Research report

Signaling pathways involved in Ca^{2+}- and Pb^{2+}-induced vesicular catecholamine release from rat PC12 cells

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Abstract

Since Pb^{2+} substitutes for Ca^{2+} in essential steps leading to exocytosis, we have investigated whether Ca^{2+} and Pb^{2+} induce exocytosis through similar pathways. Vesicular catecholamine release was measured from dexamethasone-differentiated PC12 cells using carbon fiber microelectrode amperometry. Effects of drugs known to modulate PKC (PMA, staurosporine), calcineurin (cyclosporin A), calmodulin (W7), and CaM kinase II (KN-62) activity were investigated in intact and in ionomycin-permeabilized PC12 cells. Activation of PKC and inhibition of calmodulin decrease the frequency of exocytotic events evoked by high K^+ stimulation in intact cells. In addition, inhibition of calmodulin enhances the frequency of basal exocytosis from intact cells. Activation of PKC and inhibition of calcineurin enhance the frequency of basal exocytosis in intact as well as in ionomycin-permeabilized cells. Inhibition of PKC and of CaM kinase II cause no significant effects. None of the treatments has a significant effect on vesicle contents. 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, Pb^{2+}-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 Pb^{2+}-evoked exocytosis. Synaptotagmin I- and II-deficient PC12-F7 cells exhibit vesicular catecholamine release following depolarization or superfusion with Pb^{2+}. However, the frequency of exocytosis and the contents of vesicles released are strongly reduced as compared to PC12 cells. It is concluded that Ca^{2+}-evoked exocytosis is modulated mainly by PKC and calcineurin, whereas Pb^{2+}-evoked exocytosis is mainly modulated by CaM kinase II.
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1. Introduction

Vesicular neurotransmitter release is preceded by vesicle filling, docking and priming, and is generally 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^{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 Refs. [31,34,44]). Modulation of the activity of these

regulatory proteins is likely to affect the normal progression of the vesicle cycle and may alter exocytosis.

Rat phaeochromocytoma PC12 cells 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 [32,41]. Dopamine and norepinephrine are synthesized [15] and are stored in large dense-core vesicles [54], many of which are in the close vicinity of the plasma membrane [25]. Using carbon fiber microelectrode amperometry, which allows for the detection of vesicular catecholamine release [9], PC12 cells were shown to release the stored catecholamines in a quantal manner [6,46,47,55,56]. In addition, GFP targeted to secretory vesicles has been used to demonstrate large dense-core vesicle exocytosis in PC12 cells [24,28].

The heavy metal ion Pb^{2+} is able to trigger catecholamine secretion in PC12 cells by substituting for Ca^{2+} in essential steps of the vesicle cycle leading to exocytosis [56]. PC12 cells permeabilized with ionomycin readily display vesicular catecholamine release when the extracellular Ca^{2+} concentration is raised to ~100 μM. Elevation of the extracellular Pb^{2+} concentration to 30 nM or higher causes a similar response, but only after a concentration-dependent delay. Specific intracellular targets for Pb^{2+} involved in exocytosis remain to be identified [56]. Both Ca^{2+} and Pb^{2+} are known to activate various proteins, which have a regulatory role in the vesicle cycle. Pb^{2+} is far more potent than Ca^{2+} as an activator of protein kinase C (PKC) [33,45], calmodulin [17,27], calcineurin [26], and synaptotagmin [3]. These proteins, which are potential targets for the neurotoxic action of Pb^{2+}, have previously been implicated in the functional modulation of exocytotic processes.

Activation of protein kinase C (PKC) by phorbol esters in PC12 cells has been reported to augment K^+-evoked catecholamine release, whereas depolarization-induced Ca^{2+}-influx is inhibited [18]. PKC activation has also been reported to increase basal catecholamine release from PC12 cells dependent on extracellular Ca^{2+}, but without an apparent change in intracellular Ca^{2+} concentration [36]. 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^{2+} concentration, due to a moderate hyperpolarizing shift in the voltage dependence of activation of L-type Ca^{2+} channels [47]. Apart from effects on ion channels (for review see Ref. [51]), PKC enhances catecholamine release in 'cracked' PC12 cells by a direct action on the exocytotic machinery [7]. Pb^{2+}-induced catecholamine release from populations of intact PC12 [2] and permeabilized chromaffin cells [50] 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 [50].

