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Characterization of Pardaxin-Induced Dopamine Release from Pheochromocytoma Cells: Role of Calcium and Eicosanoids

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ABSTRACT

Pardaxin, an excitatory neurotoxin, induced dopamine release from pheochromocytoma (PC12) cells both in the presence and absence of extracellular calcium ($[Ca]_o$). In the presence of extracellular calcium, nifedipine, an L-type calcium channel blocker, did not affect dopamine release, whereas 1,2-bis (2-aminophenoxy) ethane *N*,*N*, *N*′*N*′-tetra-acetic acid (BAPTA), a chelator of cytosolic calcium, and dantrolene, a blocker of calcium release from intracellular stores, inhibited only partially (30–40%) pardaxin-induced dopamine release. In the absence of $[Ca]_o$, BAPTA and dantrolene were ineffective. Pardaxin stimulated the arachidonic acid (AA) cascade in PC12 cells independently of $[Ca]_o$. The phospholipase inhibitors mepacrine and bromophenacyl bromide inhibited both pardaxin-induced AA release and pardaxin-induced dopamine release. Dopamine release induced by pardaxin also was blocked by the lipoxygenase inhibitors nordihydroguaiaretic acid, esculetin, and 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone. Under these conditions, a parallel reduction in 5-hydroxyeicosatetranoic acid release also was observed. Suppression of pardaxin-induced dopamine release by inhibitors of phospholipase A_2 and lipoxygenase was more pronounced in calcium-free medium. These results indicate the involvement of the lipoxygenase pathway in pardaxin-induced dopamine release and suggest the use of this toxin as a novel pharmacological tool for investigating the mechanism of calcium-independent neurotransmitter release.

Neurotransmitter release from the synaptic terminal occurs via regulated secretion (exocytosis) as synaptic vesicles fuse with the neuronal plasma membrane and release their contents into the synaptic cleft. Regulated exocytosis of catecholamines has been largely investigated in bovine adrenal chromaffin cells (Burgoyne, 1991) and in rat pheochromocytoma (PC12) cells (Ahnert-Hilger et al., 1985). The secretory vesicles of these cells, the chromaffin granules, store dopamine, norepinephrine, ATP, and various proteins. The essential role of calcium in catecholamine secretion from chromaffin cells has been well established (Burgoyne, 1991). The concentration of cytosolic calcium ($[Ca]_i$) is strictly regulated, and it is thought that the increase in calcium concentration within the microdomain of the active exocytotic zone allows vesicles to fuse and release their catecholamines content (Burgoyne and Morgan, 1995). $[Ca]_i$ can be increased because of membrane depolarization, opening of receptor-operated channels, or release of calcium from intracellular stores (Burgoyne, 1991).

In PC12 cells, membrane depolarization by KCl (Greene and Rein, 1977a; DiVirgilio et al., 1987) resulted in an influx of Ca^{++} through depolarization-induced activation of voltage-sensitive calcium channels, thereby triggering exocytosis (DiVirgilio et al., 1987). One of the secretagogues largely investigated in PC12 cells is acetylcholine. Acetylcholine-induced catecholamine release in PC12 cells is mediated both by nicotinic and muscarinic receptors (Zerby and Ewing, 1996). Stimulation of nicotinic receptor in PC12 cells induced catecholamine release (Greene and Rein, 1977b) by membrane depolarization, which triggers the influx of extracellular calcium through voltage-dependent calcium channels (Zerby and Ewing., 1996). Muscarinic agonists such as

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ABBREVIATIONS: PX, pardaxin; BPB, 4-bromophenacyl bromide; CCH, carbachol; $[Ca]_o$, extracellular calcium; $[Ca]_i$, cytosolic-free calcium; Dan, dantrolene; NDGA, nordihydroguaiaretic acid; AA861, 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone; AA, arachidonic acid; 5-HETE, 5-hydroxyeicosatetranoic acid; Fura-2-AM, acetoxymethyl ester of Fura-2; DMEM, Dulbecco's modified Eagle's medium; PC12, pheochromocytoma cells; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′*N*′-tetra-acetic acid; PLA_2, phospholipase A_2; IP_3, inositol 1,4,5-trisphosphate.

muscarine (Rabe et al., 1987) and methacholine (Inoue and Kenimer, 1988; Takashima and Kenimer, 1989) through muscarinic M_2 receptors (Takashima and Kenimer, 1989), and bradykinin through the BK_2-receptor subtype (Appell and Barefoot, 1989; Weiss and Atlas, 1991) induced catecholamine release as a result of an increase in cytosolic calcium. Some studies suggested the involvement of inositol 1,4,5-trisphosphate (IP_3) formation and calcium mobilization in bradykinin- (Appel and Barefoot, 1989) and muscarine-stimulated (Rabe et al., 1987) neurotransmitter release. However, other studies presented evidences that phosphoinositide hydrolysis and neurotransmitter release elicited by muscarine (Takashima and Kenimer, 1989) and bradykinin (Weiss and Atlas, 1991) are independent, noncoupled events.

In contrast to calcium-dependent neurotransmitter release, the signal transduction pathways of calcium-independent neurotransmitter release (Schwartz, 1987, Hochner et al., 1989, Knight et al., 1989, Lazarovici and Lelkes, 1992) have received scant attention.

