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Synaptotagmin I is a molecular target for lead

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Abstract

Lead poisoning can cause a wide range of symptoms with particularly severe clinical effects on the CNS. Lead can increase spontaneous neurotransmitter release but decrease evoked neurotransmitter release. These effects may be caused by an interaction of lead with specific molecular targets involved in neurotransmitter release. We demonstrate here that the normally calcium-dependent binding characteristics of the synaptic vesicle protein synaptotagmin I are altered by lead. Nanomolar concentrations of lead induce the interaction of synaptotagmin I with phospholipid liposomes. The C2A domain of synaptotagmin I is required for lead-mediated phospholipid binding. Lead protects both recombinant and endogenous rat brain synaptotagmin I from proteolytic cleavage in a manner similar to calcium. However, lead is unable to promote the interaction of either recombinant or endogenous synaptotagmin I and syntaxin. Finally, nanomolar concentrations of lead are able to directly compete with and inhibit the ability of micromolar concentrations of calcium to induce the interaction of synaptotagmin I and syntaxin. Based on these findings, we conclude that synaptotagmin I may be an important, physiologically relevant target of lead.

Keywords: calcium, metal, phospholipid, poisoning, syntaxin.

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Human exposure to lead (Pb^{2+}), especially at a young age, adversely affects a range of bodily systems including the reproductive, nervous, gastrointestinal, immune, renal, cardiovascular, skeletal, muscular and hematopoetic systems (Silbergeld 1992; Landrigan and Todd 1994; Finkelstein *et al*. 1998). In particular, the CNS is sensitive to Pb^{2+}. Symptoms can include learning disorders, IQ reduction, hyperactive behavior, ataxia and convulsions (Needleman *et al*. 1990; Bressler and Goldstein 1991; Finkelstein *et al*. 1998). According to the Centers for Disease Control, a blood Pb^{2+} level of 10 μg/dL is 'concerning' (Centers for Disease Control 1991, Centers for Disease Control and Prevention. 1997). Approximately 8.9% of children in the United States alone exceed that threshold (Goldstein 1992; Needleman 1994, 1998; Bressler *et al*. 1999) making Pb^{2+} one of the most widely prevalent environmental causes of preventable neurological disorders (Landrigan and Todd 1994).

The clinical effects of Pb^{2+} on the CNS may be due in part to its ability to alter neuronal activity. Pb^{2+} decreases evoked and increases spontaneous neurotransmitter release in a variety of neuronal preparations including synaptosomes, cell cultures and brain slices (Manalis and Cooper 1973; Carroll *et al*. 1977; Suszkiw *et al*. 1984; Tomsig and Suszkiw 1993; Bielarczyk *et al*. 1994; Struzynska and Rafalowska 1994; Bressler *et al*. 1996; Bressler *et al*. 1999). These effects are presynaptic (Manalis and Cooper 1973) and have been observed in dopaminergic (Minnema *et al*. 1986), GABAergic (Minnema and Michaelson 1986), glutamatergic (Busselberg *et al*. 1994; Guilarte 1997a; Guilarte 1997b; Guilarte and McGlothan 1998; Ma *et al*. 1998; Savolainen *et al*. 1998) and cholinergic (Manalis *et al*. 1984; Minnema *et al*. 1988) systems.

The molecular basis for the effects of Pb^{2+} on neurotransmitter release remains poorly understood. It is thought that Pb^{2+} may alter normal synaptic activity by blocking presynaptic calcium (Ca^{2+}) channels (Nachshen 1984; Suszkiw *et al*. 1984; Wang and Quastel 1991; Audesirk 1993; Audesirk and Audesirk 1993; Busselberg

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Abbreviations used: *N*-BIDA, *N*-benzyiminodiacetic acid; GST, glutathione *S*-transferase; NTA, nitrilotriacetic acid; PC, phosphatidylcholine; PKC, protein kinase C; PS, phosphatidylserine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

et al. 1993). While this may explain a decrease in evoked release, it does not account for the increase in spontaneous release induced by Pb^{2+}. It has also been suggested that Pb^{2+} may affect spontaneous release by altering the intracellular buffering and sequestering of Ca^{2+}, increasing internal free concentrations of Ca^{2+} and therefore increasing spontaneous release (Minnema *et al*. 1986; Cooper *et al*. 1984). Alternatively, it has been suggested that Pb^{2+} may mimic Ca^{2+} on specific Ca^{2+}-binding proteins that regulate neurotransmitter release (Pounds 1984; Goering 1993; Simons 1993). For example, Pb^{2+} binds and alters the activity of Ca^{2+} binding proteins such as calmodulin (Goldstein and Ar 1983) and protein kinase C (PKC) (Markovac and Goldstein 1988a; Markovac and Goldstein 1988b).

PKC regulates numerous cellular processes by phosphorylating a range of proteins in response to binding Ca^{2+} and phospholipid. PKC contains four constant (C) domains. The second, C2, domain promotes Ca^{2+}-dependent binding of PKC to phospholipid (Luo and Weinstein 1993). Pb^{2+} activates PKC in part via the C2 domain (Tomsig and Suszkiw 1995; Sun *et al*. 1999). PKC can indirectly modulate neurotransmitter release (Majewski and Iannazzo 1998) by increasing the size of the readily releasable pool of vesicles (Gillis *et al*. 1996). Tomsig and Suszkiw (1990) have suggested that Pb^{2+} directly interacts with an intracellular target as a Ca^{2+} mimic in order to cause aberrant neurotransmitter release. They subsequently provided evidence (Tomsig and Suszkiw 1993) that neither PKC nor calmodulin were that target based on the specific inhibition of these proteins, with staurosporine and calmidazolium, respectively, in Pb^{2+}-induced neurotransmitter release assays. Numerous C2 domain and other Ca^{2+}-binding proteins besides PKC and calmodulin have been identified (Nalefski and Falke 1996; Rizo and Südhof 1998). Some of these proteins are localized to the synaptic terminal and are directly involved in the regulation of neurotransmitter release.

We hypothesized that Pb^{2+} binds to and alters the binding characteristics of one of these proteins, synaptotagmin I. Synaptotagmin I is a C2 domain-containing protein that is localized to synaptic vesicles (Südhof and Rizo 1996). Synaptotagmin I has a single transmembrane domain and a short intravesicular N-terminus. The majority of its cytoplasmic portion consists of two C2 domains (C2A and C2B) that are homologous to the C2 domain of PKC (Südhof and Rizo 1996). Synaptotagmin I interacts with the plasma membrane protein syntaxin (Chapman *et al*. 1995; Kee and Scheller 1996; Shao *et al*. 1997) and negatively charged phospholipids such as phosphatidylserine and phosphatidylcholine (Chapman and Jahn 1994). These interactions occur in a Ca^{2+} dependent manner via the C2A domain of synaptotagmin I.

We studied synaptotagmin I as a target of Pb^{2+} because of four prior observations. First, Pb^{2+} can alter neurotransmitter release by increasing spontaneous release and decreasing evoked release. Second, Pb^{2+} is known to potently activate PKC. Third, synaptotagmin I contains two C2 domains each of which are homologous to the C2 domain of PKC. Fourth, synaptotagmin I plays an integral role in neurotransmitter release. As evidence of this final observation, a number of synaptotagmin I knockout and transgenic animals have been produced in species including *M. musculus* (Geppert *et al*. 1994; Neher and Penner 1994), *D. melanogaster* (DiAntonio *et al*. 1993; Littleton *et al*. 1993; Broadie *et al*. 1994; DiAntonio and Schwarz 1994; Littleton *et al*. 1994) and *C. elegans* (Nonet *et al*. 1993). Disruption of synaptotagmin I in these animal models causes a decrease in evoked release and sometimes causes an increase in spontaneous release (Littleton *et al*. 1993; Broadie *et al*. 1994; DiAntonio and Schwarz 1994; Littleton *et al*. 1994).

