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The PC12 cell as model for neurosecretion

R. H. S. Westerink[1] and **A. G. Ewing**[2,3]

[1] *Cellular and Molecular Toxicology, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, the Netherlands* [2] *Department of Chemistry, Gothenburg University, Gothenburg, Sweden* [3] *Department of Chemistry, Penn State University, University Park, PA, USA*

Abstract

This review attempts to touch on the history and application of amperometry at PC12 cells for fundamental investigation into the exocytosis process. PC12 cells have been widely used as a model for neural differentiation and as such they have been used to examine the effects of differentiation on exocytotic release and specifically release at varicosities. In addition, dexamethasone-differentiated cells have been shown to have an increased number of releasable vesicles with increased quantal size, thereby allowing for an even broader range of applications including neuropharmacological and neurotoxicological studies. PC12 cells exhibiting large numbers of events have two distinct pools of vesicles, one about twice the quantal size of the other and each about half the total releasable vesicles. As will be outlined in this review, these cells have served as an extremely useful model of exocytosis in the study of the latency of stimulation-release coupling, the role of exocytotic proteins in regulation of release, effect of drugs on quantal size, autoreceptors, fusion pore biophysics, environmental factors, health and disease. As PC12 cells have some advantages over other models for neurosecretion, including chromaffin cells, it is more than likely that in the following decade PC12 cells will continue to serve as a model to study exocytosis.

Keywords

amperometry; catecholamines; exocytosis; modulation of vesicle fusion; PC12 cells; release characteristics

The key dynamic event in neuronal communication is exocytosis. This is a process that has been investigated extensively for several decades (Helle & Serck-Hanssen 1975, Livett *et al.* 1983, Holz 1988). The process of exocytosis can be summarized as the docking of neurotransmitter-containing vesicles (storage compartments) to the cell membrane and subsequent release of the contents by fusion of the vesicle and cell membranes (Südhof 2004, Barclay *et al.* 2005, Westerink 2006). This process allows the conversion of an electrical signal (action potential) to a chemical signal (receptor recognition), which is necessary for exocytotic communication between cells.

Methods to observe and quantify individual exocytotic events have traditionally revolved around electron microscopy and patch-clamp capacitance measurements (Neher & Marty 1982). In 1990, Wightman and coworkers (Leszczyszyn *et al.* 1990) showed that they could

Correspondence: R. H. S. Westerink, Cellular and Molecular Toxicology, Institute for Risk Assessment Sciences, Utrecht University, PO Box 80.176, NL-3508 TD Utrecht, the Netherlands. E-mail: r.westerink@uu.nl.

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There is no conflict of interest.

directly monitor individual exocytotic events involving easily oxidized messengers and occurring on the millisecond timescale by use of amperometric measurements at micro-electrodes (Wightman *et al.* 1991).

Suitability of PC12 cells as a model for neurosecretion

Most work on single-vesicle exocytosis in culture has been with the adrenal chromaffin cell model. The use of adrenal chromaffin cells for neurobiological studies, including neurosecretory studies, is extensively covered in the other chapters in this issue. An important feature of chromaffin cells is that they are derived from laboratory animals, mainly rats and mice, or from slaughterhouses in the case of bovine chromaffin cells. This not only gives rise to some ethical matters regarding animal use, but also inherently increases the variation in the data. On the other hand, a wide array of knock-out animals is nowadays available and the use of these animals considerably added to the increased insight into the process of exocytosis in the past decade. Nonetheless, the use of rat phaeochromocytoma (PC12) cells developed in parallel as an alternative model to investigate exocytosis with amperometry. The popularity of PC12 cells is mainly because of their extreme versatility for pharmacological manipulation, their ease of culture and the large amount of background knowledge on their proliferation and differentiation. Moreover, they more closely resemble neurons with smaller vesicle and quantal size than chromaffin cells.

The adrenal phaeochromocytoma (PC12) cell line was originally isolated from a tumour in the adrenal medulla of a rat in 1976 (Greene & Tischler 1976). Like adrenal chromaffin cells, PC12 cells synthesize and store DA and sometimes noradrenaline, which are released upon depolarization in a Ca^{2+}-dependent way (Greene & Rein 1977). PC12 cells resemble the phenotype of sympathetic ganglion neurons upon differentiation with nerve growth factor (NGF) and can be subcultured indefinitely. The activity of tyrosine hydroxylase (TH) in PC12 cells can be increased by NGF, insulin, glucocorticoids, cholera toxin and a high plating density (Schubert *et al.* 1980, Tischler *et al.* 1983). Through a complex interplay between these agents, NGF enhances the effects of glucocorticoids on these cells. Specifically, dexamethasone in combination with NGF leads to increases in transmitter synthesis and vesicle size (Schubert *et al.* 1980). Although the majority of catecholamine contained in PC12 cells is dopamine, addition of ascorbic acid can lead to production of limited amounts of noradrenaline in some PC12 subcultures (Schubert & Klier 1977, Tischler *et al.* 1983). PC12 cells possess large dense-core vesicles (LDCVs) which contain catecholamines, whereas acetylcholine is stored in small clear vesicles (Greene & Tischler 1976, Schubert *et al.* 1980, Travis & Wightman 1998). LDCVs in PC12 cells are slightly smaller (75–120 nm radius; Greene & Tischler 1976, Schubert *et al.* 1980, Travis & Wightman 1998) compared with chromaffin cells (170 nm radius; Coupland 1968). Release of catecholamines from PC12 cells gives rise to amperometric spikes similar to those observed for isolated chromaffin cells following stimulation to exocytosis (Clark & Ewing 1997).

Amperometry at PC12 cells to monitor secretion: cellular effects

Initial experiments at undifferentiated PC12 cells

Initial studies of PC12 cells focused on the detection of zeptomole quantities of catecholamines released in a regulated manner from the soma of undifferentiated cells (Chen *et al.* 1994). Current transients for the oxidation of catecholamines were on the timescale expected for exocytotic release (9.3 ms half-width) and were thus similar to events observed at adrenal chromaffin cells. However, catecholamines are present in much lower levels in vesicles of PC12 cells. This is at least partially because of the smaller size of the PC12 vesicle (Schubert *et al.* 1980) compared with the adrenal cell vesicle (Coupland 1968). The quantity of dopamine oxidized from a single vesicle after stimulated release was first reported to be 190 zmol (114

300 molecules) for a specific passage of PC12 cells (Chen *et al.* 1994), although it appears to vary in different PC12 cell subcultures. For the cell line obtained from the American Type Culture Collection; Rockville, MD (original experiments), it was approximately 18 times smaller than the amount of release observed from a single adrenal cell vesicle (Wightman *et al.* 1991).

