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Cyclic 3′-5′-adenosine monophosphate binds to annexin I and regulates calcium-dependent membrane aggregation and ion channel activity

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Abstract The annexin (Anx) gene family comprises a set of calcium-dependent membrane binding proteins, which have been implicated in a wide variety of cellular processes including membrane fusion and calcium channel activity. We report here that cAMP activates Ca^{2+}-dependent aggregation of both phosphatidylserine (PS) liposomes and bovine chromaffin granules driven by [des 1–12]annexin I (lipocortin I, AnxI). The mechanism of cAMP action involves an increase in AnxI-dependent cooperativity on the rate of such a reaction without affecting the corresponding $k_{1/2}$ values. Cyclic AMP causes the values of the Hill coefficient (n_H) for AnxI to change from 3 to 6 in both PS liposomes and chromaffin granules. By contrast, ATP inhibits the rate of aggregation activity without affecting the cooperativity or the extent of aggregation process. We were also able to photolabel AnxI specifically with an 8-azido analogue of cAMP by a calcium-independent process. Such a process is saturable, yielding a K_d = 0.8 μM by Scatchard analysis. Specific displacement occurs in the presence of cAMP and ATP. Finally, we found that cAMP alters the conductance of calcium channels formed by AnxI in planar lipid bilayers. We interpret these data to indicate that AnxI binds both calcium and cAMP independently, and that both actions have functional consequences. This is the first report of a nucleotide binding function for a member of the annexin gene family.

Key words: cAMP; ATP; Annexin I; Calcium; Membrane aggregation; Channel activity

1. Introduction

Annexin gene family members are becoming increasingly recognised as important mediators of a number of cell functions including regulation of membrane trafficking, transmembrane channel activity, inhibition of phospholipase A_2, inhibition of coagulation, and transduction of mitogenic signals [1]. The common property of all annexins is the ability to bind phospholipids in a Ca^{-2}-dependent manner [2]. The family is characterized by possessing a homologous C-terminal tetrad repeat (or octad in the case of annexin VI), and the individual members are distinguished from each other by a unique N-terminal domain [3]. Most annexins are abundant intracellular proteins and annexin I (lipocortin I, p35, AnxI), itself is approx. 4% of the total soluble protein in human polymorphonuclear leucocytes [4]. Annexin I is a preferred substrate for the epidermal growth factor receptor kinase [5] and may play a crucial role in mediating anti-inflammatory glucocorticoid actions [6].

Many members of the annexin gene family, including annexin VII (synexin), annexin V (endonexin II) and annexin I are known to form ion channels in lipid bilayers [7,8]. Recently, all members of the annexin family have been shown to share homology with the cystic fibrosis transmembrane regulator (CFTR) [9] in a portion of the first nucleotide-binding fold (NBF-1) containing the phenylalanine F508 of CFTR [10]. Since ATP gates anion channel activity in recombinant NBF-1 [11], we have hypothesized that nucleotides might also regulate annexin functions. We failed to detect a classical Walker A consensus sequence (GXXXXGKT) for binding of ATP or GTP in the annexin family of proteins. However, we did note motifs in a portion of annexin I spanning the first and seconds repeats corresponding to those for classical cyclic AMP binding sequences.

In the present work, we show that while calcium activates annexin I driven chromaffin granule membrane aggregation and liposome fusion, these membrane active properties are specifically and cooperatively regulated by cAMP. Further, we found that annexin I itself can be photolabelled specifically with 8-azido-[^{32}P]cAMP, and that the calcium channels formed by annexin I in planar lipid bilayers can be profoundly altered by cAMP.

2. Materials and methods

2.1. Preparation of [des 1–12]AnxI

[des 1–12]AnxI was prepared from bovine lung as described in detail [12]. Briefly, fresh bovine lung was obtained from a local slaughterhouse, cut into small pieces and frozen in liquid nitrogen. The frozen tissue was ground into fine powder in a Waring blender. About 250 g of frozen powder were suspended in a 0.3 M sucrose solution containing 2.5 mM EGTA and 2 mM PMSF, and allowed to stand on ice with occasionally stirring for 15 min. After filtering through four layers of gauze, and centrifugation at 8000 × *g* for 30 min, the supernatant was again filtered and centrifuged at 135 000 × *g* for 60 min. A 50–80% ammonium sulfate fraction was prepared, and the precipitate was solubilized in 60 ml of 1 mM benzamidine, 10 mM HEPES (pH 7.4). Following dialysis against the buffer, the fraction was bound to DEAE-Sephacel (Pharmacia LKB) and eluted with 1 mM $CaCl_2$ in 10 mM HEPES (pH 7.4). The collected fractions were further chromatographed on an FPLC preparative Mono S column in 20 mM MES (pH 6.0), 1 mM EGTA and eluted with a 0–0.3 M NaCl gradient in 20 mM MES (pH 6.0). [des 1–12]AnxI was eluted as a single protein peak and was shown to be homogeneous by SDS-PAGE. Purified [des 1–12]AnxI was stored frozen in 150 mM NaCl, 10 mM MES (pH 6.0) and 1 mM EGTA at concentrations of 0.2–0.4 mg/ml. It was found to be stable for several months and its identity was verified by solid-phase amino acid sequencing of the first 15 residues [12].

