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Synaptotagmin I- and II-deficient PC12 cells exhibit calcium-independent, depolarization-induced neurotransmitter release from synaptic-like microvesicles

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Abstract Synaptotagmin I- and II-deficient PC12 cells (Shoji-Kasai et al. [1]) were used to compare the role of this protein in the calcium-dependent exocytosis of secretory granules and synaptic-like microvesicles (SLMVs). While neither catecholamine nor protein secretion from secretory granules were altered, the depolarization-induced acetylcholine release from SLMVs was no longer calcium-dependent. We propose that within the exocytotic process of SLMVs, there exist two depolarization-induced steps. One is calcium-dependent and no longer present in synaptotagmin I- and II-deficient cells. The other is induced by depolarization, does not require calcium, and suffices to trigger neurotransmitter release from SLMVs in synaptotagmin I- and II-deficient PC12 cells.

Key words: Calcium; Exocytosis; Regulated secretion; Secretory granule; Synaptic vesicle; Synaptotagmin

1. Introduction

Synaptotagmin is an integral membrane protein implicated in calcium-dependent exocytosis [2–4] (for a recent review see [5]). Two types of calcium-dependent secretory vesicles have been shown to contain synaptotagmin; (i) synaptic vesicles [2], which mediate the release of classic neurotransmitters, and their endocrine counterpart, the synaptic-like microvesicles (SLMVs) [6]; and (ii) secretory granules [7–9], from which neuropeptides are secreted. Although numerous studies in various systems have provided several lines of evidence for an important role of synaptotagmin in neurotransmitter release from synaptic vesicles [10–18], its involvement in secretory granule exocytosis is less clear [1,19,20].

In the present study, we have directly compared, in the very same cell, the role of synaptotagmin in the exocytosis of secretory granules and SLMVs. For this purpose, we have used the neuroendocrine cell line PC12, taking advantage of the previous characterization of the contents of their secretory organelles [21] and the isolation of synaptotagmin I- and II-deficient clonal variants [1].

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Abbreviations: SLMV, synaptic-like microvesicle; SgII, secretogranin II.

2. Materials and methods

2.1. Cell culture and labeling

Cell culture. PC12 cells were propagated in growth medium (DMEM, 10% horse serum, 5% fetal calf serum) as previously described [22], except that the cells were grown on poly-L-lysine-coated dishes. 37°C and 10% CO_2 were used in all the labeling and release conditions described below. Several clonal variants of PC12 cells were used: the synaptotagmin-containing clones 251 [23] and E8 [1] and the synaptotagmin I- and II-deficient clones B3, D6 and F7 [1], kindly provided by Dr. M. Takahashi.

Short-term [^{35}S]sulfate labeling and chase. PC12 cells (150 mm dishes) were incubated on a rocker for 30 min in 5 ml of sulfate-free medium (sulfate-free DMEM supplemented with 1% dialysed horse serum and 0.5% dialysed fetal calf serum) followed by replacement of the medium with 5 ml of sulfate-free medium containing 0.8 mCi/ml carrier-free [^{35}S]sulfate (Amersham SJS.1) and incubation for 5 min on a rocker. At the end of the [^{35}S]sulfate labeling, the labeling medium was removed and the cells were chased for the indicated periods of time in 15 ml of 5K/Ca-medium (127 mM NaCl, 5 mM KCl, 2.2 mM $CaCl_2$, 0.33 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 5.6 mM glucose, and 10 mM HEPES-NaOH; pH 7.4) containing 1.6 mM Na_2SO_4. After the first 10 min of chase, the medium was discarded and replaced by fresh chase medium. At the end of the chase, cells and medium were collected and processed for the determination of SgII and heparan sulfate proteoglycan secretion as described [22].

Long-term labeling with [^{35}S]sulfate and [^{3}H]noradrenaline. PC12 cells grown on 150 mm dishes were incubated on a rocker for 30 min in 5 ml of sulfate-free medium followed by replacement of the medium with 15 ml of sulfate-free medium containing 67 μCi/ml carrier-free [^{35}S]sulfate and incubation for 6 h without rocking. At the end of the [^{35}S]sulfate labeling, the cells were incubated for 16 h in 25 ml of chase medium (growth medium containing 1.6 mM Na_2SO_4) containing 0.5 μCi/ml *l*-[7,8-^{3}H]noradrenaline (40 Ci/mmol; Amersham TRK.584).

Long-term [^{3}H]choline labeling. PC12 cells grown in 150 mm dishes were incubated for 16 h in 15 ml of growth medium containing 2.66 μCi/ml of [methyl^{3}H]choline chloride (75–85 Ci/mmol; Amersham TRK.593).