We have compared the effects of Ca^{2+} vs. Pb^{2+} on endogenous and recombinant synaptotagmin I protein in phospholipid binding assays, protease protection assays, terbium (Tb^{3+}) binding assays and synaptotagmin I/syntaxin co-immunoprecipitations. Our experiments suggest that Pb^{2+} can partially mimic the effects of Ca^{2+} on synaptotagmin I and that it can induce effects on the binding characteristics of synaptotagmin I at nanomolar concentrations indicating that synaptotagmin I is a physiologically relevant target of Pb^{2+}. We demonstrate that there is a greater affinity of Pb^{2+} vs. Ca^{2+} for synaptotagmin I, that Pb^{2+} has potent effects on synaptotagmin I/phospholipid binding, but that Pb^{2+} is unable to induce the binding of synaptotagmin I to syntaxin. These effects of Pb^{2+} on the binding characteristics of synaptotagmin I may be important components of the effects of Pb^{2+} on neurotransmitter release.

Experimental procedures

Recombinant proteins

cDNA clones encoding both full and partial portions of the cytoplasmic domain of rat synaptotagmin I (synaptotagmin I 1–5 and 1–3) and syntaxin fused to a glutathione *S*-transferase (GST) tag were obtained from the laboratory of Dr Richard Scheller (Kee and Scheller 1996). Sequencing of synaptotagmin I 1–5 nucleotide sequence identified two inconsistencies with published synaptotagmin I sequence. Published sequence (Perin *et al*. 1990; Li *et al*. 1995) identifies an aspartate encoded by nucleotide sequence 5′-GAC-3′ at amino acid residue 374 while our clone encodes a glycine with nucleotide sequence 5′-GGC-3′ at the same site. As an independent confirmation, we verified by sequence analysis that a fragment of synaptotagmin I cDNA that was PCR-amplified from rat brain cDNA also coded for a glycine (5′-GGC-3′) at amino acid 374 (data not shown) as previously shown (Davis *et al*. 1999; Osborne *et al*. 1999). The second nucleotide in our sequence which did not match reported sequence was 5′-ACT-3′ encoding a threonine at position 367, as opposed to a published isoleucine with

nucleotide sequence 5′-ATT-3′. Both of these differences may reflect sequence polymorphisms.

Two mutant constructs of the synaptotagmin I 1–5 clone were produced with the Quikchange Mutagenesis kit (Stratagene, La Jolla, CA, USA). One mutant was constructed with a serine at amino acid position 230 (D230S). The sequences of the two HPLC-purified primers used to produce this mutant were: 5′-GGTGATGGCTGTGTATAGCTTTGATCGCTTCTCC-3′ and 5′-GGAGAAGCGATCAAAGCTATACACAGCCATCACC-3′. The other mutant construct contained a lysine at amino acid position 230 (D230K). The sequences of the two HPLC-purified primers used to produce this mutant were 5′-GGTGATGGCTGTG-TATAAGTTTGATCGCTTCTCC-3′ and 5′-GGAGAAGCGAT-CAAACTTATACACAGCCATCACC-3′. The mouse anti-rat monoclonal antibody ASV-48 (gift of Dr Richard Mains) detected both the D230S and the D230K mutant constructs of synaptotagmin I 1–5 by western blotting (data not shown).

Preparation of recombinant proteins was by growth and lysis of *Escherichia coli* bacteria containing wild-type and mutant constructs followed by precipitation of proteins with glutathione sepharose beads (Pharmacia, Piscataway, NJ, USA). Thrombin cleavage for one hour at room temperature was used to dissociate proteins from their GST tags. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie blue staining were used to confirm the size, purity and concentration of the proteins.

Phospholipid binding assays

Tritiated phospholipid liposomes composed of lipid bilayers were prepared as previously described (Davletov and Südhof 1993; Chapman and Jahn 1994). Briefly, brain phosphatidylserine (PS) (Avanti Polar Lipids, Alabaster, AL, USA), brain phosphatidylcholine (PC) (Avanti Polar Lipids) and [^{3}H]PC (Amersham) were stored in chloroform at −80°C. Glass capillary tubes were used for measurement of appropriate volumes of phospholipid. Seventy-five per cent PC, 25% PS and approximately 0.1 mCi/mL of [^{3}H]PC were mixed and dried under a stream of nitrogen gas. The dried lipid mixture was resuspended in Buffer A (50 mM HEPES, 150 mM NaCl, pH 7.4) and probe sonicated for 30 s. Centrifugation for 1 min at 5000 *g* precipitated large phospholipid aggregates, yielding a supernatant containing a suspension of liposomes.

Phospholipid binding assays were performed as described (Chapman and Jahn 1994; Davletov and Südhof 1993). Chelator types and concentrations were calculated based on chelator–cation dissociation constants from Martell and Smith (1989) with the software program 'Chelator' (Schoenmakers *et al*. 1992). Chelators used were EGTA, nitrilotriacetic acid (NTA) and *N*-benzyiminodiacetic acid (*N*-BIDA). Phospholipid liposomes, synaptotagmin I 1–5, ASV-48 mouse anti-rat monoclonal synaptotagmin I antibody, Protein-G Sepharose (Pharmacia) and calculated concentrations of chelator and CaOAc or PbOAc were brought to a total volume of 100 μL with Buffer A. Reactions were incubated for 1 h at 25°C. Samples were washed three times in 500 μL/wash with incubation buffer and then re-suspended in Ecolite scintillation fluid. Levels of radioactivity in each sample were determined by liquid scintillation spectroscopy. Curves were fitted to the data with the equation

$$R = (R_{max} \times [M])/(EC_{50} + [M])$$

as previously described by Tomsig and Suszkiw (1995). R_{max} is the maximum amount of phospholipid precipitated, [M] is the concentration of metal and EC_{50} is the concentration of metal at which binding is half-maximal. The amount of radioactive isotope in 10 μL of labeled phospholipid was measured for each experiment. All experiments were performed in triplicate. In order to normalize our data, background levels of radioactivity (amount of radioactivity precipitated by synaptotagmin I 1–5 protein in the absence of cations) were subtracted from all measurements. Then, the amount of radioactivity precipitated in each condition was multiplied by 10 000 and divided by the total amount of radioactivity present in 10 μL of phospholipid liposomes.