2.2. Measurement of chromaffin granule aggregation

Chromaffin granules were prepared from bovine chromaffin glands by equilibrium centrifugation in a metrizamide gradient as described by Pollard et al. [13]. Granule aggregation driven by AnxI was measured by changes in the optical density at 540 nm. To initiate the assay, granules were suspended in a 0.3 M sucrose solution containing 1 mM

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calcium and buffered with 40 mM Na-histidine at pH 6.0. In all cases, the granule suspension had an initial absorbance of 0.3 optical density units at 540 nm. Stocks solutions of Na_2ATP and cAMP were prepared in a 10 mM HEPES buffer and adjusted to pH 7.0 with NaOH.

2.3. Measurement of liposome aggregation

Liposomes were prepared from bovine phosphatidylserine (Avanti, Polar Lipids Inc.). Briefly, 10 mg of PS were dried under N_2 in a rotary evaporator. To the dry film, 2 ml of a 10 mM HEPES solution (pH 7.2) containing 0.1 M NaCl and 0.1 mM EDTA were added and vortexed vigorously. In order to obtain a relatively uniform diameter such multilamellar liposomes were passed seven times through Nuclepore filters (pore size 0.1 μm) in an extruder apparatus (Lipex Biomembranes, Vancouver, British Columbia).

Aggregation of PS liposomes was measured by following change in the optical density at 350 nm. Liposomes were added (50 μM final lipid concentration) to a 1 ml cuvette containing a calcium buffer solution prepared with 1 mM EGTA, 0.3 M sucrose solution, 10 mM MES (pH 6.0), 0.5 mM $MgCl_2$ and 0.8 mM $CaCl_2$. Na_2ATP (Sigma) or cAMP (Sigma) were added from stock solutions prepared as described above. Free calcium concentration was adjusted to 100 μM by adding small aliquots of a concentrated stock of $CaCl_2$, as calculated by a computer program [14] and verified measured using a Ca^{2+} electrode (WPI Industries).

2.4. Measurement of Ion channel activity in planar lipid bilayers

The experimental chamber consisted of two compartments (capacity = 1 cm^3 each) separated by a thin Teflon film with a small circular hole (50–100 μm) at the center. Planar bilayers were formed by spreading on the hole a mixture (1 : 1) of palmitoyloleoylphosphatidylethanolamine and phosphatidylserine dissolved in n-decane. [des 1–12]AnxI was incorporated into the lipid bilayers as described previously [15]. Both the *cis* and *trans* compartments contained 140 mM NaCl, 1 mM $CaCl_2$ and 1 mM Na-HEPES (pH 7.2).

2.5. 8-N_3[^{32}P]cAMP binding assay

8-Azidoadenosine 3′-5′-monophosphate is a photoaffinity analogue that has been shown to be a specific probe for covalently labelling cAMP binding sites [16]. For this purpose, bovine [des 1–12]AnxI (3–5 μg) was incubated in 50 μl of a buffer solution containing 25 mM Tris (pH 7.0), 150 mM sucrose, 5 mM glutathione and different amounts of cAMP and ATP. After 15 min, 5 μM 8-N_3[^{32}P]cAMP (ICN, spec. act. = 50 Ci/mol) was added and incubated at room temperature for 5 min, before photolysis for 2 min. For displacement studies, different concentrations of cold nucleotides were added prior to the addition of the radioligand. Saturation curves for 8-N_3-[^{32}P]cAMP binding were obtained by incubation of [des 1–12]AnxI with serially diluted radioligand at a final concentrations ranging from 0.04 to 10 μM. Specific binding of the radioligand to AnxI was determined by subtracting total binding from the non-specific binding obtained by incubation of the protein with 10 mM cAMP. Photolysis was achieved by exposure of the sample to a 150 W xenon arc lamp. The photolyzed mixtures were denatured by dilution in sample buffer, and applied directly to an SDS-polyacrylamide gel (12%). Alternatively, the photolyzed mixtures were filtered through a nitrocellulose membrane (S&S, NC) using a dot-blot apparatus. The dot blots were washed five times with 200 μl Tris buffer-sucrose solutions (50 mM Tris pH 7.0). Autoradiographic images of either the dried gel or the nitrocellulose membrane were generated by exposure to a Phosphor storage screen for 48 h before scanning the film with a phosphoImager (Molecular Dynamics)

