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Pardaxin Stimulation of Phospholipases A_2 and Their Involvement in Exocytosis in PC-12 Cells

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ABSTRACT

Pardaxin (PX) is a voltage-dependent ionophore that stimulates catecholamine exocytosis from PC-12 pheochromocytoma cells both in the presence and absence of extracellular calcium. Using a battery of phospholipase A_2 inhibitors we show that PX stimulation of phospholipase A_2 (PLA_2) enzymes is coupled with induction of exocytosis. We investigated the relationship between PX-induced PLA_2 activity and neurotransmitter release by measuring the levels of arachidonic acid (AA), prostaglandin E_2 (PGE_2), and dopamine release. In the presence of extracellular calcium, the cytosolic PLA_2 inhibitor arachidonyl trifluoromethyl ketone ($AACOCF_3$) inhibited by 100, 70, and 73%, respectively, the release of AA, PGE_2, and dopamine induced by PX. The mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor 2′-amino-3′-methoxyflavone (PD98059) reduced by 100 and 82%, respectively, the release of AA and PGE_2 induced by PX. In the absence of extracellular calcium, the calcium-independent PLA_2 ($iPLA_2$) inhibitors methyl arachidonyl fluorophosphonate, $AACOCF_3$, and bromoenol lactone (BEL) inhibited by 80 to 90% PX stimulation of AA release, by 65 to 85% PX stimulation of PGE_2 release, and by 80 to 90% PX-induced dopamine release. Using vesicle fusion-based enzyme-linked immunosorbent assay we found similar levels of inhibition of PX-induced exocytosis by these inhibitors. Also, PX induced the formation of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor complexes, an effect that was augmented by *N*-methylmaleimide. This complex formation was completely inhibited by BEL. Botulinum toxins type C1 and F significantly inhibited the release of AA, PGE_2, and dopamine induced by PX. Our data suggest that PX stimulates exocytosis by activating cystolic PLA_2 and $iPLA_2$, leading to the generation of AA and eicosanoids, which, in turn, stimulate vesicle competence for fusion and neurotransmitter release.

Hormones and neurotransmitters are usually released from cells by exocytosis, when a rise in cytosolic calcium triggers fusion of the secretory vesicle membrane with the plasma membrane (Augustine et al., 1987). Exocytosis of neurotransmitter involves the assembly of complexes composed of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE proteins), formed by the synaptic vesicle VAMP (synaptobrevin) and the plasma membrane syntaxin and SNAP-25 (Sollner et al., 1993). SNAREs are targets for the botulinum and tetanus toxins (Sudhof et al., 1993). These selectively inhibit synaptic vesicle fusion by site-specific proteolysis (Montecucco and Schiavo, 1995), suggesting a central function for SNARE in exocytosis. SNARE proteins have been implicated in the fusion machinery of all cellular systems investigated to date, including PC-12 cells (Ray et al., 1993). However, other cellular elements regulating the kinetics, the extent of fusion, and the vesicle assembling for release have received scant attention.

Aside from toxins that inhibit neurotransmitter release, there are others that cause a massive release of neurotrans-

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ABBREVIATIONS: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNAP receptor); SNAP-25, synaptosomal associated protein of 25 kDa; VAMP, vesicle-associated membrane protein (synaptobrevin); PX, pardaxin; AA, arachidonic acid; PLA_2, phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; $iPLA_2$, calcium-independent phospholipase A_2; MAPK, mitogen-activated protein kinase; $AACOCF_3$, arachidonyl trifluoromethyl ketone; MAFP, methyl arachidonyl fluorophosphonate; BEL, bromoenol lactone; BSA, bovine serum albumin; NEM, *N*-ethylmaleimide; 5-HETE, 5-hydroxyeicosatetraenoic acid; HPLC, high-pressure liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; RIA, radioimmunoassay; NGF, nerve growth factor; ERK, extracellular regulated kinase; PGE_2, prostaglandin E_2; NSF, *N*-ethylmaleimide-sensitive factor; BoNT/C1, botulinum toxin type C1; BoNT/F, botulinum toxin type F; O.D., optical density; UO126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene; PD98059, 2′-amino-3′-methoxyflavone.

mitters. These include α-latrotoxin, a toxin component from black widow spider venom, and pardaxin (PX). PX, a toxin isolated from the secretion of the fish *Pardachirus marmoratus* is an ionophore, amphipathic, acidic, hydrophobic polypeptide that forms voltage-dependent channels in both artificial and biological membranes (Lazarovici et al., 1986). It was shown that this toxin induced a concentration- and time-dependent release of arachidonic acid (AA) and eicosanoids in PC-12 cells (Abu-Raya et al., 1998). Furthermore, PX induced exocytosis from several neuronal preparations, both in the presence and absence of extracellular calcium (Lazarovici and Lelkes, 1992; Abu-Raya et al., 1999). In the presence of extracellular calcium, PX induced an increase in intracellular calcium (Abu-Raya et al., 1998), apparently due to its entrance from the pores of PX, a signal responsible for a small fraction of the neurotransmitter release (Lazarovici and Lelkes, 1992). In a calcium-depleted medium PX neither affected cytosolic calcium nor mobilized calcium from intracellular stores (as opposed to thapsigargin; Abu-Raya et al., 1999). The unique ability of this toxin to markedly release neurotransmitter in the absence of extracellular calcium and without releasing calcium from intracellular stores renders PX a special pharmacological tool for investigating novel cellular mechanisms of exocytosis.

AA and its metabolites, which are known as fusogens (Creutz, 1981), are likely candidates in calcium-independent neurotransmitter release. AA is found in the *sn*-2 position of membrane phospholipids, where it can be released by the deacylation of a variety of lipases, and is converted into eicosanoid by the action of cyclooxygenases and lipoxygenases. Direct cleavage of AA from the *sn*-2 position is catalyzed by phospholipases A_2 (PLA_2; Balsinde et al., 1999). PLA_2 enzymes are divided into three major subgroups: secretory PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), and calcium-independent PLA_2 ($iPLA_2$). 1) $sPLA_2$s include a group of soluble, low-molecular mass (~14 kDa) enzymes that are calcium-dependent (millimolar range) and involved in plasma membrane repair, digestion, and inflammation (Dennis, 1994). 2) $cPLA_2$s, which have an apparent preference for arachidonate-containing phospholipids, are calcium-dependent (micromolar range) enzymes, and are translocated to the plasma membrane upon mitogen-activated protein kinase (MAPK) phosphorylation (Lin et al., 1993). 3) $iPLA_2$s are cytosolic PLA_2s that do not require calcium for catalysis (Ackermann and Dennis, 1995). It was suggested that they are important for AA metabolism, in particular for phospholipid fatty acid remodeling (Balsinde et al., 1999). Because PLA_2s are involved in a variety of cellular functions, such as lipid metabolism and membrane homeostasis, and play a crucial role in eicosanoid production, signal transduction, and exocytosis (Piomelli, 1994; Balsinde et al., 1999), PLA_2 inhibitors are being vigorously pursued. Recently, arachidonyl trifluoromethyl ketone ($AACOCF_3$) and arachidonyl fluorophosphonate (MAFP) were found to inhibit intracellular PLA_2s, i.e., both $cPLA_2$s and $iPLA_2$s. $iPLA_2$s are also strongly and specifically inhibited by bromoenol lactone (BEL; Balsinde et al., 1999). Both inclusion of MAFP and $AACOCF_3$ under calcium-independent conditions (reflecting only $iPLA_2$ activity) and using the specific $iPLA_2$ inhibitor BEL enabled us to separate the contribution of $iPLA_2$s in PX-induced exocytosis. Activation of PLA_2s may lead to the production of arachidonic acid metabolites and, consequently, may affect the fusion event. We investigated whether the action of PX involves such a mechanism.

