Modification of Annexin II Expression in PC12 Cell Lines Does not Affect Ca^{2+} -dependent Exocytosis

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The Ca^{2+} /phospholipid/cytoskeletal-binding protein annexin II has been proposed to play an important role in Ca^{2+} -dependent exocytosis; however, the evidence for this role is inconclusive. More direct evidence obtained by manipulating annexin II levels in cells is still required. We have attempted to do this by generating stably transfected PC12 cell lines expressing proteins which elevate or lower functional annexin II levels and using these cell lines to investigate Ca^{2+} -dependent exocytosis. Three cell lines were generated: one expressing an annexin II mutant which aggregates annexin II in at least a proportion of the cells, thereby removing functional protein from the cell; a mixed clonal cell line constitutively overexpressing human annexin II; and a clonal cell line capable of overexpressing annexin II in the presence of sodium butyrate. After digitonin permeabilization, Ca^{2+} -dependent dopamine release from these cell lines was compared with that from control nontransfected cells, and, in addition, release was compared in induced to uninduced cells. There were no significant differences in Ca^{2+} -dependent exocytosis between any of the transfected cell lines before or after induction and the control cells. In addition, nontransfected PC12 cells treated with nerve growth factor, which elevates annexin II levels severalfold, failed to increase Ca^{2+} -dependent exocytosis after digitonin permeabilization, compared with control cells. We conclude that annexin II is not an important regulator of Ca^{2+} -dependent exocytosis in PC12 cells.

INTRODUCTION

Annexin II (also known as calpactin 1, lipocortin II, and chromobindin 8) is a member of the annexin family of Ca^{2+} -binding proteins (Burgoyne and Geisow, 1989). It can exist as a monomer of 36 kDa (also known as p36) and as a heterotetramer formed from two subunits of annexin II bound to two subunits of an 11-kDa protein called p11 which belongs to the S-100 family of proteins. The heterotetramer is localized at the cytoplasmic face of the plasma membrane and/or the submembranous cytoskeleton (Greenberg and Edelman, 1983; Zokas and Glenney, 1987; Semich *et al.*, 1989), whereas monomeric annexin II appears to be predominantly a cytosolic protein (Zokas and Glenney, 1987; Thiel *et al.*, 1992).

The exact function of annexin II is unknown but it has been implicated in many cellular processes. It is a substrate for phosphorylation by pp60^{v-src}, protein kinase C, and certain receptor tyrosine kinases (for review, Waisman, 1995). It can bind membrane phospholipids (Creutz *et al.*, 1983) and cytoskeletal proteins such as filamentous actin and nonerythroid spectrin (fodrin) (Gerke and Weber, 1984) in a Ca^{2+} -dependent manner. These properties have led to speculation that annexin II may be involved in transmission of external signals to the cytoskeleton (Glenney, 1987) and/or membrane trafficking events such as exocytosis and endocytosis (Gruenberg and Emans, 1993; Harder and Gerke, 1993; Burgoyne and Clague, 1994; Mayorga *et al.*, 1994). A certain amount of indirect experimental evidence has accumulated to support the idea that annexin II plays an important role in exocytosis (Nakata *et al.*, 1990). Most annexins have the ability to

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bring about the aggregation and fusion of secretory vesicles at high Ca^{2+} concentrations, but annexin II only requires low physiological Ca^{2+} concentrations for the same effect (Drust and Creutz, 1988). In the presence of arachidonic acid and Mg^{2+} , chromaffin granule membranes aggregated by annexin II can undergo fusion (Drust and Creutz, 1988). In addition, in mammary cells annexin II is localized to the apical membrane where secretion of milk proteins occurs (Handel *et al.*, 1991). More direct evidence involving functional studies with permeabilized cells have also supported the hypothesis of an important role for annexin II in exocytosis. Digitonin-permeabilized chromaffin cells leak proteins essential for exocytosis, including annexin II, leading to a loss of ability to secrete in response to Ca^{2+} , a phenomenon known as run-down. Permeabilized cells incubated with exogenous annexin II showed a two- to threefold increase in exocytosis above that seen in the absence of added annexin II, upon subsequent stimulation with $10 \mu\text{M}$ Ca^{2+} (Ali *et al.*, 1989).

The reconstitution assays with permeabilized cells, although valuable, have limitations. As run-down is allowed to proceed, the ability of annexin II to stimulate exocytosis is diminished (Ali and Burgoyne, 1990; Burgoyne and Morgan, 1990; Sarafian *et al.*, 1991), suggesting that other proteins essential for exocytosis have been lost. Another means of manipulating annexin II levels in intact cells is required to provide stronger evidence of an important role for annexin II in Ca^{2+} -dependent exocytosis. We have chosen to study the role of annexin II in PC12 cells since these cells normally express very low levels of the protein unless neuronal differentiation is induced by nerve growth factor (NGF)¹ treatment (Schlaepfer and Haigler, 1990). PC12 cells should therefore provide a good model system for attempts to reduce functional annexin II levels or for examination of the consequences of overexpression. The approach we have used here involves stably transfecting PC12 cells with expression constructs encoding proteins that elevate or lower levels of functional annexin II. We have produced PC12 cell lines expressing a *trans*-dominant annexin II mutant (Harder and Gerke, 1993) that causes aggregation of endogenous annexin II and p11, thereby effectively removing functional annexin II from the cell, or that overexpresses human annexin II. Using these PC12 cell lines, we have investigated Ca^{2+} -dependent secretion in digitonin-permeabilized cells. Both overexpression of annexin II or reduction of functional annexin II-p11 heterotetramer does not appear to affect Ca^{2+} -stimulated secretion, suggesting

that annexin II is not a prime regulator of exocytosis in PC12 cells.

