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Role of Annexin A6 Isoforms in Catecholamine Secretion by PC12 Cells: Distinct Influence on Calcium Response

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ABSTRACT

Noradrenaline and adrenaline are secreted by adrenal medulla chromaffin cells via exocytosis. Exocytosis of catecholamines occurs after cell stimulation with various endogenous activators such as nicotine or after depolarization of the plasma membrane and is regulated by calcium ions. Cytosolic [Ca^{2+}] increases in response to cell excitation and triggers a signal-initiated secretion. Annexins are known to participate in the regulation of membrane dynamics and are also considered to be involved in vesicular trafficking. Some experimental evidence suggests that annexins may participate in Ca^{2+}-regulated catecholamine secretion. In this report the effect of annexin A6 (AnxA6) isoforms 1 and 2 on catecholamine secretion has been described. Overexpression of AnxA6 isoforms and AnxA6 knock-down in PC12 cells were accompanied by almost complete inhibition or a 20% enhancement of dopamine secretion, respectively. AnxA6-1 and AnxA6-2 overexpression reduced $\Delta[Ca^{2+}]_c$ upon depolarization by 32% and 58%, respectively, while AnxA6 knock-down increased $\Delta[Ca^{2+}]_c$ by 44%. The mechanism of AnxA6 action on Ca^{2+} signalling is not well understood. Experimental evidence suggests that two AnxA6 isoforms interact with different targets engaged in regulation of calcium homeostasis in PC12 cells. J. Cell. Biochem. 111: 168–178, 2010.

KEY WORDS: CATECHOLAMINE SECRETION; VESICULAR TRANSPORT; CALCIUM HOMEOSTASIS; ANNEXINS; NA^+/CA^{2+} EXCHANGER; PC12 CELLS

Upon stimulation neuroendocrine cells secrete catecholamines, noradrenaline and dopamine, that elicit pleiotropic action in a whole organism. Catecholamine secretion by chromaffin cells occurs in response to various stimuli such as nicotine or plasma membrane depolarization leading to elevation of cytosolic Ca^{2+} concentration $[Ca^{2+}]_c$ [Livett, 1984; Fornai et al., 2007]. Increase in $[Ca^{2+}]_c$ may occur due to release of Ca^{2+} from the intracellular stores (endoplasmic reticulum, ER) or by Ca^{2+} entry into cells. Release of Ca^{2+} from the ER is mediated by IP_3 and/or ryanodine receptors and leads to activation of the store operated calcium entry. On the other hand, plasma membrane depolarization evokes Ca^{2+} influx through plasma membrane voltage dependent calcium channels (VDCCs) and Na^+/Ca^{2+} exchangers (NCXs) working in the reverse mode. Resting $[Ca^{2+}]_c$ is maintained or restored due to removal of Ca^{2+} from the cytosol through the action of plasma membrane and ER Ca^{2+}-ATPases (PMCA and SERCA, respectively) and electrogenic transporters, NCXs [Philipson and Nicoll, 1992; Reeves, 1992; Duman et al., 2008]. Elevation of $[Ca^{2+}]_c$ is a prerequisite for transport of chromaffin granules carrying catecholamines, their fusion with plasma membrane and release of catecholamines to the extracellular milieu [Burgoyne and Morgan, 2003; Barclay et al., 2005].

The PC12 cell line, derived from rat adrenal medulla pheochromocytoma, is frequently used as a model to study Ca^{2+}-dependent catecholamine secretion [Westerink and Ewing, 2008]. Among the five subclasses of VDCCs [García et al., 2006], the L-type predominates in PC12 cells [Janigro et al., 1989; Colston et al., 1998].

Annexins, a family of Ca^{2+}/cholesterol-dependent phospholipid-binding proteins, are considered as candidate recruiters/scaffolding molecules organizing signaling protein complexes and controlling Ca^{2+}-dependent cellular processes [Gerke et al., 2005]. The effects of annexin A6 (AnxA6) on $[Ca^{2+}]_c$ were investigated in various cell types. It was shown that overexpression of AnxA6 in cardiomyocytes resulted in decreased resting levels of $[Ca^{2+}]_c$ and lower $[Ca^{2+}]_c$ amplitude ($\Delta[Ca^{2+}]_c$) upon stimulation

Abbreviations used: 2-APB, 2-aminoethoxydiphenyl borate; ACN, acetonitryle; AnxA6, annexin A6; ER, endoplasmic reticulum; HPLC, high pressure liquid chromatography; KB-R7943, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]i-sothiourea mesylate; NCX, Na^+/Ca^{2+} exchanger; SOC, store-operated calcium entry; VDCC, voltage-dependent calcium channel.

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[Gunteski-Hamblin et al., 1996]. Furthermore, modulation of $[Ca^{2+}]_c$ and K^+ conductance by AnxA6 was also reported in spinal cord and dorsal root ganglion neurons [Naciff et al., 1996]. Moreover, decrease in AnxA6 synthesis in transgenic mice resulted in anomalies in Ca^{2+} clearance and cardiomyocyte mechanical function [Song et al., 2002]. So far there is only one publication demonstrating distinct influence of AnxA6 isoforms on calcium fluxes; in A431 cells, that lack endogenous AnxA6, synthesis of exogenous AnxA6-1 inhibited voltage-dependent Ca^{2+} influx upon stimulation with epidermal growth factor (EGF) [Fleet et al., 1999]. A recent publication demonstrates an inhibition of store-operated calcium entry by membrane-bound AnxA6 [Monastyrskaya et al., 2009]. Taken together, these results suggest modulation of calcium transport systems by AnxA6. It is also known that AnxA6 can form ion channels in vitro at pH 6.0 or in the presence of GTP at pH 7.4 [Golczak et al., 2001; Kirilenko et al., 2002; Bandorowicz-Pikula et al., 2003]. This pore-forming property of AnxA6 may correspond with its ability to increase transmembrane Ca^{2+} conductance.

Due to alternative mRNA splicing AnxA6 exists in mammalian tissues as two isoforms, the full-length AnxA6-1 and AnxA6-2 lacking the 524-VAAEIL-529 sequence [Crompton et al., 1988; Moss and Crumpton, 1990]. AnxA6-1 prevails in normal cells [Kaetzel et al., 1994] while AnxA6-2 can be more abundant in some neoplastic ones, as it was observed in Swiss 3T3 fibroblasts subjected to neoplastic transformation [Edwards and Moss, 1995]. So far little is known about differences in physiological functions of these isoforms.

The aim of the present study was to dissect the role of AnxA6 isoforms in Ca^{2+} transport and their influence on catecholamine secretion. To address this problem the PC12 cell lines overexpressing each AnxA6 isoform were established. In another experimental setup the AnxA6 level in PC12 cells was lowered using the siRNA technique. We found that cells with enhanced AnxA6 isoforms level exhibited largely reduced dopamine secretion while in the AnxA6 knock-down cells secretion of dopamine was stimulated by approximately 20%. Moreover, AnxA6-1 and AnxA6-2 overexpression significantly reduced $\Delta[Ca^{2+}]_c$ upon depolarization, while AnxA6 knock-down increased $\Delta[Ca^{2+}]_c$. The mechanism of AnxA6 action on Ca^{2+} signalling is not well understood. Experimental evidence suggests that two AnxA6 isoforms interact with different targets engaged in regulation of calcium homeostasis in PC12 cells.

