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Annexin-I inhibits PMA-induced c-*fos* SRE activation by suppressing cytosolic phospholipase A2 signal

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Abstract Annexin-I (ANX-I) is a 37-kDa protein with a calcium-dependent phospholipid-binding property. Previously we have observed the inhibition of cytosolic phospholipase A2 (cPLA2) by ANX-I in the studies using purified recombinant ANX-I, and proposed a specific interaction model for the mechanism of cPLA2 inhibition by ANX-I [Kim et al. (1994) FEBS Lett. 343, 251–255]. Here we have studied the role of ANX-I in the cPLA2 signaling pathway by transient transfection assay. The stimulation of Rat2 fibroblast cells with phorbol 12-myristate 13-acetate (PMA) induced the c-*fos* serum response element (SRE). The SRE stimulation by PMA was dramatically reduced by (1) pretreatment with a cPLA2-specific inhibitor, arachidonyltrifluoromethyl ketone, or (2) co-transfection with antisense cPLA2 oligonucleotide, indicating that the SRE activation was through cPLA2 activation. Co-transfection with an ANX-I expression vector also reduced the SRE stimulation by PMA, suggesting the inhibition of cPLA2 by ANX-I. The active domain of ANX-I was mapped using various deletion mutants. ANX-I(1–113) and ANX-I(34–346) were fully active, whereas ANX-I(114–346) abolished the activity. Therefore the activity was in the amino acid 34 to 113 region, which corresponds to the conserved domain I of ANX-I.

Key words: Annexin-I; Cytosolic phospholipase A2; Phorbol 12-myristate 13-acetate; c-*fos* serum response element

1. Introduction

Annexins (also called lipocortins) are structurally related, calcium-dependent phospholipid-binding proteins that have been implicated in various physiological roles including anti-inflammation, cell growth and differentiation, adhesion and membrane fusion [1–4]. Annexin-I (ANX-I), a 37-kDa member of the annexin family of proteins, has been shown to mediate anti-inflammatory actions of glucocorticoids. The anti-inflammatory property has been proposed to be related to the ability of ANX-I to inhibit phospholipase A2 (PLA2) activity [5]. ANX-I has also been proposed to play regulatory roles in the mitogenic signal transduction [6–8]. However, the mechanism by which ANX-I plays a regulatory role in the mitogenic signal transduction remains to be determined.

PLA2 represents a growing family of enzymes that have been proposed to play roles in inflammation, cytotoxicity and mitogenesis [9,10]. The mammalian PLA2 family includes at least three types of PLA2s, secretory PLA2 (sPLA2), cytosolic PLA2 (cPLA2) and Ca^{2+}-independent PLA2 (iPLA2). Increasing reports suggest that cPLA2, an ubiquitously distributed 85–100-kDa protein, is a key enzyme responsible for agonist-induced arachidonic acid release [9]. The activity of cPLA2 is upregulated by an increase in intracellular Ca^{2+} concentration and by protein kinase-catalyzed phosphorylation [11]. Enzymatic studies have shown that ANX-I inhibits cPLA2 by specific interaction with cPLA2 [12]. Suppression of cPLA2 activity by ANX-I has also been shown in cultured cells [13]. These results suggest that ANX-I may function as an endogenous negative regulator of cPLA2. Thus, inhibition of cPLA2 by ANX-I may constitute a plausible mechanism for the regulation of mitogenic signal transduction by ANX-I.

The serum response element (SRE) in the c-*fos* promoter has been shown to be necessary and sufficient for rapid induction of the c-*fos* gene by serum or TPA [14]. SRE is a primary nuclear target for many diverse signals including growth factors, cytokines, and stresses. In order to investigate whether ANX-I regulates signaling pathways controlled by cPLA2, the effect of ANX-I on the signaling pathways leading to the activation of c-*fos* SRE by phorbol 12-myristate 13-acetate (PMA) was analyzed. In this contribution, we show that ANX-I attenuates PMA-induced c-*fos* SRE activation by suppressing the activation of cPLA2.

