Involvement of Extracellular Signal-Regulated Kinase (ERK) in Pardaxin-Induced Dopamine Release from PC12 Cells

EUGENIA BLOCH-SHILDERMAN, HAO JIANG, SALEH ABU-RAYA, MICHAL LINIAL, and PHILIP LAZAROVICI

Department of Pharmacology and Experimental Therapeutics, School of Pharmacy, Faculty of Medicine (E.B.-S., S.A.-R., P.L.), and Department of Biological Chemistry, Life Sciences Institute (M.L.), The Hebrew University of Jerusalem, Jerusalem, Israel; William T. Gossett Neurology Laboratories, Henry Ford Health Sciences Center, and John D. Dingell Veterans Affairs Medical Center, Detroit, Michigan (H.J.)

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ABSTRACT

Pardaxin (PX), an ionophore-peptide neurotoxin isolated from the fish Pardachirus marmoratus, induces neurotransmitter release from neuronal preparations by both calcium-dependent and calcium-independent mechanisms. The aim of the present study was to investigate the role of extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) in pardaxin-induced dopamine (DA) release. The experiments were performed on variants of the PC12 cell line, an established cellular model for investigating DA release. Time course experiments indicated that PX, at nontoxic concentrations, stimulated ERK1 and ERK2 within 5 to 15 min, measured with a dual phospho-ERK antibody. PX stimulation of ERK activity was calcium (Ca$^{2+}$)-dependent and followed by ERK translocation to the nucleus. This effect was temporally related to PX-induced exocytosis, and measured by [$^{3}$H]dopamine release as well as by a vesicle fusion-based enzyme-linked immunosorbent assay. Blocking ERK activity with the specific mitogen-activated protein kinase inhibitors PD98059 (50 μM for 45 min) and UO126 (30 μM for 30 min) inhibited PX-induced exocytosis in the presence but not in the absence of extracellular Ca$^{2+}$. These results suggest the essential role of ERKs in PX-induced DA release under physiological conditions and support the hypothesis that ERKs are involved in regulating exocytosis.

Pardaxin is an amphipathic polypeptide neurotoxin composed of 33 amino acids, which was isolated from the Red Sea sole, Pardachirus marmoratus (Lazarovici et al., 1986). PX belongs to a family of five PX isoforms isolated from Pardachirus species living in the Pacific Ocean and the Red Sea (Adermann et al., 1998). In the present study we used mainly Asp-31-PX (P5) due to its lower cytotoxicity compared with that of Gly-31-PX (P4) (Adermann et al., 1998). Pardaxins, which have been shown to form voltage-dependent pores in liposomes (Loew et al., 1985; Lazarovici et al., 1986) and artificial lipid membranes (Lazarovici et al., 1992; Shi et al., 1995), act as polypeptide ionophores. They are considered unique pharmacological tools for studying neurotransmitter release (Lazarovici, 1994; Bloch-Shilderman et al., 1997), based on their ability to stimulate exocytosis in different neuronal systems, including brain slices (Wang and Friedman, 1986), neuromuscular junction (Renner et al., 1987), neurosecretory chromaffin cells (Lazarovici and Lelkes, 1992), and synaptosomes (Arribas et al., 1993), by both Ca$^{2+}$-dependent and Ca$^{2+}$-independent mechanisms (Lazarovici and Lelkes, 1992; Abu-Ray a et al., 1999). PX is thought to act by insertion into the neuronal plasma membrane, leading to the opening of poorly selective cation channels, culminating in depolarization, Ca$^{2+}$ entry, and neurotransmitter release (Lazarovici and Lelkes, 1992; Abu-Ray a et al., 1999). In an attempt to elucidate the signal transduction pathways involved in PX-induced DA release we recently showed that PX stimulates the arachidonic acid cascade in a Ca$^{2+}$-dependent and Ca$^{2+}$-independent manner in PC12 cells (Abu-Ray a et al., 1998). A direct relationship was also proposed between PX-stimulation of DA release and the arachidonic acid cascade (Abu-Ray a et al., 1999).