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 [5,7]. Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) is able to phosphorylate synapsin I [23], synaptotagmin [52], VAMP [20], syntaxin and SNAP-25 [37]. Activation of CaM kinase II, but not of PKC, increases the readily releasable pool of vesicles in mouse pancreatic β-cells [16]. Inhibition of CaM kinase II activity 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 [38]. 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 [10], but potentiates depolarization-induced Ca^{2+} influx and exocytosis in rat lactotrophs [12]. Cyclosporin A enhances the frequency of miniature endplate potentials in mouse motor nerve terminals [30] as well as 4-aminopyridine- and α-latrotoxin-evoked GABA release from rat brain synaptosomes [43]. However, inhibition of calcineurin by antibodies results in decreased noradrenaline and neuropeptide secretion from streptolysin-O-permeabilized rat brain synaptosomes [19]. 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 Ref. [1]). 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 [3]. 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 [42].

To investigate the intracellular mechanisms of Pb^{2+}-induced exocytosis, we have compared 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 [9,57]. As many of the signaling pathways mentioned above act on multiple specific targets, including voltage-activated Ca^{2+} channels (for review see Ref. [4]), 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.

2. Materials and methods

2.1. Chemicals

NaCl, KCl, $Mg(NO_3)_2$, D-glucose, sucrose, HEPES, and

NaOH (Aristar quality) were obtained from BDH Laboratory Supplies (Poole, Dorset, UK), while $Pb(NO_3)_2$ was obtained 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-filtered water (Milli-Q®; resistivity>10 MΩ cm). Immediately after preparation, all saline solutions were filtered using a 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.

2.2. Cell culture

PC12 cells (ATCC CRL-1721) [14] as well as synaptotagmin I- and II-deficient cells of the clone PC12-F7 (generously donated by Dr Shoji-Kasai, Mitsubishi-Kagaku Institute of Life Sciences, Tokyo, Japan) [42] were grown essentially as described previously [56] for a maximum of ten 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 days after subculture in 35-mm culture dishes (Nunc, 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 days. All experiments were performed 7–10 days after subculture, i.e. 5–8 days after initiating differentiation.

2.3. Carbon fiber microelectrode amperometry

Carbon fiber microelectrode (10 μm diameter) fabrication was as described previously [55]. 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 D-glucose, and 36.5 sucrose at pH 7.3 at room temperature 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, positioned ~250 μm from the cell, 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). 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+}. Data recording and analysis were as described previously [55]. Briefly, amperometric current was recorded using an EPC-7 patch clamp (List Electronic, Darmstadt, Germany), filtered at 2 kHz (eight pole Bessel filter), sampled at 4 kHz, and stored on disk for off-line analysis. Current transients associated with vesicular catecholamine release were automatically identified and analysed using custom-designed Labview routines (National Instruments, Austin, TX, USA). Peak amplitude, 50–90% rise time, event duration and event width at half height were determined from the original signal. The original signal was digitally filtered (low-pass 100 Hz, second order Bessel filter) for integration of the detected events. Vesicle contents were calculated from the total charge (Q) transferred during the event, i.e. the integral of the event, according to Q/nF, where $n=2$ electrons for the oxidation of one catecholamine molecule and F is Faraday's constant. Since distributions of vesicle contents obtained from single cells are skewed to the right, the median value of vesicle contents, instead of the mean value, was determined for each cell.

2.4. 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 two 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 and Instruments, Munich, Germany; kit no. 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 9980×*g* (12 000 rpm) for 1 min. The pellet was resuspended in washing solution and the procedure repeated two times. Finally, the catechol-

amines were stripped with 50 μl glacial 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, 100×2.1 mm; Aurora Borealis Control, Schoonebeek, The Netherlands), an INTRO electrochemical detector and a cell (ANTEC Leyden, Leiden, The Netherlands) set at a potential of 675 mV versus an Ag/AgCl reference electrode. A column oven set at 30 °C, integrated in the INTRO, was used for both the reversed-phase column and the electrochemical cell. The mobile phase consisted of 5 g/l $(NH_4)_2SO_4$, 200 mg/l heptane sulphonic acid sodium salt, 500 mg/l EDTA, 2.5% w/v 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 of 3) for both dopamine and norepinephrine was 1 pg/25 μl sample.

All experiments were performed at room temperature (21–23 °C). Because single cells were superfused with drugs, only one experiment was performed per culture dish. All reported values are mean±S.D. of n cells and results are compared using Student's t-test for unpaired values, Student's t-test for paired values, or the Mann–Whitney non-parametric test where appropriate.