3. Results

3.1. The effect of cAMP and ATP on Anx I-driven aggregation of chromaffin granules and PS liposomes

Both AnxI and its [des 1–12]AnxI derivative aggregate intact chromaffin granules in a calcium-dependent manner. In the chromaffin granule assay (Fig. 1), [des 1–12]AnxI-induced aggregation ($k_{1/2,Ca^{2+}}$ = 230 μM) is positively cooperative. In terms of protein concentration dependence, the Hill coefficient (n_H) is 3.2 ± 0.5, and the $k_{1/2}$ is 4.6 ± 0.3 μg/ml (120 nM). Upon addition of 1 mM cAMP to the reaction mixture, the $k_{1/2}$ was

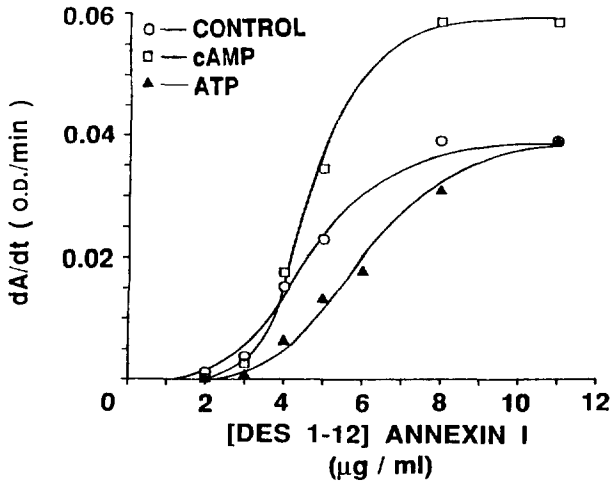

Fig. 1. The effect of cAMP and ATP on the rate of aggregation of chromaffin granules by [des 1–12]AnxI at increasing concentrations. The initial changes in optical density at 540 nm were determined after mixing increasing [des1–12]AnxI concentrations with a dilute suspension of chromaffin granules (see section 2). (○) Control, no nucleotide; (▲) 1 mM ATP; (□) 1 mM cAMP. All experimental points (average of duplicate experiments) were fitted to the Hill's cooperativity equation and the corresponding Hill index values (n_H) were calculated.

unaffected, although there was a substantial increase both in cooperativity (n_H = 6.5 ± 0.5) and in the maximum velocity. By contrast, inclusion of 1 mM ATP (Fig. 1) resulted in a reduction of the free calcium concentration from 1 mM to 0.5 mM and only a slight increase in the $k_{1/2}$ for [des 1–12]AnxI (Fig. 1). However, the extent of the aggregation reaction and the cooperativity were unchanged. Thus, cAMP and ATP have distinct effects, and ATP inhibition is unrelated to chelation of free calcium.

A critical question at this point was whether cyclic AMP was acting on the AnxI molecules, per se, or on a site associated with the target chromaffin granules. We reasoned that if the nucleotide acted on the protein, then AnxI-driven aggregation of phosphatidylserine (PS) liposomes should be activated in an equivalent manner to that observed for granules. In the liposome assay (see section 2), aggregation induced by [des 1–12]Anx I was found to be cooperative with a Hill coefficient n_H of 3.0 ± 0.3 and a $k_{1/2}$ of 7.3 ± 0.9 μg/ml (n = 4). In this case (Fig. 2), we fitted the initial rates of liposome aggregation to a normalized % change as we have previously done (cf. [7]). Upon addition of 0.8 mM cAMP (Fig. 2A), the cooperativity index n_H increased to 6.8 ± 1.5 (n = 3), although the half-maximal value $k_{1/2}$ for [des 1–12]AnxI-induced aggregation appeared unchanged (7.0 ± 0.3 μg/ml). By contrast, addition of ATP did not change the cooperativity (n_H = 3.1 ± 0.7, n = 3), but did cause the $k_{1/2}$to shift significantly to the right (Fig. 2B). Thus, upon addition of 2 mM ATP, the $k_{1/2}$ for [des 1–12]AnxI-induced aggregation shifted from 7.3 ± 0.9 μg/ml (n = 4) to 10.3 ± 0.8 μg/ml (n = 3). On this basis, we conclude that the protein seems to be the principal site of nucleotide action and that PS liposomes can be used in place of chromaffin granules to assess the effect of cAMP and ATP on [des 1–12]AnxI.