MATERIALS AND METHODS

MATERIALS

Cell culture medium and additives as well as ionomycin and nifedipine were purchased from Sigma (St. Louis, MO). KB-R7943 and 2-APB were from Tocris Cookson Inc. (Ellisville, MI). Fura-2/AM was from Molecular Probes (Carlsbad, CA). Nifedipine, ionomycin, KB-R7943, and Fura-2/AM were dissolved in dimethylsulfoxide (DMSO) and diluted so that the final DMSO concentration did not exceed 0.1%. Restriction enzymes were from Promega (Madison, WI). X-tremeGENE siRNA Transfection Reagent was from Roche Applied Science (Mannheim, Germany) and siRNAs were from Ambion (Austin, TX). Reagents for HPLC were from Sigma (HPLC grade) and the HPLC column from Merck (Darmstadt, Germany).

CELL CULTURE

PC12 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, heat-inactivated 5% fetal bovine serum and 10% horse serum (Gibco, Invitrogen). Cells were grown in adhesion in humidified atmosphere of 5% CO_2/95% air at 37°C. Catecholamine secretion was evoked by plasma membrane depolarization. For that purpose cells were incubated at room temperature in 10 mM $NaHCO_3$, 1 mM KH_2PO_4, 10 mM glucose, 20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$ with either 125 mM NaCl, and 5 mM KCl (resting Locke's solution) or with 71 mM NaCl and 59 mM KCl (stimulating Locke's solution) or with 118 mM KCl and without NaCl (the K-Locke's solution).

RNAI

Silencing of the AnxA6 gene expression and hence reduction of the endogenous AnxA6 synthesis was achieved by transfection of PC12 cells with pre-designed siRNA against rat *ANXA6* mRNA (NM_024156) from Ambion. PC12 cells transfected with scrambled siRNA (Ambion) were used as a control. Briefly, PC12 cells were seeded in 35 mm diameter dishes at 3×10^5 cells per dish (at 40–50% confluence) the day before transfection. The siRNAs were added to the cells at a final concentration of 30 nM in complexes with X-tremeGENE siRNA Transfection Reagent (Roche Applied Science), according to the manufacturer's protocol. Transfection efficiency was evaluated using a fluorescence microscope (Observer.Z1, Zeiss, Goettingen, Germany) on cells transfected with Cy3-labeled siRNA against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) and was close to 100%. The level of AnxA6 as well as calcium homeostasis and dopamine secretion were determined 48 h after transfection.

OVEREXPRESSION OF ANXA6

To generate PC12 cells overexpressing human AnxA6 we used the pEGFP-N3 vector (Clontech Laboratories, Takara Bio Company, Mountain View, CA). For the construction of pEGFP-AnxA6-1 and pEGFP-AnxA6-2 the respective cDNAs originally contained in the pRSET-5d vector were subcloned into pEGFP-N3 at the *Hin*dIII/*Xho*I restriction sites as previously described [Strzelecka-Kiliszek et al., 2008]. Due to the fact that EGFP may interfere with Fura-2 fluorescence, pN3 and pAnxA6-1or2 lacking the EGFP cDNA were used during measurements of $[Ca^{2+}]_c$. These plasmids were obtained by digesting the original pEGFP-N3, pEGFP-AnxA6-1 or pEGFP-AnxA6-2 vectors with *Not*1 and *Psp*OMI restriction enzymes, which excise EGFP cDNA. PC12 cells were transfected using the X-tremeGENE siRNA Transfection Reagent according to the manufacturer's protocol. For selection of stable transfectants the cells were grown in a medium containing G418 (1 mg/ml, Sigma). Experiments were performed after a month of selection. Transfection efficiency was controlled in cells overexpressing EGFP and EGFP-AnxA6 and was about 85–90%. Stable overexpression of AnxA6 isoforms was confirmed by RT-PCR and Western blotting.

INTRACELLULAR $[CA^{2+}]$

Time-dependent changes in $[Ca^{2+}]_c$ of PC12 cells were measured by means of Fura-2 (Molecular Probes) fluorescence using a Shimadzu

RF5000 spectrofluorimeter. Before measurements, 5×10^6 PC12 cells were incubated with 1 μM Fura-2/AM in 3 ml of DMEM without serum at 37°C for 15 min. At the end of incubation cells were washed in a resting Locke's solution, suspended in 1.585 ml of the same solution and $[Ca^{2+}]_c$ was monitored. Cell membrane depolarization was evoked by addition of 1.415 ml of the K-Locke's solution leading to extracellular $[K^+]$ elevation to 59 mM while $[Na^+]$ decreased to 66 mM. To inhibit NCX activity the cells were suspended in a resting Locke's solution in which 125 mM NaCl was substituted with 125 mM choline chloride (the Na^+-free Locke's solution). In some experiments 10 μM nifedipine, 10 μM KB-R7943, 100 nM thapsigargin or 20 μM 2-APB (or 0.05% DMSO as a control) were added prior to depolarization and the cells were incubated until the new steady-state level was established. The fluorescence signal was calibrated in each run by the addition of 8 μM ionomycin followed by 100 mM EGTA, pH 7.5. For measurement of relative Ca^{2+} content in intracellular calcium stores in PC12 cell line the addition of 8 μM ionomycin was followed by the addition of 1.5 mM $CaCl_2$ and 1 mM EGTA for calibration. $[Ca^{2+}]_c$ was calculated as in [Grynkiewicz et al., 1985]. The excitation wavelengths for Fura-2 were 340 and 380 nm changing every second and the emission signal was collected at 510 nm.

DOPAMINE SECRETION

Dopamine concentration was determined using high-performance liquid chromatography with electrochemical detection (HPLC-ECD, Merck, Darmstadt, Germany). Briefly, cells were seeded 1 day before experiment on 35 mm diameter dishes in DMEM supplemented with serum. At the beginning of the experiment cells were incubated for 5 min in darkness in 800 μl of the resting Locke's solution at room temperature. Then, 15% acetonitrile (ACN) and 25% methanol were added to an aliquot of 100 μl of the solution which was immediately placed on ice. The resting Locke's solution was changed for the stimulating Locke's solution and cells were incubated for another 10 min. Aliquots of 100 μl of the solution were taken at time intervals of 1, 5, and 10 min and treated with ACN/methanol as stated above. After filtration through syringe filters (Millipore, Bedford, MA) the supernatants were subjected to HPLC analysis using a 25 cm Purospher reverse phase C-18 column (Ø 5 μm, LiChroCART 250-4, Merck). Twenty microliter aliquots were separated in a mobile phase consisting of 107 mM NaH_2PO_4, 1 mM EDTA, 0.6 mM 1-octanesulfonic acid (OSS) and 8% (v/v) ACN, pH 4.1, at a flow rate of 0.7 ml/min at 25°C. For detection of dopamine an amperometric detector was used (L-3500A, LaChrom, Merck). Results were expressed in nmoles of dopamine per mg of protein. To gain the reference protein concentration, after each experiment the cells from the dish were harvested in a remaining (400 μl) Locke's solution supplemented with ACN and methanol. The samples were centrifuged at 13,000*g* for 15 min at 4°C. Resulting pellets were dissolved in 1 M NaOH and protein concentration was determined by the Bradford assay.