In the present study we describe PX activation of PLA_2 enzymes and their involvement in PX-induced exocytosis from PC-12 cells. We propose that activated $iPLA_2$ generates AA and/or derived eicosanoids, which, in turn, alter SNARE core-complex assembly and subsequently induce dopamine exocytosis. These results suggest the existence of calcium-independent PX-induced exocytosis, and provide evidence supporting a possible role for the AA cascade in the exocytosis process.

Experimental Procedures

Materials. [^{3}H]Dopamine (34 Ci/mmol) and [^{3}H]norepinephrine (52 Ci/mmol) were purchased from Amersham Biosciences (Piscataway, NJ); HEPES, trypan blue, BSA, EGTA, $MgCl_2$, $CaCl_2$, NaCl, NaOH, Na_2CO_3, $NaHCO_3$, KH_2PO_4, Tris-Cl, Triton X-100, glycerol, sucrose, D-glucose, propyl gallate, ethanol, HCl, petroleum ether, ethyl acetate, acetic acid, acetonitrile, 4-(2-aminoethyl) benzenesulfonyl fluoride, protease inhibitor cocktail 2 for mammalian cells, β-mercaptoethanol, bromphenol blue, *N,N,N′,N′*-tetramethylethylenediamine, ammonium persulphate, luminol, *p*-coumaric acid, *N*-ethylmaleimide (NEM), SDS, anti-synaptotagmin antibody, monoclonal anti-syntaxin antibody (HPC1), and poly-L-lysine were purchased from Sigma-Aldrich (St. Louis, MO); methanol, KCl, and ascorbic acid were purchased from Merck (Darmstadt, Germany); PD98059, BEL, MAFP, and $AACOCF_3$ were purchased from Biomol Research Laboratories (Plymouth Meeting, PA); UO126 was purchased from Promega (Madison, WI); 5-HETE (for HPLC standards) was purchased from Cayman Chemicals (Ann Arbor, MI); octadecyl functionalized silica gel was purchased from Aldrich Chemical (Milwaukee, WI); and DMEM, horse and calf serum, antibiotics, normal goat serum, and rat tail type 1 collagen were purchased from Beit Ha'emek (Naharia, Israel). An ELISA tetramethylbenzidine developing kit was purchased from Clark Laboratories (Jamestown, NY); ELISA blocking reagent was purchased from Sorin Biomedica (Saluggia, Italy); peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L) was purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA); recombinant protein-G Sepharose 4B was purchased from Zymed Laboratories (South San Francisco, CA); anti-VAMP (synaptobrevin) and anti-SNAP-25 polyclonal antibodies were the kind gift of Alomone Laboratories (Jerusalem, Israel); and polyethylenimine was synthesized and kindly provided by Prof. A. Domb (Department of Medicinal Chemistry, School of Pharmacy, The Hebrew University of Jerusalem, Israel).

Toxins. Native PX (P4) was purified by liquid chromatography from the lyophilized secretion of the flatfish *Pardachirus marmoratus* (collected in Eilat, Israel) (Lazarovici et al., 1986). Synthetic pardaxins P4 and P5 were prepared on a 433A peptide synthesizer (PerkinElmer Life Sciences, Boston, MA) using standard Fmoc solid-phase chemistry, as described previously (Adermann et al., 1998). The toxins were purified by HPLC, using a Vydac C_{18} column and analyzed by electrospray mass spectrometry (Adermann et al., 1998). Synthetic pardaxins were kindly provided by Dr. Knut Adermann (Institute for Peptide Research, Pharmaceuticals GmbH, Hannover, Germany). Botulinum toxin type C was kindly provided by Prof. DasGupta (Institute of Food Technology, Madison, WI). Botulinum toxin type F was purchased from Calbiochem (La Jolla, CA).

Cell Viability. Cytotoxicity was determined by trypan blue exclusion method. Cell death was also measured by lactate dehydrogenase release assay, as described previously (Abu-Raya et al., 1999). The concentration of PX was considered subcytotoxic when cell death was less than 10%.

PC-12 Pheochromocytoma Cell Cultures. PC-12 cells were grown in DMEM supplemented with 7% fetal calf serum, 7% horse serum, 100 μg/ml streptomycin, and 100 units/ml penicillin (Bloch-

Shilderman et al., 2001). The cultures were maintained in an incubator at 37°C in an atmosphere of 6% CO_2. The medium was changed twice weekly, and the cultures were split at a 1:6 ratio once a week. In all experiments, unless specified otherwise, the cells were plated on six-well plates coated with equal parts collagen (0.01 mg/ml collagen in 0.1 M acetic acid), poly(L-lysine) (0.01 mg/ml), and polyethylenimine (0.1 mg/ml) at a density of 2×10^6 cells/well in DMEM supplemented with serum and antibiotics as described above, and allowed to equilibrate overnight before the initiation of the experiments.

Arachidonic Acid Release. PC-12 cells were grown in six-well plates in DMEM with serum for 24 h at 37°C (Abu-Raya et al., 1999). The growth medium was then removed and replaced with serum-free DMEM to which [^{3}H]AA (0.5 μCi/ml) was added, and the plates were incubated for an additional 4 h. The medium (containing the nonincorporated isotope) was removed and the cells were washed three times with phosphate-buffered saline (PBS) containing Ca^{2+} buffer (138 mM NaCl, 8 mM Na_2HPO_4, 0.5 mM $MgCl_2$, and 0.9 mM $CaCl_2$, pH 7.4) or Ca^{2+}-free buffer (138 mM NaCl, 8 mM Na_2HPO_4, 0.5 mM $MgCl_2$, pH 7.4, and 1 mM EGTA). The rinsed cells were then incubated with 1 ml of Ca^{2+}-free PBS supplemented with 20 mM glucose and 1 mg/ml fatty acid-free BSA for 10 min at 37°C, to remove cell surface-associated radioactivity. The cultures were then exposed to different inhibitors for 45 min, at 37°C, followed by treatment with 5 μM PX for an additional 20 min. In general, PLA_2 inhibitors concentration was 25 μM, unless otherwise stated, to achieve maximal PLA_2 inhibition, as reflected from dose-response experiments and as also used by others (Balsinde and Dennis, 1997). Upon termination of the experiment, the medium was removed and centrifuged at 4°C for 10 min at 1000*g*. Aliquots (200 μl) were removed, 4 ml of scintillation fluid was added, and radioactivity was estimated in a β-scintillation analyzer. The amount of arachidonic acid released was expressed as cpm relative to the number of cells in the well of the tissue culture plate.

PGE_2 and 5-HETE Radioimmunoassay (RIA). PC-12 cells were grown in six-well plates in DMEM with serum for 24 h at 37°C. The growth medium was then removed and replaced with calcium- and serum-free DMEM. The cultures were then exposed to different inhibitors for 45 min, at 37°C, followed by treatment with 5 μM PX for an additional 20 min. Upon termination of the experiment, the medium was removed, centrifuged at 4°C for 10 min at 1000*g*, and aliquots were removed for RIAs of the 5-HETE products (Abu-Raya et al., 1999). Standard curves were generated for the respective eicosanoids. After an 18- to 24-h incubation of samples (or standard) with the appropriate antiserum and radioligands, free and bound compounds were separated by dextran coated with activated charcoal and the radioactivity was measured.