In order to gain insight into the possible mechanism of action of cAMP and ATP, we next measured liposome aggregation rates in different concentrations of ligand, and with [des

1–12]AnxI concentrations of 4.2 and 6.5 μg/ml. The inverse plots of the inhibitory actions were clearly linear in both cases (data not shown). The calculated values of K_i were 0.8 ± 0.1 mM (n = 3) for cAMP and 1.4 ± 0.5 mM (n = 3) for ATP, and were consistent with the fact that cAMP only became activating in liposomes at [des 1–12]AnxI protein concentrations greater than 7 μg/ml. These data certainly do not exclude multiple binding sites for these nucleotides on [des1–12]AnxI, but do tend to argue against cooperative interactions among them if they do occur.

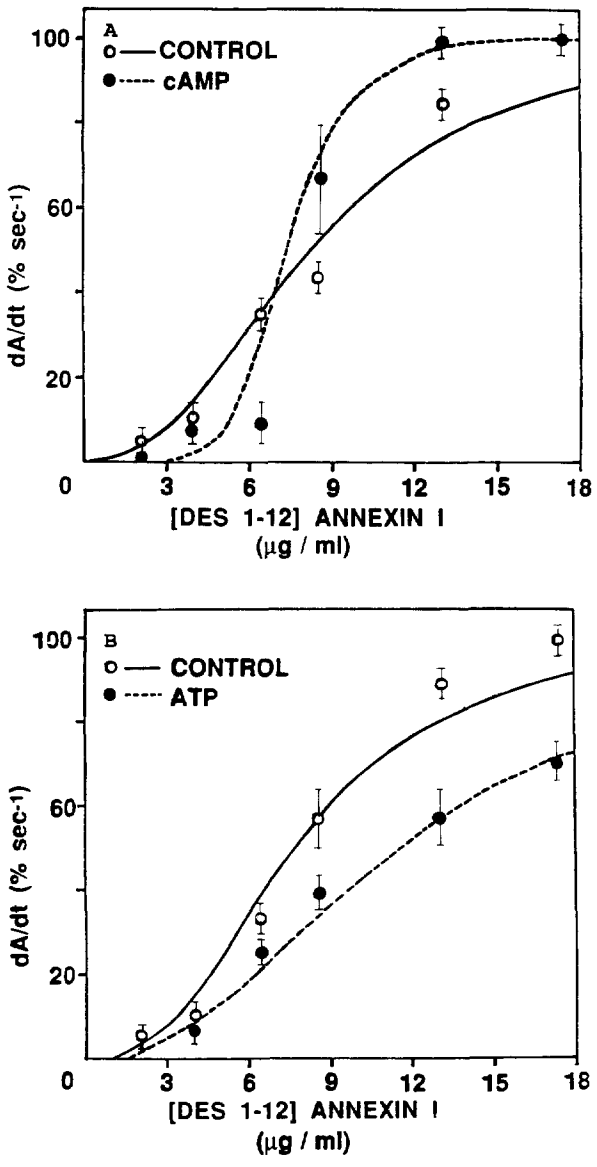

Fig. 2. The effect of ATP and cAMP on the rate of aggregation of phosphatidylserine liposomes by [des 1–12]AnxI at increasing concentrations. [des 1–12]AnxI was added at different final concentrations to PS liposomes (50 μM final lipid concentration) suspended in a Ca^{2+}/EGTA buffer solution (final calcium concentration = 100 μM) containing or not containing nucleotides (see section 2). (A) The effect of cAMP on the initial rate of the protein-driven aggregation of liposomes. (B) The effect of ATP on the initial rate of protein-driven aggregation of liposomes. The bars indicated standard deviation of three independent experiments. All experiments were fitted to a normalized % change by using the Hill cooperativity equation. (——) Control curves; (– – –) in the presence of cAMP (0.8 mM) or ATP (2 mM).

