MODULATION OF VESICULAR CATECHOLAMINE
RELEASE FROM RAT PC12 CELLS

Modulatie van Vesiculaire Catecholamine
Afgifte van Rat PC12 Cellen

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE UNIVERSITEIT UTRECHT
OP GEZAG VAN DE RECTOR MAGNIFICUS, PROF. DR. W.H. GISPEN
INGEVOLGE HET BESLUIT VAN HET COLLEGE VAN PROMOTIES
IN HET OPENBAAR TE VERDEDIGEN
OP WOENSDAG 29 MEI 2002 DES MIDDAGS TE 12.45 UUR.

door
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Geboren op 28 februari 1975 te Apeldoorn
Westerink, Remco H.S.
Modulation of Vesicular Catecholamine
Release from Rat PC12 Cells.
Utrecht: Utrecht University, Faculty of Biology
Thesis Utrecht University - with a summary in Dutch.

ISBN 90 - 393 - 2983 - 4

The studies described in this thesis were carried out at the Institute for Risk Assessment Sciences, P.O. Box 80176, 3508 TD Utrecht, The Netherlands.

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General introduction

This thesis deals with the process of vesicular neurotransmitter release and its modulation by endogenous and exogenous factors. Historically, neurotransmission and its modulation are studied from a postsynaptic view. The development of new techniques led to a new, complementary view and resulted in the exploration of the presynaptic mechanisms underlying neurotransmission. This general introduction provides a basic introduction into neurotransmission, focusing on the presynaptic cell and the processes and proteins underlying vesicular neurotransmitter release. In addition, techniques used to measure neurotransmitter release and the outline of this thesis are described.

Neurotransmission

Intercellular communication is of vital importance. This holds in particular for the nervous system, since the nervous system is the main coordinating system in animals. Nerve cells communicate with each other and with target cells by transmitting electrical signals and by releasing chemical messengers. Nerve cells contain one or more synapses, specialized structures essential for intercellular communication. Synapses link the presynaptic, information transmitting nerve cells to the postsynaptic, information receiving cells. There are two types of synapses: electrical synapses and the more common chemical synapses. At a chemical synapse, the pre- and post-synaptic cell are separated by the synaptic cleft. The presynaptic cell contains vesicles filled with neurotransmitter molecules. Upon stimulation of the presynaptic cell these vesicles are emptied in the synaptic cleft, a process called exocytosis. The neurotransmitter molecules diffuse the short distance across the synaptic cleft and reach the postsynaptic membrane. There, the neurotransmitters bind to postsynaptic receptors and trigger the postsynaptic response (Fig. 1) (for review see Südhof, 1995; Lin and Scheller, 2000). Thus, intercellular communication is initiated by the release of neurotransmitters from the presynaptic nerve cell. The nature and strength of the presynaptic signal is determined by the type of neurotransmitter released, the number of vesicles that fused and the amount of neurotransmitter released per vesicle. Intercellular communication and its modulation not only affect basic functions such as the coordination of muscles and the endocrine system, but also cognitive functions such as learning and memory.
Figure 1. Schematic representation of a presynaptic neuron projecting on a postsynaptic neuron by means of a chemical synapse. Inset shows the presynaptic nerve ending projecting on the postsynaptic neuron in more detail.

The vesicle cycle
The actual release of neurotransmitter molecules from a secretory vesicle, exocytosis, is an extremely rapid process, which takes only a few milliseconds (Almers et al., 1991). This release event is part of a much slower, complex ensemble of processes, known as the vesicle cycle (Fig. 2; for review see Südhof, 1995). The vesicle cycle starts in the cell cytoplasm, where empty lipid vesicles are filled with neurotransmitters. Once filled, these vesicles migrate towards the cell membrane, where they become docked. A docked vesicle is in close contact with the cell membrane, a state achieved by complex interactions between proteins on the vesicle membrane and on the cell membrane. After a ‘priming’ step the vesicle is ready to fuse with the cell membrane and has become part of the so-called readily releasable pool. A subsequent increase in the intracellular calcium concentration is the adequate stimulus for the fusion between the vesicle and the cell membrane, resulting in the release of the vesicle contents.
Figure 2. The vesicle cycle. Exocytosis (5), the actual release of neurotransmitter molecules, is part of an ensemble of processes, known as the vesicle cycle. 1) Vesicular neurotransmitter (NT) uptake. 2) Vesicle translocation. 3) Docking. 4) Priming. 5) Ca\(^{2+}\)-dependent vesicle fusion, exocytosis. 6) Endocytosis. 7) Translocation and forming of a proton-gradient required for neurotransmitter uptake. 8) Endosome fusion. 9) Budding of new empty vesicles (after Südhof, 1995).

Exocytosis is followed by endocytosis, the internalization of empty vesicles from the cell membrane. Apart from the selective and efficient retrieval of the specialized membrane structures of secretory vesicles, endocytosis prevents continuous swelling of the cell caused by exocytosis and regulates cell size. The empty vesicles are transported to the cell interior where the vesicles are re-filled with neurotransmitter or fuse with the endosome. Subsequent budding of new empty vesicles and neurotransmitter uptake by these new vesicles closes the vesicle cycle and allows successive rounds of exocytosis. Neurotransmission continues as long as there is enough neurotransmitter to fill the empty vesicles and an elevation in intracellular calcium concentration, which is sufficient to trigger exocytosis. In addition, every step in the vesicle cycle is initiated and coordinated by sophisticated interactions between a large number of specialized proteins, which need to function properly to prevent disturbances in intercellular communication.
Proteins involved in exocytosis

The first step in the vesicle cycle, vesicular neurotransmitter uptake, requires a proton-gradient across the vesicle membrane, generated by the vacuolar H+-ATPase, and the presence of neurotransmitter transporters on the vesicle membrane. In case of catecholamine-containing vesicles, this transporter is the vesicular monoamine transporter (VMAT). Once transported into the vesicle, catecholamines are bound to a chromogranin matrix to keep the osmolarity of the vesicle low. Modulation of the availability of neurotransmitters or matrix molecules, or modulation of the proton-gradient or of the vesicular neurotransmitter transporter affects the amount of vesicular neurotransmitter uptake and, eventually, also the amount of neurotransmitter secreted during vesicle fusion (for review see O’Connor, 1999; Gasnier, 2000; Pothos et al., 2000; Sulzer and Pothos, 2000).

Filled vesicles either reside in a so-called reserve pool, or they are translocated to the plasma membrane to become docked. The actin network acts as a barrier for vesicles and prevents them from docking (for review see Doussau and Augustine, 2000; Trifaró et al., 2000). Vesicles are kept in the reserve pool, i.e., linked to the actin network, by a class of vesicle-associated proteins named synapsins. The number of vesicles available for release depends on the phosphorylation state of synapsin. Phosphorylation of synapsin decreases the interaction with actin, resulting in an increased availability of vesicles to become docked, and thereby increases exocytosis. Thus, changes in the actin network or the phosphorylation state of synapsin regulate the number of vesicles in the reserve pool and the number of docked vesicles. The phosphorylation state of synapsin is regulated by interplay between kinases and phosphatases (Hilfiker et al., 1999; Hosaka et al., 1999; Humeau et al., 2001; Jovanovic et al., 2001). Protein kinase A (PKA), protein kinase C (PKC), Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM kinase II) and mitogen-activated protein kinase (MAPK) are all able to phosphorylate synapsin. On the other hand, protein phosphatase 2A and Ca\(^{2+}\)-calmodulin-dependent protein phosphatase 2B (calcineurin) are able to dephosphorylate synapsins.

Vesicle docking, priming and fusion require multiple protein-protein interactions. Once a secretory vesicle reaches the plasma membrane specific proteins on the vesicle, v-SNAREs, interact with specific t-SNAREs on the plasma membrane. The v-SNARE synaptobrevin/VAMP binds to the t-SNAREs syntaxin and SNAP-25 to form the so-called SNARE complex. This complex is sufficient to establish fusion of the vesicle and plasma membranes (Fig. 3; for review see Chen and Scheller, 2001).
Figure 3. The basic machinery for vesicle fusion. Interaction of v-SNAREs with t-SNAREs leads to the formation of a four-helix bundle that zips up concomitant with bilayer fusion (after Chen and Scheller, 2001).

To prevent uncoordinated vesicle fusion a number of regulators is required (see Fig. 4). The t-SNARE syntaxin is normally bound to n-sec1/Munc18, preventing formation of the SNARE complex. Phosphorylation of n-sec1 by PKC decreases its affinity for syntaxin, thus allowing the interaction of syntaxin with SNAP-25 and synaptobrevin (Jahn, 2000; Zhang et al., 2000). The newly formed SNARE complex may be stabilized by a Ca\(^{2+}\)-binding protein, possibly the proposed Ca\(^{2+}\)-sensor synaptotagmin. This stabilizing protein prevents the SNARE complex from further interactions. Eventually, an increase in the intracellular Ca\(^{2+}\) concentration displaces the Ca\(^{2+}\)-sensor resulting in membrane fusion (for review see Hazuka et al., 1999; Desai et al., 2000; Garcia et al., 2000; Verona et al., 2000).
After fusion the SNARE complex remains stable in the plasma membrane. Subsequently, the actions of NSF/α-SNAP are required to dissociate the SNARE complex, so the SNAREs can be primed for future rounds of vesicle fusion (reviewed in Hazuka et al., 1999). The protein interactions underlying vesicle exocytosis can in turn be modulated by a number of cytoplasmic proteins, making it even more complicated. For example, Rab3A acts as a negative regulator of fusion, downstream of SNARE complex formation. The activity of Rab3A is in turn regulated by several other proteins (Rabphilin, RIM, Noc2 and calmodulin). The activity of these Rab3A regulating proteins can also be modulated by a variety of proteins (e.g., several kinases and cAMP-GEFII) adding to the complexity of exocytosis (reviewed in Hazuka et al., 1999).

Figure 4. A selection of the proteins involved in the vesicle cycle is shown with the distribution in the plasma and vesicle membranes and in the cytoplasm.
The endocytotic part of the vesicle cycle is just as intricate as the exocytotic part. In brief, endocytosis requires GTP, interaction of dynamin with microtubuli, the influx of Ca$^{2+}$ and a large number of regulatory proteins. Ca$^{2+}$ influx activates calmodulin, and subsequently calcineurin. Calcineurin dephosphorylates dynamin, amphiphysin, and synaptojanin, eventually leading to invagination of the plasma membrane and the formation of endocytotic vesicles (for review see Zhang and Ramaswami, 1999; Brodin et al., 2000; Hinshaw, 2000; Jarousse and Kelly, 2001).

The activity of the proteins mentioned in this section largely depends on a fine-tuned balance between phosphatases and kinases. Kinases like PKA, PKC and CaM kinase II are able to phosphorylate e.g. Ca$^{2+}$ channels, SNAP-25, synapsin, rabphilin3A, synaptobrevin, syntaxin, synaptophysin, NSF, GAP-43, dynamin, α-SNAP, n-sec1, MARCKS and synaptotagmin. Phosphatases like calcineurin subsequently dephosphorylate these proteins to restore the original balance (Ämmälä et al., 1994; Schweitzer et al., 1995; Gillis et al., 1996; Billiard et al., 1997; Misonou et al., 1998; Lin and Lin-Shiau, 1999; Oleskevich and Walmsley, 2000; Verona et al., 2000; Becherer et al., 2001; Matveeva et al., 2001). It is obvious that a distortion of one of the kinases or phosphatases, or one of the proteins acting in the cascade of interactions, could result in a severe impairment of vesicle exocytosis. One classic example is impairment of vesicle exocytosis caused by botulinum neurotoxin, produced by Clostridium botulinum. Exposure to botulinum neurotoxins (there are at least 7 serotypes) normally occurs by ingestion of contaminated food. Depending on the type of botulinum neurotoxin, one of the SNARE proteins is cleaved, resulting in failure of synaptic transmission, paralysis and, in severe cases, death (Montecucco and Schiavo, 1995; Mochida, 2000).

**Detection of neurotransmitter release**

Considering the amount of detailed data on neurotransmitter release it is obvious that, since the first demonstration of chemical neurotransmission in 1921 by Otto Loewi, much research has been performed and several techniques have been developed to detect neurotransmitter release. Roughly, neurotransmitter release can be measured at two levels: at the level of cell populations and at the single-cell level. The common technique to measure neurotransmitter release from a cell population (or an organ like the adrenal) is by superfusion of the population with saline containing a stimulant or a toxicant. After subsequent collection the superfusate is analyzed using HPLC, if necessary combined with electrochemical end-detection (Sharp and Zetterström, 1991). This technique can be used to characterize the type and amount of neurotransmitter released. Another way of measuring neurotransmitter release is by incubation of the cell population with radioactive
neurotransmitters or radioactive neurotransmitter precursors. The radioactive transmitters are taken up by the cell and after washing off the excess of radiolabeled compound the release of radioactive neurotransmitters can be initiated. Analysis of the amount of radioactivity in the superfusate and of the amount of radioactivity remaining in the cell enables determination of the percentage of neurotransmitter released following stimulation or exposure to toxicants. The major advantages of measuring neurotransmitter release at the level of cell populations are the relative ease of the analysis procedure and the possibility to detect relatively small effects. The disadvantages are the poor temporal resolution (generally $\geq 15$ s) and the lack of the possibility to discriminate between vesicular neurotransmitter release and non-vesicular release, e.g., transmitter leakage caused by cell death or by carrier-mediated neurotransmitter outflow. In addition, it is difficult to establish whether the observed effects are caused by changes in the frequency of vesicle exocytosis or by changes in the amount of neurotransmitter released per vesicle.

For measuring single-cell neurotransmitter release there are three general possibilities. A classic possibility is to measure the activity of the postsynaptic cell. Neurotransmitter released from the presynaptic cell will elicit a postsynaptic response, which can be measured using electrophysiological techniques (Fatt and Katz, 1952). The binding of quanta of released neurotransmitter molecules to postsynaptic receptors elicits quantal responses in the postsynaptic cell. Changes in the frequency of these postsynaptic responses are due to changes in the presynaptic release machinery. However, changes in the size of the response could also have a postsynaptic origin, e.g., desensitization of postsynaptic receptors or an altered number of functional postsynaptic receptors. Although this method is suitable to demonstrate the vesicular nature of neurotransmitter release and has a high temporal resolution, it is not very suitable for quantitative studies investigating the presynaptic modulation of neurotransmitter release, since many modulators are also able to affect the postsynaptic response.

Another possibility to determine neurotransmitter release is by measuring the capacitance of the presynaptic cell membrane (Gillis, 1995). The phospholipid bilayer of the cell membrane has a capacitance ($C_M$) which is defined by the equation: $C_M = \varepsilon A/d$, where $\varepsilon$ is the dielectric constant, $A$ is area of the membrane, and $d$ is its thickness. During exocytosis vesicle membranes are incorporated in the plasma membrane resulting in an increased membrane area, which can be resolved as an increase in membrane capacitance in the whole-cell patch-clamp configuration. Since membrane capacitance decreases during endocytosis, membrane capacitance measurements reveal the net result of exocytosis and endocytosis. In addition, the sensitivity of this technique is generally too low to resolve single vesicle fusions, unless mast cells are used or capacitance measurements are carried out in the cell-attached mode. Another disadvantage is that by using the whole-cell
configuration of the patch-clamp technique, the intracellular conditions become disturbed. Finally, although capacitance measurements can be used to estimate the number of vesicles released, the nature and the amount of neurotransmitter released per vesicle remains to be determined. Therefore, the use of this technique to study vesicular neurotransmitter release is limited.

An alternative technique to investigate single vesicle release at the single cell level is by means of carbon fiber microelectrode amperometry (Fig. 5; Wightman et al., 1991; Chow and von Rüden, 1995). This technique has been used in most of the research described in this thesis. In brief, the carbon fiber microelectrode is set at a potential of ~700 mV, which is sufficiently high to oxidize easily oxidizable neurotransmitters, e.g., dopamine, noradrenaline, adrenaline, serotonin and some peptides (inset A). By placing the electrode against the surface of a secretory cell, an electrochemical current can be detected when exocytosis occurs (inset B). This current is caused by the transfer of electrons during the oxidation of the secreted neurotransmitter on the electrode surface and, therefore, the size of the current is proportional to the amount of neurotransmitter released. Since exocytosis is an extremely rapid process, each exocytotic event will result in a sharp current peak, proportional to the number of neurotransmitter molecules oxidized over time. The contents of the fused vesicles can be calculated from the total charge (Q, the time integral of the current) transferred during the event according to Faraday’s law: Q/nF, where n = 2 electrons for the oxidation of one catecholamine molecule (Ciolkowski et al., 1994) and F is Faraday’s constant. So by counting the number of current peaks and by taking the integral of each current peak, it is possible to determine the number of vesicles released, and the amount of neurotransmitter secreted per vesicle (for details see the Materials and methods section in Chapter 2). Major advantages of carbon fiber microelectrode amperometry are the high temporal resolution and the high sensitivity. It is possible to measure exocytotic events at millisecond resolution and with a detection threshold of ~15 zeptomole (zepto = 10^{-21}), or ~9000 dopamine molecules, which is sufficient to detect transmitter leakage through a not yet fully formed fusion pore (foot signal; inset C). Another advantage is that endocytosis does not affect the detection of vesicular release events and that the cell interior is not disturbed (cf. capacitance measurements). Disadvantages are that exocytosis is detected only if it occurs near/under the electrode, which covers a small part of the cell surface only, and that the technique can not be used to detect neurotransmitters that are not readily oxidized, e.g., acetylcholine, GABA and glutamate.
Figure 5. Experimental set-up for amperometric recording of vesicular catecholamine release from a secretory cell using a carbon fiber microelectrode. A) Schematic drawing of the electrochemical reaction, i.e. oxidation of secreted neurotransmitter molecules, occurring during amperometric detection of neurotransmitter release. B) Amperometric recording from a PC12 cell showing vesicular catecholamine release during superfusion with high K⁺ saline (upper trace). The bar on top indicates the period of superfusion with high K⁺ saline. A single current peak, corresponding to the release of the contents of one single vesicle, is shown on an extended time scale (lower trace). The integral of the current peak corresponds with the charge (Q) transferred during the event, which is a measure for the amount of neurotransmitter secreted. C) Example of an amperometric current transient on an expanded time scale. The transient represents single vesicle release with a so-called foot signal (arrow), indicative of transmitter leakage through a not yet fully formed fusion pore.
Outline of this thesis
The CNS is a main target for many toxic substances and drugs. Historically, much neurotoxicological research focused on the postsynaptic cell. It was found that a large number of toxicants affects the postsynaptic response by modulating channel activity or by affecting ligand-binding and a large number of studies have been undertaken to elucidate the postsynaptic mechanisms underlying the observed effects. Although the knowledge on the presynaptic mechanisms of neurotransmission steadily increases and although there are several classes of environmental toxicants known to affect neurotransmitter release or neurotransmitter levels in brain (e.g. heavy metals, PCBs and certain classes of pesticides), the neurotoxicology of the presynaptic response has received little attention so far. The development of new and more sensitive techniques to measure neurotransmitter release, in combination with recent developments in genetics and biochemistry, could greatly enhance our knowledge about the neurotoxicology of exocytosis and related processes.

The research described in this thesis was conducted to gain insight into the mechanisms of modulation of neurotransmitter release by endogenous factors and toxic substances. Therefore, a model system to study vesicular neurotransmitter release was required. The rat phaeochromocytoma (PC12) cell line, derived from a rat adrenal gland phaeochromocytoma, is commonly used as an in vitro model for both neurosecretory and neuronal cells. These cells synthesize dopamine and norepinephrine (Greene and Rein, 1977a), which are presumably stored in large dense-core vesicles (Wagner, 1985). Since PC12 cells display vesicular catecholamine release upon stimulation (Chen et al., 1994), the cells were chosen as model system in the functional studies described in this thesis.

Chapter 2 provides a detailed description of the process of vesicular catecholamine release from rat PC12 cells, as measured by means of amperometry. The results serve as a characterization of the model system used in subsequent experiments of this thesis. Based on the results, it is concluded that PC12 cells release only a limited number of catecholamine-containing vesicles because of a slow rate of vesicle cycling. Furthermore, it was shown that catecholamines are released from a heterogeneous population of vesicles. The appendix describes some additional experimental results, demonstrating that exocytosis is not only limited by a slow rate of vesicle cycling, but that Ca²⁺ channel inactivation also limits the number of vesicles that can be released by depolarization. To overcome the limited number of releasable vesicles, PC12 cells were differentiated with dexamethasone into a more chromaffin-like cell type. The use of dexamethasone differentiated PC12 cells opened the door to the more neurotoxicological-oriented research as described in chapters 3-6.
Chapter 3 describes the effect of Pb\(^{2+}\) on exocytosis using PC12 cells permeabilized by ionomycin. It was shown that Pb\(^{2+}\) exerts its effect on the exocytotic machinery even in the absence of Ca\(^{2+}\). The results obtained with amperometry were combined with Ca\(^{2+}\)- and Pb\(^{2+}\)-imaging experiments using the fluorescent dye Indo-1 in confocal laser scanning microscopy. The experiments show that PC12 cells contain a considerable Pb\(^{2+}\) buffering capacity. Partial saturation of this high-affinity buffer with Pb\(^{2+}\) is sufficient to evoke exocytosis. Furthermore, mechanisms, which may be responsible for Pb\(^{2+}\)-evoked exocytosis, are suggested.

Chapter 4 describes the effects of modulation of key proteins involved in exocytosis in order to address several possible mechanisms of Pb\(^{2+}\)- and Ca\(^{2+}\)-evoked exocytosis. It was shown that modulation of PKC, CaM kinase II, calcineurin, calmodulin and synaptotagmin affects vesicular neurotransmitter release. However, the effects of modulation depend to some degree on the type of stimulus used to evoke exocytosis, suggesting distinct, but partly overlapping, pathways for Pb\(^{2+}\)- and Ca\(^{2+}\)-evoked exocytosis.

Chapter 5 describes the effect of the neurotoxic organic solvent toluene on vesicular neurotransmitter release. The results demonstrate that toluene concentration-dependently increases the basal frequency of exocytosis, due to an increase in intracellular Ca\(^{2+}\) concentration. It is concluded that the increase in basal exocytosis, which occurs at neurotoxicological relevant concentration, is caused by enhanced influx of extracellular Ca\(^{2+}\) and not by a direct effect of toluene on the exocytotic machinery.

Chapter 6 describes the effects of three selected PCBs on exocytosis. The results demonstrate that acute (<15 min) exposure to PCB 4 and PCB 126 enhances the basal frequency of vesicular neurotransmitter release, whereas the amount of neurotransmitter secreted per vesicle is unaffected. PCB 128 does neither affect the release frequency, nor the amount of neurotransmitter secreted per vesicle. Subchronic (3 days) exposure to PCB 4, PCB 126 or PCB 128 did not affect any of the parameters investigated. It is concluded that the effects of PCBs on neurotransmitter release previously found using cell populations cannot be explained by the slight enhancement of basal release. In addition, the effects on basal release frequency are observed at PCB concentrations apparently ineffective in changing dopamine transport or tyrosine hydroxylase activity.

The general discussion (Chapter 7) highlights the findings described in this thesis and is an attempt to put these findings in the context of our current understanding of exocytosis and presynaptic mechanisms of neurotoxicity.
Heterogeneity of catecholamine-containing vesicles in PC12 cells

Remco H.S. Westerink • Aart de Groot • Henk P.M. Vijverberg


Abstract

Vesicular catecholamine release has been measured amperometrically from undifferentiated rat PC12 cells using carbon fiber microelectrodes. During superfusion with high K⁺ saline, vesicular release was detected from ~50% of 200 cells investigated. On repeated stimulation the releasable pool of vesicles is rapidly depleted, while vesicle contents remains constant. Vesicular catecholamine release is not restored within 1 hr after depletion of the releasable pool. Although the distribution of the cube root of vesicle contents of many cells is apparently Gaussian, maximum likelihood analysis of single cell data demonstrates double Gaussian distributions with median vesicle contents of 141 and 293 zeptomole. It is concluded that the releasable pool of vesicles in PC12 cells is heterogeneous. In the presence of L-DOPA mean vesicle contents increases, but cessation of release cannot be prevented, indicating that the number of releasable vesicles in PC12 cells is limited by a slow rate of vesicle cycling.

Keywords: rat phaeochromocytoma cells - vesicular neurotransmitter release - ionomycin - catecholamine - l-DOPA - carbon fiber microelectrodes - amperometry
Introduction

The exocytosis of neurotransmitter-filled vesicles plays a major role in intercellular communication and involves intracellular fusion of the vesicle and plasma membranes, the formation of a fusion pore, and the subsequent release of vesicle contents. The actual release of the contents of a neurotransmitter vesicle takes a few milliseconds only (Almers et al., 1991). Exocytosis is preceded by slower processes, which proceed on a seconds to minutes time scale and include vesicle filling, translocation, docking, and priming, and is followed by the endocytosis of empty vesicles. The ensemble of these processes is known as the vesicle cycle (for review see Südhof, 1995).

PC12 cells, derived from a rat adrenal gland phaeochromocytoma, are commonly used as an in vitro model for both neurosecretory and neuronal cells. PC12 cells express a variety of receptors and ligand-gated ion channels, as well as T-, L-, N-, and P/Q-type Ca\(^{2+}\) channels (Shafer and Atchison, 1991; Liu et al., 1996). Dopamine and norepinephrine are synthesized (Greene and Rein, 1977a) and are presumably stored in large dense-core vesicles (Wagner, 1985), many of which are in the close vicinity of the plasma membrane (Kasai et al., 1999).

Real-time amperometric detection of catecholamines using carbon fiber microelectrodes (Chow and von Rüden, 1995) has revealed vesicular catecholamine release from PC12 cells on exposure to elevated K\(^+\) concentrations in the presence of extracellular Ca\(^{2+}\) (Chen et al., 1994; Taylor et al., 1999) with an average vesicle contents of \(\sim200\) zeptomole (Chen et al., 1994; Pothos et al., 1996; Zerby and Ewing, 1996a; Zerby and Ewing, 1996b; Pothos et al., 1998). Distributions of vesicle contents are skewed, but cube root-transformed data show an apparently normal distribution (Finnegan et al., 1996; Pothos et al., 1996; Zerby and Ewing, 1996a; Zerby and Ewing, 1996b; Taylor and Peers, 1999a; Taylor et al., 1999). Under the assumption that the vesicular catecholamine concentration is constant (Wightman et al., 1991), these results may suggest that catecholamines are released from a homogenous population of vesicles. These conditions appear to hold for catecholamine release from chromaffin cells (Albillos et al., 1997, but see also Glavinovic et al., 1998). However, it is uncertain whether the same conditions apply to PC12 cells, since morphometric evidence on vesicle size (Schubert et al., 1980) is not conclusive and intravesicular catecholamine concentration in PC12 cells is unknown. Here we demonstrate that catecholamines are released from a heterogeneous population of vesicles in PC12 cells.
Materials and methods

Cell culture. PC12 cells (CRL-1721; ATCC, Manassas VA, USA) and PC12 cells from the Hubrecht Laboratory (Utrecht, the Netherlands) were grown in RPMI-1640 medium (Gibco, Grand Island NY, USA) at 37 °C in a 5% CO₂ atmosphere. The culture medium was supplemented with 5% fetal calf serum (ICN, Costa Mesa CA, USA), 10% heat-inactivated horse serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin (all from Gibco). Cells were seeded in 25 cm² flasks at a density of 5·10⁵ cells/flask and subcultured in 35 mm diameter dishes at a density of 5·10⁴ cells/dish. Cell culture flasks and dishes (Nunc Inc., Naperville IL, USA) were coated with 5 µg/cm² poly-L-lysine (Sigma, St. Louis MO, USA). The culture medium was refreshed every 2-3 days. Experiments were performed 3-8 days after subculture.

Experimental protocol. Carbon fiber microelectrodes were pulled from borosilicate glass capillaries (GC150; Clark Electromedical Instruments, Pangbourne Reading, UK) containing a 10 µm Ø carbon fiber (Thornel, P-55W-2K, Amoco Corp. Greenville SC, USA). The glass-carbon fiber junction was insulated with Sylgard® elastomer (Dow Corning 182) and the carbon fiber was trimmed close to the glass junction using a scalpel blade. Pipettes were back-filled with 3 M KCl and tested for rapid and stable responses using saline solution containing 500 µM norepinephrine. Before experiments cells were washed with saline solution containing (in mM) 125 NaCl, 5.5 KCl, 1.8 CaCl₂, 0.8 MgCl₂, 20 HEPES, 24 glucose, and 36.5 sucrose at pH 7.3. The carbon fiber was polarized to 750 mV and was placed gently on the membrane surface of a PC12 cell under continuous superfusion with saline through one barrel of a theta superfusion pipette (Clarke TGC150; pipette tip Ø 80 µm) at a rate of ~150 µl/min. In general, exocytosis was evoked by superfusion with high K⁺ saline (K⁺ elevated to 125 mM and Na⁺ reduced to 5.5 mM). Following each stimulus the cell was allowed to recover for at least 5 min, during which saline was superfused. For specific experiments other drugs were added to the saline immediately before the experiment. All experiments were performed at room temperature (21-23 °C).