Catecholamine Release. Dopamine release from undifferentiated PC-12 cells and norepinephrine release from differentiated PC-12 cells incubated with nerve growth factor (NGF; 50 ng/ml) for 10 days were determined, with slight modifications, as described previously (Abu-Raya et al., 1999). Briefly, fresh DMEM was added, and the cells were allowed to equilibrate at 37°C for 30 min. The cells were then loaded with [^{3}H]dopamine or [^{3}H]norepinephrine (0.3–1 μCi/ml) and incubated for 2 h at 37°C. The medium was removed and the cells were washed once with serum-supplemented medium and twice with serum-free medium containing 1 mM ascorbic acid or calcium- (+1 mM EGTA) and serum-free medium containing 1 mM ascorbic acid. Fresh medium was added, and the cultures were preincubated with different inhibitors for 45 min in the presence (1.8 mM) or absence of calcium (+1 mM EGTA). PX (5 μM) was then added for 20 min. Basal release was measured in untreated cultures incubated for similar intervals at 37°C. Samples of 0.2 ml were removed from the medium, centrifuged for 10 min (1000*g*) to remove floating cells, and the radioactivity was measured. To measure total uptake, the cells were washed with PBS and dissolved in 1 ml of 0.5 N NaOH. Aliquots of 0.2 ml were measured for radioactivity. The data are presented as [^{3}H]dopamine release (cpm per 2×10^6 cells), calculated relative to the control.

Vesicle Fusion-Based ELISA. The procedure was essentially as described previously (Bloch-Shilderman et al., 2001). Briefly, PC-12 cells were grown in 48-well plates. Control (untreated) cells or cells pretreated with different inhibitors for 45 min were then exposed to 5 μM PX or PBS for an additional 20 min in the presence of polyclonal anti-synaptotagmin I antibody, which was raised against the N-terminal luminal 19 amino acids of synaptotagmin I. This SNARE protein is briefly exposed on the cell surface during the exocytotic process, enabling detection with the antibody. The optimal antibody concentration was 6 μg/ml. Upon termination of incubation, the cells were washed three times with PBS at 37°C, with an interval of 15 min between washes, and then fixed with 4% paraformaldehyde in PBS for an additional 20 min at room temperature. The cells were then washed once with 0.5 ml of PBS and twice with 0.5 ml of PBS containing 0.1% Triton X-100, and incubated with 3% H_2O_2 in PBS for 5 min. The cells were then incubated for 30 min at 25°C with blocking solution (5% normal goat serum, 2% BSA, and 0.1% Triton X-100 in PBS), and then with goat anti-rabbit antibody conjugated to peroxidase (0.35 ml, diluted 1:7500) for 1 h at 25°C. The cells were further washed three times with PBS containing 0.1% Triton X-100, and incubated with 1:1 Chromagen and substrate solution (150 μl) for 5 min. Every 10 s, 75-μl samples from each well were removed and mixed with termination solution (150 μl, 1 N sulfuric acid). The intensity of the blue color formed was monitored at $O.D._{450}$ nm with the aid of an ELISA reader, Kinetic analyzer V-MAX with SOFTmax attached software (Molecular Devices, Menlo Park, CA). The values obtained in control experiments with secondary antibody alone were used to subtract the background value. Staining with nonrelevant antibodies was included routinely. Total immunoreactivity was measured by fixing the cells and incubating with synaptotagmin I antibodies in blocking solution (0.2 μg/ml), followed by incubation with secondary antibody as described above.

Analysis of Core Complexes. The procedure was essentially as described previously (Otto and Jahn, 1997). Briefly, PC-12 cells were grown in T-200 flasks. On the day of the experiment, the cells were preincubated with different inhibitors for 45 min at 37°C in calcium-free (+1 mM EGTA) and serum-free medium, followed by a 20-min incubation with 5 μM PX. The cells were collected and centrifuged for 10 min (310*g*) and the supernatant was removed. Each sample pellet was processed in a glass homogenizer in 100 μl of buffer [0.05% Triton X-100, 0.3 M sucrose, 1 mM $MgCl_2$, 25 mM Tris-Cl, pH 8.3, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, and protease inhibitor cocktail 2 (1:100)]. Cell homogenates were then centrifuged for 3 min (1000*g*) at 4°C. The supernatants (100 μl) were collected and the volume was completed to 400 μl with 1% Triton X-100 in a calcium-free physiological buffer (118 mM NaCl, 2.4 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 10 mM HEPES, pH 7.4, 20 mM D-glucose, and 1.8 mM $CaCl_2$) and incubated with or without 10 mM *N*-ethylmaleimide for 1 h at 4°C on a rocking table. The samples were then centrifuged for 20 min (4000*g*) at 4°C. For immunoprecipitation, supernatants were incubated overnight with 0.01% protein G-Sepharose 4B and monoclonal anti-syntaxin antibody (HPC-1; 1:660) at 4°C with gentle rocking. Samples were centrifugated (1550*g*) three times for 3 min at 4°C, and washed with PBS. The samples were divided into two and were boiled for 10 min or incubated for 30 min at 37°C in sample buffer (0.125 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-β-mercaptoethanol, and 0.06% bromphenol blue). Afterward, aliquots (20 μl) were electrophoresed through SDS-polyacrylamide gel electrophoresis (12%), and transferred (in buffer containing 0.5 M $NaHCO_3$ and 150 mM Na_2CO_3) to a nitrocellulose membrane. The membranes were immunoblotted overnight at 4°C, using polyclonal antibodies against SNAP-25 and synaptobrevin. Immunoblots were reacted with peroxidase-conjugated secondary antibodies, and analyzed according to the Enhanced Chemoluminescence System (Amersham Biosciences). After visualization on film, they were quanti-

fied by densitometry, using NIH 159 software and the intensity index was recorded.

Extraction of Culture Medium for HPLC Analysis of 5-HETE. The procedure was essentially as described previously (Powell, 1982). Briefly, PC-12 cells were grown to confluence (2×10^7 cells) in T-800 flasks. The medium was removed and the cells were washed twice with calcium- (+1 mM EGTA) and serum-free medium containing 1 mM ascorbic acid, and left to equilibrate for 30 min. The cells were then preincubated with 25 μM BEL for 45 min, followed by treatment with 5 μM PX for an additional 20 min at 37°C. The medium was immediately collected and ethanol and propyl gallate were added to culture medium aliquots, to a final concentration of 15% (v/v) and 0.25 mM, respectively. The aliquots were then acidified to pH 3.0 with 1 N HCl before loading on prewashed (7 ml of methanol and 7 ml of water) octadecyl functionalized silica gel columns (2.2 g/column, trache suction sets; UnoPlast, Hudested, Denmark). The columns were eluted quickly and successively under N_2 pressure (10 psi) with 7 ml of 15% ethanol, 7 ml of water, 2 ml of petroleum ether, and finally, 10 ml of ethyl acetate. The ethyl acetate fraction was collected, evaporated to dryness under a stream of N_2, and the dry material was dissolved in 500 μl of ethyl acetate. The recovery of 5-HETE standards, added to fresh medium before extraction, was ~80%.

HPLC Analysis of 5-HETE. Extract aliquots (20 μl) were analyzed by reverse phase HPLC in an L6200 Merck-Hitachi chromatography system, using a Lichrosphere pr-18 pre column (5 μm, 4 × 4 mm) and a column (5 μm, 250 × 4 mm; Merck) connected to an L-4200 UV-visible detector (235 nm). Elution was allowed to proceed at a flow rate of 1.5 ml/min with a three-solvent isocratic and gradient mixture (solvent A, 0.01%, acetic acid; solvent B, acetonitrile; and solvent C, methanol) as follows: the initial solvent mixture was 33% A, 10% B, and 57% C. A convex gradient over 25 min was then followed by 10% B and 90% C. This was followed by an isocratic elution with 10% B and 90% C for an additional 10 min. The system was then regenerated over 10 min to the initial solvent ratio with a linear program. Using this program, 5-HETE was eluted at 16.9 min, as confirmed with pure 5-HETE standards as well as with an HETEs mixture for HPLC calibration.

Statistics. Statistical differences between band intensities were determined by analysis of variance ($p \leq 0.05$). The significance of the statistical differences between the results obtained from AA, PGE_2, dopamine, and norepinephrine release was analyzed by Kruskal-Wallis and Dunn 2 tests ($p \leq 0.05$). The Mann-Whitney test was used for statistical analysis of the HPLC results ($p \leq 0.05$).

