Modulation of Ca^{2+}-independent, Pb^{2+}-induced exocytosis from rat PC12 cells by CaMK II.

Remco H.S. Westerink[a] and Henk P.M. Vijverberg[b]

a. Swammerdam Institute for Life Sciences, Neurobiology, University of Amsterdam.
b. Institute for Risk Assessment Sciences (IRAS), Utrecht University,
The Netherlands.

Correspondence: Dr. Remco Westerink, University of Amsterdam,
SILS-Section of Neurobiology, Kruislaan 320, 1098 SM Amsterdam, The Netherlands.
Phone: 31-20-5257630; **Fax:** 31-20-5257709; **Email:** rwesteri@science.uva.nl

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Exocytosis is a complex, Ca^{2+}-dependent process involving docking and priming of vesicles, the formation of a fusion pore and fusion of vesicle and plasma membrane and the release of vesicle contents. Apart from Ca^{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[1-3]. Several Ca^{2+}-dependent proteins associated with exocytosis are activated by Pb^{2+} as well. Pb^{2+} is a potent activator of calmodulin[4], PKC[5], calcineurin[6], and synaptotagmin[7]. Additionally, Pb^{2+} has been reported to cause an enhancement of spontaneous neurotransmitter release, which occurs irrespective of the presence of extracellular Ca^{2+} and has been attributed to intracellular effects of Pb^{2+} [8-9]. Previous reports demonstrate that addition of >5 μM Pb^{2+} to external saline is required to induce neurotransmitter release from undifferentiated PC12 cell populations[8]. The aim of this study was to determine the origin of Pb^{2+}-induced neurotransmitter release, to investigate whether Pb^{2+} stimulates release directly or indirectly, and to investigate the intracellular signaling pathways involved in Pb^{2+}-induced exocytosis.

MATERIALS AND METHODS

PC12 cells (ATCC CRL-1721) were grown essentially as described previously[10]. 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 days after subculturing. Culture dishes and coverslips were coated with 5 μg/cm^2 poly-L-lysine (Sigma, St. Louis MO, USA). Experiments were performed 5-8 days after initiating differentiation. Carbon fiber microelectrode (∅10 μm) fabrication and data recording and analysis were as described previously[10-11]. Exocytosis was evoked by superfusion with nominal Ca^{2+}-free saline containing > 30 nM Pb^{2+}. Experiments were performed at room temperature (21-23 °C).

RESULTS AND DISCUSSION

To investigate the direct intracellular action of Pb^{2+}, cells were permeabilized by superfusion with saline containing 5 µM ionomycin Ca^{2+}-salt. Pores formed by ionomycin have been shown to be highly permeable to Pb^{2+} as well as to Ca^{2+} [12]. Permeabilized cells were superfused with saline containing various concentrations of Pb^{2+}. Superfusion with 30 nM Pb^{2+} resulted in detectable vesicular catecholamine release after a delay of several minutes (Fig. 1A), whereas 30 min of superfusion with nominal Pb^{2+}-free saline or with 10 nM Pb^{2+} was without effect (data not shown). At increased concentrations of 100 nM or 1 µM Pb^{2+} the delay to the onset of release decreased (Fig. 1A) from 4.8 ± 4.2 to 3.8 ± 1.3 and 0.9 ± 0.8 min, respectively. In addition, the maximum frequency of vesicles released increased with increasing Pb^{2+} concentration with a maximum at 1 µM Pb^{2+}. Switching back to nominal Pb^{2+}-free saline reversed the Pb^{2+}-induced release only slowly (Fig. 1A). The concentration-dependent effects demonstrate that the threshold concentration of extracellular Pb^{2+} to induce vesicular neurotransmitter release in permeabilized PC12 cells is close to 30 nM.

Using calcium and heavy metal chelators it was shown that 1 mM Ca^{2+}-evoked release is readily reversed by superfusion with nominal Ca^{2+}-free saline containing the membrane-impermeable chelator EGTA (500 µM; not shown), demonstrating that Ca^{2+} rapidly diffuses out of the permeabilized cell. Pb^{2+}-induced release is reversed only slowly by superfusion with saline containing 500 µM EGTA (Fig. 1B), 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. 1B), whereas TPEN did not affect basal, spontaneous release or 1 mM Ca^{2+}-evoked exocytosis (not shown). 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+}.