An example of an amperometric recording of depolarization-induced exocytosis from a single PC12 cell is shown in Figure 1a. Quantification is accomplished by integrating the charge (Q) under individual current transients, representing single vesicle release, obtained using amperometry. Using Faraday's law: $Q = nFN$, where n is the number of electrons in the electrooxidation ($n = 2$ for catecholamines), and F is Faraday's constant (96 485 C/mole), the number of moles (N) of oxidized catecholamines can be determined. The reported quantity of catecholamines from individual vesicles in a cell type is an average value. Histograms generated by plotting the percentage of total release events versus vesicle content in each bin demonstrated the distribution of event sizes. Figure 1b shows a typical histogram generated from exocytotic events measured from PC12 cells. The average vesicle content for this set of PC12 cells was 199 ± 14 zmol (Zerby & Ewing 1996a); however, the distribution ranges from 20 to 800 zmol (Fig. 1b). Histograms plotted in this manner give a skewed (non-Gaussian) distribution and have been modelled mathematically by Wightman *et al.* (1991) for adrenal cells. The most feasible explanation for this distribution is that vesicles have a range of radii with relatively uniform concentration of catecholamines (Wightman *et al.* 1991). Thus, if the cube root of the vesicular content ($Q^{1/3}$ or mole$^{1/3}$) is utilized for generation of the histograms, then a normal (Gaussian) distribution is obtained (Fig. 1c).

The initial experiments to examine distributions of the cube root of vesicle contents for release from PC12 cells used pooled data from many cells and have been fitted using single Gaussian functions (Finnegan *et al.* 1996, Zerby & Ewing 1996b, Taylor & Peers 1999). However, it is not always accurate to pool data from individual event at many cells as cell-to-cell variation in the number of events can bias pooled means to only a few cells (Colliver *et al.* 2000a, Westerink *et al.* 2000). Thus, it is usually more accurate to examine pooled means than to pool data from many events covering many cells. In addition, it has been shown that in cells that release large numbers of events, the majority of these cells contain two distinct classes of catecholamine-containing vesicles (Fig. 2). The mean vesicle contents between these two classes of vesicles varies by approximately twofold, at 141 and 293 zmol, and each makes up roughly half of the total release events observed (Westerink *et al.* 2000). It is therefore likely that PC12 cells contain distinct classes of vesicles, although at present it cannot be excluded that the observed results are because of the release of aggregate vesicles or compound fusion.

Latency of release following chemical stimulation

Amperometry has been used to investigate the effect of different mechanisms of stimulation on the latency of exocytosis from PC12 cells following stimulation (Zerby & Ewing 1996b). PC12 cells possess both nicotinic and muscarinic receptors that can trigger exocytosis through two different mechanisms upon activation. Application of nicotine causes opening of sodium channels, which results in sufficient depolarization of the cell membrane to open voltage-sensitive calcium channels, thereby allowing rapid influx of calcium and subsequent exocytosis (Stallcup 1979). On the other hand, the application of muscarine activates muscarinic receptors that act through intracellular second messengers to release calcium from intracellular stores triggering exocytosis (Berridge & Irvine 1984). To obtain rapid membrane depolarization of the cell, KCl can be applied to promote exocytosis by direct membrane depolarization. The average catecholamine content of the vesicles is unaltered by these different stimuli, but the latencies (time between application of the stimulant and secretion events) vary significantly (Fig. 3; Zerby & Ewing 1996b). The mean latencies for each type of stimulation have been

observed to be 6 ± 1 s (105 mM K^+); 37 ± 5 s (1 mM nicotine); and 103 ± 11 s (1 mM muscarine). The 6-s delay until release following potassium stimulation apparently represents the diffusion time from the stimulation pipette as the delay is generally <1 s when a continuous, fast superfusion system is used to apply KCl (Westerink *et al.* 2000). The relatively long latencies until the onset of exocytosis after nicotine and muscarine are surprising as one usually expects latencies comparable with that observed following KCl-induced membrane depolarization. The longer times might reflect a relatively low number of sodium channels on these cells or a slow step in the G-protein coupling by these receptors in PC12 cells, as well as other potential rate-limiting mechanisms.

Experiments at differentiated (NGF-treated) PC12 cell varicosities

PC12 cells can be differentiated with NGF to better resemble a neuronal phenotype. Upon treatment with NGF, PC12 cells extend processes and along these processes or neurites varicosities form (bulbous regions, 1–2 μm in diameter). Varicosities have been previously shown to contain aggregates of small vesicles (20–70 nm in diameter) (Greene & Tischler 1976). Experiments carried out on days 10–14 of NGF-treated PC12 cell cultures showed no release from the cell body, only very infrequent responses from the smooth regions of the neurites, and frequent release when the electrode was located at a varicosity (Zerby & Ewing 1996a). This response was most frequently observed at varicosities located at the intersections of several neurites. The average vesicular catecholamine content observed for exocytosis at varicosities was 178 ± 9 zmol (107 000 molecules) and was not significantly different from that observed at undifferentiated cells; however, a more narrow distribution was observed at the former (Fig. 4). This study demonstrated that functional changes occur during differentiation, specifically the relocation of the sites of exocytosis without significant alteration in the overall mean vesicle catecholamine content, although the narrow distribution of vesicle content might indicate that a tighter distribution of vesicle radii is necessary to pack them into the relatively small varicosities.

Dexamethasone increases calcium channel function and exocytosis

Dexamethasone, a potent synthetic glucocorticoid, is used to treat a wide variety of conditions as an anti-inflammatory agent or immunosuppressant. Dexamethasone has also been used to differentiate PC12 cells into neuroendocrine chromaffin-like cells. These dexamethasone-differentiated cells have been monitored with patch clamp and amperometry and it was found that treatment for 5–7 days dramatically increases quantal size, excitability and coupling between calcium channels and vesicle release sites, leading to rapid exocytosis and endocytosis (Elhamdani *et al.* 2000).

These findings can be extended by comparison of amperometric measurements of exocytosis in undifferentiated and dexamethasone-differentiated PC12 cells from the same research group (Westerink *et al.* 2000, Westerink & Vijverberg 2002a). Approximately 90% of dexamethasone-differentiated PC12 cells responded with vesicular neurotransmitter release when depolarized with KCl, compared with ~50% in undifferentiated cells. During stimulation the release frequency in dexamethasone-differentiated PC12 cells amounts to ~5 Hz, whereas it averages only ~2 Hz in undifferentiated cells. The amount of releasable vesicles in dexamethasone-differentiated PC12 is also much larger (~500 vs. ~50). Finally, vesicle content increased from ~200 to ~650 zmol following differentiation with dexamethasone. Surprisingly, the cube root of vesicle content is distributed normally in single dexamethasone-differentiated PC12 indicating a more homogenous vesicle population. These characteristics allowed dexamethasone-differentiated PC12 to become a preferred model for studying modulation of neurotransmitter release at the single-cell level in neuropharmacological and neurotoxicological studies.

Amperometry at PC12 cells to monitor secretion: manipulation of exocytotic proteins

The use of amperometry at PC12 cells has led to considerable insight into the function of proteins underlying vesicle fusion. The basic molecular machinery underlying vesicle fusion is well summarized in a large number of reviews (Burgoyne & Morgan 2003, Südhof 2004, Westerink 2006). Therefore, in this section only an overview of recent achievements using amperometry at PC12 cells is given.