Pardaxin is a neurotoxin isolated from the Red Sea flatfish *Pardachirus marmoratus* (Lazarovici et al., 1986). This single-chain, acidic, amphipathic, and hydrophobic polypeptide is composed of 33 amino acids (Shai et al., 1988). Pardaxin induces extensive neurotransmitter release from a variety of neuronal preparations, including brain slices (Wang and Friedman, 1986), the neuromuscular junction (Renner et al., 1987), neurosecretory chromaffin cells (Lazarovici and Lelkes, 1992), and synaptosomes (Arribas et al., 1993). This presynaptic toxin has been used to investigate the quantal release of acetylcholine (Renner et al., 1987, Arribas et al., 1993) and the properties of voltage-dependent channels formed by this toxin in liposomes and planar lipid bilayers (Shi et al., 1995). Elucidation of pardaxin's mode of action is essential to its effective use as a pharmacological tool and also is expected to provide insights into the steps involved in the mechanism of neurotransmitter release. Pardaxin is thought to act by insertion into the neuronal plasma membrane, leading to the opening of poorly selective cation channels, culminating in depolarization, calcium entry, and neurotransmitter release (Lazarovici and Lelkes, 1992; Nikodijevic et al., 1992).

In addition, pardaxin stimulates catecholamine release from chromaffin cells in calcium-free medium (Lazarovici and Lelkes, 1992). The mechanisms responsible for this effect have not been investigated. Recently, we demonstrated that pardaxin stimulates the arachidonic acid (AA) cascade in PC12 cells, as expressed by AA release and eicosanoid production, independent of calcium (Abu-Raya et al., 1998). Therefore, in the present study we investigated the role of calcium in pardaxin-induced dopamine release from PC12 cells, focusing on the relationship between stimulation of the AA cascade and catecholamine release.

Materials and Methods

[^{3}H]Dopamine (47 Ci/mmol) and [^{3}H]arachidonic acid (210 Ci/mmol) were purchased from Amersham (Oakville, Canada); 5-hydroxyeicosatetranoic acid (5-HETE) kits were purchased from Advanced Magnetics, Inc. (Cambridge, MA); nordihydroguaiaretic acid (NDGA), bradykinin, trypan blue, esculetin, 4-bromophenacyl bromide (BPB), mepacrine, carbachol, EGTA, bovine serum albumin, 1,2-bis (2-aminophenoxy) ethane *N,N,N′N′*-tetra-acetic acid (BAPTA), collagen, thapsigargin, and poly-L-lysine were purchased from Sigma Chemical Co. (St. Louis, MO); acetoxymethyl ester of Fura-2 (Fura-2-AM) was purchased from Molecular Probes Inc. (Junction City, OR); 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone (AA861) was purchased from Biomol (Plymouth Meeting, PA); dantrolene and nifedipine were the kind gift from Alomone Laboratories (Jerusalem, Israel); methanol, KCl, and ascorbic acid were purchased from Merck (Darmstadt, Germany); and pardaxin was prepared in our laboratory from the lyophilized secretion of the flatfish *P. marmoratus* (collected in Eilat, Israel) by liquid chromatography (Lazarovici et al., 1986).

PC12 Cultures. PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% fetal calf serum, 7% horse serum, 100 μg/ml streptomycin, and 100 U/ml penicillin (Beit Haemek, Israel). 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, the cells were plated on six-well dishes coated with equal parts of collagen (0.01 mg/ml collagen in 0.1 M acetic acid) and poly-L-lysine (0.01 mg/ml) as described previously (Abu-Raya et al., 1993b).

[^{3}H]Dopamine Release. Dopamine release from PC12 cells was determined with slight modifications, as described previously (Nikodijevic et al., 1990). Briefly, fresh medium was added and the cells were allowed to equilibrate at 37°C for 30 min. The cells then were loaded with [^{3}H]dopamine (0.5 μCi/ml) for 3 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 pardaxin or other secretagogues in the presence or absence of $[Ca]_o$ (+1 mM EGTA) for various intervals. Basal release was measured in cultures incubated for similar intervals at 37°C and left untreated. Sample (0.2 ml) was removed from the medium and centrifuged for 10 min (1000*g*) to remove floating cells, and the radioactivity was measured in a liquid scintillation counter. To measure total radioactivity, the cells were washed with phosphate-buffered saline and solubilized in 1 ml of 0.5 N NaOH, and 0.2 ml was measured for radioactivity.

AA Release. PC12 cells were grown in six-well dishes in serum-containing DMEM for 24 h at 37°C. The growth medium then was removed and replaced with serum-free DMEM to which [^{3}H]AA (0.5 μCi/ml) was added for 4 h. The medium (containing nonincorporated isotope) was removed and the cells were washed three times with buffer containing NaCl (138 mM), Na_2HPO_4 (8 mM), $MgCl_2$ (0.5 mM), $CaCl_2$ (0.9 mM), and 1 mg/ml fatty acid-free bovine serum albumin, pH 7.4. The rinsed cells were incubated with 1 ml of the same buffer supplemented with glucose (20 mM) for 10 min at 37°C (Abu-Raya et al., 1998). AA release was initiated by the addition of pardaxin in the presence or absence of phospholipase A_2 (PLA_2) inhibitors, and the cultures were incubated further at 37°C for 15 min. On termination of the experiment, 200 μl of incubation medium was removed from each well and centrifuged for 10 min (1000*g*), and the supernatant was collected. Release of [^{3}H]AA was measured in 100-μl aliquots in a liquid scintillation counter. The amount of protein in each well was determined according to Lowry et al. (1951).

5-HETE Release. PC12 cells were exposed to pardaxin for 15 min at 37°C in serum-free DMEM. The medium then was removed and centrifuged for 10 min at 1000*g*, and aliquots were removed for radioimmunoassay, as described previously (Abu-Raya et al., 1993a). Samples were incubated for 18 to 24 h with the appropriate antiserum and radioligands. Free and bound compounds were separated on dextran coated with activated charcoal. Radioactivity was counted in a scintillation counter (LKB, Wallac OY, Finland).