2.2. Depolarization-induced SgII, noradrenaline and acetylcholine release

Cell stimulation. PC12 cells were long-term labeled with [^{35}S]sulfate plus [^{3}H]noradrenaline, or with [^{3}H]choline. At the end of the labeling, the cells were chased 3 times for 10 min with 5 ml of either 5K/Ca-medium (for conditions 1 and 2, see Figs. 2 and 4) or 5K/Mg-medium (5K/Ca-medium in which 2.2 mM $CaCl_2$ was replaced by 10 mM $MgCl_2$) (for conditions 3 and 4). After the chase, the medium was replaced by 5 ml of 5K/Ca-medium (condition 1), 55K/Ca-medium (5K/Ca-medium in which 127 mM NaCl and 5 mM KCl were replaced by 77 mM NaCl and 55 mM KCl) (condition 2), 5K/Mg-medium (condition 3), or 55K/Mg-medium (5K/Mg-medium in which 127 mM NaCl and 5 mM KCl were replaced by 77 mM NaCl and 55 mM KCl) (condition 4), and the cells were incubated for 15 min for SgII and noradrenaline release or 5 min for acetylcholine release. At the end of the stimulation, the dishes were placed on ice, the release medium was removed, and the cells were scraped off the dish in 10 ml of ice-cold phosphate-buffered saline and pelleted by centrifugation at $800 \times g_{av}$ for 10 min. The pellet was resuspended in 1 ml of SDS-PAGE-sample buffer, and analyzed for [^{35}S]sulfate-labeled SgII by SDS-PAGE (see

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below) and for [^{3}H]noradrenaline or total [^{3}H]choline-labeled material by liquid scintillation counting. The release medium was centrifuged for 7 min at 1,800 rpm in a Heraeus minifuge. The supernatant was centrifuged for 30 min at 35K rpm in a Beckman Ti70.1 rotor. Aliquots of the high-speed supernatant were analyzed for [^{3}H]noradrenaline by liquid scintillation counting and for [^{3}H]choline/acetyl[^{3}H]choline by liquid scintillation counting and agarose gel electrophoresis (see below). Other aliquots (1 ml) of the high-speed supernatant were acetone-precipitated and analyzed for [^{35}S]sulfate-labeled SgII by SDS-PAGE and fluorography.

Agarose gel electrophoresis. To separate acetyl[^{3}H]choline and [^{3}H]choline from other [^{3}H]choline-labeled molecules, we used agarose gel electrophoresis at pH 3.5 instead of the previously described two-dimensional cellulose thin-layer electrophoresis first at pH 1.9 and then at pH 3.5 [21]. The high-speed supernatant prepared from the release medium was diluted (1:1, v/v) with electrophoresis buffer (5% (v/v) acetic acid, 0.5% (v/v) pyridine, pH 3.5) and applied onto an agarose gel (1% in electrophoresis buffer). Electrophoresis was carried out at 100 V for 1 h. [^{3}H]Acetylcholine (NEN, NET-113) was used as a standard. The agarose gel was sectioned into pieces (0.5-cm in length), the gel pieces were melted in a microwave oven, mixed with 10 ml Ecoscint H (National Diagnostics) and ^{3}H-radioactivity was determined by liquid scintilation counting.

2.3. Subcellular fractionation and immunoblotting

Post-nuclear supernatants were prepared from unlabeled PC12 cells, subjected to preparative sequential differential centrifugation to yield P40 pellets, and the P40 pellets analyzed by equilibrium sucrose gradient centrifugation, using previously described procedures (see [21] and refs. therein). Fractions were analysed by immunoblotting as previously described [24] except that blocking of the nitrocellulose sheets was in PBS containing 10% (w/v) low fat milk powder and 0.1% (w/v) Tween-20 for 2 h at room temperature. For SgII, the rabbit anti $hSgII_{1-20}$ peptide antiserum [25], a kind gift of Dr. H.-H. Gerdes, was used at a dilution of 1:200, followed by [^{125}I]protein A (0.12 μCi/ml final; NEN). For synaptophysin, the mouse monoclonal antibody SY38 [26] (Boehringer-Mannheim) was used at 0.2 μg/ml final concentration. For synaptotagmin, the mouse monoclonal antibody 41.1 [4], a kind gift of Dr. R. Jahn, Yale University, New Haven, was used at 1 μg/ml final concentration. Both monoclonal antibodies were detected by incubation with 1 μg/ml of rabbit anti-mouse IgG and [^{125}I]protein A (0.12 μCi/ml final; NEN). Immunoreactivity was quantitated by densitometric scanning of the autoradiogram derived from the immunoblots. For qualitative analysis (Fig. 1A), blots were incubated with the above primary antibodies followed by horseradish peroxidase-coupled secondary antibodies and detection using the ECL system (Amersham).