3. Results

3.1. 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 quantal release of catecholamines [56]. Effects of drugs known to modulate Ca^{2+}-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 saline (control, Fig. 1A, upper trace) or with drugs for a period of 15 min. At the end of this 15-min period, a second exocytotic response was evoked by high K^+ stimulation. Fig. 1A (lower trace) depicts the result of a representative experiment in which calmodulin activity was inhibited by 10 μM W7. As can be seen, the basal frequency of vesicular catecholamine release was strongly increased by application of W7. However, the frequency of exocytosis during the second high K^+ stimulation was reduced as compared to the first stimulation. Using a similar paradigm, cells were exposed to 100 nM of the phorbol ester PMA and to 100 nM staurosporine to investigate the role of PKC in exocytosis. In additional experiments, calcineurin activity was inhibited by 10 μM cyclosporin and CaM kinase II activity
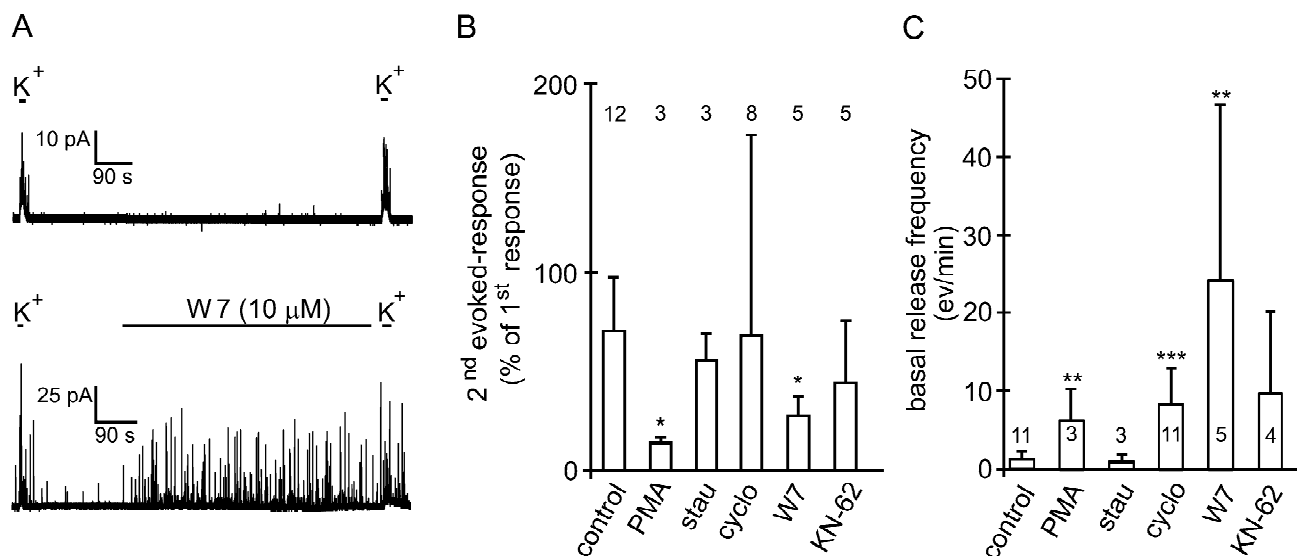

Fig. 1. Effects of modulation of PKC, calcineurin, calmodulin and CaM kinase II activity on the frequency of catecholamine-containing vesicles released from intact PC12 cells by high K^+ stimulation (A, B) and under basal conditions (A, C). (A) Amperometric recordings of basal release from intact PC12 cells superfused with saline (upper trace) and with saline containing 10 μM W7 (lower trace) 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 at the start of the experiment. (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±S.D. for the numbers of cells indicated. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

was inhibited by 10 μM KN-62. Summary graphs of the results of these experiments are shown in Fig. 1B,C. Vesicle contents during the second evoked response did not differ significantly from that during the control response recorded from the same cell (Table 1, upper part). This result shows that, within the 15-min exposure period, none of the drugs used affected vesicle contents.

In control cells, superfused with saline, the frequency of exocytotic events during the second high K^+ stimulation was reduced to $72 \pm 31\%$ (paired t-test; $P < 0.01$; $n = 12$) of that during the initial response. Therefore, drug effects were determined by comparing evoked 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. Exposure to cyclosporin, staurosporine and KN-62 did not lead to significant changes in the frequency of events evoked by the second high K^+ stimulation (Fig. 1B). However, the effect of cyclosporin on the frequency of evoked events varies strongly. In six out of eight cells tested, the frequency of evoked events was strongly reduced, on average 6.5-fold. However, the other two cells displayed a more than 3-fold enhancement. The results indicate that activation of PKC and inhibition of calmodulin activity lead, either directly or indirectly, to a change in vesicular catecholamine release during depolarization of intact PC12 cells.

