FEBS Letters 409 (1997) 300–306 FEBS 18682

Interaction of annexins IV and VI with ATP

An alternative mechanism by which a cellular function of these calcium- and membrane-binding proteins is regulated

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Received 27 February 1997; revised version received 22 April 1997

Abstract Annexin VI from porcine liver can be photoaffinity-labeled with 8-azido-[γ-^{32}P]ATP in a concentration-dependent, saturable manner. The extent of labeling varied with the concentration of calcium. The dissociation constant for the nucleotide was found to be in the range reported for ATP-binding proteins. The ATP analog, 2′-(or 3′)-*O*-(2,4,6-trinitrophenyl)-adenosine 5′-triphosphate, also bound to AnxVI, as indicated by shift in its fluorescence spectra in the presence of protein. Any significant 8-azido-ATP or TNP-ATP binding was not observed with AnxIV. ATP modulated the binding of AnxVI to erythrocyte membrane and increased the Ca^{2+} concentration required for half-maximal binding of AnxVI to F-actin.

Key words: ATP; Annexins IV and VI; Calcium; Membrane binding; F-actin

1. Introduction

In several mammalian organs (e.g., placenta, brain, heart and liver) annexins, which belong to a unique family of Ca^{2+}-binding proteins, exhibit a high level of cellular expression; however, their functions in these organs are poorly understood [1,2]. The prevailing opinion is that annexins become functionally active when the local concentrations of calcium are elevated [1]. The calcium signal causes binding of annexins to membrane phospholipids, and the interaction of Anx molecules with membrane may influence locally its structure, resulting in altered membrane–cytoskeleton interactions, and membrane permeability to ions. Some Anx isoforms have been found in vivo to be associated with membranes but in a Ca^{2+}-independent manner [3,4]. Tagoe et al. [5] have demonstrated that in the presence of millimolar concentrations of ATP, binding of annexins to membranes is potentiated. It has also been shown that adrenal AnxVI can be separated from other bovine annexins by affinity chromatography on ATP-agarose [6], and that ATP inhibits, driven by AnxI of bovine lung, the aggregation of chromaffin granules and PS liposomes [7]. These findings suggest that some annexins bind certain nucleotides which may affect their physiological functions. We show here that AnxVI but not AnxIV of porcine liver is specifically photoaffinity-labeled with 8-azido-[γ-^{32}P]-ATP in a concentration-dependent and saturable manner. The annexin labeling was found to vary with calcium concentration and ATP modulated the binding of AnxVI to membranes, and its interaction with actin.

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Abbreviations: Anx, annexin; B_{max}, maximal binding; cAMP, cyclic 3′-5′-adenosine monophosphate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; IC_{50}, concentration of an effector for half-maximal inhibition of an effect; $K_{1/2}$, free ligand concentration for half-maximal response; K_d, dissociation constant; PC, phosphatidylcholine; PS, phosphatidylserine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TNP-ATP, 2′-(or 3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate

2. Materials and methods

2.1. Preparation of porcine annexins IV and VI and human erythrocyte ghosts

AnxIV and AnxVI were purified from porcine liver as described in [8], and stored at 1–2 mg protein/ml in 20 mM Tris-HCl, pH 7.0, 1 mM EGTA at −70°C until used. Human erythrocyte ghosts were prepared according to Steck and Kant [9], then frozen in 10 mM Tris-HCl, pH 7.4, containing 0.1 mM EDTA, 0.1 mM PMSF, 1.4 mM β-mercaptoethanol, and 50 μM butylated hydroxytoluene, at a protein concentration of 9–10 mg/ml, and stored at −20°C.

2.2. Photoaffinity labeling of annexins with 8-azido-ATP

8-Azido-[γ-^{32}P]ATP (specific activity 10.6 Ci/mmol) was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA, USA) and used within a month. Labeling with 8-azido-ATP was performed at room temperature, under the conditions described for the 90-kDa canalicular transporter protein of rat hepatocytes [10], with some modifications. In the present experiments 8-azido-ATP was added from a methanolic stock solution prior to the addition of other components, and the solvent from the reaction mixture was evaporated to dryness. Labeling was achieved in a solution containing in 20 μl 20 mM Tris-HCl, pH 7.4, 0.5 mM EGTA, various concentrations of $CaCl_2$, 0.1–0.5 μg protein, in the presence or absence of 12 μg asolectin/cholesterol (4:1, w/w) or PS liposomes. Where indicated, the concentration of 8-azido-ATP was varied, otherwise it was kept at 2 μM (specific activity 0.5 Ci/mmol). Samples were irradiated for 3 min with a hand-held UV lamp (UVSL25, Ultra-Violet Product Inc., San Gabriel, CA, USA), denatured with SDS sample buffer, for 30 min at 37°C, and subjected to SDS-PAGE. Gels were dried and the peptide-associated radioactivity was visualized by exposure of gels to a storage phosphorus screen for 4–72 h, or for 18–140 h (for AnxIV). The results were evaluated using a PhosphorImager (Molecular Dynamics), and the Image Quant program (version 3.3). Non-irradiated samples served as controls for non-specific binding.