Results

Pardaxin Stimulation of $cPLA_2$ and $iPLA_2$. Previous work (Bloch-Shilderman et al., 2001) indicated that PX both stimulates PLA_2 and activates MAPK. The latter effect requires the presence of extracellular calcium. To further investigate the mechanism involved in PX-induced stimulation of the AA cascade in PC-12 cells, inhibitors of PLA_2 and inhibitors of MAPK/ERK kinase were used. MAFP and $AACOCF_3$, known as $cPLA_2$ and $iPLA_2$ inhibitors, were used. PC-12 cultures were preincubated for 45 min with 25 μM $AACOCF_3$ and thereafter treated with 5 μM PX for additional 20 min (Table 1). As shown in Table 1, $AACOCF_3$ completely inhibited PX-induced AA release, and inhibited prostaglandin E_2 (PGE_2) production and dopamine release by ~70%. Similar results were obtained using MAFP (25 μM; data not shown). MAPK has been shown to activate $cPLA_2$ in a calcium-dependent manner (Lin et al., 1993). Therefore, we also examined the effect of MAPK/ERK kinase inhibitors on PX stimulation of the AA cascade. As seen in Fig. 1A, in the presence of extracellular calcium, 50 μM PD98059 completely blocked the release of [^{3}H]AA induced by PX, and inhibited by 82% the release of PGE_2 (Fig. 1B). Also, 30 μM UO126 inhibited by 62% the release of PGE_2 induced by PX (Fig. 1B). In the absence of extracellular calcium, PD98059 did not affect PX-induced release of AA (Fig. 1A) or PGE_2 (data not shown). These results suggest that in the presence of extracellular calcium, $cPLA_2$ activation is involved in PX stimulation of the AA cascade.

TABLE 1

Inhibitory effect of $cPLA_2$ inhibitor $AACOCF_3$ on PX-induced release of AA, PGE_2, and dopamine (DA) in the presence of extracellular calcium

PC-12 cells (2×10^6/well), labeled to steady state with [^{3}H]AA, [^{3}H]DA, or left unlabeled, were preincubated for 45 min with 25 μM $AACOCF_3$ or left untreated. Thereafter, cultures were treated for 20 min with 5 μM PX or left untreated (control) in calcium-containing medium (1.8 mM). Samples of the culture medium were removed to measure radioactivity or for RIA, as described under *Experimental Procedures*. The values are the mean ± S.E.M. of three independent experiments ($n = 3$ in each experiment).

Compound	[^{3}H]AA Release	PGE_2 Release	[^{3}H]DA Release
	cpm/2 × 10^6 cells	*pg/ml*	*cpm/2 × 10^6 cells*
PX	1862 ± 162	2600 ± 250	2470 ± 200
PX + $AACOCF_3$	758 ± 45**	780 ± 100*	1141 ± 110**

* $p < 0.05$, ** $p < 0.01$ compared with PX alone (the basal release was subtracted from all the values). The control, basal release of AA, PGE_2, and dopamine was 700 ± 75 cpm/2 × 10^6 cells, 10 pg/ml, and 650 ± 40 cpm/2 × 10^6 cells, respectively.

The ability of PX to stimulate the AA cascade in the absence of extracellular calcium and without any increase in cytosolic calcium (Abu-Raya et al., 1999) indicated the possibility of $iPLA_2$ involvement because under these conditions this is the only PLA_2 that can be activated (Balsinde and Dennis, 1996). To verify the involvement of $iPLA_2$ in PX-induced AA cascade we used $AACOCF_3$, MAFP, and BEL, which exhibits a predilection for inhibiting $iPLA_2$, and performed the experiments in the absence of extracellular calcium. As shown in Fig. 2A, in the absence of extracellular calcium, MAFP, $AACOCF_3$, and BEL inhibited by 80, 95, and 80%, respectively, the release of [^{3}H]AA induced by PX, and by 80, 85, and 69%, respectively, the release of PGE_2 (Fig. 2B). Because under these conditions $cPLA_2$ is not active (Lin et al., 1993) the inhibitory effect of these compounds results, presumably, from the blocking of $iPLA_2$. It is worth noting that 0.1 to 10 μM BEL inhibited by 60 to 70% the release of the AA and PGE_2 in PC-12 cells (data not shown). Also, the basal release of the AA and PGE_2 in PC-12 cells treated with these PLA_2 inhibitors was unaffected (data not shown). In the presence of extracellular calcium BEL markedly inhibited AA release induced by PX (Abu-Raya et al., 1998), indicating that also in the presence of extracellular calcium $iPLA_2$ is the main PLA_2 activated by PX. These results suggest the involvement of $iPLA_2$ in the PX-induced AA cascade, in the presence as well as in the absence of extracellular calcium.

Relationship between PX Stimulation of $iPLA_2$ and Exocytosis. To verify the involvement of $iPLA_2$ in PX-induced dopamine release, we examined the effect of MAFP, $AACOCF_3$, and BEL on PX-induced dopamine release in the absence of extracellular calcium. Under these conditions, most probably, $iPLA_2$ is the only PLA_2 that remains active. As can be seen in Fig. 3A, MAFP, $AACOCF_3$, and BEL inhibited by 40, 90, and 85% PX-induced [^{3}H]dopamine release, respectively. The inhibition of [^{3}H]dopamine release was dose-dependent (Fig. 3B). Also, in the presence of extracellular calcium, 25 μM BEL significantly inhibited the re-

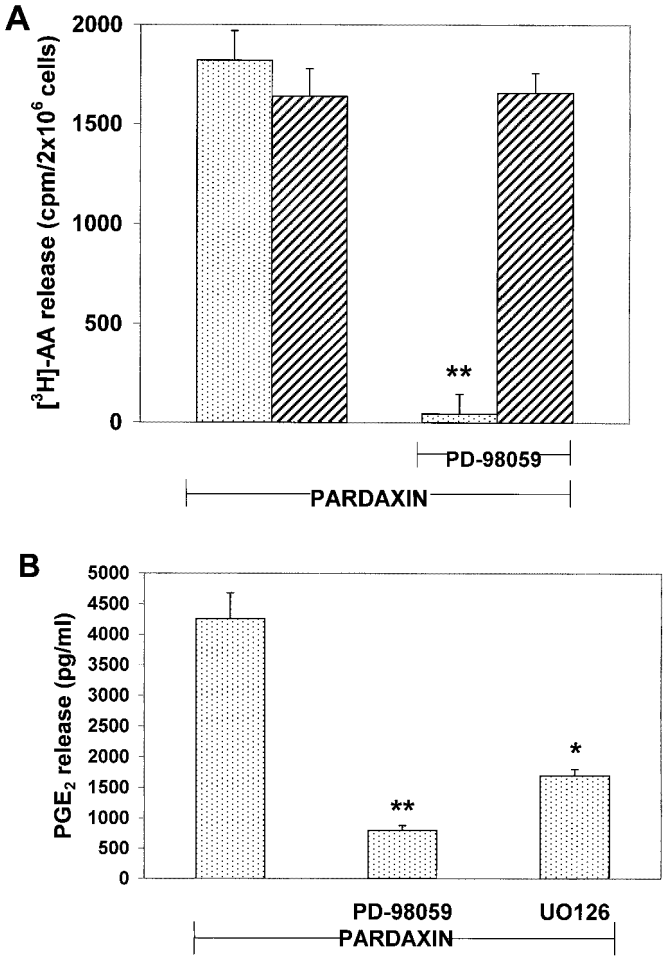

Fig. 1. MAPK/ERK kinase inhibitors block PX stimulation of the arachidonic acid cascade in the presence and absence of extracellular calcium in PC-12 cells. PC-12 cells (2×10^6/well), labeled to steady state with [^{3}H]AA (A), or unlabeled (B), were preincubated for 45 min with the MAPK/ERK kinase inhibitors PD98095 (50 μM) and UO126 (30 μM), or left untreated. Thereafter, cultures were treated for 20 min with 5 μM PX, in the presence (▨) or absence of calcium (▨) or left untreated. Samples of the culture medium were removed to measure radioactivity (A) or for RIA (B), as described under *Experimental Procedures*. The results are presented as the mean ± S.E.M. of three independent experiments ($n = 3$ in each experiment). Control values for basal release of AA (A) and PGE_2 (B) were 650 ± 30 cpm/2 $\times$ 10^6 cells and not detected, respectively. ⋆, $p < 0.05$ compared with PX alone (the basal release was subtracted from all values); ⋆⋆, $P < 0.01$ compared with PX alone (the basal value was subtracted from all values).