3. Results and discussion

3.1. Synaptotagmin I- and II-deficient PC12 cells contain both secretory granules and SLMVs

The synaptotagmin I- and II-deficient PC12 cell clones B3, D6 and F7 [1] (Fig. 1A, top) were first analyzed for the presence of secretogranin II (SgII) and synaptophysin, which are markers for secretory granules [27] and SLMVs [28], respectively. All three clones were found to express normal levels of these two marker proteins when compared with two synaptotagmin-containing control PC12 cell clones, i.e. E8 [1] and 251 [23] (Fig. 1A, middle and bottom). Because in all the experiments described below similar results were obtained for the control clones 251 and E8 and the synaptotagmin I- and II-deficient clones B3, D6 and F7, respectively, we have illustrated here only results for clones 251 and B3.

Equilibrium sucrose gradient centrifugation of a subcellular fraction enriched in small vesicles (P40) [21] revealed that in the synaptotagmin I- and II-deficient clone B3, as in the control clone 251, SgII peaked in the denser fractions of the gradient in the position characteristic of mature secretory granules (Fig. 1B,E), whereas synaptophysin was found in the lighter fractions in a distribution characteristic of SLMVs (fractions No. 14–15) and early endosomes (fractions No. 10–12) (Fig. 1C,F). Synaptotagmin was undetectable in the clone B3 (data not shown), but was associated with both SLMVs and secretory granules in the control PC12 cell clone 251 (Fig. 1D), as expected from previous reports [6–9]. We conclude that the synaptotagmin I- and II-deficient PC12 cells contain secretory granules and SLMVs which are indistinguishable from the corresponding vesicles of control cells upon subcellular fractionation. These observations imply that synaptotagmin I and II are not essential for the formation of secretory granules and SLMVs and allowed us to analyze the exocytosis of these neurosecretory vesicles in synaptotagmin I- and II-deficient PC12 cells.

3.2. Depolarization-induced secretion of catecholamines and SgII is normal in synaptotagmin I- and II-deficient PC12 cells

In PC12 cells, catecholamines are stored in secretory granules, but not in SLMVs [21]. Depolarization-induced secretion of catecholamines from PC12 cells therefore reflects their release from secretory granules, not SLMVs, and the ability of synaptotagmin I- and II-deficient PC12 cells to perform this release [1] implies that synaptotagmin I and II are not essential for this process. In contrast, microinjection of synaptotagmin antibodies and soluble synaptotagmin I fragments into PC12 cells was found to result in an inhibition of secretory granule exocytosis, as measured by the cell surface exposure of the membrane-anchored form of dopamine β-hydroxylase [19].

One possible explanation to reconcile these apparently contradictory observations with each other is based on the existence of a fusion pore as an intermediate step in vesicle fusion [29–31]. It has been postulated that molecules such as classic neurotransmitters, biogenic amines and nucleotides are released from vesicles through the fusion pore [30,32,33]. Given its size, the release of such small molecules through the fusion pore would presumably not be accompanied by the release of larger soluble constituents of the vesicle such as secretory proteins, and the fusion pore would probably not allow lumenal domains of vesicle membrane proteins to be accessible to extracellular probes such as antibodies. Thus, if one assumed that synaptotagmin acted after the formation and opening of the fusion pore, its absence, or functional blockade, would still allow the fusion pore to open but would prevent the exocytotic process from proceeding beyond this step. Catecholamines would be released through the opened fusion pore (possibly explaining the data of Shoji-Kasai et al. [1]) which, however, would not allow dopamine β-hydroxylase to be detected by an extracellular antibody (perhaps explaining the results of Elferink et al. [19]).

To investigate this possibility, we compared the secretion of catecholamines from secretory granules with that of SgII, a 68 kDa secretory protein [34] of the secretory granule matrix [27] which should be too large to be released through a fusion pore with the width of a gap junction channel [30,32]. As shown in Fig. 2, depolarization of the synaptotagmin I- and II-deficient PC12 cells in the presence of extracellular Ca^{2+} (Fig. 2, condition 2) resulted in the release not only of catecholamines (dark columns) but also of SgII (light columns), which was indistinguishable from that observed with control PC12 cells. For both compounds, this evoked release was strictly calcium-dependent (compare conditions 2 and 4). Thus, the observations reported