CELL LYSIS AND WESTERN BLOTTING

In order to estimate changes in AnxA6 level after transfection the cells were incubated in a lysis solution containing 0.5% Triton X-100, 50 mM Tris–HCl, pH 7.5, 80 mM NaCl, 20 mM EGTA and 10 μg/ml protein inhibitor cocktail (PIC) for 30 min at 4°C. Then, the lysates were centrifuged at 1,000*g*, for 5 min. Protein concentration in the supernatant was determined by the Bradford assay and the presence of AnxA6 was analyzed by Western blotting. Aliquots of cell lysates containing 30–50 μg of protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then electro-transferred into nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk and incubated with primary anti-AnxA6 (1:1,500, Abcam, Cambridge, UK), anti-NCX1 (1:2,000, Abcam), anti-L-VDCC α1 subunit (1:2,000, Abcam) or anti-β-actin (1:10,000, Calbiochem, Darmstadt, Germany) antibody at room temperature for 1 h. A secondary antibody conjugated to horseradish peroxidase was visualized with the enhanced chemiluminescence reagent (Amersham, Piscataway, NJ). Band intensities were analyzed densitometrically by means of the Ingenius Bioimaging system with the GeneTools 3.06 software (Syngene, Cambridge, UK).

STATISTICAL ANALYSIS

All experiments were repeated 3–4 times and conducted in triplicates unless otherwise stated. The results were expressed as the mean ± SD. Statistical significance of existing differences was assessed using Student's *t*-test for comparison of two groups and ANOVA for comparison of three groups of results.

RESULTS

CALCIUM-DEPENDENT RELEASE OF DOPAMINE BY PC12 CELLS

Figure 1 shows that stimulation of PC12 cells (depolarization of the plasma membrane) was followed by dopamine secretion; extracellular dopamine concentration increased almost twofold after 5 min, and 3 times after 10 min of stimulation. In PC12 cells plasma membrane depolarization resulted in rapid influx of Ca^{2+} (data not

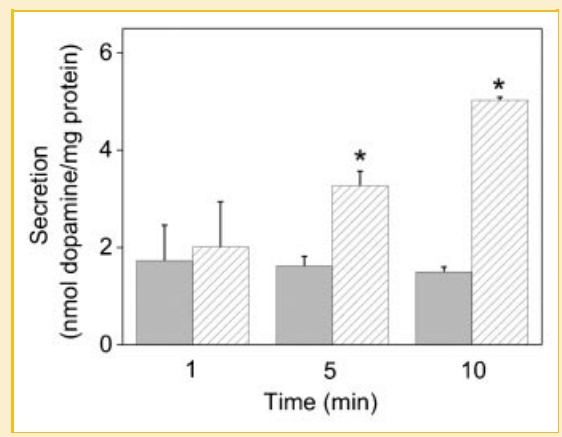

Fig. 1. Influence of PC12 cell stimulation on catecholamine secretion. Dopamine secretion from resting (filled bars) and stimulated cells (striped bars) was measured in the extracellular fluid by HPLC-ECD. Plasma membrane depolarization was evoked by elevation of KCl concentration to 59 mM for 10 min and extracellular dopamine content was determined at the indicated time points, as described in Materials and Methods Section. Bars represent mean values of three experiments ± SD. ANOVA was used for comparison of extracellular dopamine content at different time points after stimulation; $^*P \leq 0.05$.

TABLE I. Effect of Nifedipine or KB-R7943 on Ca^{2+} Influx to Stimulated Non-Transfected PC12 Cells*

Inhibitor concentration (μM)	$\Delta[Ca^{2+}]_c$ (nM)	
	+ Nifedipine	+ KB-R7943
0	312 ± 26	312 ± 26
1	116 ± 14	225 ± 11
5	88 ± 10	170 ± 18
10	29 ± 13	157 ± 28
20	22 ± 3	150 ± 20

*Cells were preincubated in resting Locke's solution supplemented with appropriate inhibitors for 5 min. For plasma membrane depolarization KCl concentration was elevated to 59 mM, as described in Materials and Methods Section. $\Delta[Ca^{2+}]_c$ corresponds to the difference between the resting $[Ca^{2+}]_c$ and the highest $[Ca^{2+}]_c$ recorded immediately after plasma membrane depolarization. Mean values of three experiments ± SD are shown.

shown). Presence of 10 μM nifedipine, an inhibitor of the L-type VDCC [Triggle, 2006], reduced the increase in $[Ca^{2+}]_c$ ($\Delta[Ca^{2+}]_c$) by 91% (Table I). Presence of 10 μM KB-R7943, an inhibitor of NCX [Takai et al., 2004], exerted a smaller effect and reduced the calcium influx by 50% (Table I). When added together, 10 μM nifedipine and 10 μM KB-R7943 completely inhibited $[Ca^{2+}]_c$ elevation upon cell stimulation (data not shown). These results indicate that Ca^{2+} influx into PC12 cells is mediated mainly by VDCCs [Janigro et al., 1989; Duman et al., 2008] and possibly by NCX working in the reverse mode [Mahapatra et al., 2004], similarly to what was described in muscle cells [Kupittayanant et al., 2006]. As it could be expected, changes in intracellular $[Ca^{2+}]$ affect dopamine secretion. In fact, cells stimulated in the presence of 10 μM nifedipine secreted reduced amount of dopamine. This inhibition reached 90% and 54% of the control value after 2.5 and 10 min of stimulation, respectively (data not shown).

OVEREXPRESSION OF *ANXA6* REDUCES ELEVATION OF $[CA^{2+}]_c$ UPON STIMULATION OF PC12 CELLS

To test a putative effect of AnxA6 overexpression on calcium homeostasis the PC12 cells were stably transfected with plasmids carrying the coding sequence of AnxA6-1 (pAnxA6-1) or AnxA6-2 (pAnxA6-2) or with a plasmid without any cDNA insertion (pN3). Enhanced AnxA6 gene expression in transfected cells was confirmed by Western blotting (Fig. 2A). Cells transfected with the pN3 vector as well as non-transfected cells have similar $[Ca^{2+}]_c$ under resting conditions and upon stimulation (Table II) while overexpression of either AnxA6 isoform led to significantly higher $[Ca^{2+}]_c$ under resting conditions in comparison to that in the pN3 transfected cells. Moreover, depolarization-induced maximal