SNARE proteins

Secretory vesicles become docked at the cell membrane through formation of the SNARE-complex consisting of vesicle-associated synaptobrevin and plasma membrane-associated synaptosomal-associated protein of 25 kDa (SNAP-25) and syntaxin. Obviously, disruption of the SNARE complex or cleavage of SNARE proteins prevents vesicle docking, priming and release. This was elegantly demonstrated in PC12 cells transfected with Botulinum neurotoxin C1 light chain (BoNT/C1), which cleaves syntaxin and SNAP-25. In PC12 cells expressing BoNT/C1, ATP-evoked exocytosis was inhibited almost completely as revealed using amperometry (Fisher & Burgoyne 1999). More recently, syntaxin was shown to be involved in fusion pore formation as well. The fusion pore is a channel-like structure connecting the vesicle and plasma membranes, which is formed during the initial phase of exocytosis. Leakage of catecholamines through the fusion pore results in the detection of foot signals that precede the actual exocytotic event (Chow *et al.* 1992, Alvarez de Toledo *et al.* 1993). An amperometric study showed the influence of specific proteins on the process of release via the pore. Point mutations in syntaxin, a protein thought to form transmembrane segments in the pore, reduced the flux of catecholamines through the fusion pore as well as its conductance (Han *et al.* 2004), underlining the importance of syntaxin in regulated exocytosis.

Synaptotagmin

The proposed Ca^{2+}-sensor synaptotagmin (Syt), which exists in several isoforms, prevents the SNARE complex from further interactions until an increase in the intracellular Ca^{2+} concentration displaces Syt and catalyses membrane fusion. Using amperometry, it was shown that silencing Syt-I in PC12 cells significantly reduced evoked exocytosis. The reduction in exocytosis was reversed following rescue with human Syt-I (Moore *et al.* 2006), indicating that Syt-I plays a major role in regulated secretion. It is not clear if Syt-I is regulatory by enhancing or inhibiting full expansion of the pore. As it appears that membrane mechanics provide enough energy for expansion of the pore (Cans *et al.* 2003), it is highly possible that protein regulation is inhibitory. However, as exocytosis is not completely abolished, these findings also indicate that one or more proteins can replace Syt-I. PC12 cells over-expressing Syt-I display a prolonged open time of the fusion pore, whereas Syt-IV decreases the fusion pore open time (Wang *et al.* 2001). Transfection with Syt-IV, or increasing endogenous Syt-IV by forskolin treatment, increases the frequency and duration of kiss-and-run events. Full fusion is inhibited by mutation of a Ca^{2+} ligand in the C2A domain of Syt-I, whereas kiss-and-run exocytosis is inhibited by mutation of a homologous Ca^{2+} ligand in the C2B domain of Syt-IV (Wang *et al.* 2003).

In permeabilized PC12 cells, elevating the intracellular Ca^{2+} concentration reduced the fusion pore lifetime, indicating that Ca^{2+} acts during the actual fusion process. Both, opening of the fusion pore and dilatation of the pore were accelerated by Ca^{2+}, suggesting separate Ca^{2+} control over each of these steps. This was further confirmed by the fact that Ca^{2+} ligand mutations in either the C2A or C2B domains of Syt-I reduced fusion pore opening, but had opposite actions on the rate of fusion pore closure (Wang *et al.* 2006). Exactly how the Syt proteins regulate pore is still a matter of debate and ongoing interest.

SCAMP2

Another protein critically involved in fusion pore formation is secretory carrier membrane protein 2 (SCAMP2). Expression of a point mutant of SCAMP2 in PC12 cells resulted in the inhibition of exocytosis. Interestingly, dilatation of the opened fusion pores was also hampered, resulting in a relative abundance of stand-alone foot signals, thus linking SCAMP2 to the process of fusion pore formation (Liu *et al.* 2005). As such, amperometry at (transfected) PC12 cells is a powerful tool to further complete the detailed picture of the molecular regulatory mechanisms underlying exocytosis.

Amperometry at PC12 cells to monitor secretion: vesicular changes

L-DOPA increases quantal size

Several early studies used PC12 cells to investigate the effects of different pharmacological agents on the average catecholamine content of PC12 cell vesicles. For example, the quantal size of release events can be increased by treatment of the cells with the dopamine precursor L-3, 4-dihydroxyphenylalanine (L-DOPA) (Kozminski *et al.* 1998). In one set of experiments, treatment of cells with 50 μM L-DOPA for 40–90 min increased the average quantal size for release by 251% (Pothos *et al.* 1996). When release events are examined at different times following exposure to L-DOPA, this treatment steadily increases vesicle content, already within 15 min of exposure, and after 60 min of exposure apparently saturates at ~175% of the contents before incubation with L-DOPA (Fig. 5A). Interestingly, L-DOPA initially increases the mean number of exocytosis events in undifferentiated PC12 cells (Fig. 5b). This increase, however, is transient and the release frequency rapidly declines following repeated stimulations indicating depletion of the pool of releasable vesicles (Westerink *et al.* 2000).

Autoreceptors on PC12 cells

Besides upregulation of vesicle contents by L-DOPA, vesicle content can also be reduced by exposure to amphetamine or reserpine (Sulzer *et al.* 1995, Kozminski *et al.* 1998). Importantly, Pothos *et al.* (1998) used amperometry to demonstrate that quantal size can also be modulated physiologically, e.g. by activation of D_2 autoreceptors. In these experiments, PC12 cells were treated with the D_2 agonist quinpirole resulting in a ~50% decrease in the quantal size of release events. In contrast to the results for amphetamine and reserpine, which appear to deplete vesicular stores by operating on the vesicle monoamine transporter (VMAT), the experiments with quinpirole provide evidence for a receptor-mediated mechanism that can deplete or modulate quantal release, probably through feedback on the TH-mediated dopamine synthesis pathway.

Variability of vesicle size and the fusion pore

PC12 cells can be loaded with L-DOPA to increase quantal size (Pothos *et al.* 1996, Kozminski *et al.* 1998, Colliver *et al.* 2000b, Westerink *et al.* 2000). However, it was unclear by what means this enhancement was taking place. Colliver *et al.* (2000b) used amperometry and transmission electron microscopy to demonstrate that the vesicle size in PC12 increased in the presence of excess L-DOPA and decreased in the presence of reserpine. This was later corroborated for adrenal chromaffin cells by Lindau and coworkers using patch-amperometry (Gong *et al.* 2003).

More recently, Sombers *et al.* (2004) have used the ability to vary vesicle size to examine the effect of vesicle size on release via the fusion pore. Although L-DOPA and reserpine increase and decrease vesicle size, respectively, the dense core of the vesicle is mostly unchanged (Colliver *et al.* 2000b) and it appears that the increased amount of dopamine in the vesicle following L-DOPA exposure is mainly in the clear halo of the vesicle (Sombers *et al.* 2005). The dense core consists primarily of the catecholamine-storage protein chromogranin A and

is thought to expand during exocytosis, facilitating fusion pore expansion (Amatore *et al.* 2005). Although this is a likely outcome with adrenal chromaffin cell vesicles, it appears that the fusion pore in PC12 cell vesicles can pass through a transition state involving a lipid nanotube. Under conditions where the dense core expands, placing tension on the vesicular membrane, the nanotube is stabilized and constricts. This mechanism has been proposed to explain a higher occurrence of events with a foot for the smaller vesicles with larger ratio of dense core to vesicle volume, following treatment with reserpine (Sombers *et al.* 2004) and might provide a mechanism for forced release of dopamine from the halo of the vesicle through the fusion pore.

