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Inhibition of annexin V-dependent Ca^{2+} movement in large unilamellar vesicles by K201, a new 1,4-benzothiazepine derivative

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Abstract

Examination was made of the effect of annexin V on Ca^{2+} movement into large unilamellar vesicles (LUV) using fura-2, a calcium-sensitive fluorescent dye. To avoid the possible difficulties relating to the addition of annexin V and/or Ca^{2+} in fura-2-loaded LUV, the burst method was used. LUV, preincubated with rat annexin V in the presence of Ca^{2+}, were collected by centrifugation and resuspended, and then burst with Triton X-100 in the presence of fura-2. Inward Ca^{2+} movement across the artificial lipid membrane was measured by determination of fura-2 fluorescence due to the leaked Ca^{2+} from ruptured LUV. The observed Ca^{2+} signal increased dependent on annexin V and Ca^{2+} concentrations, whereas bovine serum albumin did not affect this signal up to 1 μM. Thus, annexin V shows Ca^{2+} channel activity in LUV. K201, a novel 1,4-benzothiazepine, inhibited inward Ca^{2+} movement into LUV caused by annexin V in a dose-dependent manner. In the presence of 50 nM annexin V and 400 μM Ca^{2+}, 3 μM K201 showed significant inhibition of Ca^{2+} movement due to annexin V, and 50% inhibition was achieved at 25μM K201. On the other hand, diltiazem had no such effect even at 30 μM. K201 is thus shown to have inhibitory activity on inward Ca^{2+} movement due to annexin V in artificial vesicles and may prove useful as a probe for elucidating the functions of annexin V in vivo. © 1997 Elsevier Science B.V.

Keywords: K201; Annexin V; Ca^{2+} movement; Large unilamellar vesicle; Fura-2

1. Introduction

Annexin V is a member of the annexin family which binds to phospholipid and actin, in a Ca^{2+}-dependent manner [1,2]. This protein has been suggested to have such actions as anticoagulant activity [3], anti-inflammatory activity [4] and inhibition of protein kinase C [5] in vitro.

Annexin V has been found to express calcium channel activity by the patch clamp method [6], and its molecular structure as an ion channel model has been proposed based on the crystal structure [7]. The biological roles of annexin V have yet to be fully determined in vivo, but if a low molecular weight compound that binds to annexin V and inhibits its function can be made available, considerable clarification in this regard should be possible.

A novel 1,4-benzothiazepine derivative, 4-[3-{1-(4-benzyl) piperidinyl} propionyl]-7-methoxy-2,3,4,5-tetrahydro-1,4-benzothiazepine, K201 (Fig. 1), has been found to have potential for preventing

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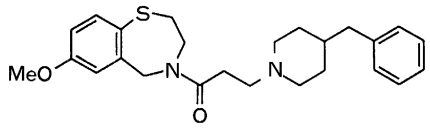

4-[3-[1-(4-benzyl)piperidinyl]propionyl]-7-methoxy-2,3,4,5-tetrahydro-1,4-benzothiazepine

Fig. 1. Chemical structure of K201.

cardiac cell damage due to calcium overload [8]. It was also found capable of protecting the myocardium from damage induced by six hour ischemia using a rat Langendorff model [9]. On the other hand, K201 exhibited an inhibitory effect on binding of cardiac annexin V with actin in a Ca^{2+}-dependent manner [8] and was demonstrated to bind to annexin V by X-ray crystallography (unpublished observation, submitted for publication).

In order to clarify the calcium ion channel activity of annexin V, the present study was conducted to examine the inhibitory effect of K201 on inward Ca^{2+} movement across large unilamellar vesicles (LUV) due to annexin V using fura-2 [10], a Ca^{2+}-dependent fluorescent agent. The effect of K201 was also compared with that of diltiazem [11], a 1,5-benzothiazepine derivative. For this purpose, the burst method was employed to measure the amount of influxed Ca^{2+} in the collected LUV after preincubation with annexin V and Ca^{2+}, to avoid possible difficulties such as LUV disintegration caused by annexin V and/or Ca^{2+}.

2. Materials and methods

2.1. Purification of annexin V

As in our previous study [12], annexin V was purified from the hearts of 50 mature male Wistar strain rats using a Sephacryl S-300 (Pharmacia, Uppsala, Sweden) column (3.3×55 cm), and then a DEAE Bio-Gel Agarose (Bio Rad Laboratories, Richmond, CA, USA) column (1.0×10 cm). Identification of annexin V was made by partial amino acid sequencing. Quantitative protein analysis was conducted according to the modified method of Lowry et al., using bovine serum albumin (BSA) as a standard protein [13].