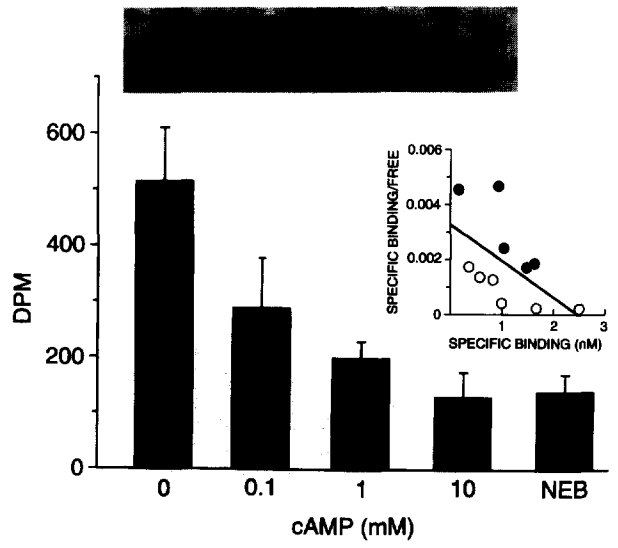

Fig. 3. Inhibition of 8-N_3[^{32}P]cAMP binding to [des 1–12]AnxI by cAMP. Bovine [des 1–12]AnxI (3 μg/ml) was incubated with various concentrations of cAMP in the presence of 2 μM 8-N_3[^{32}P]cAMP for 1 h at room temperature as described under section 2.The photolyzed mixtures containing sample buffer were applied directly to an nitrocellulose membrane using a dot-blot apparatus. The autoradiographic image of a representative example of the dried membrane is shown. The calculated values of radioactivity (in dpm) incorporated into the protein are shown as a function of the cAMP concentration. The bars indicate standard deviation of three independent experiments. NEB = non-specific binding obtained when protein sample (no cold nucleotide added) was not illuminated. Inset: Scatchard plot representing specific binding of 8-N_3[^{32}P]cAMP to [des 1–12]AnxI. The saturation curve was obtained by incubating [des1–12]AnxI (0.5 μM) with increasing concentrations of 8-N_3[^{32}P]cAMP. The K_d and B_{max} values calculated from this plot were 0.8 μM and 2.4 nmol/l, respectively. The latter value corresponds to 0.005 mol bound 8-N_3[^{32}P]cAMP per mol of protein. Points are means of two separate experiments indicated by filled and empty circles.

3.2. *Effect of adenine and guanine nucleotides on the aggregation activity of PS liposomes*

We also evaluated nucleotide specificity by testing different adenosine and guanosine nucleotides at an AnxI concentration which clearly exhibited a cAMP-activating effect on liposome aggregation (Fig. 2A). As shown in Table 1, neither GTP nor cGMP had any appreciable effect on the magnitude of the initial rate of liposome aggregation driven by 8.7 μg/ml of [des 1–12]AnxI. Among adenosine nucleotides, AMP was half as potent as cAMP in activating the same Ca^{2+}-dependent aggregation process. ADP was only slightly less potent than ATP at inhibiting the same aggregation process. Thus AnxI binds adenosine nucleotide with one phosphate group in a manner qualitatively different from the manner in which it binds nucleotides with two or three phosphates.

3.3. *Specific binding of 8-N_3[^{32}P]cAMP by [des 1–12]Anx I*

At the biochemical level, we have carried out direct binding studies with a photo-activated 8-azido[^{32}P]cAMP probe. (see section 2). As shown in Fig. 3, we found that the incorporation of the probe into [des 1–12]AnxI decreased in the presence of increasing concentrations of cold cAMP, reaching a minimum value at 10 mM cAMP. In fact, the maximal probe displacement measured at this nucleotide concentration is equivalent to the non-specific binding of the azido probe obtained in the