In some cases the fusion pore opens and closes without a full exocytosis event occurring. This phenomenon, termed 'kiss and run' has been observed in dopaminergic midbrain neurons using amperometry (Staal *et al.* 2004). If these events represent a significant communication pathway, then the cell's ability to modify vesicle size could be a mechanism of neuronal plasticity (learning and memory).

Amperometry at PC12 cells to monitor secretion; environmental factors

Heavy metals

A number of recent studies indicate that environmental and chemical factors can strongly modulate exocytosis in PC12 cells. Of these environmental factors, heavy metals are probably best studied.

Heavy metals have been known for many years to disturb neurotransmission and ion channel function (reviewed in, e.g. Cooper & Manalis 1983, Vijverberg *et al.* 1994). In PC12 cells, the heavy metal Cd^{2+} has been shown to block voltage-gated Ca^{2+} channels (VGCCs; Shafer 1998) and, consequently, inhibit neurotransmitter release (Taylor & Peers 1998, Westerink & Vijverberg 2002b). Additionally, using a combination of amperometry and membrane capacitance measurements, it has been shown that Cd^{2+}, as well as Sr^{2+} and Ba^{2+}, can induce vesicular catecholamine release in PC12 cells, probably by acting as a partial agonist of the proposed Ca^{2+}-sensor synaptotagmin (Kishimoto *et al.* 2001).

The neurotoxic heavy metal Pb^{2+} was shown to not only block VGCCs (Shafer 1998), but also to enhance spontaneous neurotransmitter release in populations of PC12 cells (Bressler *et al.* 1996). This effect was delayed and, as it occurred irrespective of the presence of extracellular Ca^{2+}, was attributed to intracellular effects of Pb^{2+} (reviewed in Suszkiw 2004, Toscano & Guilarte 2005). Recent amperometric recordings, in combination with the imaging of the intracellular Ca^{2+} and Pb^{2+} concentrations, demonstrated the vesicular origin of the enhancement of spontaneous neurotransmitter release in dexamethasone-differentiated PC12 cells (Fig. 6). Pb^{2+} concentration-dependently induced exocytosis from intact as well as from ionomycin-permeabilized PC12 cells through direct effects on intracellular mechanisms following a concentration-dependent delay (Fig. 6a). Pb^{2+}-induced exocytosis occurred only after partial saturation of intracellular high-affinity buffer components (Fig. 6b; Westerink & Vijverberg 2002a). Pb^{2+}-induced exocytosis could be blocked almost completely by inhibition of Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II), indicating that CaM kinase II plays a major role in Pb^{2+}-induced catecholamine exocytosis from PC12 cells (Fig. 6c) (Westerink *et al.* 2002), although synaptotagmin I is also likely to be involved (Bouton *et al.* 2001). Thus, PC12 cells have successfully been used to demonstrate that heavy metals can exert inhibitory (block of VGCCs) as well as stimulatory (mimicking Ca^{2+} as a trigger for exocytosis) effects on neurotransmission.

Organic solvents

Organic solvents, particularly toluene, are occasionally used as a drug of abuse and are known to cause a variety of neurotoxic and neuropathological effects. Exposure to toluene causes post-synaptic effects on a variety of receptors (reviewed in Bowen *et al.* 2006). Using amperometry at PC12 cells, it was shown that toluene induced catecholamine exocytosis in dexamethasone-differentiated PC12 cells, which depended on the influx of extracellular Ca^{2+} through VGCCs (Westerink & Vijverberg 2002b). However, in another study, it was shown that toluene dose-dependently inhibits the depolarization-induced rise in intracellular Ca^{2+} in NGF-differentiated PC12 cells (Tillar *et al.* 2002). These rather contradictory results might be explained by the different effects of NGF and dexamethasone on the density and expression patterns of VGCCs. Nonetheless, the increase in catecholamine release as observed in the amperometric study is in agreement with elevated levels of extracellular dopamine in rat brain (Riegel & French 1999).

Persistent organic pollutants

Several persistent environmental compounds, like polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), are known to accumulate in biological tissues and cause behavioural symptoms as well as alteration in neurotransmission. Potential mechanisms underlying the effects of PCBs on the catecholaminergic system include changes in dopamine homeostasis, inhibition of vesicular catecholamine uptake and alteration of the intracellular Ca^{2+} concentration (reviewed in Fonnum *et al.* 2006, Mariussen & Fonnum 2006). Surprisingly, to date there is only one study in which amperometry was used to demonstrate that acute exposure of PC12 cells to the non-planar congener PCB 4 and to the coplanar congener PCB 126, but not exposure to the non-planar congener PCB 128, increases spontaneous catecholamine exocytosis. Unexpectedly, acute and subchronic exposure to these PCBs failed to cause changes in the contents of catecholamine-containing vesicles in this study (Westerink & Vijverberg 2002c), suggesting 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 its functional consequences.

Brominated flame retardants, among them PBDEs, have been shown to inhibit vesicular neurotransmitter uptake and induce neuronal cell death (reviewed in Fonnum *et al.* 2006, Mariussen & Fonnum 2006). Only recently, it was shown that a single exposure of young mice to BDE-47 reduced long-term potentiation recorded in hippocampal slices. This reduction in long-term potentiation was probably caused by a post-synaptic mechanism and coincided with a decrease in post-synaptic glutamate receptor subunits NR2B and GluR1 and autophosphorylated-active αCaM kinase II. The involvement of a post-synaptic mechanism was further underlined by *in vitro* research in dexamethasone-differentiated PC12 cells revealing that acute exposure to high concentrations of BDE-47 (20 μM) induced only a modest increase in intracellular Ca^{2+} and basal catecholamine exocytosis (Dingemans *et al.* 2007).

These combined findings illustrate the usefulness of neuroendocrine PC12 cells as models for past and future neurotoxicological studies.

Amperometry at PC12 cells to monitor secretion: relation to health and disease

The successful combination of amperometry and PC12 cells is now in use for over a decade to study the mechanisms of exocytosis, although the field is still expanding. In recent years, PC12 cells were used to shed some light on the mechanisms underlying the diseased brain.

