Interactions of Benzodiazepine Derivatives with Annexins*

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Human annexins III and V, members of the annexin family of calcium- and membrane-binding proteins, were complexed within the crystals with BDA452, a new 1,4-benzodiazepine derivative by soaking and co-crystallization methods. The crystal structures of the complexes were analyzed by x-ray crystallography and refined to 2.3- and 3.0-Å resolution. BDA452 binds to a cleft which is located close to the N-terminus opposite to the membrane binding side of the proteins.

Biophysical studies of the interactions of various benzodiazepine derivatives with annexins were performed to analyze the binding of benzodiazepines to annexins and their effects on the annexin-induced calcium influx into phosphatidylserine/phosphatidylethanolamine liposomes. Different effects were observed with a variety of benzodiazepines and different annexins depending on both the ligand and the protein. Almost opposite effects on annexin function are elicited by BDA250 and diazepam, its 7-chloro-derivative. We conclude that benzodiazepines modulate the calcium influx activity of annexins allosterically by stabilizing or destabilizing the conducting state of peripherally bound annexins in agreement with suggestions by Kaneko (Kaneko, N., Ago, H., Matsuda, R., Inagaki, E., and Miyano, M. (1997) J. Mol. Biol., in press).

Benzodiazepines are well known pharmaceuticals used in the short time therapy of insomnia and stress induced anxiety (2, 3). Psychopharmacological effects are also reported for these substances, but the molecular mechanism of their action is not yet well understood. Benzodiazepines have been found to bind with high affinity to a defined receptor population in the brain, that has been identified as the GABA_A receptor. The affinity of different benzodiazepine derivatives to this receptor correlates well with their pharmacological potency and their binding site is apparently localized on the receptor close to the GABA-binding site (4, 5). The models of mechanism of action proposed so far suggest a cooperative effect of both GABA and benzodiazepine on the opening of the chloride channel. Recently, diazepam was shown to increase the conductance of GABA_A channel up to 7-fold in rat cultured hippocampal neurons (6). Benzodiazepine-related compounds are one of the most important classes of bioavailable therapeutic agents with widespread biological activities including anxiolytic, anticonvulsant, and antihypnotic activities (7), cholecystokinin receptor A and receptor B antagonists (8), opioid receptor ligands (9), platelet-activating factor antagonists (10), human immunodeficiency virus trans-activator Tat antagonists (11), GPIbIIa inhibitors (12), reverse transcriptase inhibitors (13), and Ras farnesyltransferase inhibitors (14).

To these multiple actions of benzodiazepine compounds was added recently the finding that the cardiac protective agent K201, a benzothiazepine derivative, inhibits annexin V binding to actin in vitro (15). Its effect on annexin-induced calcium influx has also been studied and its binding site defined (11).

Based on these observations we analyzed in detail a potential interaction between annexins and benzodiazepines. We report here that complex formation occurs between annexins and various benzodiazepines among which are the newly synthesized cholecystokinin-A and cholecystokinin-B receptor antagonists, as well as known pharamaceuticals like diazepam. Since the physiological function of annexins is still not fully understood, the interaction of these proteins with benzodiazepines might open new lines of investigation of the role of annexins in vivo.

EXPERIMENTAL PROCEDURES

Materials

Purine annexin I was purified from bacteria expressing the recombinant protein (16). Recombinant human annexin II containing a N-terminal elongation of six residues (MRGSFK) was purified from the appropriately transformed bacteria as described (17). Human annexin III (18), human annexin V (19), and human annexin VI, VIa, and VIb (20) were purified as described. The N-terminal deletion mutants AV-N1 (Δ1–6), AV-N3 (Δ1–13), and AV-N4 (Δ1–14) were made by introducing mutations in the annexin V wild-type cDNA, expressed and purified according to the wild-type protocol. The synthesis and biological properties of the benzodiazepine derivatives BDA452, BDA250, and BDA753 (see Fig. 1) will be discussed elsewhere.

Diazepam (DZM) and 4-bromo-A23187 were purchased from Sigma (Deisenhofen, Germany), N-acetyltryptophan-amine (Trp) from Bachem (Switzerland), and the pentasodium salt of PURA-2 was from Cabiotech (San Diego, CA).

