

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

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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 cycle¹⁻³. Several Ca^{2+} -dependent proteins associated with exocytosis are activated by Pb^{2+} as well. Pb^{2+} is a potent activator of calmodulin⁴, PKC⁵, calcineurin⁶, and synaptotagmin⁷. 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 $>5 \mu\text{M}$ Pb^{2+} to external saline is required to induce neurotransmitter release from undifferentiated PC12 cell populations⁸. 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 previously¹⁰. For all experiments cells were differentiated in culture medium (RPMI 1640, Gibco, Grand Island NY, USA) supplemented with $5 \mu\text{M}$ dexamethasone (Genfarma, Zaandam, The Netherlands) starting 2 days after subculturing. Culture dishes and coverslips were coated with $5 \mu\text{g}/\text{cm}^2$ poly-L-lysine (Sigma, St. Louis MO, USA). Experiments were performed 5-8 days after initiating differentiation. Carbon fiber microelectrode ($\varnothing 10 \mu\text{m}$) fabrication and data recording and analysis were as described previously¹⁰⁻¹¹. Exocytosis was evoked by superfusion with nominal Ca^{2+} -free saline containing $> 30 \text{ nM}$ Pb^{2+} . Experiments were performed at room temperature ($21-23 \text{ }^\circ\text{C}$).

RESULTS AND DISCUSSION

To investigate the direct intracellular action of Pb²⁺, cells were permeabilized by superfusion with saline containing 5 μM ionomycin Ca²⁺-salt. Pores formed by ionomycin have been shown to be highly permeable to Pb²⁺ as well as to Ca²⁺ ¹². Permeabilized cells were superfused with saline containing various concentrations of Pb²⁺. Superfusion with 30 nM Pb²⁺ resulted in detectable vesicular catecholamine release after a delay of several minutes (Fig. 1A), whereas 30 min of superfusion with nominal Pb²⁺-free saline or with 10 nM Pb²⁺ was without effect (data not shown). At increased concentrations of 100 nM or 1 μM Pb²⁺ 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 Pb²⁺ concentration with a maximum at 1 μM Pb²⁺. Switching back to nominal Pb²⁺-free saline reversed the Pb²⁺-induced release only slowly (Fig. 1A). The concentration-dependent effects demonstrate that the threshold concentration of extracellular Pb²⁺ 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 Ca²⁺-evoked release is readily reversed by superfusion with nominal Ca²⁺-free saline containing the membrane-impermeable chelator EGTA (500 μM; not shown), demonstrating that Ca²⁺ rapidly diffuses out of the permeabilized cell. Pb²⁺-induced release is reversed only slowly by superfusion with saline containing 500 μM EGTA (Fig. 1B), demonstrating that the effect of Pb²⁺ is independent of intracellular Ca²⁺. Conversely, 10 μM of the membrane-permeable heavy metal chelator TPEN quickly reversed the Pb²⁺-induced release (Fig. 1B), whereas TPEN did not affect basal, spontaneous release or 1 mM Ca²⁺-evoked exocytosis (not shown). These findings demonstrate that exocytosis is caused by binding of Pb²⁺ to an intracellular binding site and that Pb²⁺-induced exocytosis continues in the absence of Ca²⁺.