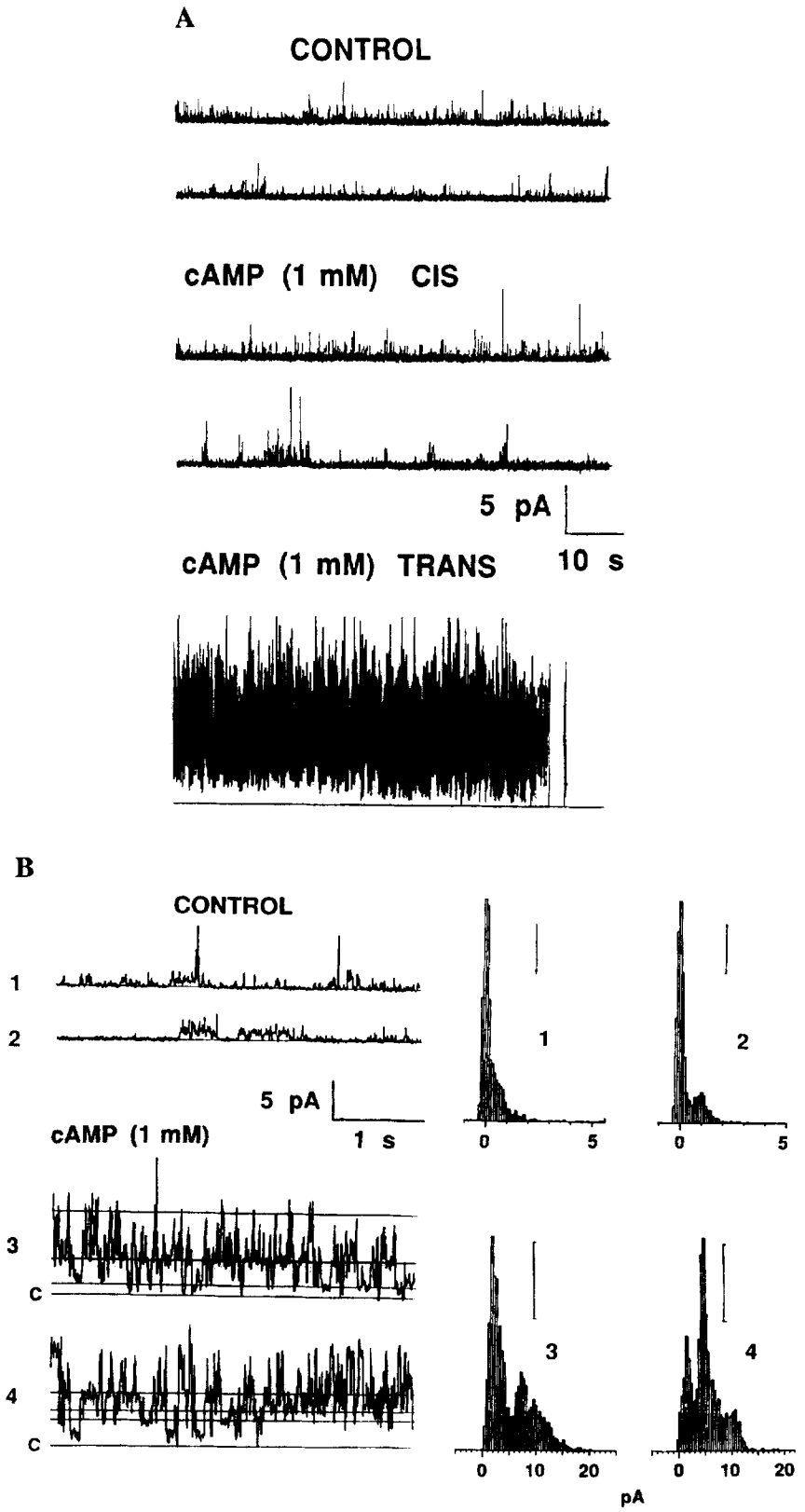

Fig. 4. The effect of cyclic cAMP on the channel activity of bovine [des 1–12]AnxI across planar lipid bilayers. (A) The cation channel activity of [des 1–12]AnxI recorded in the absence of cyclic nucleotide (control, upper traces) and after adding 1 mM cAMP to the *cis* (middle traces) or *trans* compartment (bottom trace). (B) An expanded detail of the current traces shown in A (time × 10). Numbered traces are records obtained in the absence of cyclic nucleotide (traces 1 and 2) and after adding 1 mM cAMP to the *trans* compartment (traces 3 and 4). The corresponding analyses of amplitude histograms are shown on the right side of each set of records. The bar on the abscissa of each histogram represents 100 events.

Conductance (obs.), pS		Conductance (pred.), pS	
Control (△)	+cAMP (▲)	Pred #1	Pred #2
34		29	31
47	43	43*	47*
60		65	71
	107	96	106
	150	145	159
	190	217	
	240		240
	317	326	
	353		360
	507	489	
	607		540

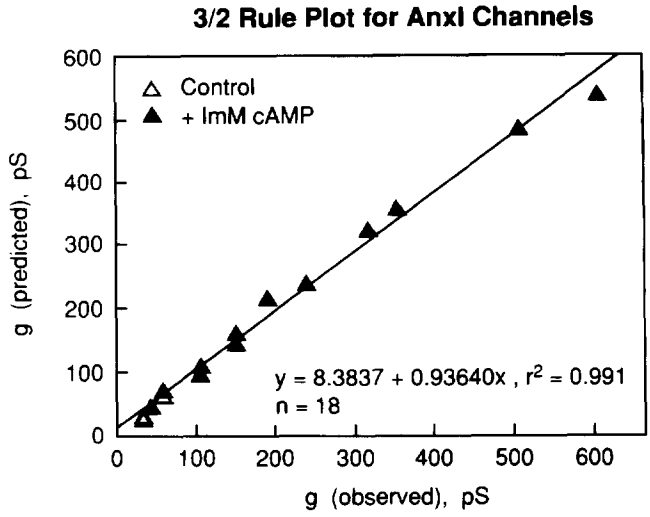

Fig. 5. Analysis of AnxI channels in the absence or presence of cAMP. In the absence of cAMP, three conductances are observed. In the presence of cAMP, nine conductances were observed. The analysis of this set of conductances by the 3/2 rule is based on expanding the conductances marked by an asterisk (*), according to the equation $g_n = g_o(3/2)^n$. A comparison of observed and predicted conductances in shown in the graph on the right. The effect of cAMP is to increase the number of observed conductances and to elicit heterogeneity in the AnxI channels.

absence of UV illumination (Fig. 3). It can also be seen in Fig. 3 that addition of 100 μM cAMP to the annexin led to a 50% inhibition in the specific binding of the photoprobe. By using this binding assay and 10 mM cAMP to achieve maximal displacement of the probe, we obtained a saturation curve. The corresponding Scatchard plot indicated that $K_d = 0.8$ μM and $B_{max} = 2.5$ nmol/l (inset, Fig. 3). The latter value corresponds to 1 mol of bound label per 200 mol of protein, and is consistent with the well known inefficiency of the photolabelling process [16].