2. Materials and methods

2.1. Chemicals and reagents

PMA, lysophosphatidic acid (LPA), hydrogen peroxide and mepacrine were purchased from Sigma (St. Louis, MO, USA). Arachidonyltrifluoromethyl ketone ($AACOCF_3$) was from BioMol (Plymouth Meeting, PA, USA). Fetal bovine serum (FBS), gentamicin, modified Eagle's medium, non-essential amino acids solution, Dulbecco's modified Eagle's medium (DMEM) and geneticin were from Gibco-BRL (Gaithersburg, MD, USA). Monoclonal antibody to ANX-I was purchased from Transduction Laboratories (Lexington, KY, USA). Antisense oligonucleotide (GsTsGCTGGTAAGGATCTsAsT), of which two linkages were phosphorothioated at both 5′ and 3′ ends, was directed against codons 4–9 of the human cPLA2. A scrambled sequence oligonucleotide (GsTsGCTCCAAGTTTCTsAsT) was designed and used for control. Both oligonucleotides were purchased from Biobasic Co. (Ontario, Canada).

2.2. Plasmid preparation

The reporter plasmid pSRE-Luc has been described previously (Fig. 1A) [15]. The SRE oligonucleotide (23mer) is inserted at −53 of the

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truncated c-*fos* promoter fused to the luciferase gene. The ANX-I cDNA (346 amino acids) fragment was cloned into *Bam*HI/*Not*I sites of pcDNA3 (Invitrogen, Groningen, the Netherlands) to construct pANX. The deletion mutants of the ANX-I expression plasmid, pANX(1–114), pANX(1–195), pANX(1–273), pANX(1–311), pANX-(33–346) and pANX-I(114–346), were constructed by generating the deletion DNA fragment by polymerase chain reaction and cloning into pcDNA3 (Fig. 1B). All the ANX-I mutant clones were verified by nucleotide sequence analysis.

2.3. Cell culture, DNA transfection and luciferase assay

Rat2 fibroblast cells (ATCC, CRL 1764) were grown in DMEM supplemented with 2 mM glutamine, non-essential amino acids solution and 10% FBS at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For analysis by transient transfection, cells (1×10^5) were seeded on 35-mm dishes and cultivated for 24 h. The cells were transfected with 1.8 μg of DNA/dish using Lipofectamine plus (Gibco-BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. The 1.8 μg of DNA was made up from 0.6 μg of reporter plasmid (pSRE-Luc), 0.6 μg of internal control (pCMV-βGAL) and 0.6 μg of sample (pANX) or control (pcDNA3). The transfection was stopped after 3 h by changing the culture medium to 0.5% FBS. After 36 h incubation, cells were lysed in 0.15 ml of lysis buffer (8 mM Tricine, pH 7.8, 10 mM NaCl, 0.4 mM EDTA, 1 mM DTT, 0.2% Triton X-100). The lysates were centrifuged and the supernatants were used for the luciferase assay. The luciferase activity was measured with the luciferase assay kit (Promega, Madison, WI, USA) as indicated by the manufacturer. β-Galactosidase activity was measured spectrophotometrically by the generation of *o*-nitrophenol from *o*-nitrophenyl-β-D-galactopyranoside, and was used as an internal standard to normalize the luciferase activity.

Stable Rat2 fibroblast cell lines expressing ANX-I were established by using pANX. Rat2 cells were transfected with pANX and clones expressing ANX-I were selected by cultivating the cells in the presence of geneticin (400 μg/ml) for 3–4 weeks. The expression of ANX-I in the clones was confirmed by Western blot analysis using anti-ANX-I antibody.

2.4. Western blot analysis

The cells were lysed in the lysis buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonylfluoride, 5 mM benzamidine) and were disrupted by sonication (3×20 s). The lysates were centrifuged for 10 min at 4°C to remove debris, and the protein concentrations were determined using the protein assay kit (Bio-Rad, Richmond, CA, USA). Equivalent amounts of samples (30 μg of protein) were separated by SDS–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and probed with anti-ANX-I antibody.