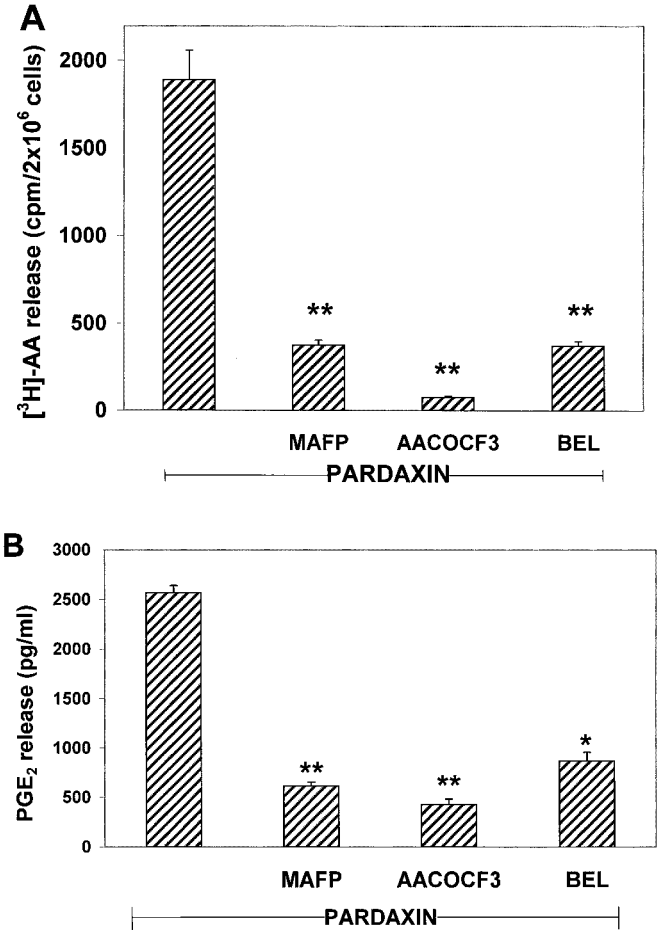

Fig. 2. $iPLA_2$ inhibitors block PX stimulation of the AA cascade in the absence of extracellular calcium. PC-12 cells (2×10^6/well), labeled to steady state with [^{3}H]AA (A), or unlabeled (B), were preincubated for 45 min with $iPLA_2$ inhibitors (25 μM), or left untreated (control). The cultures were then treated for 20 min with 5 μM PX or left untreated. Samples of the culture medium were removed to measure radioactivity (A) or for RIA (B), as described under *Experimental Procedures*. The results are the mean ± S.E.M. of three independent experiments ($n = 3$ in each experiment). Control basal release of AA (A) and PGE_2 (B) were 750 ± 30 cpm/2 $\times$ 10^6 cells and 10 pg/ml, respectively. ⋆, $P < 0.05$, ⋆⋆, $p < 0.01$ compared with PX alone (the basal release was subtracted from all values).

lease of dopamine induced by PX, although to a lesser extent (by 60%; data not shown). These findings suggest the involvement of $iPLA_2$ in PX-induced calcium-dependent and -independent dopamine release. To test whether under these conditions PX acts to release dopamine from the vesicular pool, we used a vesicle fusion-based ELISA, in which we quantified exocytotic vesicular release by the exposure of the luminal domain of synaptotagmin to an antibody present in the extracellular medium (Bloch-Shilderman et al., 2001). An antibody lacking a luminal epitope was used as control. As shown in Fig. 4, PX increased synaptotagmin exposure on the cell surface by about 260% over that of the control. PX-induced synaptotagmin exposure was blocked by 70% by the $iPLA_2$ inhibitors MAFP (25 μM), $AACOCF_3$ (25 μM), and BEL (25 μM). In a previous study, it was found that PX increased the release of AA metabolites such as PGE_2 and 5-HETE produced by cyclooxygenase and lipoxygenase pathways (Abu-Raya et al., 1999). It was observed that the lipoxygenase inhibitors AA861, esculetin, and nordihydroguaiaretic acid, but not the cyclooxygenase inhibitor indomethacin, blocked PX-induced dopamine release (Abu-Raya et al., 1999). Therefore, we investigated the effect of 5 μM nordihydroguaiaretic acid on PX-induced synaptotagmin exposure, and found that it was inhibited by 77% (Fig. 4). Under these conditions, using HPLC, we found that the basal release of 5-HETE was 12.9 ng/10^6 cells, which was increased by exposure to 6 μM PX to 32.25 ng/10^6 cells. BEL (25 μM) completely inhibited the PX-induced 5-HETE release. Therefore, it is reasonable to assume that lipoxygenase metabolites, such as 5-HETE, may be also involved in PX-induced exocytosis.

Involvement of $iPLA_2$ in PX-Induced SNARE Complex Formation. Neurotransmitter release involves assembly of a synaptic core complex, which constitutes the hub of a

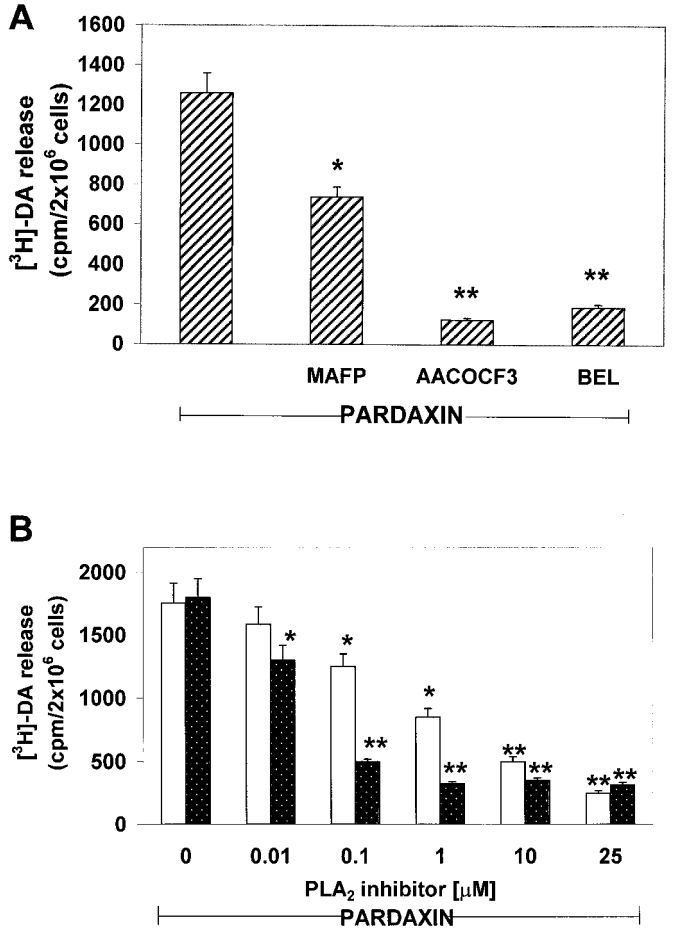

Fig. 3. iPLA$_2$ inhibitors block PX-induced dopamine (DA) release from PC-12 cells in the absence of extracellular calcium. A, comparison between iPLA$_2$ inhibitors. B, dose response of iPLA$_2$ inhibitors: AACOCF$_3$ (□) and BEL (▩). PC-12 cells (2×10^6/well), labeled to steady state with [^{3}H]dopamine, were preincubated for 45 min with 25 μM (A) increasing concentrations (0–25 μM; B) of the different compounds, or left untreated. The cultures were then treated for 20 min with 5 μM PX or left untreated. Samples of culture medium were removed to measure radioactivity, as described under *Experimental Procedures*. The results are presented as the mean ± S.E.M. of three independent experiments (n = 3 in each experiment). Control basal release of dopamine was 450 ± 50 cpm/2×10^6 cells (A) and 500 ± 35 cpm/2×10^6 cells (B). ⋆, $p < 0.05$, ⋆⋆, $p < 0.01$ compared with PX alone (the basal release was subtracted from all values).