An additional signal transduction circuit that may contribute to PX-induced DA release is the Ras-mitogen-activated protein kinase (MAPK) pathway (Blenis, 1993). MAPKs/ERKs are a family of protein-serine/threonine kinases with a diverse array of cellular targets, suggesting that they are key regulators of many cellular responses. They are activated by a wide variety of extracellular stimuli affecting eukaryotic cells, including hormones, growth factors, radicals, mitogens, and synaptosomes (Arribas et al., 1993), by both Ca$^{2+}$-dependent and Ca$^{2+}$-independent mechanisms (Lazarovici and Lelkes, 1992; Abu-Ray a et al., 1999). PX is thought to act by insertion into the neuronal plasma membrane, leading to the opening of poorly selective cation channels, culminating in depolarization, Ca$^{2+}$ entry, and neurotransmitter release (Lazarovici and Lelkes, 1992; Abu-Ray a et al., 1999). In an attempt to elucidate the signal transduction pathways involved in PX-induced DA release we recently showed that PX stimulates the arachidonic acid cascade in a Ca$^{2+}$-dependent and Ca$^{2+}$-independent manner in PC12 cells (Abu-Ray a et al., 1998). A direct relationship was also proposed between PX-stimulation of DA release and the arachidonic acid cascade (Abu-Ray a et al., 1999).

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UV, and toxins (Force and Bonventre, 1998). These kinases require phosphorylation of both the serine/threonine and tyrosine residues in the catalytic domain for activation and to be translocated into the nucleus (Treisman, 1996). The ERKs are activated by an upstream activator kinase, MEK (MAPK kinase) (Blenis, 1993), which is selectively blocked by PD98059 and U0126 inhibitors (Alessi et al., 1995; Favata et al., 1998). The potential role of ERKs in secretagouge-induced norepinephrine release from bovine adrenal chromaffin cells was recently proposed (Cox et al., 1996; Cox and Parsons, 1997). However, the basic regulatory role of ERKs and their substrates involved in neurotransmitter release have not been fully characterized.

The aim of the present study was to assess the role of ERKs in regulating PX-induced DA release from PC12 cells. Our results indicate that PX stimulates Ca2+-dependent ERK activity in PC12 cells. This process is essential to PX-induced DA release in Ca2+-containing medium. Our findings support the importance of ERKs in neurotransmitter release.

**Experimental Procedures**

**Materials**

[^3H]Dopamine (47 Ci/mmol), 45Ca2+ (5–50 mCi/mg), and [y-32P]ATP were purchased from Amersham, Arlington Heights, IL; HEPES, myelin basic protein (MBP), trypsin blue, sorbitol, bovine serum albumin, EGTA, MgCl2, CaCl2, NaCl, NaOH, Triton X-100, glycerol, dithiothreitol, aprotinin, leupeptin, rabbit protein kinase inhibitor, sodium vanadate, phenylmethylsulfonyl fluoride, b-mercaptoethanol, bromphenol blue, ATP, SDS, Nonidet P-40, choline chloride, anti-synaptotagmin antibody, and poly(t-lysine) were purchased from Sigma Chemical Co., St. Louis, MO; methanol, KCl, and ascorbic acid were purchased from Merck, Darmstadt, Germany; PD98059 was purchased from BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA; U0126 was purchased from Promega, Madison, WI; anti-phospho ERK and anti-pan ERK antibodies were a generous gift from Dr. Erik Schaefer, QC8, Hopkinson, MA; and nerve growth factor (NGF) was kindly provided by Alomone Labs, Jerusalem, Israel. DMEM, horse and calf serum, antibiotics, normal goat serum, and rat tail type 1 collagen were purchased from Alomone Labs, Jerusalem, Israel. DMEM was added and the cells were allowed to equilibrate at 37°C for 30 min. The cells were then loaded with [3H]dopamine (0.3–1 uCi/ml) for 12 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. Fresh medium was added, and the cultures were incubated with PX (4 uM) or KCl (80 mM) in the presence (1.8 mM) or absence of Ca2+ (+1 mM EGTA) for 20 min. Basal release was measured in cultures incubated for similar intervals at 37°C and left untreated. Samples of 0.2 ml were removed from the medium, centrifuged for 10 min (1000g) to remove floating cells, and the radioactivity was measured. To measure total radioactivity, the cells were washed with PBS and dissolved in 1 ml of 0.5 N NaOH and 0.2-ml aliquots were measured for radioactivity.

The data are presented as [3H]dopamine release, calculated as percentage of control.