Measurement of Cytosolic Calcium Level. The concentration of cytosolic-free calcium was measured using the fluorescent calcium chelator Fura-2-AM, as described previously (Lazarovici and Lelkes, 1992). PC12 cells were collected and incubated for 45 min in the dark at 37°C with Fura-2-AM. Subsequently, the cells were washed several times to remove extracellular, nonincorporated probe and

resuspended to a final density of 10^8 cells/ml. The fluorescence experiments were carried out in UV-transparent, acrylic cuvettes at room temperature, using a concentration of about 10^6 cells/ml (for pardaxin experiments) or 5×10^6 cells/ml (for thapsigargin and KCl experiments) in an SLM-Aminco SPF 500-C spectrofluorometer equipped with a stirred cuvette holder. Intracellular conversion of Fura-2-AM to Fura-2 was verified by running the excitation (340 nm) and emission (510 nm) spectra. A 435-nm cut-off filter was used to reduce light scattering. After 3 to 5 min of initial equilibration of the fluorescent signal, the baseline remained stable over the duration of the experiment (15 min). When the experiments were performed in the presence of extracellular calcium, the calcium-containing buffer was composed of 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 15 mM Na-HEPES, and 10 mM glucose (pH 7.4). The calcium-free solution was as above, without $CaCl_2$, containing, in addition, 1 mM EGTA.

Cytotoxicity. In the present study subcytotoxic concentrations of pardaxin were used. Pardaxin concentration was considered subcytotoxic when <10% cell death was observed, as determined by trypan blue exclusion (Abu-Raya et al., 1993b).

Statistics. The results are presented as the mean ± S.E.M. of three different experiments. In each experiment the number of replicates was between 3 and 6. The mean for each individual replication in a single experiment was calculated, and, thereafter, an overall mean of all experiments of the same type was determined and is presented in *Results* as overall mean ± S.E.M. Determination of statistically significant differences between experimental groups was performed using analysis of variance, and they were considered significant when p values <.05 were obtained.

Results

Role of Calcium in Pardaxin-Induced Dopamine Release. The ability of pardaxin versus other secretagogues to induce dopamine release from PC12 cells in the presence or absence of $[Ca]_o$ is presented in Fig. 1. By subtracting the basal release, pardaxin (6 μM) stimulated dopamine release by 26 ± 2% and 20 ± 1% of total content in the presence or absence of $[Ca]_o$, respectively. In calcium-containing medium, carbachol (10 μM), bradykinin (1 μM), and KCl (50 mM) stimulated dopamine release by 9.5 ± 2%, 13 ± 3%, and 15 ± 2.5% of total content, respectively. In the absence of $[Ca]_o$ these compounds did not induce dopamine release.

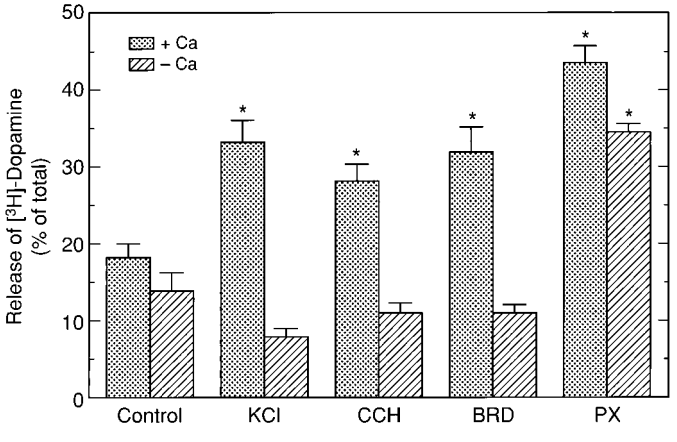

Fig. 1. The effect of $[Ca]_o$ on pardaxin-, KCl-, carbachol-, and bradykinin-induced dopamine release from PC12 cells. PC12 cells (5×10^5 cells/well) preloaded with [^{3}H]dopamine were incubated for 20 min at 37°C in the presence (+Ca) or absence (−Ca + 1 mM EGTA) of $[Ca]_o$ with KCl (50 mM), carbachol (CCH, 10 μM), bradykinin (BRD, 1 μM), or pardaxin (PX, 6 μM) or were left untreated (control). The dopamine release was measured as described under *Methods*. The values presented are the mean ± S.E.M. of three independent experiments (n = 6 in each experiment). *p < .01 compared with the respective control (basal release).

To further characterize pardaxin's effect, the dose and time course dependence of pardaxin-induced dopamine release were determined in calcium-containing medium. As shown in Fig. 2, pardaxin caused dopamine release in a concentration- and time-dependent manner. The most pronounced effect was obtained at 10 μM, the highest concentration used. At this concentration, pardaxin stimulated dopamine release by 37 ± 3%, 51 ± 2.5%, and 58 ± 2.5% of total content after 15, 30, and 60 min, respectively.

To determine the involvement of L-type calcium channels in pardaxin-induced dopamine release, experiments with nifedipine (L-type calcium channel blocker) were performed. As shown in Fig. 3, nifedipine (100 μM) did not affect dopamine release by pardaxin, but markedly blocked KCl-induced dopamine release. This finding suggests that L-type calcium channels are not involved in pardaxin-induced dopamine release.

Because pardaxin can induce dopamine release in the absence of extracellular calcium, experiments were performed to test the possibility that pardaxin mobilizes calcium from intracellular stores. For this purpose, we examined the effect of dantrolene, a blocker of calcium release from the endoplasmic reticulum (Guo et al., 1996), on pardaxin-induced dopamine release. As shown in Fig. 4, dantrolene (10 μM) partially (30%) inhibited pardaxin-induced dopamine release in the presence of $[Ca]_o$. In the absence of $[Ca]_o$, dantrolene had no effect on pardaxin-induced dopamine release (Fig. 4).