MATERIALS AND METHODS

Materials

High-purity digitonin was obtained from Novabiochem (Nottingham, United Kingdom). Fetal calf serum, horse serum, RPMI 1640, and G418 sulfate were all obtained from Life Technologies (Paisley, United Kingdom). The pcDNA3 plasmid was obtained from Invitrogen (San Diego, CA) and the restriction enzymes and ligase were obtained from Promega (Madison, WI). $[7,8\text{-}^3\text{H}]\text{dopamine}$, biotinylated anti-mouse Ig, and Texas Red streptavidin were all obtained from Amersham (Buckinghamshire, United Kingdom). All other reagents were of analytical grade from Sigma (Poole, United Kingdom).

Cell Culture

PC12 cells were cultured in suspension in RPMI 1640 containing 5% fetal calf serum, 10% horse serum, and 25 U/ml penicillin with 25 $\mu\text{g}/\text{ml}$ streptomycin. Neomycin-resistant cell lines were maintained in the presence of 400 $\mu\text{g}/\text{ml}$ G418 sulfate. Cells required for dopamine release assays and for SDS-PAGE were dissociated in trypsin/EDTA buffer and plated into 24-well tissue culture dishes precoated with collagen at a density of 0.5×10^6 . All cells were cultured in a humidified atmosphere of 5% $\text{CO}_2/95\%$ air.

Recombinant DNA

A chimeric construct (known as XM) encoding the 18 NH_2 -terminal amino acids of human annexin II fused to the complete human p11 molecule (with the starting methionine of p11 changed to aspartate residue) had previously been cloned into the pLKneo expression vector containing the neomycin-resistant gene and the mouse mammary tumour virus (MMTV) promoter (known as pLKXM; Harder and Gerke, 1993). The cDNA encoding human annexin II with the replacement of Ala⁶⁵ by glutamic acid (known as A65E) was originally cloned into the pCMV5 expression vector (Thiel *et al.*, 1992). This insert was excised using restriction endonucleases *EcoRI* and *XbaI* and ligated into the *EcoRI/XbaI*-linearized pcDNA3 expression vector. The vector possesses the neomycin-resistance gene and the human cytomegalovirus (CMV) promoter. The resulting plasmid is referred to as pcDNA3 AnxII.

Transfections

Transfected PC12 cell lines were generated by electroporation using a Progenitor II PG200 (Hoefer, San Francisco, CA) at 1080 μF , 260 V, and three discharges per sample in a 0.4-cm cuvette in the presence of pLKXM or pcDNA3 AnxII (linearized with *XbaI* and *BglIII* restriction enzymes, respectively). For electroporation, PC12 cells in suspension were dissociated with trypsin, and 1 ml of media containing 5×10^6 cells was placed in an electroporation cuvette with 10 μg of plasmid. After electroporation cells were rapidly diluted in fresh media to a density of $0.5 \times 10^6/\text{ml}$ and then plated into collagen-coated 6-cm tissue culture Petri dishes. The following day the medium was replaced by fresh medium initially containing 500 $\mu\text{g}/\text{ml}$ G418 sulfate but subsequently containing 400 $\mu\text{g}/\text{ml}$. Fresh media and G418 were added as required. Resistant colonies were isolated and cultured. Clones were analyzed for expression of transfected cDNA and for induction by 1 μM dexamethasone (for the chimeric XM protein) or 5 mM sodium butyrate (for human A65E annexin II). Cell samples were analyzed by SDS-PAGE and transfected proteins were detected by Western blotting.

¹ Abbreviations used: CMV, cytomegalovirus; MMTV, mouse mammary tumour virus; NGF, nerve growth factor.

Dopamine Release Assay

Cells were cultured in 24-well trays for at least 3 d before assaying and were induced to express protein by the addition of dexamethasone (MMTV promoter-inducing agent), sodium butyrate (CMV promoter-inducing agent) overnight, or with a 5-h treatment with sodium butyrate followed by overnight incubation which was as effective as the continuous presence of sodium butyrate immediately before assaying. Assays involving a comparison between different cell types or treatments were always performed within one 24-well tray. PC12 cells were labeled for 2 h in RPMI 1640 containing 0.5 μ Ci of [7,8- 3 H]dopamine and 0.5 mM ascorbic acid. After labeling, cells were washed three times in Krebs-Ringer buffer (145 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4 , 10 mM glucose, 20 mM HEPES, 3 mM $CaCl_2$ at pH 7.4). Cells in each well were permeabilized by the addition of 300 μ l of permeabilization buffer (139 mM potassium glutamate, 5 mM EGTA, 20 mM PIPES, 2 mM ATP, 2 mM $MgCl_2$, 20 μ M digitonin at pH 6.5) for 6 min. After the removal of the permeabilization buffer, cells were challenged by the addition of buffer with either 0 Ca^{2+} or with 10 μ M free Ca^{2+} in the absence of digitonin. After stimulation for 5 or 15 min, the challenge buffer was removed, spun for 1 min at high speed in a microcentrifuge, and supernatant samples were counted in duplicate in a scintillation counter. Total dopamine content of the cells was determined by release with 1% Triton X-100. Released dopamine was calculated as a percentage of total cellular dopamine. All experiments were performed at room temperature. Each treatment was performed at least in triplicate wells per tray.