2.5. Assay of cPLA2 activity

The cPLA2 activity was determined using 1-stearoyl-2-[1-^{14}C]-arachidonyl-*sn*-glycero-3-phosphocholine as the substrate, as described previously [12].

3. Results

3.1. Inhibition of PMA-induced c-fos SRE activation by ANX-I

Regulatory role of ANX-I in the signaling pathway leading to c-*fos* SRE activation was investigated by using the transfection reporter assay. Rat2 fibroblast cells were co-transfected with the pSRE-Luc, the luciferase reporter (Fig. 1A) and pCMV-GAL, which was used as an internal control. The cells were serum-starved in DMEM containing 0.5% FBS for 36 h and then treated with PMA or 0.05% DMSO (vehicle). To determine the optimum conditions for PMA-induced SRE activation the cells were cultured under various PMA concentrations. As shown in Fig. 2A,B, SRE was maximally activated at 5–10 ng/ml PMA and 2 h after addition of PMA. Therefore, all experiments were performed under these conditions. Effects of ANX-I on the PMA-induced SRE activation were determined by co-transfection assay. Rat2 fibroblast cells were co-transfected with pSRE-Luc, pCMV-GAL and pANX, the expression vector for human ANX-I (Fig. 1B). The luciferase assay showed that ANX-I inhibited PMA-induced SRE activation, whereas ANX-I had no effect on LPA-induced SRE activation (Fig. 3A). When a variable amount of pANX is used, the inhibition of PMA-induced SRE activation decreased with a decreasing amount of pANX (Fig. 3B).

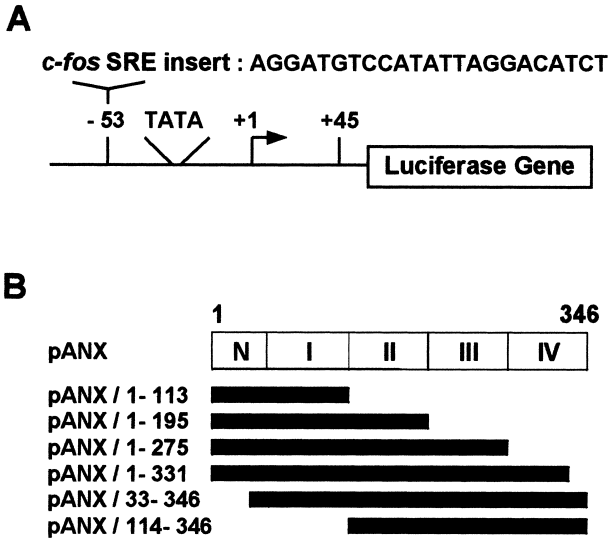

Fig. 1. Schematic presentation of plasmids used in this study. A: The c-*fos* SRE reporter plasmid. The SRE oligonucleotide (23mer) is inserted at −53 of the truncated c-*fos* promoter fused to the luciferase gene [15]. B: ANX-I and the deletion mutants expression vectors. Roman numerals represent the conserved repeat domains [1].

3.2. cPLA2 activity is necessary for SRE activation induced by PMA

While LPA signaling to c-*fos* SRE activation occurs primarily through a Rho-PLA2 cascade, epidermal growth factor signaling to the SRE activation can occur through a Rac-PLA2 cascade [15,16]. Signaling pathways leading to SRE activation by PMA, however, remain unknown.