X-ray Structure Determination

Annexin V—Rhombohedral annexin V wild-type crystals were grown by vapor diffusion at room temperature against 1 mM CaCl_2, 1 M (NH_4)_2SO_4, 0.1 M Tris, pH 8.0. Crystals were then soaked with 5 mM benzodiazepine in the harvesting buffer for several days. Co-crystallization was also attempted, but failed as no crystal growth was observed. The crystals with space group P2_1 have cell constants a = b = 160.93 Å, c = 36.90 Å and contain one molecule per asymmetric unit (21, 22).
were measured on a MAR image plate system (MAR Research, Hamburg) mounted on a Rigaku rotating anode generator (λ = 1.5418 Å). Data analysis was performed with the MOSFLM program package (23) and data reduction with the CCP4 program suite (24). Data statistics are summarized in Table I, the soaked crystals were isomorphous to wild-type annexin V. Starting from the annexin V-WT structure, refinement was initiated with X-PLOR (25), using the conjugate gradient minimization. A 2Fo — Fc map was calculated and used for inspection and model building of the benzodiazepine on a graphics terminal with FRODO (26). Further rounds of refinement were done to obtain good geometry which was checked with the program PROCHECK (27). Table II summarizes the refinement results. The topology of BDA452 was constructed using an AM1 calculation with MOPAC (28).

Annexin III—Soaking of pre-formed crystals in BDA452 solutions proved impossible and co-crystallization was therefore attempted. Best crystals were obtained from a solution of 15 mg/ml protein in 50 mM Tris-HCl buffer, pH 7.5, 20 mM CaCl₂, 2 mM benzodiazepine, and 1 M (NH₄)₂SO₄ in the drop, in vapor diffusion against a well containing a 1.5418 Å.

Data analysis was performed with the program DENZO and SCALEPACK (29, 55) and the data reduction with the CCP4 program suite (24). The data statistics are summarized in Table I. Since the data were nonisomorphous with both wild-type annexin III data, as well as data of annexin III co-crystallized with inositol phosphate (30), the structure analysis had to be started using the rigid-body refinement option in X-PLOR (25). Subsequent refinement was performed with REFMAC from the CCP4 program suite (24), and the data, as well as data of annexin III co-crystallized with inositol phosphate (30), the structure analysis had to be started using the rigid-body refinement option in X-PLOR (25). Subsequent refinement was performed with REFMAC from the CCP4 program suite (24), and the geometry which was checked with the program PROCHECK (27). Table II summarizes the refinement results. The topology of BDA452 was constructed using an AM1 calculation with MOPAC (28).

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Fig. 1. Structures of substances mentioned. BDA452, 3-(R,S)-(l-tryptophanyl)-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepine-2-one. BDA753, 3-(R,S)-all-l-(NH-Trp-Gly-Tyr-Ala-H)-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepine-2-one. BDA250, 1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepine-2-one. DZM, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepine-2-one (diazepam). TRP, (±)-N-acetyl-l-tryptophan-amide. K201, 4-(3-(1-(4-benzyl)piperidinyl)propionyl)-7-methoxy-2,3,4,5-tetrahydro-1,4-benzothiazepine (14).

Vesicle Preparation

Phospholipid vesicles were prepared according to Reeves and Dowben (32) by mixing phosphatidylserine and phosphatidylethanolamine (Avanti Polar Lipids) at a molar ratio of 3:1 in chloroform (total lipid amount for centrifugation assay: 10 μmol, for all other experiments: 1 μmol). The solution was dried under a stream of nitrogen for 30 min and then exposed to a stream of water-saturated nitrogen for another 30 min. Lipids for the centrifugation assay were covered with a 0.2 M saccharose solution and the vesicles were allowed to swell overnight at 19 °C. For the use in fluorescense titration experiments, the lipids were covered with buffer (180 mM saccharose, 10 mM HEPES, pH 7.4) and incubated at 37 °C for 2 h. The liposomes for the calcium influx assay were covered with 2 ml of buffer F1 (100 mM FURA-2, 180 mM EDTA, 162 mM saccharose, 5 mM HEPES, pH 7.4) and incubated at 37 °C for 2 h. The vesicles were pelleted by centrifugation at 12,000 × g for 30 min. After resuspension in 200 μl of buffer F2 and applied to a S200 spin column (Pharmacia). After two additional centrifugation steps, the liposome pellet was finally resuspended in 200 μl of F2. Aliquots of 20 μl were used for the calcium influx assay. All final collection steps for the different liposome preparations were done by centrifugation at 12,000 × g for 30 min.