Electrophoresis and Western Blotting

The chimeric annexin II-p11 derivative and the A65E annexin II protein were detected in transfected cells by Western blotting. Cells were plated out and incubated with inducers overnight as described for the dopamine release assay. Cells were washed twice with Krebs-Ringer buffer and then solubilized in SDS-PAGE dissociation buffer (150 μ l/well). Cell samples were run on 15% acrylamide gels and then transferred to nitrocellulose paper. Transfer buffer containing 40% methanol (as opposed to the usual 20% methanol) in Tris-glycine (pH 8.6) was used to transfer the p11 subunit and the chimeric protein. Nitrocellulose paper was incubated in phosphate-buffered saline (PBS) containing 3% powdered milk for 45 min followed by the primary antibody diluted in PBS plus 3% powdered milk for 1 h. The monoclonal antibodies H21 (for p11) and H28 (for human A65E annexin II) were used at a 1:1000 (Osborn *et al.*, 1988) dilution, and the HH7 monoclonal (for rat annexin II and human A65E annexin II) was used at a 1:200 dilution (Thiel *et al.*, 1992). Anti-mouse peroxidase at a 1:400 dilution in PBS plus 3% powdered milk and 0.5% Tween 20 was used for second antibody incubations for 1 h. The reaction was visualized using enhanced chemiluminescence (Amersham).

Immunofluorescence

Trypsin-dissociated PC12 cells were plated at a density of 0.1×10^6 cells/well onto collagen-coated glass coverslips in 24-well trays and induced with dexamethasone and sodium butyrate as described previously. At 3 d *in vitro*, cells were fixed with 3.7% formaldehyde in PBS. Cells were permeabilized by incubation in 0.1% Triton X-100 and 0.3% bovine serum albumin in PBS (PBT) for 30 min. Incubation with the primary antibody was for 1 h at a 1:50 dilution in PBT for both H21 and HH7 antibodies. The second incubation was for 1 h with anti-mouse Ig (biotinylated) at a 1:100 dilution in PBT followed by Texas Red streptavidin at 1:50 in PBT for 30 min.

RESULTS

Generation of a Stably Transfected Cell Line Expressing the Chimeric Annexin II-p11 Protein

A chimeric annexin II-p11 cDNA coding for the NH₂-terminal domain (residues 1–18) of annexin II fused to the NH₂-terminus of the entire human p11 chain was transfected into PC12 cells using the pLK expression vector, which contains the cDNA insert under the control of the dexamethasone-inducible MMTV promoter (Hirt *et al.*, 1992; Harder and Gerke, 1993). A neomycin-resistant, stably transfected clone displaying dexamethasone-inducible expression of the chimeric protein was isolated. The chimeric protein (XM) was detected by Western blotting using the monoclonal antibody H21 (Osborn *et al.*, 1988), which recognizes the human p11 component of XM but does not cross-react with the endogenous rat p11 of PC12 cells (Figure 1). Figure 1 also reveals that the expression of XM is increased 2.3-fold by dexamethasone treatment. Western blotting of the PC12-XM cells with HH7 antibody (which detects endogenous rat annexin II) showed that levels of annexin II were also increased compared with control PC12 cells (and other transfected PC12 cell lines not expressing the XM protein; our unpublished observations; Figure 1), suggesting that XM expression stimulated annexin II overexpression. However, in contrast to the levels of the XM protein the expression of endogenous annexin II was not further increased following dexamethasone treatment.

Figure 1. Western blot of PC12-XM cells. Solubilized cells were run on a 15% acrylamide electrophoresis gel and transferred to nitrocellulose paper for Western blotting. Left panel shows the presence of the XM chimeric protein (the first 18 residues of the NH₂-terminus of human annexin II fused to the entire human p11 molecule) in PC12 cells stably transfected with the pLKXM plasmid. The H21 monoclonal antibody used for the detection reacts only with the transfected protein (the human p11 component of the chimera). Cells incubated in the presence of 1 μ M dexamethasone overnight (lane b) show increased XM protein expression. The XM protein runs slightly slower than p11 on SDS-PAGE. Right panel shows a Western blot using the HH7 antibody which detects endogenous rat annexin II. The transfected PC12-XM cells (lanes b and c) show increased annexin II expression compared with control nontransfected PC12 cells (lane a). The presence of dexamethasone did not further increase annexin II expression in PC12-XM cells.