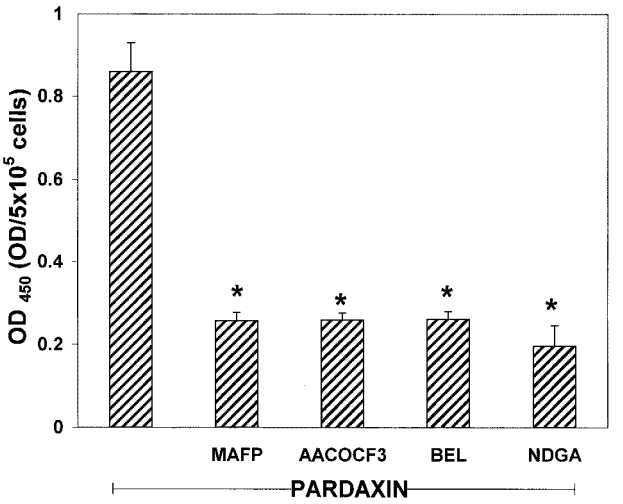

Fig. 4. Effect of AA cascade inhibitors on PX-induced exocytosis in the absence of extracellular calcium. Exocytosis was measured by vesicle fusion-based ELISA, as described under *Experimental Procedures*. Control, untreated cells, or cells pretreated for 45 min with different inhibitors (25 μM) were exposed to 5 μM PX for 20 min in the presence of anti-synaptotagmin I antibody. The cells were then washed, fixed, and incubated with blocking solution. Blue color formation was monitored at O.D.$_{450}$ nm with the aid of an ELISA reader. The values presented are the mean ± S.E.M. of three independent experiments (n = 6 in each experiment). Control basal release was 0.250 ± 0.020 O.D./5×10^5 cells. ⋆, $p < 0.05$ compared with PX alone (the basal release was subtracted from all values).

series of protein interactions occurring immediately upstream or downstream of calcium-dependent exocytosis (Sudhof, 1995). The main molecules participating in exocytosis are the SNAREs and NEM-sensitive fusion protein (NSF). The latter is an ATPase that dissociates low-energy SNARE complexes. NSF is inhibited by *N*-ethylmaleimide and consequently primes SNARE proteins for fusion (Morgan and Burgoyne, 1995). In addition, NSF plays a major role in disassembly of the SNARE complex fusion. Ternary synaptic complexes, composed of SNAP-25 and syntaxin, display temperature-dependent resistance to dissociation by SDS, a property that can be used to assay complexes preexisting in membranes (Otto et al., 1997). We examined the sensitivity of PX-induced SNARE core-complex formation to the iPLA$_2$ inhibitor BEL. Western blots of PC-12 cell homogenates probed with antibodies against VAMP, SNAP-25, or syntaxin (Fig. 5) revealed high molecular mass complexes (100 and 70 kDa) with identical electrophoretic mobility that were stable in SDS at 37°C but dissociated at 100°C (Fig. 5). SNARE core-complex formation is estimated by the accumulation of a 70-kDa tetrameric complex and the appearance of a 100-kDa complex (Fig. 5). In control untreated cells, we observed a basal level of core complex (Fig. 5, A and C) at 37°C, which represents the steady state of postfusion and primed vesicles. Under these conditions and by using fresh cells, NSF is fully active. In the presence of *N*-ethylmaleimide, which stabilizes the SNARE complexes by preventing NSF-ATPase activity, the amount of 100- and 70-kDa protein bands was increased by 30% (Fig. 5, A and C). Disassembly of SNARE complexes by heating at 95°C (Fig. 5A, bottom) caused a breakdown of both 100- and 70-kDa protein bands and recovery of the individual proteins in bands corresponding to their molecular mass (19 kDa). Upon treating the cells with 6 μM PX, both in the absence (Fig. 5A) and presence (Fig. 5B) of *N*-ethylmaleimide at 37°C, an increase in SNARE core complex formation was observed (relative intensity 2 and 2.5, respectively; Fig. 5C). Preincubation of the cells with 25 μM BEL inhibited PX-induced SNARE core-complex formation to control values, both in the presence and absence of *N*-ethylmaleimide (Fig. 5, A–C). Similar experiments were performed in the presence of calcium to evaluate the extent of core-complex formation as a result of PX application. Our results indicated that under calcium-containing medium PX-induced core-complex formation was increased only by about 20% in comparison with that of calcium-depleted medium. These data are in accordance with the main role of PX in induction of calcium-independent exocytosis.

Additional evidence for linking the PX-induced AA cascade to vesicle fusion is presented in Fig. 6 and Table 2. We treated PC-12 cells with botulinum toxin type C1 (BoNT/C1) to cleave syntaxin and SNAP-25 and type botulinum toxin F

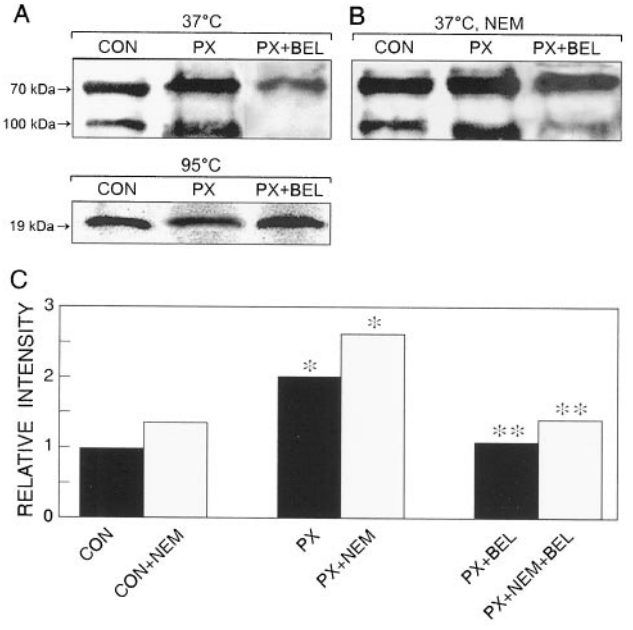

Fig. 5. BEL inhibits PX-induced SNARE-complex formation, in the absence of extracellular calcium. PC-12 cells grown to confluence (1×10^7 cells), untreated or pretreated with BEL (25 μM) for 45 min, were incubated for 20 min with 5 μM PX or left untreated (CON, A). NEM (2 mM) was added 10 min before termination of the experiment or after the cells were collected and homogenized (10 mM) (B). The supernatants were collected by centrifugation and their protein content was measured, as described under *Experimental Procedures*. Similar protein aliquots were used for immunoprecipitation with anti-syntaxin antibody (1:660). The immunoprecipitates were electrophoresed on 12% SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes, which were immunoblotted with anti-synaptobrevin antibodies. Bands of proteins visualized on film were quantified by densitometry. The relative density values of the bands of representative experiments are presented (C). The location of accumulated tetrameric complexes (70 kDa) and of complexes of higher molecular masses (100 kDa) is indicated by arrows. The recovery of individual proteins in bands corresponding to their molecular masses, after core disassembly at 95°C, is indicated by arrows (19 kDa; A, bottom). *, $p < 0.05$ compared with control; **$p < 0.05$ compared with PX alone (the basal release was subtracted from all values).