**Vesicle Fusion-Based ELISA**

The protocol followed was essentially as previously described (Parnas and Linial, 1998). Briefly, PC12 cells were grown on 48-well plates. Control (untreated) cells or cells pretreated with PD98059 for 45 min were further exposed to PX or PBS for an additional 20 min in the presence of polyonal anti-synaptotagmin I antibody, which was raised against the N-terminal luminal 19 amino acids of synaptotagmin I. This soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor (SNARE) is briefly exposed on the cell surface during the exocytotic process (Parnas and Linial, 1998), enabling detection with the antibody. We optimized the antibody concentration to 6 ug/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 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% H2O2 in PBS for 5 min. The cells were then incubated for 30 min at 25°C with blocking solution (5% normal goat serum, 2% bovine serum albumin, 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. Cells were further washed three times with PBS containing 0.1% Triton X-100. After washing, the cells were incubated with 1:1 chromagen and substrate solution (150 uL) for 5 min. Every 10 s, 75-µl samples from each well were removed and mixed with the stop solution (150 uL, 1 N sulfuric acid). The intensity of the blue color formed was monitored at OD450 nm with the aid of an ELISA reader-Kinetic analyser V-MAX with the attached software SOFMax (Molecular Devices Corporation, Menlo Park, CA). Control experiments were performed to ensure that the antibody was working. Normal rabbit IgG was used as control to confirm the specificity of the antibody. A positive control was included to ensure that there was no cross-reactivity of the antibody with the attached substrate.
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.

**Calcium Uptake**

The procedure is described in detail in Jiang et al. (1997). Briefly, cells were grown in six-well plates in DMEM containing 10% fetal bovine serum. After 24 h at 37°C, cells were washed once with Ca²⁺ and serum/antibiotic-free medium and incubated in the same medium for 30 min. ⁴⁵Ca²⁺ (1 μCi) was then added to each well along with PX (4 μM) or KCl (80 mM) and the cells were incubated at 37°C for an additional 4 min. The medium was rapidly aspirated, and the cells were washed twice with 3 ml of wash buffer, pH 7.2, containing 20 mM HEPES, 50 mM choline chloride, and 2.5 mM EGTA. The cells were lysed in 0.5 ml of NaOH for 2 h. A 0.4-ml sample of the cell lysate was used to estimate the cell-associated radioactivity and 0.05 ml was used for protein determination. The final counts were normalized per milligram of protein (cpm/mg of protein) and the data are presented as percentage of control.

**ERK Assays**

Detection of Activated ERKs in PC12 Cell Extracts by Western Blotting Using Anti-Phospho ERK Polyclonal Antibodies. Activation of MAPKs/ERKs requires that these enzymes are dually phosphorylated by MEK (Blenis, 1993) on both the Thr and the Tyr residues in the Thr-Glu-Tyr consensus sequence within the catalytic core of the enzyme (Payne et al., 1991). The phospho-ERK antibody (anti-active ERK) used in the present study was developed against a dually phosphorylated synthetic peptide encompassing residues Thr-183 and Tyr-185 of p42/MAPK2/erk2, corresponding to the active form of the ERK enzymes (Khokhlatchev et al., 1997). The antibody was purified, using a negative adsorption step to remove antibody recognizing the nonphosphopeptide, followed by positive selection-affinity purification with the dually phosphorylated peptide to select for antibody preferentially recognizing ERK1 and ERK2. PC12 cells were treated with 50 ng/ml NGF for 5 min, or with 0.5 M sorbitol for 5 min or PX for 15 min, unless otherwise indicated, or left untreated. The cells were lysed and aliquots (50 μg) of each extract were analyzed by SDS-PAGE (10% gel, under reducing conditions) and transferred to a nitrocellulose membrane. The membranes were probed with the indicated anti-phospho antibody, which recognizes the active (phosphorylated) forms of ERKs or with anti-pan ERK antibody, a polyclonal antibody that recognizes the inactive (nonphosphorylated) form of ERKs, at a dilution of 1:10,000 (Lazarovici et al., 1998).