Annexin and Benzodiazepine Binding to Phospholipid Vesicles

Samples of 500 μl for binding assays contained the appropriate concentration of the given benzodiazepine, a 20-μl aliquot of phospholipid vesicles suspended in 5 mM TRIS, pH 7.4, 180 mM saccharose, and 1 mM CaCl₂. The components of the sample were mixed and after 10 min the phospholipid vesicles were separated by centrifugation at 130,000 × g for 30 min (4 °C). Binding of benzodiazepines to the vesicles was quantified by measuring the UV absorbance of the supernatant at 280 nm with a Perkin-Elmer Lambda 17 UV/Vis spectrophotometer. Control experiments were performed in the absence of lipid vesicles. For calcium-dependent annexin binding 100 μg of annexin V (6 μg) from a highly concentrated stock solution were added to the sample containing 1 μmol of lipid suspended in the above mentioned buffer, the appropriate amount of CaCl₂ and 100 μM BDA452. Centrifugation and measurements followed the same protocol. As a control the annexin binding assay was repeated in the absence of BDA452.

Calcium Influx Assay

The calcium influx into liposomes was monitored by using the calcium-sensitive dye FURA-2 (33) and the FURA assay was performed.
following the protocol described by Berendes et al. (34). To increase the stability of the FURA liposomes, all solutions were saturated with Ar. A 20-μl aliquot of the FURA-loaded liposome suspension was mixed with 475 μl of buffer F2, and 5 μl of a 50 mM CaCl2 solution was added. The fluorescence intensity was measured at 510 nm with the sample excited at 430 and 280 nm at time intervals of 1 min. After an equilibration time of 4 min the protein was added from a concentrated stock solution and so was the benzodiazepine derivative from a Me2SO-containing stock solution. The MeSO content of the sample did not exceed 1% of the total sample volume in any experiment. Fluorescence measurements were carried out in 1-min intervals. At t = 36 min, 3 μl of a solution of Br-A23187 (0.1 mg/ml) was added to determine the maximal possible calcium signal. Intensity measurements were continued until t = 40 min. Data analysis was performed by normalizing the fluorescence ratio \( \frac{F(340 nm)}{F(380 nm)} \) with respect to the maximal possible fluorescence ratio obtained from the values after addition of the ionophore Br-A23187 (36–40 min). The normalized fluorescence ratio \( f(\text{annexin,MeSO}) \) is plotted vs. time, thereby yielding an influx curve. For further analysis the slope of the time interval 15–35 min was used as an activity parameter (*steady state*). Alternatively, the initial slope \( \beta \) of the influx curve, starting at \( t = 4 \) min, was analyzed. The percentage of inhibition/activation was calculated using the steady state slope and the initial slope, respectively, of the influx experiment in the absence of benzodiazepine. Fluorescence measurements were performed on a Perkin-Elmer 650-40 fluorescence spectrophotometer with a spectral bandwidth of 5 nm (excitation slit) and 5 nm (emission slit). The shutter was closed between the measurements to avoid photobleaching effects.

**Fluorescence Titration**

Binding of benzodiazepine derivatives to annexin was monitored by quenching of the protein fluorescence, using a Perkin-Elmer 650-40 fluorescence spectrophotometer. Protein was added to 500 μl of buffer (5 mM TRIS, 0.01% NaNO3, pH 7.4) and the change in fluorescence intensity was examined as a function of benzodiazepine concentration. The benzodiazepine derivatives were added in 1-μl aliquots from stock solutions (1 mM, 10 mM) in MeSO. The data were normalized with respect to protein fluorescence intensity at an excitation wavelength of 280 nm. A control experiment was performed recording the concentration-dependent fluorescence of the benzodiazepine derivative and, similarly, the protein was titrated with MeSO in 1-μl aliquots to yield the maximal possible fluorescence intensity in each titration step. These binding experiments were also performed in the presence of PS/PE liposomes (36) following an analogous protocol. The buffer used for the liposome containing experiments (F3) consists of 180 mM saccharose, 10 mM HEPES, pH 7.4. Assuming a simple complex formation,

\[
\text{Annexin + ligand} \rightleftharpoons [\text{annexin-ligand}]
\]