2.2. Preparation of LUV

LUV were prepared by the procedure of Reeves and Dowben [14], with modification and by that of Berendes et al. [15]. Briefly, a solution of phosphatidylserine and phosphatidylethanolamine (w/w 9:1) dissolved in methanol/chloroform (v/v 1:2) to a total volume of 1115 μl was poured into a round bottom flask. Treatment of the mixture with a rotary evaporator (Tokyo-Rikakikai, type N-1N, Tokyo) led to the formation of a dehydrated film under N_2 gas. This film, comprised of lipids, was further dehydrated for 30 min by the evaporator under water-saturated nitrogen. After gently pouring 2 ml buffer A [180 μM EGTA, 162 mM sucrose, 10 mM HEPES, pH 7.4 (tetramethylammonium hydroxide; TMA)] into the flask, the lipid film was saturated with nitrogen gas and the flask was sealed with a rubber cap and left to stand at 37°C for 2 h to produce LUV. The multilamellar vesicles were removed by centrifugation. The LUV solution was centrifuged in a Beckman Avanti 30 compact centrifuge at $12\,000 \times g$ for 10 min at 4°C. To the sediment containing LUV, dilution buffer B [180 μM EGTA, 160 mM sucrose, 10 mM HEPES, pH 7.4 (TMA)] was added to adjust the total volume to 100 μl. This procedure was performed twice, and the suspension of LUV (200 μl) was used to perform at least twenty experiments. The total lipid concentration of liposomes was estimated as inorganic phosphate by phosphate analysis [16].

2.3. Burst method

The burst method was used to examine inward Ca^{2+} movement into LUV due to annexin V. To 442 μl dilution buffer B containing ca. 0–450 μM Ca^{2+}, a suspension containing LUV (8 μl) and annexin V ca. 0–100 nM (50 μl) were added, followed by preincubation at 26°C for 10 min. After adding 400 μM EGTA, the solution was centrifuged at 4°C for 10 min at $7000 \times g$ to collect LUV. The collected LUV were suspended in buffer B solution (480 μl) containing 1 μM fura-2. After incubation in a cuvette

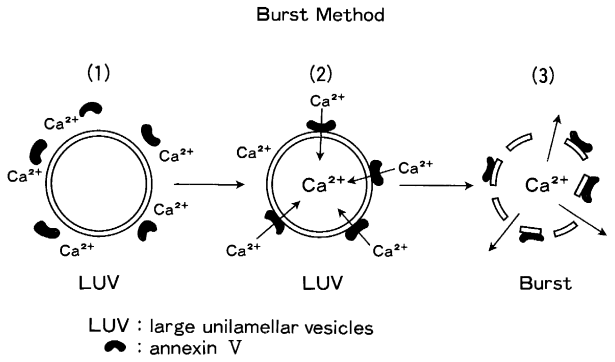

Fig. 2. Burst method: (1) LUV were added to buffer solution containing Ca^{2+} and then annexin V with or without reagent was added. Concentration of phospholipid in LUV suspension was 20 mM. The final amount of phospholipid used in each experiment was 0.125 μmol $cell^{-1}$. (2) Incubation was conducted at 26°C for 10 min. LUV were recollected by centrifugation following the addition of 400 μM EGTA. (3) Resuspended LUV were burst by addition of 0.2% Triton X-100 in 1 μM fura-2 solution.

at 37°C for 1 min, a burst was made with 0.2% Triton X-100 (10% w/w, 10 μl) (Fig. 2). The effect of BSA (ca. 0–1000 nM) on Ca^{2+} movement into LUV was also investigated.

To prove that there is no influence of K201 on the liposomes, Ca^{2+}-incorporated LUV were made. Ca^{2+}-incorporated LUV were prepared by addition of 400 μM Ca^{2+} in buffer A at the time of preparation of LUV. These were incubated with ca. $0–10^{-4}$ M K201 at 26°C for 10 min, and then recollected and burst as above.

The influence of K201 on the ability of annexin V to bind to liposomes in the presence of Ca^{2+} was estimated by measurement of the amount of annexin V binding to LUV incubated in various concentrations of K201. LUV (containing only buffer A) were incubated with 50 nM annexin V and ca. $0–10^{-4}$ M K201 in the presence of 400 μM Ca^{2+} at 26°C for 10 min. Recollected LUV were burst with 0.2% Triton X-100 and then the concentrations of annexin V were measured by ELISA, as previously reported [17].

Ca^{2+} concentration was measured by a JASCO CAF-110 fluorescence spectrophotometer (Nihon Bunkou, Tokyo, Japan). Data were obtained at excitation wavelengths of 340 and 380 nm and at an emission wavelength of 500 nm. K201 or diltizem at ca. 0.1–100 μM was first incubated with annexin V at 26°C for 30 min, and then these were added to the reaction buffer B solution containing LUV, and the effects of both drugs were examined.

2.4. Materials

Phosphatidylserine, phosphatidylethanolamine, BSA (fatty acid free) and TMA were obtained from Sigma (St. Louis, MO). HEPES and EGTA were from Nacarai Tesque (Kyoto), fura-2 from Dojin Laboratories (Kumamoto, Japan) and Triton X-100 from Millipore (San Francisco, CA). The reagents used were K201 (Japan Tobacco, Central Pharmaceutical Research Institute, Osaka, Japan) and diltiazem (Sigma), which were dissolved in distilled water for use in the experiments.