**Measurement of ERK Activity, Using MBP as Substrate.** In another approach we measured ERK activity by protein phosphorylation assay, using the substrate MBP. MBP serves as a general nonselective substrate for a variety of protein kinases (Chan and Lazarovici, 1987). The first step was the immunoprecipitation of ERKs from the cell lysates, using anti-ERK polyclonal antibody (at a dilution of 1:200 for 2-h incubation at 37°C with continuous agitation). Thereafter, triple lysates of the various samples were incubated with washed protein A agarose (50-μl suspension) for additional 2-h incubation at 4°C. The immunoprecipitated ERK, bound to protein A agarose was washed twice with 0.5 ml of kinase assay buffer. In the second step, the washed immunoprecipitated ERK, bound to protein A agarose, was suspended in 30 μl of kinase assay buffer (7.5 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 2.5 μM protein kinase A inhibitor peptide PKI(6-22)-amide, 225 μM cold ATP, 25 μCi of [γ-³²P]-ATP, and 500 μg/ml MBP), and incubated for 30 min at room temperature. The reaction was terminated by the addition of 30 μl of SDS sample buffer, heated for 5 min and electrotransferred on SDS-PAGE. Phosphorylated MBP was visualized by autoradiography (XAR film; Eastman Kodak, Rochester, NY). The bands were quantified with a laser scanner.

Immunoblotting was performed as previously described (Lazarovici et al., 1998). Briefly, cells were plated on 10-cm Petri dishes at 20 h before the experiment. After treatment with PX or other reagents, triple cultures were washed twice with Tris-buffered saline (20 mM Tris-HCl, pH 8.0, 137 mM NaCl) and then treated with lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl, 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit/ml aprotinin, 20 mM leupeptin, and 1 mM sodium vandate, at 4°C for 20 min. Insoluble material was removed by centrifugation for 10 min at 12,000g. Triplicate lysate aliquots were diluted in SDS sample buffer (0.06 mM Tris-HCl, pH 6.8, 12.5% glycerol, 1.25% SDS, 5% β-mercaptoethanol, 0.002% bromphenol blue), boiled for 5 min in SDS sample buffer, and subsequently electrophoresed through 7.5% SDS-PAGE. The proteins on the gel were electrotransferred to nitrocellulose. Blots were probed overnight at 4°C with the different anti-ERK antibodies and analyzed, using the enhanced chemiluminescence system and horseradish peroxidase-conjugated secondary antibody. After visualization on film, they were quantified by densitometry.

**Visualization of ERK by Confocal Laser Scanning Fluorescence Microscopy**

PC12 cells were plated onto collagen- and poly(lysine)-coated two-well chambers (Nalgene) 1 day before each experiment. The cells were washed with PBS and then incubated for 2 h at 4°C with rabbit anti-phospho ERK antibody diluted 1:10,000 in PBS containing 10% fetal bovine serum and 0.2% Triton X-100. The cultures were then washed three times, 10 min each, at room temperature with 0.2% Triton X-100 in PBS containing 10% fetal bovine serum. The cultures were then incubated with a goat-anti-rabbit IgG-conjugated with Cy3 for 1 h at 37°C. The cells were washed twice with 1 ml of fresh medium and used immediately. Cells were treated with PX (5 μM) for 1 to 30 min or left untreated. The fluorescence of Cy3 was quantified with the aid of confocal laser scanning fluorescence microscopy (Leica TCS-4D; Leica Lasertechnik, Heidelberg, Germany), using excitation and emission wavelengths of 535 and 570 nm, respectively. Gray scale images with 0 to 255 steps were collected at different time points before and up to 30 min after the addition of PX by using a 512 × 512 pixel format and archived as image files for quantitative analysis. The intensity of the fluorescence in the individual cells was measured using Leica quantitation software. Background levels of immunoreactivity were very low in the control cells and this level of nonspecific signal was subtracted from the digitized images, measured in the PX-treated cells.

**Statistics**

Images obtained on X-ray film following autoradiography or chemiluminescence were scanned and the protein bands were quantified by densitometry, using NIH Image software. Significant statistical differences between band intensities were determined by ANOVA analysis (p ≤ 0.05). Also, the significance of the fluorescence value, obtained by confocal microscopy was analyzed by ANOVA (p ≤ 0.01). The significance of the statistical differences between the results obtained from DA release and vesicle fusion-based ELISA were analyzed by Kruskal-Wallis and Dunn tests (p ≤ 0.05).