To investigate whether PLA2 is involved in the activation of SRE by PMA in Rat2 cells, the effect of PLA2 inhibitors on the PMA-induced SRE activation was examined. Both mepacrine and $AACOCF_3$ inhibited the PMA-induced SRE activation (Fig. 4A). Inhibition by mepacrine, which inhibits both cPLA2 and sPLA2, had a stronger effect than $AACOCF_3$, a specific cPLA2 inhibitor. The treatment of Rat2 cells with $AACOCF_3$ inhibited the PMA-induced SRE activation to the level observed in the unstimulated cells, while the inhibitor alone had little effect on the expression of the SRE reporter. On the other hand, mepacrine inhibited the SRE reporter both in PMA-stimulated and -unstimulated cells. These results show that cPLA2 activity is necessary for the PMA-induced SRE activation and that PLA2s other than cPLA2 may be involved in maintaining the basal level of SRE activity. To confirm further that cPLA2 is involved in the PMA-induced SRE activation, the effect of antisense oligonucleotide for cPLA2 on the SRE activity was examined. Fig. 5A reveals that 100 nM (0.6 μg) antisense oligonucleotide suppressed almost completely the PMA-induced SRE activation to the level of control cells while scrambled oligonucleo-

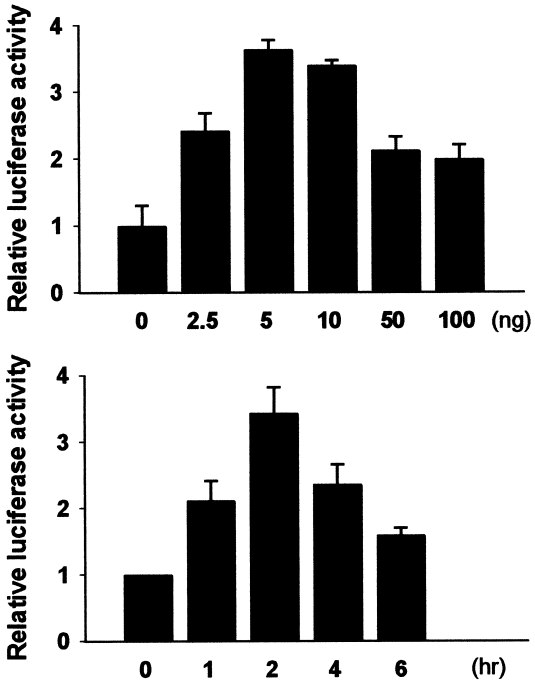

Fig. 2. Activation of c-*fos* SRE by PMA. Rat2 fibroblast cells were transfected with 1.8 μg DNA (0.6 μg pSRE-Luc, 0.6 μg pCMV-βGAL and 0.6 μg pcDNA3) as described in Section 2. The cells were serum-starved in DMEM containing 0.5% FBS for 36 h and then treated with PMA or 0.05% DMSO (vehicle). A: Cells were cultured in the presence of 2.5–100 ng/ml PMA for 2 h. B: Cells were cultured in the presence of 10 ng/ml PMA for 0–6 h. Luciferase activity was normalized with β-galactosidase activity. Data shown are the averages of triplicate values.

tide had little effect on the SRE activity. The antisense oligonucleotide had no effect on LPA-induced SRE activation. These results further indicate that PMA-induced SRE activation is mediated by cPLA2.

3.3. Inhibition of PMA-induced SRE activation by ANX-I is mediated by suppressing cPLA2

Results shown in Figs. 3–5 demonstrate that ANX-I inhibits cPLA2-mediated SRE activation. The inhibition by ANX-I was investigated using stable Rat2 cells expressing ANX-I (Rat2/ANX-I). Because the expression level of ANX-I was very low in Rat2 cells as determined by Western blot analysis (Fig. 6A), Rat2/ANX-I is a suitable model system to study the gain of function by ANX-I. In the stable Rat2/ANX-I cells, SRE activation was not induced by PMA, whereas SRE was activated by LPA, which occurs through cPLA2-independent pathways (Fig. 6B). To determine whether the attenuation of PMA-induced SRE activation in stable Rat2/ANX-I cells is ascribed to the suppression of cPLA2 by ANX-I, cPLA2 activity was determined. The $cPLA_2$ activity in Rat2/ANX-I cells was much lower than in Rat2 cells in both PMA-unstimulated and -stimulated cells (Fig. 6C).