2.3. Enhancement of TNP-ATP fluorescence in the presence of annexins

The fluorescence emission spectra of TNP-ATP (Molecular Probes, Inc., Eugene, OR, USA) were measured using a F-4500 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) at 23°C in a buffer in a total volume of 1 ml 50 mM Tris-HCl, pH 7.4, 0.5 mM EGTA, containing 0–5 mM $CaCl_2$, in the presence or absence of asolectin/cholesterol liposomes (0.25 mg/ml). If present, annexins were at concentration ranging from 1.3–7.5 μM. TNP-ATP concentration ranged from 0–10 μM. The samples were excited at 412 nm (excitation slit

PII S0014-5793(97)00534-6

5 nm), and emission spectra were recorded between 500 and 600 nm (emission slit 20 nm). Data were acquired at a scanning speed of 240 nm/min. The value of fluorescence intensity of an emission peak at 535 nm, after subtracting the fluorescence intensity of TNP-ATP measured in the absence of protein, was used for calculations.

2.4. Binding of annexins to ATP-agarose

ATP insolubilized on 4% beaded agarose (Sigma) was used throughout (3.0 μmol ATP/ml packed gel). Prior to the experiments ATP-agarose (100-μl aliquots) was pelleted by centrifugation and washed (×3) with 1 ml portions of 10 mM Tris-HCl, pH 7.4; this procedure gave 25 μl of packed resin. The resin was then incubated with 200 μl of 10 mM Tris-HCl, pH 7.4, 0.5 mM EGTA, 0–5 mM $CaCl_2$, and 19–22 μg of annexin, for 60 min at 23°C. After incubation, samples were centrifuged at 3000×*g* for 10 min, 100-μl aliquots from supernatants were lyophilized, solubilized in SDS sample buffer, and subjected to SDS-PAGE. In parallel, the resin was washed, incubated with 5 mM ATP in 20 mM Tris-HCl, pH 7.4, for 30 min, and the composition of peptides eluted from ATP-agarose analyzed by electrophoresis. The gels were scanned, their computer images were created using Eagleeye II (Stratagene, La Jolla, CA, USA), and quantified with the aid of the Image Quant program. The samples incubated without the resin or with agarose without covalently linked ATP served as controls.

2.5. Binding of annexins to erythrocyte membrane and to F-actin

Binding of annexins to erythrocyte ghosts or liposomes was performed according to [8], or as otherwise stated in the text. Quantitative analysis of the obtained results was performed as described in Section 2.4. F-actin from bovine muscle insolubilized on cyanogen-bromide-activated Sepharose 4B (Sigma) resin (0.9 mg bound F-actin/ml packed resin) was prepared according to the procedure described by Singhal et al. [11]. Binding of annexin to the F-actin/Sepharose was performed in 2 mM Tris-HCl, pH 7.4, 50 mM KCl, 0.5 mM dithiothreitol, 2 mM $MgCl_2$ (BB buffer), supplemented with 0.5 mM EGTA, various concentrations of $CaCl_2$, 15–18 μg of AnxIV or VI, with or without 2 mM ATP. To the reaction mixture (total volume 0.2 ml), 60–70 μl of packed resin was added. Before use the resin was washed thrice in BB buffer, then incubated with Anx for 60 min at 23°C. Samples were centrifuged at 1300×*g* for 2 min, 100-μl aliquots of the supernatants were lyophilized, subjected to SDS-PAGE, and the amount of protein bound to the resin was determined by subtraction from the total the amount of protein found in the supernatant. The gels stained with Coomassie brilliant blue were subjected to densitometric analysis.

2.6. Other procedures

Free calcium concentrations were calculated with the aid of a computer program and the log K_a^{EGTA} values of Vianna [12], and verified using a Ca^{2+}-selective electrode (Orion, Res. Inc., USA). Protein concentration was determined by the method of Bradford [13], with bovine serum albumin as a standard. SDS-PAGE under reducing conditions was performed on 3% stacking and 12.5% resolving gels, according to Laemmli [14]. Gels were stained with Coomassie brilliant blue (G-250) or silver, as described in [14] and [15], respectively.