**Results**

**Pardaxin-Induced Activation of ERKs in PC12 Cells.** Using anti-phospho ERKs antibody we observed strong stimulation of ERK1 (p44 MAPK) and ERK2 (p42 MAPK) by NGF (Fig. 1A, top) and to a lesser extent by osmotic shock with sorbitol (Fig. 1A, top). The same NGF- and sorbitol-treated cell lysates were also analyzed using immunoblotting with an anti-pan ERK antibody to confirm that the amount of ERK
Effect of extracellular calcium on ERK stimulation. PC12 cells were treated for 5 min with 50 ng/ml NGF or for 15 min with 5 μM PX or left untreated (CON). The cells were then collected and 10-μg protein aliquots were analyzed by SDS-PAGE (10% gel, reducing conditions) and electrotransferred to nitrocellulose membranes. The membranes were probed with anti-phospho ERK antibody (Fig. 2I), anti-ERK antibody (Fig. 2II), or, after stripping, re-probed with antibody for unphosphorylated ERK. NGF (lane 2) and sorbitol (lane 4). Controls (lanes 1 and 3). Top, anti-phospho ERK antibody; bottom, anti-ERK antibody. The location of ERK1 and ERK2 is indicated by arrows. B, time course of ERK stimulation by 5 μM PX. Cells were collected and treated as described above. CON, control. NGF, PC12 cells were treated for 5 min with 50 ng/ml NGF.

Involvement of ERK in Pardaxin-Induced Dopamine Release.

Fig. 1. Pardaxin stimulation of ERK activity in PC12 cells. A, PC12 cells were treated (+) for 5 min with 50 ng/ml NGF or 0.5 M sorbitol, or left untreated (−). The cells were then collected and 10-μg protein aliquots were analyzed by SDS-PAGE (10% gel, reducing conditions) and electrotransferred to nitrocellulose membranes. The membranes were probed with anti-phospho (anti-active) ERK antibody or, after stripping, re-probed with antibody for unphosphorylated ERK. NGF (lane 2) and sorbitol (lane 4). Controls (lanes 1 and 3). Top, anti-phospho ERK antibody; bottom, anti-ERK antibody. The location of ERK1 and ERK2 is indicated by arrows. B, time course of ERK stimulation by 5 μM PX. Cells were collected and treated as described above. CON, control. NGF, PC12 cells were treated for 5 min with 50 ng/ml NGF.

Relationship between the Inhibitory Effect of PD98059 and U0126 on ERK Activity and PX-Induced Dopamine Release. Recently, it was found that PX induces in a time-dependent manner DA release from PC12 cells,
both in the presence and absence of extracellular Ca\textsuperscript{2+} (Abu-Raya et al., 1999), as well as activation of ERK (Fig. 1). Therefore, we examined the relationship between DA release and ERK activation. PC12 cells were pretreated with the selective MEK inhibitor PD98059 (50 \text{mM}) for 45 min. The cells were then stimulated with NGF (50 \text{ng/ml}) or PX (4 \text{mM}) or left untreated (Fig. 4). PD98095 completely blocked the basal activity of ERK1 and ERK2. Both NGF- and PX-induced ERK activity was inhibited by 60 and 100\%, respectively (Fig. 4). In the presence of extracellular Ca\textsuperscript{2+} the complete inhibition of PX-induced ERK activity was accompanied by 83 and 53\% inhibition of PX-induced DA release by PD98095 and UO126, respectively (Fig. 5A). However, both MEK inhibitors did not affect significantly PX-induced DA release in Ca\textsuperscript{2+}-free medium (Fig. 6). To further investigate the involvement of ERKs in neurotransmitter release, we designed an exocytotic assay, which quantifies vesicular release, reflected by exposure of the vesicular protein synaptotagmin to the extracellular matrix, using vesicle fusion-based ELISA (Parnas and Linial, 1998). As shown in Fig. 5B, PX increased synaptotagmin exposure on the cell surface by 50\%.

Fig. 3. PX-induced translocation of active ERKs to the nucleus. Phase contrast micrographs of PC12 cells before treatment (I) and immunofluorescence micrographs of the same cells treated with PX for 1 min (II) or 30 min (III). The cells were stained with anti-phospho ERK antibody and detected by the fluorescent CY3 dye.