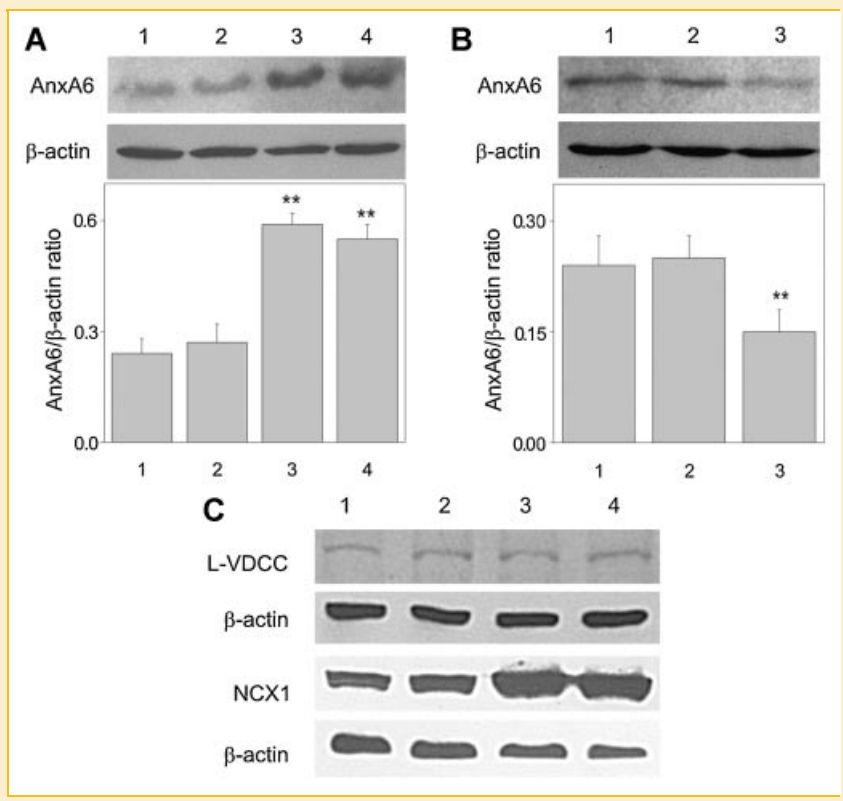

Fig. 2. AnxA6 content in *ANXA6*-transfected (A) or siRNA-transfected (B) PC12 cells. A: Protein level of AnxA6 was analyzed by Western blotting (upper panel) and quantified densitometrically (lower panel). Order in the gel: non-transfected cells (lane 1), pN3 (lane 2), pAnxA6-1 (lane 3), and pAnxA6-2-transfected (lane 4) cells. B: PC12 cells were transfected with siRNA recognizing AnxA6 mRNA (AnxA6 siRNA, lane 3) or control siRNA (lane 2); non-transfected cells (lane 1). The AnxA6 level (lower panel) is shown as the ratio of AnxA6 to β-actin band intensity. The bars represent mean values ± SD of three experiments. A: ANOVA or (B) Student's *t*-test was used for comparison of transfected cells; $^{**}P \leq 0.01$. (C) L-VDCC and NCX1 content in PC12 cells. Protein level of L-VDCC α1 subunit (upper panel) and of NCX1 (lower panel) was analyzed by Western blotting. Order in the gel: non-transfected cells (lane 1), and cells transfected with pN3 (lane 2), with pAnxA6-1 (lane 3), or with pAnxA6-2 (lane 4).

TABLE II. $[Ca^{2+}]_c$ in Resting or Stimulated PC12 Cells Overexpressing AnxA6*

Inhibitor	Vector used for cells transfection	$[Ca^{2+}]_c$ (nM)		
		Resting conditions	Stimulating conditions	$\Delta [Ca^{2+}]_c$
None	Non-transfected cells	55 ± 20	394 ± 34	312 ± 20
	pN3	65 ± 22	365 ± 38	301 ± 22
	pAnxA6-1	113 ± 26***	318 ± 42	205 ± 19***
	pAnxA6-2	96 ± 28***	223 ± 40***	127 ± 12***
Nifedipine (10 μM)	Non-transfected cells	70 ± 6	99 ± 16	29 ± 13
	pN3	76 ± 6	104 ± 10	30 ± 11
	pAnxA6-1	87 ± 15	101 ± 12	14 ± 6*
	pAnxA6-2	96 ± 11	104 ± 7	8 ± 7*
KB-R7943 (10 μM)	Non-transfected cells	130 ± 30	287 ± 37	157 ± 28
	pN3	125 ± 17	282 ± 16	159 ± 29
	pAnxA6-1	124 ± 22	268 ± 26	144 ± 18
	pAnxA6-2	125 ± 8	194 ± 10	70 ± 12***

The effect of nifedipine or KB-R7943.
*Cells were pre-incubated in resting Locke's solution without or supplemented with appropriate inhibitors for 5 min. For plasma membrane depolarization KCl concentration was elevated to 59 mM, as described in Materials and Methods Section. $\Delta[Ca^{2+}]_c$ corresponds to the difference between the resting $[Ca^{2+}]_c$ and the highest $[Ca^{2+}]_c$ recorded immediately after plasma membrane depolarization. Mean values of three experiments ± SD are shown. Student's *t*-test was used for comparison of pN3 with pAnxA6-1 or pAnxA6-2-transfected cells; $^{***}P \leq 0.005$; $^{*}P \leq 0.05$.

$[Ca^{2+}]_c$ was lower in pAnxA6-transfected cells than in the appropriate control (318 and 223 nM in AnxA6-1 and AnxA6-2 overexpressing cells, respectively vs. 365 nM in pN3-transfected cells) but the difference was statistically significant only in the case of pAnxA6-2-transfected cells. Hence, depolarization-induced $[Ca^{2+}]$ rise in pAnxA6-1-transfected cells was reduced by 32% ($\Delta[Ca^{2+}]_c$ amounted to 205 nM vs. 301 nM in control pN3-transfected cells). In case of pAnxA6-2-transfected cells the effect was even more pronounced, as $\Delta[Ca^{2+}]_c$ was lowered by 58% in comparison to that observed in pN3-transfected cells (Table II).

To test an effect of AnxA6 isoforms 1 and 2 overexpression on secretory properties of PC12 cells, depolarization-induced increase in dopamine concentration in the extracellular milieu of cells transfected with appropriate plasmids was measured (Table III). It was found that transfection of cells with the pN3 plasmid did not affect resting dopamine secretion (Table III). Elevation of AnxA6-1 content resulted in decreased dopamine secretion by cells under resting as well as stimulating conditions by 57% and 52%, respectively. In cells with altered AnxA6-2 content dopamine secretion was below the detection level (Table III).

CA^{2+} SIGNALING IN PC12 CELLS WITH DECREASED CONTENT OF ANXA6 BY SIRNA

To support the observations described hitherto an alternative approach based on siRNA induced knock-down of AnxA6 encoding genes was applied. Table IV shows an effect of AnxA6 gene silencing on $[Ca^{2+}]_c$ transients in PC12 cells challenged by high KCl

TABLE III. Dopamine secretion from resting or stimulated PC12 cells overexpressing AnxA6*

Vector used for cells transfection	Dopamine (nmol/mg protein)		
	Resting conditions	Stimulating conditions	Δ
Non-transfected cells	1.61 ± 0.05	3.27 ± 0.10	1.65 ± 0.10
pN3	1.62 ± 0.06	2.64 ± 0.05	1.02 ± 0.03
pAnxA6-1	0.69 ± 0.02***	1.27 ± 0.07***	0.58 ± 0.05***
pAnxA6-2	0.00	0.00	0.00

*Extracellular dopamine content measurement after 5 min of incubation was performed as described in Materials and Methods Section. Mean values of three experiments ± SD are shown. Student's *t*-test was used for comparison of pN3 with pAnxA6-1-transfected cells; $^{***}P \leq 0.005$.