Terbium metal binding assay

Recombinant synaptotagmin I 1–5 GST-fusion protein was purified from bacterial lysate using glutathione sepharose affinity chromatography followed by the addition of thrombin protease to cleave the protein from the GST tag. Pure synaptotagmin I 1–5 protein was obtained by successive fractionation on a Superdex-75 gel filtration column equilibrated with 100 mM *N*,*N*-*bis*[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES) (pH 7.4), 200 mM NaCl and 2 mM EDTA (Sutton *et al*. 1995). Synaptotagmin I 1–5 protein was judged to be > 95% pure when visualized on a 15% SDS–PAGE gel. The purified protein was exhaustively dialyzed against EDTA (2 mM) to remove metal ions and then into 10 mM Bis-Tris (pH 7.0) and 100 mM KCl. Bis-Tris was selected as a buffer because it weakly chelates Pb^{2+} ($\log_\beta = 4.3$) (Scheller *et al*. 1980) and hence helps to prevent precipitation of Pb^{2+} hydroxides. In this way, the Bis-Tris mimics the natural metal-buffering role of glutathione in cells (Vallee and Wacker 1970; Kagi and Hapke 1984; Payne *et al*. 1999). Amount of protein used was standardized by determination of the extinction coefficient (280 = 29.5 mM −1 cm − 1)(Keck Biophysical Facility, Yale University). Tb^{3+} binding titrations were performed according to the method developed by Horrocks and Sudnick (1981) that has been used to determine the affinity of Pb^{2+} for the C2 domain of PKC (Walters and Johnson 1990). Spectra were recorded by exciting the protein at 280 nm and measuring the fluorescence from bound Tb^{3+} over the range of 470–570 nm in 1 nm increments with a 2-s integration time. Slit widths of 2 mm were used on the excitation and emission channels that corresponded to a bandwidth of 10 nm full-width at half maximum. A 455-nm cut-on filter was placed in the emission channel to block scattered light from the detector. At the end of representative titrations, the sample was centrifuged and the absorbance spectrum of the filtrate was checked to ensure that no protein precipitation had occured during the course of the titration. Fluorescence measurements were recorded with an ISS PC1 fluorimeter equipped with a cell holder thermostated at 25°C and a computer-controlled autotitrator in the Keck Biophysics Facility, North western University. We further confirmed that 0.5 mM Pb^{2+} did not alter the secondary structure of synaptotagmin I by performing circular dichroism measurements (wavelength = 210 nm to 300 nm; Jasco J715 spectropolarimeter) using 1.6 μg/μL protein in 10 mM Tris HCl (pH 8), 25 mM NaCl at 20°C (data not shown).

Synaptotagmin I/syntaxin co-immunoprecipitation

Co-immunoprecipitations of endogenous synaptotagmin I and syntaxin were performed in a manner similar to those previously

described (Chapman *et al*. 1995). One adult rat brain was homogenized in 10 mL of Buffer A containing 1% Triton-X-100 (Fisher Scientific, Pittsburgh, PA, USA) and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 5 μg/mL leupeptin and 5 μg/mL pepstatin A) with a Dounce homogenizer. The detergent-solubilized, cytosolic fraction was isolated by differential centrifugation for 15 min at 10 000 *g* with a JA-20 rotor in a Beckmann J2–21 centrifuge and then for 15 min in a TLA 120.2 rotor at 100 000 *g* in a Beckmann Optima TLX Ultracentrifuge. The sample was dialyzed in Spectra/Por (MWCO 6–8000) dialysis tubing for 48 h against two successive volumes of 2 L Buffer A. 5 μL of the mouse anti-rat monoclonal syntaxin antibody HPC-1 (Sigma, St Louis, MO, USA) or 5 μL of the mouse anti-rat monoclonal Golgi-localized 58K protein antibody were incubated with a bead volume of 20 μL Protein-G sepharose (Pharmacia) on ice for 30 min and subsequently with 300 μL of the dialyzed, detergent solubilized cytosolic fraction of rat brain homogenate in the presence of EGTA, Mg^{2+}, Ca^{2+} or Pb^{2+}. Samples were incubated for 3 h on ice. Three 500 μL washes were performed with incubation buffer. Following the final wash, sepharose beads were re-suspended in 10 μL of protein loading buffer, boiled for 10 min at 95°C and electrophoresed on SDS–PAGE. Transfer of protein to nitrocellulose membrane was performed for 3 h at 50 V. Ponceau S staining was used to confirm protein transfer and compare protein loading. The nitrocellulose membrane was rinsed in phosphate buffered saline with 0.1% Tween-20 (PBS-T) and then blocked for 20 min in 5% non-fat dry milk in PBS-T. Incubation with 1 : 1000 rabbit anti-rat polyclonal synaptotagmin I antibody VAS-SV008 (Stressgen, Collegeville, PA, USA) for 2 h at room temperature was followed by three 10 min washes in PBS-T. Incubation with protein-A peroxidase (Pierce, Rockford, IL, USA) conjugated secondary at 1 : 1000 or goat anti-rabbit peroxidase conjugated secondary (Jackson Immuno-research) at 1 : 10 000 for 1 h at room temperature was followed by three 10 min washes in PBS-T. Immersion in the chemiluminescent substrate ECL (Amersham, Pharmacia Biotech, Piscataway, NJ, USA) was followed by exposure to Kodak Biomax MR film and subsequent film development.

Co-immunoprecipitations of recombinant synaptotagmin I 1–5 and syntaxin were performed in a manner similar to those previously described (Kee and Scheller 1996). 0.5 μg of the synaptotagmin I 1–5 and syntaxin proteins were combined with calculated volumes of chelator, cation(s), 20 μL bead volume of Protein-A sepharose beads and 2 μL of the rabbit polyclonal syntaxin antibody VAP-SV064 (Stressgen) in a total volume of 500 μL Buffer A. Reactions were incubated for 1 h on ice and subsequently washed three times with 500 μL of incubation buffer. The reactions were electrophoresed and western blotted as described above except that 1 : 1000 of the mouse anti-rat monoclonal synaptotagmin I antibody ASV-48 and 1 : 5000 of the goat anti-mouse antibody (Jackson Immuno-Research, West Grove, PA, USA) were used as the primary and secondary antibodies, respectively.

Gel films were digitized by scanning and saved as TIFF files. Intensity values for protein bands were calculated using the software package Scion Image (http://www.scioncorp.com). In order to standardize intensity values between experiments, the intensity for each synaptotagmin I band in a given experiment was multiplied by the average intensity for the syntaxin protein bands in that experiment (data not shown). In order to normalize the synaptotagmin I signal in Figs 8 and 9, the standardized synaptotagmin intensity values were divided by the intensity of each corresponding syntaxin protein band.

Protease protection assays

Protease protection assays of endogenous synaptotagmin I were performed as previously described (Davletov and Südhof 1994). Briefly, one adult rat brain was homogenized in 10 mL Buffer A containing 1% Triton-X-100 (Fisher) and 1 mM EGTA with a Dounce homogenizer. The cytosolic fraction of the homogenate was isolated by centrifugation as described above. Trypsin (5 μg/mL; Sigma) was added to samples and incubated at room temperature in the presence of approximate free concentrations of 1 mM Mg^{2+}, 11 μM Ca^{2+} and 2 μM Pb^{2+}. Digestions were stopped by addition of 5 μL of protein loading buffer, followed by boiling for 10 min at 95°C. The reactions were electrophoresed and western blotted as described above using 1 : 1000 of the mouse anti-rat monoclonal synaptotagmin I antibody ASV-48 and 1 : 5000 of the goat anti-mouse antibody (Jackson Immuno-Research) as the primary and secondary antibodies, respectively.

Protease protection assay of recombinant synaptotagmin I 1–3 was performed in a manner similar to that previously described (Davletov and Südhof 1994). Synaptotagmin I 1–3 (26 ng/μL) were combined with 66 ng/μL trypsin (Sigma), Buffer A and calculated amounts of chelator and cation in a total volume of 15 μL. Reactions were incubated at room temperature for 2 h and stopped by addition of 5 μL of protein loading buffer, followed by boiling for 10 min at 95°C. The reactions were electrophoresed and western blotted as described above using 1 : 1000 of the mouse anti-rat monoclonal synaptotagmin I antibody ASV-48 and 1 : 5000 of the goat anti-mouse antibody (Jackson Immuno-Research) as the primary and secondary antibodies, respectively.