3. Results

3.1. Photoaffinity labeling of AnxVI with 8-azido-ATP

Photoaffinity labeling of AnxIV and AnxVI with 8-azido-ATP was examined in order to characterize the nucleotide binding site of these proteins. Annexins in the cell alternate between two major forms—a soluble form, when cytosolic Ca^{2+} concentration is 50–100 nM, and a membrane-bound form, when calcium concentrations become elevated upon cell activation [1,2]. Therefore, we incubated and UV-irradiated AnxIV and AnxVI separately with 8-azido-[γ-^{32}P]ATP and either 0.5 mM EGTA or 0.5 mM $CaCl_2$, with or without liposomes. The results of this experiment showed that only AnxVI was photoaffinity-labeled with the ATP analog (Fig. 1). No labeling was observed with AnxVI bound to either asolectin/cholesterol or PS liposomes. The labeling of AnxVI was dependent on UV activation of the photoaffinity probe, was saturable with respect to 8-azido-ATP concentration (Fig. 2A; K_d 4.6 μM at 0.5 mM EGTA), and diminished gradually with increasing ATP concentration in the reaction mixture (Fig. 2B); however, the competition of ATP with 8-azido-ATP labeling was not complete. Among other nucleotides, 20 μM ADP and UTP had no effect on labeling, 20 μM CTP inhibited it by about 30%, the effect of 20 μM GTP was the same as that of 20 μM ATP, and 16 μM TNP-ATP inhibited it completely. At 100 μM Ca^{2+} the 8-azido-ATP labeling of AnxVI was as efficient as in the presence of 0.5 mM EGTA, and decreased on raising $CaCl_2$ concentrations in the assay mixture. Under the described conditions there was none or minimal labeling of AnxIV, observed only in the presence of relatively high calcium concentrations, and after 6 days of exposure of the gels in the PhosphorImager.

3.2. Binding of TNP-ATP to annexins

Upon excitation at 412 nm, TNP-ATP in aqueous solution exhibits a fluorescence emission spectrum with a maximum at approximately 550 nm. Addition of AnxVI (with 0.5 mM EGTA) markedly increased the probe fluorescence intensity with a concomitant 15-nm blue shift of the emission peak maximum to 535–540 nm. The differential spectrum, corresponding to the fluorescence of the protein-bound TNP-ATP, exhibited maximal emission at 535 nm (Fig. 3). Under the same conditions AnxIV did not affect the emission spectra of TNP-ATP, further confirming the inability of AnxIV to bind nucleotides. The binding of TNP-ATP to AnxVI under various conditions (Fig. 4) was concentration-dependent and saturable. The binding was also saturable with respect to the protein concentration, and was calcium concentration-dependent. The affinity of AnxVI for TNP-ATP was found to be higher than for 8-azido-ATP. Binding of TNP-ATP to protein in the presence of 0.5 mM EGTA gave, however, opposite results to labeling of AnxVI with 8-azido-ATP

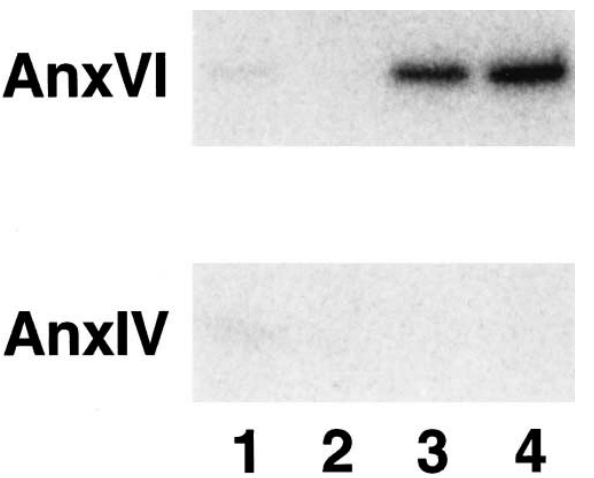

Fig. 1. Labeling of annexins IV and VI with 8-azido-ATP. Annexins (40 μg/ml) were preincubated in the presence of various effectors: 0.5 mM $CaCl_2$ (lane 1), 0.5 mM $CaCl_2$/0.5 mg asolectin liposomes (lane 2), 0.5 mM EGTA (lane 3) or 0.5 mM EGTA/0.5 mg asolectin liposomes (lane 4) in 20 mM Tris-HCl, pH 7.4. Aliquots of 20 μl were irradiated for 3 min in the presence of 2 μM 8-azido-[γ-^{32}P]-ATP. The samples were subjected to SDS-PAGE, and the radioactivity associated with AnxVI or AnxIV was visualized using a PhosphorImager after 24 h exposure.