To clarify the partial inhibition exerted by dantrolene, we examined the effect of pardaxin on $[Ca]_i$. For this purpose, Fura-2-AM-loaded PC12 cells were treated with pardaxin in the presence or absence of $[Ca]_o$ (Fig. 5). Removal of extracellular calcium reduced the baseline level of $[Ca]_i$ (Fig. 5), as described previously (Lelkes and Pollard, 1987). After the addition of pardaxin to calcium-containing medium, there was a gradual and sustained increase in cytosolic calcium (Fig 5A, +Ca). In the absence of extracellular calcium, pardaxin did not augment $[Ca]_i$ (Fig. 5A, −Ca). To examine the possibility that the intracellular calcium stores were depleted under these conditions (absence of extracellular calcium and presence of 1 mM EGTA), experiments with thapsigargin and KCl were performed in the presence or absence of extracellular calcium (Fig. 5B). Thapsigargin, an inhibitor of the endoplasmic reticulum pump, is a compound known to release calcium from intracellular stores (Berridge, 1995). KCl causes an influx of calcium through depolarization-induced activation of voltage-sensitive calcium channels (DiVirgilio et al., 1987). As shown in Fig. 5B, treatment of the cells in calcium-containing buffer with 30 mM KCl resulted in a rapid increase in $[Ca]_i$, which plateaued after 1 min and then gradually declined. In calcium-free medium KCl did not lead to an elevation in $[Ca]_i$. Stimulation of PC12 cells with thapsigargin (1 μM) caused a sustained rise in $[Ca]_i$, the magnitude of which is similar to KCl-induced signal, while the onset is slightly delayed (Fig. 5B). In calcium-free medium, thapsigargin yielded a transient elevation of $[Ca]_i$, peaked after 1 min, and then rapidly returned to basal level, confirming that calcium redistribution from intracellular stores is maintained. Taken together, these data indicate that pardaxin induces an influx of calcium but does not mobilize calcium from intracellular stores.

The influx of calcium elicited by pardaxin in the presence of extracellular calcium should enhance dopamine release. To

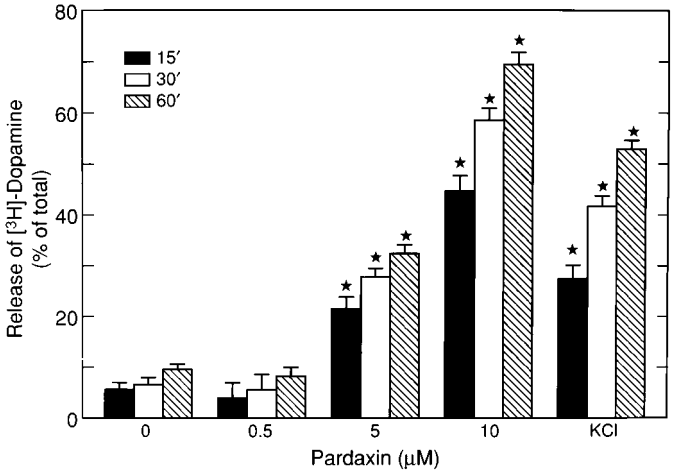

Fig. 2. Time course and dose dependence of pardaxin-induced dopamine release from PC12 cells. PC12 cells (5×10^5 cells/well) preloaded with [^{3}H]dopamine were incubated at 37°C with 50 mM KCl or various concentrations of pardaxin for the indicated times in the presence of $[Ca]_o$. The values presented are the mean ± S.E.M. of three independent experiments ($n = 6$ in each experiment). *$p < .01$ compared with the basal release.

test this possibility, we determined the effect of BAPTA-AM, a membrane-permeant chelator of cytosolic calcium (Yang et al., 1994). In the presence of $[Ca]_o$, BAPTA (10 μM) inhibited KCl-induced dopamine release by 63% and pardaxin-induced dopamine release by 40%. However, pardaxin-induced dopamine release in the absence of $[Ca]_o$ was not affected by BAPTA treatment (Fig. 6). These data suggest that calcium influx is partially involved in pardaxin-induced dopamine release.

Relationship Between Pardaxin-Induced Dopamine Release and Eicosanoid Production. Recently, we reported that pardaxin stimulates the AA cascade in PC12, as expressed by AA release and eicosanoid production (Abu-Raya et al., 1998). Because AA and eicosanoids may act as

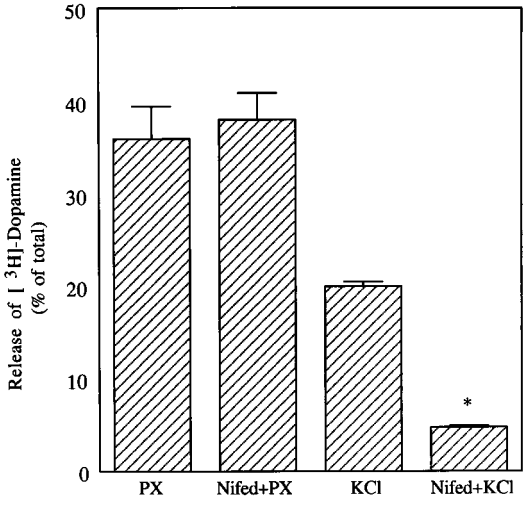

Fig. 3. The effect of nifedipine on pardaxin- and KCl-induced dopamine release in calcium-containing medium. PC12 cells (5×10^5 cells/well) preloaded with [^{3}H]dopamine were incubated for 15 min at 37°C with nifedipine (Nifed, 100 μM). Pardaxin (PX, 6 μM) or KCl (50 mM) was added for an additional 20 min. Spontaneous release (~5%) was subtracted from all values presented as the mean ± S.E.M. of three independent experiments ($n = 6$ in each experiment). *$p < .01$ compared with the agent alone.