(Eq. 1)

the fraction of complexed annexin is \( f(\text{AL}) \) corresponding to the normalized fluorescence \( f(\text{AL}) \) which can be calculated by Equation 2,

\[
f(\text{AL}) = f_{0}(A) - f(A) = \frac{F(\text{annexin,MeSO})}{F(\text{annexin,280 nm})} - \frac{F(\text{annexin,ligand}) - F(\text{ligand})}{F(\text{annexin,280 nm})}
\]

(Eq. 2)

where \( F(\text{annexin,MeSO}) \) is the fluorescence intensity of annexin in the presence of MeSO, \( F(\text{annexin, ligand}) \) the measured intensity during the titration and \( F(\text{ligand}) \) the fluorescence intensity of the benzodiazepine. Division by \( F(\text{annexin, 280 nm}) \) normalizes the experimental values with respect to protein fluorescence without ligand and MeSO.

The dissociation constant \( K_{D} \) was determined by nonlinear least-squares fit of the data to a binding model with a Hill coefficient of \( n = 1 \).

The quenching of fluorescence intensity was also analyzed in terms of the Stern-Volmer equation (35),

\[
\frac{I_{0}}{I} = \frac{F(\text{annexin,MeSO})}{F(\text{annexin,ligand})} = 1 + K_{q}c(\text{ligand})
\]

(Eq. 3)

where \( I \) represents the protein fluorescence intensity in the presence of the ligand and \( I_{0} \) the intensity in its absence.

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2 The abbreviations used are: MeSO, dimethyl sulfoxide; PE, phosphatidylethanolamine; PS, phosphatidylserine; CF, carboxyfluorescein; DZM, diazepam.

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**RESULTS**

Crystal Structure—The crystal form of annexin V used, as well as annexin III, have the Trp-187 containing loop of domain III exposed on the surface of the protein. As described previously (37), five α-helices (A to E) form one domain, with the axes of helices A, B, D, and E almost anti-parallel to each other, whereas the connecting helix C lies approximately perpendicular to the A-B axis. The four domains (I to IV) are arranged in a cyclic array with domains IV and II/III forming two modules with pseudo 2-fold symmetry. In the center of the molecule a prominent pore is created by helices IIA, IIB, IVA, and IVB, lined with highly conserved charged or polar residues. The calcium-binding sites are located on the convex side of the protein within a 17-amino acid sequence called the endonexin-

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**FIG. 2.** Surface representation of the annexin-BDA452 complexes. A, annexin III-BDA452. B, annexin V-BDA452. The molecular surface of the protein is colored according to the electrostatic surface potential (red, negative; blue, positive). The BDA452 ligand is depicted as skeleton. Figure was prepared with GRASP (51).

Carboxyfluorescein Leakage Assay

Liposomes for the leakage assay were prepared as described (34), except that 50 mM carboxyfluorescein (CF; obtained from Sigma, Deisenhofen, Germany) was included into the buffer to monitor leakage (36). Nonencapsulated CF was separated by gel filtration runs on S200 microspin columns (Pharmacia). Leakage was investigated by adding aliquots of the benzodiazepine derivatives to the vesicle suspension directly in the cuvette used for fluorescence determination. Excitation was set to 480 nm and the emission was detected at 540 nm. The results are expressed as,

\[
\text{CF-leakage(} \% \text{)} = \frac{F - F_{t}}{F_{0} - F_{t}} \times 100
\]

(Eq. 4)

where \( F_{t} \) is the initial fluorescence intensity before adding the protein, \( F \) is the fluorescence reading at different times, and \( F_{0} \) is the final fluorescence determined after adding Triton X-100 to the liposome suspension (final concentration 0.1%).

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**REFERENCES**

1. The abbreviations used are: MeSO, dimethyl sulfoxide; PE, phosphatidylethanolamine; PS, phosphatidylserine; CF, carboxyfluorescein; DZM, diazepam.
fold (38), which has been shown to bind to the membrane (39). The N and C termini lie on the opposite, concave side of the molecule.