by Shoji-Kasai et al. [1] cannot be reconciled with those of Elferink et al. [19] by assuming an 'arrested' fusion pore caused by synaptotagmin I and II deficiency. Rather, our data indicate that synaptotagmin I and II are not essential for regulated exocytosis of secretory granules in PC12 cells, perhaps because related proteins such as synaptotagmin III [35], synaptotagmin IV [36] and rabphilin [37] compensate for synaptotagmin I and II deficiency in this process. Hence, with respect to regulated exocytosis of secretory granules, the only known phenotype of synaptotagmin I and II deficiency is the one recently reported by Shoji-Kasai et al. [38], i.e. the loss of the α-latrotoxin-induced calcium-independent catecholamine release [39] that is thought to be mediated via the direct interaction of the α-latrotoxin receptor [40,41], a plasma membrane protein of the neurexin family [42], with synaptotagmin.

3.3. *Synaptotagmin I and II deficiency does not result in an increased basal secretion from secretory granules*

Synaptotagmin has been proposed to be an inhibitor of exocytosis unless cytoplasmic calcium is elevated [43]. If so, one might expect an increased basal exocytosis of secretory granules in synaptotagmin I- and II-deficient PC12 cells. To investigate this possibility, we studied the secretion of SgII from newly formed secretory granules by pulse-labeling these cells with [^{35}S]sulfate for 5 min and chasing for up to 100 min, without any stimulation (compare [22] and [44]). As shown in Fig. 3, no significant release of SgII was detectable, while that of the heparan sulfate proteoglycan, a marker for the constitutive pathway of protein secretion in PC12 cells [22,44], was virtually complete by 100 min (Fig. 3B), as has been previously observed for synaptotagmin-containing PC12 cells [22,44]. Thus, the lack