The basal frequency of exocytotic events, in the absence of stimulation, was also assessed in intact cells. 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 (Fig. 1A) significantly enhanced the basal release frequency, whereas the 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 the direct effects of the drugs on the exocytotic machinery.

3.2. Modulation of exocytosis in ionomycin-permeabilized cells

Vesicular catecholamine release from ionomycin-permeabilized PC12 cells is Ca^{2+}-dependent. The threshold extracellular Ca^{2+} concentration for vesicular release was previously demonstrated to be close to 100 μM [56]. Since stimulation of permeabilized cells by superfusion with saline containing a high concentration of Ca^{2+} results in less stable responses than the high K^+-evoked responses in intact cells [56], 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

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

	Median vesicle contents during control response (zmol)	Median vesicle contents after drug exposure (% of control)	n	P
	First high K^+ response in intact cell	Second high K^+ response in intact cell		
Saline control	391 ± 132	108 ± 31	10	0.33
100 nM PMA	602 ± 325	103 ± 50	3	0.62
100 nM staurosporine	497 ± 121	109 ± 8	3	0.18
10 μM cyclosporin A	484 ± 90	90 ± 27	5	0.42
10 μM W7	506 ± 144	91 ± 22	5	0.30
10 μM KN-62	549 ± 110	93 ± 29	4	0.72
	First high K^+ response in intact cell	Pb^{2+} response in permeabilized cell		
100 nM PMA	560 ± 70	80 ± 32	3	0.42
100 nM staurosporine	614 ± 130	80 ± 16	3	0.18
10 μM cyclosporin A	417 ± 75	87 ± 8	3	0.12
10 μM W7	478 ± 213	116 ± 18	3	0.31
10 μM KN-62	356 ± 140	120 ± 61	4	0.56

Effects (right column) are relative to the median values of the contents of vesicles released during an initial depolarizing stimulus (left column) in the same cell. The upper part describes effects of 15-min superfusion of intact cells with drug-containing saline on high K^+-evoked exocytotic events. The lower part describes the vesicle contents obtained from Pb^{2+}-evoked exocytotic events after 15-min superfusion with ionomycin- and drug-containing saline. All values are mean$\pm$S.D. from the number of cells (n) analyzed. The result of a t-test for the paired observations (P) is also presented. Note that none of the drug treatments caused a significant effect on vesicle contents.

4–5-min permeabilization period. The cells were continuously superfused with 5 μM ionomycin Ca^{2+} salt added to nominal Ca^{2+}-free saline. Under these experimental conditions (i.e. ~5.4 μM Ca^{2+}), the basal release frequency in ionomycin-permeabilized PC12 cells amounted to 0.1 ± 0.1 events/min ($n = 8$). In similar experiments, ionomycin was applied simultaneously with the different drugs at the same concentrations as used in experiments on intact cells, to assess the effects of the drugs on the basal release frequency in permeabilized cells. The comparison with control cells shows that PMA and cyclosporin cause a significant increase in basal release frequency (Fig. 2A), 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 increase the basal frequency of exocytosis, and indicate that calmodulin and CaM kinase II exert indirect effects.

3.3. 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 [56]. Therefore, ionomycin-permeabilized PC12 cells were used to investigate the influence of intracellular signaling pathways on Pb^{2+}-induced exocytosis. Each of the cells was first stimulated with high K^+ saline for ~5 s to assess the responsiveness and to obtain control values for the parameters of vesicular