Results

Pb^{2+} induces binding of synaptotagmin I to phospholipid

We employed an *in vitro* phospholipid-binding assay to compare the effects of Pb^{2+} vs. Ca^{2+} on the recombinant synaptotagmin I 1–5 protein. [^{3}H]PC/PS/PC phospholipid liposomes were incubated with synaptotagmin I 1–5 protein and chelated concentrations of Ca^{2+} or Pb^{2+}. The Ca^{2+} concentration used in initial experiments (85 μM) was chosen based on doses used in a previous study (Davletov and Südhof 1993), while the Pb^{2+} concentration (6 nM) was chosen based on the effects of lead on PKC (Markovac and Goldstein 1988a). In the presence of synaptotagmin I 1–5, [^{3}H]-phospholipid liposomes were precipitated in the presence of either 85 μM Ca^{2+} or 6 nM Pb^{2+} (Fig. 1), while phospholipids were not precipitated in the absence of synaptotagmin I 1–5 under any cation condition (Fig. 1). This suggests that the cation-dependent precipitation of phospholipid liposomes requires the synaptotagmin I 1–5 protein.

Binding of synaptotagmin I 1–5 to [^{3}H]-phospholipid liposomes occured in the presence of Ca^{2+} with a

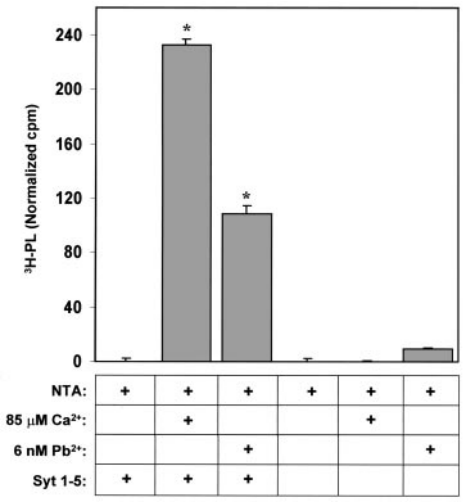

Fig. 1 Ca^{2+} and Pb^{2+} promote synaptotagmin I 1–5 binding to phospholipid. Synaptotagmin I 1–5 co-immunoprecipitates with [^{3}H]-phospholipid liposomes in the presence of Pb^{2+} (6 nM) or Ca^{2+} (85 μM) but not in the absence of cation (1 mM NTA). In the absence of synaptotagmin I 1–5, [^{3}H]-phospholipid liposomes are not immunoprecipitated under any cation condition. Asterisks (*) denote a significant effect (two-tailed Student's *t*-test: $p < 0.005$) relative to no cation condition. Normalized cpm values (*y*-axis) were calculated by subtracting background levels of precipitated phospholipid and then dividing by the total radioactivity present in 10 μL of [^{3}H]-phospholipid liposomes. Results are typical of an experiment performed three independent times, each time in triplicate. Error bars represent standard error of the mean [i.e. (standard deviation of the mean)/($\sqrt{n}$) where *n* is the number of samples].

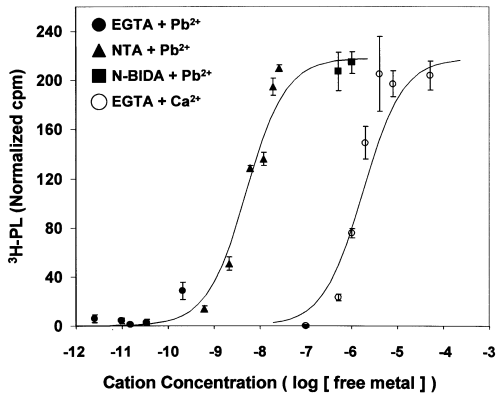

Fig. 2 Dose responses of synaptotagmin I 1–5/phospholipid binding in the presence of Ca^{2+} or Pb^{2+}. Dose response of synaptotagmin I 1–5 binding to [^{3}H]-phospholipid liposomes in the presence of increasing chelated concentrations of either Ca^{2+} or Pb^{2+} (*x*-axis). Chelators used to achieve specific cation concentrations are indicated by the symbols used at each point. Solid symbols represent Pb^{2+} concentrations. Open symbols represent Ca^{2+} concentrations (○). Circles (●) indicate the use of EGTA, triangles (▲) indicate the use of NTA and squares (■) represent the use of *N*-BIDA. Curves were generated by fitting the data to the equation $R = R_{max}*[M]/(EC50 + [M])$ according to the method of Tomsig and Suszkiw (1995) as described in the text. Normalized cpm values (*y*-axis) were calculated by subtracting background levels of precipitated phospholipid and then dividing by the total radioactivity present in 10 μL of [^{3}H]-phospholipid liposomes. Results are typical of an experiment performed three independent times, each time in triplicate. Error bars represent standard error of the mean.

half-maximal cation effect (EC_{50}) at a concentration of approximately 5 μM Ca^{2+} (Fig. 2). This result is consistent with previous reports of an EC_{50} of 3–6 μM Ca^{2+} (Davletov and Südhof 1993; Li *et al*. 1995). We measured the binding of synaptotagmin I 1–5 to [^{3}H]-phospholipid liposomes in the presence of a range of concentrations of Pb^{2+} (Fig. 2). Pb^{2+} induced binding of synaptotagmin I 1–5 to phospholipid at subnanomolar concentrations, with half-maximal binding at approximately 8 nM and binding saturation at approximately 10 μM. Thus, the promotion of synaptotagmin I 1–5 binding to phospholipid by Pb^{2+} is nearly 1000 times more potent than Ca^{2+}. At the EC_{50} of each cation, comparable amounts of synaptotagmin I/phospholipid binding were detected with either Ca^{2+} or Pb^{2+} (Fig. 2).

To test whether Pb^{2+} activates synaptotagmin I by interacting with the Ca^{2+} binding region of the C2A domain, we performed site-directed mutagenesis of the aspartate 230 (D230) residue, converting it to either a serine (D230S) or a lysine (D230K). Previous NMR, X-ray crystallography and thermal stability studies on synaptotagmin I (Shao *et al*. 1996; von Poser *et al*. 1997; Ubach *et al*. 1998) have demonstrated an integral role for D230 in the binding of Ca^{2+}. Also, synaptotagmins IV and XI constitute a subclass of synaptotagmin isoforms that do not bind phospholipid in a Ca^{2+}-dependent manner due to the substitution of a serine for an aspartate at the 230 position (von Poser *et al*. 1997) in those proteins. Therefore the D230S mutation mimics a Ca^{2+}-independent but otherwise functional protein. We constructed the D230K mutant to abolish the ability of the C2A domain to bind positively charged cations. In [^{3}H]-phospholipid binding assays, wild-type synaptotagmin I 1–5 protein provided typical results in response to both 85 μM Ca^{2+} and 6 nM Pb^{2+}. However, the D230S protein displayed a reduced and non-significant response to both 85 μM Ca^{2+} and 6 nM Pb^{2+} (Fig. 3). The D230K protein was unable to bind phospholipid in response to either 85 μM Ca^{2+} or 6 nM Pb^{2+} (Fig. 3). From previous experiments we determined that the ASV-48 antibody used to precipitate synaptotagmin I proteins in these experiments was able to recognize both of the mutant proteins. This finding ruled out the possibility that the mutant proteins were not precipitated by the antibody during the experiment. These data suggest that single amino acid substitutions in the C2A domain of the synaptotagmin I 1–5 protein caused reductions in cation-dependent phospholipid binding.