3.4. Domain I in ANX-I is responsible for the inhibition of PMA-induced SRE activation

The structure of ANX-I comprises a 43-amino acid-long N-terminal and four conserved repeat domains of about 70 amino acids each as schematically shown in Fig. 1 [1]. To determine which domain is responsible for the inhibition of

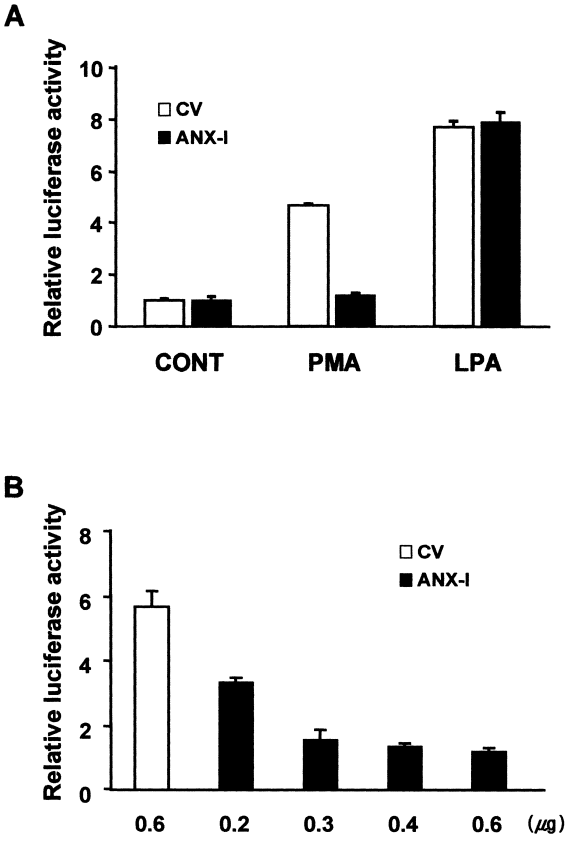

Fig. 3. Inhibition of PMA-induced c-*fos* SRE activation by ANX-I. A: Cells were transfected with 1.8 μg of DNA (0.6 μg pSRE-Luc, 0.6 μg pCMV-βGAL, and 0.6 μg pANX or 0.6 μg of pcDNA3) and cultured in the presence of 10 ng/ml PMA or 10 μM LPA for 2 h. B: Cells were transfected with 1.2 μg of the reporter DNA (0.6 μg of pSRE-Luc and 0.6 μg of pCMV-βGAL) and a variable amount of pANX. CV, control vector (pcDNA3); ANX-I, pANX. Data shown are the averages of triplicate values and are representative of at least three independent experiments.

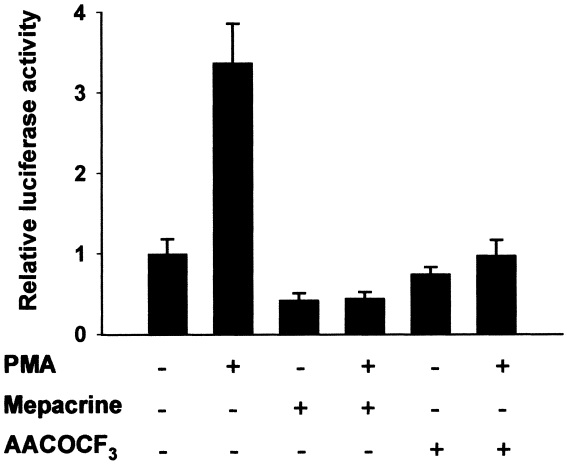

Fig. 4. Inhibition of PMA-induced SRE activation by PLA2 inhibitors. After 36 h serum starvation, cells were treated with 1 μM mepacrine and/or 10 μM $AACOCF_3$ for 30 min, followed by treatment with 10 ng/ml PMA for 2 h. Other details were as in Figs. 2 and 3.