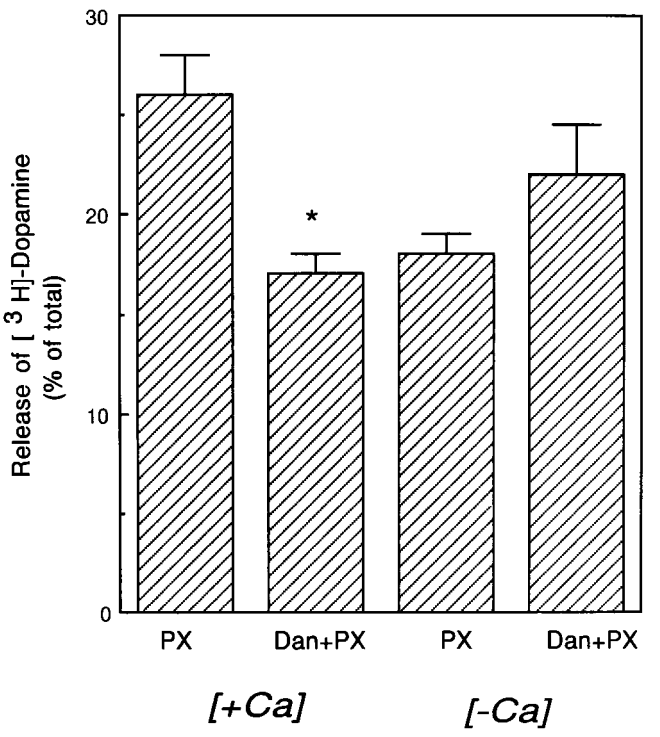

Fig. 4. The effect of dantrolene on pardaxin-induced dopamine release from PC12 cells in the presence or absence of $[Ca]_o$. PC12 cells (5×10^5 cells/well) preloaded with [^{3}H]dopamine were incubated for 20 min at 37°C with 10 μM dantrolene (Dan). The cultures then were exposed to 5 μM pardaxin in the presence of extracellular calcium (+Ca) or to 6 μM pardaxin in the absence of extracellular calcium + 1 mM EGTA (−Ca) for an additional 20 min. The values presented are the mean ± S.E.M. of three independent experiments ($n = 4$ in each experiment) after subtraction of the spontaneous release (7%). *$p < .01$ compared with the agent alone.

intracellular second messengers and affect synaptic transmission (Piomelli, 1994), we examined the effect of inhibitors for the AA cascade in PC12 cells on pardaxin-induced dopamine release. In PC12 cells treated with indomethacin (a cyclooxygenase inhibitor; Ray et al., 1993) there was an increase by about 10 to 20% in dopamine release in response to 1 μM and 5 μM pardaxin in the presence (Fig. 7A) or absence (Fig. 7B) of $[Ca]_o$. At 10 μM pardaxin, the indomethacin effect was not significant (Fig. 7). However, indomethacin did not affect KCl-induced dopamine release in the presence (Fig. 7A) or absence (Fig. 7B) of $[Ca]_o$. Indomethacin treatment caused complete inhibition of pardaxin-stimulated release of prostaglandin E_2, thromboxane $_2$, and 6-keto-prostaglandin $F1_\alpha$ (Abu-Raya et al., 1998). Also, a small increment in amount of released 5-HETE was measured (data not shown). These results suggest that the cyclooxygenase pathway is not involved in pardaxin-induced dopamine release.

To determine the involvement of the lipoxygenase pathway in pardaxin-induced dopamine release, several lipoxygenase inhibitors were tested. As indicated in Table 1, esculetin (20 μM), AA861 (10 μM), and NDGA (100 nM) inhibited pardaxin-induced dopamine release by about 50% in calcium-containing medium. A parallel inhibition of pardaxin-induced 5-HETE release by these compounds also was observed (Table 1). At 1 μM and 5 μM, NDGA further inhibited (by about 85%) pardaxin-induced dopamine release, which was accompanied by a marked reduction (by about 90–95%) in