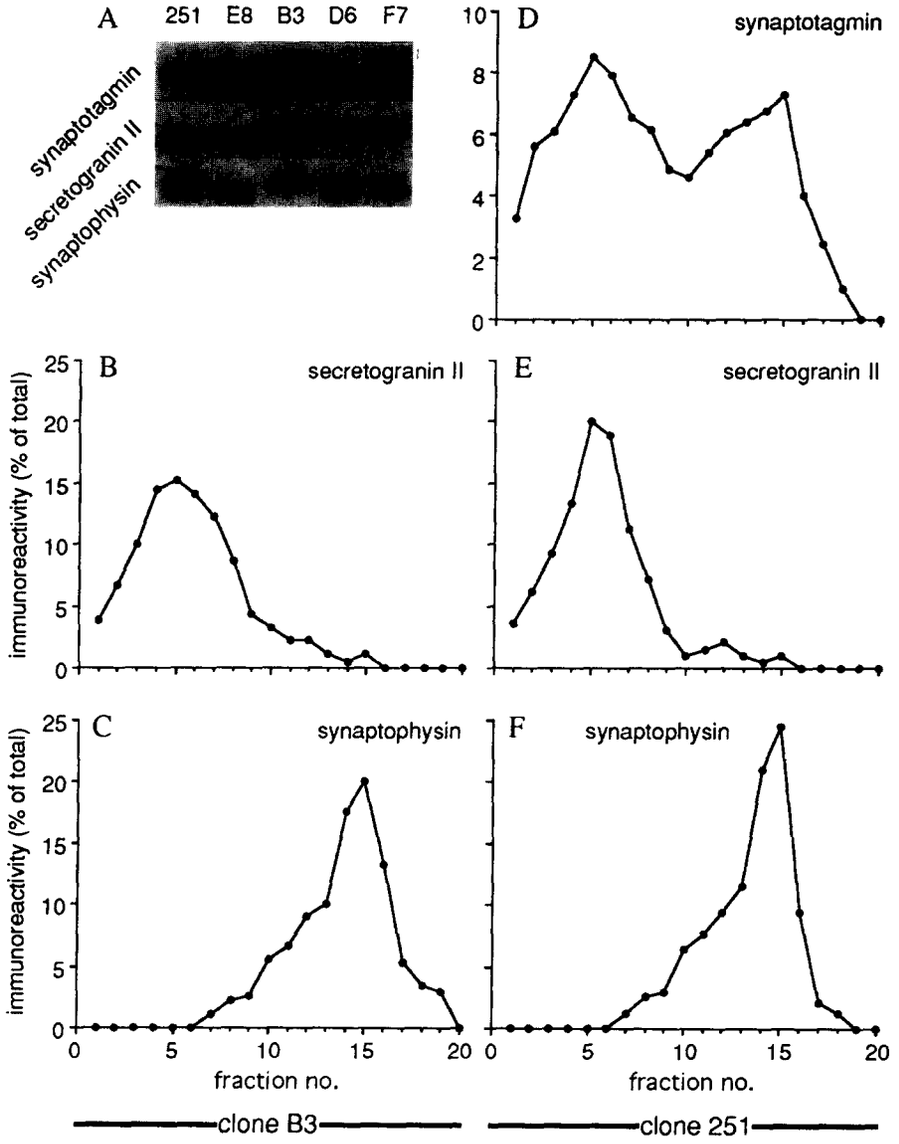

Fig. 1. Synaptotagmin I- and II-deficient PC12 cells contain both secretory granules and SLMVs. (A) Cell homogenates (15 μg protein) of the PC12 cell control clones 251 and E8 and of the synaptotagmin I- and II-deficient clones B3, D6, and F7 were analysed by immunoblotting for the presence of synaptotagmin, SgII and synaptophysin. (B–F) Membrane pellets enriched in small vesicles (P40) were prepared from the PC12 cell control clone 251 (D–F) and the synaptotagmin I- and II-deficient clone B3 (B and C), and subjected to equilibrium sucrose gradient centrifugation. The gradient fractions (fraction 1 = bottom) were analyzed by immunoblotting for the presence of synaptotagmin (D), the secretory granule marker SgII (B and E) and the SLMV marker synaptophysin (C and F). For each marker, values are expressed as percentage of total recovered in the sum of the gradient fractions.

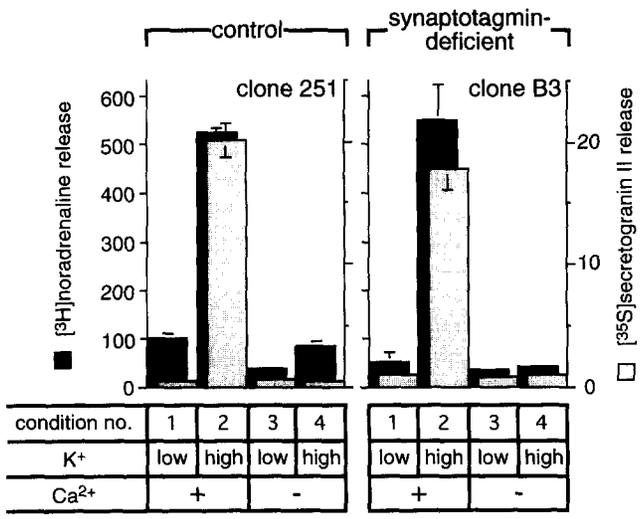

Fig. 2. Depolarization-induced secretion of noradrenaline and SgII from secretory granules of synaptotagmin I- and II-deficient PC12 cells is indistiguishable from that of control PC12 cells. After long-term labeling with [^{3}H]noradrenaline and [^{35}S]sulfate, control (clone 251) and synaptotagmin I- and II-deficient (clone B3) PC12 cells were incubated in various conditions and analyzed for the secretion of [^{3}H]noradrenaline (dark columns) and [^{35}S]SgII (light columns) induced by 55 mM KCl. Condition 1: 5K/Ca-medium; condition 2: 55K/Ca-medium; condition 3: 5K/Mg-medium; condition 4: 55K/Mg-medium. For each marker secreted, individual values were calculated as percentage of total recovered in cells plus medium. The means of four independent experiments are shown. For [^{3}H]noradrenaline, the mean of the unstimulated control cells (clone 251, condition 1) was arbitrarily set to 100 and the other means expressed relative to this. Bars indicate S.E.; for some columns, these were too small to be depicted.

of synaptotagmin I and II does not result in an increased basal secretion from secretory granules.

3.4. *Depolarization-induced acetylcholine release from SLMVs of synaptotagmin I- and II-deficient PC12 cells is no longer calcium-dependent*

To investigate a possible effect of synaptotagmin I and II deficiency on the exocytosis of SLMVs, we took advantage of our previous observation that after [^{3}H]choline-labeling of PC12 cells, these organelles (but not secretory granules) contain biosynthetic acetyl[^{3}H]choline [21]. To prevent acetyl[^{3}H]choline which is present in early endosomes [21] and which may be secreted from these organelles via constitutive membrane recycling to the plasma membrane [45] from contributing to the acetyl[^{3}H]choline release, we reduced the endosomal acetyl[^{3}H]choline pool by a chase prior to stimulation. As shown in Fig. 4A, the subsequent basal acetyl[^{3}H]choline release observed in the absence of stimulation (condition 1) was virtually identical for the control and the synaptotagmin I- and II-deficient PC12 cell clone. Upon depolarization, both the control and the synaptotagmin I- and II-deficient PC12 cell clone exhibited a similar increase in acetyl[^{3}H]choline release (condition 2). However, whereas in the control PC12 cell clone the depolarization-induced acetyl[^{3}H]choline release was strictly calcium-dependent, in the synaptotagmin I- and II-deficient clone this release occurred also in the absence of extracellular calcium (Fig. 4A, compare conditions 2 and 4).