release. After 15 min of exposure of these cells to ionomycin in nominal Ca^{2+}-free saline, superfusion with 1 μM Pb^{2+}-containing saline results in exocytosis after a delay of 1–2 min (Fig. 3, upper trace, Fig. 4, top left panel). The frequency of vesicles released gradually increased to a maximum of 125 ± 67 events/min ($n = 15$) after ~4.5 min of superfusion with 1 μM Pb^{2+}. Pb^{2+}-induced exocytosis was also observed following exposure of cells to drugs which modulate signaling pathways simultaneously with ionomycin for 15 min. The frequency of exocytotic events recorded with a carbon fiber microelectrode generally varied 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 ($n = 4$) which caused a consistent strong decrease in the frequency of exocytosis (Fig. 3). KN-62 caused a significant decrease in the 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 (Figs. 4 and 5). Conversely, exocytosis in W7-treated cells ($n = 3$) was already consistently evident within the first 30 s of superfusion of the cells with Pb^{2+}-containing saline. Exposure of the cells to PMA ($n = 5$), to staurosporine ($n = 3$), and to cyclosporin ($n = 3$) caused neither systematic nor differential effects (Figs. 3–5). 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 median value of the contents of vesicles released during Pb^{2+}-evoked exocytosis did not differ from that of vesicles released by the same cells during high K^+ stimulation at the start of the experiment. Table 1 (lower part) demonstrates that none of the drug treatments affected the contents of vesicles released by the subsequent action of Pb^{2+}. The results
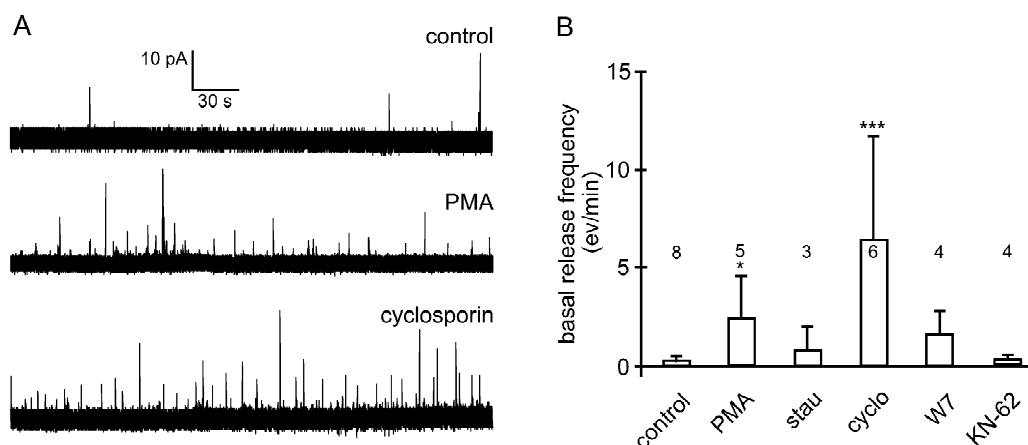

Fig. 2. Effects of modulation of PKC, calcineurin, calmodulin and CaM kinase II activity on the frequency of catecholamine-containing vesicles released from ionomycin-permeabilized PC12 cells under basal conditions. (A) Amperometric recordings from permeabilized PC12 cells continuously superfused with 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 from permeabilized PC12 cells 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±S.D. for the numbers of cells indicated. *$P < 0.05$; ***$P < 0.001$.
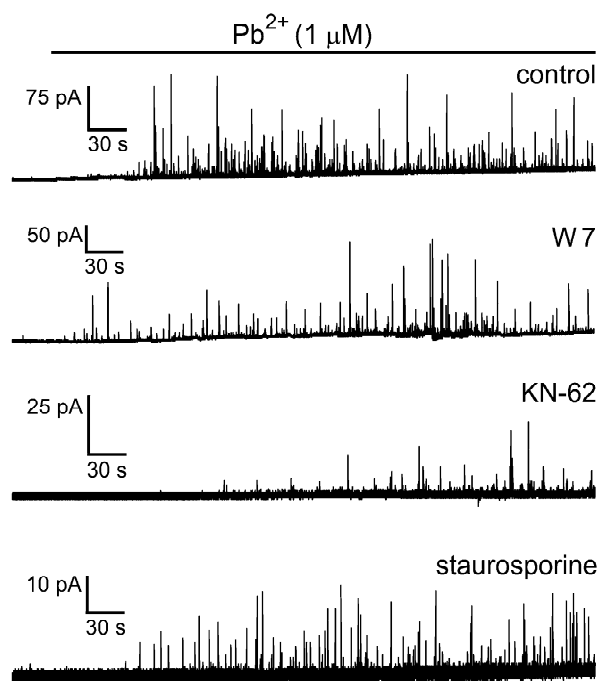
Fig. 3. Modulation of Pb^{2+}-induced vesicular catecholamine release from ionomycin-permeabilized PC12 cells by inhibitors of calmodulin, CaM kinase II and PKC. Amperometric recordings show vesicular catecholamine release induced by superfusion of the permeabilized cell with saline containing 1 μM Pb^{2+} (control) as indicated by the bar on top of the recordings and, additionally, 10 μM W7, 10 μM KN-62, and 100 nM staurosporine, as indicated. All cells were pretreated 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.

presented in Figs. 3–5 further show that Pb^{2+}-induced exocytosis is modulated by calmodulin and CaM kinase II and that Pb^{2+}-induced exocytosis is not affected by modulation of PKC and calcineurin activity.