Data recording and analysis. Amperometric current was recorded using an EPC-7 patch clamp (List Electronic, Darmstadt, Germany), filtered at 2 kHz (8 pole Bessel filter), sampled at 4 kHz, and stored on disk for off-line analysis. Current transients associated with vesicular catecholamine release were identified and analyzed using custom-designed Labview routines (National Instruments, Austin, Texas, USA). The peak amplitude and the 50-90% rise time of events were determined from the original signal. The original signal was digitally filtered (low-pass 100 Hz, 2nd order Bessel filter) for integration of the detected events. All events and their integrals were inspected visually to remove overlapping events and artifacts in the amperometric data (<5% of the total number of events detected). Vesicle contents was calculated from the total charge (Q) transferred during the event according to Q/nF, where n = 2 electrons for the oxidation of one catecholamine molecule (Ciolkowski et al., 1994) and F is Faraday’s constant (96,485 C/mole). In general, this method allowed for the detection of quanta ≥15 zeptomole of catecholamines.
**Chemicals.** NaCl, KCl, and HEPES were obtained from Merck (Whitehouse Station NJ, USA), MgCl$_2$ and CaCl$_2$ from BDH Laboratory Supplies (Poole Dorset, UK). All other chemicals were obtained from Sigma. Saline solutions were prepared with Milli-Q® (Millipore, Bedford MA, USA) distilled water. Stock solutions of 1 mM ionomycin in dimethylsulfoxide (DMSO) and of L-DOPA in distilled water were kept at -20°C and thawed before the experiment. DMSO alone (0.5% v/v) did not affect release.

**Statistical analysis.** In order to relate vesicle contents to vesicle volume (Wightman *et al*., 1991) distributions of the cube root of vesicle contents were constructed and analyzed using custom-designed Matlab routines (The Math Works Inc., Natick, MA, USA). Cube root-transformed data were tested for normality using the Kolmogorov-Smirnov test. Maximum-likelihood estimates of the parameters of Gaussian distributions were calculated using the Matlab simplex optimization routine. A correction was made for events that were too small to be detected according to the method described by Colquhoun and Sigworth (1995). The goodness-of-fit of a double versus a single Gaussian distribution was tested by the log likelihood-ratio test (Colquhoun and Sigworth, 1995). Results were compared with Student’s *t*-test and tests for correlations were performed using Spearman’s rank correlation test. All values are reported as mean ± SD.

**Results**

During superfusion of PC12 cells with saline the frequency of quantal catecholamine release, recorded with the 10 µm diameter carbon fiber microelectrodes, was very low (<1/min). Following a switch of the superfusion to high K$^+$ saline quantal events were recorded from ~50% of the cells (*n* = 200). Exocytosis was strongly linked to depolarization, as the first events were usually detected within the first second after switching the superfusion to high K$^+$ saline and exocytosis stopped within seconds after switching back to normal saline (Fig. 1A). The amperometric events displayed typical characteristics of quantal catecholamine release. The evoked current transients had a rapid rising phase with 50-90% rise times that were generally <2.5 ms and a relatively slow exponential decay. The release of single vesicle contents was generally complete within 10-50 ms. Occasionally, foot signals, which are assumed to represent early transmitter leakage during formation of the fusion pore (Chow and von Rüden, 1995), could be resolved (Fig. 1B). The distribution of cube root of vesicle contents obtained from data pooled from 18 cells with mean vesicle contents in the range of 118 - 315 zeptomole is apparently Gaussian (Fig. 1C) with a mean ± SD of 5.67 ± 1.16 zeptomole$^{1/3}$, corresponding to a median value of 182 zeptomole. This confirms previously reported results suggesting that catecholamines are released from a homogenous population of vesicles in PC12 cells (Finnegan *et al*., 1996; Pothos *et al*., 1996; Zerby and Ewing, 1996a; Zerby and Ewing, 1996b; Taylor and Peers, 1999a; Taylor *et al*., 1999).
Factors limiting vesicular release. The number of quanta that could be released from single PC12 cells by stimulation with high K⁺ saline was limited and varied markedly with total numbers ranging between 1 and 603 (mean = 53; median = 25; n = 117 cells). In only ~14% of the cells >100 amperometric events could be detected. The vesicle contents also differed strongly between cells with mean values in the range of 90-600 zeptomole (n = 72 cells).

Since few cells released large numbers of vesicles, efforts were made to characterize the factors underlying this apparent limitation. The number of quanta released by repeated depolarizations decreased on each subsequent stimulus, despite a 5-10 min recovery interval between successive depolarizations (Fig. 2A). The average decrease of the number of exocytotic events evoked by repeated stimulation in 12 cells is illustrated in Fig. 2B. It is readily seen that the number of events decreases by approximately 50% on each subsequent stimulus, resulting in the depletion of releasable vesicles after up to six stimuli.
Reappearance of catecholamine release did not occur within the time span of experiments. When cells were repeatedly depolarized until exocytosis stopped completely, recovery of the exocytotic response was not observed within 1 hr, during which cells were superfused with saline solution \((n = 3)\). Control experiments showed that the superfusion itself had no effect on exocytosis. Cells superfused for 1 hr with saline still responded with exocytosis when stimulated with high K\(^+\) saline \((n = 3\); results not shown\). Limited numbers of events, which were comparable to those recorded from cells repeatedly stimulated with high K\(^+\) saline, were also recorded from cells which were stimulated with ionomycin \((n = 10)\) as well as from cells that were repeatedly stimulated with 200 \(\mu\)M ATP \((n = 6)\). After depletion of releasable vesicles by repeated depolarizations, superfusion with saline containing 1-5 \(\mu\)M ionomycin was unable to evoke additional release in 5 cells, and in a 6\(^{th}\) cell ionomycin evoked a marginal response only. This indicates that depletion of the depolarization-sensitive pool of vesicles parallels depletion of the Ca\(^{2+}\)-sensitive pool (results not shown).

**Figure 2.** Depletion of releasable vesicles during repeated depolarizing stimuli. (A) Amperometric traces obtained from a single PC12 cell during repeated 2 min depolarizations alternated by 5 min recovery periods. The bar on top indicates the period of superfusion with high K\(^+\) saline. (B) Relation between the exocytotic frequency and the number of successive stimulations. The exocytotic frequency is expressed as the fraction of the value obtained during the first response. Each bar represents mean ± SD of 12 cells.
Cessation of release could also be caused by depletion of catecholamines following exocytosis, resulting in less efficient vesicle filling and in an increase in the number of quanta below the detection limit. Although vesicle contents differed considerably between cells, there appeared to be no relation between vesicle contents and event number of the quanta released. Testing for trends did not reveal a statistical significant relation between vesicle contents and event number for 5 cells from which >300 events were recorded (Spearman’s rank correlation test, \( P > 0.8 \)). Since vesicle contents remains unaffected until depletion of all releasable vesicles, the intracellular concentration of catecholamines appears not to be a limiting factor for the number of releasable quanta.

To further elucidate the role of the intracellular catecholamine concentration, cells were superfused with saline containing 100 \( \mu \text{M} \) L-DOPA in between high K\(^+\) stimuli, which were repeated every 15 min. Within the first 15 min period of superfusion with L-DOPA mean vesicle contents significantly increased (\( t \)-test, \( P < 0.01 \)) and remained increased for at least 60 min when L-DOPA was superfused in between depolarizations. The kinetics of the increase in vesicle contents are described by a single exponential function with a time constant of 21 min (Fig. 3A). In addition to the increase in mean vesicle contents, L-DOPA initially caused a three-fold increase in the mean number of quanta released by the depolarizing stimulus (Fig. 3B; \( t \)-test, \( P < 0.05 \)). However, after the initial increase the number of quanta decreased with successive stimulations and releasable vesicles were depleted in a way comparable to that observed in control cells (Fig. 3B). Since the apparent charging time constant of vesicles is 21 min (Fig. 3A), these results rule out the possibility that a reduction in the availability of intracellular catecholamines causes the cessation of vesicular release in PC12 cells. Therefore, it is concluded that the depletion of the releasable vesicle pool is due to a very slow rate of vesicle cycling.
Vesicle heterogeneity in PC12 cells

Figure 3. (A) Relation between mean vesicle contents and the duration of superfusion with saline containing 100 µM l-DOPA. Release was evoked by superfusion with high K⁺ saline for 15 s, shortly after l-DOPA was removed. During the 15 min intervals of superfusion with l-DOPA-containing saline, the carbon fiber electrode voltage clamp was switched off. Each point represents mean ± SD (n = 8) and at all time points the mean vesicle contents was increased significantly as compared to control (t-test, P <0.01). The drawn line is an exponential curve fitted to the data with a maximum increase amounting to 76% and an exponential time constant of increase of 21 min.

(B) Relation between the exocytotic frequency and the number of successive stimulations with high K⁺ saline for 15 s at 10-15 min intervals, during which 100 µM l-DOPA-containing saline was superfused. The exocytotic frequency is expressed as the fraction of the value obtained during the first response, which was evoked before exposure to l-DOPA. Each bar represents mean ± SD (n = 8). During the first stimulus after l-DOPA superfusion the frequency of events was significantly higher than the control value (t-test, P <0.05).

Single-cell distributions of vesicle contents. Although it has been common practice before, the pooling of data to obtain distributions of vesicle contents containing large numbers of events appears incorrect because of an up to 7-fold difference in mean vesicle contents between cells. Therefore, only those cells from which more than 300 events were recorded during high K⁺ saline stimuli (n = 5) were used for a detailed analysis of single cell distributions of vesicle contents.

Statistical analysis demonstrates that the distributions of the cube root of vesicle contents in 4 of the 5 cells (Fig. 4) cannot be described by a single Gaussian function (Kolmogorov-Smirnov test, P <0.05). Likelihood analysis showed that fitting the four distributions by double Gaussian functions resulted in a significant improvement of the goodness-of-fit as compared to a single Gaussian (log likelihood-ratio test, P <0.005). Post-
hoc testing of the binned data presented in Fig. 4 by the $\chi^2$ goodness-of-fit test for the same four cells also showed significant deviations from single Gaussian distributions ($P < 0.05$), whereas the fits by double Gaussian functions could not be rejected ($P = 0.21 - 0.74$). The double Gaussian distribution of the cube root of vesicle contents indicates the presence of distinct populations of large and small quanta with a mean of $5.20 \pm 0.39$ zeptomole$^{1/3}$ and $6.64 \pm 0.58$ zeptomole$^{1/3}$ ($n = 4$; $t$-test, $P < 0.01$). The fitted mean values correspond to median vesicle contents of 141 and 293 zeptomole, respectively. The number of large events, as a fraction of the total number of events, is $0.50 \pm 0.09$ for the cells presented in Fig. 4B-E. The distribution in Fig. 4A, which was fitted by a single Gaussian with a mean value of 6.89 zeptomole$^{1/3}$, corresponding to a median value of 327 zeptomole, may be composed of large events mainly.

Since the cells used for analyzing the distribution of vesicle contents released many more vesicles than found on average, it is possible that the quanta detected in these experiments originate from multiple sites. Therefore, the possibility that the heterogeneity in the distributions in Fig. 4 originates from different distances between multiple sites of catecholamine release and the carbon fiber surface should be ruled out. It has been pointed out before that event rise time increases steeply with the distance between the site of release and the electrode surface (Chow and von Rüden, 1995) and that the amount of charge detected with a large flat electrode surface remains constant for distances up to 5 µm (Schroeder et al., 1992). Analysis of the events used for the distributions in Fig. 4 showed that all events had 50-90% rise times $< 2.5$ ms. If the small events originate from distant release sites, they should have slow rise times and a negative correlation between rise time and vesicle contents would be expected. Conversely, for the data presented in Fig. 4A, C, D and E rise time and vesicle contents showed a weak positive correlation (Spearman’s rank correlation test, $R_S = 0.12$, 0.11, 0.21, 0.47; $P < 0.05$), and a negative correlation was apparent only for the data of the cell presented in Fig. 4B ($R_S = -0.21; P < 0.01$). Therefore, the results demonstrate that catecholamines are released from a heterogeneous pool of vesicles in PC12 cells.
Vesicle heterogeneity in PC12 cells

Figure 4. Single cell distributions of the cube root of the vesicle contents of 5 PC12 cells from which >300 events were recorded during repeated depolarizing stimuli with high K⁺ saline. The distributions are fitted by double Gaussian functions for 4 of the 5 cells (B-E) and by a single Gaussian function for the cell in (A). The dashed lines represent the sum of the Gaussian distributions (solid lines) with the maximum likelihood estimates of mean (μ) and variance (σ) indicated in each panel. The estimated number of small events (A₁) is indicated as a percentage of the total number of events (n).
Discussion

Availability of catecholamines and vesicle cycling. PC12 cells release only a limited number of catecholamine-containing vesicles. Although previous amperometric studies have not specifically addressed the question of how many vesicles can be released from single PC12 cells, all results published thus far show a similar limitation of vesicular catecholamine release (Pothos et al., 1996; Zerby and Ewing, 1996a; Pothos et al., 1998; Taylor et al., 1999). From the increase of membrane capacitance evoked by rapid photolysis of caged Ca\(^{2+}\), undifferentiated PC12 cells have been estimated to contain a readily releasable pool of 1000 large dense-core vesicles (Kasai et al., 1996). Since the carbon fibers used in the present experiments (10 \(\mu\)m Ø) will cover up to ~20% of the cell surface, the release of 1000 vesicles from the cell would result in the detection of no more than 200 events. The present results show that, despite the competence of PC12 cells for exo- and endocytosis (Kasai et al., 1996) and despite the demonstrated expression of many of the proteins essential for the different steps of the vesicle cycle (reviewed in Malosio et al., 1999), the releasable pool of vesicles is rapidly depleted by exocytotic stimuli, e.g., high K\(^+\) saline, ionomycin, and ATP. If the availability of catecholamines would be a limiting factor, vesicle contents would be expected to decrease before cessation of release, since quantal size is reduced by inhibition of tyrosine hydroxylase (Pothos et al., 1998). However, vesicle contents remains constant over time until complete cessation of release and the cessation of release cannot be prevented by increasing the availability of catecholamines by continuous superfusion with L-DOPA (Fig. 3). These results show that, although quantal size appears to be related to catecholamine availability, the availability of catecholamines is not responsible for the cessation of vesicular release. Since the depletion of the releasable pool of vesicles by depolarization parallels the depletion of the ionomycin-releasable pool and since recovery of the releasable pool takes much longer than the time constant for charging of the vesicles in the presence of L-DOPA (Fig. 3A), it is concluded that appreciable cycling of large dense-core vesicles does not occur within the time span of the experiment.

Vesicle heterogeneity. In previous studies, distributions of the cube root of vesicle contents of PC12 cells, chromaffin cells, mast cells and pancreatic \(\beta\) cells have been fitted using single Gaussian functions (Wightman et al., 1991; Finnegan et al., 1996; Zerby and Ewing, 1996a; Taylor and Peers, 1999a). All distributions of vesicle contents of PC12 cells were based on data from multiple cells in order to obtain sufficiently large numbers of events. However, the present results demonstrate large (6- to 7-fold) differences in mean vesicle contents between cells. Maximum likelihood analysis of single cell data (Fig. 4) shows that
double Gaussian curves are generally required to describe the distribution of the cube root of vesicle contents of single PC12 cells and demonstrates the existence of two distinct classes of catecholamine-containing vesicles, which differ in their median vesicle contents by a factor of ~2. This difference is much smaller than the difference in mean vesicle contents between cells, and may be obscured in the distributions of pooled data that were considered previously (cf. Fig. 1C).

Vesicle heterogeneity should be reflected in either differences in vesicle size or in differences in vesicular catecholamine contents. Morphometric measurements did not reveal distinctly sized classes of large dense-core vesicles in PC12 cells, as the distribution of vesicle diameter is approximately Gaussian (Schubert et al., 1980). However, like previous results on the distribution of vesicle contents, the distribution of vesicle size is also based on data pooled from multiple cells. If it is assumed that the observed two-fold difference in vesicle contents is exclusively caused by differences in vesicle size, the mean diameter of the large and small populations of vesicles would differ by a factor of 1.28. Such a difference would be difficult to resolve from the published distribution of vesicle diameter, since its standard deviation is approximately 25% of the mean (Schubert et al., 1980).

The two-fold difference in vesicle contents could be explained by release of aggregated vesicles, as observed at a relatively high frequency of 30% in mast cell degranulation (Alvarez de Toledo and Fernandez, 1990), or by compound fusion of vesicles, which may occur at the rare incidence of ~0.4% in chromaffin cells (Oheim et al., 1999). In order to obtain a number of large events equal to 50% of the total number of events (Fig. 4), 67% of the vesicles should be aggregated or be released simultaneously. Although this seems an unlikely high percentage, vesicle aggregation and compound fusion cannot be excluded at present.

It is known that PC12 cells release the catecholamines dopamine and norepinephrine (Greene and Rein, 1977a), and that the large dense-core vesicles in these cells contain catecholamines as well as ATP (Wagner, 1985). The question whether the different classes of vesicles contain different types of catecholamines or contain ATP and catecholamines at different ratios cannot be answered at present and would require a quite advanced analysis of single vesicle contents.

Acknowledgements

We thank Dr. Leon Tertoolen (Hubrecht Laboratory, Utrecht, The Netherlands) for donation of PC12 cells, Gina van Kleef for assistance in cell culture and Dr. Sam Agulian (Yale University, West Haven CT, USA) for donation of carbon fibers.
Ca\textsuperscript{2+} channel inactivation limits exocytosis during maintained depolarization in PC12 cells

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Abstract

Vesicular release of catecholamines from undifferentiated rat PC12 cells has been measured by amperometry with carbon fiber microelectrodes. Exocytosis was evoked by whole-cell superfusion of intact cells with saline containing 125 mM K\textsuperscript{+}. Vesicular release requires the presence of external Ca\textsuperscript{2+} and stimuli \( \geq 60 \text{ mM K}^+ \), corresponding to a voltage threshold close to -20 mV. During maintained depolarization, the event frequency gradually declines to zero, due to voltage-dependent inactivation of Ca\textsuperscript{2+} channels. Vesicle cycling limits the releasable pool of vesicles in PC12 cells (Westerink et al., 2000), whereas inactivation of high voltage-activated Ca\textsuperscript{2+} channels limits release during maintained depolarization.

Keywords: rat phaeochromocytoma cells - vesicular neurotransmitter release - ionomycin - catecholamine - Ca\textsuperscript{2+} channels - carbon fiber microelectrodes - amperometry
Introduction

Advances in amperometric techniques have enabled real-time electrochemical detection of vesicular catecholamine release using carbon fiber microelectrodes (Chow and von Rüden, 1995; Kelly and Wightman, 1986). Quantal release has been studied from a variety of secretory and neuronal cells by this technique, including adrenal chromaffin cells (Wightman et al., 1991), mast cells (Alvarez de Toledo et al., 1993), PC12 cells (Chen et al., 1994), invertebrate neurons (Bruns and Jahn, 1995; Chen and Ewing, 1995), pancreatic cells (Zhou and Misler, 1996), gonadotroph and melanotroph cells (Billiard et al., 1997; Paras and Kennedy, 1995), glomus cells (Urena et al., 1994) and rat midbrain neurons (Pothos et al., 1996).

PC12 cells, derived from a rat adrenal gland phaeochromocytoma, are commonly used as an in vitro model both for neurosecretory and for neuronal cells. Undifferentiated PC12 have a 5-12 µm soma diameter and few extensions of the plasma membrane (Greene and Tischler, 1976). These cells synthesize and store dopamine and norepinephrine, which are released upon depolarization in a Ca\(^{2+}\)-dependent way (Baizer and Weiner, 1985; Greene and Rein, 1977a; Greene and Rein, 1977b; Rabe et al., 1987). The catecholamines are presumably stored in large dense-core vesicles (Wagner, 1985), many of which are in close vicinity of the plasma membrane (Kasai et al., 1999). Vesicular catecholamine release, observed on exposure of PC12 cells to elevated K\(^{+}\) concentrations, requires the presence of extracellular Ca\(^{2+}\) (Chen et al., 1994; Taylor et al., 1999). Apart from voltage-gated Na\(^{+}\)-channels and several types of K\(^{+}\)-channels, PC12 cells express T-, L-, N-, and P/Q-type Ca\(^{2+}\)-channels (reviewed in Shafer and Atchison, 1991; and Liu et al., 1996). In addition to voltage-gated channels, PC12 cells express a variety of receptors and ligand-gated ion channels, e.g., nicotinic and muscarinic ACh receptors, and P\(_{2}\) subtypes of ATP receptors (Shafer and Atchison, 1991). Elevation of the intracellular Ca\(^{2+}\) concentration caused by activation of these receptors leads to quantal catecholamine release, either directly or indirectly (Fisher and Burgoyne, 1999; Zerby and Ewing 1996a).

In this study, quantal catecholamine release was measured from undifferentiated PC12 cells using carbon fiber microelectrode amperometry. The results demonstrate that exocytosis in PC12 cells is limited by inactivation of high voltage-activated Ca\(^{2+}\) channels during maintained depolarization.
Materials and methods

Cell culture, carbon fiber microelectrode fabrication, data recording and analysis were as described previously (Westerink et al., 2000). Before the experiment a culture dish containing PC12 cells was washed twice with saline solution containing (in mM) 125 NaCl, 5.5 KCl, 1.8 CaCl2, 0.8 MgCl2, 20 HEPES, 24 glucose, and 36.5 sucrose at pH 7.3. The culture dish was placed on the stage of an inverted phase-contrast microscope and the carbon fiber was placed gently on the cell surface membrane. The cell of interest was superfused continuously with saline at a rate of ~150 µl/min through one barrel of a theta superfusion pipette (Clarke TGC150; pipette tip Ø 80 µm). In general, exocytosis was evoked by switching to the other barrel of the theta superfusion capillary, containing saline with K+ elevated to 125 mM and Na+ reduced to 5.5 mM (125 mM K+ saline), using a step-motor-controlled micromanipulator. Each stimulus was followed by a recovery period of at least 5 min, during which the cell was superfused with saline. All experiments were performed at room temperature (21-23 °C).

NaCl, KCl, and HEPES were obtained from Merck (Whitehouse Station NJ, USA), MgCl2 and CaCl2 from BDH Laboratory Supplies (Poole Dorset, UK). All other chemicals were obtained from Sigma. Saline solutions were prepared with Milli-Q® (Millipore, Bedford MA, USA) distilled water. Aliquots of a 1 mM stock solution of ionomycin in dimethylsulfoxide (DMSO) were mixed with saline to obtain a final concentration of 1-5 µM ionomycin immediately prior to the experiments. DMSO alone (0.5% v/v) did not affect release. All values are reported as mean ± SD.

Results

In order to record quantal catecholamine release from PC12 cells a 10 µm diameter carbon fiber microelectrode was gently placed against the intact cell, which was continuously superfused with saline. Placement of the electrode generally did not evoke exocytosis. In few cases of mechanical stimulation of the cell by the carbon fiber, exocytotic events were observed. These cells were not used for analysis. In general, exocytosis was strongly linked to depolarization, as the first events were usually detected within the first second after switching the superfusion to 125 mM K+ saline and exocytosis stopped within seconds after switching back to normal saline. As shown previously (Westerink et al., 2000), only a limited number of exocytotic events could be evoked in PC12 cells due to a slow rate of vesicle cycling. Further efforts were made to investigate the existence of additional factors responsible for the gradual decrease in event frequency and the eventual complete cessation of vesicular catecholamine release in PC12 cells.

During a maintained depolarization of cells by superfusion with 125 mM K+ saline the frequency of exocytotic events gradually decreased to zero (Fig. 1A). In order to determine the role of membrane potential, cells were depolarized by superfusion with 125 mM K+, nominal Ca2+-free saline for a 3 min period. Exocytotic events were not observed in the
Ca$^{2+}$ channel inactivation limits exocytosis

absence of extracellular Ca$^{2+}$, despite the membrane depolarization. Subsequent switching
to 125 mM K$^+$, Ca$^{2+}$-containing saline also failed to evoke exocytosis, whereas addition of
5 µM of the Ca$^{2+}$ ionophore ionomycin, which permeabilizes the membrane for Ca$^{2+}$, caused a robust exocytotic response (Fig. 1B). Superfusion with saline containing 125 mM K$^+$ and 1-5 µM ionomycin failed to evoke exocytosis under nominal Ca$^{2+}$-free conditions, whereas switching to normal external Ca$^{2+}$ did evoke exocytosis. Exocytosis rapidly ceased after switching back to nominal Ca$^{2+}$-free saline, despite the continued presence of 125 mM K$^+$ and ionomycin (Fig. 1C). These findings confirm that the influx of external Ca$^{2+}$ is required for exocytosis and also demonstrate that prolonged depolarization prevents the influx of Ca$^{2+}$, but does not eliminate the Ca$^{2+}$ requirement of exocytosis.

To further explore the nature of the Ca$^{2+}$ influx pathway, the voltage threshold for exocytosis was determined. The exocytotic response was greatly attenuated when cells were stimulated with saline containing 55-60 mM K$^+$. Responsive cells (n = 12) were stimulated for ~15 s by superfusion with saline containing 55-60 mM K$^+$ and, after 5 min of wash, were stimulated again for ~15 s by superfusion with 125 mM K$^+$ saline. All cells showed an exocytotic response during the 125 mM K$^+$ stimulus. However, during stimulation with 55-60 mM K$^+$, 5 cells failed to respond at all (Fig. 1D). The other 7 cells showed exocytosis during the modest depolarizing stimulus, but in 5 cells the number of events was greatly attenuated (<15%) as compared to the number of events evoked by the second stimulus. In the remaining 2 cells the number of events during the first stimulus amounted to 50% and 120% of the number recorded during the second stimulus. When cells were stimulated by superfusion with 65-70 mM K$^+$-containing saline, the number of events was approximately equal to that evoked during a subsequent stimulus with 125 mM K$^+$ saline (116 ± 35%; n = 10). Thus, it appears that depolarization by 55-60 mM K$^+$ is close to the voltage threshold for exocytosis in PC12 cells. Assuming an intracellular K$^+$ concentration within the range of 100-150 mM, the voltage threshold is calculated to be close to -20 mV. The combined results demonstrate that Ca$^{2+}$ required for exocytosis enters the cell predominantly through high voltage-activated Ca$^{2+}$ channels. The observed cessation of release during maintained depolarization appears to be caused by slow inactivation of these high voltage-activated Ca$^{2+}$ channels.
Figure 1. Voltage and Ca$^{2+}$ dependence of vesicular catecholamine release from PC12 cells. (A) Cessation of release during prolonged depolarization. Amperometric recording from a single cell during a prolonged depolarization with 125 mM K$^+$ saline. During the depolarization the exocytotic frequency gradually decreases and finally vesicular release stops completely. The same result was obtained in 7 other cells. (B) Prolonged depolarization with 125 mM K$^+$, nominal Ca$^{2+}$-free saline fails to evoke catecholamine release. After re-introduction of 1.8 mM Ca$^{2+}$, release is not evoked despite the continued presence of 125 mM K$^+$ until the Ca$^{2+}$ ionophore ionomycin (5 µM) is superfused. The same result was obtained in 5 other cells. (C) Exocytotic events are observed only in the presence of external Ca$^{2+}$, whereas 125 mM K$^+$ and 3 µM ionomycin in the absence of Ca$^{2+}$ do not evoke release. The same result was obtained in 2 other cells. (D) Voltage dependence of Ca$^{2+}$ influx required for exocytosis. A depolarization with 55 mM K$^+$ fails to evoke vesicular catecholamine release, whereas a subsequent depolarization of the same cell with 125 mM K$^+$ after a 5 min recovery period evokes a characteristic burst of events.
Ca\textsuperscript{2+} channel inactivation limits exocytosis

Discussion

Throughout the experiments it was observed that only a limited number of catecholamine-containing vesicles could be released from PC12 cells. The present results indicate that inactivation of Ca\textsuperscript{2+} channels limits the release of catecholamine-containing vesicles during maintained depolarization. The depolarization-induced release depends on the influx of extracellular Ca\textsuperscript{2+} through high voltage-activated Ca\textsuperscript{2+} channels and the observed cessation of release during maintained depolarization appears due to inactivation of these channels (Fig. 1).

The ability of ionomycin to evoke Ca\textsuperscript{2+}-dependent exocytosis after prolonged depolarization (Fig. 1B) and the possibility to evoke multiple responses by repeated depolarizations, provided that the cell is allowed to recover in normal saline (Westerink et al., 2000), demonstrate that the cessation of vesicular release during maintained depolarization is due to inactivation of Ca\textsuperscript{2+} channels. PC12 cells have been shown to express low voltage-activated (LVA) T-type, and high voltage-activated (HVA) L-, N-, and P/Q-type Ca\textsuperscript{2+} channels (reviewed in Shafer and Atchison, 1991; and Liu et al., 1996). In undifferentiated PC12 cells, dihydropyridine-sensitive, L-type Ca\textsuperscript{2+} channels are the major type of HVA Ca\textsuperscript{2+} channels (Usowicz et al., 1990) and these channels appear to form the major pathway for the influx of Ca\textsuperscript{2+} required for catecholamine release from undifferentiated PC12 cell cultures (Avidor et al., 1994; Watanabe et al., 1999). The present results indicate that the voltage threshold for activation of the Ca\textsuperscript{2+} channels mediating the influx of Ca\textsuperscript{2+} required for vesicular release is close to –20 mV (Fig. 1D).