To investigate whether the ^{3}H-labeled material released from [^{3}H]choline-labeled cells was indeed acetyl[^{3}H]choline, we analyzed the medium of the synaptotagmin I- and II-deficient clone B3 in conditions 2 and 4 by agarose gel electrophoresis at pH 3.5 (Fig. 4B). We have previously shown that after [^{3}H]choline-labeling of cells, the only radioactive compounds migrating towards the cathode at pH 3.5 are acetyl[^{3}H]choline and [^{3}H]choline itself, with [^{3}H]choline running slightly ahead of acetyl[^{3}H]choline [21]. As shown in Fig. 4B, in both condition 2 and 4 the vast majority of the ^{3}H-radioactivity was recovered in a peak that comprised both the position of the acetyl[^{3}H]choline standard and that of free [^{3}H]choline, and hence most likely corresponded to a mixture of [^{3}H]choline plus acetyl[^{3}H]choline. The presence of [^{3}H]choline in the release medium can be explained by (i) substantial amounts of choline, in addition to acetylcholine, being present in SLMVs [21] and

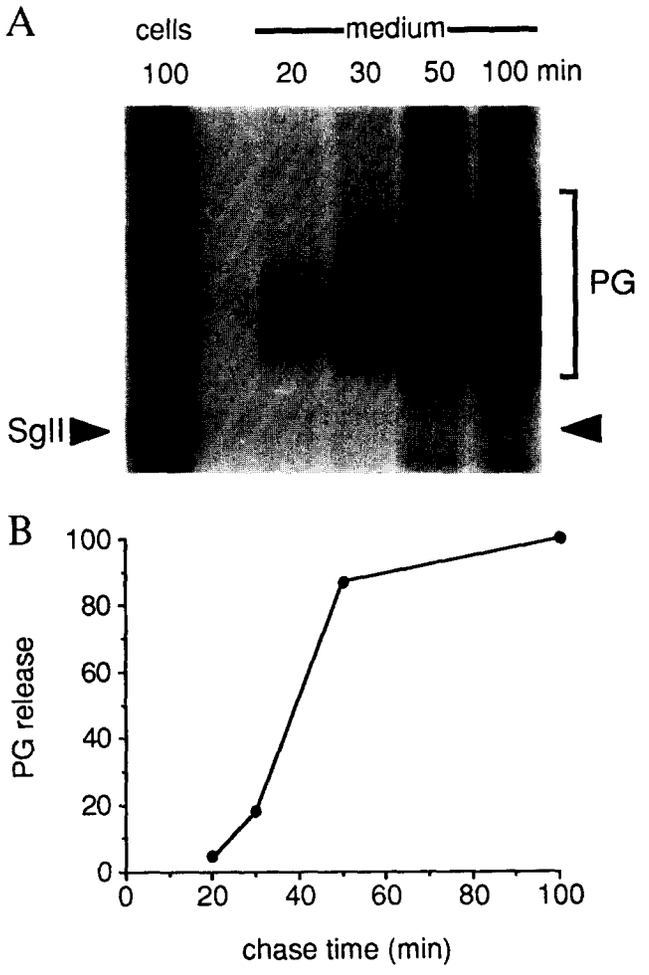

Fig. 3. Synaptotagmin I- and II-deficient PC12 cells do not exhibit an increased basal release of SgII. Synaptotagmin I- and II-deficient PC12 cells (clone B3) were short-term labeled with [^{35}S]sulfate and then chased for the indicated time periods. Aliquots of the various chase media (one fifth) and of the cells at the end of the chase (one tenth) were analyzed by SDS-PAGE followed by fluorography (A). The heparan sulfate proteoglycan (PG), a marker for the constitutive pathway of protein secretion, was quantified and is expressed as percent of that found in the medium after a 100 min chase (B). Note that over a chase period which results in the secretion of the bulk of the constitutively secreted heparan sulfate proteoglycan, SgII is stored in the cells and not detectable in the medium (arrowheads).

(ii) the extracellular degradation of released acetyl[^{3}H]choline by the PC12 cell acetylcholine esterase [46].