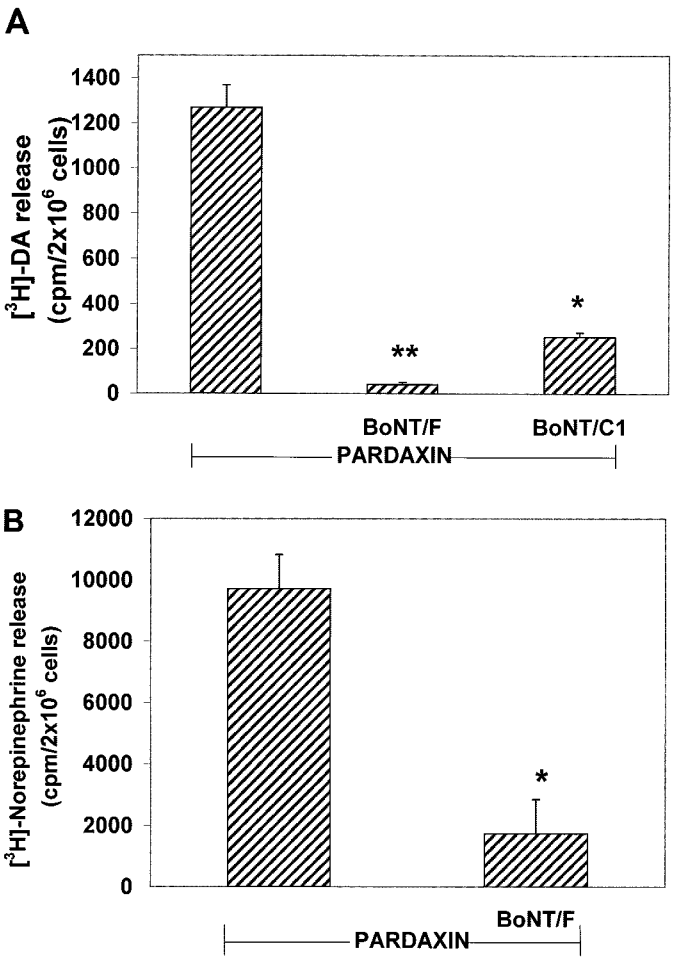

Fig. 6. Botulinum toxins C1 and F inhibit PX-induced catecholamine release in the absence of extracellular calcium. Undifferentiated PC-12 cells (2×10^6/well) (A) or NGF-differentiated cells (2×10^5, 50 ng/ml, 10-day treatment) (B) were labeled to steady state with [^{3}H]dopamine (DA) (A) or with [^{3}H]norepinephrine (B). The cells were left untreated (control) or preincubated for 2 h with botulinum toxins (2 nM), followed by treatment for 20 min with 6 μM PX. Samples of culture medium were removed to measure radioactivity, as described under *Experimental Procedures*. The results are presented as the mean ± S.E.M. of three independent experiments ($n = 3$ in each experiment). Control basal release of dopamine and norepinephrine were 450 ± 50 cpm/2 $\times$ 10^6 cells (A) and 2800 ± 100 cpm/2 $\times$ 10^6 cells (B), respectively. ⋆, $p < 0.05$, ⋆⋆, $p < 0.01$ compared with PX alone (the basal release was subtracted from all values).

(BoNT/F) to cleave VAMP (Montecucco and Schiavo, 1995). As can be seen in Fig. 6A, in the absence of extracellular calcium, BoNT/F and C1 blocked by 100 and 80%, respectively, the PX-induced release of dopamine. Similar results were obtained in experiments with NGF-differentiated PC-12 cells (Fig. 6B). Under the same conditions, BoNT/F and C1 significantly inhibited PX-induced release of AA and PGE_2, as shown in Table 2. Cumulatively, these results suggest the involvement of the AA cascade in PX-induced exocytosis.

Discussion

In the present study, we show that PX-induced exocytosis is a calcium-independent process. This was coupled to the stimulation of the AA cascade, indicating that $iPLA_2$ is the main enzyme involved in this calcium-independent process, whereas $cPLA_2$ is involved in the calcium-dependent process. This conclusion is supported by the following findings: 1) PX-induced calcium-independent activation of the AA cascade and dopamine release were markedly inhibited by the $iPLA_2$ inhibitors MAFP, $AACOCF_3$, and BEL; 2) PX-induced calcium-independent SNARE core-complex formation was temporally correlated with the PX-induced AA cascade and dopamine release, and was also inhibited by BEL; 3) In the presence of extracellular calcium the dual $cPLA_2$/$iPLA_2$ inhibitors MAFP and $AACOCF_3$ markedly blocked the PX-induced release of AA, its metabolites, and dopamine; and 4) The MAPK/ERK kinase inhibitor PD98059, which was found to inhibit PX activation of ERKs (Bloch-Shilderman et al., 2001), and UO126 markedly inhibited PX induction of the AA cascade and dopamine release in the presence of extracellular calcium, but not in its absence. To the best of our knowledge, this is the first report of the involvement of $iPLA_2$ in PX-induced calcium-independent exocytosis mediated by SNARE proteins. Our results are consistent with other studies suggesting a role for PLA_2 in neurotransmitter release in the nervous system (Frye and Holz, 1984; Ray et al., 1993, 1999), and support the hypothesis that both calcium-dependent exocytosis and calcium-independent exocytosis occur via

TABLE 2

Inhibitory effect of botulinum toxins C1 and F on PX activation of the AA cascade in the absence of extracellular calcium

PC-12 cells (2×10^6/well), labeled to steady state with [^{3}H]AA or left unlabeled, were preincubated for 2 h with BoNT F and C1 (2 nM) or left untreated (control). The cultures were then treated for 20 min with 5 μM PX or left untreated (control). Samples of the culture medium were removed to measure radioactivity or for RIA, as described under *Experimental Procedures*. The values are the mean ± S.E.M. of three independent experiments (n = 3 in each experiment).

Compound	[^{3}H]AA Release	PGE_2 Release
	cpm/2 × 10^6 cells	*pg/ml*
PX	1578 ± 140	2600 ± 250
PX + BoNT F	760 ± 50**	598 ± 100*
PX + BoNT C1	915 ± 90*	1222 ± 110*

* $p < 0.05$, ** $p < 0.01$ compared with PX alone (the basal release was subtracted from all values). The control basal release of AA (100%) and PGE_2 was 670 ± 40 cpm/2×10^6 cells and 10 pg/ml, respectively.

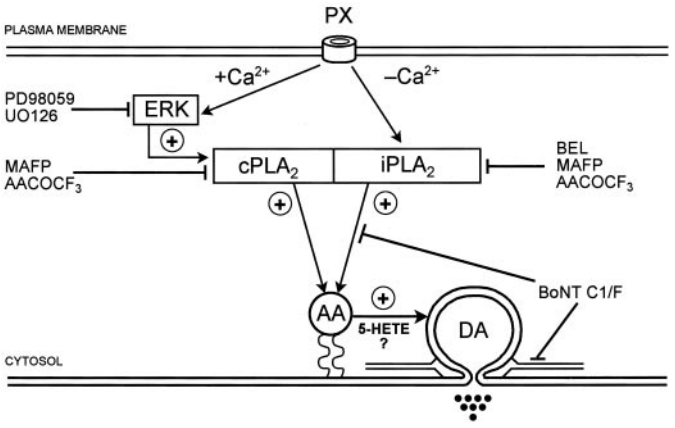

Fig. 7. Proposed model for PX-induced exocytosis, with emphasis on the AA pathway. The role of PLA_2s in PX-induced dopamine (DA) release in the presence and absence of extracellular calcium. Arrows indicate the flow of enzymatic pathways. $+Ca^{2+}$, a calcium-activated pathway in calcium-containing medium. $-Ca^{2+}$, a calcium-independent pathway, in the absence of extracellular calcium. ⊕, stimulatory effect. ⊥, inhibitory effect.

a SNARE-dependent mechanism (Glenn and Burgoyne, 1996).