The structure of annexin V in complex with the ligand BDA452 reveals that the ligand is bound in a cavity (Fig. 2) which is located at the interface of domains II, III, and IV at the concave side of the molecule. The strongly bent BDA452 molecule (Fig. 3B) interacts with all domains in that cleft in a hydrophobic manner, sharing a contact surface of about 373 Å² with the protein. The most prominent contact residues are Thr-118 (loop IIB/IIC), Pro-119 (IIC), Glu-120 (IIC), Arg-161 (loop IIE/IHIA), Asp-164 (loop IIE/IHIA), Val-203 (IIC), Arg-207 (IIC), Ser-243 (IIIE), and Ser-247 (IVA) (Fig. 3A). Despite a prevalent positive charge of the protein in the binding cleft no notable polar interactions could be identified and the closest distance of BDA452 to the protein atoms is above 3.1 Å. The e-imino group of Arg-207 faces the 5-phenyl group of the ligand.

Several attempts were made to solve the crystal structure of annexin V with other benzodiazepine derivatives analyzed in this study. Although there is a high electron density peak in all of these structures no reliable model building was possible for complexes AV-DZM, AV-BDA250, and AV-BDA753, probably due to substantial disorder. This observation is supportive of a remarkable flexibility of the benzodiazepine derivatives even in the protein-bound state. Additionally, the binding site might only be occupied partially.

The annexin III-BDA452 complex electron density shows a peak of difference density in the analogous region as annexin V. This peak is, however, not sufficiently well defined to give detailed information on the ligand structure. The best fit of the benzodiazepine obtained indicates a nonspecific interaction, with the 5-phenyl group of the ligand facing the side chain of Phe-206, with Arg-164 close by (annexin III numbering). The indole moiety of BDA452 points toward the N terminus, which is well defined up to Ser-2. The interaction with BDA452 provokes slight displacements of the connecting segments between domains II and III, and domains III and IV which line the binding cavity.

Binding of Benzodiazepine Derivatives to Phospholipid Membranes—Lipid membranes and benzodiazepines may interact directly as suggested by recent experiments, which show that different benzodiazepine derivatives insert into lipid bilayers to...
Fig. 4. A, BDA452 is attached to liposomes. A suspension containing PS/PE liposomes (3:1) (about 1 μmol total lipid content), 1 mM CaCl₂, and the appropriate amount of BDA452 in 180 mM saccharose, 5 mM TRIS, pH 7.4, is separated by centrifugation and the UV absorbance of the supernatant is measured at 280 nm (filled squares). Above a concentration of 5 μM BDA452, significant attachment of the benzodiazepine to the liposomes is observed. The filled circles represent the UV absorbance in the supernatant in the absence of liposomes. The results shown are the mean of three independent preparations. B, annexin V binding to phospholipid membranes is not affected by BDA452. The calcium dependent binding of 100 μM of annexin V (6 μg) to PS/PE liposomes (3:1) in the presence (filled triangles) and absence (filled circles) of 100 μM BDA452 was measured by a centrifugation assay. Shown on the ordinate is 1 - a₂₈₀ nm which represents the ratio of binding of annexin to liposomes, a₂₈₀ nm, to the absorbance of the supernatant at 280 nm normalized to the pure annexin V absorbance. The dot-and-dash line was obtained by considering the UV absorbance of 100 μM BDA452 in the presence of phospholipids and the appropriate amount of CaCl₂ in the supernatant.

Fig. 5. A, fluorescence quenching of annexin V upon addition of BDA452. Aliquots of BDA452 (stock solution in Me₂SO) are added to a solution of 52 μg of annexin V (3 μg) in 5 mM TRIS, pH 7.4, 1% i-PrOH, 0.01% NaN₃. The ordinate values of f(AL) are calculated as described under “Experimental Procedures” from the fluorescence intensities F at 310 nm (excitation 280 nm). The solid line was obtained by fitting a binding equation to the experimental data, yielding K_d = 26.1 μM. B, the addition of N-acetyltryptophan-amide does not affect annexin V fluorescence significantly. N-Acetyltryptophan-amide is added stepwise to an annexin V solution (3 μM). Buffering and calculation of the f(AL) values as in A. Emission was measured at 310 nm (excitation 280 nm). In the concentration range tested no significant fluorescence quenching of annexin V occurs. Measurements at higher N-acetyltryptophan-amide concentrations are not reliable due to its high intrinsic fluorescence.