Fig. 4. Effect of the MEK inhibitor PD98059 on NGF and PX stimulation of ERK. To measure ERK activity, cells were pretreated for 45 min with 50 \text{mM} PD98059 (PD) or left untreated. The cells were then stimulated with NGF (50 \text{ng/ml}) or PX (4 \text{mM}) or left untreated (CON). Cell lysates (25 \text{\mu g/sample}) were separated, blotted, and analyzed, using anti-phospho ERK antibodies, and further processed, as described in the legend to Fig. 1. The location of ERK1 and ERK2 is indicated by arrows.

Fig. 5. Effect of PD98059 and UO126 on pardaxin-induced neurotransmitter release in Ca\textsuperscript{2+}-containing medium. A, PC12 cells (1 \times 10^6 cells/well) preloaded with [\text{H}]dopamine were treated for 45 min with 50 \text{mM} PD98059 (PD), or for 30 min with 30 \text{mM} UO126, or left untreated. The cells were then exposed to 4 \text{mM} PX for an additional 20 min or left untreated (control). DA release was measured as described under Experimental Procedures. The values presented are the mean \pm S.E.M. of three independent experiments (n = 3 in each experiment). *p < 0.05 compared with the control (basal release). **p < 0.05 compared with PX alone. B, exocytosis was measured by vesicle fusion-based ELISA, as described under Experimental Procedures. Control (untreated) cells or cells pretreated for 45 min with 50 \text{mM} PD98059 (PD) were exposed to 4 \text{mM} PX or left untreated for an additional 20 min in the presence of anti-synaptotagmin 1 antibody. The cells were washed, fixed, and incubated with blocking solution. Blue color formation was monitored at OD\text{_{450}} \text{nm}, with the aid of an ELISA reader. The values presented are the mean \pm S.E.M. of three independent experiments (n = 6 in each experiment). *p < 0.05 compared with the control (basal release). **p < 0.05 compared with PX alone.

Fig. 6. Effect of PD98059 and UO126 on pardaxin-induced neurotransmitter release in Ca\textsuperscript{2+}-free medium. PC12 cells (1 \times 10^6 cells/well) preloaded with [\text{H}]dopamine were treated for 45 min with 50 \text{mM} PD98059 (PD), or for 30 min with 30 \text{mM} UO126, or left untreated. The cells were then exposed to 4 \text{mM} PX for an additional 20 min or left untreated (control). DA release was measured as described under Experimental Procedures. The values presented are the mean \pm S.E.M. of three independent experiments (n = 3 in each experiment). *p < 0.05 compared with the control (basal release).
over that of the basal. However, PD98095 (PD + PX) completely inhibited PX-induced synaptotagmin exposure (Fig. 5B). Cumulatively, these results suggest the involvement of ERK1 and ERK2 in PX-induced Ca\(^{2+}\)-dependent DA release.

**Effect of Pardaxin on Dopamine Release in Dominant-Negative Ras Cells.** One of the important pathways of ERK activation involves a small guanine nucleotide-binding protein, p21Ras, a well known mediator in growth factor receptor tyrosine kinase signaling pathways. To determine whether PX-induced DA release via activation of ERKs is Ras-dependent, experiments with a dominant-negative variant PC12 cell line, M-M17-26 (Lazarovici et al., 1997, 1998), were performed. In these cells the endogenous native Ras cannot stimulate the MAPK pathway because of the large excess of inactive dominant-negative Ras protein competing for raf, which is required for ERK activation. Indeed, in these variant cells, NGF-induced ERK activation, as well as differentiation, were completely blocked (Lazarovici et al., 1997).

As can be seen in Fig. 7A, depolarization with KCl increased DA release in WT cells by 2.2-fold. This effect was completely blocked in M-M17-26 cells, as was previously obtained in other study with PC12 cells (Rosen et al., 1994). In contrast, PX stimulated by 2.5-fold and by 2.2-fold, DA release from WT and M-M17-26 cells, respectively, compared with the corresponding controls (Fig. 7A). To determine whether dominant-negative Ras cells are defective in their voltage-dependent Ca\(^{2+}\) channels, which may explain the inability of KCl to induce dopamine release, Ca\(^{2+}\) uptake was measured. KCl and PX induced similar Ca\(^{2+}\) uptake in both the WT and the M-M17-26 cells (Fig. 7B). These findings indicate that the inability of KCl to induce DA release was not due to a lack of depolarization-induced Ca\(^{2+}\) entry in these cells. In summary, it appears that although depolarization-induced DA release by KCl is fully dependent on Ras, PX-induced DA release is Ras-independent.