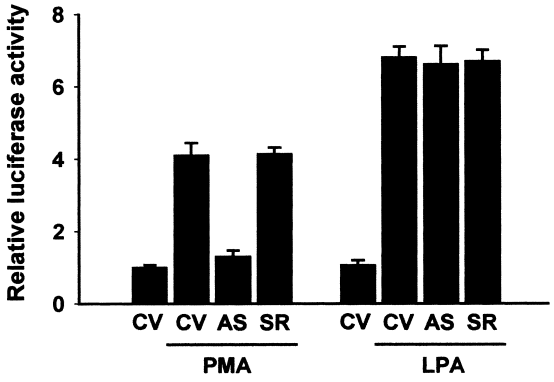

Fig. 5. Effect of cPLA2 antisense oligonucleotide on the PMA-induced SRE activation. An oligonucleotide with antisense cPLA2 sequence (GsTsGCTGGTAAGGATCTsAsT), of which two linkages were phosphorothioated at both the 5′ and 3′ ends was directed against codons 4–9 of the human cPLA2. Scrambled sequence oligonucleotide (GsTsGCTCCAAGTTTCTsAsT) was used for the control. Rat2 cells were transfected with 1.8 μg DNA (0.6 μg pSRE-Luc, 0.6 μg pCMV-βGAL and 0.6 μg oligonucleotide). Other details were as in Figs. 2 and 3. AS, antisense oligonucleotide; SR, scrambled oligonucleotide; CV, control vector.

PMA-induced SRE activation, various deletion mutants of ANX-I were used (Fig. 1B). Transient transfection of these mutants attenuated PMA-induced SRE activation. ANX-I C-terminal deletion mutants ANX/1–273, ANX/1–195 and ANX/1–113 attenuated PMA-induced SRE activation to the comparable level of wild type ANX-I (Fig. 7). In the case of deletion of N-terminal (ANX/33–346) or of C-terminal RACK (receptor for activated C-kinase) binding sites (ANX/1–322) [17], PMA-induced SRE activation was inhibited to the basal level, which is lower than the full-length ANX-I and other deletion mutants. These results suggest that the N-terminal and C-terminal ends might be involved in the positive regulation of the signaling pathway leading to SRE activation by PMA. In fact, the C-terminal 13 peptide has been shown to play a role in activating PKC [17,18]. On the other hand, the deletion of the N-terminal and domain I (ANX/114–346) abolished most of the inhibitory activity (15% inhibition of PMA-induced SRE activation). Taken together, these results showed that domain 1 of ANX-I is responsible for the inhibitory action of PMA-induced SRE activation.

4. Discussion

In this contribution we have demonstrated that (1) cPLA2 is involved in the activation of c-*fos* SRE activation, (2) ANX-I inhibits this process and (3) domain I (amino acids 34–143) of ANX-I is responsible for this inhibition. In addition, we provide evidence suggesting that PMA stimulates c-*fos* SRE via cPLA2-dependent cascade. The signaling cascade by which PMA causes stimulation of c-*fos* SRE remains to be determined. It has been shown that extracellular signal regulated kinases (ERKs), including ERK1 and ERK2, also known as $p44^{mapk}$ and $p42^{mapk}$, are induced by PMA [11]. ERKs phosphorylate and regulate the activity of certain enzymes, including $cPLA_2$ and $p90^{rsk}$, and nuclear proteins, such as the ternary complex factor $p62^{TCF}$ or Elk-1 [11]. The latter represents a critical event in controlling the expression of several genes, including c-*fos*. Therefore, the potential pathway involving ERKs-$p62^{TCF}$/Elk-1 or ERKs-cPLA2/$p90^{rsk}$-linked