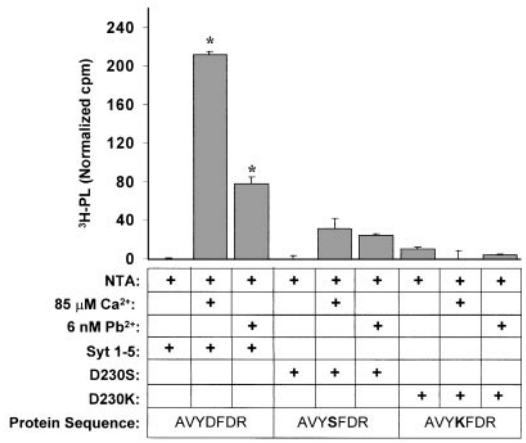

Fig. 3 Mutagenesis of aspartate 230 diminishes interaction of synaptotagmin I 1–5 with phospholipid in the presence of both Ca^{2+} and Pb^{2+}. Site-directed mutagenesis of aspartate 230 in the C2A domain of synaptotagmin I 1–5 to a serine (D230S) reduces the ability of 85 μM Ca^{2+} or 6 nM Pb^{2+} to promote binding to [^{3}H]-phospholipid liposomes. Mutagenesis of the aspartate 230 to a lysine (D230K) eliminates binding to phospholipids. Partial amino acid sequences indicating mutations are represented in 'Protein Sequence.' Asterisks (*) denote a significant effect (two-tailed Student's *t*-test: $p < 0.005$) relative to no cation condition (1 mM NTA). Normalized cpm values (*y*-axis) were calculated by subtracting out background levels of precipitated phospholipid and then dividing by the total radioactivity present in 10 μL of [^{3}H]-phospholipid liposomes. Results are typical of an experiment performed three independent times, each time in triplicate. Error bars represent standard error of the mean.

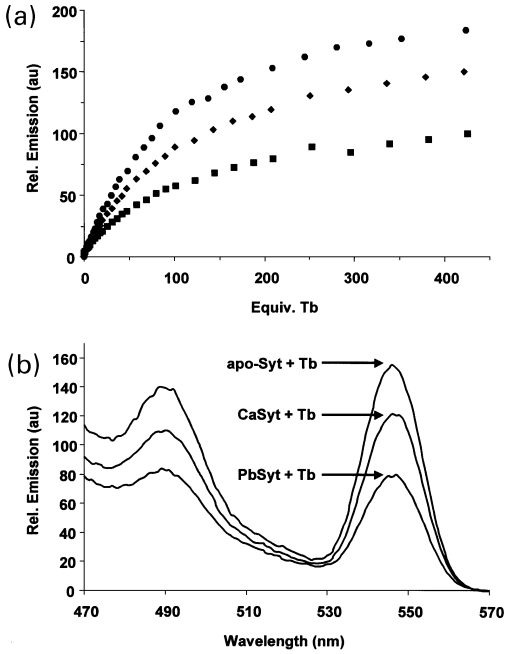

Fig. 4 Competition experiments with the luminescent reporter ion Tb^{3+} reveal that synaptotagmin I 1–5 binds Pb^{2+} more tightly than Ca^{2+}. (a) Progress of the addition of Tb^{3+} to solutions of synaptotagmin I 1–5 (5 μM) in the presence of either no added metal (●), Ca^{2+} (1.95 mM, ◆) or Pb^{2+} (1.95 mM, ■) monitored by the luminescent emission from bound Tb^{3+} at 545 nm. (b) Fluorescence spectra of synaptotagmin I 1–5 with approximately 210 equivalents Tb^{3+} and with no competing metal ion (apo-Syt + Tb), with 400 equivalents Ca^{2+} (CaSyt + Tb), and with 404 equivalents Pb^{2+} (PbSyt + Tb). The protein solution is irradiated at 280 nm (10 nm FWHM) in 10 mM BisTris (pH 7.0), 100 mM KCl.

Furthermore, they suggest that the D230 residue is critical for the binding of Pb^{2+} just as it is critical for the binding of Ca^{2+}. Therefore, we conclude that Pb^{2+} interacts with the same site on the C2A domain of synaptotagmin I that Ca^{2+} interacts with in order to induce phospholipid binding.

Pb^{2+} binds more tightly than Ca^{2+} to synaptotagmin I

We employed competition-binding experiments using Tb^{3+} to study the relative affinities of Pb^{2+} and Ca^{2+} for recombinant rat synaptotagmin I 1–5 and to assess whether Pb^{2+} binds to the same site as Ca^{2+}. The ability of Tb^{3+} to displace Ca^{2+} or Pb^{2+} from synaptotagmin I 1–5 was monitored by the fluorescence emission intensity of Tb^{3+} bound to synaptotagmin I 1–5 (545 nm) when a Tb^{3+} stock solution was added to solutions containing synaptotagmin I 1–5 and either Ca^{2+} or Pb^{2+} (Fig. 4a). More Tb^{3+} was required to displace Pb^{2+} than Ca^{2+}, suggesting that Pb^{2+} binds more tightly than Ca^{2+} to synaptotagmin I 1–5. The greater affinity of Pb^{2+} for synaptotagmin I 1–5 was evident both in the titration curves (Fig. 4a) and in the amount of bound Tb^{3+} observed at the endpoint of each titration. When Tb^{3+} was added to two solutions containing synaptotagmin I 1–5 and equal amounts of either Pb^{2+} or Ca^{2+}, less Tb^{3+} bound to synaptotagmin I 1–5 in the presence of Pb^{2+} (Fig. 4b). Although these data could also be explained by non-competitive binding between Tb^{3+}, Pb^{2+}, and Ca^{2+}, the simplest explanation is that all three metals bind to the same site(s). This suggestion is consistent with the observation that Pb^{2+} binds to the high affinity Ca^{2+}-binding site of C2A when Pb^{2+} is soaked into crystals of synaptotagmin I (Sutton *et al.* 1995; Sutton *et al.* 1999; Sutton RB, personal communication) and with the mutagenesis studies reported herein (Fig. 3).

Pb^{2+} protects a fragment of synaptotagmin I from tryptic proteolysis

Ca^{2+} induces a structural stabilization upon binding to the C2A domain of synaptotagmin I (Rizo and Südhof 1998). This structural stabilization may explain the ability of Ca^{2+} to protect a ~32 kDa fragment of synaptotagmin I from tryptic proteolysis (Davletov and Südhof 1994). We tested whether Pb^{2+} as compared to Ca^{2+} can protect both recombinant and endogenous synaptotagmin I from tryptic proteolysis.