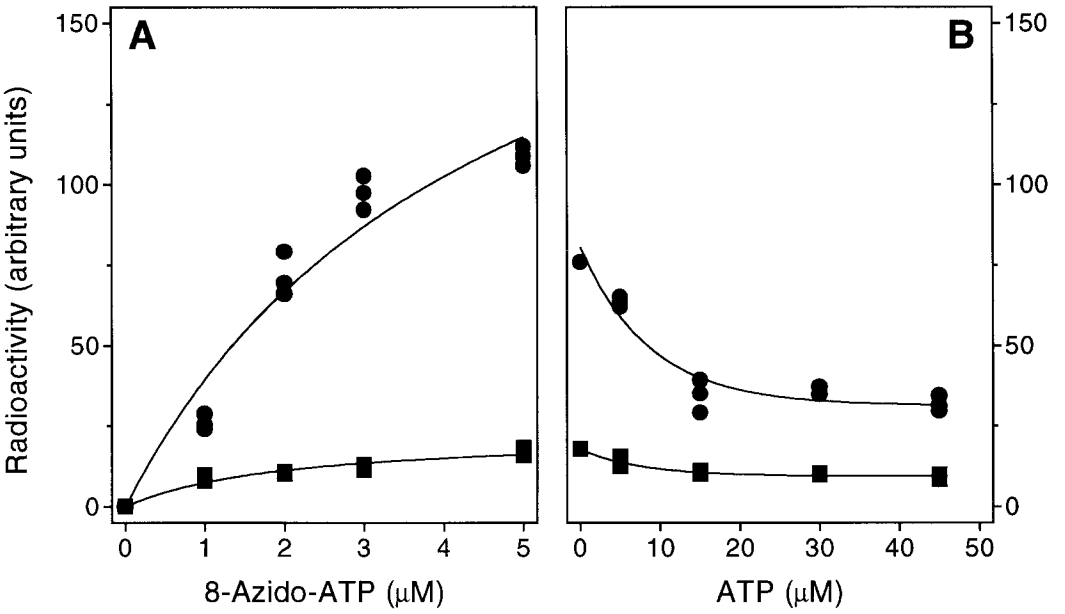

Fig. 2. Characteristic features of 8-azido-ATP labeling of annexins. A: Saturation curve for annexins. AnxIV (380 ng) (■) + 0.5 mM $CaCl_2$ or AnxVI (440 ng) (●) + 0.5 mM EGTA samples were UV-irradiated in the presence of increasing concentrations of 8-azido-[γ-^{32}P]ATP. B: Competition with ATP. AnxIV in 0.5 mM $CaCl_2$ or AnxVI in 0.5 mM EGTA was supplemented with 2 μM 8-azido-[γ-^{32}P]ATP and with ATP at a concentration range from 0–45 μM. Radioactivity associated with proteins was estimated using a PhosphorImager; the control value (no ATP) was taken as 100%; symbols are the same as in panel A. The experiment described was repeated 3 times and the results obtained are shown on both panels.

(Fig. 1). The enhancement of TNP-ATP fluorescence in the presence of AnxVI and 0.5 mM EGTA was 50% lower than that observed for AnxVI incubated in the presence of 0.5 mM $CaCl_2$ (Fig. 4A). No change in protein affinity for the nucleotide was observed under these conditions (K_d 1.28 and 0.75 μM, respectively). The reasons for such a discrepancy are given in Section 4. In agreement with the results of experiments with 8-azido-ATP labeling, the binding of TNP-ATP to AnxVI in the presence of 0.5 mM $CaCl_2$ was abolished by more than 35% in the presence of liposomes, with no change in affinity for the nucleotide (K_d 0.73 μM). Furthermore, the binding of TNP-ATP to AnxVI in the presence of EGTA was not affected by liposomes. This points to partial competition of nucleotide binding and binding of AnxVI to membranes in the presence of calcium.

The competition between ATP and TNP-ATP for binding to AnxVI (Fig. 4B) was similar to that found for 8-azido-ATP labeling and other ATP-binding proteins. This effect, pronounced at low TNP-ATP concentrations, points to competition between ATP and its analog for the same binding site. It is worth noting that AnxIV did not interact with TNP-nucleotide, except when it was incubated with asolectin liposomes in the presence of 0.5 mM $CaCl_2$ (Fig. 4A, K_d for TNP-ATP 7.01 μM), under conditions when binding of Anx to liposomes [1] and self-association of protein molecules might occur [16].

3.3. Binding of annexins to ATP-agarose

The binding of AnxVI to ATP-agarose is calcium-dependent with K_d of 0.56 μM for Ca^{2+} and B_{max} of 11 nmol of annexin per 1 ml of packed resin at 100 μM Ca^{2+} (Fig. 5). The latter value corresponds approximately to 4 μmol protein/mol ATP. The binding was observed even in the presence of 0.5 mM EGTA (20% of B_{max} at 100 μM Ca^{2+}). Under these conditions AnxIV did not bind to ATP-agarose; however, minimal binding was observed at calcium concentrations exceeding 1 mM.

3.4. Effect of ATP on Ca^{2+}-dependent binding of annexins to erythrocyte ghosts and F-actin

Porcine AnxIV and AnxVI bind to ghost membranes with B_{max} of 64.5 and 29.5 nmol/mg protein, and K_d 1.0 μM and 0.35 μM at 0.5 mM $CaCl_2$, respectively [8]. Both proteins interact with PS [17] in a calcium-dependent manner, and when associated with membranes induce extensive clustering of acidic phospholipids [18,19]. Upon addition of ATP to the reaction mixture, $K_{1/2}$ for Ca^{2+} of AnxVI to erythrocyte ghost binding increased gradually from 1.54 μM in the absence of ATP to 4.48 and 21.1 μM in the presence of 2 and 4 mM ATP, respectively (Fig. 6A). No changes were, however, observed in maximal binding capacity. On the other hand, for AnxIV (Fig. 6B) $K_{1/2}$ values for Ca^{2+} amounted to 20 ± 6 μM, irrespective of the presence or absence of ATP. AnxIV and AnxVI bound also to F-actin insolubilized on Sepharose 4B, which is consistent with previously reported binding of AnxVI to F-actin in solution [20]. The $K_{1/2}$ values for calcium for F-actin binding to AnxIV and AnxVI were found to be 98.7 and 5.8 μM, respectively, in the absence of nucleotides (Fig. 7). In the presence of 2 mM ATP, $K_{1/2}$ for Ca^{2+} increased to a 10-fold higher value for AnxVI (66.5 μM; Fig. 7A), while it remained practically unchanged for AnxIV (85.0 μM; Fig. 7B). These results suggest that ATP may attenuate the binding of AnxVI to filamentous actin.