Hypoxia is a serious condition that can result in ischaemia. Hypoxia results in the inhibition of O_2-sensitive K^+ channels and, consequently, depolarization. As PC12 cells contain these O_2-sensitive channels, they can be used as a chemosensory model to study the cellular effects of hypoxia. In a series of studies, Peers *et al.* showed that acute hypoxia stimulated basal and depolarization-induced exocytosis in PC12 cells. The enhancement of exocytosis resulted from hypoxia-induced inhibition of the Kv1.2 K^+ channel (Taylor & Peers 1998). Additionally, it was shown that chronic hypoxia increased exocytosis in response to acute hypoxia, at least partly by increasing O_2-sensitive K^+ channel-mediated depolarization. Importantly, the amount of catecholamines released per vesicle also increased (Taylor & Peers 1999), which is probably because of increased expression of the rate-limiting enzyme in dopamine synthesis TH. On the other hand, prolonged hypoxia resulted in the formation of amyloid β-peptides (AβPs). These AβPs cause not only selective augmentation of calcium influx through L-type Ca^{2+} channels but also induce the formation of a Cd^{2+}-resistant Ca^{2+} influx pathway (Green & Peers 2001). The hypoxic enhancement of catecholamine exocytosis was markedly reduced by inhibitors of Alzheimer's AβPs and mimicked by direct application of AβPs under normoxic conditions (Taylor *et al.* 1999). The formation of AβP and generation of reactive oxygen species from AβP were prerequisites for the hypoxic effect. Consequently, the hypoxia-induced enhancement of exocytosis could be inhibited by antioxidants like ascorbic acid and melatonin (Green *et al.* 2002). Thus, using PC12 cells as a model it was shown that acute hypoxia induces exocytosis, whereas prolonged hypoxia can induce formation of Ca^{2+}-permeable AβP channels resulting in excessive exocytosis. This excessive secretion in turn probably contributes to AβP pathophysiology following cerebral ischaemia.

Notably, AβPs have a marked resemblance to some prion proteins. Prion diseases, like Creutzfledt Jakob disease, are characterized by neuro-degeneration and arise from infection with the protease-resistant scrapie form of prion. PC12 cells were used to investigate the cellular mechanisms by which prion protein fragments cause neuronal dysfunction. In PC12 cells prion protein fragment 106–126 also generated a Cd^{2+}-resistant Ca^{2+} influx pathway, thereby augmenting catecholamine exocytosis in a concentration-dependent manner. This mechanism of action was remarkably comparable with the enhancement of exocytosis following formation of AβPs, although prion protein fragment 106–126 did not increase the amount of catecholamines released per vesicle (Taylor *et al.* 2001).

Some forms of familial Parkinson's disease, a neurodegenerative disorder characterized by a loss of dopaminergic neurons in the substantia nigra and the presence of Lewy bodies are caused by mutations in α-synuclein (α-syn) or by the over-expression of wild-type α-syn. Recently, PC12 cells were used to investigate how these mutations or the enhanced expression of α-syn affects catecholamine homeostasis and neurotransmission. Using intracellular patch amperometry (see below), it was demonstrated that PC12 cells over-expressing wild-type or mutated α-syn display elevated levels of cytosolic catecholamines. This effect appeared to result from leakage of catecholamines from the vesicles (Mosharov *et al.* 2006). Importantly, these PC12 cells displayed impaired depolarization-evoked dopamine release. This is rather surprising as the number of morphologically docked vesicles was increased (Larsen *et al.* 2006). These findings suggest that exocytosis under these conditions is inhibited by interference with a step following vesicle docking, but preceding vesicle fusion.

The cellular mechanisms underlying the antimanic properties of lithium have also been investigated using PC12 cells. Exposure of NGF-differentiated PC12 cells to lithium results in an increase in mRNA encoding for secretogranin II and VMAT1, proteins associated with filling of LDCVs with catecholamines. Surprisingly, this upregulation is absent in undifferentiated PC12 cells (Cordeiro *et al.* 2000). The selective increase in mRNA in NGF-differentiated PC12 cells correlated with an increase in regulated secretion of ^{3}H-labelled dopamine from PC12 cell populations (Cordeiro *et al.* 2004). More recently, using

amperometry, it was found that lithium exposure increased K^+-evoked exocytosis. The increase in exocytosis was without effects on vesicle contents and spike characteristics, although vesicle diameter was increased by ~15% (Umbach *et al.* 2005). This is rather surprising as an increase in vesicle diameter and an increase in VMAT are usually correlated and accompanied by an increase in vesicle contents (Colliver *et al.* 2000b). Either way, the mechanisms underlying the antimanic action of lithium are probably more complex than initially thought, although the future use of PC12 cells will most probably contribute to resolving the underlying mechanisms.

The future of amperometric measurements at PC12 cells

As science and technology progress, new applications become available to further elucidate the mechanism of exocytosis. Additionally, techniques already in use for other cell types may be used to resolve the modulation of regulated secretion in PC12 cells. One of the older applications is voltammetry at carbon paste (Wightman *et al.* 1976), and later carbon fibre (Gonon *et al.* 1980, Dayton *et al.* 1981, Ewing *et al.* 1983) electrodes. These were originally used to measure levels of neurotransmitters and metabolites in cerebrospinal fluid and in defined brain areas *in vivo*. Using voltammetry, the concentration profiles, instead of the amount, and identity of neurotransmitters and metabolites can be resolved with high temporal resolution. In the 1990s, cyclic voltammetry was applied by Wightman *et al.* (1991) to identify the neurotransmitters secreted by chromaffin cells as catecholamines, whereas amperometry was used to determine the amount of secreted catecholamines per vesicle with enhanced temporal resolution. In 1998, it was proven in PC12 cells that the secreted neurotransmitters, as detected with amperometry, were indeed catecholamines, most probably dopamine (Kozminski *et al.* 1998). In the same study, it was shown that following exposure to L-DOPA, the amount of secreted catecholamines increased, although the measured catecholamine concentration was rather similar in all vesicles clearly suggesting that the concentration of catecholamines within a vesicle is tightly regulated.

Further evidence for the tight regulation of the intravesicular catecholamine concentration was obtained using patch-amperometry. Patch-amperometry is an elegant combination of cell-attached membrane capacitance measurement, thus providing single-vesicle resolution, with amperometry (Dernick *et al.* 2005). Patch-amperometry was first used in 1997 to simultaneous determine the opening of individual fusion pores and of the kinetics of catecholamine release from the same vesicle in chromaffin cells. This study demonstrated that fusion pore diameter stays at <3 nm for a variable period (up to seconds) before expanding and, importantly, the existence of kiss-and-run exocytosis (Albillos *et al.* 1997). Several years later, patch amperometry was used with chromaffin cells (Gong *et al.* 2003) to substantiate previous work combining amperometry and TEM of PC12 cells (Colliver *et al.* 2000b) in both cases showing that a decrease and increase in quantal size, induced by incubation with reserpine or L-DOPA, are associated with a respective decrease and increase in vesicle size. Thus, vesicles can change size in response to changes in transmitter content to maintain a stable intravesicular neurotransmitter concentration.

Using patch-amperometry in a slightly different way, i.e. intracellular patch electrochemistry, it was possible to measure directly the concentration of oxidizable molecules in the cytosol of chromaffin cells. This study demonstrated that the strict regulation of the catecholamine concentration is not restricted to the vesicle, but also holds for the cytosol (Mosharov *et al.* 2003). Up to now, because of their smaller vesicle size and cytosolic catecholamine content, it has not been possible to use these techniques at PC12 cells. Realizing the advances in sensitivity needed to carry out patch amperometry at PC12 cells is probably only a matter of time.