Binding of Benzodiazepine Derivatives to Annexins—Fig. 5A shows the titration of 3 μg annexin V with BDA452 (0–280 μM). Binding of the benzodiazepine to the protein results in substantial quenching of fluorescence emission intensity of the protein excited at 280 nm. Data analysis according to Equation 2 yields the dissociation constants K_d, which are summarized in Table I. Although the data shown in Fig. 5A might suggest a biphasic interaction of BDA452 with annexin V, a monophasic model was applied to all binding experiments, since neither the crystallographic results nor data analysis according to Stern-Volmer relations indicate a biphasic behavior. To ensure that the fluorescence quenching is due to a specific interaction of the benzodiazepine derivative with annexin, a control titration experiment was done with N-acetyltryptophan-amide at concentrations from 0 to 32 μM. The addition of N-acetyltryptophanamide to an annexin solution does not result in any specific fluorescence quenching within the concentration range tested (Fig. 5B). Considerable scattering of data is observed in the presence of higher N-acetyltryptophan-amide concentrations presumably due to the high intrinsic fluorescence of this derivative. It has to be noted that reproducible quenching data were only obtained with benzodiazepine derivatives carrying a fluorophore group. Measuring of binding parameters was therefore limited to BDA452 and BDA753. As mentioned above, control titration experiments were also performed with annexins and Me₂SO revealing that the protein fluorescence is affected by the presence of Me₂SO in the sample solution (Fig. 6A). The quenching effect by the organic solvent, however, is strongly decreased, if PS/PE liposomes and 200 μM CaCl₂ are present in the buffer (Fig. 6B). This indicates that the protein is accessible to the quencher to a much lower extent in the membrane-bound state. As concluded from the K_d values, BDA452 is bound by one order of magnitude better than BDA753 for each annexin tested. This might be due to the steric interference of the tetrapeptide. Binding of BDA452 to annexins was not affected by the presence of phospholipid...
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vesicles, whereas binding of BDA753 was much tighter in the presence as compared with the absence of phospholipid membranes. We also attempted data analysis according to Stern-Volmer Equation 3 where the quenching constant $K_q$ is obtained by fitting the plot of $l/l_0$ versus $c$ (ligand) to a linear equation (Fig. 7). Since $K_q$ represents an association constant the values are much higher for BDA452 than for BDA753 (Table III), which is in agreement with the conclusions drawn from the $K_d$ determination. Likewise the tendencies in the binding behavior with and without lipids, respectively, are the same as indicated by the $K_q$ values. The Stern-Volmer analysis in terms of an association constant works well in the case of tight binding between annexin and the ligand since one can assume predominantly static quenching. In the case of weak binding (high dissociation constants), the $K_q$ values show less agreement with the $K_d$ values. Presumably, other effects than the formation of a nonfluorescent complex might apply for the observed quenching behavior.

**Effects of Benzodiazepines on the Annexin-induced Calcium Influx into Lipid Vesicles**—The calcium influx activity of different annexins and the influence of benzodiazepines was examined using FURA-2 loaded lipid vesicles and recording time dependent excitation spectra after the addition of annexin and/or benzodiazepine derivatives. Calcium influx curves were obtained by plotting the normalized fluorescence ratio $f$ versus time (Fig. 8). A control experiment was performed with DZM to exclude a possible membrane damage by the benzodiazepine derivative itself. Within the concentration range of the annexin/benzodiazepine experiments no significant membrane permeabilization was detected. At much higher concentrations (above 300 μM) the benzodiazepine leads to a considerable membrane damage (Fig. 9A). As shown by different runs of the CF-leakage assay, the benzodiazepine derivatives used in this work do not cause significant membrane damage (Fig. 9B and C). Only BDA452 at higher concentrations is able to increase the CF fluorescence intensity.

The effect of different benzodiazepine derivatives on different annexins is not uniform (Table IV). BDA452 was found to inhibit calcium fluxes induced by annexins AV, AV-N1, AVI, and AVb, whereas with annexins AI, AIII, and AVI rates of calcium fluxes are increased upon addition of the benzodiazepine (Fig. 10). A similar effect was observed with the N terminally truncated mutants AV-N3 and AV-N4. Consequently, the question arose, whether the contact of the ligand with the N-terminal region of the protein is essential and sufficient for the macroscopic effect in the calcium influx assay. However, no general correlation between the length of the N-terminal domain of annexin V and the mode of effect of the benzodiazepine is observed. While the truncated mutants AV-N1, AV-N3, and AV-N4 are differently affected in the calcium influx assay albeit being unable to contact the ligand, AIII and AI both have a longer N-terminus than AV but are activated by addition of BDA452. Similarly, AVI, a two-monomer annexin, is activated upon BDA452 addition. Annexin II shows a more complex behavior. Analysis of the steady state slope revealed a clearly inhibitory effect of BDA452 on this annexin, whereas the initial slope indicates an activation (Fig. 11A).