The chimeric protein contains the entire p11-binding domain of annexin II [i.e., amino acids 1–14 (Johnsson *et al.*, 1988)] fused to the NH₂-terminus of the whole p11 protein. This allows for three binding sites, i.e., a p11-binding site in the NH₂-terminal annexin II domain, a p11-binding site in the p11 chain (dimerization), and an annexin II-binding site in the p11 chain (Harder and Gerke, 1993). High expression of XM thus leads to the formation of aggregates containing endogenous annexin II and p11 in addition to the chimeric protein. This should result in a sequestration of annexin II and p11, removing them from the functional pool. Immunofluorescence studies of PC12-XM cells using the HH7 antibody indeed showed the presence of large annexin II aggregates in a proportion (10–20%) of the cells after dexamethasone induction (Figure 2). These results are similar to those found previously in other cell lines, especially 208F rat fibroblasts and MDCK cells, although the fraction of transfected cells containing visible aggregates is somewhat higher in the latter cases (Harder and Gerke, 1993).

The annexin II monomer and/or the heterotetramer, i.e., annexin II-p11 complex are thought to play a role in regulated exocytosis since the purified proteins partially retard secretory run-down in permeabilized bovine chromaffin cells (Ali *et al.*, 1989). The chimeric protein can be considered to be a *trans*-dominant mutant of the annexin II-p11 heterotetramer (Harder and Gerke, 1993); therefore, secretion studies using the PC12-XM cell line would be useful to investigate the role of annexin II in Ca²⁺-dependent exocytosis in PC12 cells. PC12-XM cells and control PC12 cells were incubated overnight with the MMTV inducer, dexamethasone (1 μ M). To examine Ca²⁺-dependent exo-

cytosis directly, the cells were then briefly permeabilized with digitonin before stimulation with 0 or 10 μ M Ca²⁺. The permeabilized cells were assayed for Ca²⁺-dependent dopamine exocytosis over 5 min or 15 min. These data are shown in Figure 3A. Both cell types displayed Ca²⁺-stimulated exocytosis at the two time points. The basal levels of release of dopamine were lower for the PC12-XM cell line but the extent of Ca²⁺-dependent release of dopamine is similar for the PC12-XM and control cells at both time points. Since only a single XM-expressing cell line was available, we attempted to rule out problems due to clonal differences by using the PC12-XM cell line as its own control by comparing exocytosis with or without induction by dexamethasone. As shown in Figure 3B, essentially identical levels of exocytosis were seen with or without induction of the chimeric protein after overnight or 2-d treatment with dexamethasone. These results suggest that disrupting the normal annexin II distribution by expression of the chimeric protein has little effect on Ca²⁺-dependent secretion. It should be noted however that annexin II aggregates were only clearly observed in a proportion of the cells.

Production of Stably Transfected PC12 Cells which Overexpress Human A65E Annexin II

PC12 cells were transfected with the pcDNA3 AnxII plasmid and stably transfected clones were selected in the presence of neomycin. The annexin II cDNA coded for human p36 with the substitution of alanine 65 by glutamic acid (A65E; Thiel *et al.*, 1992). This substitution allowed the transfected protein to be detected with H28 monoclonal antibody [H28 does not detect

Figure 2. Immunofluorescence of PC12-XM cells. Cells were grown on collagen-coated glass coverslips for 3 d, fixed with 3.7% formaldehyde, and stained with HH7 antibody to detect both endogenous and transfected annexin II. Cells in the absence of dexamethasone (a) show cytoplasmic staining. A proportion of the cells which have been induced to express the chimeric protein by incubation with 1 μ M dexamethasone for 2 d (b) show lower cytoplasmic staining and large aggregates of precipitated annexin II. Bar, 20 μ m.

rodent or wild-type human annexin II (Osborn *et al.*, 1988)], which recognizes a discontinuous epitope with a crucial residue at position 65. The presence of Glu at this site is essential for H28 immunoreactivity (Johnson *et al.*, 1988). The A65E mutation does not alter the biochemical properties of annexin II (Thiel *et al.*, 1991).

Selection of a clonal cell line can be problematical since any observed differences may be due to clonal differences rather than expression of the transfected protein. We attempted to overcome this problem by initially making populations of stably transfected cells containing a mixture of clones. All of the mixtures isolated expressed high levels of human A65E annexin II as detected by Western blotting using the H28 antibody. None could be induced to significantly increase expression after sodium butyrate treatment which has previously been used to enhance expression from the CMV promoter (Gorman *et al.*, 1983). We wished to establish whether the levels of annexin II expressed in transfected cells were comparable to those seen due to physiological stimulation. NGF is known to elevate annexin II levels significantly in PC12 cells (Schlaepfer and Haigler, 1990). Transfected PC12 cells were incubated overnight in the presence or absence of sodium butyrate, and control nontransfected cells were incubated for 3 d with or without NGF (100 ng/ml). Expression of total annexin II (endogenous rat plus transfected human) was investigated by Western blotting with the HH7 antibody. Similar levels of annexin II were found in both the NGF-treated control cells and transfected cells (i.e., around 2.9-fold above control levels), although sodium butyrate treatment did not increase expression of annexin II in the transfected cells (Figure 4A). Immunofluorescence staining of the overexpressing cells with HH7 antibody was carried out to determine whether or not all cells in this mixed clonal population overexpressed annexin II. All transfected cells showed an increase in fluorescence staining and also a marked change in annexin II distribution with essentially all cells showing intense staining near the plasma membrane (Figure 4, b and c).