The displacement of the 8-N_3[^{32}P]cAMP binding to [des 1–12]AnxI by cAMP and ATP was confirmed by directly applying the photolyzed mixtures containing sample buffer to an SDS-polyacrylamide gel (12%). Autoradiographic images of the dried gel show that the radioligand bound to [des 1–12]AnxI band could be displaced by an excess of ATP as well as cAMP (10 mM each). 1 mM cAMP was only slightly more potent than 1 mM ATP and substantial displacement could be achieved by 100 μM cAMP (data not shown). On the basis of these data, we can only conclude that the potentiating site is specific for cAMP, but that ATP can also bind, albeit with the opposite functional consequences.

Table 1
The effect of nucleotides on the rate of aggregation of liposomes by des[1–12] annexin I

Nucleotide	$dA/dt \times 10^3$ (OD/s)	%	Relative to control
None	9.5 ± 0.6		
cAMP	15.4 ± 3.7	+62.1[a]	$P < 0.05$
cGMP	9.3 ± 1.5	NS	NS
AMP	12.8 ± 1.7	+34.7[a]	$P < 0.05$
ADP	7.8 ± 0.9	−17.9[b]	$P < 0.05$
GTP	9.0 ± 0.6	NS	NS
ATP	7.5 ± 0.2	−21.0[b]	$P < 0.05$

The concentration of [des 1–12]Anx I was 8.7 μg/ml. All nucleotide concentrations were 0.8 mM. For the rest of experimental details see the legend to Fig. 2. Mean values of experiments ($n = 3$, ± standard deviation) are given. The means of the nucleotide values indicated by an [a] or a [b] were found to be different with respect to the control at the $P < 0.05$ level (non-paired t-test). Experiments labelled [a] were not statistically different from one another. Similar experiments labelled [b] were not statistically different from one another. NS = not significant.

3.4. *The effect of cAMP on the ion channel activity exhibited by AnxI across lipid bilayers*

We have also investigated whether nucleotides might have any direct effect on the channel properties exhibited by [des 1–12]AnxI in planar lipid bilayers [15]. In the experiment shown in Fig. 4, the AnxI channels were incorporated into a planar lipid bilayer separating symmetric solutions of 140 mM NaCl. Under nucleotide-free conditions, we observed conductances of 34, 47 and 60 pS. They were calculated from the amplitude histograms of the current records (Fig. 4B). We found that the activity of these channels did not change significantly upon adding cAMP to the *cis* side of the membrane (Fig. 4A, middle). By *cis* we mean the chamber to which the protein was added. However, upon addition of cAMP (1 mM) to the *trans* chamber (Fig. 4A,bottom) we observed a significant alteration of the channel activity, not only in terms of frequency but also in amplitude. The new conductances measured were 43 107150 190240 317, 353 507 and 607pS (Fig. 4B). ATP also altered AnxI channel activity in a manner similar to that of cAMP but GTP was without effect (data not shown). Thus, evidence for functional interaction of cAMP and ATP with [des 1–12]AnxI rests upon at least two types of measurements: aggregation of membranes and alteration of AnxI ion conductance through a membrane bilayer.

4. Discussion

In the present work, we have demonstrated that [des 1–12]AnxI is both a calcium binding protein, and a nucleotide binding protein, which appears to interact differentially with cAMP and ATP. The interaction between cAMP and AnxI is manifest by the specificity of the incorporation of azido-cAMP

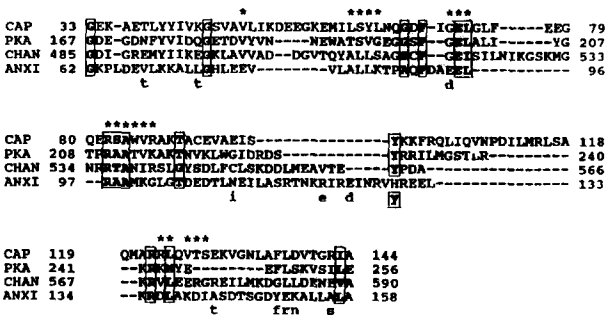

Fig. 6. Sequence homologies between AnxI and several cAMP-binding proteins. Amino acid residues 33–144 (in one letter code) of the catabolite activating protein of *E. coli* (CAP) were aligned using the MACAW program [29] with the sequences of the regulatory chain of the human cAMP-dependent kinase type I (PKA), the cAMP-gated channel from bovine olfactory epithelium (CHANN) and bovine AnxI. Amino acids replaced in bovine AnxI by different residues from those in human AnxI were printed below the bovine amino acid sequence. In order to facilitate structural comparisons, the original AnxI sequence has been renumbered as in [28]. All protein sequences were taken from the Swiss-Prot data base with the exception of bovine AnxI [4]. The corresponding accession numbers of the sequence entries were P03020 (CAP), P10644 (PKA), Q03041(CHANN) and P04083 (human AnxI). Positions of residues that interact or lie near the cAMP molecule in CAP are marked by asterisks above the CAP sequence. Identical or homologous residues among the four proteins are boxed. The definition of homology is that of the Swiss-Prot data bank, in which amino acids in the following groups are homologous: [S,T,A,G,P]; [N,D,E,Q]; [R,K,H]; [M,I,L,V]; and [F,Y,W].