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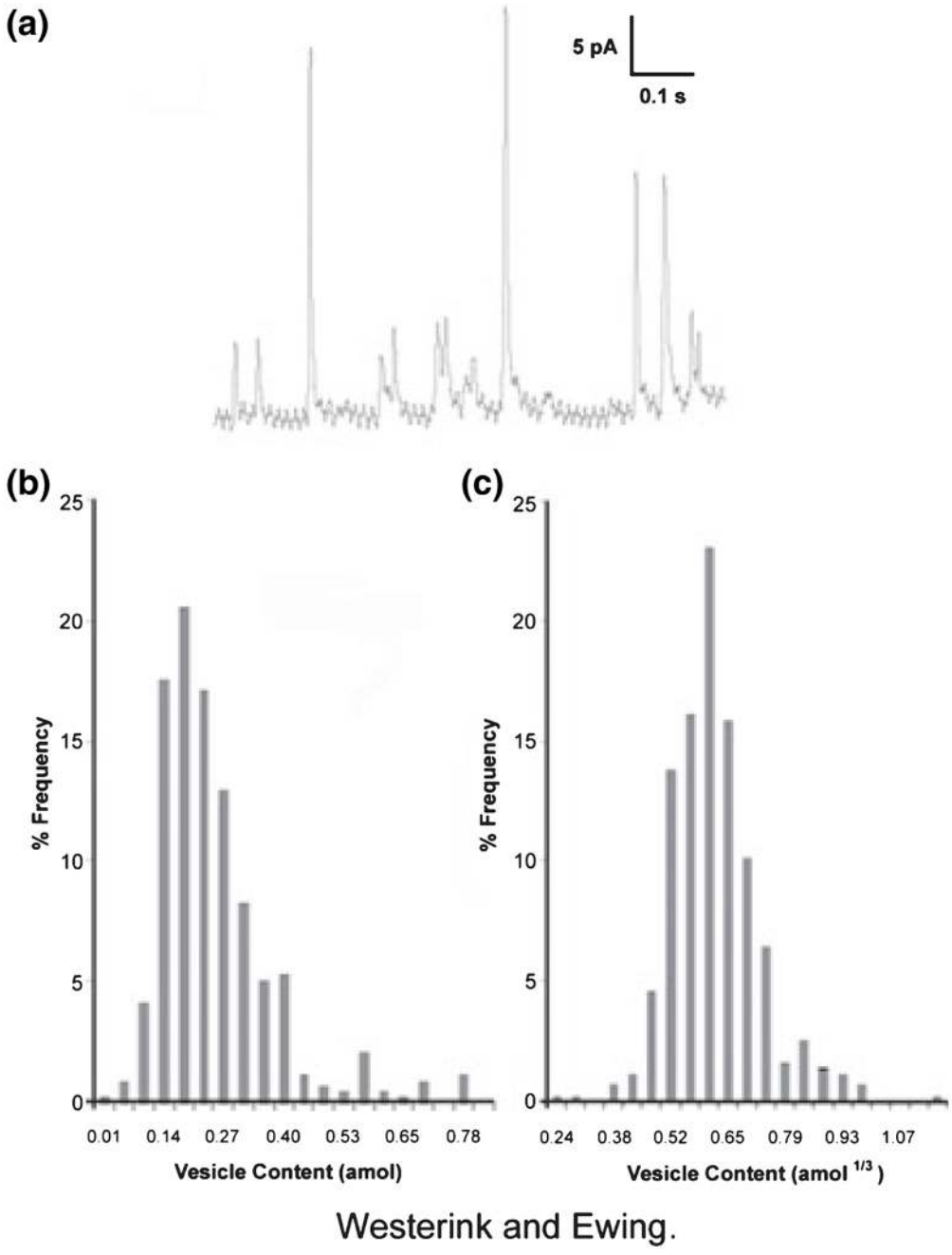

Figure 1.

(a) Example of a current–time trace for exocytosis at a single undifferentiated PC12 cell following depolarization with KCl. The resulting current transients correspond to the oxidation of catecholamine at the electrode tip upon release from the cell. The area under each current transient is equivalent to the total charge produced by the oxidation of the catecholamine content of one vesicle. (b) Distribution of the amount of catecholamine released following potassium stimulation of undifferentiated PC12 cells. The total number of moles of catecholamine detected for each exocytosis event observed in the first 40 s of KCl-stimulated release is collected into bins and plotted as the per cent of the total number of vesicles

undergoing exocytosis. (c) Cubed-root histogram for amperometric charges from PC12 cells shown in (b).