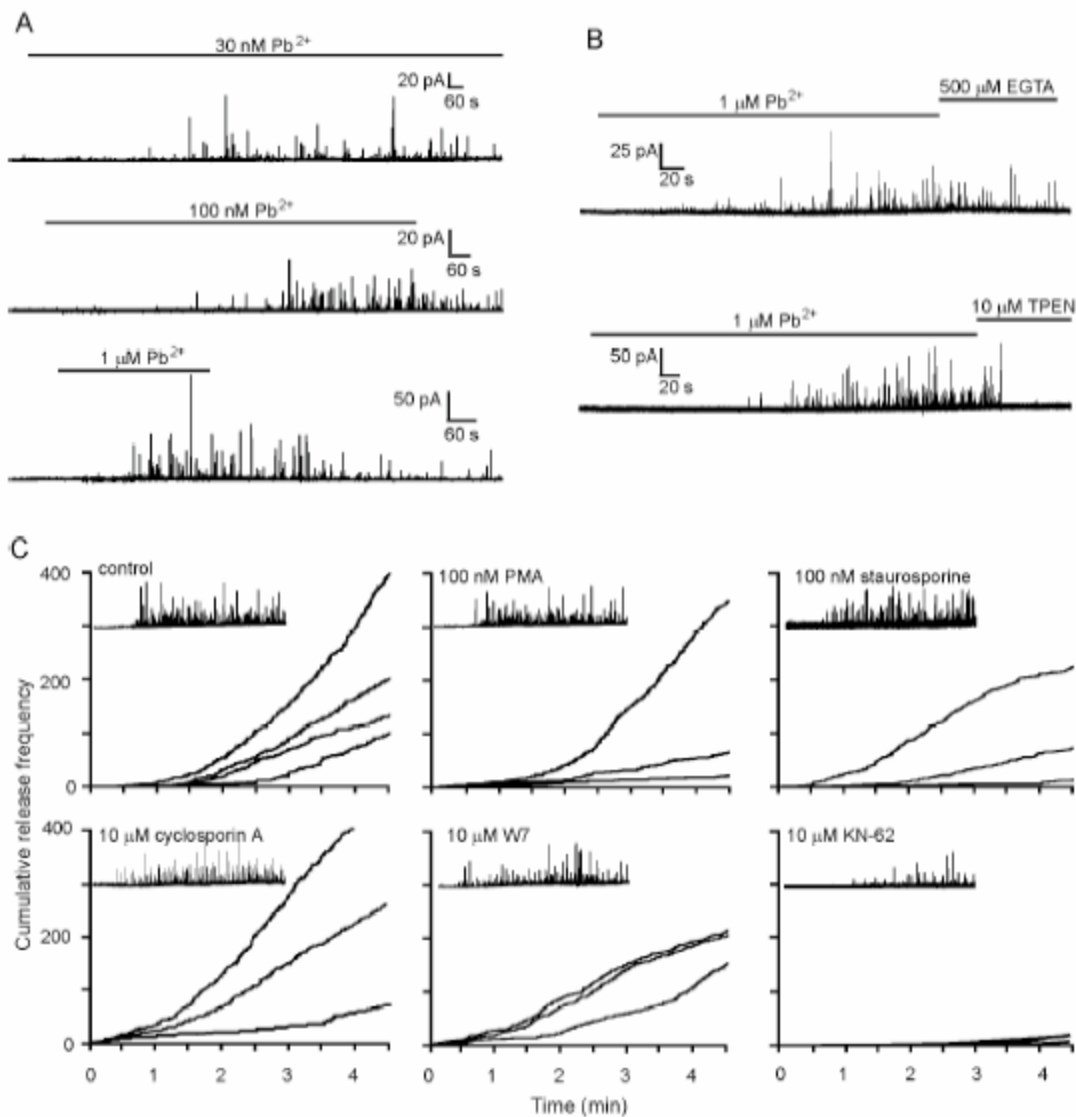


Figure 1 A) Concentration-dependence of Pb²⁺-evoked exocytosis in permeabilized PC12 cells. Amperometric recordings from permeabilized PC12 cells superfused with Ca²⁺-free saline containing 5 μM ionomycin Ca²⁺-salt and 0.03, 0.1 and 1 μM Pb²⁺, as indicated by the bars on top of the recordings. B) Ca²⁺-independence of Pb²⁺-evoked exocytosis in permeabilized PC12 cells. Pb²⁺-induced (1 μM) exocytosis is not reversed by the membrane-impermeable chelator EGTA at concentrations sufficient to rapidly reduce the intracellular Ca²⁺ 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 $1 \mu M Pb^{2+}$ 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 exocytosis¹³. 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 cells¹⁰, 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 function¹³. 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 PKC⁵, and activation of PKC enhances basal

exocytosis in permeabilized PC12 cells¹³. The calmodulin inhibitor W7, which does not cause significant effects on basal release in permeabilized PC12 cells¹³, 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 I¹⁴, synaptotagmin¹⁵, and the t- and v-SNAREs¹⁶. Furthermore, CaMK II-induced phosphorylation is associated with an increase in the number of releasable vesicles¹⁷. 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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