into [des 1–12]AnxI, by its dependence on UV illumination, by the observation that the covalent binding of the probe to the protein decreased in the presence of increasing concentrations of cAMP (Fig. 3) and ATP (not shown), and by demonstration of saturation kinetics (Fig. 3, inset). By contrast, the action of ATP is to reduce the affinity of calcium-activated AnxI for the aggregation reaction of both chromaffin granules and pure PS liposomes. Cyclic AMP increases the cooperativity of [des 1–12]AnxI in the aggregation process. Such cooperativity is characteristic of other annexins, such as synexin [17] (annexin VII), and has been previously interpreted in terms of formation of bifunctional polymers which are able to crosslink target membranes [17–19]. Thus the increase in cooperativity of [des 1–12]AnxI by cAMP could be consistently interpreted in terms of cAMP binding to the AnxI, and a concomitant increase in the efficiency of self-association of AnxI monomers into active polymers. The fact that equivalent changes in kinetics of membrane aggregation could be detected using either biological membranes or phospholipid bilayers is evidence that the protein itself is the principal site of cAMP action in this system.

This conclusion regarding the possible direct interaction of cAMP with AnxI is borne out by the effects of cAMP on AnxI ion channels. We find here that cAMP and ATP selectively alters ion channel activity only when the nucleotide is added to the *trans* chamber of the planar lipid bilayer system. It would thus appear that a specific AnxI domain, able to interact with cAMP apparently, penetrates the bilayer and is exposed to the bulk phase on the *trans* side. This conclusion is consistent with the transmembrane organization model of Guy et al. [19,20] in which the annexin molecules penetrates the target membrane in an asymmetric manner. The nature of the alteration of the channel activity by cAMP is also consistent with predictions from the '3/2 Rule' [21]. As summarized in Fig. 5, the three conductances noted in the control condition and the additional conductances noted upon addition of cAMP can be interpreted in terms of two slightly different geometric sequences. In the presence of cAMP, two of the three lower conductances are lost and eight others are gained.

Although the binding constant for cAMP on AnxI is in the μM range, concentrations of cAMP in the mM range are required to modify the observed AnxI-induced effects on chromaffin granules and PS liposome aggregation (Figs. 1 and 2A), and on ion channel activity (Fig. 4). It is possible that the affected membranes may be having an effect on the interaction between cAMP and AnxI. However, a survey of the literature indicates that while the resting intracellular concentration of cAMP may be in the micromolar range, quantitative conversion of cytosolic millimolar concentrations of ATP to cAMP has been observed in stimulated adipocytes [22] and melanocytes [23] using HPLC and NMR, respectively. The quantitative flow of ATP to cAMP has also been the subject of extensive studies by mass spectrometry [24]. Thus, while other cellular factors may act on AnxI to reduce the binding constant for effector interactions, the membrane data as they stand can be considered physiologic.

We can now turn to the question of where cAMP and ATP might act on the AnxI. As shown in Fig. 6, regions of homology occur between AnxI and the three classical cAMP-binding proteins [25], many of which are in regions crystallographically associated with cAMP interactions [26,27]. The most striking of these includes the E72 and R82 of CAP which are coincident with E95 and R97 of AnxI. Interestingly, R97 in AnxI is the location of the RAA motif which in CAP is crucial for binding cAMP [26,27]. A characteristic RAA motif have also been observed in all known cAMP-dependent kinases, but not in cGMP-dependent kinases where the first alanine of the motif is replaced by a threonine [25]. In this respect, the observation of the RAA motif in the segment of AnxI, which is homologous to CAP and other well characterized cAMP-binding proteins (Fig. 6), is consistent with our finding of a selective action by cyclic AMP on the membrane aggregation activity of AnxI as compared to cyclic GMP (Table 1). However, we must conclude that these site predictions are necessarily only suggestive until additional crystallographic data sets for AnxI [28] can be collected with cAMP present. Nonetheless, based on the strong chemical data presented here, we can conclude that AnxI is a protein for which the regulation of function by cyclic nucleotides may rank in equal importance to that of calcium and phospholipid binding. The applicability of this observation to other members of the annexin gene family awaits further study.

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