This is similar to the activation threshold of a slowly inactivating Ba\textsuperscript{2+} current in whole-cell voltage clamp experiments on undifferentiated PC12 cells. Steady-state inactivation of this current is virtually complete at 0 mV (Streit and Lux, 1990). In cells superfused with 125 mM K\textsuperscript{+}, nominal Ca\textsuperscript{2+}-free saline at least 2 min of pre-depolarization were required before vesicular release in response to addition of extracellular Ca\textsuperscript{2+} was inhibited completely (Fig. 1B), essentially confirming that Ca\textsuperscript{2+} current inactivation is very slow in the intact PC12 cell. The slow time course of inactivation and the duration of exocytotic responses observed are consistent with the slow Ca\textsuperscript{2+} transients observed in undifferentiated PC12 cells using Ca\textsuperscript{2+} sensitive fluorescent dyes. The depolarization-evoked initial rise of internal Ca\textsuperscript{2+}, mediated by dihydropyridine-sensitive Ca\textsuperscript{2+} channels, was markedly reduced by pre-depolarization under Ca\textsuperscript{2+}-free conditions, as was the initial rate of release of \textsuperscript{3H}-dopamine from a population of PC12 cells (Di Virgilio et al., 1987). The combined results indicate that the slow initial Ca\textsuperscript{2+} transient is responsible for catecholamine release and the rapid initial phase of \textsuperscript{3H}-dopamine release actually represents vesicular release. Although the present results are consistent with L-type channels mediating Ca\textsuperscript{2+} influx required for...
vesicular catecholamine release in undifferentiated PC12 cells, mainly N-type Ca\textsuperscript{2+} channels appear to be involved in the release from dexamethasone-treated PC12 cells (Taylor and Peers, 1999b). The difference may not simply be due to enhanced expression of N-type channels in dexamethasone-treated cells, as in PC12 cells differentiated with nerve growth factor, which also enhances the expression of N-type channels (Usowicz et al., 1990), the release of catecholamines from the cell population depends mainly on Ca\textsuperscript{2+} influx through L-type channels (Kanwal et al., 1997). Thus, it appears that different types of Ca\textsuperscript{2+} channels are able to support vesicular catecholamine release and that the role of specific subtypes of Ca\textsuperscript{2+} channels in release may depend on the state of differentiation of PC12 cells.

**Acknowledgements**

We want to thank Dr. Leon Tertoolen (Hubrecht Laboratory, Utrecht, The Netherlands) for donation of PC12 cells, Gina van Kleef for assistance in cell culture, Dr. Sam Agulian (Yale University, West Haven CT, USA) for donation of carbon fibers, and Aart de Groot for excellent technical assistance and software development.
Ca\(^{2+}\) channel inactivation limits exocytosis
Ca\textsuperscript{2+}-independent vesicular catecholamine release in PC12 cells by nanomolar concentrations of Pb\textsuperscript{2+}

Remco H.S. Westerink • Henk P.M. Vijverberg


Abstract

Effects of Pb\textsuperscript{2+} on vesicular catecholamine release in intact and ionomycin-permeabilized PC12 cells were investigated using carbon fiber microelectrode amperometry. Changes in intracellular Pb\textsuperscript{2+} and Ca\textsuperscript{2+} were measured from indo-1 fluorescence by confocal laser scanning microscopy. Depolarization of intact cells and superfusion of permeabilized cells with saline containing $\geq 100$ $\mu$M Ca\textsuperscript{2+} rapidly evokes quantal catecholamine release. Superfusion with up to 10 $\mu$M Pb\textsuperscript{2+}-containing saline evokes release of similar catecholamine quanta after a concentration-dependent delay. Thresholds to induce exocytosis within 30 min of exposure are between 1 and 10 $\mu$M Pb\textsuperscript{2+} in intact cells and between 10 and 30 nM Pb\textsuperscript{2+} in permeabilized cells. Additional inhibition of exocytosis occurs in permeabilized cells exposed to 10 $\mu$M Pb\textsuperscript{2+}. Using membrane-impermeable and -permeable chelators it is demonstrated that intracellular Ca\textsuperscript{2+} is not required for Pb\textsuperscript{2+}-induced exocytosis. In indo-1-loaded cells Pb\textsuperscript{2+} reduces the fluorescence intensity after a concentration-dependent delay, whereas the fluorescence ratio, indicating intracellular Ca\textsuperscript{2+} concentration, remains unchanged. The delay to detect an increase in free intracellular Pb\textsuperscript{2+} ($\geq 30$ nM) is much longer than the delay to Pb\textsuperscript{2+}-induced exocytosis, indicating that cytoplasmic components buffer Pb\textsuperscript{2+} with high affinity. It is concluded that Pb\textsuperscript{2+} acts as a high-affinity substitute for Ca\textsuperscript{2+} to trigger essential steps leading to vesicular catecholamine release, which occurs when only $\sim 20\%$ of the intracellular high-affinity binding capacity ($\sim 2$ attomol/cell) is saturated with Pb\textsuperscript{2+}.

Keywords: rat phaeochromocytoma cells - vesicular catecholamine release - ionomycin - carbon fiber microelectrode amperometry - indo-1 - Pb\textsuperscript{2+}-imaging - Ca\textsuperscript{2+}-imaging
Exocytosis is a complex, Ca\textsuperscript{2+}-dependent process involving docking and priming of vesicles, intracellular fusion of the vesicle and plasma membranes, the formation of a fusion pore, and the release of vesicle contents. Exocytosis is tightly regulated by a variety of highly conserved proteins (for review see Südhof, 1995; Martin, 1997; Lin and Scheller, 2000). Several proteins associated with exocytosis, which require Ca\textsuperscript{2+} as a cofactor, are also activated by Pb\textsuperscript{2+}. Pb\textsuperscript{2+} is far more potent than Ca\textsuperscript{2+} as an activator of calmodulin (Habermann et al., 1983; Kern et al., 2000), PKC (Tomsig and Suszkiw, 1995; Sun et al., 1999), calcineurin (Kern and Audesirk, 2000), and synaptotagmin (Bouton et al., 2001). Furthermore, extracellular Pb\textsuperscript{2+} inhibits voltage-gated Ca\textsuperscript{2+} currents (Reuveny and Narahashi, 1991; Audesirk and Audesirk, 1993; Oortgiesen et al., 1993; Sun and Suszkiw, 1995). Under low Ca\textsuperscript{2+} conditions, Pb\textsuperscript{2+} may permeate through Ca\textsuperscript{2+} channels (Tomsig and Suszkiw, 1991) and reduce inactivation of Ca\textsuperscript{2+} channels at an intracellular site (Sun and Suszkiw, 1995).

Two main effects of Pb\textsuperscript{2+} on neurotransmitter release have been reported. The first effect observed is inhibition of evoked neurotransmitter release, which has been attributed to a reduced influx of Ca\textsuperscript{2+} due to Ca\textsuperscript{2+} channel block. The second, delayed effect of Pb\textsuperscript{2+} is an enhancement of spontaneous neurotransmitter release, which occurs irrespective of the presence of extracellular Ca\textsuperscript{2+} and has been attributed to intracellular effects of Pb\textsuperscript{2+} (Manalis and Cooper, 1973; Tomsig and Suszkiw, 1993; Bressler et al., 1996; Braga et al., 1999). The enhancement of the frequency of miniature endplate potentials at the frog neuromuscular junction (Manalis and Cooper, 1973) and of excitatory postsynaptic currents in rat hippocampal neurons (Braga et al., 1999) indicate that Pb\textsuperscript{2+} facilitates synaptic vesicle release. However, measurements from chromaffin cell populations provide only circumstantial evidence for a vesicular origin of catecholamines released by the action of Pb\textsuperscript{2+} (Tomsig and Suszkiw, 1996).

Intracellular effects of Pb\textsuperscript{2+} have been investigated mainly in bovine chromaffin cells. Release of [\textsuperscript{3}H]-norepinephrine from populations of digitonin-permeabilized chromaffin cells exposed to a saline solution containing Pb\textsuperscript{2+} buffered by EGTA occurs at a calculated threshold concentration of ~1 nM free Pb\textsuperscript{2+}. In addition, in fura-2-loaded, intact chromaffin cell populations exposed to saline solution containing Pb\textsuperscript{2+} buffered by citric acid the estimated intracellular free Pb\textsuperscript{2+} concentration required to induce release is ≤10 pM, whereas the extracellular free Pb\textsuperscript{2+} concentration demonstrated to induce neurotransmitter release is in the micromolar range (Tomsig and Suszkiw, 1990). It should be noted that these results have been obtained under rather complex experimental conditions, in which extracellular Pb\textsuperscript{2+} was buffered with citric acid or EGTA, which have micromolar and...
picomolar affinity for Pb\(^{2+}\), respectively (Martell and Smith, 1974), i.e., much lower and similar to the affinity of several intracellular proteins involved in exocytosis. In addition, fura-2 has picomolar affinity for Pb\(^{2+}\) and its spectrum in the presence of Pb\(^{2+}\) is very similar to that in the presence of Ca\(^{2+}\) (Tomsig and Suszkiw, 1990; Kerper and Hinkle, 1997). Thus, fura-2 is likely to compete with intracellular proteins for Pb\(^{2+}\) and is not suited to be used in combination with low-affinity Pb\(^{2+}\) buffers, like citric acid. More recently, facilitation of Pb\(^{2+}\) entry by Ca\(^{2+}\) store depletion was shown by using indo-1 to indicate changes in intracellular free Pb\(^{2+}\) concentration (Kerper and Hinkle, 1997).

The PC12 cell line is an extensively characterized in vitro model for neurotransmitter release. PC12 cells synthesize dopamine and norepinephrine (Greene and Rein, 1977a), which are presumably stored in large dense-core vesicles (Wagner, 1985). PC12 cells express a variety of receptors, voltage- and ligand-gated ion channels (Shafer and Atchison, 1991; Liu et al., 1996) and can be differentiated with glucocorticoids towards a chromaffin-like cell type with increased Ca\(^{2+}\) current, catecholamine synthesis and release, and rapid endocytosis following exocytosis, (Schubert et al., 1980; Tischler et al., 1983; Elhamdani et al., 2000). Previous reports demonstrate that addition of >5 µM Pb\(^{2+}\) to external saline is required to induce neurotransmitter release from undifferentiated PC12 cell populations (Bressler et al., 1996). However, so far Pb\(^{2+}\)-induced exocytosis has not been quantitatively coupled with an increase in intracellular Ca\(^{2+}\) or Pb\(^{2+}\) concentration. The aim of this study was to determine the origin of Pb\(^{2+}\)-induced neurotransmitter release and to investigate whether Pb\(^{2+}\) stimulates release directly or indirectly through an increase in intracellular Ca\(^{2+}\) concentration.

Effects of Pb\(^{2+}\) on vesicular catecholamine release from single PC12 cells were investigated using carbon fiber microelectrode amperometry (Wightman et al., 1991; Chow and von Rüden, 1995). Direct intracellular effects were measured in ionomycin-permeabilized cells exposed to Pb\(^{2+}\). In addition, the quenching of indo-1 fluorescence was used to monitor intracellular Pb\(^{2+}\) and changes in the ratio of the emission spectrum were used to monitor intracellular Ca\(^{2+}\) concentration. The results demonstrate that nanomolar concentrations of intracellular Pb\(^{2+}\) evoke vesicular catecholamine release from PC12 cells, which appear to contain a considerable capacity for intracellular high-affinity binding of Pb\(^{2+}\).
Materials and methods

**Materials.** NaCl, KCl, Mg(NO$_3$)$_2$, glucose, sucrose, HEPES, and NaOH (Aristar quality) were obtained from BDH Laboratory Supplies (Poole Dorset, UK); indo-1 pentapotassium salt, indo-1 AM, calcium calibration buffer kit with magnesium #2, and tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) from Molecular Probes (Leiden, The Netherlands); Pb(NO$_3$)$_2$ from Alfa Aesar (Johnson Matthey, Karlsruhe, Germany); Ca$^{2+}$-free ionomycin from Calbiochem (Darmstadt, Germany). All other chemicals were obtained from Sigma (St. Louis MO, USA). Saline solutions were prepared with de-ionized millipore water (Milli-Q®; resistivity >10 MΩ·cm). Immediately after preparation all saline solutions were filtered using a Millipore GSWP 0.22 µm filter (Millipore, Bedford MA, USA) and stored in thoroughly cleaned and rinsed glass bottles at -20 °C until use. Stock solutions of 2 mM ionomycin Ca$^{2+}$-salt in DMSO and 100 mM TPEN in ethanol were kept at -20 °C, and were thawed before the experiment. Stock solutions of 1.4 mM Ca$^{2+}$-free ionomycin in DMSO and 100 µM indo-1 pentapotassium salt in distilled water were kept at 4 °C. Stock solutions of 1 mM indo-1 AM in DMSO and 50 mM Pb(NO$_3$)$_2$ in distilled water were prepared immediately before use, and diluted in external solution to obtain the desired concentrations. DMSO alone (0.5% v/v) did not affect release.

**Cell culture.** PC12 cells (Greene and Tischler, 1976; ATCC CRL-1721; cultured for 10 passages) were grown essentially as described previously (Westerink et al., 2000), with some modifications. For confocal laser scanning microscopy (CLSM) cells were subcultured on 24 mm diameter borosilicate glass coverslips (Merck, Whitehouse Station NJ, USA) at a density of 4·10$^4$ cells/coverslip. For all experiments cells were differentiated in culture medium (RPMI 1640, Gibco, Grand Island NY, USA) supplemented with 5 µM dexamethasone (Genfarma, Zaandam, The Netherlands) starting 2 d after subculturing. Culture dishes and coverslips were coated with 5 µg/cm$^2$ poly-L-lysine (Sigma, St. Louis MO, USA). The culture medium was refreshed every 2-3 d. Experiments were performed 7-10 d after subculturing, i.e., 5-8 d after initiating differentiation.

**Carbon fiber microelectrode amperometry.** Carbon fiber microelectrode (Ø 10 µm) fabrication and data recording and analysis were as described previously (Westerink et al., 2000). Before experiments cells were washed twice with saline solution containing (in mM) 125 NaCl, 5.5 KCl, 2 CaCl$_2$, 0.8 MgCl$_2$, 10 HEPES, 24 glucose, and 36.5 sucrose at pH 7.3 adjusted with NaOH. The carbon fiber, polarized to 700 mV, was placed gently on the membrane surface of a PC12 cell under continuous superfusion with saline through one barrel of a theta superfusion pipette (Clarke TGC150; pipette tip Ø 80 µm) at a rate of ~150 µl/min. In intact cells, exocytosis was evoked by superfusion with high K$^+$ saline (KCl elevated to 125 mM and NaCl reduced to 5.5 mM). Ca$^{2+}$, Pb$^{2+}$, and drugs were added to the saline immediately before the experiment. Ca$^{2+}$ and Pb$^{2+}$ concentrations indicated in the results refer to the metal concentrations added to nominal Ca$^{2+}$- and Pb$^{2+}$-free saline, which contained ~0.4 µM Ca$^{2+}$ and ~20 nM Pb$^{2+}$ (see below). All experiments were performed at room temperature (21-23 °C).
**Confocal laser scanning microscopy.** For imaging experiments, cells grown on glass coverslips were incubated with 10 µM indo-1 AM in saline solution at 37 °C for 45 min. After incubation cells were washed twice with nominal Ca²⁺-free saline and kept at 37 °C to allow intracellular de-esterification of indo-1 AM. After 30 min cells were placed on the stage of a Nikon Diaphot 300 inverted microscope, connected to a Nikon RCM 8000 confocal laser scanning unit equipped with an argon UV laser (excitation wavelength 351 nm) and a water immersion objective (Nikon CF Fluor 40x, 1.15 numerical aperture, 0.20 mm working distance). Ratio images were recorded with two photomultipliers and corrected for background and shading. Optics and settings of the system were standardized for proper comparison of the ratio images and intensities from different experiments. For the pinhole size used (2.0 µm confocality) laser power was set at 5-10 µW with a voltage ratio of 490/600. Emission filters of 380-440 nm and 440-630 nm were used for photomultipliers 1 and 2, respectively. Images were collected by averaging the ratio of 8 scans, one scan taking 64 ms. The ratio images were displayed in real-time. Intensities recorded by the two photomultipliers and the ratios of these intensities were calculated with Nikon/Os9 software.

**Calibration of indo-1.** Emission spectra of indo-1 pentapotassium salt (300-500 nM) were recorded using a LS 50B Perkin-Elmer spectrofluorometer and analyzed using FL Winlab (Perkin-Elmer, Norwalk CT, USA). Fluorescence was measured at room temperature with an excitation wavelength of 351 nm and 5 nm slit width. Emission was measured in the wavelength range of 360-630 nm. Slit widths were 8 and 5 nm for 300 and 500 nM indo-1, respectively. Apart from indo-1, none of the chemicals used showed detectable autofluorescence.

Emission spectra of 500 nM indo-1 pentapotassium salt in nominal Ca²⁺-free saline and after addition of various concentrations of Ca²⁺ are shown in Fig. 1A. After addition of 5 mM EGTA (curve f in Fig. 1A) and in 10 mM EGTA-containing calibration buffers (Molecular Probes) (Fig. 1B) the emission spectrum of indo-1 displayed an approximate 20 nm blue-shift and enhanced sensitivity to Ca²⁺. Since most chelators are membrane impermeable and, moreover, have several orders of magnitude higher affinities for Pb²⁺ than for Ca²⁺, chelators were not used in imaging experiments. The emission spectrum of intracellular indo-1 was also determined in differentiated PC12 cells. Cells were harvested mechanically, resuspended in saline at a concentration of 2·10⁶ cells/ml, loaded with indo-1 AM as described above, washed twice with nominal Ca²⁺-free saline, and diluted to a concentration of 1·10⁶ cells/ml in nominal Ca²⁺-free saline at 37 °C. After 30 min cells were placed in a cuvette with stir bar at room temperature to record the emission spectrum of indo-1. The spectrum of intracellular indo-1 had the same isosbestic point as spectra recorded in saline, but differed markedly from spectra recorded in the presence of EGTA (c.f., curve g in Fig. 1A, B). Permeabilizing the cells by addition of 5 µM ionomycin Ca²⁺-salt did not change the isosbestic point of the indo-1 spectrum and yielded a fluorescence ratio (intensity at 380-440 nm/intensity at 440-630 nm) of 0.96 ± 0.07 (n = 21) identical to the value of 0.94 ± 0.03 (n = 3) obtained in 5 µM Ca²⁺-containing saline. The calibration curves demonstrate that spectra recorded in the absence of EGTA are suitable for comparison with intracellular conditions. Emission spectra of 500 nM indo-1 pentapotassium salt in 5 µM ionomycin Ca²⁺-salt containing, nominal Pb²⁺-free saline and after addition of various concentrations of Pb²⁺ (Fig. 1D) show concentration-dependent quenching of indo-1 fluorescence by Pb²⁺. At ≥300 nM Pb²⁺ a slight change in spectral properties was observed.
Figure 1. (A) Emission spectra of indo-1 with Ca\textsuperscript{2+} in the absence of chelators. Spectra were obtained in nominal Ca\textsuperscript{2+}-free saline with 500 nM indo-1 (a) and after subsequent additions of Ca\textsuperscript{2+} to obtain nominal concentrations of 5, 25, 100, and 250 µM Ca\textsuperscript{2+} (b-e) or after addition of 5 mM EGTA (f). The fluorescence spectrum from an indo-1-loaded cell suspension in external saline was also recorded (g). (B) Emission spectra of indo-1 in the presence of 10 mM EGTA, using a calibration solution (Molecular Probes) with 500 nM indo-1, 1 mM free Mg\textsuperscript{2+} and 0, 0.065, 0.150, 0.602, 1.35 and 39 µM free Ca\textsuperscript{2+} (a-f). For comparison the intracellular spectrum of indo-1 (curve g in A) is also reproduced. Note the increased Ca\textsuperscript{2+}-sensitivity in the presence of EGTA and a ~20 nm shift in isosbestic point, which is not observed for the intracellular spectrum of indo-1. (C) Relation between the Ca\textsuperscript{2+} concentration and the intensity ratio (\(F_{\text{max}} - F_{\text{min}}\)) (\(n = 3\); SD bars are smaller than the symbol size). The drawn line is based on a Hill equation with slope 2 fitted to the data with EC\textsubscript{50} = 47.3 µM, \(F_{\text{max}} = 1.13, F_{\text{min}} = 0.80\) and with an estimated background concentration of 0.4 µM Ca\textsuperscript{2+}. (D) Emission spectra of indo-1 with Pb\textsuperscript{2+}. Spectra were obtained in a cuvette containing nominal Ca\textsuperscript{2+}- and Pb\textsuperscript{2+}-free saline with 5 µM ionomycin Ca\textsuperscript{2+}-salt and 500 nM indo-1 (a) and after subsequent additions of Pb\textsuperscript{2+} to obtain nominal concentrations of 0.03, 0.1, 0.3, 1, and 10 µM Pb\textsuperscript{2+} (b-f). (E) Relation between the Pb\textsuperscript{2+} concentration and indo-1 fluorescence intensity (380-440 nm) at 5 µM Ca\textsuperscript{2+} (closed circles; \(n = 3\)) and at 1 mM Ca\textsuperscript{2+} (open circles; \(n = 3\)). The drawn lines are based on a Hill equation with slope 2 fitted to the data with IC\textsubscript{50} = 140 nM and 170 nM, \(F_{\text{max}} = 0.95\) and 0.92, \(F_{\text{min}} = 0.09\) and 0.10, respectively, and with an estimated background concentration of 25 nM Pb\textsuperscript{2+}. (F) Relation between the Pb\textsuperscript{2+} concentration and indo-1 fluorescence intensity (380-440 nm) at 5 µM Ca\textsuperscript{2+} for 300 nM and for 500 nM indo-1 (\(n = 3\)). The drawn lines are based on a Hill equation with slope 2 fitted to the data with IC\textsubscript{50} = 47 nM and 134 nM, \(F_{\text{max}} = 0.97\) and 0.93, \(F_{\text{min}} = 0.11\) and 0.09 and with an estimated background concentration of 15 and 26 nM Pb\textsuperscript{2+}, respectively. The dashed line is interpolated based on the estimated intracellular indo-1 concentration of 380 nM with IC\textsubscript{50} = 72 nM, \(F_{\text{max}} = 0.95, F_{\text{min}} = 0.10\) and with an estimated background concentration of 20 nM Pb\textsuperscript{2+}. 
Spectra as depicted in Figs. 1A and D were used to construct calibration curves for Ca\textsuperscript{2+}-dependent changes in indo-1 fluorescence ratio and for Pb\textsuperscript{2+}-dependent quenching of indo-1 fluorescence. A Hill equation (modified after Grynkiewicz \textit{et al.}, 1985) was fitted to the data describing the dependence of indo-1 fluorescence ratio on Ca\textsuperscript{2+} concentration using SigmaPlot 5.0 software (SPSS Inc., Richmond CA, USA) to obtain estimates of $F_{\text{min}}$, $F_{\text{max}}$, EC50, and of the background concentration of Ca\textsuperscript{2+} in nominal Ca\textsuperscript{2+}-free saline (Fig. 1C). A similar equation was used to fit the concentration-dependent quenching of indo-1 fluorescence by Pb\textsuperscript{2+} (Fig. 1F). The IC50 of Pb\textsuperscript{2+} appeared to depend on indo-1 concentration. An approximate 3-fold left shift of the curve obtained at 300 nM indo-1 was observed as compared to the curve obtained at 500 nM indo-1. The interpolated dashed line is based on an estimated intracellular indo-1 concentration of 380 nM (see Results) and is used to quantify intracellular Pb\textsuperscript{2+} concentrations as measured using CLSM. As demonstrated in Fig. 1E, only a marginal right shift of the quenching curve was observed when the Ca\textsuperscript{2+} concentration was increased from 5 µM to 1 mM. All reported values are mean ± SD of $n$ cells and results are compared using Student’s $t$-test.

### Results

**Vesicular catecholamine release from dexamethasone-differentiated PC12 cells**

From 135 dexamethasone-differentiated PC12 cells depolarized with high K\textsuperscript{+} saline ~90% responded with vesicular neurotransmitter release. The number of exocytotic events during the first 5 s of depolarization-evoked release varied between 3 and 104 ($23 ± 17$ events; median = 18; $n = 123$). Repeated stimulations with high K\textsuperscript{+} saline for 10-30 s, separated by a 5 min recovery interval, did not result in cessation of release within eight successive stimulations (Figs. 2A and 2B). Average vesicle contents from 49 cells from which >50 events were recorded during depolarizations with high K\textsuperscript{+} saline varied between 238 and 1236 zeptomol ($645 ± 256$ zmol; median = 601 zmol). For a homogenous population of catecholamine-containing vesicles, the cube root of vesicle contents is proportional to vesicle volume and should be distributed normally (Wightman \textit{et al.}, 1991). In 7 out of 7 cells tested the cube root of the vesicle contents was normally distributed (Fig. 2C; Kolmogorov-Smirnov test, all P-values >0.07). The results show that dexamethasone-differentiated PC12 cells have an increased number of releasable vesicles with increased vesicle contents as compared to undifferentiated cells (Westerink \textit{et al.}, 2000). With the apparently normal distribution of the cube root of vesicle contents, these cells are a suitable model for studying modulation of neurotransmitter release at the single cell level.
Pb\textsuperscript{2+}-induced exocytosis in PC12 cells

**Figure 2.** Characteristics of vesicular catecholamine release from dexamethasone-differentiated PC12 cells. (A) Amperometric traces obtained from a single cell during repeated 5 s depolarizations by superfusion with high K\textsuperscript{+} saline (indicated by the bar on top of the traces) alternated by 5 min recovery periods. Shown are traces from the 1\textsuperscript{st}, 2\textsuperscript{nd}, 4\textsuperscript{th}, 6\textsuperscript{th} and 8\textsuperscript{th} depolarization. (B) Relation between the exocytotic response and the number of successive stimulations. The frequency of exocytotic events is expressed as the fraction of the value obtained during the first response. Each bar represents mean ± SD of 7 cells. (C) Distribution of the cube root of the vesicle contents of a single cell from which 672 events were recorded during repeated depolarization with high K\textsuperscript{+} saline. The distribution is fitted by a single Gaussian (smooth line), with an estimated mean and variance indicated.

**Pb\textsuperscript{2+}-induced vesicular catecholamine release**

During superfusion of intact differentiated PC12 cells with saline the frequency of spontaneous vesicular catecholamine release was very low (0.2 ± 0.3 events/min; \( n = 8 \)). To test whether Pb\textsuperscript{2+} evokes vesicular catecholamine release, single cells were first superfused with high K\textsuperscript{+} saline for 5-15 s to determine their responsiveness and basic release characteristics. Responsive cells, which displayed >20 events within the first 5 s of depolarization, were allowed to recover for 5 min and were subsequently superfused with saline containing 1 or 10 µM Pb\textsuperscript{2+} for up to 30 min. Cells exposed to 1 µM Pb\textsuperscript{2+} (\( n = 9 \)) did not show a detectable increase in the frequency of vesicle exocytosis (0.3 ± 0.5 events/min; \( P = 0.7 \)) as compared to control cells. On the other hand, cells exposed to saline containing 10 µM Pb\textsuperscript{2+} (\( n = 4 \)) displayed a significant increase in release frequency becoming apparent several minutes after exposure (Fig. 3A; Table 1). Following a delay of several minutes the frequency of vesicles released, determined using a sliding 15 s time window, gradually increased up to a maximum of 20 ± 4 events/min after 12.5 ± 1.8 min (\( n = 4 \)). These results demonstrate that extracellularly applied Pb\textsuperscript{2+} evokes vesicular catecholamine release in intact dexamethasone-differentiated PC12 cells and that the threshold concentration to evoke exocytosis within the 30 min time span of the experiments is between 1 µM and 10 µM Pb\textsuperscript{2+}.
Chapter 3

Figure 3. Pb²⁺-induced exocytosis in intact PC12 cells. (A) Amperometric recordings from intact PC12 cells superfused with saline containing 1 µM or 10 µM Pb²⁺. The bars on top indicate the period of superfusion with Pb²⁺-containing saline. Scale bars apply to both traces. Note that 1 µM Pb²⁺ fails to induce exocytosis in an otherwise responsive cell. (B) Distribution of the cube root of the vesicle contents of a single cell from which 285 events were recorded during exposure to saline containing 10 µM Pb²⁺. The distribution is fitted by a single Gaussian (smooth line), with an estimated mean and variance indicated.