To complete the general view on annexin-induced calcium influx we used the smaller, commercially available benzodiazepine derivative DZM (diazepam). Annexins AIII, AV, and AV-N3 are activated upon addition of this derivative, whereas AI is not affected. It is very surprising in this respect that the addition of BDA250 instead of DZM has an almost opposite effect on the annexin behavior in the FURA assay, most likely due to the missing 7-chloro substituent in BDA250. Annexin V is not influenced by BDA250, whereas AI and AIII are inhibited (Fig. 11, B and C).

**Table III**

| Excitation wavelength: 280 nm, emission was monitored at 310 nm (AI, AII, and AV-WT) and 340 nm (AIII), respectively |
|--------------------------------------------------|
| **TABLE III**                                    |
| BDA452 with liposomes                            |
| **K_q in μM**                                    |
| **K_q in 10^5 M^{-1}**                           |
| AI                                               |
| 65.6                                             |
| 51.8                                             |
| 4.23                                             |
| 434                                              |
| AII                                              |
| 932                                              |
| 21.5                                             |
| ND°                                              |
| ND°                                              |
| AVI                                              |
| 34.8                                             |
| 18.3                                             |
| 89.2                                             |
| 18.5                                             |
| AIII                                             |
| 26.1                                             |
| 35.6                                             |
| 27.0                                             |
| 14.2                                             |
| AV-WT                                            |
| 1.00                                             |
| 222                                              |

$^a$ NA, not analyzable.

$^b$ ND, not determined.

[Image 103x220 to 253x358]
Ordinate values are normalized to the highest

\[ F_{380}/F_{340} \]

ratio. At \( t = 4 \) min, 6.4 \( \mu \text{g} \) of annexin III (0.4 \( \mu \text{M} \)) is added. Data acquisition continues until \( t = 35 \) min, where 5 \( \mu \text{l} \) of a solution of Br-A23187 (0.1 \( \text{mg/ml} \)) is used to yield the maximal possible fluorescence.

**DISCUSSION**

Annexin V is known to display an ion channel-type activity under certain conditions (37, 41, 42). Other annexins were also found to cause cation fluxes through artificial membranes. On the other hand, high annexin concentration leads to formation of two-dimensional crystals on the membrane surface (39), a state that is conduction-incompetent. Many parameters may influence the surface concentration of annexin, e.g., the calcium concentration (43, 44), transmembrane potential (45), membrane composition (46) etc.

The molecular mechanisms of annexin function on membrane surfaces are not completely understood. There are several data supporting a membrane stabilization by annexins at high concentrations, like the two-dimensional crystal formation (EM), membrane rigidification (NMR), and increase of seal resistance (patch clamp) (40, 45, 47, 49).3 A particular interesting effect on membranes is the permeabilization elicited by annexins, since these proteins do not insert into the bilayer.

**Influx Mechanism**—We need to distinguish between three different effects: (i) the interaction of the benzodiazepines with the lipid membrane, (ii) the interaction of annexins with the membrane, and (iii) the interaction of the annexin-BDA complex with the membrane in the presence of excess benzodiazepine. It is known from previous work (40) that benzodiazepines insert into lipid membranes to varying extents depending on their particular structure. The binding assay conducted in the present work indicates that about 80% of the total benzodiazepine amount is attached to lipid vesicles at an initial concentration of 100 \( \mu \text{M} \), respectively.