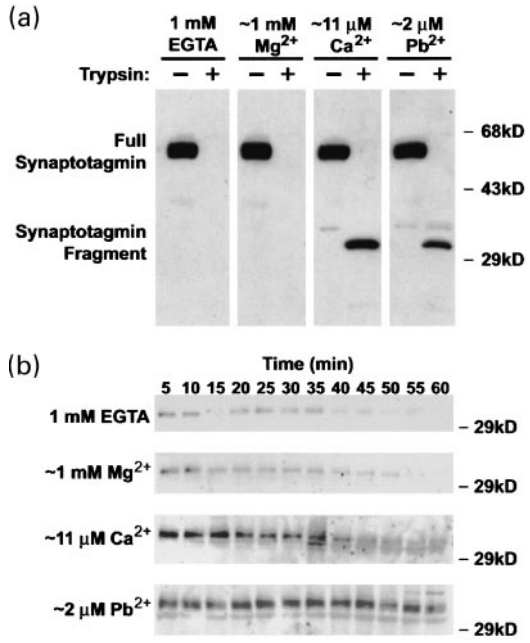

Fig. 5 Pb^{2+} and Ca^{2+} protect a fragment of endogenous rat brain synaptotagmin I from proteolytic cleavage. (a) Western blot of synaptotagmin I protein detected in rat brain homogenate treated with or without trypsin (5 μg/mL, 30 min, 22°C) in the presence of approximate free metal concentrations of either 11 μM Ca^{2+} or 2 μM Pb^{2+}. Brain samples treated with either Ca^{2+} or Pb^{2+} contain a fragment of synaptotagmin I which is the same size in both conditions (~ 32 kDa). Synaptotagmin I is completely degraded in the EGTA and 1 mM Mg^{2+} conditions prohibiting the detection of any protein. Reactions were stopped with the addition of sample buffer, boiled (10 min, 95°C), electrophoresed and western blotted. (b) Cation conditions in the reactions were the same as in (a). An equivalent amount of trypsin was added to all reactions. At 5 min time points reactions were stopped by the addition of sample buffer, boiled (10 min, 95°C), electrophoresed and western blotted. The ~ 32 kDa fragment of synaptotagmin I is shown for each time point. 11 μM Ca^{2+} and 2 μM Pb^{2+} protect the fragment longer than do 1 mM EGTA or 1 mM Mg^{2+}. Results are typical of an experiment performed three independent times.

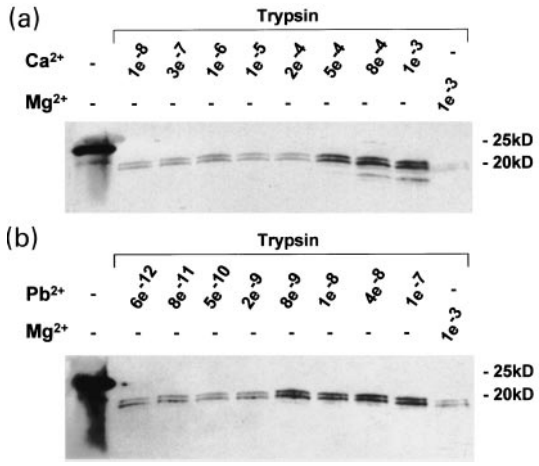

Fig. 6 Pb^{2+} and Ca^{2+} protect a fragment of synaptotagmin I 1–3 from proteolytic cleavage. Western blot of synaptotagmin I 1–3 protein using the mouse anti-rat monoclonal antibody ASV-48. Samples were treated with or without trypsin (66 ng/μL, 2 h, 22°C) in the presence of indicated free concentrations of either (a) Ca^{2+} or (b) Pb^{2+}. Synaptotagmin I 1–3 protein in the absence of trysin is approximately 25 kDa. Samples containing trypsin, treated with 500 μM, 800 μM or 1 mM Ca^{2+} or 8 nM, 10 nM, 40 nM or 100 nM Pb^{2+} contain a fragment of synaptotagmin I 1–3 which is the same size in both conditions (~20 kDa). Reactions were stopped with the addition of sample buffer, boiled (10 min, 95°C), electrophoresed and western blotted. Results are typical of an experiment performed three independent times.

To test whether endogenous rat brain synaptotagmin I is protected, the cytosolic fraction of whole rat brain homogenate was treated with 5 μg/mL trypsin in the presence of 1 mM EGTA and either Pb^{2+}, Ca^{2+}, or Mg^{2+}. In the presence of approximately 2 μM free Pb^{2+}, a fragment of synaptotagmin I which was the same size (~32 kDa) as that observed in the presence of approximately 11 μM free Ca^{2+} (Fig. 5a) was protected from proteolytic degradation. EGTA (1 mM) and approximately 1 mM Mg^{2+} conditions failed to protect synaptotagmin I from proteolytic cleavage, leading to full degradation of the protein.

Protease protection reactions were performed over the course of an hour. Reactions were stopped with the addition of protein loading buffer at 5-min intervals. Pb^{2+} (2 μM) and Ca^{2+} (11 μM) were able to protect the 32 kDa fragment of synaptotagmin I for a longer period of time than 1 mM EGTA or 1 mM Mg^{2+} (Fig. 5b). In order to control for the possibility that either Ca^{2+} or Pb^{2+} alters the activity of trypsin, Ponceau S staining was used to confirm equivalent loading and degradation of protein samples under all conditions (data not shown). These results suggest that Pb^{2+} can bind to endogenous synaptotagmin I and cause a structural stabilization in a manner similar to Ca^{2+}. They also suggest that micromolar concentrations of Pb^{2+} do not denature the synaptotagmin I molecule; on the contrary Pb^{2+} appears able to stabilize the secondary structure of synaptotagmin I in a manner similar to Ca^{2+}.

We also tested the ability of Pb^{2+} as opposed to Ca^{2+} to protect the recombinant synaptotagmin I 1–3 protein from proteolytic cleavage. The synaptotagmin I 1–3 protein contains the first but not the second C2 domain of synaptotagmin I. Previously, Davletov and Südhof (1994) used a similar recombinant protein in order to determine whether the C2A domain is sufficient for the Ca^{2+}-dependent protease protection of a fragment of synaptotagmin I. We used the synaptotagmin I 1–3 protein to test whether the C2A domain is sufficient for the Pb^{2+}-dependent protease protection of synaptotagmin I. Another advantage of using a recombinant synaptotagmin I protein was the ability to use chelators to control the concentrations of Pb^{2+} and Ca^{2+} present in the reactions across a

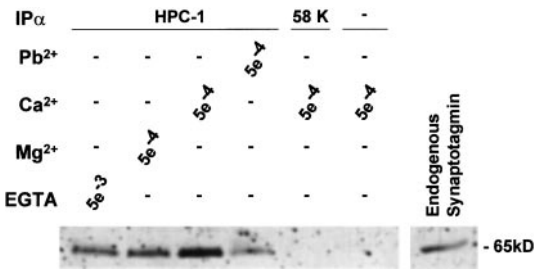

Fig. 7 Pb^{2+} fails to promote the binding of endogenous rat brain synaptotagmin I and syntaxin. The mouse anti-rat monoclonal syntaxin antibody HPC-1 (Sigma) was used to immunoprecipitate syntaxin from rat brain homogenate in the presence of either 5 mM EGTA, 500 μM Mg^{2+}, 500 μM Ca^{2+} or 500 nM Pb^{2+}. Western blotting detected the synaptotagmin I protein (~65 kDa) using the rabbit anti-rat polyclonal antibody VAS-SV008 (Stressgen). Synaptotagmin I was not detected in the absence of primary antibody or in the presence of a non-specific mouse anti-rat monoclonal antibody to the Golgi-specific 58K protein indicating the specificity of the immunoprecipitation. A low level of cation-independent binding was observed in the presence of either 5 mM EGTA or 500 μM Mg^{2+}. Five hundred micromolar Ca^{2+} conditions significantly enhanced binding. However, 500 μM Pb^{2+} failed to induce binding. Ten microliters of brain homogenate was used as a positive control to confirm the position of each protein and the amounts used. Results are typical of an experiment performed three independent times.