TABLE IV. $[Ca^{2+}]_c$ and Dopamine Secretion From Resting and Stimulated PC12 Cells With AnxA6 Knock-Down*

siRNA used for cells transfection	Resting conditions	Stimulating conditions	Δ
$[Ca^{2+}]_c$ (nM)			
Negative siRNA	75 ± 28	379 ± 38	304 ± 15
AnxA6 siRNA	88 ± 21	525 ± 42***	438 ± 30***
Dopamine (nmol/mg protein)			
Negative siRNA	1.02 ± 0.07	2.96 ± 0.07	1.94 ± 0.09
AnxA6 siRNA	1.00 ± 0.08	3.59 ± 0.10***	2.59 ± 0.07***

*Cells were pre-incubated in resting Locke's solution for 5 min. For plasma membrane depolarization KCl concentration was elevated to 59 mM, as described in Materials and Methods Section. $\Delta[Ca^{2+}]_c$ corresponds to the difference between the resting $[Ca^{2+}]_c$ and the highest $[Ca^{2+}]_c$ recorded immediately after plasma membrane depolarization. Extracellular dopamine content measurement after 5 min of preincubation was performed as described in Materials and Methods Section. Mean values of three experiments ± SD are shown. Student's *t*-test was used for comparison of pN3 with pAnxA6-1 or pAnxA6-2-transfected cells; $^{***}P \leq 0.005$.

concentration. It has previously been reported that cardiomyocytes from the *ANXA6* knock-out mice had altered intracellular signaling and mechanical properties [Song et al., 2002]. PC12 cells transfected with siRNA against AnxA6 had AnxA6 protein content decreased by about 40% 48 h after transfection (Fig. 2B). As shown in Table IV knock-down of AnxA6 did not significantly affect the resting $[Ca^{2+}]_c$ but resulted in substantially higher (reaching 144% of control) Ca^{2+} rise upon cell depolarization.

Stimulation of the calcium response in cells with silenced *ANXA6* gene was accompanied by enhanced (reaching 121% of control) dopamine secretion (Table IV).

THE EFFECT OF ANXA6 ISOFORMS ON VDCC AND NCX ACTIVITY

Depolarization of the plasma membrane may activate two different Ca^{2+} routes allowing Ca^{2+} to enter cells. They are the voltage-gated calcium channel route, which needs depolarization of the plasma membrane to get opened, and the sodium/calcium exchanger route operating in the reverse mode. The latter is designed to remove an excess of Ca^{2+} from post-stimulated cells, but in depolarized cells it may support Ca^{2+} entry driven by reversed electrochemical potential of sodium cations. To define which route of Ca^{2+} entry is modulated by AnxA6 isoforms, the cells transfected with appropriate plasmids were stimulated by KCl in the presence of 10 μM nifedipine (L-VDCC inhibitor) or 10 μM KB-R7943 (NCX inhibitor). As shown in Table II depolarization-induced $[Ca^{2+}]_c$ rises are substantially reduced (from 301 to 30 nM, i.e., by 91%) in cells treated with nifedipine.

A relatively similar effect was observed in AnxA6-1 and AnxA6-2 overexpressing cells. Nifedipine-evoked reduction in $\Delta[Ca^{2+}]_c$ reached approx. 90% (from 205 to 14 nM and 127 to 8 nM in AnxA6-1 or AnxA6-2 overexpressing cells, respectively; see Table II). It must be stressed that the maximal levels of $[Ca^{2+}]_c$ are the same in all cells tested, thus any difference in $[Ca^{2+}]_c$ transients results from different $[Ca^{2+}]_c$ under resting conditions. It also indicates that the contribution of the nifedipine-independent Ca^{2+} entry route is very similar in both control and pAnxA6-transfected cells. Lower $\Delta[Ca^{2+}]_c$ values in pAnxA6-transfected cells resulting from higher resting $[Ca^{2+}]_c$ might suggest decreased capability of these cells to maintain the basal Ca^{2+} content rather than inhibition of other, nifedipine-independent Ca^{2+} transporting systems. Data shown in Table II also indicate that L-VDCC is a major route of plasma membrane depolarization-induced Ca^{2+} entry into PC12 cells.

Preincubation of pN3-transfected cells with KB-R7943 results in substantial elevation of the resting $[Ca^{2+}]_c$ (from 65 to 125 nM; see Table II). This might suggest that NCX is to some extent involved in maintaining the resting Ca^{2+} level despite its relatively low affinity to Ca^{2+}. The same resting $[Ca^{2+}]_c$ was observed in the pAnxA6-transfected cells (approx. 125 nM). This indicates that the effect of KB-R7943 on resting $[Ca^{2+}]_c$ was far less pronounced in cells transfected with pAnxA6-1 or pAnxA6-2 than in controls. In addition, the results summarized in Table II indicate that depolarization-induced $[Ca^{2+}]_c$ transients are very similar in control and pAnxA6-1-transfected cells pretreated with KB-R7943 while in the case of pAnxA6-2-transfected cells this relative inhibitory effect is much lower, mainly because of initially decreased $\Delta[Ca^{2+}]_c$ in pAnxA6-2-transfected cells (127 nM in comparison with 301 nM in pN3- and 205 nM in AnxA6-1-transfected cells).

Comparison of the $\Delta[Ca^{2+}]_c$ values found for all cell lines studied in the absence and presence of KB-R7943 shows that KB-R7943 is a less potent inhibitor of calcium entry into AnxA6-1 overexpressing cells ($\Delta[Ca^{2+}]_c$ decreased by 30%) than in controls ($\Delta[Ca^{2+}]_c$ decreased by 50% and 47% for non-transfected and pN3-transfected cells, respectively), and in AnxA6-2 overexpressing cells ($\Delta[Ca^{2+}]_c$ reduced by 45%; see absolute values in Table II).

In summary, (i) inhibition of NCX only slightly affects the initially increased resting $[Ca^{2+}]_c$ in AnxA6 overexpressing cells while it significantly increases $[Ca^{2+}]_c$ in controls. (ii) Experiments based on the inhibition of NCX by KB-R7943 revealed that contribution of this cation exchanger to Ca^{2+} entry is relatively more pronounced in control than in transfected cells. Almost complete inhibition by nifedipine of Ca^{2+} entry into all cells tested indicates that the contribution of NCX is rather negligible. On the other hand, significantly decreased calcium transient in AnxA6-2 cells under conditions of inhibited NCX in comparison to controls and AnxA6-1 overexpressing cells convincingly indicates that other Ca^{2+} transport routes are sensitive to the excess of AnxA6-2. Since, these effects are observed upon addition of KCl, voltage-gated calcium channels should be taken into account. This suggestion is supported by the result showing very low or no effect of plasma membrane depolarization in nifedipine treated pAnxA6-2-transfected cells on $\Delta[Ca^{2+}]_c$ (Table II).

Figure 2C demonstrates that the content of L-VDCC α1 subunit was the same in all cell types indicating that increased AnxA6 content modified its activity rather than gene expression. Unexpectedly, the amount of NCX was elevated by 60% in pAnxA6-1-transfected cells and by 80% in pAnxA6-2-transfected cells. This may reflect some adaptive changes which counteract an elevated resting $[Ca^{2+}]_c$ in cells with increased AnxA6 level (Table II). These changes may involve remodeling of other systems controlling calcium homeostasis including up-regulation of store operated calcium entry.