4. Discussion

In the present report we provide evidence that AnxVI of porcine liver interacts with ATP. AnxVI is specifically photolabeled with 8-azido-[γ-^{32}P]ATP, and the K_d of the process is

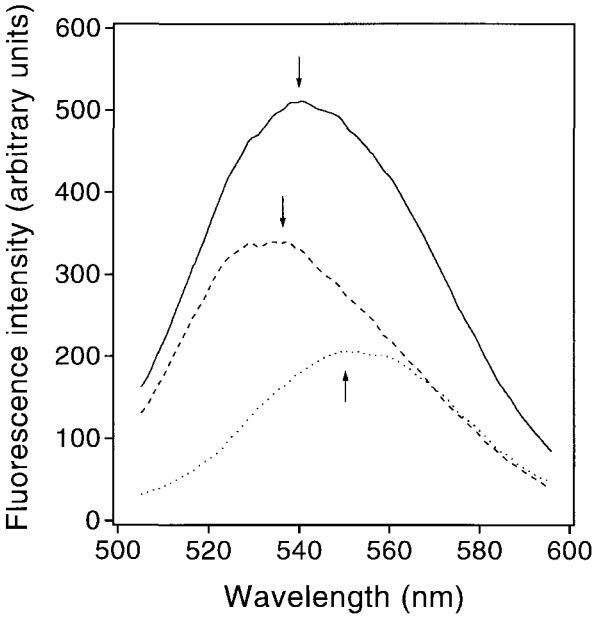

Fig. 3. Spectral features of TNP-ATP binding to AnxVI upon excitation at 412 nm. The extrinsic fluorescence of 9.6 μM TNP-ATP was measured in the presence of 0.5 mM EGTA, in the absence of protein (lower curve) or in the presence of 4.4 μM AnxVI (upper curve). The differential spectrum corresponding to net TNP-ATP bound to AnxVI is represented by the middle curve. The arrows indicate the wavelengths of maximal emissions.

in the range comparable to that for ATP-binding proteins. TNP-ATP also binds to AnxVI, as indicated by enhancement in its fluorescence upon addition of the protein. Since TNP-ATP has recently been used to characterize the nucleotide binding properties of other ATP-dependent proteins, such as P-glycoprotein [21], cystic fibrosis transmembrane conductance regulator [22], rat liver multispecific organic anion transporter [10], the epidermal growth factor receptor protein-tyrosine kinase [23], mitochondrial F1-ATPase [24], Na^+,K^+-ATPase [25], Ca^{2+}-ATPase [26], and F-actin [27], our results support the idea that nucleotides do indeed bind to annexin. AnxVI binds to ATP-agarose, and this binding was further promoted by calcium ions. We have not been able, however, to observe similar behavior of liver AnxIV. These observations suggest that ATP is an important ligand which may, upon interaction with AnxVI, regulate cellular function of the protein. Present studies indicate that the extent of binding of ATP to porcine AnxVI varied with the calcium concentration, pointing to an interrelationship between Ca^{2+}- and nucleotide-binding sites within an AnxVI molecule. There is, however, an inconsistency with the results of labeling with 8-azido-ATP and the binding experiments with TNP-ATP with regard to the presence of calcium and liposomes. The 8-azido-ATP labeling of AnxVI has been found to be completely prevented in the presence of 0.5 mM $CaCl_2$ and liposomes while binding of TNP-ATP was only partially inhibited. Moreover, photoaffinity labeling was decreased in the presence of 0.5 mM $CaCl_2$, while the interaction of protein with TNP-ATP was significantly enhanced. These differences can be explained by a lower affinity of AnxVI for 8-azido-ATP than for TNP-ATP and a different mechanism of interaction for AnxVI with these ATP analogs (covalent, non-stoichiometric attachment and non-covalent interaction, respectively). In fact, the results of our preliminary investigations into the effects of nucleotides on the intrinsic fluorescence of AnxVI reveal that TNP-ATP is the most potent, among the adenine nucleotides tested, in quenching fluorescence. In the presence of EGTA it was accompanied by a fluorescence energy transfer from AnxVI tryptophans to a fluorophore, and