**Inhibition Mechanism**—Among the benzodiazepine derivatives analyzed in this study, BDA452 inhibits calcium influx activity of annexins AV, AV-N1, and AV-Ib, and BDA250 annexins I and III. Taking into account the N-terminal sequences shown in the crystal structure. Such binding may affect the inter-module angle and the flexibility in different ways depending on the annexin and the benzodiazepine derivative leading to activation or inhibition of calcium influx.
of annexins, BDA452 might be able to contact the N terminus of AV and AVIb both of which have an alanine in neighboring positions at the N terminus. This argument, on the other hand, is not true for annexin V-N1 where residues 1 to 6 are missing. Additionally, the contact is only possible with BDA452 because of its tryptophan moiety being exposed to the protein exterior. It is therefore surprising that BDA250 also displays inhibitory effects on AI and AIII. These annexins, however, contain a longer N-terminal region than AV and might be able to contact the ligand at other sites. Moreover, both of them have a conserved serine (Ser-27; Fig. 12).

Crystal Structures—The BDA452 ligand bound to annexin III appears to have a somewhat different conformation when compared with the annexin V-BDA452 structure (Fig. 13), although it is impossible to make detailed comparisons due to the poor density of the ligand bound to annexin III. The orientation of the ligand is similar in both proteins, while its shape is more open in annexin III. In both structures the tryptophan moiety is pointing toward the N-terminal region of the protein, but the length and conformation of the N termini is different in the different annexins studied. The N terminus seems to define the space within which the ligand can bind: thus Val-4 (annexin V) prevents the ligand from a closer contact with domain I, which is accomplished in annexin III, and the ligand is slightly rotated. On the other hand, the change in position of domains II

### TABLE IV

|        | BDA452 | BDA250 | DZM | BDA753 |
|--------|--------|--------|-----|--------|
| $a/a_0$| $\downarrow$ | 0.5 | 3.8 | $\uparrow$ |
| $b/b_0$| $\downarrow$ | ND | ND | $\uparrow$ |
| $a/a_0$| ND | ND | 57 | 44 |
| $b/b_0$| 6.2 | 3.8 | ND | ND |
| AI     | $\uparrow$ | ND | ND | $\Rightarrow$ |
| AV-N1  | $\downarrow$ | ND | ND | $\Rightarrow$ |
| AVIa   | $\downarrow$ | ND | ND | $\Rightarrow$ |
| AVIb   | $\downarrow$ | ND | ND | $\Rightarrow$ |
| AIII   | $\uparrow$ | "NA" | 5.3 | ND |
| AV-N3  | $\downarrow$ | ND | ND | $\Rightarrow$ |
| AV-N4  | $\uparrow$ | ND | ND | $\Rightarrow$ |
| AVI    | $\uparrow$ | 46 | 33 | $\Rightarrow$ |

- ND, not determined.
- NA, not analyzable.

![Graph A](image1.png)

**Fig. 10. Activation of annexin VI-induced calcium influx by BDA452.** The steady state slope $\alpha$ (closed circles, solid line) and the initial slope $\beta$ (open triangles, dashed line) are plotted against the concentration of BDA452 used in the FURA assay with annexin VI. Both parameters indicate the strong activation of annexin VI due to BDA452. The curves follow a saturation equation.

![Graph B](image2.png)

**Fig. 11. A, contradictory effects of BDA452 on annexin II.** Data analysis of BDA452-dependent calcium influx assays with 21 μg of annexin II (1 μM). Whereas the steady state slope (filled circles) indicates an inactivation of annexin II with increasing amounts of BDA452, the initial slopes (open squares) point to an activation. In terms of the total amount of calcium crossing the phospholipid membrane annexin II is not influenced by BDA452. Normalization was done against the influx activity of 1 μM annexin II without BDA452. B and C, BDA250 and DZM display almost opposite effects. Whereas DZM enhances the annexin-induced calcium influx (dose dependent), BDA250 has no effect on the annexin V-induced membrane permeabilization. The membrane function of annexin III is even inhibited. B, annexin III; C, annexin V. Filled circles, BDA250; open squares, DZM.

and III in annexin III as compared with annexin V seems to be designed to ensure their similar contacts with the ligand as in annexin V. Thus, the side chain of Arg-164 (annexin III) maintains a similar contact with the 7-position of the benzodia-
zepine ligand in both structures. This side chain could be important as a possible sensor for substitutions in the 7-position of these ligands, since it is conserved throughout nearly all annexins (except annexin VII). Surprisingly, the binding pocket in both proteins is not as hydrophobic as one would expect (Fig. 2) but is positively charged which might be attractive for the polarizable seven-membered ring of the benzodiazepine derivative.

Binding of Benzodiazepine Derivatives to Annexins—Gener-
Annexins and Benzodiazepines

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