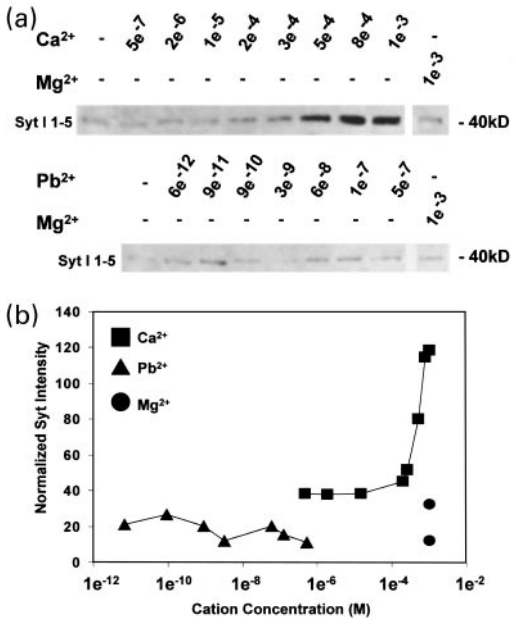

Fig. 8 Ca^{2+} but not Pb^{2+} promotes the binding of synaptotagmin I 1–5 and syntaxin. (a) The rabbit anti-rat polyclonal syntaxin antibody VAP-SV064 was used to immunoprecipitate recombinant syntaxin in the presence of specific free Ca^{2+}, Pb^{2+} or Mg^{2+} concentrations. Western blotting for recombinant synaptotagmin I 1–5 (~ 40 kDa) revealed the amount of syntaxin/synaptotagmin I 1–5 binding under each condition. (b) Normalized synaptotagmin I 1–5 intensity values (see Methods) were plotted against the concentrations of Ca^{2+} (■), Pb^{2+} (▲) or Mg^{2+} (●) used. Two hundred micromolar Ca^{2+} and higher concentrations induced interaction of the proteins. All concentrations of Pb^{2+} failed to induce interaction of the proteins. Background levels of binding were observed in the presence of 1 mM Mg^{2+}. Results are typical of an experiment performed three independent times.

physiologically relevant concentration range for each cation. Different concentrations of trypsin or incubation times will lead to variable amounts of protease protection of the synaptotagmin I fragment before the eventual full degradation of both protected and unprotected fragments. Under our conditions, we observed the protection of a fragment of the synaptotagmin I 1–3 protein in the presence of 500 μM or more Ca^{2+} (Fig. 6a). Pb^{2+} was able to protect synaptotagmin I 1–3 from proteolytic cleavage at concentrations five orders of magnitude lower than Ca^{2+}, starting at a concentration of 8 nM (Fig. 6b). Because there are only two proteins present during the recombinant protease protection assay, synaptotagmin I and trypsin, these results indicate that Pb^{2+} is able to bind directly to the synaptotagmin I protein to protect a fragment from proteolytic cleavage. Furthermore, because the synaptotagmin I 1–3 protein only contains the C2A domain of synaptotagmin I, these results suggest that the Pb^{2+}-mediated protease protection of synaptotagmin I involves a direct interaction of Pb^{2+} with the C2A domain of synaptotagmin I.

Pb^{2+} fails to induce the interaction of synaptotagmin I and syntaxin

The binding of synaptotagmin I to syntaxin is enhanced by Ca^{2+} (Chapman *et al*. 1995; Kee and Scheller 1996; Shao *et al*. 1996; Shao *et al*. 1997). We compared the effects of Ca^{2+} vs. Pb^{2+} on the co-immunoprecipitation of both recombinant and endogenous synaptotagmin I and syntaxin proteins.

In order to investigate the interaction of endogenous rat brain synaptotagmin I and syntaxin, a monoclonal syntaxin antibody was used to immunoprecipitate syntaxin from rat brain homogenate followed by the detection of synaptotagmin I by western blotting. In the presence of total concentrations of either 5 mM EGTA or 500 μM Mg^{2+} a low level of cation-independent synaptotagmin I/syntaxin binding was observed in rat brain homogenate as previously described (Chapman *et al*. 1995). 500 μM Ca^{2+} caused an enhanced level of binding of synaptotagmin I to syntaxin as expected. However, 500 μM Pb^{2+} failed to enhance synaptotagmin I co-immunoprecipitation with syntaxin (Fig. 7). High concentrations of both Ca^{2+} and Pb^{2+} were used because it was assumed that cation-binding proteins present in the homogenate would chelate a large proportion of each of these cations. Immunoprecipitation with a non-specific monoclonal mouse antibody for the Golgi-localized

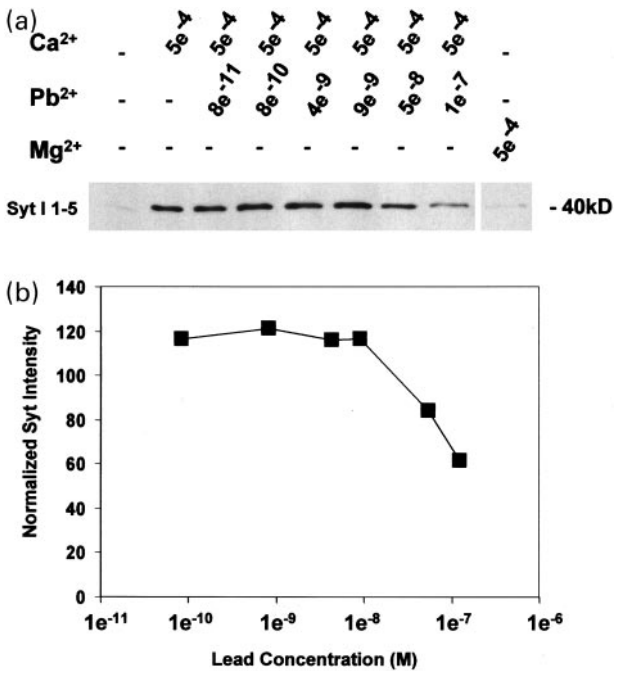

Fig. 9 Nanomolar Pb^{2+} competes with Ca^{2+} induced synaptotagmin I 1–5/syntaxin binding. (a) The rabbit anti-rat polyclonal syntaxin antibody VAP-SV064 was used to immunoprecipitate recombinant syntaxin. Increasing free concentrations of Pb^{2+} were titrated into a constant free concentration of 500 μM Ca^{2+}. Relative to the no cation condition, 500 μM Ca^{2+} in the absence of Pb^{2+} induced a significant level of syntaxin/synaptotagmin I 1–5 interaction. Pb^{2+} concentrations of 50 and 100 nM competed with the 500 μM Ca^{2+} induced interaction of the proteins. (b) Normalized synaptotagmin I 1–5 intensity values (see Methods) were plotted against the concentrations of Pb^{2+} used. Background levels of binding were observed in the presence of 500 μM Mg^{2+}. Results are typical of an experiment performed three independent times.

rat 58K protein or with no primary antibody failed to precipitate synaptotagmin I or syntaxin.

We performed co-immunoprecipitations of recombinant synaptotagmin I 1–5 and syntaxin so that we could examine the interaction of the proteins across physiologically relevant concentration ranges of both Ca^{2+} and Pb^{2+}. In agreement with the endogenous co-immunoprecipitation results, there was only a small degree of cation-independent binding in no cation or 1 mM Mg^{2+} conditions (Figs 8a and b). In agreement with a previous report (Kee and Scheller 1996), specific free Ca^{2+} concentrations from 200 μM to 1 mM were able to enhance the binding of synaptotagmin I 1–5 and syntaxin (Figs 8a and b). However, reactions containing physiologically relevant free concentrations of Pb^{2+} ranging from 6 pM to 500 nM did not display enhanced binding of synaptotagmin I 1–5 to syntaxin (Figs 8a and b).