**Discussion**

In the present study we show that ERKs participate in Ca\(^{2+}\)-dependent DA release from PC12 cells stimulated by PX. This is supported by the following findings: 1) PX-induced activation of ERKs in PC12 cells; 2) Ca\(^{2+}\)-dependent stimulation of ERKs by PX was temporally correlated with PX-induced DA release; and 3) treatment of the cells with the MEK inhibitor PD98059 completely blocked ERK activation and markedly inhibited the induced DA release, which was also inhibited by UO126. To the best of our knowledge, this is the first report of the involvement of MEK/MAPK in PX-induced DA release, supporting the hypothesis that ERKs participate in the catecholamine secretory process (Cox and Parsons, 1997).

Ras-MAPK is well characterized as a major signal transduction pathway activated by tyrosine kinase receptors such as NGF-tyrosine kinase A receptor (Kaplan and Stephens, 1994). Cross talk between MAPK pathways and Ca\(^{2+}\) pathways has been demonstrated in numerous cell types (Ely et al., 1990; Chao et al., 1992; Rosen et al., 1994; Clapman, 1995), but the precise mechanism by which Ca\(^{2+}\) mediates activation of ERK is unknown. Since PX did not activate protein kinase C (Bloch-Shilderman et al., 1996) and we do not have any evidence that PX directly stimulates tyrosine kinases, we assume that PX stimulation of ERKs reflects cross talk between Ca\(^{2+}\) pathway(s) and the MAPK cassette. Indeed, evidence for this concept was provided by molecular studies (Hawes et al., 1995; Lopez-llasaca, 1998) on G protein-coupled receptors. Activation of such receptors induces disassociation of the G protein to \(\beta\gamma\) and \(\alpha\)-subunits. \(\beta\gamma\)-subunits in turn, stimulate ERK activation, most probably increasing intracellular Ca\(^{2+}\) and activating protein kinase C. In other studies with PC12 cells it was reported that the influx of Ca\(^{2+}\) ions, resulting from depolarization, leads to Ras-dependent MAPK/ERK activation (Rosen et al., 1994; Rusnescu et al., 1995). It was suggested that the protein tyrosine kinase PYK2, which is activated by an increase in intracellular Ca\(^{2+}\) and by protein kinase C, leads to activation of ERK (Lev et al., 1995). The possibility that PX may stimulate PYK2 kinase, and as a result activate ERKs, requires further investigation, since PYK2 may be a candidate kinase that functions upstream of MEK/MAPK. PD98095 is known to selectively inhibit MEK (Alessi et al., 1995), and also blocks PX stimulation of ERKs (Fig. 4). It is reasonable to assume that ERK activation was not due to the direct binding of PX to MEK enzymes. Rather, PX may have acted upstream of the MEK cassette.

An important function of ERKs is to regulate gene expression. Upon activation, ERKs translocate to the nucleus where they phosphorylate transcription factors (Blenis, 1993). In the present study, we found that following PX activation ERKs translocated to the nucleus. Therefore, transcription factors phosphorylated by ERKs may be of significance in the action of PX. Indeed, experiments in our laboratory show that PX stimulates c-fos early gene expression (P. Lazarovici, unpublished data). This probably requires both Ca\(^{2+}\) and the MAPK pathway, as recently reported for depolarization-induced c-fos activation in PC12 cells (Lee et al., 2000). Further investigation of whether DA release induced by PX is inhibited by interference with c-fos or with ERK translocation to the nucleus is required.
There are very few studies describing the interaction of toxins with ERKs. Palytoxin, a nonphorbol ester tumor promoter, sodium ionophore, produced by *Palythoa tuberculosa*, strongly stimulates another member of the MAPK family, c-Jun NH2 terminal kinase (Kuroki et al., 1997). In addition to MAPK-activating toxins such as PX and palytoxin there is also a MAPK inhibitory toxin, Anthrax lethal factor. This toxin, produced by *Bacillus anthracis*, is a metalloprotease that cleaves the amino terminus of MEKs, and thus, like PD98059, inhibits MAPK activation (Duesbery et al., 1998). These various studies suggest that toxins, which affect the MAPK cassette, are potential pharmacological tools for investigating the role of ERKs in signal transduction.