We assessed the specific PLA_2 isoform involved in this process by using chemical inhibitors. Both $AACOCF_3$ and MAFP, which were designed as site-directed $cPLA_2$ inhibitors (Balsinde and Dennis, 1997), also strongly inhibit $iPLA_2$; therefore, they lack the required selectivity. To overcome this difficulty we omitted extracellular calcium and used a fairly selective $iPLA_2$ inhibitor, BEL. Under these conditions we ruled out the contribution of calcium-dependent PLA_2s. Therefore, the inhibition of the effect of PX under these conditions strongly suggests the involvement of $iPLA_2$ in PX action. BEL is also known to inhibit other important effectors in signal transduction, e.g., phosphatidate phosphohydrolase, which may be responsible for the AA release (Balboa et al., 1998). However, this mechanism did not play a role in our experiments because we previously demonstrated that this enzyme is not involved in AA release by PX (Abu-Raya et al., 1998). Thus, our present findings indicate that in the presence of extracellular calcium both $cPLA_2$ and $iPLA_2$ are involved in PX-induced AA cascade, whereas in the absence of extracellular calcium, the only phospholipase A_2 activated by PX is $iPLA_2$.

It was already shown in neuronal preparations that PX induction of neurotransmitter release occurs via exocytosis (Lazarovici and Lelkes, 1992; Arribas et al., 1993). The novel finding is that in the absence of extracellular calcium, and without any increase in the cytosolic calcium level (Lazarovici and Lelkes, 1992), PX stimulates exocytosis, involving the formation of SNARE core complexes, the ubiquitous physiological system known in all cells (Sudhof, 1995), and enables a massive release (about 3-fold of the basal level; Fig. 3). Furthermore, cleavage of the SNARE proteins by botulinum toxins (Montecucco and Schiavo, 1995) blocked the catecholamine release induced by PX (Fig. 6), indicating the sensitivity of the PX effect to these toxins, further supporting the essential role of SNAREs in exocytosis. Furthermore, we provide evidence (Figs. 5 and 6) that calcium-independent exocytosis depends on SNARE complex formation and thus rely on vesicle fusion. In this study PX-induced SNARE core-complex formation was calcium-independent, a unique property of this neurotoxin. Although the basic role of calcium in neurotransmitter release is well established (Burgoyne and Morgan, 1995), the precise event(s) in which this second messenger is involved in exocytosis is not known. Recent studies reassure the involvement of calcium in the last steps of exocytosis (Chen et al., 1999), but several other studies reported calcium-independent neurotransmitter release (Lonart and Zigmond, 1991; Bauerfeind et al., 1995). Because SNARE core-complex assembly is a prerequisite for exocytosis (Lonart and Sudhof, 2000), it is reasonable to suggest that its induction by PX, independently of calcium, reflects the involvement of other mediators in the process of exocytosis. Such mediators could include AA itself, which is a known fusogen (Ray et al., 1993), or certain derived eicosanoids, such as lipoxygenase metabolites (Abu-Raya et al., 1999), and/or other lipid mediators. It is tempting to propose that PX-induced SNARE core-complex formation involves lipid second messengers directly or indirectly and thus enables calcium-independent exocytosis. Our findings indicate that $iPLA_2$ is a likely candidate, and thus suggest for this enzyme a role in the release of AA, as proposed previously (Balsinde et al., 1999; Ramanadham et al., 1999). Several studies reported the involvement of phospholipases A_2 in calcium-dependent exocytosis (Ray et al., 1993, 1997; Yang et al., 1994; Kudo et al., 1996; Matsuzawa et al., 1996). However, PLA_2 may also be involved in calcium-independent exocytosis. Indeed, Nishio et al. (1996) showed calcium-independent fusion of synaptic vesicles in PLA_2-treated presynaptic membranes in vitro. Karly et al. (1990) documented the fusion of neurotransmitter vesicles with target membrane upon exposure to PLA_2. The involvement of $iPLA_2$ in calcium-independent secretion was recently reported by Ramanadham et al. (1999), supporting our present finding of $iPLA_2$ involvement in exocytosis. Cumulatively, these findings indicate an important role for $iPLA_2$ in PX-induced exocytosis, independently of calcium. In the present study BEL also inhibited PX-induced dopamine release under physiological conditions (in the presence of extracellular calcium), suggesting again $iPLA_2$ involvement in secretion as previously mentioned for insulin secretion (Ramanadham et al., 1999). This finding indicates a physiological role of $iPLA_2$ in PX-induced dopamine release.

As mentioned previously, $cPLA_2$s are calcium-dependent (μM range) enzymes, which are translocated to the plasma membrane upon MAPK phosphorylation (Lin et al., 1993).

MAPKs/ERKs are a family of protein-serine/threonine kinases that are activated by an upstream activator kinase, MAPK/ERK kinase, which is selectively blocked by PD98059 and UO126 (Alessi et al., 1995; Favata et al., 1998). $cPLA_2$s have been identified in a variety of cells and are involved in the regulation of AA release and signaling processes (Balsinde et al., 1999). We now show that MAFP and AA-$COCF_3$ markedly inhibit PX-induced release of AA and PGE_2 (Table 1), and that PD98059 and UO126 significantly inhibit PX-induced release of AA and PGE_2 in the presence, but not in the absence, of extracellular calcium (Fig. 1). We have previously found in time-course experiments that PX, at nontoxic concentrations, stimulated ERK1 and ERK2 within 5 to 15 min, measured with a dual phospho-ERK antibody (Bloch-Shilderman et al., 2001). This activity of ERKs in PC-12 cells was obtained only in calcium-containing medium and was completely blocked by 50 μM PD98059 (Bloch-Shilderman et al., 2001). Because PX was characterized as a pore-forming ionophore toxin, it is reasonable to assume that ERK1 and ERK2 stimulation by PX is due to its ability to increase the level of intracellular calcium, as was also described for other ionophores (Atherfold et al., 1999). Taken together, these findings suggest that in the presence of extracellular calcium, $cPLA_2$ is involved in the PX-induced AA cascade.

In addition to the effect of botulinum toxin (BoNT/F and C1) on the exocytotic process (evident from the inhibition of catecholamine release; Fig. 6) our results, as well as those reported by Ray et al. (1999), suggest an inhibitory effect (direct?) of botulinum toxin on PLA_2 itself: in the report of Ray et al. (1999) inhibition of calcium-dependent PLA_2 and in our experiments (Table 2) inhibition of calcium-independent PLA_2.

According to the results presented in this study, we propose a model for PX action (Fig. 7): In the presence of extracellular calcium, PX most probably stimulates all PLA_2 isoforms. The stimulation of $cPLA_2$ by PX may explain the contribution of AA to PX-induced catecholamine release when calcium is present in the medium. Also, in the presence of extracellular calcium PX stimulated $sPLA_2$ (S. Yedgar and S. Abu-Raya, personal communication), supporting previous finding demonstrating the involvement of $sPLA_2$ in the AA cascade and catecholamine secretion by PC-12 cells (Matsuzawa et al., 1996). In the absence of extracellular calcium PX did not activate ERK (Bloch-Shilderman et al., 2001); therefore, $cPLA_2$ was not activated. PX activates $iPLA_2$ (BoTX/F- and C1-sensitive), which leads to AA release and the generation of eicosanoids such as 5-HETE. These metabolites conceivably induce the assembly of the SNARE core complex, leading to exocytotic neurotransmitter release.

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