Ca²⁺-dependent secretion was assayed from transfected mixed clonal cells following digitonin permeabilization. Both control cells and transfected cells were treated with sodium butyrate overnight. Ca²⁺-dependent dopamine release was assayed over 5 and 15 min after an initial short digitonin permeabilization period. No significant differences in Ca²⁺-dependent dopamine release over basal values were found between the cell types (Figure 5). Thus, it seems that constitutive elevation of annexin II in PC12 cells does not affect Ca²⁺-dependent dopamine release from permeabilized PC12 cells.

Figure 3. Release of dopamine from digitonin-permeabilized control and PC12-XM cells. Cells were grown on collagen-coated 24-well plates for at least 3 d. In A, control nontransfected cells and PC12-XM cells were incubated overnight with 1 μM dexamethasone, labeled with [³H]dopamine for 2 h, and washed three times with Krebs-Ringer buffer. Cells were permeabilized for 6 min with digitonin and then stimulated for 5 or 15 min with buffer containing 0 or 10 μM Ca²⁺. [³H]dopamine release was calculated as a percentage of total [³H]dopamine in the cells. Although absolute secretion values differed between cell types, the Ca²⁺-dependent secretion was not significantly different. Each treatment was performed in triplicate. In B, XM cells were treated with no additions (N/A), 1 μM dexamethasone overnight (O/N Dex), or 1 μM dexamethasone for 2 d. Release of dopamine was assayed as described in A, except the stimulation period was for 15 min only. Again, Ca²⁺-dependent release was not significantly different between treatments. Each data point is the mean of four values. There were no significant differences in [³H]dopamine uptake between PC12 cell clones or as a consequence of dexamethasone treatment.

Figure 4. Western blot and immunofluorescence photomicrograph of a mixed PC12 cell population stably expressing human A65E annexin II. PC12 cells were transfected with the pcDNA3 AnxII plasmid. A population of cells containing a mixture of clones was cultured and analyzed by SDS-PAGE. After transfer to nitrocellulose paper, proteins were probed with HH7 antibody which detects both transfected annexin II and endogenous rat annexin II (panel a). The transfected cells expressed higher levels of annexin II than control nontransfected cells (lanes a and b compared with duplicate control lanes c and d) and levels of annexin II similar to those seen in control PC12 cells given 100 ng/ml NGF for 3 d (duplicate lanes e and f). The addition of 5 mM sodium butyrate overnight to transfected cells did not increase annexin II expression further (lane b compared with lane a). For immunofluorescence, cells were grown on collagen-coated glass coverslips for 3 d, fixed with 3.7% formaldehyde, and stained with HH7 antibody to detect both endogenous and transfected annexin II. Panel b shows nontransfected control cells and panel c shows transfected cells, both cell types incubated in the presence of sodium butyrate (5 mM overnight). Note the increased staining, loss of punctate staining, and the translocation of the stain to the plasma membrane in panel c. The micrographs in panels b and c were taken and processed under identical conditions. Bar, 20 μ m.

Figure 5. Release of dopamine from a mixed PC12 cell population stably expressing human A65E annexin II. Cells were transfected with the pcDNA3 AnxII plasmid and cultured as a population containing a mixture of clones. Cells were grown on collagen-coated 24-well trays for at least 3 d. All cells were incubated overnight with 5 mM sodium butyrate, labeled for 2 h with [^3H]dopamine, and washed three times with Krebs-Ringer buffer. Cells were permeabilized with digitonin for 6 min and then challenged with buffer containing either 0 or 10 μM Ca^{2+} for 5 or 15 min. [^3H]dopamine release was calculated as a percentage of total [^3H]dopamine. The percentage of increase in dopamine release over basal due to Ca^{2+} was similar in transfected cells (annexin II) and control nontransfected cells. Each treatment was made in triplicate and the assay was performed twice with similar results. Total [^3H]dopamine uptake was higher ($15,424 \pm 1,162$ cpm) for A65E compared with control ($10,689 \pm 251$ cpm) cells.

Isolation of a Stably Transfected Clonal PC12 Cell Line Showing Sodium Butyrate-inducible A65E Annexin II Expression

To analyze the effects of an acute rise in annexin II on exocytosis, we needed to select a clonal PC12 cell line which would only overexpress A65E annexin II in an inducible manner, e.g., in response to sodium butyrate. This was done by selecting a series of stably transfected clonal cell lines from the mixed clonal PC12 cells transfected with pcDNA3 Anx II. Clones were analyzed for sodium butyrate-inducible human annexin II expression using Western blotting with H28 and HH7 antibodies (Figure 6). Three of the clones (C4, C5, and C9) showed expression of the human A65E annexin II detected with H28 and an increase in total annexin II levels detected with HH7 with or without sodium butyrate treatment. One of the clones, referred to as C6, proved to be reproducibly responsive to induction by sodium butyrate, with A65E annexin II increasing 27-fold and total annexin II levels 3-fold, and was used for further investigations.