How can we explain that, irrespective of the absence or presence of extracellular calcium, equal amounts of [^{3}H]choline/acetyl[^{3}H]choline are released upon high K^+-induced depolarization of synaptotagmin I- and II-deficient PC12 cells? One possibility is that in these cells, the release was not due to exocytosis of SLMVs but to a reversal of the sodium-dependent choline transport across the plasma membrane [47–49]. The latter may have been caused by the reduction in the sodium concentration of the high K^+ release medium which was done to keep its osmolarity constant. This explanation implies that (i) the ^{3}H-labeled material released from the synaptotagmin I- and II-deficient cells was mostly [^{3}H]choline (with some of it trailing in the electrophoresis); (ii) the reversal of sodium-dependent choline transport across the plasma membrane in the high K^+ medium occurred only in the synaptotagmin I- and II-deficient, but not the control, PC12 cells (as there was no increase in release in condition 4 with clone 251); and (iii) most importantly, the synaptotagmin I- and II-deficient PC12 cells, in contrast to control PC12 cells, did not exhibit [^{3}H]choline/acetyl[^{3}H]choline release from SLMVs in the presence of extracellular calcium (as the release in conditions 2 and 4 was equal for clone B3). In other words, the high K^+-induced release of [^{3}H]choline/acetyl[^{3}H]choline from control PC12 cells reflected exocytosis of SLMVs whereas that from synaptotagmin I- and II-deficient cells was due to transport of cytosolic [^{3}H]choline across the plasma membrane. In this scenario, our results would suggest an essential role of synaptotagmin I in the exocytosis of SLMVs induced by depolarization.

Alternatively, if we interpret the high K^+-induced release of [^{3}H]choline/acetyl[^{3}H]choline from the synaptotagmin I- and II-deficient PC12 cells in the context of SLMV exocytosis, we are left with two possibilities. First, the release in the absence of extracellular calcium was due to a depolarization-induced mobilization of calcium from intracellular stores. We find this unlikely because this should also have resulted in the exocytosis of secretory granules, which are known to require lesser increases in cytosolic calcium than synaptic vesicles [50]. However, no depolarization-induced noradrenaline and SgII release was detected in this condition (compare Fig. 2, condition 4). Furthermore, as there was no depolarization-induced release of [^{3}H]choline/acetyl[^{3}H]choline from control PC12 cells (Fig. 4A left, condition 4), one would have to assume that calcium mobilization from intracellular stores occurred only in the synaptotagmin I- and II-deficient cells.

Second, in the synaptotagmin I- and II-deficient PC12 cells, the depolarization-induced exocytosis of SLMVs was no longer calcium-dependent. This conclusion is distinct from the various interpretations of data obtained with neurons of synaptotagmin-deficient animals [11–15,17,18] including mice [16,51]. The fusion of synaptic vesicles with the presynaptic membrane in neurons differs in several aspects from the exocytosis of SLMVs in neuroendocrine cells, and hence even for mammalian cells the phenotype of synaptotagmin deficiency may well depend on the system studied. With respect to SLMV exocyto-