To what extent ERKs contribute to neurotransmitter release has not been clearly established. In previous reports on chromaffin cells (Cox et al., 1996; Cox and Parsons, 1997), it was suggested that ERKs are one of the multiple components in the signaling cascades that upon stimulation with nicotine or other secretagogues regulate optimal secretory activity. In the present study, we found a direct temporal correlation between PX stimulation of ERKs and DA release. In addition, the ability of PD98059 to inhibit both PX-induced ERK activation and DA release suggests that ERKs play an important role in DA secretion from PC12 cells, as was also found for norepinephrine secretion from chromaffin cells (Cox and Parsons, 1997). One way of MAPK activation is via Ras (Blenis, 1993). To examine the role of the Ras-MAPK pathway in DA secretion from PC12 cells we used the dominant-negative Ras, PC12 cell line M-M17-26. The lack of Ras activity in these cells resulted in the absence of ERK activity (Lazarovici et al., 1997). Secretagogue-induced DA release was markedly reduced in M-M17-26 cells compared with that in WT cells. This was not due to a lack of Ca2+ uptake by the cells (Fig. 7B), indicating that Ca2+ itself is not sufficient for release. Furthermore, there was no difference in DA uptake between PC12 and M-M17-26 cells (data not shown). It is reasonable to assume that the reduced DA release observed in M-M17-26 cells is due to the disruption of one or more steps critical to the exocytotic machinery. Although, quantitatively, DA release was low in M-M17-26 cells, PX, but not KCl, still induced DA release (Fig. 7A). Other studies with PC12 cells (Rosen et al., 1994) and chromaffin cells (Cox and Parsons, 1997) have also shown that depolarization-induced neurotransmitter release is blocked in dominant-negative Ras cells. One plausible interpretation of this finding is that PX and KCl induce DA release by different signal transduction pathways: although KCl-induced neurotransmitter release is completely Ras-dependent, PX activates signal transduction component(s) essential to DA release that are Ras-independent but ERK-dependent.

The involvement of intracellular Ca2+ in neurotransmitter release has been well established (Burgey and Morgan, 1995). However, Ca2+-independent neurotransmitter release has also been reported in different systems (Nicholls et al., 1987; Lonart and Zigmond, 1991; Abu-Raya et al., 1999). ERK activation by NGF and PX was markedly and completely abolished, respectively, in Ca2+-free medium (Fig. 2I). As seen in Fig. 6, the inhibitors PD98059/UYO126 did not significantly affect PX-induced DA release. Therefore, unlike the ERK-dependence of PX-induced DA release in the presence of extracellular Ca2+, PX stimulation of DA release in the absence of extracellular Ca2+ is ERK-independent. Recently, we showed (Abu-Raya et al., 1998) that PX stimulates the activity of phospholipase A2, and the release of eicosanoids, independently of Ca2+. Inhibitors of phospholipase A2 and lipoxygenases markedly blocked DA release induced by PX (Abu-Raya et al., 1999). PX stimulated the release of arachidonic acid from M-M17-26 and WT PC12 cells. This process was not affected by PD98059, indicating that arachidonic acid release by PX is Ras/MAPK-independent (E. Bloch-Shilderman, S. Abu-Raya, V. Trembolov, M. Linial, H. Boschwitz, A. Gruzman, S. Sasson, and P. Lazarovici, in preparation). Therefore, it is tempting to suggest that in the absence of ERK activation by PX, as occurred in Ca2+-free medium (Fig. 2F), the arachidonic acid cascade plays an essential role in PX-induced DA release. This aspect is now under investigation in our laboratory. Further clarification of the interaction of PX with other MAPK members, such as c-Jun NH2 terminal kinase, and p38 kinases and Ca2+-dependent tyrosine kinases such as PYK should provide insights into the mechanism of action of PX and may contribute putative targets for drug development in synaptic transmission.

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References


Involvement of ERK in Pardaxin-Induced Dopamine Release


Send reprint requests to: Prof. Lazarović Philip, Department of Pharmacology and Experimental Therapeutics, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, 91120, Israel. E-mail: lazph@md2.huji.ac.il

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