The cube root of the contents of vesicles released from cells exposed to 10 µM Pb²⁺ displayed a single Gaussian distribution (Fig. 3B; Kolmogorov-Smirnov test, all P-values >0.06; n = 4). In order to investigate possible effects of Pb²⁺ on vesicle contents the average vesicle contents in control cells stimulated by depolarization with high K⁺ saline was determined and compared with the average contents of vesicles released from cells exposed to 10 µM Pb²⁺. Vesicle contents from control cells, averaging 645 ± 256 zmol (n = 49), did not differ significantly from the contents of vesicles released during Pb²⁺ exposure (698 ± 147; n = 4; P = 0.54). This result demonstrates that Pb²⁺ has no effect on vesicle contents.
Table 1. Concentration-dependent effects of extracellular Pb^{2+} on vesicular catecholamine release from ionomycin-permeabilized and intact PC12 cells. Values are mean ± SD obtained from n cells.

<table>
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<th>extracellular [Pb^{2+}] (µM)</th>
<th>delay to onset release (min)</th>
<th>delay to max. release (min)</th>
<th>max. frequency (events/min)</th>
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<td>4.8 ± 4.2</td>
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<td>3.8 ± 1.3</td>
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<td>106 ± 54</td>
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<tr>
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Ca^{2+} and Pb^{2+}-induced exocytosis in ionomycin-permeabilized cells
To investigate the direct intracellular action of Pb^{2+}, cells were permeabilized by superfusion with saline containing 5 µM ionomycin. Pores formed by ionomycin have been shown to be highly permeable to Pb^{2+} as well as to Ca^{2+} (Erdahl et al., 2000). Before performing experiments with Pb^{2+}, vesicular catecholamine release from permeabilized cells in response to Ca^{2+} was characterized. At the onset of permeabilization with nominal Ca^{2+}-free saline containing 5 µM Ca^{2+}-free ionomycin (not shown) or 5 µM ionomycin Ca^{2+}-salt a transient (<30 s) burst of exocytotic events was observed in ~50% of the cells. After this initial exocytotic activity the frequency at which catecholamine-containing
vesicles were released in the continuous presence of ionomycin Ca\(^{2+}\)-salt was very low (0.2 ± 0.2 events/min; \(n\) = 4), indicating that up to 5 µM Ca\(^{2+}\) is insufficient to evoke catecholamine release. An increase in extracellular Ca\(^{2+}\) concentration to 1 mM evoked a rapid and robust exocytotic response, even in cells that had already been superfused with saline containing 5 µM ionomycin Ca\(^{2+}\)-salt for 30 min. The threshold concentration of Ca\(^{2+}\) to induce vesicular catecholamine release is ~100 µM, since 3 out of 4 cells tested showed a marginal release response when the superfusate was switched from 5 µM Ca\(^{2+}\)-containing to 100 µM Ca\(^{2+}\)-containing saline, whereas superfusion with 50 µM Ca\(^{2+}\)-containing saline (\(n\) = 4) failed to induce vesicular catecholamine release (Fig. 4A). Additional experiments were performed to determine the cause of the transient exocytotic response observed on permeabilization with ionomycin. Responsive cells (\(n\) = 6), kept in Ca\(^{2+}\)-free saline containing 500 µM EGTA (i.e., estimated free Ca\(^{2+}\) concentration <1 nM), were superfused with Ca\(^{2+}\)-free saline containing 500 µM EGTA and 5 µM Ca\(^{2+}\)-free ionomycin. Five out of six cells showed transient vesicular catecholamine release upon permeabilization. Subsequent superfusion with saline containing 5 µM Ca\(^{2+}\) and 5 µM Ca\(^{2+}\)-free ionomycin failed to cause exocytosis, whereas 500 µM Ca\(^{2+}\) caused release in all cells (data not shown). These results show that the transient response observed on permeabilization is caused by an effect of ionomycin and not by Ca\(^{2+}\), confirming previous results (Chen et al., 2001), and that 5 µM Ca\(^{2+}\) is not sufficient to induce an exocytotic response. Repeated applications of 1 mM Ca\(^{2+}\)-containing saline to permeabilized PC12 cells resulted in repeated exocytotic responses, with a gradually declining number of vesicles released with successive stimulations (Fig. 4B). Average contents of vesicles released during stimulation of permeabilized cells with 1 mM Ca\(^{2+}\)-containing saline (757 ± 206 zmol; \(n\) = 11) did not differ significantly from that of intact cells from which exocytosis was evoked by stimulation with high K\(^+\) saline (\(P\) = 0.14). Single cell distributions of the cube root of vesicle contents of 4 cells tested, were described by a single Gaussian for 3 cells (Fig. 4B; Kolmogorov-Smirnov test, all P-values ≥0.06) and not for 1 cell (\(P\) = 0.02). These results indicate that the exocytotic machinery of ionomycin-permeabilized cells remains operational and relatively stable for at least 30 min, and requires high Ca\(^{2+}\) concentrations for vesicular catecholamine release.
Figure 4. Characteristics and Ca\(^{2+}\) dependence of vesicular catecholamine release in ionomycin-permeabilized PC12 cells. (A) Representative amperometric traces (top) showing a transient burst of vesicular catecholamine release shortly after the onset of superfusion with nominal Ca\(^{2+}\)-free saline supplemented with 5 µM ionomycin Ca\(^{2+}\)-salt. In the continuous presence of ionomycin, superfusion with saline containing 0.1 mM Ca\(^{2+}\) evokes the release of few events only, whereas superfusion with saline containing 1 mM Ca\(^{2+}\) evokes a robust release response in the same cell. Superfusion periods are indicated by the bars on top, and traces were recorded at 5 min intervals. The relation between Ca\(^{2+}\) concentration and release (bottom) shows the relative release frequency expressed as the fraction of the value obtained during stimulation with 1 mM Ca\(^{2+}\). Points represent mean ± SD (n = 4). (B) Amperometric traces (top) recorded from a single ionomycin-permeabilized cell during three successive 30 s applications of Ca\(^{2+}\) alternated by 5 min recovery periods. The bar on top indicates the period of superfusion with 1 mM Ca\(^{2+}\)-containing saline. The relation between the exocytotic frequency and the number of successive stimulations (middle). The exocytotic frequency is expressed as the fraction of the value obtained during the first response. Bars represent mean ± SD (n = 9). The distribution of the cube root of the vesicle contents of a single permeabilized cell from which 175 events were recorded during repeated stimulation with 1 mM Ca\(^{2+}\)-containing saline (bottom) is fitted by a single Gaussian (smooth line) with an estimated mean and variance indicated.
Intracellular effects of Pb²⁺ on vesicular catecholamine release were investigated in cells permeabilized by superfusion with saline containing 5 μM ionomycin Ca²⁺-salt and subsequently superfused with the same saline containing various concentrations of Pb²⁺. Superfusion with 30 nM Pb²⁺ resulted in detectable vesicular catecholamine release in 3 out of 4 cells tested after a delay of several minutes (Fig. 5A), whereas 30 min of superfusion with nominal Pb²⁺-free saline (n = 4) or with 10 nM Pb²⁺ (n = 4) was without effect (data not shown). At increased concentrations of 100 nM or 1 μM Pb²⁺ the delay to the onset of release (Fig. 5A) and the time to maximum release (Table 1) decreased. In addition, the maximum frequency of vesicles released increased with increasing Pb²⁺ concentration with a maximum at 1 μM Pb²⁺ (Fig. 5B; Table 1). Switching back to nominal Pb²⁺-free saline reversed the Pb²⁺-induced release only slowly (see Fig. 5A).

**Figure 5.** Concentration dependence of Pb²⁺-induced exocytosis in permeabilized PC12 cells. (A) Amperometric recordings from permeabilized PC12 cells superfused with Ca²⁺-free saline containing 5 μM ionomycin Ca²⁺-salt and 0.03, 0.1, 1 and 10 μM Pb²⁺, as indicated by the bars on top of the recordings. (B) Relation between the Pb²⁺ concentration and the maximum release frequency (events/min) plotted for 30 cells. (C) Distribution of the cube root of the vesicle contents of a single permeabilized cell from which 279 events were recorded during continuous superfusion with saline containing 1 μM Pb²⁺. The distribution is fitted by a single Gaussian (smooth line), with an estimated mean and variance indicated.
At the highest concentration of 10 µM, a short delay between Pb^{2+} application and the onset of release was observed. However, within 1 min after onset the Pb^{2+}-induced release suddenly ceased in 4 out of 5 cells tested and switching back to nominal Pb^{2+}-free saline did not result in a rapid reappearance of release (see Fig. 5A). Superfusion with 2 mM Ca^{2+}-containing saline after a 5 min wash-out period did not evoke catecholamine release in the cells that had responded to 10 µM Pb^{2+} (n = 4; data not shown), confirming the inhibitory effect of 10 µM Pb^{2+}. The cube root of the contents of vesicles released from cells exposed to 1 µM Pb^{2+} displayed a single Gaussian distribution (Fig. 5C; Kolmogorov-Smirnov test, all P-values > 0.10; n = 3). The average contents of vesicles released during superfusion with 1 µM Pb^{2+} amounted to 712 ± 225 zmol (n = 10), indicating that, as in intact cells, Pb^{2+} does not affect vesicle contents in permeabilized cells. The concentration-dependent effects demonstrate that the threshold concentration of extracellular Pb^{2+} to induce vesicular neurotransmitter release in permeabilized PC12 cells is between 10 and 30 nM.

Figure 6. Ca^{2+}-independence of Pb^{2+}-evoked exocytosis in permeabilized PC12 cells. Amperometric recordings showing vesicular catecholamine release during superfusion with 2 mM Ca^{2+} saline or nominal Ca^{2+}-free saline containing 1 µM Pb^{2+}. The concentration of the membrane-impermeable chelator EGTA is sufficient to rapidly reduce the intracellular Ca^{2+} concentration below the threshold for release (top trace), but does not reverse Pb^{2+}-induced exocytosis (middle trace). The membrane-permeable heavy metal chelator TPEN rapidly reduces the intracellular Pb^{2+} concentration below the threshold for release (lower trace). Superfusion periods are indicated by the bars on top of each recording.

Calcium and heavy metal chelators were used to investigate whether exocytosis in ionomycin-permeabilized cells requires the continuous presence of Ca^{2+} or Pb^{2+}. Ca^{2+}-evoked release is readily reversed by superfusion with nominal Ca^{2+}-free saline containing 500 µM EGTA (Fig. 6; n = 3). Estimated free Ca^{2+} and Pb^{2+} concentrations in the saline
containing 500 µM EGTA and 5 µM ionomycin Ca\(^{2+}\)-salt are <1 nM and <1 pM, respectively. Since EGTA is a membrane-impermeable chelator, the result demonstrates that Ca\(^{2+}\) rapidly diffuses out of the permeabilized cell. Pb\(^{2+}\)-induced release is reversed only slowly by superfusion with saline containing the membrane-impermeable chelators EDTA (50 µM; not shown; \(n = 3\)) or EGTA (500 µM; Fig. 6; \(n = 4\)), demonstrating that the effect of Pb\(^{2+}\) is independent of intracellular Ca\(^{2+}\). Conversely, 10 µM of the membrane-permeable heavy metal chelator TPEN quickly reversed the Pb\(^{2+}\)-induced release (Fig. 6; \(n = 6\)). The same concentration of TPEN did not affect the spontaneous release or 1 mM Ca\(^{2+}\)-evoked exocytosis (not shown; \(n = 3\)). These findings demonstrate that exocytosis is caused by binding of Pb\(^{2+}\) to an intracellular binding site and that Pb\(^{2+}\)-induced exocytosis continues in the absence of Ca\(^{2+}\).

**Determination of intracellular Pb\(^{2+}\) and Ca\(^{2+}\) concentrations**

In order to investigate the quantitative relation between the concentration of Pb\(^{2+}\) required to induce exocytosis and intracellular Ca\(^{2+}\) and Pb\(^{2+}\) concentrations, PC12 cells were loaded with indo-1, permeabilized, and subsequently exposed to various concentrations of Pb\(^{2+}\). Immediately after permeabilization with 5 µM ionomycin Ca\(^{2+}\)-salt the cells were brightly fluorescent and, after 5 min of equilibration, Pb\(^{2+}\) was applied and the quenching of indo-1 fluorescence was measured from confocal images recorded at intervals of 1-5 min. Fig. 7A shows that exposure of permeabilized PC12 cells to 100 nM Pb\(^{2+}\) resulted in detectable quenching (>10% reduction of the initial intensity) after a considerable delay of 18 ± 10 min (\(n = 13\); Table 2). After 45 min of exposure to 100 nM Pb\(^{2+}\) the remaining fluorescence amounted to 42 ± 11% (\(n = 13\)). In control experiments the intensity of indo-1 fluorescence in cells exposed to nominal Pb\(^{2+}\)-free saline amounted to 98.5 ± 5.3 % (\(n = 6\)) of the initial value after 45 min of recording images at an average interval of 1.4 min. The fluorescence ratio in control and in 100 nM Pb\(^{2+}\)-exposed permeabilized cells remained constant at 0.95 ± 0.03 (\(n = 19\)) (equivalent to ~6 µM Ca\(^{2+}\)) over the entire recording period (not shown). Since the quenching by Pb\(^{2+}\) depends to some degree on the concentration of indo-1 (see Fig. 1F) it is necessary to estimate the intracellular indo-1 concentration. Assuming that after 45 min of superfusion of the permeabilized cells with 100 nM Pb\(^{2+}\) intracellular and extracellular Pb\(^{2+}\) concentrations have become equal, the mean of 42% remaining fluorescence corresponds to 100 nM free Pb\(^{2+}\). Using this value to calculate an interpolated calibration curve (see Fig. 1F) indicates that the average intracellular indo-1 concentration is ~380 nM. The interpolated calibration curve was used to convert the measured quenching of indo-1 fluorescence to intracellular free Pb\(^{2+}\) concentration (Fig. 7A). The detection threshold was estimated to be 30 nM free Pb\(^{2+}\) (i.e., 10% quenching). Exposure of
permeabilized cells to saline containing 1 µM and 10 µM Pb\textsuperscript{2+} resulted in a significant shortening of the delay between Pb\textsuperscript{2+} application and a detectable increase of the intracellular free Pb\textsuperscript{2+} concentration (P < 0.001). The rate of increase in intracellular free Pb\textsuperscript{2+} concentration was also enhanced when cells were exposed to saline containing 1 and 10 µM Pb\textsuperscript{2+} (Fig. 7A; Table 2).

**Figure 7.** Time-dependent increase in intracellular Pb\textsuperscript{2+} concentration in permeabilized (A) and intact (B) PC12 cells and its relation with exocytosis. (A) Relation between quenching of indo-1 fluorescence, the estimated intracellular Pb\textsuperscript{2+} concentration and the duration of exposure to saline containing 5 µM ionomycin Ca\textsuperscript{2+}-salt and 0.1 µM (closed circles), 1 µM (closed squares) and 10 µM Pb\textsuperscript{2+} (open circles). All points represent mean ± SD (n = 3 for each experimental condition). The bars on top mark the intervals in which the onset of exocytosis in permeabilized cells exposed to the same concentrations of Pb\textsuperscript{2+} is observed (n = 5-11). (B) Quenching of indo-1 fluorescence in intact PC12 cells during exposure to nominal Pb\textsuperscript{2+}-free saline (closed squares) and 10 µM Pb\textsuperscript{2+}-containing saline (open circles). The bar on top marks the interval in which the onset of exocytosis is observed (n = 4). The normalized intracellular Ca\textsuperscript{2+} concentration as a function of the duration of exposure to saline containing 10 µM Pb\textsuperscript{2+} is also depicted. All points represent mean ± SD (n = 3 for each experimental condition). See Table 1 for data summary and Fig. 1F for calibration curves used.
Exposure of intact cells to saline containing 10 µM Pb\(^{2+}\) also caused a decrease of indo-1 fluorescence (Fig. 7B) after a considerable delay of 12 ± 2 min (n = 8). Control cells kept in nominal Pb\(^{2+}\)-free saline did not show a detectable decrease in fluorescence (n = 5; Fig. 7B), demonstrating that, under the experimental conditions used, significant bleaching or leakage of indo-1 does not occur. In the intact cells, Pb\(^{2+}\) entry appeared to be slow under resting conditions (Table 2). After 25 min of exposure to 10 µM Pb\(^{2+}\) fluorescence quenching amounted to 50 ± 14%, corresponding to 85 nM of intracellular free Pb\(^{2+}\). In control cells (not shown) as well as in cells exposed to 10 µM Pb\(^{2+}\) significant changes in intracellular free Ca\(^{2+}\) concentration were not observed (Fig. 7B), demonstrating that Pb\(^{2+}\)-induced exocytosis is caused by Pb\(^{2+}\) itself and is not due to a Pb\(^{2+}\)-induced elevation of intracellular free Ca\(^{2+}\) to a level required for exocytosis. Depolarization appeared to facilitate Pb\(^{2+}\) entry in intact cells (not shown), basically confirming previous results based on measurements of cell populations (Simons and Pocock, 1987; Tomsig and Suszkiw, 1991; Kerper and Hinkle, 1997; Legare et al., 1998).

### Table 2

The relations between extracellularly applied Pb\(^{2+}\), the time to observe a detectable increase in the intracellular concentration of free Pb\(^{2+}\), and the maximum rate of Pb\(^{2+}\) entry (V\(_{\text{max}}\)) in ionomycin-permeabilized and intact PC12 cells. The mean intracellular high-affinity Pb\(^{2+}\) buffering capacity and the mean total amount of Pb\(^{2+}\) required intracellularly were estimated from these data and from the data in Table 1, as indicated in the results section. Values are mean ± SD obtained from n cells.

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<th>V(_{\text{max}}) (nmol l(^{-1}) min(^{-1}))</th>
<th>buffer capacity (amol/cell)</th>
<th>total Pb(^{2+}) required for release (amol/cell)</th>
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**Pb²⁺-induced exocytosis in PC12 cells**

**Intracellular Pb²⁺ required for exocytosis**

As indicated by the bars on top of Figs. 7A and 7B, Pb²⁺-induced release occurs before an increase in the intracellular free Pb²⁺ concentration is detected. However, as soon as intracellular free Pb²⁺ is detected, the concentration rises rapidly (Table 2). Thus, it appears that intracellular Pb²⁺ initially binds to cytoplasmic components in the permeabilized cell with a much higher affinity than that for the dye indo-1, thereby preventing the detection of free Pb²⁺ by indo-1 until the intracellular high-affinity binding capacity is saturated. Since the rate of Pb²⁺ entry will be maximal and constant until an appreciable level of intracellular free Pb²⁺ is reached, the total intracellular Pb²⁺ concentration at early times, i.e., before detecting a change in free Pb²⁺ with indo-1, can be calculated from the product of the maximum rate of entry ($V_{\text{max}}$ in mol·l⁻¹·min⁻¹) and time of exposure. $V_{\text{max}}$ values, calculated from the data in Figs. 7A and 7B, for the extracellular Pb²⁺ concentrations applied were used to calculate the total intracellular concentration of Pb²⁺ at the time of onset of release (Table 2). The results show that, in cells exposed to 100 nM, 1 µM, and 10 µM extracellular Pb²⁺, the estimated total intracellular Pb²⁺ concentration at the onset of release amounts to 19 nM, 16 nM, and 12 nM, respectively. Thus, it appears that release is triggered when the total intracellular Pb²⁺ concentration is raised to values between 10 and 20 nM, irrespective of the extracellular Pb²⁺ concentration. This is consistent with the result that the extracellular threshold concentration of Pb²⁺ to induce vesicular catecholamine release in permeabilized PC12 cells within 30 min is between 10 and 30 nM.

From the rate of Pb²⁺ entry and the delay between Pb²⁺ application and the detection of an increase in intracellular free Pb²⁺ concentration (Table 2), the high-affinity buffer capacity of the cell can also be estimated. Using an average cell volume of 2.24·10⁻¹¹ l, corresponding to an average cell diameter of 35 µm, the estimated high-affinity buffer capacity in intact as well as in permeabilized cells is in the range of 2-3 attomol (amol). This is almost an order of magnitude more than the total amount of intracellular Pb²⁺ required to induce vesicular catecholamine release, which is estimated to amount to 0.3-0.4 amol in permeabilized cells and 0.7 amol in intact cells. Thus, it appears that only a fraction of the high-affinity buffering capacity in the cytoplasm is saturated with Pb²⁺ at the time of onset of Pb²⁺-induced release.
Discussion

The results from this study provide a direct demonstration that Pb\(^{2+}\)-induced catecholamine release has a vesicular origin (Fig. 3). The concentration of extracellular Pb\(^{2+}\) required to induce exocytosis of catecholamine-containing vesicles in intact, dexamethasone-differentiated PC12 cells is comparable with previous results using suspensions of undifferentiated PC12 cells (Bressler et al., 1996) and bovine chromaffin cells (Tomsig and Suszkiw, 1990). The present results show that Pb\(^{2+}\) enhances neurotransmitter release by inducing exocytosis and not by changing vesicle contents.

The direct effects of Pb\(^{2+}\) on exocytosis are mediated by intracellular mechanisms. Firstly, the threshold concentration of 1-10 \(\mu\)M Pb\(^{2+}\) to evoke release in intact cells is orders of magnitude higher than the threshold concentration of 10-30 nM Pb\(^{2+}\) required to evoke release in permeabilized cells (Figs. 3 and 5A). Thus, the membrane forms a barrier for Pb\(^{2+}\) permeation, but transmembrane signaling appears not to be involved in Pb\(^{2+}\)-induced exocytosis. Secondly, the extracellular application of membrane-impermeable chelators EGTA (Fig. 6) and EDTA rapidly reduces the intracellular Ca\(^{2+}\) concentration below threshold levels and thereby reverse Ca\(^{2+}\)-evoked exocytosis. The membrane-impermeable chelators used, at concentrations sufficient to rapidly reduce the extracellular Pb\(^{2+}\) concentration below the pM level, leave the Pb\(^{2+}\)-induced release unaffected. Conversely, the membrane-permeable heavy-metal chelator TPEN rapidly reverses Pb\(^{2+}\)-induced exocytosis, indicating that Pb\(^{2+}\) is tightly associated with an intracellular binding site. Finally, the simultaneous imaging of intracellular Pb\(^{2+}\) and Ca\(^{2+}\) shows that an increase in intracellular free Pb\(^{2+}\) is not accompanied by detectable changes in the concentration of intracellular free Ca\(^{2+}\) (Fig. 7B). Although the Ca\(^{2+}\) sensitivity of indo-1, as used under the present experimental conditions, is relatively low, the results exclude that Pb\(^{2+}\) induces sufficient Ca\(^{2+}\) influx or Ca\(^{2+}\) release from intracellular stores to elevate intracellular Ca\(^{2+}\) to a level required for exocytosis. The combined results show that Pb\(^{2+}\)-induced exocytosis involves a direct high-affinity interaction with an intracellular site. A low affinity intracellular effect of Pb\(^{2+}\), which leads to inhibition of exocytosis in permeabilized cells (Fig. 5A), is also observed. This indicates that Pb\(^{2+}\) interacts with multiple intracellular targets, which are functionally involved in the exocytosis of catecholamine-containing, large dense-core vesicles in PC12 cells.

The use of indo-1 in the absence of chelators is not without consequences for the imaging of intracellular Ca\(^{2+}\). Careful calibration (Fig. 1) shows that, in the absence of EGTA which is used routinely for dye calibrations, the emission spectrum of indo-1 is shifted and the sensitivity of indo-1 to Ca\(^{2+}\) (Fig 1A,B) as well as that to Pb\(^{2+}\) (c.f., Kerper and Hinkle, 1997) are greatly reduced. However, the spectrum of intracellular indo-1 is
very similar to that in solutions free of EGTA, but differs from that in solutions containing EGTA (Fig. 1A, B). Despite the low Ca\(^{2+}\)-sensitivity of indo-1, imaging shows that in the presence of 5 µM ionomycin Ca\(^{2+}\) salt the estimated intracellular free Ca\(^{2+}\) concentration is close to 5 µM. An exocytotic response is not observed in permeabilized cells exposed to up to 50 µM Ca\(^{2+}\) and threshold exocytosis is observed during superfusion with 100 µM Ca\(^{2+}\)-containing saline (Fig. 4). Previous studies suggest that the threshold for catecholamine release from permeabilized PC12 cells is <1 µM free Ca\(^{2+}\) (Ahnert-Hilger et al., 1987; Chen et al., 2001). The difference in Ca\(^{2+}\) threshold for release, which was not confirmed by intracellular imaging in the previous studies, may originate from the fact that catecholamine release was measured from cell populations instead of measuring vesicular release from single cells. Moreover, we applied Ca\(^{2+}\) in the absence of chelators, whereas in previous studies Ca\(^{2+}\) was buffered to micromolar levels with total Ca\(^{2+}\) in the millimolar range. The recently reported Ca\(^{2+}\) threshold of ~6 µM free intracellular Ca\(^{2+}\) for flash photolysis-induced catecholamine release in PC12 cells (Kishimoto et al., 2001) is still an order of magnitude smaller than the presently found value. The remaining difference is likely attributable to the presence of intracellular buffers, i.e., ≥10 mM DM-nitrophen and 0.2 mM of the low-affinity Ca\(^{2+}\) indicator BTC, and to the whole-cell patch clamp configuration (Kishimoto et al., 2001) as compared to the buffer-free and the intracellularly less disturbed situation in the ionomycin-permeabilized cells.

The results of the experiments on permeabilized cells show that exocytosis is induced when 30 nM Pb\(^{2+}\) and not when 10 nM Pb\(^{2+}\) is added to nominal Ca\(^{2+}\)-free (~0.4 µM Ca\(^{2+}\)) and Pb\(^{2+}\)-free (~20 nM Pb\(^{2+}\)) saline. This demonstrates that the total Pb\(^{2+}\) concentration in the external saline required for release is ≥30 nM and appears to be at variance with the notion that picomolar concentrations of intracellular Pb\(^{2+}\) induce catecholamine release (Tomsig and Suszkiw, 1990; 1991). An independent estimate from the present imaging experiments yield 12-19 nM of total intracellular Pb\(^{2+}\) required for release. The main difference between the present, single cell study and the previous experiments on chromaffin cell suspensions are the use of fura-2 instead of indo-1 for imaging and the use of chelators to buffer extracellular free Pb\(^{2+}\) concentration in the previous study. It is unknown whether the intracellular affinity of fura-2 for Pb\(^{2+}\) is ~3.5 pM as determined in EGTA-containing Pb\(^{2+}\) buffers (Tomsig and Suszkiw, 1990), since the properties of fura-2, like that of indo-1, may change in the presence of EGTA. Further drawbacks of using fura-2 imaging for measuring intracellular Pb\(^{2+}\), particularly when extracellular Pb\(^{2+}\) is buffered, have been outlined in the introduction. The sensitivity of indo-1 for Pb\(^{2+}\) under the experimental conditions used is relatively low, such that at the time at which exocytosis is observed binding of Pb\(^{2+}\) to indo-1 is minimal and the binding of Pb\(^{2+}\) to intracellular components is not disturbed. The effect of Pb\(^{2+}\) on the intensity and that of Ca\(^{2+}\) on the ratio
of indo-1 fluorescence are largely independent (Fig. 1). The slight change in spectral properties observed at Pb²⁺ concentrations ≥300 nM (Fig. 1D) is unimportant, since the dynamic sensitivity range of indo-1 for Pb²⁺ is limited at ~300 nM. The observation that the delay between Pb²⁺ application and the onset of release is much shorter than the delay to a detectable increase of intracellular free Pb²⁺ (Fig. 7) indicates that the intracellular high-affinity binding capacity is much higher than the amount of Pb²⁺ required to induce release (Table 2). Back-extrapolation, based on the measured rates of Pb²⁺ entry, indicates that a total concentration of 12-19 nM Pb²⁺ is present intracellularly at the time of onset of exocytosis, irrespective of the extracellular Pb²⁺ concentration (100 nM - 10 µM). Together with the observation that a maximum release response to Pb²⁺ is observed at 1 µM extracellular Pb²⁺ (Fig. 5) this shows that it is highly unlikely that vesicular catecholamine release in PC12 cells is triggered by subnanomolar concentrations of intracellular Pb²⁺. The present finding that the amount of Pb²⁺ required to induce exocytosis in intact cells is approximately twice the amount required in permeabilized cells, may be due to differences in sequestration and extrusion of Pb²⁺, which may be impaired in ionomycin-permeabilized cells.

The finding that exocytosis is triggered by higher concentrations of Pb²⁺ than initially believed does not distract from the toxicological hazard of this heavy metal. Since high-affinity buffering facilitates the intracellular accumulation of Pb²⁺, much depends on the availability of extracellular Pb²⁺, i.e., free Pb²⁺ concentration as well as the amount of remaining extracellular Pb²⁺ and the affinity by which it is bound. If the conditions are such that a critical amount of Pb²⁺, estimated to be 2-3 amol for PC12 cells, accumulates intracellularly, sustained exocytosis may be triggered to cause the adverse effect.