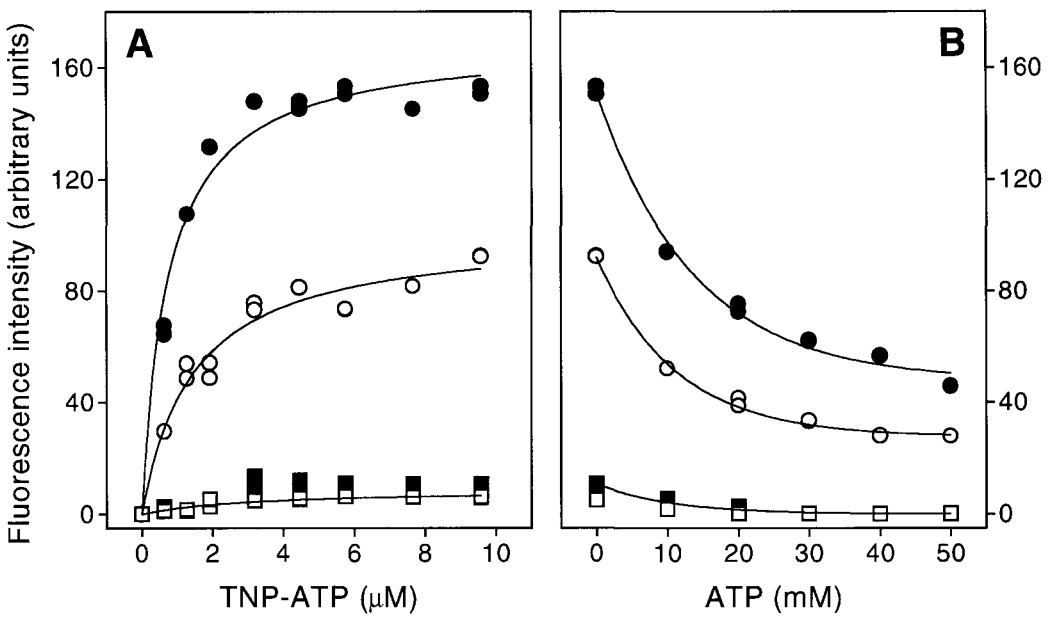

Fig. 4. Binding of TNP-ATP to annexins. A: Fluorescence enhancement at 535 nm of various concentrations of TNP-ATP was determined in the presence of 2.1 μM of AnxIV + 0.5 mM EGTA (□) or + 0.5 mM Ca^{2+} (■) or in the presence of 1.3 μM of AnxVI + 0.5 mM EGTA (○) or + 0.5 mM Ca^{2+} (●), and fluorescence intensity of TNP-ATP in buffer in the absence of protein was subtracted. B: Increasing concentrations of ATP were added to the samples in the presence of 9.6 μM TNP-ATP. Symbols are the same as in panel (A). Mean values of two experiments are shown; they varied by 3–5%.

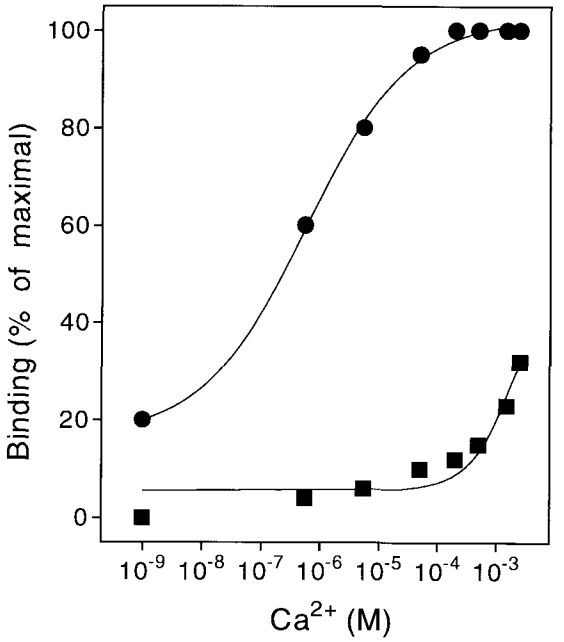

Fig. 5. The effect of calcium on the binding of annexins to ATP-agarose. AnxIV (■) or AnxVI (●) (19–22 μg of protein) was preincubated for 60 min at 23°C with 25 μl of packed ATP-agarose resin in a total volume of 200 μl of 10 mM Tris-HCl, pH 7.4, 0.5 mM EGTA, with various concentrations of $CaCl_2$ (0–5 mM). The incubation mixtures were centrifuged for 10 min at 4000 rpm and 100-μl aliquots from supernatants were lyophilized and subjected to SDS-PAGE. In parallel, the resin was treated for 30 min at room temperature with the buffer containing 5 mM ATP, and the peptides present in the eluate were analyzed by SDS-PAGE gels stained with Coomassie brilliant blue (R-250), as described in the text. Binding was quantified from the difference between samples incubated with and without resin. Mean values of two experiments which varied by 5% are shown.

this effect was not observed in the presence of millimolar $CaCl_2$ concentrations. One may, therefore, speculate that the interaction of AnxVI with TNP-ATP is a sum of relatively weak but numerous forces and is affected by binding of AnxVI to the membrane in the presence of calcium and self-association of protein molecules on the membrane surface. Further studies are required to clarify this point.