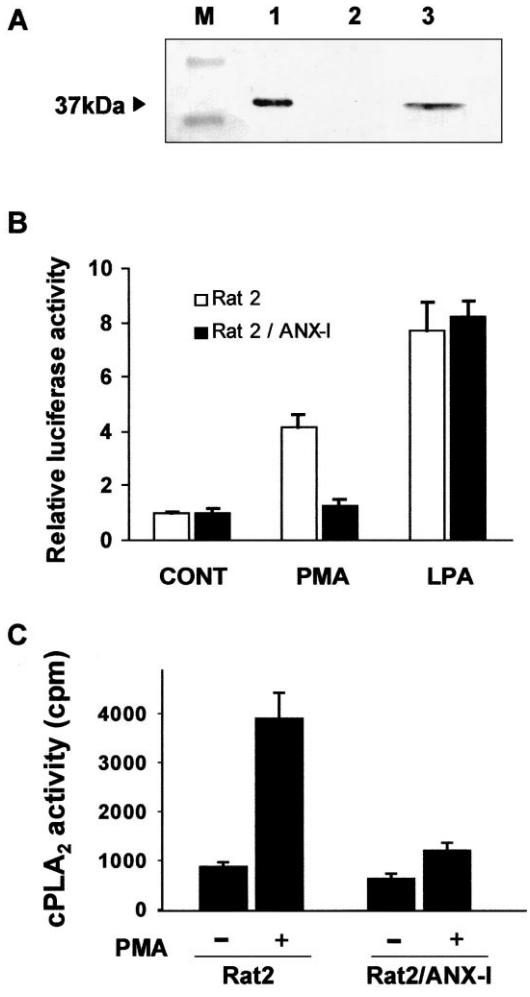

Fig. 6. ANX-I inhibits PMA-induced SRE activation in stable Rat2/ANX-I cells. Rat2 cells were transfected with pANX and clones expressing ANX-I were selected by cultivating the cells in the presence of geneticin (400 μg/ml) for 3–4 weeks. A: Western blot analysis using anti-ANX-I antibody. M, size marker; 1, purified ANX-I; 2, Rat2 cells; 3, Rat2/ANX-I cells. B: Inhibition of PMA-induced SRE activation in Rat2/ANX-I cells. SRE activation was determined as in Figs. 2 and 3. C: cPLA2 activity in Rat2 and Rat2/ANX-I cells. cPLA2 activity was determined as described in Section 2.

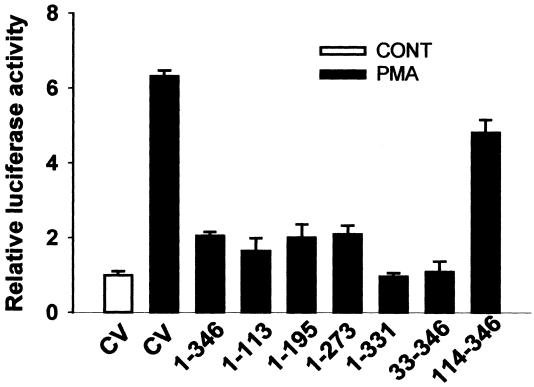

Fig. 7. Inhibition of PMA-induced SRE activation by ANX-I deletion mutants. Deletion mutants described in Fig. 1 were used. Details are as in Figs. 2 and 3.

cascades may possibly mediate the stimulation of c-*fos* SRE. Our data provide an essential role of cPLA2 in PMA-signaling to SRE (Figs. 4,2 and 5). In addition, we present a key role of ANX-I in PMA-induced nuclear signaling. However, the exact mechanism by which ANX-I regulates PMA-signaling to SRE remains to be determined, although ANX-I is known to regulate cPLA2 negatively. Because of a lack of evidence for the direct interaction of ANX-I with cPLA2 in cells, the hypothesis that ANX-I may be a specific endogenous inhibitor of cPLA2 has been controversial. Our previous in vitro studies have shown that ANX-I interacts with cPLA2 [12]. The results shown in Figs. 6 and 7 support the hypothesis. Whether inhibition by ANX-I is a major mechanism of cPLA2 regulation or not needs further study.

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