The PC12-C6 cells were treated with 100 ng/ml NGF for 1 d and 2 d to establish whether levels of annexin II found in response to sodium butyrate were similar to those obtained after NGF stimulation, i.e.,

Figure 6. Selection of clonal cell lines stably transfected with pcDNA3 AnxII. Neomycin-selected clones were cultured and analyzed by Western blotting. The top panel shows a blot using the monoclonal antibody H28 which detects human A65E annexin II, which is present in transfected PC12 cells only. Each clone was incubated in the absence (–) or presence (+) of sodium butyrate (5 mM overnight) to assess transfected protein inducibility. Note the complete absence of staining in the control nontransfected cells. Of the transfected clones, C6 appears to be most sensitive to induction as seen by the large increase in protein expression in response to sodium butyrate. The C4, C5, and C9 clones all expressed large amounts of A65E annexin II in the absence of sodium butyrate. The lower panel shows a Western blot using the HH7 monoclonal antibody which detects both endogenous rat annexin II and transfected human A65E annexin II. Again, the transfected C6 clone shows a large increase in total (transfected and endogenous) annexin II expression in response to sodium butyrate.

under more physiological conditions. Western blotting with the HH7 antibody to detect total annexin II (Figure 7) showed that, in this experiment, sodium butyrate increased expression by 4.6-fold to levels similar to those found after 1 d of NGF treatment (4.1-fold above control levels).

The distribution of total annexin II in C6 cells was examined by immunofluorescence, both before and after sodium butyrate treatment and compared with control nontransfected cells given the same treatment. As expected, the levels of annexin II in PC12-C6 cells increased in response to sodium butyrate as shown by markedly brighter fluorescence staining (Figure 8), but in addition there was also a marked apparent translocation of immunoreactivity from the cytoplasm to the plasma membrane (Figure 8d) as previously noted for the mixed clonal cells overexpressing annexin II. This phenomenon was also observed in control PC12 cells treated with NGF (our unpublished observations). Sodium butyrate did not affect the intensity or localization of immunofluorescence in control cells (Figure 8, a and b).

Figure 7. Western blot of stably transfected PC12-C6 cells using the HH7 antibody (to detect transfected and endogenous annexin II). Cells were treated with NGF (100 ng/ml for 1 d or 2 d) or 5 mM sodium butyrate overnight. There was a large increase in annexin II in response to NGF (lanes c and d) and in response to sodium butyrate (lane e) compared with cells with no additions (N/A, lanes a and b). The level of increased annexin II expression in the sodium butyrate cells was similar to that seen in response to NGF for 1 d.

The release of dopamine from the clonal PC12-C6 cell line after sodium butyrate treatment was compared with that from similarly treated control nontransfected PC12 cells. The percentage of increase in secretion over basal due to Ca^{2+} stimulation from permeabilized cells was not significantly different between the two cell lines, after either a 5-min (our unpublished observations) or 15-min stimulation (Figure 9A). Since annexin II was inducible in the PC12-C6 cells, we were also able to use these cells as their own controls to avoid problems of interpretation due to clonal variation. Release of dopamine was compared in cells with or without treatment with sodium butyrate. Induction of annexin II overexpression did not affect the extent of release found (Figure 9B). These results show that an acute increase in annexin II levels does not affect Ca^{2+} -dependent exocytosis in permeabilized PC12 cells.

Release of Dopamine from Control Nontransfected PC12 Cells Treated with NGF

Treatment of PC12 cells with NGF leads to a prolonged elevation of annexin II levels (Schlaepfer and Haigler, 1990, and confirmed here in Figures 4 and 7). Although NGF is known to induce differentiation of PC12 cells to a more neuronal-like phenotype, the PC12 cell line used in the present secretion studies shows little change in morphology and little neurite outgrowth. NGF treatment therefore can be used as an additional model for investigating the effects of a rise in annexin II upon PC12 cell exocytosis. Nontransfected PC12 cells were treated with 100 ng/ml NGF for 4 d. Ca^{2+} -dependent dopamine release after digitonin permeabilization was compared with the same cells not given NGF. Ca^{2+} -dependent dopamine release over 5 and 15 min was slightly reduced in the NGF-treated cells compared with the control cells (Figure 10). This result provides further evidence that an elevation of annexin II does not increase Ca^{2+} -dependent exocytosis from PC12 cells.

Figure 8. Immunofluorescence of control and PC12-C6 cells. Cells were grown on collagen-coated glass coverslips for 3 d, fixed with 3.7% formaldehyde, and stained with HH7 antibody to detect both endogenous and transfected annexin II. a and b show control nontransfected cells and c and d show PC12-C6 cells. Each cell type was incubated in the presence (b and d) and absence (panels a and c) of 5 mM sodium butyrate overnight. PC12-C6 cells given sodium butyrate (d) showed increased intensity of annexin II staining and in addition showed a marked translocation of staining from the cytoplasm to the plasma membrane compared with c. The micrograph in d was printed to demonstrate the plasma membrane staining and underestimates the increased intensity of fluorescence after sodium butyrate treatment. Bar, 20 μ m.