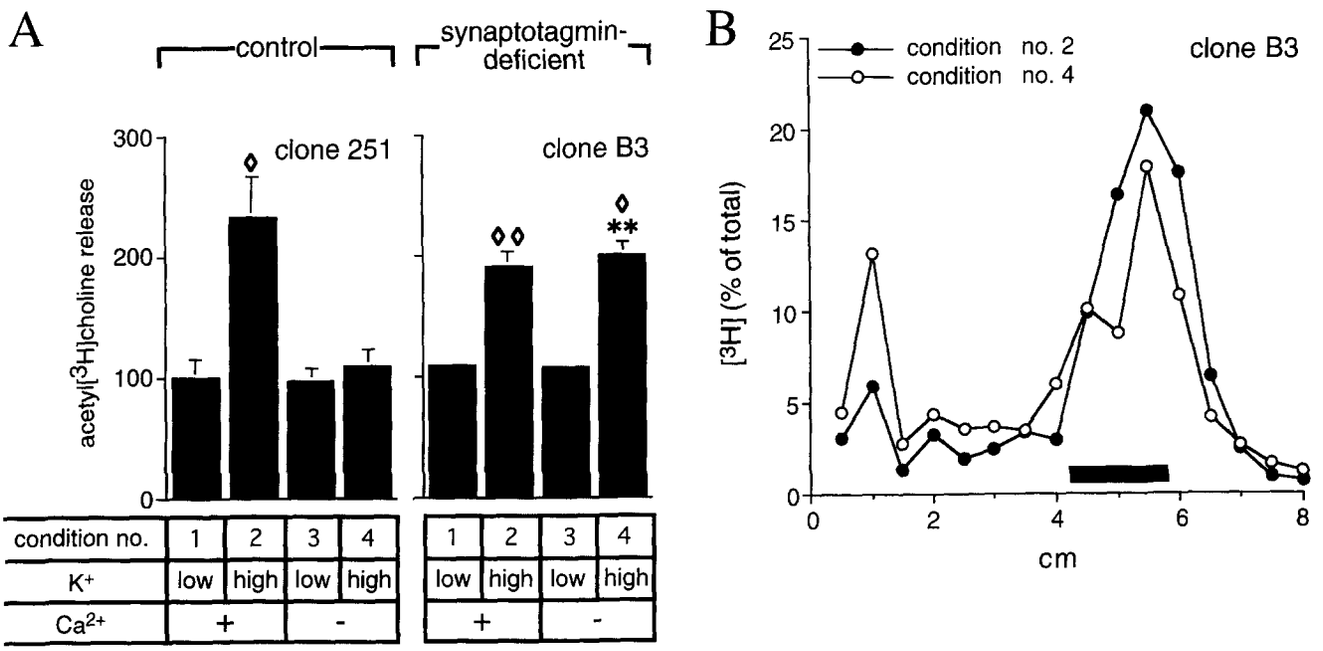

Fig. 4. Depolarization-induced, calcium-independent release of acetylcholine from SLMVs of synaptotagmin I- and II-deficient PC12 cells. (A) After long-term labeling with [^{3}H]choline, control (clone 251) and synaptotagmin I- and II-deficient (clone B3) PC12 cells were incubated in various conditions and analyzed for the secretion of acetyl[^{3}H]choline induced by 55 mM KCl. Condition 1: 5K/Ca-medium; condition 2: 55K/Ca-medium; condition 3: 5K/Mg-medium; condition 4: 55K/Mg-medium. ^{3}H-Radioactivity in the medium (largely [^{3}H]choline/acetyl[^{3}H]choline, see panel B) was calculated as percentage of total recovered in cells plus medium. The means of four independent experiments are shown, with that of the unstimulated control cells (clone 251, condition 1) being arbitrarily set to 100 and the other means expressed relative to this. Bars indicate S.E.; for some columns, these were too small to be depicted. Open diamonds indicate values statistically different from those of condition 1, asterisks those different from condition 3, as calculated using the paired Student's t-test (◊ $P < 0.05$; ◊◊ $P \leq 0.005$; **, $P \leq 0.005$). (B) Agarose gel electrophoresis (origin on the left, cathode on the right) of the release media from condition 2 and 4 of clone B3 (compare panel A). Values are expressed as percentage of the total ^{3}H-radioactivity recovered in the gel. The bar indicates the position of the acetyl[^{3}H]choline standard.

sis, our data would imply that the stimulation-induced event within the fusion process of SLMVs consists of at least two distinct steps. One is calcium-dependent and no longer present in synaptotagmin I- and II-deficient cells. Given the properties of synaptotagmin [3,4], it is appealing to relate the lack of the calcium-dependent step in these cells to the absence of synaptotagmin I and II, although it cannot be excluded that synaptotagmin deficiency is not the only difference between these cells and control PC12 cells. The other step is induced by depolarization, does not require calcium, and suffices to trigger SLMV (but not secretory granule) exocytosis in synaptotagmin I- and II-deficient PC12 cells.

The role of depolarization in neurotransmitter release has been controversial [52–55]. According to the calcium hypothesis (see [52] and refs. therein), membrane depolarization is necessary only for opening calcium channels. The calcium-voltage hypothesis (see ref. 54 and refs. therein), on the other hand, postulates that depolarization induces a conformational change in a membrane protein, rendering it sensitive to calcium. Upon calcium entry into the synapse (which is also induced by depolarization), the binding of calcium to the thus sensitized form of this protein triggers neurotransmitter release. The present results support the calcium-voltage hypothesis [54], if modified such that distinct, but interacting, proteins are the targets for calcium and depolarization. Considering current models of the molecular components of the docking/fusion machinery and their interactions with each other [56–58], synaptotagmin and related proteins, as previously proposed (for reviews see [5,43]), would be the calcium targets mediating the calcium-dependence of neurotransmitter release. However, the depolarization-induced, but calcium-independent acetylcholine release from SLMVs occurring in synaptotagmin I- and II-deficient PC12 cells leads us to propose that there exists an additional regulated step in the fusion process, which is mediated by proteins other than synaptotagmin I and II that are sensitive to membrane depolarization. Perhaps some of the plasma membrane proteins implicated in synaptic vesicle (and, by analogy, SLMV) exocytosis such as syntaxin [59–61], SNAP-25 [60,62] and the neurexins [40–42] need to undergo a conformational change in the course of the fusion process, and depolarization induces this conformational change. After all, depolarization-induced conformational changes are well established for membrane proteins such as voltage-gated ion channels.

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