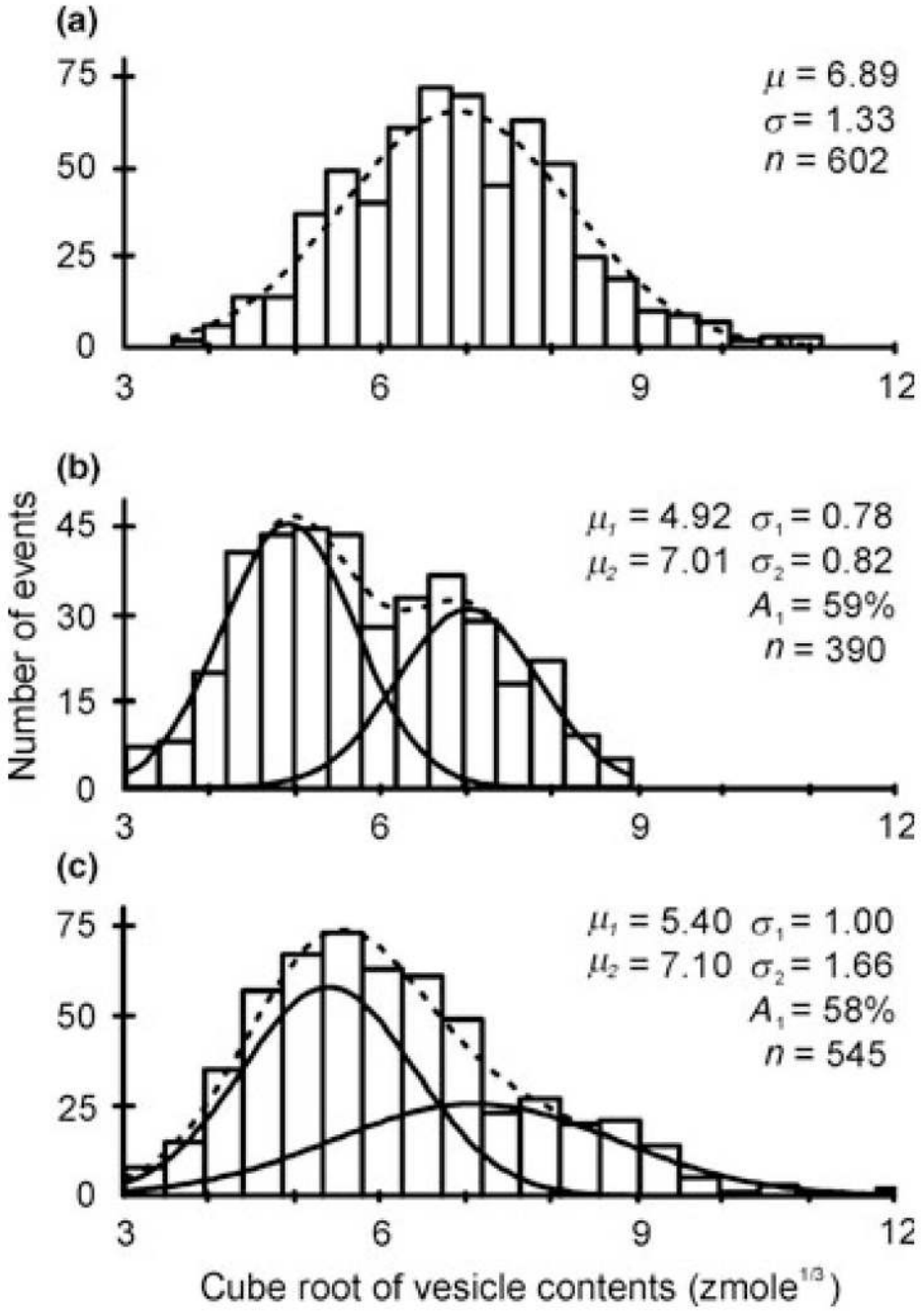

Figure 2.

Distributions of the cube root of the vesicle contents of three single undifferentiated PC12 cells from which >300 events were recorded during repeated depolarizing with KCl. The distributions were best fitted by double Gaussian functions in 80% of the cells (see, e.g. b and c) 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 (A_1) is indicated as a percentage of the total number of events (n). Modified after Westerink *et al.* (2000).

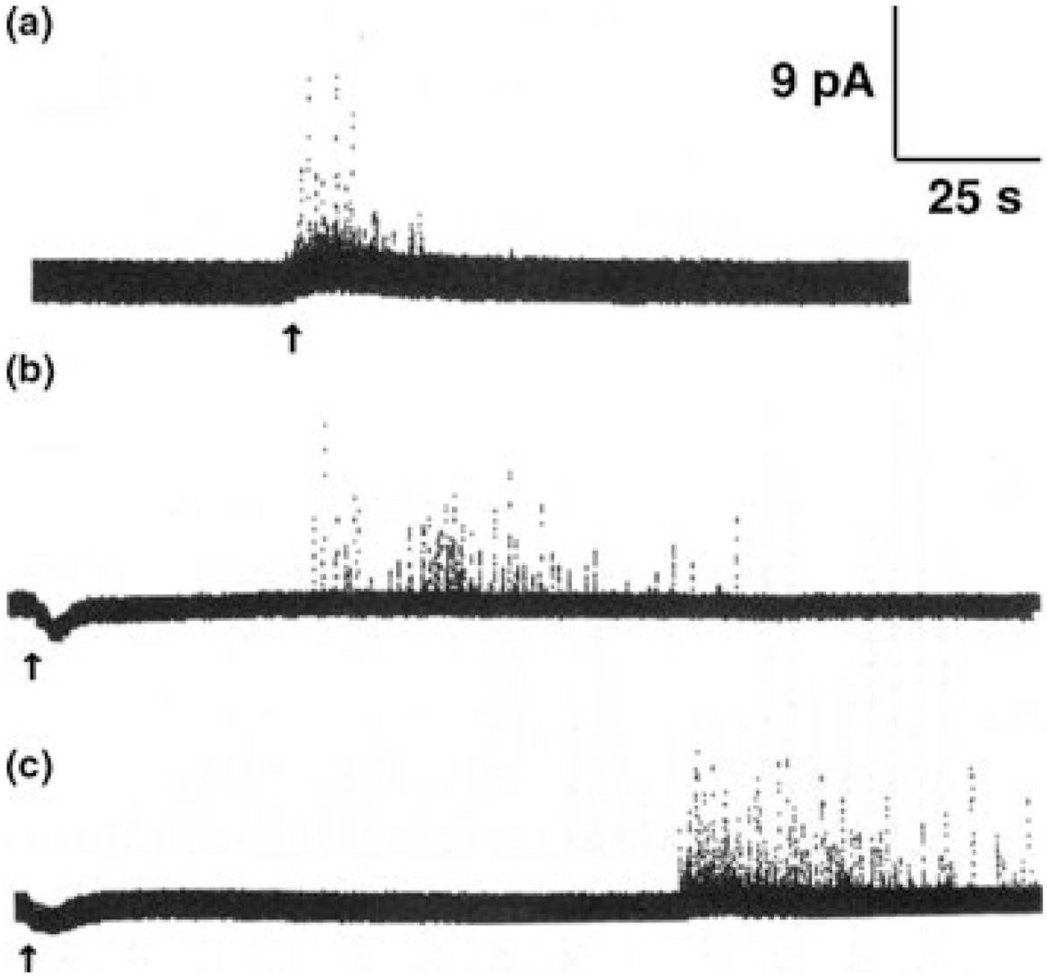

Figure 3.
Current–time traces for exocytosis at single PC12 cells. A 6-s ejection of stimulant (105 mM K^+ (a), 1 mM nicotine (b) or 1 mM muscarine (c) from a microinjector was administered at each arrow. The resulting current transients correspond to the oxidation of dopamine at the electrode tip as it is released from the cell. Reproduced with permission from Zerby & Ewing (1996b).

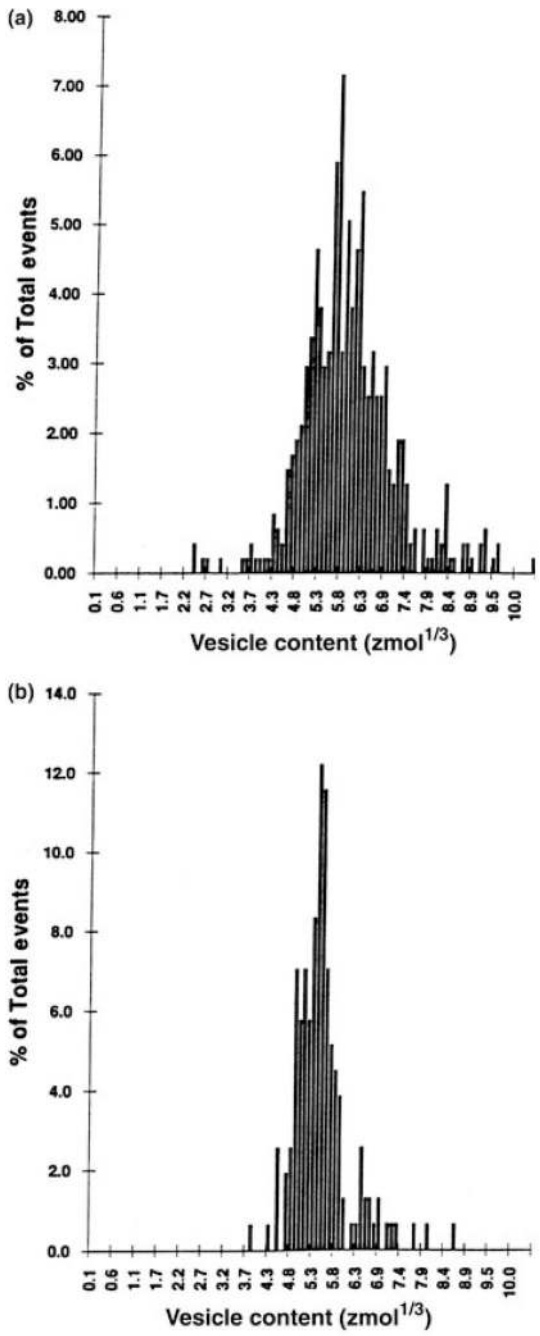

Figure 4.
Distribution of the cube root of vesicle catecholamine content for KCl-stimulated release plotted as the percent of the total number of vesicles undergoing exocytosis from (a) the soma of undifferentiated cells (n = 17 cells, 475 events) and from (b) varicosities of NGF-differentiated PC12 cells (n = 16 cells, 156 events). Reproduced with permission from Zerby & Ewing (1996a).