THE EFFECT OF OVEREXPRESSION OF *ANXA6* IN PC12 CELLS ON RESTING $[CA^{2+}]_C$

The NCX-dependent Ca^{2+} removal from cells may interfere with measurements of activity of other routes of Ca^{2+} entry. Therefore, the putative participation of store-operated calcium channels in the intracellular Ca^{2+} homeostasis was determined in the Na^+-free Locke's solution (described in the Materials and Methods Section) in which the NCX activity was completely abolished. The aim of this experiment was to verify the concept that AnxA6 may influence store-operated Ca^{2+} entry (SOCE) into PC12 cells. Figure 3A,C shows that Ca^{2+} entry was significantly enhanced in cells overexpressing AnxA6 isoform 2 in comparison to pN3-transfected cells. Moreover, the effect was independent of the Na^+ presence in the solution (Fig. 3B). These results indicate that SOCE is stimulated in pAnxA6-2-transfected cells and/or Ca^{2+} removal from the cytosol to the ER and to the extracellular space is inhibited. Addition of 20 μM 2-APB, a SOCE inhibitor [Bootman et al., 2002], effectively inhibited Ca^{2+} entry in pN3-transfected and AnxA6-1 and -2-transfected cells (Fig. 4) resulting in the similar Ca^{2+} transient in all cell types.

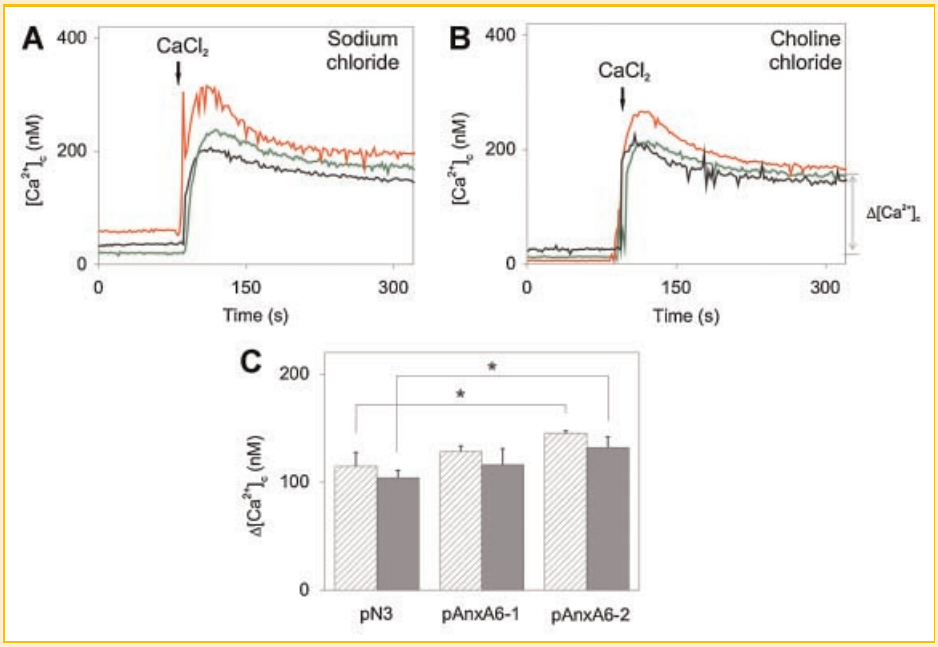

Fig. 3. Effect of Na^+ on SOCE in PC12 cells with AnxA6 isoforms overexpression. $[Ca^{2+}]_c$ was measured spectrofluorimetrically in Fura-2 loaded cells suspended and pre-incubated for 10 min in the resting Locke's solution without $CaCl_2$, supplemented with 100 nM thapsigargin. The solution contained sodium chloride (A and striped bars in C) or choline chloride (B and gray bars in C). Addition of 1.5 mM $CaCl_2$ is indicated with arrow. $\Delta[Ca^{2+}]_c$ stands for the difference in $[Ca^{2+}]_c$ before addition of $CaCl_2$ and the new steady state. Upper panels (A,B) present representative traces: pN3 (black line), pAnxA6-1 (green line) and pAnxA6-2-transfected (red line) cells. Bars in (C) present mean values of $\Delta[Ca^{2+}]_c$ of three experiments ± SD; $^*P \leq 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

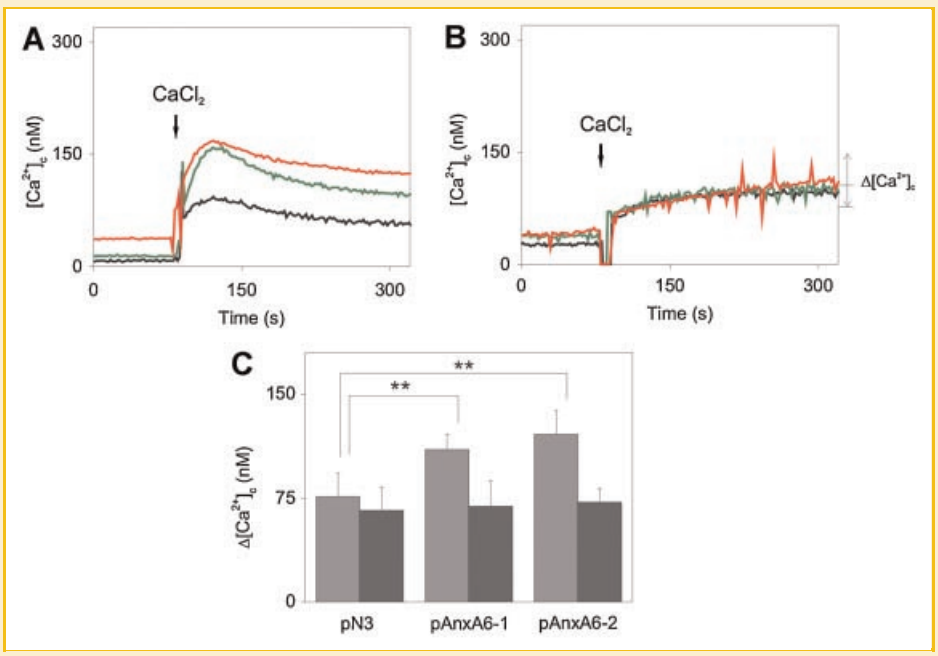

Fig. 4. Effect of 2-APB on SOCE in PC12 cells with AnxA6 isoforms overexpression. $[Ca^{2+}]_c$ was measured spectrofluorimetrically in Fura-2 loaded cells in the resting Locke's solution without $CaCl_2$, in which sodium chloride was substituted by choline chloride. The cells were pre-incubated for 5 min without inhibitor (A and light gray bars in C), or in the presence of 20 μM 2-APB (B and dark gray bars in C). Addition of 1.5 mM $CaCl_2$ is indicated with arrow. $\Delta[Ca^{2+}]_c$ stands for the difference in $[Ca^{2+}]_c$ before addition of $CaCl_2$ and the new steady state. Upper panels (A,B) present representative traces: pN3 (black line), pAnxA6-1 (green line) and pAnxA6-2-transfected (red line) cells. Bars in (C) present mean values of $\Delta[Ca^{2+}]_c$ of three experiments ± SD; $^{**}P \leq 0.01$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