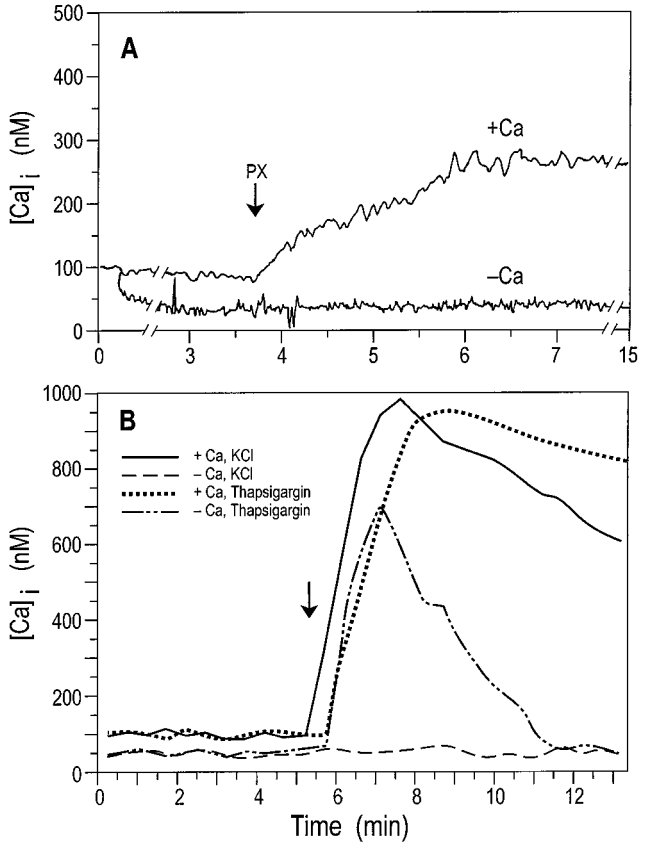

Fig. 5. The effect of pardaxin, thapsigargin, and KCl on $[Ca]_i$ in the presence or absence of $[Ca]_o$. PC12 cells were loaded for 45 min with 5 μM Fura-2-AM (A) or 10 μM (B). The cells then were washed several times. The fluorescence experiments were carried out at room temperature using a concentration of about 10^6 cells/ml (A) or 5×10^6 cells/ml (B) in the presence of 2 mM $CaCl_2$ (+Ca), or calcium-free buffer supplemented with 1 mM EGTA (−Ca). Pardaxin (PX, A) and thapsigargin and KCl (B) were added at concentrations of 6 μM, 1 μM, and 30 mM, respectively. Arrow indicates the time addition of the compound. The data presented are representative of at least three independent experiments.

5-HETE release (Table 1). In the absence of extracellular calcium, NDGA (1 μM) and AA861 (10 μM) inhibited pardaxin-induced dopamine release by about 90% (data not shown).

To further confirm the involvement of arachidonic acid cascade in pardaxin-induced dopamine release, we tested the effect of two PLA_2 inhibitors. BPB (30 μM) and mepacrine (50 μM) inhibited by 47 and 60%, respectively, the pardaxin-induced dopamine release in the presence of $[Ca]_o$ and by 73 and 87%, respectively, in the absence of $[Ca]_o$. In parallel cultures, BPB and mepacrine inhibited by 75 to 90% pardaxin-induced AA release independently of $[Ca]_o$ (Table 2).

Discussion

The aims of the present study were to 1) determine the role of calcium in pardaxin-induced dopamine release and 2) clarify the relationship between pardaxin-induced release of AA and dopamine from PC12 cells.

Pardaxin, in contrast to KCl, carbachol, and bradykinin, induced dopamine release in the absence of $[Ca]_o$ (Fig. 1) similarly to pardaxin-stimulated, calcium-independent catecholamine release from bovine adrenal medullary chromaffin cells (Lazarovici and Lelkes, 1992). Because KCl was found to depolarize the membrane and stimulate calcium influx, thereby evoking neurotransmitter release, this compound did not induce dopamine release in calcium-free medium (Fig. 1). Carbachol, a nonselective cholinergic agonist, induced catecholamine release by stimulation of both nicotinic and muscarinic receptors. Carbachol-induced neurotransmitter release mediated by nicotinic receptors requires an influx of extracellular calcium through voltage-sensitive calcium channels; thus, it is expected that nicotinic agonist-induced neurotransmitter release would be inhibited in calcium-free medium. Previous studies with PC12 cells have indicated that muscarinic agonist-induced catecholamine release was also inhibited when the experiments were performed in the absence of $[Ca]_o$ (Rabe et al., 1987; Inoue and Kenimer, 1988; Takashima and Kenimer, 1989). To explain this observation it was suggested that muscarinic agonist-induced catecholamine release is mediated by calcium influx, which occurs through a channel different from the voltage-sensitive channel activated by nicotine (Inoue and Kenimer, 1988; Takashima and Kenimer, 1989). Alternatively, it was suggested that the reduction of basal intracellular-free calcium that was observed in the absence of extracellular calcium (as shown also in Fig. 5) may inhibit calcium-dependent phospholipase C. This would reduce the amount of IP_3 formed upon muscarinic stimulation and, in turn, reduce the rise in $[Ca]_i$ and neurotransmitter release (Rabe et al., 1987). Taken together, these data suggest that extracellular calcium is required for catecholamine release induced by nicotinic as well as by muscarinic agonists in PC12 cells and may explain the inability of carbachol to induce dopamine release in calcium-free medium (Fig. 1). As shown in Fig. 1, bradykinin also did not induce dopamine release in calcium-free medium, as previously reported (Weiss and Atlas, 1991), suggesting an essential role for calcium influx in bradykinin-induced dopamine release from PC12 cells. Since bradykinin did not induce release in the absence of $[Ca]_o$, it appears that IP_3, which is produced under these conditions (Fasolato et al., 1988), is not sufficient to induce neurotransmitter release. However, in another study with PC12 cells, bradyki-

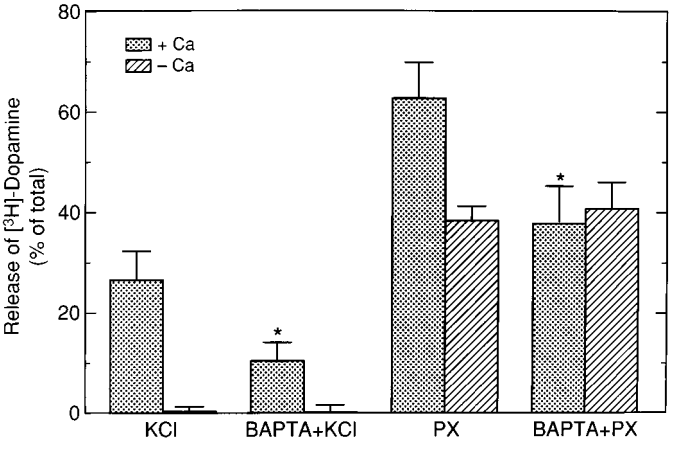

Fig. 6. The effect of BAPTA-AM on pardaxin- and KCl-induced dopamine release from PC12 cells in the presence or absence of $[Ca]_o$. PC12 cells (5×10^5 cells/well), preloaded with [^{3}H]dopamine, were incubated for 1 h at 37°C with BAPTA-AM (10 μM). The cells then were treated with KCl (50 mM) or pardaxin (PX, 6 μM) for an additional 20 min. The values presented are the mean ± S.E.M. of three independent experiments (n = 4 in each experiment) after subtraction of the spontaneous release (5%). *$p < .05$ compared with the agent alone.