Tagoe et al. [5] have demonstrated that in the presence of physiological concentrations of ATP the association of annexins to hepatocyte plasma membrane is potentiated. Furthermore, calcium-dependent binding of adrenal medulla AnxVI has been found to be promoted by ATP [28]. Lin et al. [29] have shown that cytosol-dependent activation of Anx required for budding of clathrin-coated pits occurred in the presence of both ATP and Ca^{2+}. Recently, Cohen et al. [7] have reported that ATP binding to AnxI of bovine lung is accompanied by inhibition of Ca^{2+}-dependent aggregation of PS liposomes and bovine chromaffin granules driven by AnxI, and alteration of the conductance of calcium channels formed by AnxI in planar lipid bilayers. Considering the physiological significance of ATP binding by AnxVI, we have tested several experimental models in which, as we expected, the effect of nucleotides would be visible. Since Plager and Nelsestuen [30] proposed that binding of AnxVI to membrane could serve as one of the regulators of cell function, we checked the effect of ATP on binding of AnxVI to erythrocyte ghosts and found that nucleotide modulates this process. Taking into account that AnxVI is able to bind F-actin [20], dissociates brain spectrin from F-actin in a Ca^{2+}/PS-dependent manner [31], or colocalizes with actin in enamel- and dentin-related portions of dental tissue, where it is postulated to play a role in exocytotic and endocytotic events [32], we checked whether AnxVI could interact with F-actin and whether this process was modulated by ATP. We found that ATP indeed raised the Ca^{2+} concentration required for half-maximal binding of Anx to F-actin.

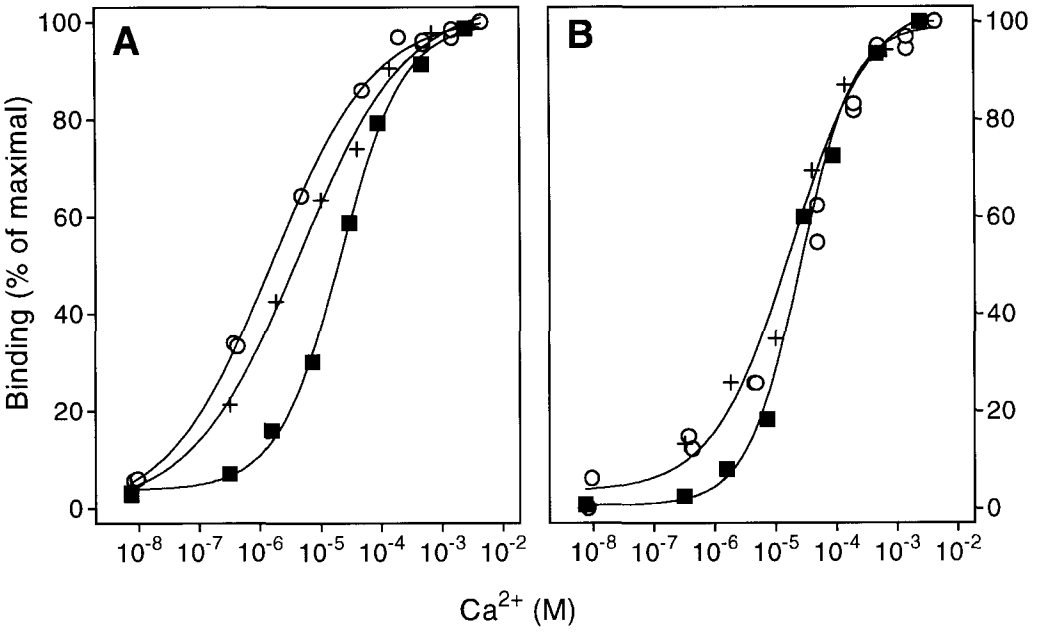

Fig. 6. The effect of ATP on binding of annexin VI (A) and annexin IV (B) to human erythrocyte membrane. Ca^{2+}-dependent binding of annexins to erythrocyte ghosts was performed as described in the text, in the presence of 2 mM ATP (+), 4 mM ATP (■), or absence of ATP (○), at various Ca^{2+} concentrations. Mean values of two experiments are shown; they varied by 5–7%.