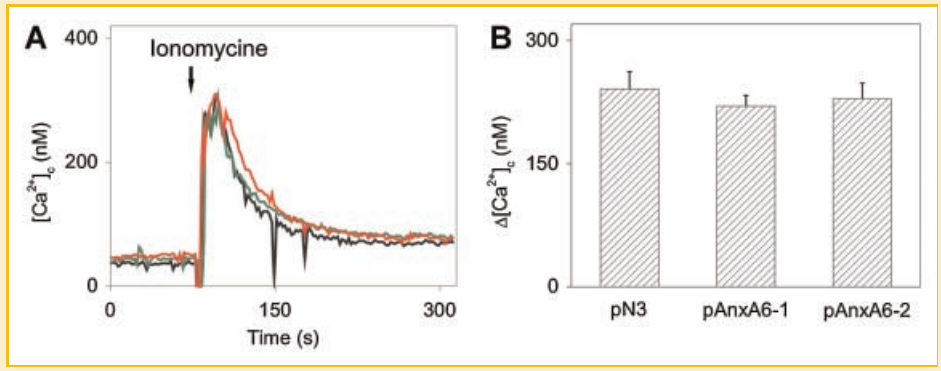

Fig. 5. Relative Ca^{2+} content in intracellular calcium reservoirs in PC12 cell line. $[Ca^{2+}]_c$ was measured spectrofluorimetrically in Fura-2 loaded cells pr-incubated for 5 min in the resting Locke's solution without $CaCl_2$. Addition of 8 μM ionomycin is indicated with arrow. $\Delta[Ca^{2+}]_c$ stands for the difference in $[Ca^{2+}]_c$ before ionomycin addition and the highest Ca^{2+} response. Representative traces are presented in (A): pN3 (black line), pAnxA6-1 (green line), pAnxA6-2-transfected (red line) cells. B: Bars represent mean values of $\Delta[Ca^{2+}]_c$ of three experiments ± SD. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Inhibition of Ca^{2+} entry into all three cell lines and more importantly, the abolishment of differences in a new $[Ca^{2+}]$ steady state by 2-APB, suggest a prominent role of SOCE in the altered Ca^{2+} homeostasis in AnxA6-transfected cells.

Intensity of store-operated Ca^{2+} influx may depend on the amount of Ca^{2+} deposited in ER. To test the possible effects of AnxA6 isoforms on ER Ca^{2+} stores the relative calcium content within ER was determined. For this purpose ionomycin was added to the cells suspended in resting Ca^{2+}-free Locke's solution. Addition of ionomycin resulted in a fast and transient increase in $[Ca^{2+}]_c$ (Fig. 5). As the total surface of the ER membranes is much bigger than the surface area of the plasma membrane and ionomycin is distributed proportionally between these membranes, $[Ca^{2+}]_{ER}$ and $[Ca^{2+}]_c$ equilibrate much faster than the external $[Ca^{2+}]$ and $[Ca^{2+}]_c$. Thus, the height of the Ca^{2+} peak appearing upon ionomycin addition reflects the relative calcium content of ER [Evtodienko et al., 1995]. There were no differences between the pN3-transfected and AnxA6 overexpressing cells indicating that Ca^{2+} accumulation in ER is not affected by AnxA6 overexpression. Thus, the higher $[Ca^{2+}]_c$ steady state (Fig. 3) in pAnxA6-2-transfected cells could be caused by stimulation of the store-operated Ca^{2+} entry.

DISCUSSION

Due to the fact that catecholamine secretion from PC12 cells depends mostly on Ca^{2+} influx upon depolarization of the plasma membrane and because AnxA6 isoforms differ in their influence on Ca^{2+} influx [Fleet et al., 1999] we investigated how over-expression of AnxA6 isoforms affects calcium influx in PC12 cells and, in consequence, catecholamine secretion.

It is established that in PC12 cells the nifedipine-sensitive VDCC is the major route of Ca^{2+} entry [Janigro et al., 1989; Colston et al., 1998]. The reverse mode of NCX also accounted for Ca^{2+} entry upon depolarization in myocytes [Kupittayanant et al., 2006] and chromaffin cells [Mahapatra et al., 2004]. Our results, showing the inhibitory effect of nifedipine and KB-R7943 on Ca^{2+} entry, also indicate the involvement of L-VDCC as well as NCX working in the reverse mode as the routes of the Ca^{2+} influx to PC12 cells upon plasma membrane depolarization (Table I).

We found that the increase in the cellular content of both AnxA6 isoforms was accompanied by decreased $[Ca^{2+}]_c$ amplitude upon depolarization (Table II), similarly to what was observed in cardiomyocytes [Gunteski-Hamblin et al., 1996]. The decrease in $\Delta[Ca^{2+}]_c$ was accompanied by decreased dopamine secretion (Table III). However, in contrast to data for A431 epithelial cells [Fleet et al., 1999], in PC12 cells AnxA6-2 appeared to have a stronger impact on calcium influx and dopamine secretion than AnxA6-1. This apparent discrepancy may arise from differences between electrically excitable and non excitable cells, in terms of their calcium signaling machinery composition. This observation was supported by experiments performed on cells with silenced AnxA6 gene. PC12 cells with AnxA6 knock-down exhibited a higher $[Ca^{2+}]_c$ amplitude upon depolarization and elevated dopamine secretion (Table IV) than control.

Available data characterizing the effects of AnxA6 expression on intracellular calcium homeostasis are contradictory. Increased AnxA6-1 and -2 content resulted in elevated resting $[Ca^{2+}]_c$ in PC12 cells, whilst there was a significant difference in the influence of the two AnxA6 isoforms on Ca^{2+} transient (Table II). AnxA6-2 but not the AnxA6-1 isoform significantly affected $[Ca^{2+}]_c$ in stimulated cells (Table II). In contrast, elevated expression of AnxA6 targeted to heart resulted in altered contractile properties and Ca^{2+} dynamics of cardiomyocytes [Gunteski-Hamblin et al., 1996]. Those cells had lower basal $[Ca^{2+}]_c$ and a reduced rise in $[Ca^{2+}]_c$ was observed following depolarization. Moreover, Ca^{2+} clearance from cytosol was much faster in cells overexpressing AnxA6. The authors hypothesized that such phenomenon relied on the activation of NCX or Ca^{2+} pumps or on decreased activity of VDCC or on all these factors. On the other hand, it has been shown that loss of AnxA6 in cardiomyocytes resulted in increased contraction-relaxation dynamics and accelerated Ca^{2+} clearance after caffeine stimulation [Song et al., 2002]. The authors also suggested that AnxA6 could act as a regulator of NCX or Ca^{2+}-ATPases. Co-distribution of AnxA6 with NCX and L-VDCC was recently demonstrated by other authors [Ueng et al., 2008]. Till now, no experimental evidence

demonstrating the influence of AnxA6 on a particular calcium transporter has been delivered. Interestingly, data shown in this report indicate an elevated resting $[Ca^{2+}]_c$ in cells overexpressing AnxA6. This might be a result of affected NCX activity (Table II) despite enhanced NCX1 content (Fig. 2C). Such apparent discrepancy with an earlier hypothesis [Gunteski-Hamblin et al., 1996; Song et al., 2002] might reflect serious differences between regulatory mechanisms of calcium homeostasis in PC12 and heart muscle cells.