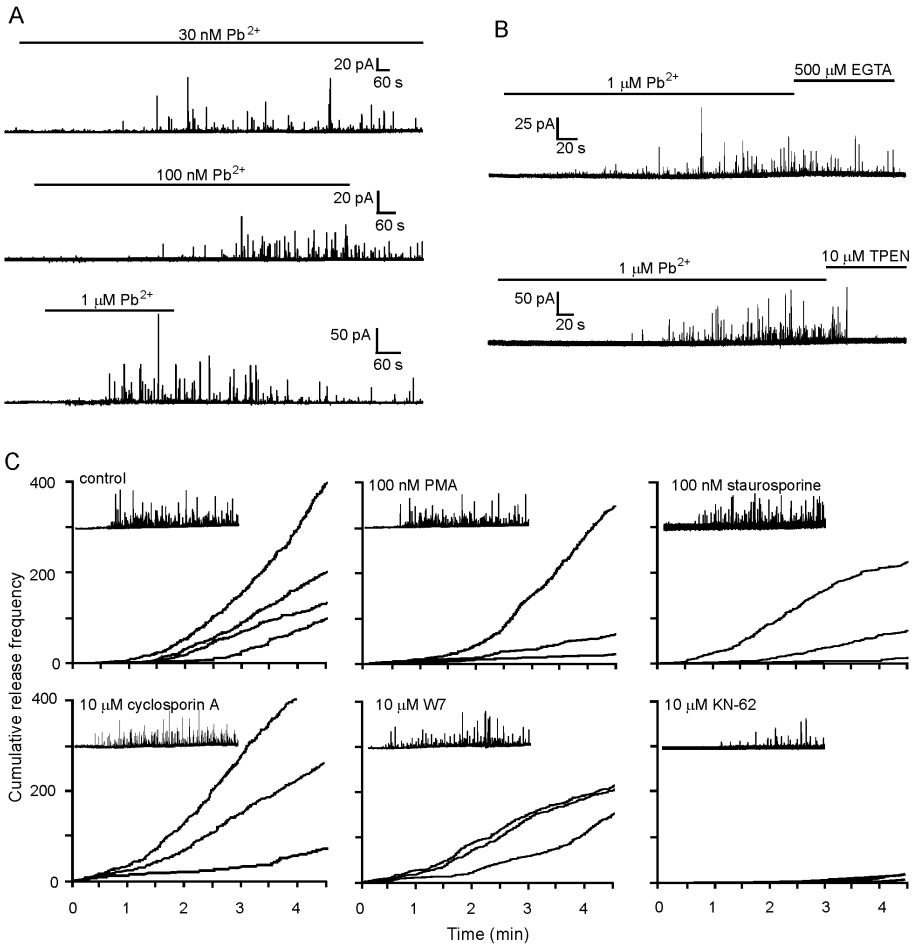

Figure 1 A) Concentration-dependence of Pb^{2+}-evoked exocytosis in permeabilized PC12 cells. Amperometric recordings from permeabilized PC12 cells superfused with Ca^{2+}-free saline containing 5 μM ionomycin Ca^{2+}-salt and 0.03, 0.1 and 1 μM Pb^{2+}, as indicated by the bars on top of the recordings. **B)** Ca^{2+}-independence of Pb^{2+}-evoked exocytosis in permeabilized PC12 cells. Pb^{2+}-induced (1 μM) exocytosis is not reversed by the membrane-impermeable chelator EGTA at concentrations sufficient to rapidly reduce the intracellular Ca^{2+} concentration below the threshold for release (upper trace). Conversely, 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. **C)** Effects of drugs modulating PKC (PMA and staurosporine), calcineurin (cyclosporin A), calmodulin (W7) and CaMK II (KN-62) activity on the frequency of Pb^{2+}-induced exocytosis in ionomycin-permeabilized PC12 cells. Drug application started 15 min before superfusion with saline containing 1 □M Pb^{2+} and the same concentrations of the drug. The panels show cumulative frequencies of exocytotic events recorded from different cells following the start of superfusion with Pb^{2+}-containing saline (t = 0) and the drugs indicated; insets show representative examples of amperometric recordings. The curves show that the frequency of exocytosis varies between cells. However, the delay to the onset of Pb^{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.