The molecular mechanism through which Pb²⁺ exerts its effects on exocytosis remains a matter of debate. The present findings (Fig. 7) suggest that the molecular targets for Pb²⁺-induced exocytosis have a higher affinity for Pb²⁺ than indo-1. Moreover, the inhibition of exocytosis in permeabilized cells by high concentrations of Pb²⁺ (Fig. 5A) provides evidence for the existence of an additional, low-affinity, intracellular target for Pb²⁺. Unfortunately, of the many proteins involved in exocytosis only few have been investigated to determine the effect of Pb²⁺. Calmodulin and calcineurin are both activated at ~100 pM free Pb²⁺, and calcineurin activity is inhibited at ~2 nM free Pb²⁺ (Kern et al., 2000; Kern and Audesirk, 2000). Biochemical analysis of Pb²⁺-induced PKC activity revealed half maximal activation at ~2 pM free Pb²⁺ and half maximal inhibition at ~2 µM free Pb²⁺ (Sun et al., 1999). In permeabilized chromaffin cells, exposed to extracellular saline containing Pb²⁺ buffers, ~1 nM free Pb²⁺ was required for maximal Pb²⁺-induced PKC activation, which facilitated neurotransmitter release (Tomsig and Suszkiw, 1995). Since Pb²⁺ exerts biphasic effects on both calcineurin and PKC, it is tempting to speculate that these are
protein targets involved in Pb$^{2+}$-induced exocytosis. However, specific activators of calmodulin, calcineurin, and PKC fail to mimic the effect of Pb$^{2+}$, since they do not induce Ca$^{2+}$-independent catecholamine release from PC12 cells or chromaffin cells (Chamberlain et al., 1995; Artalejo et al., 1996; Taylor et al., 2000). This makes it unlikely that Pb$^{2+}$ induces exocytosis through specific activation of one of these proteins. A more likely target for Pb$^{2+}$ might be synaptotagmin, which is the proposed fast Ca$^{2+}$-sensor for exocytosis (for review see Adolfsen and Littleton, 2001). Synaptotagmin is present on large dense-core vesicles in bovine chromaffin cells (Ohara-Imaizumi et al., 1997). Pb$^{2+}$, buffered at nanomolar free concentrations, induces the interaction of synaptotagmin with phospholipid liposomes and protects synaptotagmin from proteolytic cleavage in a manner similar to Ca$^{2+}$. Furthermore, tenfold higher concentrations free Pb$^{2+}$ inhibit the Ca$^{2+}$-induced interaction between syntaxin and synaptotagmin (Bouton et al., 2001). However, synaptotagmin may not be essential for large dense-core vesicle release, since synaptotagmin I- and II-deficient PC12 cells display normal Ca$^{2+}$-evoked catecholamine release (Shoji-Kasai et al., 1992; Bauerfeind et al., 1995). Thus, it appears that specific intracellular protein targets involved in the Pb$^{2+}$-induced activation and inhibition of exocytosis remain to be identified.

Acknowledgements

We thank Dr. L. Tertoolen (Hubrecht Laboratory, Utrecht) for donation of PC12 cells; Dr. S. Agulian (Yale University, West Haven, USA) for donation of carbon fibers; M. van Eijk and Dr. C. van de Lest (Dept. of Biochemistry, Cell Biology & Histology, Faculty of Veterinary Sciences, Utrecht) for using the spectrofluorometer; Dr. M. Rook (Dept. of Medical Physiology, University Medical Center, Utrecht) for CLSM facilities, excellent technical assistance, and critical comments; Dr. C. Meulenberg for helpful discussions and assistance in CLSM; and Ing. A. de Groot for excellent technical assistance.
Abstract

The modulation of vesicular catecholamine release by intracellular signaling pathways has been investigated in rat phaeochromocytoma PC12 cells using carbon fiber microelectrode amperometry. In intact PC12 cells, drugs known to modulate PKC (PMA and staurosporine), calcineurin (cyclosporin A), calmodulin (W7), and CaM kinase II (KN-62) activity, exert differential effects on the frequency of exocytotic events evoked by high K⁺ stimulation and on the basal frequency of exocytotic events. None of the drugs has a significant effect on vesicle contents. In ionomycin-permeabilized cells, PMA and cyclosporin enhance the basal frequency of exocytosis, whereas staurosporine, W7, and KN-62 cause no significant effects. The combined results indicate that PKC and calcineurin enhance and inhibit exocytosis through direct effects on the exocytotic machinery, whereas calmodulin and CaM kinase II exert indirect effects only. Conversely, Pb²⁺-evoked exocytosis in permeabilized cells is strongly reduced by inhibition of CaM kinase II, but is not sensitive to modulation of PKC and calcineurin activity. Inhibition of calmodulin only reduces the delay to onset of Pb²⁺-evoked exocytosis. In synaptotagmin-deficient PC12-F7 cells Ca²⁺- and Pb²⁺-induced catecholamine release are reduced to a similar extent. It is concluded that CaM kinase II plays a key role in Pb²⁺-induced neurotransmitter release from PC12 cells.

Keywords: exocytosis - protein kinase C - synaptotagmin - carbon fiber microelectrode amperometry - Ca²⁺/calmodulin-dependent protein kinase II - Ca²⁺/calmodulin-dependent protein phosphatase 2B
Distinct pathways of exocytosis in PC12 cells

Introduction

The fusion of a neurotransmitter-filled vesicle with the cell membrane and the subsequent release of vesicle contents, are preceded by vesicle filling, docking and priming, and followed by the endocytotic retrieval of emptied vesicles. This ensemble of processes, known as the vesicle cycle, is tightly regulated by a number of highly conserved vesicle- and membrane-associated proteins. Apart from Ca\textsuperscript{2+} ions, which are required to trigger the actual exocytotic event, a variety of cytoplasmic proteins is involved in the regulation of specific steps of the vesicle cycle (for reviews see Südhof, 1995; Martin, 1997; Lin and Scheller, 2000). Modulation of the activity of these regulatory proteins is likely to affect the normal progression of the vesicle cycle and may alter exocytosis.

The presence of a sufficiently high intracellular Ca\textsuperscript{2+} concentration is a prerequisite for functional exocytosis as demonstrated in rat chromaffin and in rat phaeochromocytoma PC12 cells (Finnegan and Wightman, 1995; Westerink and Vijverberg, 2002). The heavy metal ion Pb\textsuperscript{2+} substitutes for Ca\textsuperscript{2+} in essential steps of the vesicle cycle leading to exocytosis. PC12 cells permeabilized with ionomycin readily release catecholamine-containing vesicles when the extracellular Ca\textsuperscript{2+} concentration is raised to ~100 µM. Elevation of the extracellular Pb\textsuperscript{2+} concentration to 30 nM or higher causes a similar response, but only after a concentration-dependent delay. Specific intracellular targets for Pb\textsuperscript{2+} involved in exocytosis remain to be identified (Westerink and Vijverberg, 2002).

Both Ca\textsuperscript{2+} and Pb\textsuperscript{2+} are known to activate various proteins, which have a regulatory role in the vesicle cycle. Pb\textsuperscript{2+} is far more potent than Ca\textsuperscript{2+} as an activator of protein kinase C (PKC; Markovac and Goldstein, 1988; Sun et al., 1999), calmodulin (Habermann et al., 1983; Kern et al., 2000), calcineurin (Kern and Audesirk, 2000), and synaptotagmin (Bouton et al., 2001).

Activation of protein kinase C (PKC) by phorbol esters in PC12 cells has been reported to augment K\textsuperscript+-evoked catecholamine release, whereas depolarization-induced Ca\textsuperscript{2+}-influx is inhibited (Harris et al., 1986). PKC activation has also been reported to increase basal catecholamine release from PC12 cells dependent on extracellular Ca\textsuperscript{2+}, but without an apparent change in intracellular Ca\textsuperscript{2+} concentration (Oda et al., 1995). Recently, however, the enhancement of basal release by the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) has been shown to be associated with a rise in intracellular Ca\textsuperscript{2+} concentration, due to a moderate hyperpolarizing shift in the voltage dependence of activation of L-type Ca\textsuperscript{2+} channels (Taylor et al., 2000). Apart from effects on ion channels (for review see Vaughan et al., 1998), PKC enhances catecholamine release in ‘cracked’ PC12 cells by a direct action on the exocytotic machinery (Chen et al., 1999). Pb\textsuperscript{2+}-induced catecholamine release from populations of intact PC12 (Bressler et al., 1996) and
permeabilized chromaffin cells (Tomsig and Suszkiw, 1995) appears enhanced in the presence of phorbol esters. Although the concentration of Pb\(^{2+}\) required to induce neurotransmitter release is lowered by phorbol esters, inhibition of PKC activity resulted in a reversal of the phorbol ester effect but did not affect Pb\(^{2+}\)-induced catecholamine release (Tomsig and Suszkiw, 1995).

Calmodulin appears to enhance spontaneous and evoked catecholamine release by modulating events occurring in the triggering stage of exocytosis in populations of permeabilized chromaffin and PC12 cells (Chamberlain et al., 1995; Chen et al., 1999). Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM kinase II) is able to phosphorylate synapsin I (Jovanovic et al., 2001), synaptotagmin (Verona et al., 2000), VAMP (Hirling and Scheller, 1996), syntaxin and SNAP-25 (Risinger and Bennett, 1999). Activation of CaM kinase II, but not of PKC, increases the readily releasable pool of vesicles in mouse pancreatic \(\beta\)-cells (Gromada et al., 1999). Inhibition of CaM kinase II by KN-62 causes a 50% reduction of catecholamine release evoked from populations of intact PC12 cells, without affecting stimulus-induced intracellular Ca\(^{2+}\) signals (Schweitzer et al., 1995).

Inhibition of Ca\(^{2+}\)/calmodulin-dependent protein phosphatase 2B, calcineurin, with cyclosporin A decreases depolarization-induced Ca\(^{2+}\) influx and exocytosis in bovine chromaffin cells (Engisch and Nowycky, 1998), but potentiates depolarization-induced Ca\(^{2+}\) influx and exocytosis in rat lactotrophs (Fomina and Levitan, 1997). Cyclosporin A enhances the frequency of miniature endplate potentials in mouse motor nerve terminals (Lin and Lin-Shiau, 1999) as well as 4-aminopyridine- and \(\alpha\)-latrotoxin-evoked GABA release from rat brain synaptosomes (Storchak et al., 2001). Inhibition of calcineurin by antibodies results in decreased noradrenaline and neuropeptide secretion from streptolysin-O-permeabilized rat brain synaptosomes (Hens et al., 1998). Thus, modulatory effects of calcineurin on exocytosis remain controversial and possibly depend on the model system used.

Synaptotagmin is the proposed Ca\(^{2+}\) sensor for fast, synchronized exocytosis (for review see Adolfsen and Littleton, 2001). Pb\(^{2+}\) has been shown to be a functional substitute for Ca\(^{2+}\) on synaptotagmin I in several respects, but, unlike Ca\(^{2+}\), Pb\(^{2+}\) does not trigger the interaction of synaptotagmin with syntaxin. From these results it has been suggested that synaptotagmin is a potential target for Pb\(^{2+}\) effects on exocytosis (Bouton et al., 2001). However, results from experiments on synaptotagmin I- and II-deficient clones of PC12 cells indicate that synaptotagmin is not essential for the release of catecholamines from PC12 cells (Shoji-Kasai et al., 1992).
We have investigated modulatory effects of various intracellular signaling pathways on Ca\(^{2+}\)- and Pb\(^{2+}\)-induced vesicular catecholamine release from PC12 cells using carbon fiber microelectrode amperometry (Wightman et al., 1991; Chow and von Rüden, 1995). As many of the signaling pathways mentioned above act on multiple specific targets, including voltage-activated Ca\(^{2+}\) channels (for review see Catterall, 2000), direct and indirect effects on the exocytotic machinery are distinguished by comparing effects on exocytosis in intact PC12 cells with effects in ionomycin-permeabilized PC12 cells.

**Materials and methods**

**Materials.** NaCl, KCl, Mg(NO\(_3\))\(_2\), glucose, sucrose, HEPES, and NaOH (Aristar quality) were obtained from BDH Laboratory Supplies (Poole, Dorset, UK); Pb(NO\(_3\))\(_2\) from Alfa Aesar (Johnson Matthey, Karlsruhe, Germany). All other chemicals and drugs were obtained from Sigma (St. Louis MO, USA). Saline solutions were prepared with de-ionized millipore water (Milli-Q\(^\text{®}\); resistivity >10 MΩ·cm). Immediately after preparation all saline solutions were filtered using a Millipore GSWP 0.22 µm filter (Millipore, Bedford MA, USA) and stored in thoroughly cleaned and rinsed glass bottles at -20 °C until use. Stock solutions of 2 mM ionomycin Ca\(^{2+}\)-salt, 1 mM phorbol 12-myristate 13-acetate (PMA), 500 µM staurosporine, 1 mM 1-(N,O-bis-[5-isoquinolinesulfonyl]-N-methyl-L-tyrosyl)-4-phenylpiperazine (KN-62), 4.2 mM cyclosporin A, and 40 mM nomifensine maleate salt in DMSO as well as 50 mM N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) in methanol were kept at -20 °C, and were thawed before the experiment. Stock solutions of 50 mM Pb(NO\(_3\))\(_2\) in distilled water were prepared immediately before use, and diluted in external solution to obtain the desired concentrations. DMSO alone (0.5% v/v) did not affect release.

**Cell culture.** PC12 cells (Greene and Tischler, 1976; ATCC CRL-1721) as well as synaptotagmin I- and II-deficient cells of the clone PC12-F7 (Shoji-Kasai et al., 1992) were grown essentially as described previously (Westerink and Vijverberg, 2002) for a maximum of 10 passages. In short, cells were grown in RPMI 1640 medium (Gibco, Grand Island NY, USA) supplemented with 5% fetal calf serum (ICN, Costa Mesa CA, USA), 10% heat-inactivated horse serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin (all from Gibco). Differentiation was initiated 2 d after subculture in 35 mm culture dishes (Nunc Inc., Naperville IL, USA) in culture medium supplemented with 5 µM dexamethasone (Genfarma, Zaandam, The Netherlands). Culture flasks and dishes were coated with 5 µg/cm\(^2\) poly-L-lysine (Sigma). For measuring catecholamine release from cell populations, cells were grown and differentiated in 25 cm\(^2\) culture flasks (Nunc), as described above, until confluence (~7·10^6 cells/flask). The culture medium was refreshed every 2 - 3 d. All experiments were performed 7 - 10 d after subculture, i.e., 5 - 8 d after initiating differentiation.
Carbon fiber microelectrode amperometry. Carbon fiber microelectrode (Ø 10 µm) fabrication and data recording and analysis were as described previously (Westerink et al., 2000). Before experiments cells were washed twice with saline solution containing (in mM) 125 NaCl, 5.5 KCl, 2 CaCl₂, 0.8 MgCl₂, 10 HEPES, 24 glucose, and 36.5 sucrose at pH 7.3 adjusted with NaOH. The carbon fiber, polarized to 700 mV, was placed gently on the membrane surface of a PC12 cell under continuous superfusion with saline through one barrel of a theta superfusion pipette (TGC150; Clark Electromedical Instruments, Pangbourne, Reading, UK) with a tip diameter of 80 µm at a rate of ~150 µl/min. In intact cells, exocytosis was evoked by superfusion with high K⁺ saline (KCl elevated to 125 mM and NaCl reduced to 5.5 mM). Pb²⁺, and drugs were added to the saline immediately before the experiment. Ca²⁺ and Pb²⁺ concentrations indicated in the results refer to the metal concentrations added to nominal Ca²⁺- and Pb²⁺-free saline, which contained ~0.4 µM Ca²⁺ and ~20 nM Pb²⁺.

Catecholamine release from cell populations. Prior to release experiments, cells were washed thrice with saline. After 5 min of incubation the saline was collected and replaced by high K⁺ saline for 1 min. The high K⁺ saline was replaced by saline for 2 subsequent periods of 2 min. To prevent catecholamine reuptake all salines were supplemented with 10 µM nomifensine. The external saline samples were collected, put on ice and frozen in tightly closed test tubes at -80 °C until analysis. The amounts of dopamine and norepinephrine were determined using HPLC equipped with an electrochemical detector. A 500 µl sample was added to an aliquot of alumina oxide (RECIPE Chemicals & Instruments; kit nr. 1000), followed by 25 µl internal standard 3,4-dihydroxybenzylamine hydrobromide (0.1 mM) and 250 µl tris-(hydroxymethyl)-aminomethane (2 M). This was mixed in a rotator mixer for 5 min, and subsequently centrifuged at 9,980x g (12,000 rpm) for 1 min. The pellet was resuspended in washing solution and the procedure was repeated two times. Finally, the catecholamines were stripped with 50 µl acetic acid. From the supernatant, 25 µl was injected into the HPLC (pump: Model P580, Gynkotek Separations, H.I. Ambacht, The Netherlands; autosampler: Gilson Model 231 XL, Meyvis, Bergen op Zoom, The Netherlands), equipped with a reversed phase column (Inertsil ODS 3, 3 µm, 100 x 2.1 mm; Aurora Borealis Control, Schoonebeek, The Netherlands), an INTRO electrochemical detector and a cell (ANTEC Leyden B.V., Leiden, The Netherlands) set at a potential of 675 mV vs. Ag/AgCl reference electrode. A column oven set at 30 ºC, integrated in the INTRO, was used for both the reversed phase column and the electrochemical cell. The mobile phase consisted of 5 g/l (NH₄)₂SO₄, 200 mg/l heptane sulphonic acid sodium salt, 500 mg/l EDTA, 2.5% methanol, 30 µl triethylamine, adjusted to pH 4.4 with acetic acid. The flow rate was 0.3 ml/min. For data acquisition, the software package Chromeleon 4.32 (Gynkotek Separations) was used. The detection limit (signal to noise ratio = 3) for both dopamine and norepinephrine was 1 pg/25 µl sample.

All experiments were performed at room temperature (21 - 23 °C). All reported values are mean ± SD of n cells and results are compared using Student’s t-test or the Mann-Whitney non-parametric test where appropriate.
Distinct pathways of exocytosis in PC12 cells

Results

Effects of modulators of intracellular signaling on quantal release from intact cells

Dexamethasone-differentiated PC12 cells generally respond to stimulation with high K⁺ saline with the release of catecholamine-containing vesicles (Westerink and Vijverberg, 2002). Effects of drugs known to modulate Ca²⁺-dependent intracellular signaling pathways associated with exocytosis were investigated in intact PC12 cells. Single cells were first stimulated with high K⁺ saline for 5 - 10 s to determine the control response, and were allowed to recover for ~3.5 min. Subsequently the same cells were superfused with drugs for a period of 15 min. At the end of the drug exposure period, a second exocytotic response was evoked by high K⁺ stimulation. Experiments were performed to investigate the role of PKC with 100 nM of the phorbol ester PMA and with 100 nM staurosporine. In addition, calcineurin activity was inhibited by 10 µM cyclosporin, CaM kinase II activity was inhibited by 10 µM KN-62, and calmodulin activity was inhibited by 10 µM W7 (Fig. 1A). The contents of vesicles released during the second evoked response did not differ significantly from that during the control response (Table 1). This result shows that, within the 15 min exposure period, none of the drugs used did affect vesicle contents.

In control cells, superfused with saline, the frequency of exocytotic events during the second response was reduced to 72 ± 31% (P < 0.01; n = 12) of that during the initial response. Therefore, drug effects on release frequency were determined by comparing event frequencies in drug-exposed cells to those in control cells. Analysis of the frequency of evoked events after drug exposure (Fig. 1B) showed that PMA and W7 cause a significant reduction in the frequency of evoked events in intact PC12 cells. Cyclosporin had a dual effect. In 6 out of 8 cells tested the frequency of evoked events was reduced, on average 6.5 fold, and in 2 cells the frequency was enhanced 3.3 fold (Fig. 1B). Exposure to staurosporine and KN-62 did not lead to significant changes in the frequency of evoked events. The results indicate that activation of PKC and inhibition of calmodulin and calcineurin activity lead, either directly or indirectly, to a change in vesicular catecholamine release during depolarization of intact PC12 cells.
Figure 1. Effects of modulation of PKC, calcineurin, calmodulin and CaM kinase II activity on the frequency of catecholamine-containing vesicles released from intact PC12 cells by high K⁺ stimulation (A,B) and under basal conditions (A,C). (A) Amperometric recordings from intact PC12 cells superfused with saline (upper trace) and with saline containing 10 µM W7 for a 15 min period indicated by the bar on top of the trace. Responses to high K⁺ stimulation at the beginning and at the end of each experiment are also included. (B) The frequency of evoked events, assessed after 15 min of superfusion of 100 nM PMA, 100 nM staurosporine, 10 µM cyclosporin A, 10 µM W7, and 10 µM KN-62, compared to the frequency of evoked events after 15 min of superfusion with saline (control). All frequencies are expressed as a percentage of the control response evoked from the same cell. Note that inhibition of calcineurin by cyclosporin A resulted in a dual effect. (C) Effects of the drugs on the mean frequency of basal release during the 15 min period of exposure. Note that the pattern of modulation of basal release (C) differs from that for evoked release (B). The bar diagrams show mean ± SD for the numbers of cells indicated. *, P <0.05; **, P <0.01; ***, P <0.001.

The basal frequency of exocytotic events, in the absence of stimulation, was also assessed. In control cells the basal release frequency, as determined during a ~10 min period following the recovery period after the first stimulus, amounted to 1.2 ± 1.0 events/min (n = 10). Comparing the basal release frequency in control cells with that in drug-superfused cells (Fig. 1C) shows that PMA, cyclosporin and W7 (see Fig. 1A) significantly enhanced the basal release frequency, whereas effects of staurosporine and KN-62 were not significant. Thus, the effects of PMA and W7 on basal release are opposite to those on evoked release, as is the effect of cyclosporin for the majority of cells investigated. The differential effects suggest that multiple mechanisms contribute to the modulation of exocytotic frequency in intact cells. Therefore, additional experiments were performed on permeabilized PC12 cells to assess direct effects of the drugs on the exocytotic machinery.
Distinct pathways of exocytosis in PC12 cells

Table 1. Absence of effects of drugs, known to modulate PKC, calcineurin, calmodulin, and CaM kinase II activity, on the contents of catecholamine-containing vesicles released from PC12 cells. The median vesicle contents was determined from events recorded during the first high K⁺ stimulation of the intact cell. Subsequently the same cells were either superfused with saline containing the drug at the concentrations indicated for 15 min and challenged with high K⁺ saline for a second time, or were permeabilized with ionomycin, superfused with the drug for 15 min and challenged with saline containing the drug and 1 µM Pb²⁺ to evoke exocytosis. All values are mean ± SD from the number of cells (n) analyzed, and the result of a t-test for the paired observations (p) is also presented.

<table>
<thead>
<tr>
<th>Drug</th>
<th>1st high K⁺ response in intact cell</th>
<th>2nd high K⁺ response in intact cell</th>
<th>n</th>
<th>p</th>
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<tr>
<td>saline control</td>
<td>391 ± 132</td>
<td>108 ± 31</td>
<td>10</td>
<td>0.33</td>
</tr>
<tr>
<td>100 nM PMA</td>
<td>602 ± 325</td>
<td>103 ± 50</td>
<td>3</td>
<td>0.62</td>
</tr>
<tr>
<td>100 nM staurosporine</td>
<td>497 ± 121</td>
<td>109 ± 8</td>
<td>3</td>
<td>0.18</td>
</tr>
<tr>
<td>10 µM cyclosporin A</td>
<td>484 ±  90</td>
<td>90 ± 27</td>
<td>5</td>
<td>0.42</td>
</tr>
<tr>
<td>10 µM W7</td>
<td>506 ± 144</td>
<td>91 ± 22</td>
<td>5</td>
<td>0.30</td>
</tr>
<tr>
<td>10 µM KN-62</td>
<td>549 ± 110</td>
<td>93 ± 29</td>
<td>4</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Modulation of exocytosis in ionomycin-permeabilized cells

Ionomycin-permeabilized PC12 cells release catecholamine-containing vesicles in a Ca²⁺-dependent way. The threshold extracellular Ca²⁺ concentration for vesicular release was previously demonstrated to be close to 100 µM (Westerink and Vijverberg, 2002). Since stimulation of permeabilized cells by superfusion with saline containing a high concentration of Ca²⁺ results in less stable responses than the high K⁺-evoked responses in
intact cells, basal release was measured to assess drug effects in permeabilized cells. In control experiments, basal release was measured during a ~10 min period following a 4 - 5 min permeabilization period. The cells were continuously superfused with 5 µM ionomycin Ca²⁺ salt added to nominal Ca²⁺-free saline. Under these experimental conditions (i.e., ~5.4 µM Ca²⁺), the basal release frequency in ionomycin-permeabilized PC12 cells amounted to 0.1 ± 0.1 events/min (n = 8). The basal release frequency was also assessed in ionomycin-permeabilized cells exposed to the different drugs at the same concentrations as used in experiments on intact cells (Fig. 2A). The comparison with control cells shows that PMA and cyclosporin cause a significant increase in basal release frequency, whereas effects of staurosporine, W7, and KN-62 were not significant (Fig. 2B). The general pattern of modulation of the basal release frequency in permeabilized cells (Fig. 2) is similar to that in intact cells (Fig. 1C) except for W7 and KN-62, which appeared to be inactive in permeabilized cells. The results show that phosphorylation and dephosphorylation of intracellular proteins by PKC and calcineurin directly modulate the basal frequency of exocytosis, and also indicate that calmodulin and CaM kinase II exert indirect effects.

Figure 2. Effects of modulation of PKC, calcineurin, calmodulin and CaM kinase II activity on the frequency of catecholamine-containing vesicles released from ionomycin-permeabilized PC12 cells under basal conditions. (A) Amperometric recordings from permeabilized PC12 cells superfused with saline, with saline containing 100 nM PMA, and with saline containing 10 µM cyclosporin A, as indicated. (B) Effects of the drugs on the mean frequency of basal release during the 15 min period of exposure to the drugs at the same concentrations as used in the experiments shown in Fig. 1. PMA and cyclosporin cause a significant increase in the basal release frequency as compared to that in control cells superfused with saline. Note that the pattern of modulation of basal release in permeabilized cells (B) corresponds to that of the modulation of basal release in intact cells (Fig. 1C), except for W7 and KN-62. The bar diagrams show mean ± SD for the numbers of cells indicated. *, P <0.05; ***, P <0.001.
Distinct pathways of exocytosis in PC12 cells

Signaling pathways involved in Pb\(^{2+}\)-induced exocytosis
Pb\(^{2+}\) substitutes for Ca\(^{2+}\) in inducing vesicular catecholamine release from PC12 cells through a direct intracellular effect (Westerink and Vijverberg, 2002). Superfusion of ionomycin-permeabilized cells with 1 µM Pb\(^{2+}\)-containing saline results in exocytosis after a delay of 1 - 2 min (Fig. 3), after which the frequency of vesicle release gradually increases to a maximum of 125 ± 67 events/min (\(n = 15\)) after ~4.5 min. The frequency of exocytotic events recorded with a carbon fiber microelectrode generally varies strongly between PC12 cells and systematic effects of the drug treatment on the frequency of Pb\(^{2+}\)-evoked events were not observed, except for KN-62 which caused a consistent strong decrease in the frequency of exocytosis (Fig. 4).

Figure 3. Modulation of Pb\(^{2+}\)-induced vesicular catecholamine release from ionomycin-permeabilized PC12 cells by inhibitors of calmodulin, CaM kinase II and PKC. Amperometric recordings show vesicular catecholamine release induced by superfusion of the permeabilized cell with saline containing 1 µM Pb\(^{2+}\) (control) and, additionally, 10 µM W7, 10 µM KN-62, and 100 nM staurosporine, as indicated. All cells were pre-treated with the drugs for a period of 15 min and in the control cell saline was superfused during this period. The delay to onset of Pb\(^{2+}\)-induced release is reduced by W7 and enhanced by KN-62, whereas staurosporine does not appear to affect Pb\(^{2+}\)-induced exocytosis.

KN-62 also caused an increase in the delay to the onset of Pb\(^{2+}\)-induced release. Conversely, exocytosis in W7-treated cells was consistently evident already within the first 30 s of superfusion of the cells with Pb\(^{2+}\)-containing saline. Exposure of the cells to PMA, to staurosporine, and to cyclosporin did neither cause systematic nor differential effects. An early apparent effect in cyclosporin-treated cells is due to the enhancement of basal release by cyclosporin before the onset of Pb\(^{2+}\)-evoked release. The contents of vesicles released by
Pb\textsuperscript{2+} did not differ from that of vesicles released during control responses evoked by high K\textsuperscript+ stimulation at the start of experiments (Table 1; Westerink and Vijverberg, 2002). These results demonstrate that Pb\textsuperscript{2+}-induced exocytosis is modulated by calmodulin and CaM kinase II through direct effects on the exocytotic machinery and not by PKC and calcineurin, whereas the reverse was observed for basal release (see Fig. 2).