3.4. 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, but it also shows high sensitivity to Pb^{2+} [3] 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 [42]. Catecholamine release from populations of PC12 and PC12-F7 cells was measured using HPLC with electrochemical detection (Fig. 6A) to determine the total amount of catecholamines that can be released upon stimulation. 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 (Fig. 6B). PC12 cells are highly responsive to depolarization and the majority of the cells tested responded with vesicular catecholamine release (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±44 zmol ($n = 11$) in PC12-F7 cells. Thus, PC12-F7 cells are capable of vesicular catecholamine release, albeit at a very low level. Furthermore, superfusion with Ca^{2+}-free saline containing 1 μM Pb^{2+} resulted in the detection of vesicular release after a considerable delay. As for PC12 cells, the median value of vesicle contents of PC12-F7 cells during superfusion with Pb^{2+}-containing saline did not differ from that of vesicles released during control responses evoked by high K^+ stimulation (91±33 zmol; $n = 3$; $P = 0.52$).

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.

4. 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
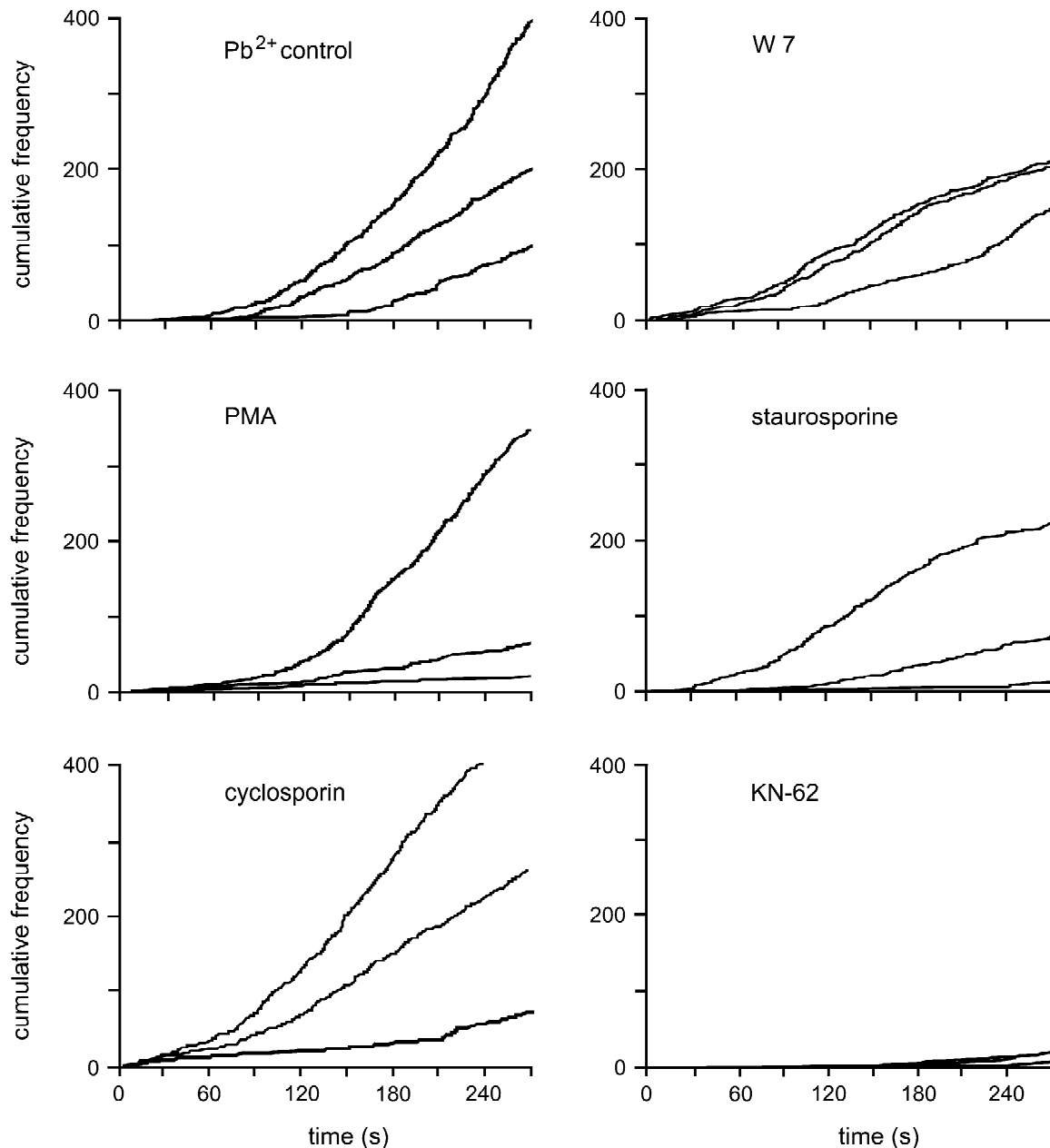
Fig. 4. Effects of drugs modulating PKC, calcineurin, calmodulin and CaM kinase II activity on the frequency of catecholamine-containing vesicles released from ionomycin-permeabilized PC12 cells superfused with saline containing 1 μM Pb^{2+}. The panels show cumulative frequencies of exocytotic events recorded following the start of superfusion with Pb^{2+}-containing saline ($t=0$) and the drugs indicated. The permeabilized cells were superfused with saline (control) or with saline containing 10 μM W7, 100 nM PMA, 100 nM staurosporine, 10 μM cyclosporin A and 10 μM KN-62 for 15 min before superfusion with saline containing 1 μM Pb^{2+} and the same concentrations of the drug. The curves, depicting release over time from different cells, show that the frequency of exocytosis varies between cells. However, the delay to the onset of Pb^{2+}-evoked release, which amounts to 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 neither resulted in systematic nor in differential modulation of the Pb^{2+}-induced exocytosis. For each experimental condition results of three representative cells are shown.