Activation of PKC by PMA or inhibition of calcineurin by cyclosporin causes a significant increase of the basal release frequency in permeabilized cells. Conversely, inhibition of PKC, calmodulin and CaMK II by staurosporine, W7 and KN-62, respectively, did not affect basal exocytosis. These results indicate that enhancement of the phosphorylation state of intracellular proteins by PKC and calcineurin directly increased the basal frequency of exocytosis[13]. In case of Pb^{2+}-induced exocytosis, systematic effects of these drug treatments on the frequency of Pb^{2+}-induced events were only observed for the inhibition of CaMK II by KN-62 and for inhibition of calmodulin by W7. Inhibition of CaMK II caused a consistent and strong decrease in the frequency of exocytosis (Fig. 1C). Consequently, KN-62 caused a significant decrease in the total number of vesicles released during the first 270 s of Pb^{2+} exposure, as well as an increase in the delay to the onset of Pb^{2+}-induced exocytosis (Fig. 1C). 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 (Fig. 1C). Exposure of the cells to PMA, to staurosporine, and to cyclosporin caused neither 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, which was not observed with W7. The results

presented in Figure 1C show that Pb^{2+}-induced exocytosis is modulated by calmodulin and CaMK II activity**.**

The results from this study provide a direct demonstration that Pb^{2+}-induced catecholamine release has a vesicular origin and that the threshold concentration to evoke exocytosis in permeabilized PC12 cells amounts to ~30 nM (Fig. 1A). The direct effects of Pb^{2+} on exocytosis are mediated by intracellular mechanisms. This is demonstrated by the fact that the extracellular application of membrane-impermeable chelators EGTA rapidly reduces the intracellular Ca^{2+} concentration below threshold levels and thereby reverse Ca^{2+}-evoked exocytosis, whereas Pb^{2+}-induced exocytosis is reversed only very slowly (Fig.1B). Conversely, the membrane-permeable heavy-metal chelator TPEN rapidly reverses Pb^{2+}-induced exocytosis (Fig. 1B), indicating that Pb^{2+} is tightly associated with an intracellular binding site. The results show that Pb^{2+}-induced exocytosis involves a direct interaction with an intracellular, high-affinity site.

The extracellular threshold concentration of ~30 nM Pb^{2+} to evoke exocytosis is slightly higher than the concentrations of Pb^{2+} found in blood after Pb^{2+}-poisoning. However, this does not distract from the toxicological hazard of this heavy metal. Since high-affinity buffering facilitates the intracellular accumulation of Pb^{2+}, much depends on the availability of extracellular Pb^{2+}, i.e., free Pb^{2+} concentration as well as the amount of remaining extracellular Pb^{2+} and the affinity by which it is bound. If the conditions are such that a critical amount of Pb^{2+}, estimated to be 2-3 attomol for PC12 cells[10], accumulates intracellularly, sustained exocytosis may be triggered and cause the adverse effect.

In ionomycin-permeabilized PC12 cells modulation of the phosphorylation state of the exocytotic machinery by PKC/calcineurin results in modulation of exocytotic function[13]. 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[5], and activation of PKC enhances basal

exocytosis in permeabilized PC12 cells[13]. The calmodulin inhibitor W7, which does not cause significant effects on basal release in permeabilized PC12 cells[13], clearly reduced the delay to onset of Pb^{2+}-induced exocytosis (Fig. 1C). Since the delay to onset of release is reduced with increasing Pb^{2+} concentration, the effect of W7 is equivalent to an apparent enhancement of the sensitivity to Pb^{2+} (cf. Fig. 1C and Fig. 1A). Inhibition of CaMK II activity by KN-62 leads to a strong reduction of the number of vesicles released and to an apparent increase in the delay to onset of Pb^{2+}-evoked exocytosis (Fig. 1C), which appears to be independent of calmodulin. These results indicate that CaMK II is required for Pb^{2+}-induced vesicular catecholamine release.

Although a direct demonstration of activation of CaMK II by Pb^{2+} is lacking, CaMK II is known to phosphorylate a range of intracellular proteins, including synapsin I[14], synaptotagmin[15], and the t- and v-SNAREs[16]. Furthermore, CaMK II-induced phosphorylation is associated with an increase in the number of releasable vesicles[17]. Therefore, CaMK II provides a novel and plausible target for the direct intracellular action of Pb^{2+} leading to neurotransmitter release.

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