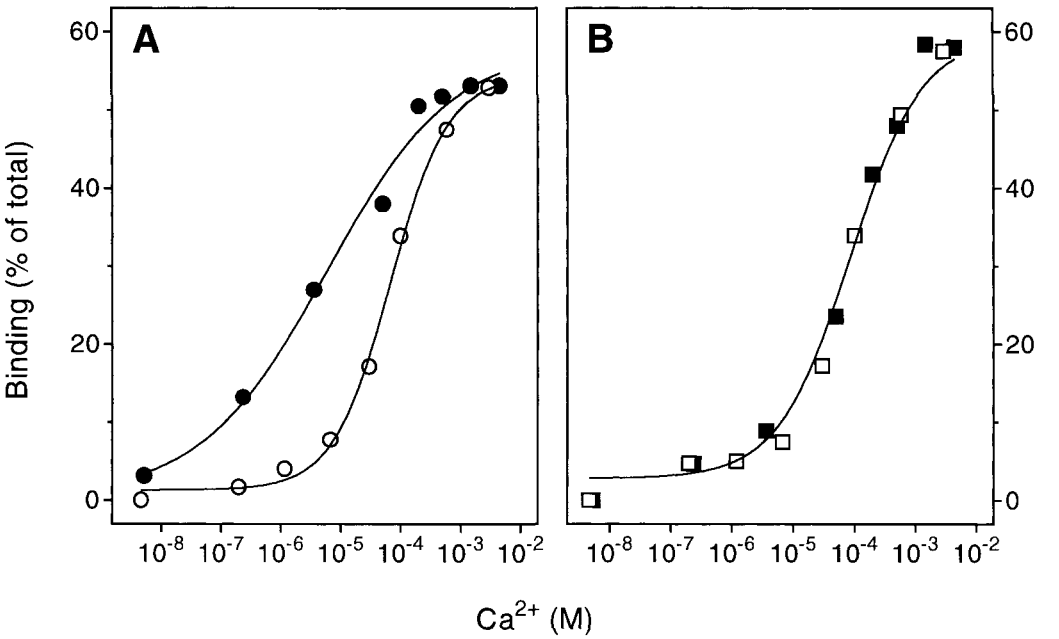

Fig. 7. Binding of annexins VI (A) and IV (B) to F-actin insolubilized on Sepharose 4B. The effect of ATP. The experiments were performed in the presence of 2 mM ATP (○, □) or absence of ATP (●, ■), at various Ca^{2+} concentrations, as described in the text. The mean values of two experiments with AnxIV (□, ■) or AnxVI (○, ●) are shown; they varied by 5–7%.

The universal importance of ATP for living organisms lies in the fact that hydrolysis of ATP releases a substantial amount of free energy, and that the ATP hydrolysis products provide opportunities for coupling of a variety of chemical reactions. ATP itself may play an important role as an intracellular signaling molecule and as a specific ligand required for proper functioning of certain proteins without being hydrolysed. ATP is also released from nerve cells, dense granules during platelet aggregation, chondrocytes and adrenal medulla, endothelial or other cells under stress, hypoxia or inflammation. It can act on P_2 purinoreceptors of hepatocytes or smooth muscle cells and on ATP-gated ion channels [33,34], including the ATP-inhibited K^+ channel in inner mitochondrial membrane [35]. The growth plate cartilage chondrocytes represent an interesting example of cells which are significantly depleted of ATP and have elevated cytosolic P_i, a prerequisite for formation of Ca^{2+}-acidic phospholipid-complexes during matrix mineralization [36]. These cells actively acquire Ca^{2+}, concentrate in the cell periphery and exfoliate it as Ca^{2+}-rich matrix vesicles, microstructures which induce mineral formation in vitro. Matrix vesicles are linked to type II and X collagens which in turn activate the influx of Ca^{2+} into these vesicles. The binding of collagens to the vesicle surface is mediated by annexins II, V and VI, relatively abundant in chondrocytes [37,38]. In addition, these cells secrete ATP which is used to regulate cell maturation, and has a profound influence on the amount and type of minerals deposited [39]. ATP may also elicit deleterious effects when its extracellular concentration rises above certain levels [40]. One may, therefore, speculate that annexins which are able to form Ca^{2+}-channels in chondrocytes and matrix vesicle membranes and bind to the matrix collagens or acidic phospholipids, act as mechano-transducers in growth plate cartilage, gating Ca^{2+} entrance into the cells, and facilitating formation of Ca^{2+} and P_i-primed matrix vesicles, as postulated by Wuthier [36]. ATP binding to annexins could serve as an additional factor influencing their interaction with a set of proteins characteristically associated with mineralization [41] and with acidic phospholipids.

We have shown recently that porcine liver AnxIV and AnxVI are indistinguishable with respect to their Ca^{2+}-dependent phospholipid binding characteristics [8,42], and specific abolishment of Ca^{2+}-induced fluidization of PS/PC liposomes [17]. The present results would point, however, to a distinct difference between these two homologous proteins in their interaction with nucleotides, suggesting that the function of various annexins is differently regulated within the cell.

Acknowledgements: The authors wish to thank Dr. Andrzej Sobota from the Nencki Institute of Experimental Biology, Warsaw, Poland, for providing G-actin. This work was supported in part by Grant No. 6 PO4A 014 08 from the Polish State Committee for Scientific Research (to J.B.-P.) and in part by the Nencki Institute of Experimental Biology and NIH Grant GM32304 (to Y.C.A.).

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