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 [13,18,36,39,40,47], indicate that uncontrolled factors play an important role in the modulatory

effects of PKC on catecholamine release, particularly in intact cells. The present results (Fig. 1B,C, Fig. 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. It is unlikely that enhanced basal catecholamine release in the presence of PMA and
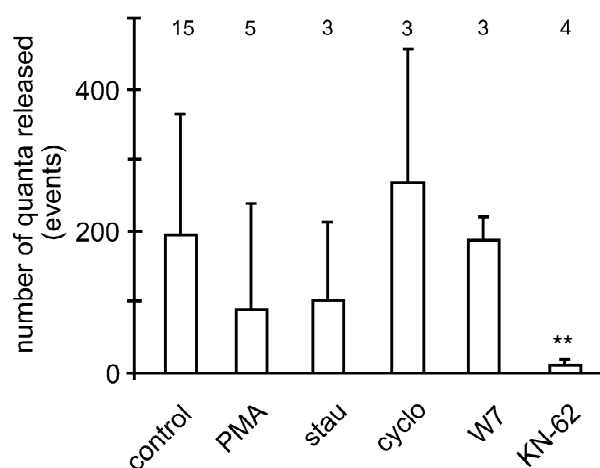
Fig. 5. Effects of drugs, known to modulate PKC, calcineurin, calmodulin, and CaM kinase II activity, on the number of quanta released from permeabilized PC12 cells during the first 270 s of superfusion with 1 μM Pb^{2+}-containing saline. The permeabilized cells were superfused with the drug indicated, at the same concentrations as used in Fig. 4, for 15 min before superfusion with saline containing 1 μM Pb^{2+} and the same concentrations of the drug. The average number of quanta released during Pb^{2+}-evoked exocytosis obtained from control cells was compared with that obtained from cells exposed to saline containing the drug and 1 μM Pb^{2+}. Note that although there is a large variation within each treatment, KN-62 treatment caused a consistent and significant decrease in the average number of quanta released. The bar diagrams show mean±S.D. for the number of cells indicated, and the result of a t-test for unpaired observations (P) is also presented. **$P < 0.01$.