Given the results of both our endogenous and recombinant synaptotagmin I and syntaxin co-immunoprecipitation experiments we hypothesized that Pb^{2+} may directly compete with the ability of Ca^{2+} to induce the interaction of synaptotagmin I and syntaxin. To test this hypothesis, we titrated increasing amounts of free Pb^{2+} into synaptotagmin I/syntaxin binding reactions containing a constant free concentration of 500 μM Ca^{2+}. Fifty and 100 nM concentrations of Pb^{2+} were able to compete away the binding of synaptotagmin I to syntaxin induced by 500 μM Ca^{2+} (Figs 9a and b). This result indicates that nanomolar concentrations of Pb^{2+} can directly compete with the ability of a micromolar concentration of Ca^{2+} to induce the binding of synaptotagmin I and syntaxin.

Discussion

Our results suggest that synaptotagmin I is a physiologically relevant, molecular target of Pb^{2+} at the synaptic terminal. We found that Pb^{2+} is able to act as an imperfect mimic of Ca^{2+} on synaptotagmin I. Even though Pb^{2+} is capable of binding to synaptotagmin I at the same site as Ca^{2+}, Pb^{2+} induces altered binding characteristics of synaptotagmin I.

We compared the effects of Pb^{2+} vs. Ca^{2+} on various Ca^{2+}-dependent activities of synaptotagmin I using both recombinant and endogenous rat synaptotagmin I proteins. Pb^{2+} promotes half-maximal binding of recombinant synaptotagmin I 1–5 to phospholipids at a half-maximal concentration (8 nM) nearly one thousand times less than that required for Ca^{2+} (5 μM). Tomsig and Suszkiw (1990, 1993) defined the range of Pb^{2+} vs. Ca^{2+} required to trigger secretion of norepinephrine from digitonin permeabilized bovine chromaffin cells. Half-maximal effects at 4.6 nM Pb^{2+} vs. 2.4 μM Ca^{2+} were observed indicating the greater potency of Pb^{2+} in the induction of release. These concentrations closely match those we have observed as being required for Pb^{2+} vs. Ca^{2+} to induce the interaction of synaptotagmin I and phospholipids. We observed that mutagenesis of the D230 residue of the synaptotagmin I 1–5 C2A domain to either a serine or lysine disrupts the ability of both Pb^{2+} and Ca^{2+} to promote synaptotagmin I 1–5 binding to phospholipids. This result suggests that both cations bind to the C2A domain to promote the interaction of synaptotagmin I with phospholipid.

Titrations using the luminescent reporter ion Tb^{3+} revealed that Pb^{2+} binds more tightly than Ca^{2+} to synaptotagmin I 1–5 and also indicate that Ca^{2+} and Pb^{2+} share a common metal-binding site. Pb^{2+} protected endogenous rat brain synaptotagmin I from proteolytic cleavage suggesting that Pb^{2+} can bind to and stabilize the secondary structure of synaptotagmin I in a manner similar to Ca^{2+}. Pb^{2+} also protected recombinant rat brain synaptotagmin I 1–3, containing the C2A but not the C2B domain, from proteolytic cleavage at a concentration five orders of magnitude lower than that required for Ca^{2+}. Since there were no other proteins present in the recombinant

protease protection assay, other than a low concentration of trypsin, this experiment provides strong evidence that Pb^{2+} binds directly to the C2A domain of synaptotagmin I at low nanomolar concentrations. Furthermore, this experiment suggests that relevant concentrations of Pb^{2+} do not denature synaptotagmin I. Instead they stabilize synaptotagmin I in a manner similar to higher concentrations of Ca^{2+}.

Pb^{2+} failed to promote the binding of synaptotagmin I to syntaxin in rat brain homogenates even though the interaction of these two proteins is enhanced in the presence of Ca^{2+}. In order to confirm this finding, two different experiments were performed with recombinant synaptotagmin I 1–5 protein. First, we showed that Ca^{2+} was able to induce the binding of synaptotagmin I and syntaxin in a range of concentrations similar to that previously reported. The physiological relevance of the effect of Ca^{2+} on the interaction of synaptotagmin I and syntaxin is not yet fully elucidated. Due to discrepencies in the literature, it is not yet clear whether Ca^{2+} causes the association or dissociation of synaptotagmin I and syntaxin *in vivo*. Some previous studies (Leveque *et al*. 2000; Verona *et al*. 2000) have suggested that Ca^{2+} may dissociate synaptotagmin I from the t-SNARE core complex, which includes syntaxin. Alternatively, a number of previous studies suggest that Ca^{2+} can induce, not dissociate, the interaction of synaptotagmin I and syntaxin (Chapman *et al*. 1995; Kee and Scheller 1996; Shao *et al*. 1997). Our studies also suggest that Ca^{2+} promotes the interaction between these two proteins. Further experimentation, beyond the scope of this work, will be required in order to resolve these discrepancies and elucidate the full extent of the effects of Ca^{2+} on the interaction of synaptotagmin I and syntaxin *in vivo*. Unlike Ca^{2+}, Pb^{2+} was unable to induce the binding of these proteins at any concentration tested even though similar concentrations of Pb^{2+} were sufficient to potently protect synaptotagmin I 1–3 from proteolytic cleavage and to induce the binding of the synaptotagmin I to phospholipid. Our results suggest that even though Pb^{2+} was able to directly bind to synaptotagmin I at low nanomolar concentrations, it was unable to induce the binding of the synaptotagmin I to syntaxin. This suggested that these concentrations of Pb^{2+} could compete with the Ca^{2+}-dependent binding of synaptotagmin I to syntaxin. In order to test this hypothesis we performed co-immunoprecipitations of synaptotagmin I and syntaxin as increasing free concentrations of Pb^{2+} were titrated into a solution containing a constant free concentration of Ca^{2+}. Nanomolar concentrations (50 and 100 nM) of Pb^{2+} were able to compete with and inhibit the binding of synaptotagmin I/syntaxin induced by 500 μM Ca^{2+}. This result provides evidence for a direct competitive interaction of Pb^{2+} vs. Ca^{2+} on synaptotagmin I 1–5.

Taken together, our data suggest that Pb^{2+} binds directly to the Ca^{2+}-binding C2A domain of synaptotagmin I and alters its Ca^{2+}-dependent binding characteristics at nanomolar concentrations. In particular, Pb^{2+} promotes the binding of synaptotagmin I to phospholipids, but impairs the ability of synaptotagmin I to bind to syntaxin. These differences in effects of Pb^{2+} on synaptotagmin I may be due to conformational (Garcia *et al*. 2000) or electrostatic changes (Shao *et al*. 1997) on the protein. Our results also indicate that synaptotagmin I may use distinct mechanisms to mediate interactions with phospholipids vs. syntaxin. In agreement with this, Chapman *et al*. (1995) proposed that distinct sites on synaptotagmin I are responsible for phospholipid vs. syntaxin binding.

Further work is required in order to determine whether alteration of the normal functioning of synaptotagmin I by Pb^{2+} may be an important component of the effects of Pb^{2+} on neurotransmitter release. Electrophysiological or neurotransmitter release studies could address this issue. Finally, the observation of effects of Pb^{2+} on both PKC and synaptotagmin I suggests that Pb^{2+} may affect other Ca^{2+} binding proteins as well. A comprehensive analysis of the effects of Pb^{2+} on Ca^{2+} binding proteins, such as other C2 domain-containing proteins in the synaptic terminal, would provide information concerning the full extent of the effects of Pb^{2+} on Ca^{2+}-mediated systems.

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