**Figure 4.** Effects of drugs modulating PKC, calcineurin, calmodulin and CaM kinase II activity on the frequency of catecholamine-containing vesicles released from ionomycin-permeabilized PC12 cells superfused with saline containing 1 \( \mu \text{M} \) Pb\textsuperscript{2+}. The panels show cumulative frequencies of exocytotic events recorded following the start of superfusion with Pb\textsuperscript{2+}-containing saline \((t = 0)\) and the drugs indicated. The cells were superfused with saline (control) or with saline containing 10 \( \mu \text{M} \) W7, 100 nM PMA, 100 nM staurosporine, 10 \( \mu \text{M} \) cyclosporin A and 10 \( \mu \text{M} \) KN-62 for 15 min before superfusion with saline containing 1 \( \mu \text{M} \) Pb\textsuperscript{2+} and the same concentrations of the drug. The curves, depicting release over time from different cells, show that the frequency of exocytosis varied strongly between cells. The delay to the onset of Pb\textsuperscript{2+}-evoked release, which amounts 1 - 2 min in control cells, is reduced to < 30 s by W7 and is increased by KN-62 to > 2 min. In addition, the number of events recorded from KN-62-treated cells was strongly reduced. Note that modulation of PKC activity by PMA and staurosporine and inhibition of calcineurin activity by cyclosporin A did neither result in systematic nor in differential modulation of the Pb\textsuperscript{2+}-induced exocytosis.
Catecholamine release from synaptotagmin-deficient PC12-F7 cells

The triggering of exocytosis is generally assumed to occur through the interaction of Ca\(^{2+}\) with a Ca\(^{2+}\) sensor. Synaptotagmin is not only the most likely candidate to serve as Ca\(^{2+}\) sensor, it also shows high sensitivity to Pb\(^{2+}\) (Bouton et al., 2001) and is a target of several of the intracellular signaling pathways addressed in this study. Therefore, the role of synaptotagmin was also investigated by comparing release from PC12 cells with release from the clone PC12-F7, selected for the absence of synaptotagmin I and also deficient in synaptotagmin II (Shoji-Kasai et al., 1992). Catecholamine release from populations of PC12 and PC12-F7 cells was measured using HPLC with electrochemical detection (Fig. 5A).

**Figure 5.** Catecholamine release from PC12 cells and from synaptotagmin I- and II-deficient PC12-F7 cells. (A) Time course of dopamine secretion from cell populations determined using HPLC with electrochemical detection. Dopamine release is low under control conditions. Following a 1 min application of high K\(^+\)-saline, as indicated by the bar on top of the diagrams, dopamine secretion is markedly increased in populations of PC12 (upper) and PC12-F7 (lower) cells. Note that release under resting conditions as well as during depolarization is strongly reduced in PC12-F7 cells as compared to PC12 cells. Each point represents mean ± SD of 3 experiments. (B) Amperometric current transients on an expanded time scale. The transients represent single catecholamine-containing vesicles released from an intact PC12 cell during high K\(^+\) stimulation (upper trace), and from intact and ionomycin-permeabilized PC12-F7 cells superfused with high K\(^+\) saline (middle trace) and with 1 µM Pb\(^{2+}\)-containing saline (lower trace), respectively. Vesicle contents are indicated below the events.
In control experiments PC12 cells showed robust dopamine release during a 1 min challenge with high K⁺ saline. Most of the catecholamines secreted appeared to be dopamine and only ~0.8% was noradrenaline, which was secreted with the same time course (not shown). The synaptotagmin-deficient PC12-F7 cells released only ~3.5% of the amount of catecholamines secreted by PC12 cells. Like PC12 cells, PC12-F7 cells released mainly dopamine and only ~0.4% of the catecholamines secreted was noradrenaline (not shown). The present result demonstrates that synaptotagmin I- and II-deficient PC12-F7 cells release catecholamines, but to an amount that is 30-fold lower than that released from PC12 cells.

The low level of catecholamine release in PC12-F7 cells was confirmed by carbon fiber microelectrode amperometry. PC12 cells are highly responsive to depolarization in releasing catecholamine-containing vesicles (88%; n = 140). The fraction of PC12-F7 cells responding to depolarization is much smaller and amounts to 21% (n = 71). The cells also differ in the frequency of events evoked by high K⁺ stimulation, which was 4.6 ± 3.4 events/s for PC12 (n = 123) and 0.3 ± 0.4 events/s (n = 15) for PC12-F7 cells. In addition, the median vesicle contents amounted to 391 ± 132 zmol (n = 10) in PC12 and to 88 ± 41 zmol (n = 14) in PC12-F7 cells. For PC12 cells the total amount of catecholamines released by exocytosis was calculated using the median vesicle contents, the mean frequency of evoked events, and the assumption that the carbon fiber covers ~20% of the cell surface. The amount calculated for single cells appeared to agree within 20% with the amount secreted from populations of PC12 cells. However, > 70% of the amount of catecholamines secreted from PC12-F7 cell populations could not be accounted for by the amount detected by carbon fiber microelectrode amperometry, indicating that many quanta were below the detection limit (~25 zmol) or that a significant proportion of catecholamines is secreted by non-vesicular mechanisms. Because of the low level of catecholamine release from PC12-F7 cells, the numbers of vesicles released per cell by superfusion of intact cells with high K⁺ saline and of ionomycin-permeabilized cells with 1 μM Pb²⁺-containing saline were too small for a statistical analysis. However, events evoked by depolarization were very similar to Pb²⁺-evoked events (Fig. 5B).
Distinct pathways of exocytosis in PC12 cells

Discussion

The results show that depolarization-evoked and basal exocytosis of catecholamine-containing vesicles from intact PC12 cells are differentially affected by drugs modulating PKC, calcineurin, calmodulin, and CaM kinase II activities (Fig. 1). Since the depolarization-evoked (125 mM K+-containing saline) release occurs at a membrane potential close to 0 mV and basal release at resting membrane potential, voltage-activated channels may play a role in the differences observed. Contradictory effects of phorbol esters on voltage-activated Ca\(^{2+}\) current reported in the literature (Harris et al., 1986; Sena et al., 1995, 1999; Oda et al., 1995; Gillis et al., 1996; Taylor et al., 2000), indicate that uncontrolled factors play an important role in the modulatory effects of PKC on catecholamine release, particularly in intact cells. The present results (Figs. 1B, C, 2) suggest that inhibition of voltage-activated Ca\(^{2+}\) current is responsible, at least in part, for the observed reduction of depolarization-evoked vesicular catecholamine release from intact cells by PMA. Whether the enhancement of basal release of catecholamine-containing vesicles from intact PC12 cells is caused by a direct intracellular effect of PMA or, indirectly, by a differential enhancement of low voltage-activated Ca\(^{2+}\) current by PMA cannot be concluded on the basis of the present results. Similar ambiguities arise when trying to interpret the effects of the other drugs modulating intracellular signaling in intact PC12 cells. In general, the reported effects of the drugs on neurotransmitter release vary (see Introduction), and firm conclusions with respect to direct or indirect effects cannot be drawn based on the results obtained from intact cells (Fig. 1).

In permeabilized PC12 cells voltage-activated ion channels are bypassed, allowing the assessment of direct effects of membrane-permeable drugs on intracellular signaling pathways. Like PMA in ionomycin-permeabilized PC12 cells (Fig. 2), TPA has been reported to enhance catecholamine release from digitonin-permeabilized chromaffin cells, an effect associated with protein phosphorylation and PKC translocation from the cytoplasm to the membrane (Lee and Holz, 1986; TerBush and Holz, 1986). In ‘cracked’ PC12 cells PKC also enhances catecholamine release by a direct action on the exocytotic machinery (Chen et al., 1999). The present results on basal release from ionomycin-permeabilized cells, showing that PKC and calcineurin exert direct, opposite effects on vesicular catecholamine release (Fig. 2), indicate that PKC-mediated phosphorylation enhances and calcineurin-mediated dephosphorylation inhibits exocytosis downstream of Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels. Conversely, calmodulin appears to exert primarily indirect effects on exocytosis and inhibition of CaM kinase II does not cause significant effects on the release of catecholamine-containing vesicles from intact and permeabilized PC12 cells (Figs. 1, 2). From the results on ionomycin-permeabilized PC12
cells it is concluded that modulation of the phosphorylation state of the exocytotic machinery by PKC/calcineurin results in modulation of exocytotic function.

Systematic effects of pretreatment with PMA, staurosporine, and cyclosporin A on Pb^{2+}-induced release from ionomycin-permeabilized PC12 cells are not observed. This is a surprising result, since Pb^{2+} has been reported to be a potent activator of PKC in various cell lines, including PC12 cells (Jadhav et al., 2000; Tian et al., 2000), and activation of PKC enhances basal exocytosis in permeabilized PC12 cells (Fig. 2) consistent with the general findings reported in the literature (see above). Although inhibitory effects of Pb^{2+} on PKC activity have also been reported (Sun et al., 1999), the absence of systematic and coherent effects of PMA, staurosporine and calcineurin inhibition indicate that PKC-mediated phosphorylation does not exert a major regulatory effect on Pb^{2+}-induced exocytosis. This is consistent with the observation that inhibition of PKC activity by staurosporine does not affect Pb^{2+}-induced catecholamine release from populations of α-toxin-permeabilized bovine chromaffin cells (Tomsig and Suszkiw, 1995). The calmodulin inhibitor W7, which does not cause significant effects on basal release in permeabilized PC12 cells, clearly reduced the delay to onset of Pb^{2+}-induced exocytosis (Figs. 3, 4). Since the delay to onset of release is reduced with increasing Pb^{2+} concentration (Westerink and Vijverberg, 2002), the effect of W7 is equivalent to an apparent enhancement of the sensitivity to Pb^{2+}. Inhibition of CaM kinase II activity by KN-62 leads to a reduction of the number of vesicles released and to an apparent increase in the delay to onset of Pb^{2+}-evoked exocytosis (Figs. 3, 4), which appears to be independent of calmodulin. The effect of 10 µM KN-62, which causes a nearly complete inhibition of CaM kinase II activity in PC12 cells (Schweitzer et al., 1995), indicates that CaM kinase II is required for Pb^{2+}-induced release. KN-62 does neither affect depolarization-evoked exocytosis in intact cells nor basal exocytosis in permeabilized cells, indicating that Ca^{2+}-induced exocytosis is little affected. The combined results obtained from permeabilized PC12 cells demonstrate that Pb^{2+}-induced exocytosis is controlled by CaM kinase II and calmodulin, unlike basal exocytosis which is controlled by PKC/calcineurin.

A straightforward conclusion with respect to the role of synaptotagmin in Ca^{2+}- and Pb^{2+}-induced exocytosis is hampered by the finding that the amount of catecholamines released from PC12-F7 cells is strongly reduced as compared to that from PC12 cells, consistent with the result of a previous study (Chen et al., 2001). This is reflected in the parameters of quantal catecholamine release, showing a reduced vesicle contents and a reduced frequency of vesicles released (Fig. 5). Whether the low frequency of exocytotic events measured from PC12-F7 cells is a consequence of the small vesicle contents, or is due to the absence of synaptotagmins I and II cannot be assessed from the present data. However, the distinct features of vesicular catecholamine release from PC12-F7 cells...
observed for both Ca\(^{2+}\) and Pb\(^{2+}\) (Fig. 5), indicate that these metal ions are able to induce neurotransmitter release from similar vesicle pools through exocytotic pathways which are independent of synaptotagmins I and II. The present results do not rule out that the pathways of Ca\(^{2+}\)- and Pb\(^{2+}\)-induced exocytosis converge at other synaptotagmins, e.g., of the subtypes III, IV, and V which are also expressed in PC12 cells (Mizuta et al., 1994; Hudson and Birnbaum, 1995; Vician et al., 1995).

In conclusion, the results demonstrate that Pb\(^{2+}\)-induced exocytosis does not depend on the modulatory effects of PKC activity on vesicular catecholamine release, but requires activation of CaM kinase II. Although a direct demonstration of activation of CaM kinase II by Pb\(^{2+}\) is lacking, CaM kinase II is known to phosphorylate a range of intracellular proteins, including synapsin I (Jovanovic et al., 2001), synaptotagmin (Verona et al., 2000), and the t- and v-SNAREs (Hirling and Scheller, 1996; Risinger and Bennett, 1999) and CaM kinase II-induced phosphorylation is associated with an increase in the number of releasable vesicles (Gromada et al., 1999). Therefore, CaM kinase II provides a novel and plausible target for the direct intracellular action of Pb\(^{2+}\) leading to neurotransmitter release.

Acknowledgements

We thank Dr. L. Tertoolen (Hubrecht Laboratory, Utrecht) for donation of PC12 cells; Dr. Shoji-Kasai (Mitsubishi-Kagaku Institute of Life Sciences, Tokyo, Japan) for the generous donation of PC12-F7 cells; Dr. S. Agulian (Yale University, West Haven, USA) for donation of carbon fibers; and Ing. A. de Groot for excellent technical assistance.
Toluene-induced, Ca\textsuperscript{2+}-dependent vesicular catecholamine release in PC12 cells

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accepted Neurosci. Lett.

Abstract

Acute effects of toluene on vesicular catecholamine release from intact PC12 cells have been investigated using carbon fiber microelectrode amperometry. The frequency of vesicles released is low under basal conditions and is enhanced by depolarization. Toluene causes an increase in basal release frequency. The threshold effect is obtained at 30 µM toluene and the maximum enhancement of basal release is 7-fold at 1 mM toluene. Toluene-induced exocytosis depends on the influx of Ca\textsuperscript{2+} through voltage-activated Ca\textsuperscript{2+} channels, which are blocked by Cd\textsuperscript{2+}. Toluene does neither affect depolarization-evoked exocytosis, nor the characteristics of release events. It is concluded that toluene-induced vesicular catecholamine release is due to an increase in intracellular Ca\textsuperscript{2+} concentration, whereas basic processes underlying exocytosis do not appear to be affected by toluene at concentrations up to 300 µM.

Keywords: rat phaeochromocytoma cells - calcium - vesicular catecholamine release - carbon fiber microelectrode amperometry - volatile organic solvent - cadmium
**Introduction**

Toluene is widely present in organic solvent mixtures and also in solvents of abuse. Long-term occupational exposure to toluene has been associated with a variety of neurotoxic effects ranging from subtle alterations in neurobehavioral parameters to gross neuropathological changes (reviewed in Schaumburg, 2000). Acute high-level exposure results in CNS excitation followed by depression (Balster, 1998; Schaumburg, 2000). Toluene-induced hyperactivity appears to occur through a dopamine-dependent mechanism, since it is inhibited by a selective dopamine D₂ receptor antagonist (Riegel and French, 1999). In addition, high concentrations of toluene have been shown to inhibit rat NMDA type glutamate receptors and potentiate responses mediated by human GABA₅ and glycine receptors expressed in *Xenopus* oocytes (Cruz et al., 1998; Beckstead et al., 2000). The presynaptic effects of toluene are unknown.

Catecholamine release from rat PC12 phaeochromocytoma cells has been characterized in detail. These cells synthesize dopamine and norepinephrine and express a variety of receptors, voltage- and ligand-gated ion channels (Shafer and Atchison, 1991). In PC12 cells, differentiated with glucocorticoids into a chromaffin-like cell type, Ca²⁺ current, catecholamine synthesis and release are enhanced, and these cells show rapid exo- and endocytosis (Tischler, et al., 1983; Elhamdani et al., 2000). The exocytotic process involves dynamic protein and membrane interactions and consists of vesicle docking and priming, intracellular fusion of the vesicle and plasma membranes, the formation of a fusion pore, and the release of vesicle contents (reviewed in Lin and Scheller, 2000). We have investigated the effects of toluene on vesicular catecholamine release in PC12 cells.

**Materials and methods**

PC12 cells (ATCC #CRL-1721) were grown as described previously (Westerink et al., 2000), with some modifications. Cells were differentiated in culture medium supplemented with 5 µM dexamethasone starting 2 d after subculturing. Culture dishes were coated with 5 µg/cm² poly-L-lysine. The culture medium was refreshed every 2 - 3 d. Carbon fiber microelectrode amperometry experiments (Chow and von Rüden, 1995) were performed 5 - 8 d after initiating cell differentiation. Carbon fiber (⌀ 10 µm) microelectrode fabrication and data recording and analysis were as described previously (Westerink et al., 2000). Before experiments, cells were washed twice with saline solution containing (in mM) 125 NaCl, 5.5 KCl, 2 CaCl₂, 0.8 MgCl₂, 10 HEPES, 24 glucose, and 36.5 sucrose at pH 7.3 adjusted with NaOH. The carbon fiber, polarized to 700 mV, was placed gently on the membrane surface of a PC12 cell under continuous superfusion with saline through one barrel of a theta superfusion pipette (⌀ ~50 µm) at a rate of ~150 µl/min. Toluene (97.7%) was dissolved in a large volume of saline solution (100 ml) by overnight stirring in a tightly closed glass bottle to
produce a saturated stock solution (6.3 mM in water (Eastcott et al., 1988)). Stock solutions of
toluene and of 50 mM Cd(NO$_3$)$_2$; prepared in distilled water immediately before use, were diluted in
saline to obtain the desired concentrations. All experiments were performed at room temperature (21-
23 °C). Exocytotic responses were evoked by superfusion with high K$^+$ saline (KCl elevated to 125
mM and NaCl reduced to 5.5 mM). Between stimuli, cells were superfused with saline for a period of
4 min. The basal frequency of quantal catecholamine release was determined during the last 2.5 min
of this 4 min period and appeared to vary between 0.3 and 4.0 events/min. Because of this
intercellular variability, values of basal release frequency were obtained under control conditions
(open bar below the traces of Fig. 1A) and in the presence of toluene (solid bar below the traces of
Fig. 1A) from the same cells and were compared using a paired Student’s t-test. All reported values
are mean ± SD of n cells.

Results

PC12 cells display a large exocytotic response on depolarization with high K$^+$ saline for 5 s
(298.7 ± 136.7 events/min, n = 36; Fig. 1A and Fig. 2), which is entirely dependent on Ca$^{2+}$
influx through voltage-activated Ca$^{2+}$ channels (Green et al., 2001; confirmed, not shown).
However, the basal frequency of quantal catecholamine release in PC12 cells superfused
with saline is low and amounts to 1.4 ± 1.0 events/min (n = 36). Continued superfusion
with saline (n = 8; Fig. 1A) or subsequent superfusion with saline containing 10 µM
toluene (n = 3; not shown) for 7.5 min did not result in a significant change in the
frequency of vesicle exocytosis as compared to the pre-exposure period (1.3 ± 0.9 vs. 1.7 ±
1.7 events/min; $P = 0.15$ and $1.3 ± 0.8$ vs. $2.5 ± 1.7$ events/min; $P = 0.24$, respectively; Fig.
1B). Conversely, superfusion with saline containing 30 µM toluene caused an increase in
basal release frequency within several seconds after application. This effect was sustained
in the presence of toluene for up to 15 min. The mean frequency of exocytotic events
during the first 7.5 min of exposure to toluene amounted to $3.5 ± 1.4$ events/min (n = 4;
pree-exposure: $1.1 ± 0.8$; $P = 0.04$). At increased concentrations of 100 µM and 300 µM
toluene, the basal release frequency further increased to $7.7 ± 7.9$ events/min (n = 10; pre-
exposure: $1.4 ± 1.1$; $P = 0.03$) and $8.8 ± 7.2$ events/min (n = 8; pre-exposure: $1.4 ± 1.1$; $P =
0.02$), respectively (Fig. 1A, B). At 1 mM toluene the effect on the basal release frequency
appeared to saturate and amounted to $9.3 ± 3.5$ events/min (n = 3; pre-exposure: $1.2 ± 0.3$;
$P = 0.01$; Fig. 1B). These results demonstrate that extracellularly applied toluene rapidly
evokes vesicular catecholamine release in dexamethasone-differentiated PC12 cells and that
the threshold concentration to evoke exocytosis is between 10 µM and 30 µM toluene.

Toluene did not affect the ability of cells to respond to high K$^+$ saline with vesicular
catecholamine release. Switching from high K$^+$ saline to 300 µM toluene-containing high
K⁺ saline ($n = 3$) did not result in observable changes in the exocytotic response. In addition, a marked exocytotic response was evoked by superfusion with high K⁺ saline in cells that had been exposed to 300 µM toluene for 7.5 (Fig. 1A) and up to 15 min (not shown). The characteristic parameters of quantal events, i.e., mean vesicle contents, as well as event rise time, amplitude, and width, were determined from events evoked by depolarization with high K⁺ saline, by superfusion with saline containing toluene, and by depolarization with high K⁺ saline in the presence of toluene. Toluene did neither cause significant changes in vesicle contents, which averaged 750 ± 184 zmol during depolarization, 633 ± 105 zmol in the presence of 300 µM toluene ($P = 0.15$), and 670 ± 121 zmol ($n = 4$) during depolarization in the presence of toluene ($P = 0.10$), nor in the other event characteristics analyzed. These results indicate that up to 300 µM toluene neither affects depolarization-induced exocytosis nor affects the release kinetics and contents of catecholamine-containing vesicles in PC12 cells.

**Figure 1.** Toluene evokes vesicular catecholamine release in PC12 cells. (A) Amperometric recordings showing the response to depolarization by superfusion with high K⁺ saline in a control cell (upper trace), and before and after 7.5 min of exposure to 30 µM and 300 µM toluene (middle and lower traces). Toluene alone enhances vesicular catecholamine release. Bars on top of the traces indicate exposure periods. The open and solid bars below the traces indicate the periods during which control and test values of the basal release frequency were obtained in each cell. (B) The concentration dependence of the effect of toluene on vesicular catecholamine release. The frequency of vesicles released during toluene exposure (solid bars) is expressed as events/min and compared to basal release frequency before exposure of the same cells to toluene (open bars). Significant enhancement of the release frequency is observed at ≥ 30 µM toluene as indicated (paired t-test; * $P < 0.05$). Each bar represents mean ± SD of a number of cells indicated in parentheses.
Since intracellular Ca\(^{2+}\) forms the trigger for exocytosis, the role of Ca\(^{2+}\) and Ca\(^{2+}\) channels in toluene-induced vesicular catecholamine release was also investigated. In cells, superfused with nominal Ca\(^{2+}\)-free saline containing 300 µM toluene, the effect of toluene on exocytosis remained absent until Ca\(^{2+}\) was added to the saline (Fig. 2A). This demonstrates that the effect of toluene depends on the presence of extracellular Ca\(^{2+}\). The pathway responsible for the influx of extracellular Ca\(^{2+}\) was determined using the non-specific voltage-activated Ca\(^{2+}\) channel blocker Cd\(^{2+}\) (Shafer, 1998; Taylor et al., 2000; Green et al., 2001). During superfusion with high K\(^{+}\) saline the exocytotic response was blocked when the superfusion medium was changed to 200 µM Cd\(^{2+}\)-containing high K\(^{+}\) saline, and vesicular catecholamine release rapidly reappeared upon switching back to high K\(^{+}\) saline (Fig. 2B), which is as expected from the characteristics of the block of voltage-activated Ca\(^{2+}\) channels in PC12 cells by Cd\(^{2+}\) (Shafer, 1998; Taylor et al., 2000; Green et al., 2001). During such short-term exposure to Cd\(^{2+}\) (30 - 60 s) no additional effects of Cd\(^{2+}\) are expected. In cells superfused with saline containing 300 µM toluene a similar effect of Cd\(^{2+}\) was observed. As with depolarization-evoked exocytosis, 200 µM Cd\(^{2+}\) blocked vesicular catecholamine release and this effect was rapidly reversed when Cd\(^{2+}\) was removed from the superfusion medium (Fig. 2B). The results with Cd\(^{2+}\) demonstrate that Ca\(^{2+}\) ions, required for depolarization- and toluene-induced exocytosis, enter the cell through voltage-activated Ca\(^{2+}\) channels.

**Figure 2.** (A) Amperometric recording illustrating the Ca\(^{2+}\) requirement of toluene-induced exocytosis in PC12 cells. After a short depolarization and a recovery period, the cell is superfused with 300 µM toluene-containing, nominal Ca\(^{2+}\)-free saline. Toluene fails to induce exocytosis until the cell superfusate is changed back to Ca\(^{2+}\)-containing saline. (B) Catecholamine release induced by superfusion with high K\(^{+}\) saline (upper trace) as well as that induced by superfusion with 300 µM toluene-containing saline (bottom trace) are inhibited by 200 µM Cd\(^{2+}\). This result demonstrates the involvement of voltage-activated Ca\(^{2+}\) channels in both depolarization- and toluene-induced exocytosis in PC12 cells. The bars on top of the amperometric traces indicate superfusion periods.
Toluene-induced exocytosis in PC12 cells

Discussion

The combined results show that toluene-induced release of catecholamine-containing vesicles in dexamethasone-differentiated PC12 cells is mediated by influx of extracellular Ca\(^{2+}\) through voltage-activated Ca\(^{2+}\) channels. The absence of an effect of toluene on depolarization-evoked vesicular catecholamine release indicates that the exocytotic process remains intact. The relatively low frequency of exocytotic events in the presence of toluene suggests that the sustained exocytosis of catecholamine-containing vesicles is maintained by a small number of activated Ca\(^{2+}\) channels. From the results it cannot be deduced whether these Ca\(^{2+}\) channels are directly activated by toluene or indirectly as a result of toluene-induced membrane depolarization.

The concentration of toluene, which enhances vesicular catecholamine release in PC12 cells, is comparable to blood levels of toluene reached in acute occupational intoxication (9 - 44 µM) (Brugnone et al., 1983; Meulenbelt et al., 1990) and acute intoxication caused by abusive inhalation of toluene (90 µM) (King, 1982). Antagonism of toluene-induced hyperactivity by the dopamine D\(_2\) receptor antagonist remoxipride (Riegel and French, 1999) indicates that an elevated level of extracellular dopamine in rat brain contributes to the toxic symptoms of toluene. Whether this involves a toluene-induced enhancement of dopamine release \textit{in vivo} remains to be established. Since catecholamine release in PC12 cells is enhanced as a result of elevated intracellular Ca\(^{2+}\), toluene may affect the release of other neurotransmitters as well.

Acknowledgements

We thank Dr. L. Tertoolen (Hubrecht Laboratory, Utrecht) for donation of PC12 cells; Dr. S. Agulian (Yale University, West Haven, USA) for donation of carbon fibers; Dr. C. Meulenberg for helpful discussions; and Ing. A. de Groot for assistance with electronics and computer analysis.
Catecholamine-Containing Vesicles Released from Rat PC12 Cells on Acute Exposure to Polychlorinated Biphenyls.

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submitted

Abstract

Effects of selected polychlorinated biphenyls (PCBs) on vesicular catecholamine release from rat PC12 phaeochromocytoma cells have been measured using carbon fiber microelectrode amperometry. Exocytotic responses were evoked by superfusion of single PC12 cells with high K⁺ saline. Subsequent exposure of the same cells to saline containing the nonplanar PCB 4 and the coplanar PCB 126 at concentrations between 5 and 25 µM for 15 min caused an enhancement of the frequency of basal release of catecholamine-containing vesicles at the lower concentrations, but not at the high concentrations tested. The nonplanar PCB 128 did not affect basal release. The PCBs caused only marginal effects on the frequency of evoked events during high K⁺ stimulation and did not affect vesicle contents. Prolonged exposure of PC12 cells to the same PCBs in the culture medium for a period of 3 days did not cause significant changes in vesicle contents. The results demonstrate that low concentrations of PCBs may cause acute vesicular catecholamine release, but do not influence the contents of catecholamine-containing vesicles, neither on acute nor after subchronic exposure.

Keywords: PCB - quantal catecholamine release - rat PC12 phaeochromocytoma cells - carbon fiber microelectrode amperometry
Introduction

The neurotoxic potential of specific polychlorinated biphenyls (PCBs) and related substances, many of which persist in the environment and may accumulate in biological tissues, is a matter of toxicological concern (Tilson and Kodavanti, 1997). Behavioral symptoms and brain neurotransmitter levels in exposed animals, as well as in vitro experimental data, indicate involvement of monoaminergic systems in PCB neurotoxicity and of the dopaminergic system in particular (for review see: Seegal, 1995).

Potential mechanisms underlying the effects of PCBs on the catecholaminergic system have been addressed using in vitro model systems. Several of the ortho-substituted, nonplanar PCBs reduce the dopamine contents of rat phaeochromocytoma (PC12) cells after 6 hr of exposure with IC50 values < 100 µM, whereas coplanar PCBs are inactive in this respect (Shain et al., 1991). In an additional study of PC12 cells, the threshold concentrations of several PCBs to affect cellular dopamine contents, and basal and evoked dopamine release were reported to be close to 10 µM (Angus and Contreras, 1996). In primary cultured bovine adrenal chromaffin cells, exposed to 50 - 100 µM of the nonplanar 2,2’,4,4’ PCB for 1 and 5 days, cellular catecholamine contents and evoked catecholamine release are reduced, and basal catecholamine release is enhanced. The coplanar 3,3’,4,4’ PCB did not cause any of these effects at concentrations up to 100 µM (Messeri et al., 1997).