cyclosporin A (Fig. 1C) leads to a significant depletion of releasable vesicles, since dexamethasone-differentiated, intact PC12 cells are capable of releasing hundreds of catecholamine-containing vesicles upon repeated depolarizing stimuli [56], as are permeabilized PC12 cells upon application of Pb^{2+} in the presence of modulators of PKC, calcineurin and calmodulin activity (Figs. 3–5). Whether the enhancement of basal exocytosis of catecholamine-containing vesicles 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 discerned from the results on intact PC12 cells. 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
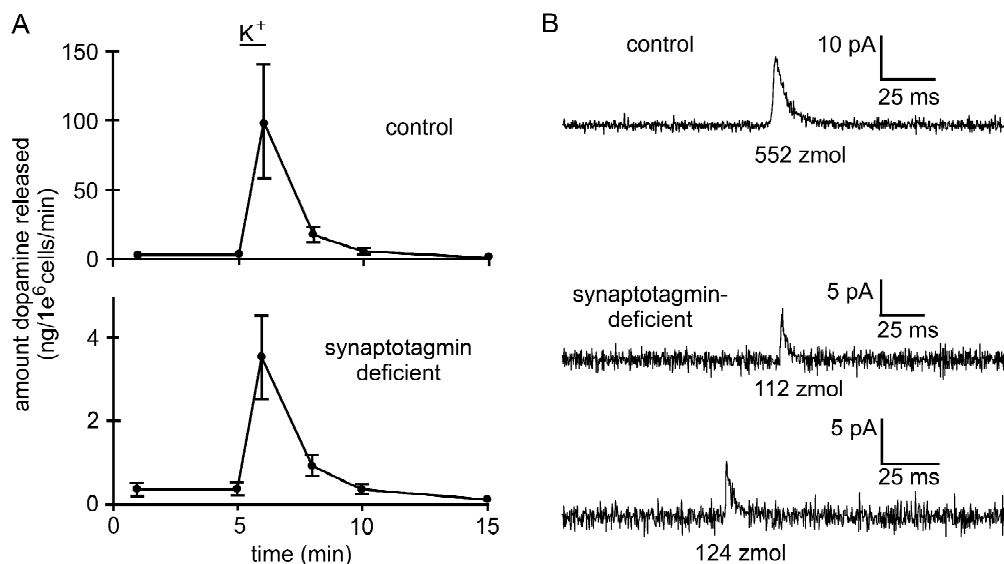

Fig. 6. Catecholamine release from PC12 cells and from synaptotagmin I- and II-deficient PC12-F7 cells. (A) Time course of dopamine secretion from cell populations determined using HPLC with electrochemical detection. Dopamine release is low under control conditions. Following a 1-min application of 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±S.D. of three 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.

[29,48]. In 'cracked' PC12 cells, PKC also enhances catecholamine release by a direct action on the exocytotic machinery [7]. 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 de-phosphorylation inhibits exocytosis downstream of Ca^{2+} influx through voltage-gated Ca^{2+} channels. The frequency of exocytosis is not affected by inhibition of PKC activity and is enhanced in the presence of inhibitors of calcineurin activity (Fig. 2). This suggests that the basal PKC activity is rather low and the basal calcineurin activity is relatively high, which would result in a low basal phosphorylation state of intracellular proteins. In contrast to PKC and calcineurin, calmodulin appears to exert primarily indirect effects on exocytosis and inhibition of CaM kinase II does not cause significant effects on the release of catechol-amine-containing vesicles from intact and permeabilized PC12 cells (Figs. 1 and 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.

The large intercellular variation in release frequency is due, at least partly, to intercellular differences in the size of the releasable pool of vesicles. However, part of the variation is inevitably due to the uncontrollable number of release sites under the surface of the carbon fiber microelectrode, resulting in the detection of a large number of released vesicles in one cell and only a moderate number of released vesicles for another cell. The influence of the number of release sites is partly compensated for by the selection of highly responsive cells. Nevertheless, this is not enough to allow for the detection of subtle effects. 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 [22,49], 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 [45], 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 [50]. 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 and 4). Since

the delay to onset of release is reduced with increasing Pb^{2+} concentration [56], 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 strong reduction of the number of vesicles released (Figs. 3–5) and to an apparent increase in the delay to onset of Pb^{2+}-evoked exocytosis (Figs. 3 and 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 [38], indicates that CaM kinase II is required for Pb^{2+}-induced vesicular catechol-amine release. KN-62 neither affects 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 mainly controlled by direct effects of CaM kinase II and calmodulin on the exocytotic machinery, 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 [8]. This is reflected in the parameters of quantal catecholamine release, showing reduced vesicle contents and a reduced frequency of vesicles released (Fig. 6). 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. 6), 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 [21,35,53].

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 instead 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 [23], synaptotagmin [52], and the t- and v-SNAREs [20,37] and CaM kinase II-induced phosphorylation is associated with an increase in the number of releasable vesicles [16]. Therefore, CaM kinase II provides a novel and plausible target for the direct intracellular action of Pb^{2+} leading to neurotransmitter release.

Acknowledgements

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