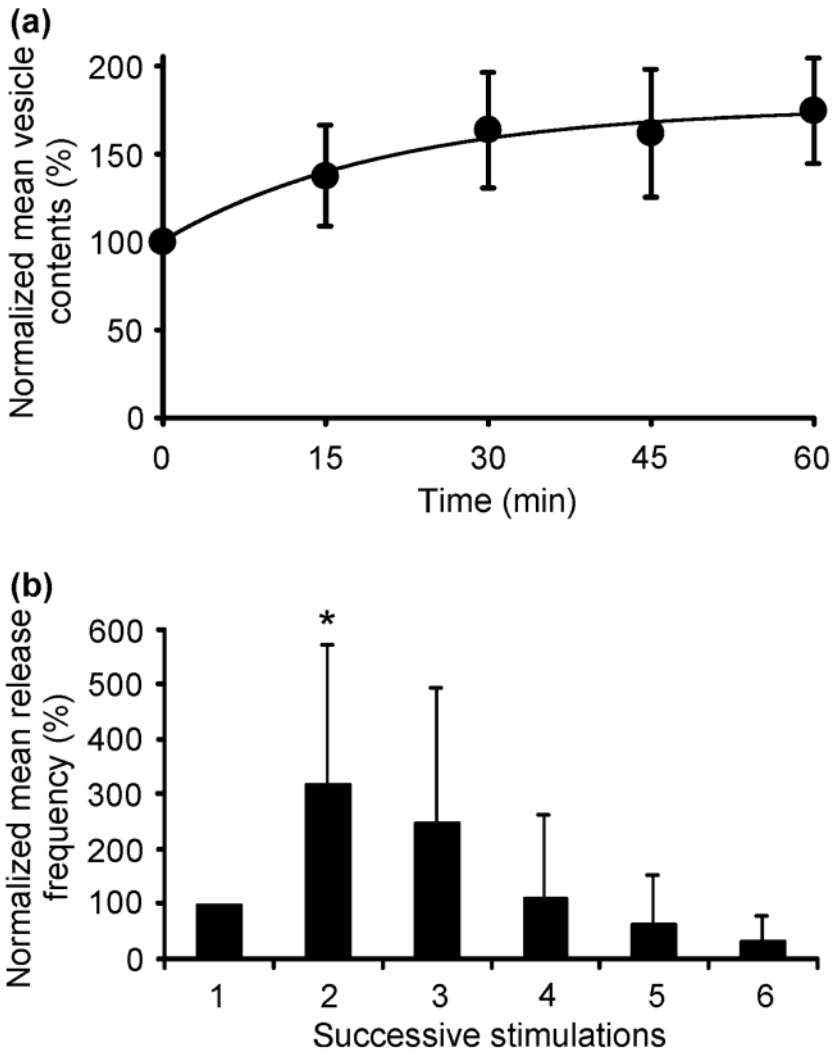

Figure 5.

The mean vesicle content of undifferentiated PC12 cells, determined following KCl-induced exocytosis, increased with increasing duration of superfusion with saline containing 100 μM L-DOPA (a). Each point represents mean ± SD (n = 8 cells) and at all time points the mean vesicle contents was increased significantly when compared with 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 with an increase of 21 min. The graph in (b) depicts the relation between the KCl-induced release frequency and the number of successive stimulations at 10–15 min intervals, during which 100 μM L-DOPA-containing saline was superfused. The exocytotic frequency is expressed as a percentage of the value obtained during the first response (set at 100%), which was evoked before exposure to L-DOPA. Each bar represents mean ± SD (n = 8 cells). During the first stimulus after L-DOPA superfusion, the frequency of events was significantly higher than the control value (t-test, $P < 0.05$). Reproduced with permission from Westerink *et al.* (2000).

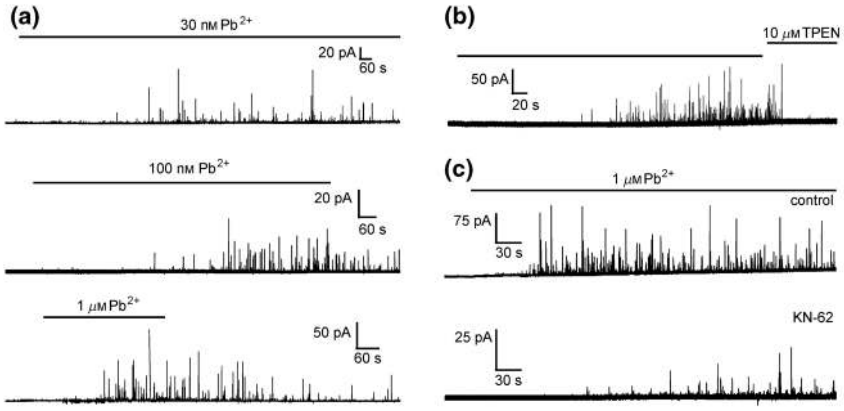

Figure 6.

Effects of the heavy metal Pb^{2+} on vesicular catecholamine release from ionomycin-permeabilized dexamethasone-differentiated PC12 cells. (a) Amperometric recordings from permeabilized PC12 cells superfused with Ca^{2+}-free saline containing 5 μM ionomycin and 0.03, 0.1 and 1 μM Pb^{2+}, demonstrating the concentration dependence of Pb^{2+}-induced exocytosis. (b) Amperometric recording from a permeabilized PC12 cells showing vesicular catecholamine release during superfusion with nominal Ca^{2+}-free saline containing 1 μM Pb^{2+}. The membrane-permeable heavy metal chelator TPEN rapidly reduces the intracellular Pb^{2+} concentration below the threshold for release, whereas the membrane-impermeable chelator EGTA is ineffective (not shown), demonstrating the Ca^{2+}-independence of Pb^{2+}-evoked exocytosis. (c) Amperometric recordings from ionomycin-permeabilized PC12 cells reveal that saline containing 1 μM Pb^{2+} induced vesicular release (control), which is strongly inhibited by co-application of an inhibitor of CaM kinase II (10 μM KN-62), whereas inhibition of Protein Kinase C does not appear to affect Pb^{2+}-induced exocytosis (not shown). Modified after Westerink & Vijverberg (2002a) and Westerink *et al.* (2002).