Experiments with rat PC12 and mouse neuroblastoma N1E-115 cells (Seegal et al., 1991) have shown that 30 - 200 µM PCB 4 (2,2'-dichlorobiphenyl) inhibits tyrosine hydroxylase. A less potent, partial inhibition of tyrosine hydroxylase activity by PCB 4 was observed in minced rat corpus striatum (Choksi et al., 1997). Uptake of dopamine into rat brain synaptosomes and into rat brain synaptic vesicles are both inhibited by micromolar concentrations of PCBs. Highly chlorinated congeners appear to inhibit the plasma membrane dopamine transporter more potently than the vesicular monoamine transporter (Mariussen et al., 1999; Mariussen and Fonnum, 2001). Exposure of primary cultured rat neocortical and cerebellar granule cells to micromolar concentrations PCBs may lead to variety of early transient, sustained, and oscillatory elevations of the intracellular Ca^{2+} concentration (Kodavanti et al., 1993; Carpenter et al., 1997; Mundy et al., 1999; Inglefield and Shafer, 2000; Inglefield et al., 2001). An elevation of the intracellular Ca^{2+} concentration and of Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) activation, i.e., common signaling pathways, appear to be involved in PCB-induced release of insulin from RINm5F rat insuloma cells (Fischer et al., 1996; 1999).
Several of the alleged mechanisms of action of PCBs would be expected to lead to changes in the characteristics of vesicular catecholamine release from PC12 cells, which can be measured using carbon fiber microelectrode amperometry (Wightman et al., 1991). An increase in the available amount of dopamine by supplementing L-DOPA causes an elevation of the contents of catecholamine-containing vesicles released from PC12 cells (Pothos and Sulzer, 1998; Westerink et al., 2000). Conversely, a decrease of dopamine availability by inhibition of tyrosine hydroxylase (Pothos et al., 1998) and by inhibition of dopamine transport across the plasma membrane (Pothos and Sulzer, 1998) are associated with a decrease in vesicle contents. In addition, a disturbance in Ca\(^{2+}\) homeostasis, e.g., induced by caffeine in PC12 cells (Taylor and Peers, 1999b), may lead to changes in basal release.

The combined literature data indicate that PCBs will exert a general effect on the probability of vesicle exocytosis and a more specific effect on the contents of catecholamine-containing vesicles. We have investigated the effects two ortho-substituted nonplanar and one coplanar PCB on the release of catecholamine-containing vesicles from single PC12 cells.

**Materials and methods**

**Materials.** NaCl, KCl, Mg\((\text{NO}_3\))\(_2\), glucose, sucrose, HEPES, and NaOH (Aristar quality) were obtained from BDH Laboratory Supplies (Poole Dorset, UK). PCB 4 (2,2'-dichlorobiphenyl, purity ≥ 99%), PCB 126 (3,3',4,4',5-pentachlorobiphenyl, purity ≥ 99%), and PCB 128 (2,2',3,3',4,4'-hexachlorobiphenyl, purity ≥ 99%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany) and DMSO from Sigma (St. Louis MO, USA). Saline solutions were prepared with de-ionized millipore water (Milli-Q\(^\text{m}\); resistivity >10 M\(\Omega\)·cm). Immediately after preparation all saline solutions were filtered using a Millipore GSWP 0.22 \(\mu\)m filter (Millipore, Bedford MA, USA) and stored in thoroughly cleaned and rinsed glass bottles at -20 °C until use. Stock solutions of 5 mM PCB 4, 5 mM PCB 126, and 5 mM PCB 128 were prepared by ultrasonication in DMSO, kept at room temperature, and diluted in external solution to obtain the desired concentrations immediately prior to the experiments. DMSO alone (0.5% v/v) has previously been shown not to affect release characteristics in acute experiments.
Cell culture. PC12 cells (Greene and Tischler, 1976; ATCC CRL-1721; cultured for 10 passages) were grown essentially as described previously (Westerink et al., 2000), with some modifications. Cells were differentiated in culture medium (RPMI 1640, Gibco, Grand Island NY, USA) supplemented with 5 µM dexamethasone (Genfarma, Zaandam, The Netherlands) starting 2 days after subculturing. Culture dishes were coated with 5 µg/cm² poly-L-lysine (Sigma, St. Louis MO, USA). The culture medium was refreshed every 2 - 3 days. Experiments were performed 7 - 10 days after subculturing, i.e., 5 - 8 days after initiating differentiation. For subchronic PCB exposure PCBs were added to culture medium during the last 3 days of culturing and the PCB-containing culture medium was refreshed daily.

Carbon fiber microelectrode amperometry and data analysis. Carbon fiber microelectrode (Ø 10 µm) fabrication and data recording and analysis were as described previously (Westerink et al., 2000). Before experiments, cells were washed twice with saline solution containing (in mM) 125 NaCl, 5.5 KCl, 2 CaCl₂, 0.8 MgCl₂, 10 HEPES, 24 glucose, and 36.5 sucrose at pH 7.3 adjusted with NaOH. The carbon fiber, polarized to 700 mV, was placed gently on the membrane surface of a PC12 cell. The cell under investigation was continuously superfused with saline solution through one barrel of a theta superfusion pipette (Clarke TGC150; pipette tip Ø 80 µm) at a rate of ~150 µl/min. Exocytotic responses were evoked by switching the superfusate to high K⁺ saline (KCl elevated to 125 mM and NaCl reduced to 5.5 mM) for a period of 5 - 15 s. Experiments were performed at room temperature (21 - 23 °C). All reported values are mean ± SD of n cells and results are compared using the Student t-test or the Mann-Whitney nonparametric test where appropriate.

Results

Acute effects
Vesicular catecholamine release was evoked from saline-superfused, single PC12 cells by switching the superfusate to high K⁺ saline for a period of 5 - 15 s. Control experiments showed that exocytotic responses to high K⁺ stimulation, recorded with a carbon fiber microelectrode on the cell surface (Fig. 1A), could be evoked repeatedly. High K⁺ stimulation evoked vesicular catecholamine release in > 80% of all cells tested, but the frequency of exocytotic events varied considerably between cells. Few very large events were often observed during the first stimulation and some rundown of the number of vesicles released per stimulus occurred over time, as reported previously (Westerink and Vijverberg, 2002). After cessation of high K⁺ stimulation, the frequency of exocytotic events rapidly declined to a low basal level of 1.2 ± 1.0 min⁻¹ (n = 10).
Figure 1. Vesicular release in PC12 cells measured by carbon fiber microelectrode amperometry. Control responses evoked by superfusion of the cell with high K⁺ saline at the start and at the end of the experiment (upper trace). The effects of 10 µM (middle trace) and 25 µM (lower trace) PCB 4. At the lower concentration a marked increase of basal vesicular release during the 15 min period of PCB exposure is observed. Note that the biphasic deflections from baseline are noise artifacts, whereas virtually all monophasic, positive deflections represent exocytotic events.

In acute experiments cells were allowed to recover for 4 min from initial high K⁺ stimulation and then superfused with saline solution containing 10 µM PCB 4, 5 µM PCB 126, or 5 µM PCB 128 for a period of 15 min. Complete ~20 min recordings of raw data for a control cell and for two cells exposed for 15 min to 10 µM and 25 µM PCB 4 are shown in Fig. 1. The result shows a marked increase in the basal release frequency during the 15 min period of exposure to 10 µM PCB 4, but not during exposure to 25 µM PCB 4. Analysis of the effects of PCBs on the release frequency in multiple cells (Fig. 2) showed that PCB 126 at a concentration of 5 µM, but not at 12.5 µM, also enhanced basal release. PCB 128 did not affect the frequency of vesicular catecholamine release from PC12 cells, either at 5 µM and at 25 µM (Fig. 2).
Figure 2. Effects of PCBs on the basal release of catecholamine-containing vesicles from PC12 cells. The panels show cumulative event frequencies (n), normalized to the total number of events observed from each cell (N), over the total experimental period. All cells were stimulated with high K⁺ saline for a period of 5 - 10 s (indicated by arrows) at the beginning and at the end of the experiment. Following the first stimulus cells were superfused with saline to recover for 4 min and subsequently superfused with the PCBs at the concentrations indicated for a 15 min period (indicated by bars). Note that during the superfusion with 10 µM PCB 4 and with 5 µM PCB 126 the basal release is enhanced. PCB 128 and the high concentrations of PCB 4 and PCB 126 do not cause marked elevations of basal release. Each type of symbol represents data from a different cell.

The median values of vesicular contents of events recorded during the control response and during the response evoked after PCB exposure were determined and were compared statistically. The frequency of evoked events during the response evoked after PCB exposure was normalized to that during the control response obtained from the same cell. The relative frequencies of events after PCB exposure were compared to that obtained from the second response in control cells. The results, summarized in Table 1, show that except for a statistically significant increase of the frequency of evoked events after exposure to 5 µM PCB 128, the characteristics of evoked vesicular catecholamine release did not change significantly on acute exposure to the PCBs.
Table 1. Characteristics of vesicular catecholamine release in PC12 cells after acute exposure (15 min) to PCB 4, PCB 126 and PCB 128 at the concentrations indicated. Values are the mean ± SD of the response evoked after 15 min of exposure expressed as a percentage of the control response from the same cell. The number of cells is given in between brackets.

<table>
<thead>
<tr>
<th></th>
<th>median vesicle contents</th>
<th>evoked event frequency</th>
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<tr>
<td></td>
<td>(% of matched controls)</td>
<td>(% of matched controls)</td>
</tr>
<tr>
<td>Control</td>
<td>108 ± 31 (10)</td>
<td>72 ± 31 (12)</td>
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<td>PCB 4</td>
<td></td>
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<tr>
<td>10 µM</td>
<td>70 ± 24 (4)</td>
<td>48 ± 15 (4)</td>
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<tr>
<td>25 µM</td>
<td>98 ± 28 (3)</td>
<td>106 ± 42 (3)</td>
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<tr>
<td>PCB 126</td>
<td></td>
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<tr>
<td>5 µM</td>
<td>82 ± 17 (4)</td>
<td>79 ± 58 (4)</td>
</tr>
<tr>
<td>12.5 µM</td>
<td>127 ± 22 (3)</td>
<td>62 ± 35 (3)</td>
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<tr>
<td>PCB 128</td>
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<tr>
<td>5 µM</td>
<td>75 ± 25 (4)</td>
<td>166 ± 93 (3)*</td>
</tr>
<tr>
<td>25 µM</td>
<td>105 ± 11 (2)</td>
<td>79 ± 44 (3)</td>
</tr>
</tbody>
</table>

*P < 0.05 compared to control cells (Mann-Whitney)

Subchronic effects

Since previously reported effects of PCB on catecholamine release generally come from studies in which cells were exposed for periods of several hours up to several days, subchronic experiments were also performed. PC12 cell cultures were exposed for 3 days to 10 µM PCB 4, 5 µM PCB 126, or 10 µM PCB 128, added to the culture medium which was refreshed daily. Exocytotic responses were evoked in exposed cells by superfusion with high K⁺ saline. Representative responses to high K⁺ stimulation recorded from a control cell, exposed to 0.2% DMSO only, and from cells exposed to 5 µM PCB 126 and to 10 µM PCB128 are shown in Fig. 3.
Acute PCB effects on quantal catecholamine release

Figure 3. Exocytotic responses evoked by high K⁺ stimulation (indicated by bar) from PC12 cells after culture for 3 days in medium containing 0.2% DMSO (control), 5 µM PCB 126, and 10 µM PCB 128.

The contents of catecholamine-containing vesicles was determined from 16 cells for each experimental condition and compared with that obtained from cells from parallel cultures exposed to DMSO only. Of the different PCB-exposed cells 13 - 16 out of the 16 cells tested responded to high K⁺ stimulation with a robust exocytotic response, as compared to 14 of the DMSO-exposed cells. This indicates that the PCB exposure did not affect the proportion of responsive cells. Analysis of the results did not reveal statistically significant changes in vesicle contents in cells exposed to the PCBs for a period of 3 days as compared to the values obtained from control cells that were exposed for the same period to DMSO only (Fig. 4). The mean frequency of exocytotic events in PCB-exposed cells (5.4, 5.6, and 4.5 s⁻¹ for PCB 4, PCB 126 and PCB 128, respectively) did not differ significantly from that in DMSO-exposed cells (5.9 s⁻¹), but the intercellular variation was too large to detect subtle effects.

Figure 4. Median vesicle contents of catecholamine-containing vesicles released by high K⁺ stimulation from PC12 cells exposed to DMSO (control) and to 10 µM PCB 4, 5 µM PCB 126 and 10 µM PCB 128 for a period of 3 days. SD bars were calculated from the median contents determined from the numbers of cells indicated. None of the PCB treatments caused a significant change in vesicle contents as compared to the vesicle contents of cells exposed to DMSO only (t-test; all P values > 0.3).
Discussion

The results demonstrate that acute exposure to low concentrations of PCB 4 and PCB 126, but not exposure to PCB 128, leads to an enhancement of the basal frequency of exocytosis of catecholamine-containing vesicles from PC12 cells. In addition, acute and subchronic exposure fail to cause changes in the contents of catecholamine-containing vesicles.

High concentrations (> 25 µM) PCB 4 and PCB 126 cause a sustained elevation of intracellular Ca\(^{2+}\) concentration in cultured rat cerebellar granule cells. The effect of PCB 4 was larger than that of PCB 126 (Kodavanti et al., 1993). PCB 4, at the same concentration of 10 µM that enhanced basal release in the present experiments (Figs. 1 and 2), has also been shown to cause an acute, slow transient elevation of the intracellular Ca\(^{2+}\) concentration in a majority of 74% of cultured rat neocortical neurons (Inglefield et al., 2001). However, 5 µM PCB 126, which caused a similar enhancement of basal release frequency in PC12 cells (Fig. 2), caused a Ca\(^{2+}\) signal in only 5% of the rat neocortical neurons (Inglefield et al., 2001). At 2.5 - 5 fold higher concentrations the effects on basal release frequency are no longer observed. This may indicate that additional effects of the PCBs influence the probability of vesicle exocytosis. Because of the differences in the reported abilities of PCB 4 and PCB 126 to elevate intracellular Ca\(^{2+}\), a straightforward relation with the presently observed enhancement of the basal frequency of exocytosis in PC12 cells is not evident. As PCB 4 and PCB 128 are ortho-substituted, nonplanar congeners and PCB 126 is a coplanar congener, the acute effects observed do not appear to obey the proposed relation between PCB structure and neurotoxic potential (Kodavanti and Tilson, 1997). However, it should be noted that, contrary to the general notion that coplanar PCBs have a low neurotoxic potential, perinatal exposure to the coplanar PCB 126 causes low-frequency hearing loss in rats (Crofton and Rice, 1999). Effects on vesicle contents may not be expected in acute experiments on PC12 cells, since previous studies have shown that vesicle cycling in these cells is slow and the total number of vesicles that can be released from a single PC12 cells in an acute experiment is limited (Westerink et al., 2000; Westerink and Vijverberg, 2002). Establishing a reduction of vesicle contents may require exposure to PCBs for more than 15 min, which was the exposure period in the acute experiments.
A subchronic exposure study of PC12 cells showed that after 3 days of exposure, basal dopamine release was selectively enhanced by 50% by 5 \( \mu \text{M} \) PCB 126 and \( \text{K}^+ \)-evoked release was selectively inhibited by 50% by 10 \( \mu \text{M} \) PCB 128. Significant cytotoxicity did not occur at these PCB concentrations (Angus and Contreras, 1996). The present results (Fig. 4) show that these effects cannot be accounted for on the basis of changes in the available amount of dopamine. In PC12 cells the amount of dopamine available in the cytoplasm is closely linked to vesicle contents (see Introduction). The concentration of PCB 4 tested is rather low as compared to that which has been reported to inhibit tyrosine hydroxylase (Seegal et al., 1991) and is also below the IC50 values for inhibition of dopamine uptake into rat brain synaptosomes and into rat brain synaptic vesicles (Mariussen et al., 1999; Mariussen and Fonnum, 2001). Therefore, an effect on vesicle contents may not have been expected. Nonetheless, the same low concentration of PCB 4 is able to enhance the basal exocytosis of catecholamine-containing vesicles from PC12 cells. These combined results indicate that the toxicological focus may need to be redirected from the turnover of catecholamines to other effects including the mechanisms underlying the subtle changes in basal neurotransmitter release and the functional consequences thereof for the developing nervous system.

Acknowledgements

We thank Dr. L. Tertoolen (Hubrecht Laboratory, Utrecht) for donation of PC12 cells; Dr. S. Agulian (Yale University, West Haven, USA) for donation of carbon fibers, T. Sinnige for help with preparation of PCB stock solutions, Prof. M. van den Berg for initial discussions and Ing. A. de Groot for excellent technical assistance.
General discussion

The objective of the investigations described in this dissertation was to develop a suitable approach to study the modulation of vesicular neurotransmitter release, exocytosis, by endogenous factors and toxicants. This general discussion highlights the findings described in this thesis. The properties and suitability of the model system used are summarized. The observed modulation of exocytosis by endogenous factors and toxicants is recapitulated and an attempt is made to put the findings in the context of our current understanding of exocytosis and presynaptic mechanisms of neurotoxicity.

PC12 cells as a model system for exocytosis

In order to study the modulation of exocytosis by endogenous factors and toxic substances, a suitable model system and a suitable technique to measure exocytosis were required. Initially, amperometry was used to study exocytosis in single undifferentiated PC12 cells. Amperometry turned out to be a suitable and sensitive technique to determine the number of vesicles released and the amount of neurotransmitter released per vesicle. However, the percentage of responsive cells, i.e., cells capable of releasing one or more quanta of neurotransmitter upon stimulation, is low. In addition, the number of releasable vesicles in responsive PC12 cells is limited and the releasable pool of vesicles in PC12 cells is heterogeneous (Chapter 2). Finally, exocytosis is limited by Ca\(^{2+}\) channel inactivation during prolonged depolarization (Chapter 2, appendix). A prerequisite for neurotoxicological studies investigating the modulation of exocytosis and underlying mechanisms is a stable model system, capable of releasing high numbers of neurotransmitter-containing vesicles upon repeated stimulations. Therefore, the use of undifferentiated PC12 cells for neurotoxicological-oriented studies is limited and focus was on finding and testing potential suitable cell lines.

Several cell lines appeared promising as model system. Populations of human (SH-SY5Y: Murphy et al., 1990; IMR-32: Sher et al., 1989) and mouse (N1E-115: Horn and Mirkin, 1990) neuroblastoma cells, small cell lung carcinoma cells (GLC8: Codignola et al., 1993) and immortalized serotonergic rat raphe cells (RN46A-B14: Eaton et al., 1995) have been shown to release easily oxidizable neurotransmitters upon stimulation. NT2 cells and primary cultures of rat cerebellar granule cells, both capable of regulated secretion (Sheridan and Maltese, 1998; Stone, 1979), as well as the cell lines mentioned above, were...
tested for secretory responses using a variety of stimuli, before and after charging with L-DOPA, dopamine or serotonin. Unfortunately, none of the cell types tested showed vesicular catecholamine release in carbon fiber microelectrode experiments.

The dexamethasone-differentiated PC12 cell used for the research described in Chapters 3-6 appeared a more robust model system. The percentage of responsive cells and the number of releasable vesicles is increased, as compared to undifferentiated PC12 cells. Moreover, average vesicle contents is increased simplifying detection of vesicular catecholamine release. Finally, distributions of the cube root of vesicle contents indicate that catecholamine-containing vesicles released upon stimulation originate from a more homogeneous population of vesicles (Chapter 3). The dexamethasone-differentiated PC12 cell made it possible to investigate presynaptic mechanisms of neurotransmission and study the effects of several toxicants and endogenous factors in considerable detail.

It has been reported that several types of primary cultures of neurons and a few types of neurons in brain slices respond with Ca\(^{2+}\)-dependent vesicular dopamine or serotonin release upon stimulation (Chen and Ewing, 1995; Pothis et al., 1996; Dunn et al., 1999; Bruns et al., 2000; Hochstetter et al., 2000; Gillis and Anctil, 2001; Ishizaki and Oka, 2001; Kilic et al., 2001). In neurons, functional vesicular neurotransmitter release and subsequent endocytosis was further established using membrane capacitance measurements, vesicle-specific fluorescent dyes and other optical techniques (Hsu and Jackson, 1996; Angleson et al., 1999; Zoccarato et al., 1999; Cochilla et al., 1999; Murthy, 1999; Beutner et al., 2001).

Since PC12 cells are easily cultured, display rapid endocytosis (Elhamdani et al., 2000) and exhibit Ca\(^{2+}\)-dependent vesicular catecholamine release as determined using optical techniques (Lang et al., 1997), membrane capacitance measurements (Kasai et al., 1996), and amperometry (this thesis, Chen et al., 1994), the PC12 cell line is a suitable model system for exocytosis. Primary cultures of rat and bovine chromaffin cells were also tested for their secretory response. As described previously (Wightman et al., 1991), chromaffin cells responded with vesicular catecholamine release upon stimulation with high K\(^+\) saline (data not shown). Since the culture of chromaffin cells is rather time consuming and the dexamethasone-differentiated PC12 cells display similar or even better secretory responses, it is obvious that the PC12 cell line was chosen as model system.

In Chapters 3 and 4 of this thesis it was shown that the PC12 cells were highly suitable to use as a permeabilized system to study the direct modulation of the exocytotic machinery and for fluorescent imaging, to determine the intracellular concentrations of Ca\(^{2+}\) and heavy metal ions simultaneously. In permeabilized cells it was shown that the threshold concentration of Ca\(^{2+}\) to induce exocytosis of catecholamine-containing vesicles is close to 100 µM. Phosphorylation through a PKC- and calcineurin-dependent mechanism inhibits depolarization-induced exocytosis from intact cells, likely by inhibiting voltage-gated Ca\(^{2+}\)
channels, and enhances the frequency of basal exocytosis in intact and permeabilized PC12 cells. CaM kinase II and calmodulin did not cause major effects on exocytosis. This demonstrates that PKC/calcineurin-mediated phosphorylation/dephosphorylation directly modulates the exocytotic machinery of PC12 cells.

So far, the PC12 cell line is the only cell line suitable for studying vesicular catecholamine release. It would be interesting to know why exocytosis is undetectable in the other cell lines tested. Since the cell lines mentioned above exhibit stimulus-induced neurotransmitter release, as measured on the level of cell populations, there are several explanations for the lack of exocytotic events at the level of single cells. If the cells are capable of exocytosis, the amount of neurotransmitter released per vesicle could be too small to detect using amperometry. Since this technique can be used to detect quanta as small as 15 zmol, this would indicate that the intravesicular neurotransmitter concentration is very low. It is also possible that vesicles contain and release considerable amounts of neurotransmitter but that the actual release of neurotransmitter from the vesicles is very slow, resulting in broadened events with strongly reduced amplitude, preventing detection. Another possibility is that the neurotransmitters are released through a non-vesicular mechanism. Non-vesicular release might be mediated by reversal of the membrane transporter, normally responsible for neurotransmitter (re-)uptake, resulting in neurotransmitter efflux. Neurotransmitter release through reversal of the membrane dopamine transporter was recently shown to occur in dopaminergic neurons in rat brain slices of the substantia nigra (Falkenburger et al., 2001). Non-vesicular release through reverse-transport of dopamine probably occurs mainly outside the synapses and might play a role in the maintenance of basal extracellular dopamine levels in striatum. Dopamine released through this atypical mode of release might act as a neurotrophic factor as well as a neuromodulator (for review see Leviel, 2001).

Although there is no evidence that reverse-transport is responsible for the stimulus-evoked neurotransmitter release observed in populations of the cell lines mentioned above, non-vesicular neurotransmitter release might play a role in PC12 cells. As shown in Chapter 4, the amount of neurotransmitter released by a population of wild-type PC12 cells is in close agreement with the expected amount of neurotransmitter release based on average release frequency and average vesicle contents. Synaptotagmin-deficient PC12-F7 cells release only ~3.5% of the amount of neurotransmitter secreted by control cells. The expected amount of neurotransmitter release is only ~30% of the amount released by populations of synaptotagmin-deficient PC12-F7 cells. The remaining ~70% is probably partly non-vesicular release. Assuming a similar level of non-vesicular neurotransmitter release in both cell types, this would indicate that non-vesicular neurotransmitter release in wild-type PC12 cells accounts for at most ~2% of the total amount of neurotransmitter
released during depolarization. Since the two modes of neurotransmitter release may co-exist in the same cell, it would be interesting to know the functional consequences of these different types of neurotransmitter release for neurotransmission, learning and memory and for the developing nervous system.

PC12 cells as a model for neurotoxic modulation of neurotransmitter release

Effects of Pb\textsuperscript{2+} on exocytosis

It has long been known that Pb\textsuperscript{2+} exerts multiple neurotoxic effects ranging from acute inhibition of evoked neurotransmitter release due to Ca\textsuperscript{2+} channel block to a delayed increase in basal neurotransmitter release due to the activation of a presently unknown intracellular target (see introduction of Chapter 3). By a combined amperometric and imaging approach (Chapter 3) it was shown that Pb\textsuperscript{2+} induces exocytosis in the absence of Ca\textsuperscript{2+} by a direct interaction with one or more high-affinity intracellular target proteins. Exocytosis is triggered when the total intracellular Pb\textsuperscript{2+} concentration is raised to values between 10 and 20 nM, irrespective of the extracellular Pb\textsuperscript{2+} concentration. In intact as well as in ionomycin-permeabilized PC12 cells, Pb\textsuperscript{2+}-induced exocytosis is observed only after the partial saturation of an intracellular high-affinity buffer with Pb\textsuperscript{2+}. The effects of modulation of several likely targets of Pb\textsuperscript{2+}, i.e., PKC, calmodulin, CaM kinase II, calcineurin, and synaptotagmin, on Pb\textsuperscript{2+}-induced release were also investigated (Chapter 4). Unlike basal release from permeabilized PC12 cells, Pb\textsuperscript{2+}-induced exocytosis is largely unaffected by modulation of PKC and calcineurin, whereas calmodulin and CaM kinase II modulate Pb\textsuperscript{2+}-induced exocytosis, but cause opposite effects. Additional experiments are required to pinpoint the specific target proteins of calmodulin and CaM kinase II involved in Pb\textsuperscript{2+}-induced exocytosis.

The combined results indicate that although Pb\textsuperscript{2+} acts as a high-affinity substitute for Ca\textsuperscript{2+} to trigger essential steps leading to exocytosis, there are important differences between Pb\textsuperscript{2+}- and Ca\textsuperscript{2+}-evoked exocytosis. One of the most striking differences is that Pb\textsuperscript{2+}-induced exocytosis is delayed, even at relatively high concentrations, whereas Ca\textsuperscript{2+}-induced exocytosis occurs rapidly, even at relatively low concentrations. This suggests that Pb\textsuperscript{2+}-induced exocytosis requires an additional step as compared to Ca\textsuperscript{2+}-evoked exocytosis. Synaptotagmin is probably a prerequisite for both types of exocytosis, since Pb\textsuperscript{2+}- and Ca\textsuperscript{2+}-induced exocytosis are reduced to a similar degree in synaptotagmin I- and II-deficient PC12-F7 cells. Another noteworthy difference is that high concentrations of Pb\textsuperscript{2+} inhibit exocytosis in permeabilized cells through a low-affinity intracellular effect, whereas inhibition of release is not observed at high intracellular Ca\textsuperscript{2+} concentrations. Since Pb\textsuperscript{2+}
enhances exocytosis at low concentrations and inhibits exocytosis at high concentrations, Pb\textsuperscript{2+} apparently interacts with multiple intracellular targets. The target responsible for the observed inhibition is selectively activated by Pb\textsuperscript{2+} and not by Ca\textsuperscript{2+}. Another indication for the presence of multiple intracellular targets for Pb\textsuperscript{2+} comes from the observation that modulation of calmodulin and CaM kinase II exerts opposite effects on Pb\textsuperscript{2+}-induced exocytosis. The differences are further emphasized by the fact that both Pb\textsuperscript{2+} and Ca\textsuperscript{2+}-induced exocytosis are differentially modulated by calmodulin, kinases and phosphatases. Nonetheless, both metal ions induce release of similar vesicles since there are no differences in the amount of neurotransmitter released per vesicle. It is likely that the described effects of Pb\textsuperscript{2+} hold for exocytosis in general and are not specific for catecholamine-containing large dense-core vesicles. Although elevated levels of dopamine and glutamate are generally believed to affect the development, maintenance, and survival of neurons (Michaelis, 1998; Ozawa et al., 1998; Rho and Storey, 2001), it remains difficult to predict functional consequences of elevated levels of these neurotransmitter in vivo.