DISCUSSION

The strategy used here was to generate stably transfected PC12 cell lines showing an altered expression or intracellular localization of annexin II to allow assessment of the function of the protein in Ca^{2+} -dependent exocytosis. Similar strategies have been successfully used to demonstrate a role in exocytosis in PC12 cells for rab3A and rab3B using stable transfections (Weber *et al.*, 1996), and rabphilin (Komuro *et al.*, 1996) and Doc2 (Orita *et al.*, 1996) using transient transfections, indicating the validity of this approach in the present study.

Annexin II was thought to play a role in Ca^{2+} -dependent exocytosis, although much of the evidence for this was indirect. The major functional evidence for a role in exocytosis has come from studies using

permeabilized chromaffin cells in which secretory run-down is retarded by the addition of exogenous annexin II or the heterotetramer, i.e., the annexin II-p11 complex (Ali *et al.*, 1989; Sarafian *et al.*, 1991). Recently, it has been suggested that annexin II is essential for exocytosis in intact chromaffin cells based on its translocation to the plasma membrane on stimulation and an inhibitory effect of a synthetic peptide (Chasserot-Golaz *et al.*, 1996). The peptide was derived from the sequence surrounding the protein kinase C phosphorylation site and, therefore, could simply have been acting as an inhibitor of protein kinase C rather than a specific inhibitor of annexin II function.

In this study, we set out to manipulate annexin II levels in intact cells. In PC12 cells in culture, the addition of NGF can elevate annexin II many fold from

Figure 9. Release of dopamine from permeabilized PC12-C6 cells. Cells were grown on collagen-coated 24-well trays for at least 3 d. In A, control nontransfected cells and PC12-C6 cells were incubated overnight with 5 mM sodium butyrate, labeled with [^3H]dopamine for 2 h, and washed with Krebs-Ringer buffer. After permeabilization with digitonin for 6 min, cells were challenged with buffer containing 0 or 10 μM Ca^{2+} for 15 min. [^3H]dopamine release was calculated as a percentage of total [^3H]dopamine. There was no significant difference in either the basal or the Ca^{2+} -stimulated levels of secretion between the transfected PC12-C6 cells overexpressing human A65E annexin II and the control cells. The results are the mean of three different assays with three replicates per treatment per assay. In B, PC12-C6 cells were treated with or without sodium butyrate for 5 h, washed, incubated overnight, and then assayed for dopamine release as described in A (except both 5- and 15-min stimulation time was used). There was no significant difference in Ca^{2+} -dependent release due to sodium butyrate treatment. The data are the means of three values. There were no significant differences in [^3H]dopamine uptake between PC12 cell clones or as a consequence of sodium butyrate treatment.

Figure 10. Comparison of the release of dopamine from digitonin-permeabilized nontransfected PC12 cells with or without NGF treatment. Cells were grown on collagen-coated 24-well trays for 4 d either in the continuous presence of 100 ng/ml NGF or with no additions (N/A). Cells were labeled with [^3H]dopamine for 2 h and then washed three times with Krebs-Ringer buffer. Cells were permeabilized with digitonin for 6 min and then challenged with buffer containing either 0 or 10 μM Ca^{2+} for 5 or 15 min. [^3H]dopamine release was calculated as a percentage of total [^3H]dopamine. The percentage of increase in dopamine release over basal due to Ca^{2+} was slightly decreased at both stimulation times in the NGF-treated cells. Each treatment was made in triplicate and the assay was performed twice with similar results. Total [^3H]dopamine uptake was higher in cells without NGF ($48,978 \pm 839$ cpm) compared with NGF-treated cells ($41,820 \pm 1,371$ cpm).

an initial low level of expression (0.02% of cellular protein) (Schlaepfer and Haigler, 1990). We found here that treatment with NGF raised annexin II levels in the cells but did not result in increased Ca^{2+} -dependent exocytosis. The confounding effects of differentiation may, however, interfere with purely annexin II effects on exocytosis. One way of overcoming these problems is to stably transfect cells with cDNA-encoding proteins that will directly alter levels of annexin II. Using this approach, we have generated stably transfected PC12 cell lines expressing different levels of functional annexin II. The PC12-XM cell line expresses a chimeric protein consisting of the entire human p11 sequence fused C-terminally to the first 18 residues of human annexin II. This protein has the ability to bind to both endogenous rat annexin II and p11 eventually resulting in the formation of large aggregates containing the proteins. Such a manipulation is thus likely to reduce the functionally available pool of annexin II and p11 in the transfected PC12 cells. It should be noted however that a visible aggregate was only detected in a proportion of the cells and therefore we cannot rule out the possibility that not all of the cells