PC12 cell plasma membrane depolarization in the presence of nifedipine or KB-R7943 (Table II) revealed that in cells with elevated AnxA6 isoform 1 content the inhibitory effect of KB-R7943 was reduced (Table II). To assess more precisely the effect of AnxA6 on calcium entry through NCX operating in the reversed mode, we tried to distinguish between two components affecting calcium influx to AnxA6 overexpressing cells challenged by KB-R7943. As mentioned above and shown in Table II the inhibition of NCX by KB-R7943 reduced calcium influx ($\Delta[Ca^{2+}]_c$ by 47% in pN3-transfected cells). Overexpression of AnxA6-1 or AnxA6-2 also resulted in reduced calcium influx by 32% and 58%, respectively (from 301 to 205 nM and 301 to 127 nM; see Table II). In the presence of KB-R7943 $\Delta[Ca^{2+}]_c$ was further reduced by 30% and 45% in the case of AnxA6-1 and AnxA6-2-transfected cells (compare data in Table II). If the effects of altered AnxA6 content and KB-R7943 were additive then summing up the reduction of $\Delta[Ca^{2+}]_c$ caused by altered AnxA6 isoforms content and KB-R7943 would give 62% (30 + 32%) in the case of pAnxA6-1-transfected cells and approx. 100% (45 + 58%) in the case of pAnxA6-2-transfected cells. However, summarized effects of all components studied (AnxA6 and KB-R7943) indicate that $\Delta[Ca^{2+}]_c$ is reduced by 52% in pAnxA6-1 and by 77% in pAnxA6-2-transfected cells in comparison to that in the pN3-transfected cells (from 301 nM in pN3-transfected cells to 144 nM and 70 nM in pAnxA6-1 and pAnxA6-2-transfected cells, respectively).

Comparison of the theoretical calculations with the experimental data seem to reveal an additivity of effects of AnxA6-1 overexpression and inhibition of NCX (62% vs. 52% decrease of $\Delta[Ca^{2+}]_c$). Contrary to that, in the case of pAnxA6-2-transfected cells effects of the increased protein content and KB-R7943 are not additive. Taken together these observations indicate that in cells overexpressing the AnxA6-1 isoform the contribution of NCX, working in reverse mode, to the overall Ca^{2+} influx upon plasma membrane depolarization is less pronounced than in the pN3-transfected cells. In the case of AnxA6-2 overexpressing cells the effect of NCX inhibition on depolarization-induced calcium entry is negligible. It indicates that elevation of AnxA6-2 content might be a cause of reduced L-VDCC contribution to the overall Ca^{2+} entry into PC12 cells.

In PC12 cells, in response to calcium influx, a readily-releasable pool of vesicles which are already docked in the sub-plasma membrane region fuses with the plasma membrane and catecholamines are released. In addition, rising $[Ca^{2+}]_c$ stimulates chromaffin granule maturation from the Golgi apparatus, the movement of secretory vesicles from a reserve pool towards the plasma membrane and formation of a readily-releasable pool of vesicles. In accordance with the fact that membrane fusion requires high Ca^{2+} concentration it was previously observed that exocytosis in chromaffin cells is enhanced in the vicinity of the L-type VDCCs [Becherer et al., 2003; Ore and Artalejo, 2004]. Therefore, inhibition of Ca^{2+} influx by nifedipine, a specific L-type VDCC inhibitor, significantly impairs catecholamine secretion [Douglas et al., 2008; Hagalili et al., 2008]. It was demonstrated that NCX activity also influences catecholamine secretion in chromaffin cells [Schneider et al., 2002; Pan et al., 2006].

According to the data shown in Figure 3 NCX may not participate in the establishment of a new steady state in thapsigargin pretreated cells. We compared the effects of AnxA6 isoforms overexpression on SOCE in the resting and Na^+-free Locke's solutions and noticed that SOCE was higher in PC12 cells overexpressing AnxA6-2. This effect was independent of the Na^+ presence in the resting Locke's solution (Fig. 3). There are several possible explanations of this phenomenon. Either there are differences in the velocity of Ca^{2+} removal from cytosol or AnxA6 indirectly stimulates Ca^{2+} entry. We eliminated the possibility that AnxA6 overexpression affects Ca^{2+} accumulation in ER by showing very similar ER relative calcium content in pN3-transfected and in AnxA6 overexpressing cells (Fig. 5). Thus, stimulation of SOCE or inhibition of Ca^{2+} removal from cells, were taken under further consideration.

Store-operated calcium channels (SOCs) and other non-store-operated cation channels (NSCCs) play a major role in the regulation of intracellular $[Ca^{2+}]$ increase in response to hormones or neurotransmitters. Under NCX-inactivating condition (the Na^+-free resting Locke's solution) the addition of $CaCl_2$ caused much higher influx of Ca^{2+} to cells overexpressing AnxA6, and the effect was stronger in the case of pAnxA6-2-transfected cells (Fig. 4). Pre-addition of the SOCE inhibitor, 2-APB, resulted in attenuation of the calcium influx in AnxA6 overexpressing cells. These results indicate that AnxA6 (particularly AnxA6-2) stimulates Ca^{2+} entry through SOC thus affecting calcium homeostasis.

The mechanism of the observed change in NCX content and activity as well as SOCE stimulation remains to be determined. Both Ca^{2+}-transporting systems may respond to changes in mitochondrial energy state [Zablocki et al., 2001]. Lack of ATP impairs plasma membrane potential (Na^+/K^+-ATPase is affected), having impact on NCX activity. Recent findings demonstrate that SOCE is inhibited by mitochondrial uncouplers [Bolanos et al., 2009]. Furthermore, it has been shown that SOC and NCX activities depend on each other. Na^+ entry through the SOCE pathway leads to activation of the reversal of Na^+/Ca^{2+} exchanger activity [Harper and Sage, 2007; Bolanos et al., 2009]. Moreover, NCX associates with F-actin through its central hydrophilic domain [Conrdrescu and Reeves, 2006]. As a result reverse mode NCX activity regulated by the actin cytoskeleton influences SOCE [Harper and Sage, 2007]. Monastyrskaya and co-workers [Monastyrskaya et al., 2009] demonstrated that AnxA6 overexpressed in HEK293 cells as a protein permanently attached to the plasma membrane inhibits store-operated calcium entry through stabilization of cortical actin cytoskeleton. Further experiments concerning the mechanism of AnxA6 influence on SOCE as well as NCX activity are necessary.

Summarizing, in this report we show that elevated level of AnxA6 isoform 1 and 2 affects calcium influx to stimulated PC12 cells, the

process which is crucial in regulation of dopamine secretion. Since the efficiency of dopamine secretion seems to be directly dependent on $[Ca^{2+}]_c$, the decreased $[Ca^{2+}]_c$ amplitude upon depolarization in pAnxA6-1 and pAnxA6-2-transfected cells may cause diminution of dopamine secretion from these cells. This conclusion, emphasizing the role of AnxA6, is further corroborated by the opposite effects observed in cells with AnxA6 knock-down. It may be concluded that the two annexin A6 isoforms may interact with different targets engaged in regulation of dopamine secretion. Data shown in this paper are the first that demonstrate that annexin A6 isoforms modulate NCX and L-VDCC activity. This finding is in line with the previously published hypothesis [Gunteski-Hamblin et al., 1996; Song et al., 2002].

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