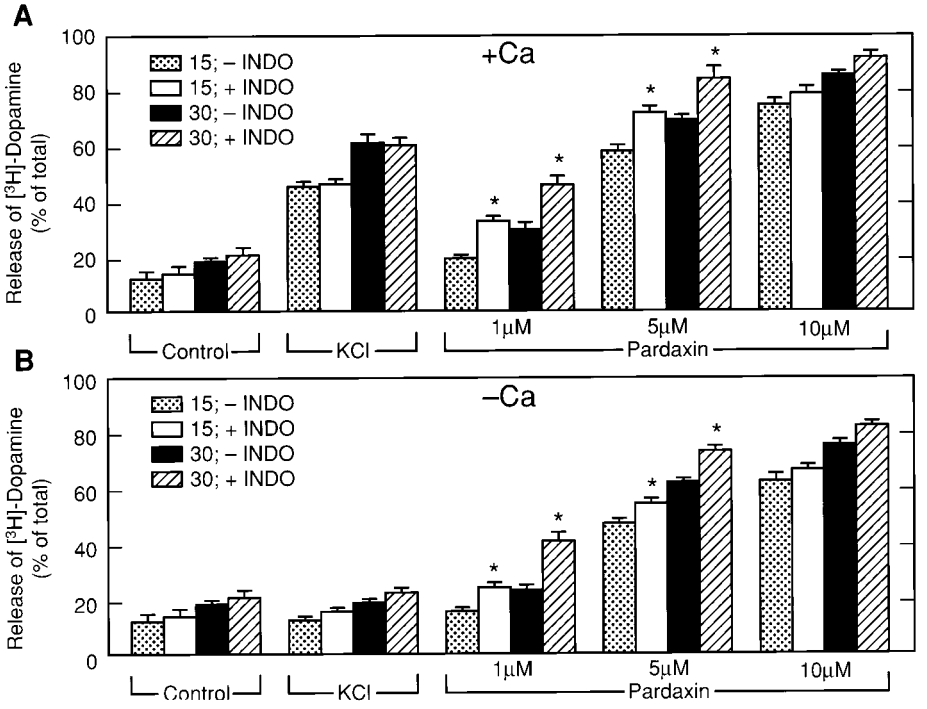

Fig. 7. The effect of indomethacin on pardaxin- and KCl-induced dopamine release from PC12 cells in the presence or absence of $[Ca]_o$. PC12 cells (5×10^5 cells/well) preloaded with [^{3}H]dopamine were treated for 30 min at 37°C with indomethacin (INDO, 50 μM). The cells then were treated with various concentrations of pardaxin or 50 mM KCl in the presence (A) or absence (B) of $[Ca]_o$ for the periods of time indicated. The values are presented as the mean ± S.E.M. of three independent experiments ($n = 4$ in each experiment). *$p < .05$ compared with the agent alone.

TABLE 1
Effect of lipoxygenase inhibitors on PX-induced release of 5-HETE and [^{3}H]dopamine

Inhibitor (concentration)	% of PX Alone	
	5-HETE Release[a]	[^{3}H]Dopamine Release[b]
Esculetin (20 μM)	48 ± 7*	51 ± 6*
AA861 (10 μM)	42 ± 2*	52 ± 5*
NDGA (100 nM)	43 ± 6*	54 ± 3*
NDGA (1 μM)	10 ± 4**	17 ± 2**
NDGA (5 μM)	5 ± 2**	15 ± 3**

[a] PC12 cells were preincubated with the lipoxygenase inhibitor in calcium-containing medium at the indicated concentration. The cells were incubated further at 37°C with PX (5 μM) for 15 min. Values for 100% 5-HETE release (PX alone) were 1580 ± 226 pg/ml. The basal release (400 ± 80 pg/ml) was subtracted from the experimental groups. The values presented are the mean ± S.E.M. of three independent experiments ($n = 3$ in each experiment). *$p < .05$, **$p < 0.01$ compared with PX alone.

[b] Cells prelabeled with [^{3}H]dopamine were incubated with the lipoxygenase inhibitor in calcium-containing medium at the indicated concentration. Then the cells were incubated further at 37°C with pardaxin (5 μM) for 15 min. Values for 100% dopamine release (PX alone) were 28 ± 2% of total content, after subtracting the basal release (6 ± 2% of total content). The values presented are the mean ± S.E.M. of three independent experiments ($n = 3$ in each experiment). *$p < 0.05$, **$p < 0.01$ compared with PX alone.

TABLE 2
Effect of PLA_2 inhibitors on PX-induced release of AA and dopamine in the presence or absence of extracellular calcium

Compound	[^{3}H]AA Release[a] (% of control)		[^{3}H]Dopamine Release[b] (% of control)	
	+Ca	−Ca	+Ca	−Ca
PX (control)	100	100	100	100
PX + mepacrine	10 ± 4*	11 ± 2*	40 ± 7*	13 ± 2*
PX + BPB	26 ± 6*	25 ± 6*	53 ± 9*	27 ± 3*

PC12 cultures were labeled with [^{3}H]dopamine or [^{3}H]AA. After washing, the cultures were treated for 30 min with mepacrine (50 μM) or BPB (30 μM) or left untreated in the presence of calcium (+Ca) or in the absence of calcium + 1 mM EGTA (−Ca). The cultures then were incubated with 5 μM PX for 15 min at 37°C.