will have been affected by the XM construct masking the effect on secretion measured in the whole population. The interpretation of lack of any difference between control and XM cells could have been difficult due to clonal variation, but by using the XM cells as their own controls it was clear that induction of XM did not lead to any changes in secretion compared with uninduced cells. Other PC12 cells were transfected with human annexin II containing the substitution of alanine at position 65 by glutamic acid (A65E). This substitution does not alter biochemical properties (Thiel *et al.*, 1991) but does allow detection by the monoclonal antibody H28 (Osborn *et al.*, 1988). A stably selected cell population was established which consisted of a mixture of clones constitutively overexpressing human A65E annexin II. Another cell line (PC12-C6) was generated which could be induced to overexpress human A65E annexin II by the addition of sodium butyrate to the culture. Thus, we had the means to mislocalize annexin II, chronically overexpress and acutely overexpress the protein. All of these manipulated cell types displayed similar Ca^{2+} -stimulated exocytosis when compared with their parent nontransfected PC12 cells. We must conclude therefore that annexin II is not an important regulator of exocytosis in PC12 cells unless very low levels of the protein are already sufficient for its effect to be maximal. These results are the first *in vivo* examination of the effect of directly manipulating annexin II levels upon Ca^{2+} -dependent exocytosis.

The reconstitution of secretion assay in permeabilized chromaffin and PC12 cells has been used to identify several proteins involved in the regulation of exocytosis. To date, annexin II (Ali *et al.*, 1989; Sarafian *et al.*, 1991), 14-3-3 proteins (Morgan and Burgoyne, 1992), calmodulin (Chamberlain *et al.*, 1995), phosphatidylinositol transfer protein (Hay and Martin, 1993), phosphatidylinositol-4-phosphate 5-kinase (Hay *et al.*, 1995), protein kinase C (Morgan and Burgoyne, 1992), protein kinase A (Morgan *et al.*, 1993), and α -soluble NSF-attachment protein (Morgan and Burgoyne, 1995) have been identified as stimulators of permeabilized chromaffin or PC12 cell exocytosis. In another report, however, the reconstituting ability of cytosol was found to be unaffected by annexin II depletion (Wu and Wagner, 1991). The main stimulatory component of run-down reconstituting cytosol in chromaffin cells was identified as Exo1, which turned out to be a family of 14-3-3 proteins (Morgan and Burgoyne, 1992), and immunodepletion of these proteins from cytosol did reduce its reconstituting ability (Wu *et al.*, 1992). 14-3-3 proteins stimulate exocytosis by reorganization of the cortical actin cytoskeleton that normally acts as a barrier to exocytosis, thus allowing increased availability of secretory granules for release (Roth and Burgoyne, 1995). One of the domains conserved in the entire range of the 14-3-3 proteins is

homologous to the C terminus of the annexins and is most similar to annexin II (Aitken *et al.*, 1990). The failure to demonstrate a major role for annexin II in transfected PC12 cell exocytosis leads us to speculate that the annexin II effects observed in run-down chromaffin cells may be due to annexin II mimicking the effects of 14-3-3 proteins. Indeed a 16-residue synthetic peptide based on the common annexin II/14-3-3 protein domain partially inhibited Ca^{2+} -dependent exocytosis in permeabilized chromaffin cells (Roth *et al.*, 1993).

It may be possible that in the permeabilized chromaffin cell run-down assay, annexin II is simply delaying the loss of other factors required for exocytosis rather than exerting a direct stimulatory effect itself, since its major effect is to retard run-down and it is not stimulatory when added at later times of run-down (Ali and Burgoyne, 1990; Burgoyne and Morgan, 1990). A difference in cell type between chromaffin and PC12 cells also cannot be ruled out as a possibility for the different effects of annexin II on exocytosis. It has not been possible to repeat the annexin II reconstitution of run-down observed in permeabilized chromaffin cells in PC12 cells, since in our hands digitonin-permeabilized PC12 cells do not exhibit the run-down phenomenon. Other laboratories have reported similar findings (Wagner *et al.*, 1993). PC12 cells permeabilized by cell cracking do exhibit run-down, which can be reconstituted by a Ca^{2+} - and ATP-dependent protein called p145, purified from brain cytosol (Walent *et al.*, 1992).

PC12-C6 cells induced with sodium butyrate (as well as the mixed clonal cell population overexpressing annexin II) exhibit a translocation of annexin II immunoreactivity from the cytoplasm to the plasma membrane. If annexin II had a major role in Ca^{2+} -dependent exocytosis, we would expect induced PC12-C6 cells to show a marked increase in Ca^{2+} -dependent dopamine release due to its increased expression localized at the site of exocytosis. This was not the case, no difference in the extent of exocytosis in induced compared with uninduced PC12-C6 cells was observed and thus further indication that annexin II is not a major regulator of Ca^{2+} -dependent exocytosis in these cells.

The data presented here suggest that annexin II is not likely to have a major role in Ca^{2+} -dependent exocytosis leading to dopamine release from PC12 cells, since manipulations leading to a decrease or an increase in functional annexin II levels, including an increase in the plasma membrane localization of annexin II, were without effect on the extent of release. Nevertheless, the stably transfected cell lines generated here may be useful for the study of other suggested functions of annexin II.

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