The present findings demonstrate that exocytosis is triggered by higher concentrations of Pb\textsuperscript{2+} than initially believed. Nonetheless, Pb\textsuperscript{2+} triggers exocytosis at relatively low concentrations. The neurotoxic effect depends on the availability of extracellular Pb\textsuperscript{2+}, since high-affinity buffering facilitates the intracellular accumulation of Pb\textsuperscript{2+}. The availability of extracellular Pb\textsuperscript{2+} is determined by the free Pb\textsuperscript{2+} concentration as well as the amount of remaining extracellular Pb\textsuperscript{2+} and the affinity by which it is bound. Although the Pb\textsuperscript{2+} levels in blood steadily decreased over the last years, the relatively low Pb\textsuperscript{2+} levels may be sufficient to result in Pb\textsuperscript{2+} accumulation in the cells of the nervous system. If the affinity by which Pb\textsuperscript{2+} is bound in erythrocytes is lower than the affinity of the intracellular high-affinity buffer in neurons, a critical amount of Pb\textsuperscript{2+} will eventually accumulate intracellularly. The concept that the availability of Pb\textsuperscript{2+} determines the eventual occurrence of adverse effects may also hold for other toxicological effects of Pb\textsuperscript{2+}, such as Pb\textsuperscript{2+}-induced alterations in gene expression. This would imply that there is no safe threshold concentration for the adverse effects of Pb\textsuperscript{2+}.

**Effects of toluene on exocytosis**

Apart from the detailed research on Pb\textsuperscript{2+}-induced exocytosis and the underlying mechanisms (Chapters 3 and 4), the dexamethasone-differentiated PC12 cells were also used to study the effects of toluene on exocytosis (Chapter 5). Although the research described in Chapter 5 is not as extensive as the research described in Chapters 3 and 4, it certainly demonstrates the potential of the model system to distinguish distinct mechanisms leading to modulation of neurotransmitter release. The neurotoxic organic solvent toluene was shown to induce exocytosis in a concentration-dependent way. One important
difference with Pb\textsuperscript{2+}-induced exocytosis is that toluene does not appear to exert direct effects on exocytosis. Toluene increases the basal frequency of exocytosis by elevating the intracellular Ca\textsuperscript{2+} concentration, which is mediated by enhanced influx of extracellular Ca\textsuperscript{2+}. Whether the toluene-induced increase in intracellular Ca\textsuperscript{2+} is caused by direct modulation of Ca\textsuperscript{2+} channels or by depolarization-mediated Ca\textsuperscript{2+} channel opening remains to be determined. In addition, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from intracellular stores might play a role in toluene-induced catecholamine release.

The concentration at which toluene enhances exocytosis is comparable with that required to inhibit GABA\textsubscript{A} receptors in human IMR-32 neuroblastoma cells, which is presently the most sensitive target of toluene (Meulenberg, 2001). Both effects require concentrations of toluene comparable with blood levels of toluene reached in acute occupational intoxication, confirming the neurotoxicological hazard of this organic solvent. In addition, since exocytosis requires an increase in intracellular Ca\textsuperscript{2+}, it is to be expected that toluene affects not only the release of catecholamines from large dense-core vesicles in PC12 cells, but the release of other neurotransmitters in other cell types as well.

Extracellular levels of dopamine and other neurotransmitters in brain affect the development and functioning of neurons (Michaelis, 1998; Ozawa et al., 1998; Rho and Storey, 2001). Since long-term occupational exposure to toluene has been associated with a variety of neurotoxic effects (Schaumburg, 2000), it is tempting to speculate that the toluene-induced enhancement of exocytosis contributes to these adverse in vivo effects. The observation that toluene-induced hyperactivity is inhibited by a dopamine D\textsubscript{2} receptor antagonist (Riegel and French, 1999), indicating that an elevated level of extracellular dopamine in rat brain contributes to the toxic symptoms of toluene, provides further support for this hypothesis.

Effects of polychlorinated biphenyls (PCBs) on exocytosis

Various PCBs are known to affect cellular dopamine contents and basal and evoked dopamine release in populations of PC12 cells and chromaffin cells after subchronic exposure (Shain et al., 1991; Angus and Contreras, 1996; Messeri et al., 1997). These effects may be caused by inhibition of tyrosine hydroxylase (Seegal et al., 1991; Choksi et al., 1997), the rate-limiting enzyme in catecholamine synthesis, resulting in a reduced dopamine availability. In addition, PCBs have been reported to inhibit the plasma membrane dopamine transporter and the vesicular monoamine transporter (Mariussen et al., 1999; Mariussen and Fonnum, 2001), thus potentially limiting cellular dopamine availability and vesicle contents and increasing extracellular dopamine levels. Finally, PCBs, e.g., PCB 4 and PCB 126, are reported to elevate the intracellular Ca\textsuperscript{2+} concentration (Kodavanti et al., 1993; Carpenter et al., 1997; Mundy et al., 1999; Inglefield and Shafer,
2000; Inglefield et al., 2001), which could affect exocytosis directly or indirectly depending on the amount of intracellular Ca^{2+}.

At the level of single cells acute exposure to low levels of PCB 4 and PCB 126 enhances the basal frequency of vesicular neurotransmitter release, whereas vesicle contents is unaffected. PCB 128 is without effects on exocytosis. None of the selected PCBs affects exocytosis following subchronic exposure (Chapter 6). Since the research described in Chapter 6 was designed as a pilot experiment, demonstrating for the first time that acute exposure to relatively low concentrations of PCBs may lead to dopamine release, many questions remain.

The effects of acute PCB exposure on basal release frequency are observed at PCB concentrations apparently ineffective in changing dopamine transport or tyrosine hydroxylase activity. An effect on vesicle contents may not have been expected, since the concentrations of PCBs tested are rather low as compared to those which have been reported to inhibit tyrosine hydroxylase, cellular dopamine uptake and vesicular dopamine uptake (Seegal et al., 1991; Mariussen et al., 1999; Mariussen and Fonnum, 2001). Since vesicle contents and depolarization-evoked exocytosis are unaffected, the effects of PCBs on neurotransmitter release previously found using cell populations cannot be explained by the slight enhancement of basal release only. Although monoaminergic systems, in particular the dopaminergic system, are involved in PCB neurotoxicity (Seegal, 1995), additional research is required to determine whether PCBs selectively modulate catecholamine release, or affect exocytosis in general.

High concentrations of PCB 4 and PCB 126 (> 25 µM) cause an elevation of the intracellular Ca^{2+} concentration in a few cell types, albeit to a different extent (Kodavanti et al., 1993; Inglefield et al., 2001). Conversely, the basal frequency of exocytosis is enhanced only during exposure to low concentrations of PCB 4 and PCB 126. Therefore, it is possible that high concentrations of PCB 4 and PCB 126 exert a direct inhibitory effect on exocytosis. Additional experiments on permeabilized cells and Ca^{2+}-imaging of intact cells could resolve this issue.

Perinatal exposure to PCB 126 causes low-frequency hearing loss in rats (Crofton and Rice, 1999) and enhances the basal release of catecholamine-containing vesicles during acute exposure in PC12 cells (Chapter 6). These in vivo and in vitro neurotoxic effects of the coplanar PCB 126 indicate that either the proposed relation between PCB structure and neurotoxic potential (Kodavanti and Tilson, 1997) requires some modifications, or that PCB 126 is an exception to this general concept. A thorough screening for the neurotoxicological potential of coplanar congeners might shine some light on this issue.

As with Pb^{2+} and toluene, the changes in neurotransmitter release might affect the development and maintenance of the nervous system since the enhancement of exocytosis...
General discussion

is likely to affect the extracellular levels of dopamine and possibly other neurotransmitters in brain. Presently, one can only speculate on the functional in vivo consequences of the observed enhancement of neurotransmitter release in vitro.

Summary and perspectives

The work described in this thesis is the result of a successful attempt to apply a novel, sensitive technique to investigate the modulation of the presynaptic mechanisms of neurotransmission in an in vitro model system. The technique and model system used, i.e. amperometry and dexamethasone-differentiated PC12 cells, allow for the real-time detection of single exocytotic events at the level of intact, single cells. In addition, the amount of neurotransmitter released per vesicle can be determined at the zeptomol level. Finally, the use of ionomycin-permeabilized cells enables discrimination between direct effects and indirect, Ca\(^{2+}\)-mediated effects on exocytosis induced by endogenous factors and toxicants.

The suitability of the technique and model system for neurotoxicological-oriented studies is demonstrated by the results described in this thesis. Different classes of toxicants exert distinct effects. Pb\(^{2+}\) exerts a direct effect on exocytosis, which is sustained in the absence of Ca\(^{2+}\), through interaction with a presently unknown target protein. On the other hand, the effect of toluene on exocytosis fully depends on the presence of extracellular Ca\(^{2+}\). The effects of PCBs on exocytosis are probably indirect and are mediated by an increase in the intracellular Ca\(^{2+}\) concentration. However, additional indirect effects on exocytosis caused by changes in dopamine availability or vesicular dopamine uptake and direct effects on the release probability cannot be excluded at present. The successful application of permeabilized PC12 cells for investigating the direct modulation of exocytosis by endogenous factors and the mechanisms underlying Ca\(^{2+}\)- and Pb\(^{2+}\)-induced exocytosis adds a powerful tool and opens new perspectives for the investigation of presynaptic mechanisms of the action of neurotoxicants.

Future challenges lie ahead and might include investigation of the effects on exocytosis of exposure to mixtures of chemicals and long-term low-level exposures. Both constitute issues of major toxicological concern and probably require the use of additional techniques such as Ca\(^{2+}\)-imaging and cultures of developing neurons to produce reliable and clear-cut conclusions. However, the ultimate challenge might just be to translate the observed in vitro effects on quantal neurotransmitter release into functional consequences for neurotransmission in general and for nervous system function in vivo.


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Samenvatting in het Nederlands

Algemeen

Een goed functionerend zenuwstelsel is van essentieel belang voor ons dagelijkse, bewuste en onbewuste handelen en denken. Het functioneren van het zenuwstelsel is afhankelijk van goede en efficiënte communicatie tussen zenuwcellen, ook wel neuronen genoemd. Om te kunnen communiceren, bezitten neuronen synapsen. De synaps verbindt de presynaptische, informatie versturende cel met de postsynaptische, informatie ontvangende cel. Om een signaal te kunnen afgeven, bevat het presynaptische neuron blaasjes gevuld met een signaalstof (≠ neurotransmitter). Als de presynaptische cel wordt geactiveerd, worden de blaasjes geleegd in de synaptische spleet, een proces dat exocytose wordt genoemd. De neurotransmitters bereiken vervolgens de postsynaptische cel en binden daar aan postsynaptische receptoren om zo een postsynaptische respons te initiëren. Als het functioneren van het presynaptische of van het postsynaptische neuron is verstoord, bijvoorbeeld door chemicaliën, heeft dat invloed op de signaaloverdracht (= neurotransmissie) en uiteindelijk ook op ons functioneren of op onze gezondheid.

Exocytose van neurotransmitter-bevattende blaasjes treedt op als de intracellulaire calciumconcentratie in het presynaptische neuron sterk toeneemt. Exocytose duurt maar een paar milliseconden en is dus een extreem snel proces. Er zijn echter nog veel andere processen die zich in het presynaptische neuron afspelen voordat exocytose op kan treden. Zo moeten de blaasjes allereerst worden gevuld met neurotransmitters, waarna ze naar gespecialiseerde afgifteplaatsen worden getransporteerd. Op die afgifteplaatsen worden de blaasjes min of meer verankerd aan de celmembraan door interactie met een groep gespecialiseerde eiwitten (“docking”). Vervolgens worden de blaasjes “op scherp gezet” door weer andere eiwitten (“priming”) en zijn ze klaar om geëxocyteerd te worden. Exocytose wordt gevolgd door endocytose, waarbij het inmiddels lege blaasje weer wordt opgenomen door de presynaptische cel. Het lege blaasje wordt vervolgens naar het invendige van de cel getransporteerd, waar het weer kan worden gevuld met neurotransmitters. Deze cyclus van processen (“the vesicle cycle”) wordt geïnitieerd en gecoördineerd door unieke interacties tussen een groot aantal gespecialiseerde eiwitten. Een lichte verstoring van slechts één van deze interacties kan al voldoende zijn om neurotransmissie te beïnvloeden.
Van oudsher is de signaaloverdracht tussen neuronen, en de eventuele modulatie daarvan door toxische (= giftige) en/of lichaamseigen stoffen, bestudeerd d.m.v. experimenten waarbij werd gekeken naar de postsynaptische respons. Door recente wetenschappelijke ontwikkelingen is het nu ook mogelijk om de afgifte van neurotransmitter-bevattende blaasjes in het presynaptische neuron te onderzoeken. Met de nieuwste elektrochemische technieken, zoals microelectrode amperometrie, is het bijvoorbeeld mogelijk om op het niveau van één enkele cel te kijken naar de afgifte van neurotransmitter uit individuele blaasjes. Door deze technieken is onze kennis over de presynaptische mechanismen van neurotransmissie sterk toegenomen. Echter, tot nu toe zijn deze technieken niet of nauwelijks toegepast om te onderzoeken of, en hoe, toxische en/of lichaamseigen stoffen presynaptische processen moduleren, terwijl wel bekend is dat diezelfde stoffen de neurotransmitter niveaus in de hersenen kunnen veranderen.

Het onderzoek beschreven in dit proefschrift had als doel het inzicht te vergroten in de mechanismen verantwoordelijk voor de modulatie van exocytose door toxische en/of lichaamseigen stoffen. Om dat doel te bereiken is gebruik gemaakt van amperometrie, op dit moment de gevoeligste techniek om exocytose van individuele cellen te meten. Deze techniek is geschikt om naast het aantal afgegeven blaasjes ook de hoeveelheid neurotransmitter die per blaasje is vrijgekomen te bepalen, en dat met milliseconde resolutie en een detectiegrens van ~15 zeptomol, ofwel ~9000 moleculen! Aangezien het meten van presynaptische modulatie van neurotransmissie in het zenuwstelsel erg complex is, werd bij het onderzoek gebruik gemaakt van een modelsysteem. Het gebruikte modelsysteem is een cellijn die oorspronkelijk is geïsoleerd uit een bijniertumor in een rat, de phaeochromocytoma PC12 cellijn. Deze makkelijk te kweken cellen worden al jaren gebruikt voor allerlei soorten onderzoek. Er is daardoor veel bekend over op de celmembraan aanwezige receptoren en over intracellulaire enzymen en processen. Na differentiatie met het hormoon dexamethason lijken PC12 cellen sterk op neuroendocriene bijniermergcellen en zijn ze in staat om gedurende stimulatie grote hoeveelheden catecholamine-bevattende blaasjes af te geven. Naast Ca\(^{2+}\)-afhankelijke exocytose vertonen de PC12 cellen ook endocytose, net als de neuronen in ons zenuwstelsel. Verondersteld wordt dat exocytose en de modulatie daarvan in PC12 cellen grote overeenkomsten vertoont met exocytose en de modulatie daarvan in neuronen. De combinatie van amperometrie en PC12 cellen bleek bijzonder geschikt om de modulatie van exocytose door toxische en/of lichaamseigen stoffen te bestuderen.
Exocytose van catecholamine-bevattende blaasjes in PC12 cellen

Hoofdstuk 2 geeft een gedetailleerde beschrijving van vesiculaire catecholamine afgifte (= exocytose) in ongedifferentieerde PC12 cellen gemeten met amperometrie. De resultaten dienen als karakterisering van het modellsysteem zoals dat gebruikt is in latere experimenten. Op grond van de resultaten kan worden geconcludeerd dat PC12 cellen exocytose vertonen na stimulatie met “hoog K⁺ medium” (= depolarisatie). Het percentage responsieve cellen, net als het aantal blaasjes dat per cel wordt afgegeven, is echter gelimiteerd. Deze limitatie blijft gehandhaafd na het opladen van cellen met L-DOPA, een grondstof voor catecholaminen. De resultaten suggereren dat de limitatie niet wordt veroorzaakt door een beperkte beschikbaarheid van catecholaminen, maar doordat de zogenaamde “vesicle cycle” traag verloopt. Bovendien tonen de resultaten aan dat de blaasjes niet afkomstig zijn van één homogene groep van blaasjes, maar uit tenminste 2 verschillende groepen die op basis van hoeveelheid catecholamine per blaasje kunnen worden onderscheiden. Aanvullende experimenten (Hoofdstuk 2, appendix) tonen aan dat exocytose Ca²⁺-afhankelijk is. Ca²⁺ komt de cel binnen tijdens depolarisatie via “high voltage-activated” Ca²⁺ kanalen. Deze kanalen inactiveren langzaam gedurende een aanhoudende depolarisatie, wat ertoe leidt dat er uiteindelijk geen Ca²⁺ meer de cel in komt en exocytose ophoudt.

Een vereiste voor neurotoxicologisch onderzoek naar exocytose en de modulatie daarvan is een modellsysteem dat in staat is om relatief grote hoeveelheden blaasjes af te geven gedurende stimulatie. In dexamethason-gedifferentieerde PC12 cellen (Hoofdstuk 3 - 6) is het percentage responsieve cellen en het aantal blaasjes dat per cel kan worden afgegeven toegenomen. Verder is in dexamethason-gedifferentieerde cellen de detectie van vesiculaire catecholamine afgifte vergemakkelijkt doordat de hoeveelheid catecholamine die per blaasje wordt afgegeven, is toegenomen. Tenslotte zijn de afgeven blaasjes afkomstig uit een meer homogene groep blaasjes en op grond van de hoeveelheid catecholamine per blaasje zijn er geen duidelijk onderscheidbare groepen blaasjes binnen één cel. Deze kenmerken maken de dexamethason-gedifferentieerde PC12 cel tot een geschikt modellsysteem om de presynaptische mechanismen van neurotransmissie te onderzoeken en om de effecten van verschillende toxische en/of lichaamseigen stoffen in aanzienlijk detail te bestuderen.
Effecten van Pb$^{2+}$ op exocytose

Hoofdstuk 3 beschrijft de effecten van Pb$^{2+}$ op exocytose in intacte en in ionomycine-gepermeabiliseerde (= poreus gemaakte) PC12 cellen. Deze gepermeabiliseerde cellen zijn bijzonder geschikt om intracellulaire effecten van metaalionen te onderzoeken, omdat de ioniën makkelijk de poreuze cel in kunnen zonder daarbij te worden gehinderd door de cellmembran. De resultaten tonen aan dat Pb$^{2+}$ in staat is om Ca$^{2+}$-onafhankelijke exocytose te veroorzaken via een direct intracellulair effect op niet nader omschreven processen die zijn betrokken bij exocytose. Dit effect treedt in gepermeabiliseerde cellen al op bij een extracellulaire Pb$^{2+}$ concentratie van 30 nM. In vergelijking met Ca$^{2+}$-geïnduceerde exocytose is Pb$^{2+}$-geïnduceerde exocytose sterk vertraagd. Als de externe Pb$^{2+}$ concentratie toeneemt, neemt de frequentie waarmee catecholamine-bevattende blaasjes worden afgegeven toe en is Pb$^{2+}$-geïnduceerde exocytose minder vertraagd. Als de concentratie Pb$^{2+}$ nog verder wordt opgehoogd tot ~10 µM, treedt inhibitie van exocytose op. Dit geeft aan dat Pb$^{2+}$ op ten minste twee processen aangrijpt. De combinatie van amperometrische data met data uit confocale laser scanning microscopie experimenten met de fluorescente kleurstof indo-1 toont aan dat PC12 cellen een aanzienlijke Pb$^{2+}$ buffer capaciteit hebben. Een gedeeltelijke verzadiging van deze hoge affiniteit buffer met Pb$^{2+}$ is voldoende om exocytose te veroorzaken.

Hoofdstuk 4 beschrijft de effecten van modulatie van verschillende cruciale eiwitten betrokken bij exocytose in een poging de mechanismen verantwoordelijk voor Pb$^{2+}$- en depolarisatie-geïnduceerde exocytose te identificeren. De resultaten tonen aan dat modulatie van protein kinase C (PKC), calcineurine, calmoduline en Ca$^{2+}$/calmodulin-dependent protein kinase II (CaM kinase II) in staat is exocytose te beïnvloeden. Synaptotagmine beïnvloedt Pb$^{2+}$- en depolarisatie-geïnduceerde exocytose in dezelfde mate, wat aangeeft dat synaptotagmine in beide typen exocytose eenzelfde, waarschijnlijk essentiële, functie vervult. Een toename van intracellulaire fosforylering via modulatie van PKC en calcineurine zorgt voor een toename van de frequentie van basale exocytose, terwijl PKC en calcineurine geen, of slechts een ondergeschikte, rol lijken in te spelen bij Pb$^{2+}$-geïnduceerde exocytose. Dit is opmerkelijk aangezien PKC activatie leidt tot exocytose en PKC al bij zeer lage concentraties Pb$^{2+}$ kan worden geactiveerd. De resultaten geven verder aan dat Pb$^{2+}$-geïnduceerde exocytose vooral wordt gemoduleerd door calmoduline en CaM kinase II. Hoewel nog niet bekend is of Pb$^{2+}$ CaM kinase II kan activeren, is wel aangetoond dat CaM kinase II in staat is om v-SNAREs, t-SNAREs, synapsine en synaptotagmine te fosforyleren. Aangezien CaM kinase II activatie tevens leidt tot een toename van het aantal blaasjes dat kan worden afgegeven, is het zeer goed mogelijk dat de interactie tussen Pb$^{2+}$ en CaM kinase II de cruciale gebeurtenis is die uiteindelijk resulteert in de waargenomen neurotransmitter afgifte.
Samenvatting in het Nederlands

Aangezien lage concentraties Pb\(^{2+}\) exocytose veroorzaken, terwijl hoge concentraties Pb\(^{2+}\) exocytose inhiberen, interacteert Pb\(^{2+}\) waarschijnlijk met meerdere, voor exocytose relevante, intracellulaire aangrijplingspunten. Inhibitie van exocytose door hoge concentraties intracellulair Ca\(^{2+}\) is niet waargenomen. Een ander verschil tussen Pb\(^{2+}\)-geïnduceerde en Ca\(^{2+}\)-geïnduceerde exocytose, naast het feit dat ze door verschillende mechanismen worden gemoduleerd, is dat Pb\(^{2+}\)-geïnduceerde exocytose vertraagd optreedt.

De neurotoxiciteit van Pb\(^{2+}\) hangt af van de beschikbaarheid van Pb\(^{2+}\). De beschikbaarheid van Pb\(^{2+}\) wordt niet alleen bepaald door de vrije concentratie Pb\(^{2+}\), maar ook door de hoeveelheid resterend extracellulair Pb\(^{2+}\) en de affiniteit waarmee het is gebonden. Een relatief laag Pb\(^{2+}\) niveau kan voldoende zijn om te resulteren in intracellulaire Pb\(^{2+}\) accumulatie in neuronen, mits de affiniteit van de intracellulaire buffer voor Pb\(^{2+}\) hoger is dan de affiniteit van de andere in het lichaam aanwezige buffers. Zodra er een kritische hoeveelheid Pb\(^{2+}\) intracellulair is geaccumuleerd, zal Pb\(^{2+}\)-geïnduceerde exocytose optreden. Dit duidt erop dat er eigenlijk geen veilige drempelconcentratie is vast te stellen voor bijvoorbeeld de concentratie van Pb\(^{2+}\) in bloed.

Effecten van tolueen op exocytose

Hoofdstuk 5 beschrijft de effecten van het neurotoxische oplosmiddel tolueen op exocytose. De resultaten tonen aan dat tolueen de frequentie van basale exocytose verhoogt. Dit effect is concentratie-afhankelijk en wordt veroorzaakt door een toename in de intracellulaire Ca\(^{2+}\) concentratie. De depolarisatie-geïnduceerde exocytose en de basale processen die normaal gesproken essentieel zijn voor exocytose worden niet beïnvloed door tolueen. De toename in de frequentie van basale exocytose treedt op bij neurotoxicologisch relevante concentraties en wordt veroorzaakt door influx van extracellulair Ca\(^{2+}\) door spanningsafhankelijke Ca\(^{2+}\) kanalen. Aangezien tolueen de intracellulaire Ca\(^{2+}\) concentratie verhoogt, is het aannemelijk dat de effecten niet beperkt blijven tot de exocytose van catecholamine-bevattende blaasjes, maar dat tolueen ook afgifte van andere typen neurotransmitters kan veroorzaken.
Effecten van PCBs op exocytose

Hoofdstuk 6 beschrijft de effecten van drie geselecteerde polygechlorineerde bifénylen (PCBs) op exocytose. De resultaten laten zien dat acute (<15 minuten) blootstelling aan lage concentraties PCB 4 (10 µM) en PCB 126 (5 µM) de basale frequentie van exocytose verhoogt. Dit effect is afwezig bij hogere concentraties en ook tijdens blootstelling aan PCB 128 (5 - 25 µM). Acute blootstelling aan deze PCBs heeft geen effecten op de hoeveelheid afgegeven catecholamine per blaasje en slechts marginale effecten op depolarisatie-geïnduceerde exocytose. Subchronische (3 dagen) blootstelling aan lage concentraties van deze PCBs heeft geen effect op de hoeveelheid afgegeven catecholamine per blaasje en ook niet op depolarisatie-geïnduceerde exocytose. De eerder gevonden effecten op populaties PC12 cellen na subchronische blootstelling aan PCBs, kunnen niet worden verklaard door enkel de geringe toename in exocytose die wordt waargenomen tijdens acute blootstelling aan lage concentraties PCBs. Bovendien zijn de concentraties PCBs die een verhoging van de basale frequentie van exocytose veroorzaken, schijnbaar zonder effect op tyrosine hydroxylase activiteit en dopamine transport. Er zijn dus nog veel vragen omtrent de werkingsmechanismen van PCBs.

Uit de in dit proefschrift beschreven resultaten blijkt dat lichamsvreemde, toxische stoffen in staat zijn om exocytose te beïnvloeden. Hetzelfde geldt voor enkele lichaamseigen, intracellulaire eiwitten, zoals PKC, CaM kinase II, calcineurine en calmoduline. Op dit moment is voor geen van deze stoffen het exacte presynaptische werkingsmechanisme bekend. Op grond van andere onderzoeken is al wel duidelijk geworden dat veranderingen in de extracellulaire niveaus van catecholaminen de ontwikkeling en het normale functioneren van ons zenuwstelsel beïnvloeden. Dit geldt met name voor dopamine, maar ook voor andere typen neurotransmitters, zoals glutamaat. De grootste uitdaging is waarschijnlijk het vertalen van de beschreven in vitro effecten op exocytose in PC12 cellen naar functionele consequenties voor neurotransmissie in het algemeen en voor het functioneren van het zenuwstelsel in vivo.
LIST OF PUBLICATIONS

Papers


Abstracts


Leuk hè? Promoveren. Inderdaad, wel leuk, maar niet altijd even gemakkelijk (vooral die laatste loodjes niet....). Vandaar dat er velen zijn die een steentje hebben bijgedragen. Op deze bladzijde (de enige bladzijde die écht iedereen leest) wil ik dan ook iedereen bedanken die op enige wijze heeft bijgedragen aan de totstandkoming van dit proefschrift. Allereerst Henk, jij hebt in je eentje genoeg steentjes bijgedragen om in Drente een heel hunebed te bouwen. Aart, voor alle hulp bij statistiek, computer- en technische-problemen, en voor de prettig kritische werksfeer (inclusief een “gezellig” muziekje....). Mede-AIO’s Cécil en Chantal, Gina, en oud collega’s Ruud en Anna, voor alle hulp bij chemicaliën, celkweek, experimentele technieken, bierbrouwen/-drinken, wetenschappelijke feedback, een luisterend oor en de ontspannen werkomgeving. Willem, voor de fietsen (die ik vervolgens aan gort reed), de wetenschappelijke feedback en het snelle nakijkwerk. Iedereen van de werkplaats voor de vele technische aanpassingen aan mijn opstelling. En, om deze pagina niet al te groot te maken, iedereen bij het IRAS voor alle collegialiteit en voor alle wetenschappelijke-, secretariële-, grafische-, technische-, experimentele-, en andere vormen van ondersteuning. Om niet de afzonderlijke “acknowledgements” te resumeren en uit te breiden, wil ik ook iedereen bedanken die niet werkzaam is bij het IRAS, maar wel een bijdrage heeft geleverd aan het onderzoek beschreven in dit proefschrift.

Verder gaat mijn dank uit naar alle familie, vrienden en kennissen voor de interesse, aanmoedigingen, steun, afleiding, broodnodige relativering, en voor de snelle en kritische feedback. Dit geldt natuurlijk in het bijzonder voor mijn (“schoon”-) ouders en (“schoon”-) broers/zussen. Bedankt voor alle onvoorwaardelijke steun en interesse die jullie me gaven in dat vreemde, wetenschappelijke wereldje, en gelukkig ook daar buiten.

Tenslotte, Linda. Sta je weer onderaan in het lijstje (maar wel in een “eigen alinea”), en dat terwijl jouw steun, interesse, begrip en aandacht zo belangrijk waren…. Bedankt voor alles, zowel in de goede als in de slechte(re) tijden.