[a] Values for 100% AA release were 2400 ± 351 cpm/mg protein and 1909 ± 62 cpm/mg protein in the presence and absence of $[Ca]_0$, respectively.

[b] Values for 100% dopamine release were 31 ± 3% of total content and 22 ± 7% of total content in the presence and absence of $[Ca]_0$, respectively. The values presented are the mean ± S.E.M. of three independent experiments ($n = 6$ in each experiment). *$p < .01$ compared with PX alone.

nin-induced dopamine release was partially inhibited in calcium-free medium (Appell and Barefoot, 1989).

Although the basic role of calcium in neurotransmitter release is well established (Burgoyne, 1991; Burgoyne and Morgan, 1995), $[Ca]_o$-independent neurotransmitter release has been reported in different systems. Depolarization of brain slices or synaptosomes can induce the release of adrenaline (Adam-Vizi and Ligeti, 1984), γ-aminobutyric acid (Schwartz, 1987), glutamate (Nicholls et al., 1987), and dopamine (Lonart and Zigmond, 1991) independently of $[Ca]_o$.

One explanation for calcium-independent neurotransmitter release is the induction of a conformational change in certain cellular proteins by membrane depolarization, rendering them sensitive to $[Ca]_i$ and triggering exocytosis (Hochner et al., 1989). According to this hypothesis, the mobilization of intracellular calcium is a prerequisite for neurotransmitter release. Therefore, we investigated the involvement of $[Ca]_i$ in pardaxin-induced dopamine release. The membrane-permeant calcium chelator BAPTA-AM (Yang et al., 1994) partially inhibited pardaxin-induced dopamine release in the presence of $[Ca]_o$ (Fig. 6), most probably because of the chelation of cytosolic calcium after its influx (Fig. 5A) through pardaxin pores but not through L-type calcium channels (Nikodijevic et al., 1992). Treatment of PC12 cells with dantrolene, a blocker of calcium release from the endoplasmic reticulum (Guo et al., 1996), did not affect pardaxin-induced dopamine release in the absence of $[Ca]_o$ (Fig. 4). Pardaxin also induced dopamine release in cultures treated with BAPTA-AM in calcium-free medium (Fig. 6). Therefore, it is reasonable to assume that mobilization of calcium from intracellular stores is not the mechanism by which pardaxin stimulates dopamine release. This notion is supported by the finding that pardaxin did not increase $[Ca]_i$ in the absence of $[Ca]_o$ in Fura-2-AM-loaded PC12 cells (Fig. 5A). Under these conditions, thapsigargin (Fig. 5B) and bradykinin (Fasolato et al., 1988; Appel and Barefoot, 1989) did induce a rise in

$[Ca]_i$, confirming that calcium intracellular stores are releasable under these conditions. Because pardaxin did not cause the direct release of calcium from intracellular stores, the effect of dantrolene on dopamine secretion in calcium-containing medium may be due to its inhibition of calcium mobilization from intracellular stores elicited by calcium influx via the pardaxin pores.

Recently, we reported that pardaxin stimulates the AA cascade in PC12 cells, as expressed by AA release and eicosanoid production (Abu-Raya et al., 1998). Pardaxin (1–10 μM) induced AA release in a dose-dependent manner. Stimulation of AA release by pardaxin (5 μM) was detected after 5 min of incubation, whereas maximal stimulation was measured after 30 min of incubation. In the present study we investigated the role of the AA cascade in pardaxin-induced dopamine release. The PLA_2 inhibitors (Ray et al., 1993) BPB (30 μM) and mepacrine (50 μM) inhibited pardaxin-induced dopamine release by about 50 to 60% in the presence of $[Ca]_o$ and by about 73 to 87% in the absence of $[Ca]_o$ (Table 2). These results suggest the involvement of PLA_2 activation on neurotransmitter release by pardaxin. In other studies with PC12 cells, it was suggested that PLA_2 is involved in exocytosis (Ray et al., 1993; Yang et al., 1994; Matsuzawa et al., 1996). Therefore, it is very tempting to assume that AA or derived eicosanoid mediates pardaxin-induced dopamine release. Indomethacin strongly inhibited the release of cyclooxygenase products in response to pardaxin (Abu-Raya et al., 1998), whereas dopamine release was slightly stimulated rather than inhibited under these conditions (Fig. 7). These data rule out the involvement of the cyclooxygenase pathway in pardaxin-induced dopamine release. However, the lipoxygenase inhibitors NDGA, esculetin (Barja-Fidalgo et al., 1991), and AA861 (Harish and Poo, 1992) strongly inhibited both pardaxin-induced dopamine release and 5-HETE release (Table 1). These results indicate that the lipoxygenase products may be involved in pardaxin-induced dopamine release. The involvement of the lipoxygenase pathway in hormone and neurotransmitter release has been reported in several studies (Metz, 1985; Naor et al., 1985; Barja-Fidalgo et al., 1991). However, in these and others investigations the involvement of the lipoxygenase pathway was examined under physiological conditions (in calcium-containing medium). In the present experiments, we presented evidences that the lipoxygenase pathway may be involved in dopamine release also in the absence of $[Ca]_o$ and without any increase in $[Ca]_i$. The slight stimulation of dopamine release under cyclooxygenase inhibition may be a result of the diversion of the AA released by pardaxin from the cyclooxygenase pathway to the lipoxygenase pathway.

Although the role of calcium in catecholamine release has been widely investigated, the intracellular signaling pathways whereby eicosanoids activate release in the absence of an increase in $[Ca]_i$ are unknown. It would be interesting to know whether AA metabolites act as intracellular second messengers or in an autocrine fashion. The possibility that the generation of lipid products such as eicosanoids directly regulate the fusion process, independent of calcium, also merits careful examination. Pardaxin can provide a new pharmacological tool for clarifying these issues.

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