textsuperscript{2+}-independent high affinity interaction (EC\textsubscript{50} ~ 3 nM). Its action was inhibited by low concentrations of La\textsuperscript{3+}, indicating a specific mechanism (Fig. 2). Sucrose caused massive transient neurotransmitter release (data not shown). Preliminary studies showed that sucrose is more potent in releasing neurotransmitters from synaptosomes than KCl depolarization but that both secretagogues act on the same neurotransmitter pools.\textsuperscript{2} Sucrose-triggered release similar to electrophysiological studies is partially inhibited by tetanus toxin.\textsuperscript{2} Together these experiments suggest that the synaptosomes can be used to probe secretion of different types of neurotransmitters elicited by stimuli acting on distinct parts of the release machinery.

Effect of Blocking PIP and PIP\textsubscript{2} Synthesis on Neurotransmitter Release—We treated synaptosomes with 3 μM PAO or control buffer and measured norepinephrine and glutamate release stimulated by KCl, ionomycin, and α-latrotoxin (Fig. 3A). After PAO treatment, norepinephrine secretion was blocked whereas glutamate release was unchanged. This was a surprising result because it suggests that glutamate and norepinephrine release may be mechanistically different, although both are Ca\textsuperscript{2+}-dependent (Fig. 2). To exclude the possibility that the distinct effects of PAO were caused by differences between experimental conditions, we measured norepinephrine and glutamate release in the same preparation of PAO-treated and control synaptosomes (Fig. 3B). The PAO concentrations used were high enough to assure a virtually total inhibition of PI and

\textsuperscript{2} G. Lonart and T. C. Südhof, unpublished observation.
PIP phosphorylation (Fig. 1). Again, norepinephrine release was inhibited whereas glutamate release was not. Thus PAO has opposite effects on the exocytosis of norepinephrine- and glutamate-containing vesicles.

The possibility that norepinephrine release but not glutamate release is inhibited under conditions that block PI and PIP phosphorylation is interesting because it implies that PI and PIP phosphorylation is not universally required for exocytosis. In addition, if the components of the release machinery differ between types of neurotransmitters, their exocytotic mechanisms must be distinct. To affirm these conclusions, we performed a large-scale study of the effects of PAO on synaptic vesicle exocytosis. Three transmitters, norepinephrine, glutamate, and GABA, were analyzed in parallel under PAO treatment conditions that block PI and PIP phosphorylation. Because PAO is also a tyrosine phosphatase inhibitor (11, 12), we acquired their results, and expressed the integrated release as percent of control (Figs. 4 and 5). Overall, the data confirm that PAO selectively inhibits exocytosis of norepinephrine containing synaptic vesicles independent of which secretory agent is used, without a consistent inhibitory effect on GABA or glutamate release.

When release was induced by KCl depolarization, PAO at low concentrations (3 μM) severely depressed norepinephrine secretion but had no effect on glutamate release even at 10-fold higher levels (30 μM PAO; Fig. 4). PAO also partially inhibited GABA release stimulated by KCl. Norepinephrine release triggered by ionomycin, the second Ca2+-dependent and probably most powerful secretagogue used here (Fig. 2), was also inhibited by PAO. Again, glutamate secretion was unaffected. With ionomycin, GABA release was also unchanged by PAO (Fig. 4). In addition, norepinephrine secretion triggered by α-latrotoxin or by sucrose was severely inhibited by 3 μM PAO (Fig. 5). With both stimulation agents, we again observed no effect of PAO at concentrations of up to 30 μM on glutamate secretion. Furthermore, PAO exerted no significant inhibition of GABA release stimulated by either hypertonic sucrose or α-latrotoxin.

In the design of our experiments, PAO treatments preceded the uptake of labeled neurotransmitters for the release measurements. To exclude the possibility that PAO changes the disposition of neurotransmitters after uptake into the synaptosomes, we performed release measurements in which the order of PAO treatment and neurotransmitter uptake was reversed. With this protocol, PAO still did not inhibit glutamate release but strongly suppressed norepinephrine secretion (data not shown). It is also unlikely that “glutamategic” and “GABAergic” nerve terminals are selectively protected from the inhibitory effects of PAO. These two transmitters account for more than 90% of all synapses in brain cortex. Thus the changes in PIP and PIP2 observed by TLC with PAO treatment must affect glutamategic and GABAergic synaptosomes.

**DISCUSSION**

In the current experiments, we use synaptosomes to study the release of three neurotransmitters, norepinephrine, glutamate, and GABA. We applied PAO to inhibit PIP and PIP2 synthesis and studied the effect of this inhibition on release. Our data show that norepinephrine secretion elicited with four stimulation protocols is severely inhibited by PAO. In contrast, PAO had no effect on glutamate secretion stimulated by all four stimulation protocols. PAO did not inhibit GABA release stimulated by ionomycin, sucrose, and α-latrotoxin, and moderately inhibited GABA release evoked by KCl. We used PAO concentrations that cause a nearly complete inhibition of PI and PIP phosphorylation. Thus glutamate and GABA secretion do not require newly synthesized PIP and PIP2. In contrast, similar to PC12 and chromaffin cells (9–12), PI and PIP phosphorylation is essential for norepinephrine secretion. The fact that we employed different means of stimulating release ensures that the effects observed are not artifacts of a particular stimulation method.

These results have implications for our understanding of neurotransmitter release and membrane fusion. First, the data show that there are transmitter-specific differences in the mechanism of exocytosis. No such differences have been observed in the protein composition of synapses (e.g., see Refs. 5–8). The selective requirement for phosphoinositides in norepinephrine but not glutamate and GABA release may reflect fundamental differences between exocytosis of dense-core synaptic vesicles containing catecholamines and clear synaptic vesicles containing glutamate and GABA.

Second, exocytosis of norepinephrine-containing vesicles requires PIP phosphorylation at a step that precedes Ca2+-action. We found that PAO equally inhibits Ca2+-dependent release induced by KCl and ionomycin and Ca2+-independent release evoked by hypertonic sucrose, which triggers exocytosis of all docked synaptic vesicles by an unknown mechanism prior to Ca2+-entry (27).

Third, phosphorylation of PI is not universally involved in exocytosis. Although PAO could have multiple actions, our data document that it acts as an effective inhibitor of PI and PIP phosphorylation in synaptosomes. The undiminished capacity of synaptosomes to secrete glutamate and GABA in the absence of significant PI and PIP phosphorylation is striking. It thus is unlikely that PIP and PIP2 function as general signals or lipid mediators in membrane fusion.

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