2.5. Statistics

Student's *t*-test was conducted for statistical analysis of the data; *P* values less than 0.05 were considered significant.

3. Results

The amino acid segment of annexin V purified from rat heart was shown to be YMTIS-GFQIEETIDR, corresponding to 213–227 of previously reported annexin V from human placenta [3]. Examination of the effects of K201 and diltiazem on

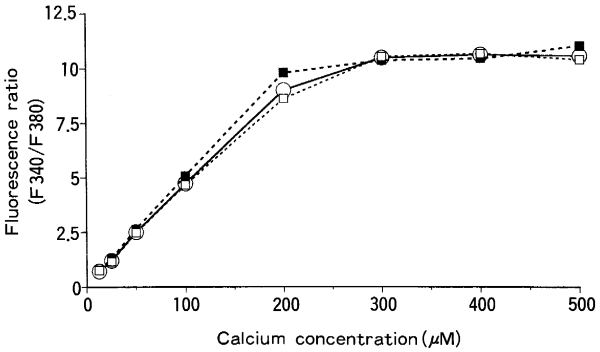

Fig. 3. Effects of K201 and diltiazem on fluorescence intensity in fura-2 solution. Composition of solution: 180 μM EGTA, 160 mM sucrose, 10 mM HEPES, 1 μM fura-2, pH 7.4 (TMA). Open circles: control. Open squares: 100 μM K201. Closed squares: 100 μM diltiazem. Both K201 and diltiazem had no effect on the fluorescence ratio.

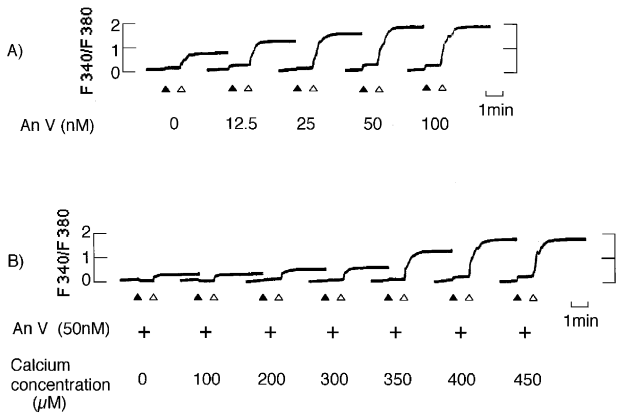

Fig. 4. (A) Effects of annexin V on fluorescence intensity. LUV were preincubated with annexin V (ca. 0–100 nM) in the presence of Ca^{2+} (400 μM). (B) Effects of calcium on fluorescence intensity. LUV were incubated with annexin V (50 nM) in the presence of Ca^{2+} (ca. 0–450 μM). Closed triangles: Time of resuspension of LUV. Open triangles: Time of addition of Triton X-100. An V: Annexin V

fura-2-calcium complex was made by addition of Ca^{2+} to buffer B solution containing 1 μM fura-2. The 340/380 fluorescence ratio indicated neither K201 nor diltiazem to have any effect (Fig. 3).

Fluorescence signals due to the leakage of trapped Ca^{2+} were recognized by the burst of LUV preincubated with annexin V in the presence of Ca^{2+}. The degree of fluorescence signal emission was dependent on annexin V concentration. When the annexin V concentration in the incubation solution increased, the fluorescence ratio increased dose dependently and reached the maximum at 50 nM in the presence of 400 μM Ca^{2+} (Fig. 4A). LUV, following preincubation with 50 nM annexin V, also showed an increase in fluorescence ratio with Ca^{2+} concentration. Fluorescence ratio reached a plateau at Ca^{2+} concentrations higher than 400 μM (Fig. 4B). BSA did not affect this fluorescence signal up to 1 μM in the presence of 400 μM Ca^{2+} (Fig. 5).

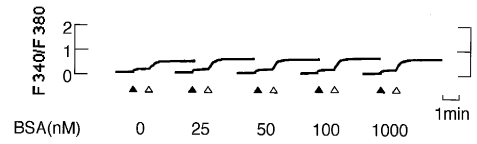

Fig. 5. Effects of BSA (ca. 0–1000 nM) on fluorescence intensity. LUV were incubated with BSA (ca. 0–1000 nM) in the presence of Ca^{2+} (400 μM). Closed triangles: Time of resuspension of LUV. Open triangles: Time of addition of Triton X-100.

The effect of K201 and diltiazem on inward Ca^{2+} movement into LUV was measured in the presence of 50 nM annexin V and 400 μM Ca^{2+}. K201 was shown to inhibit inward Ca^{2+} movement dose dependently (Fig. 6A). Under the same conditions (in the presence of 50 nM annexin V and 400 μM Ca^{2+}), K201 at 0.1 or 1 μM had no significant effect, but

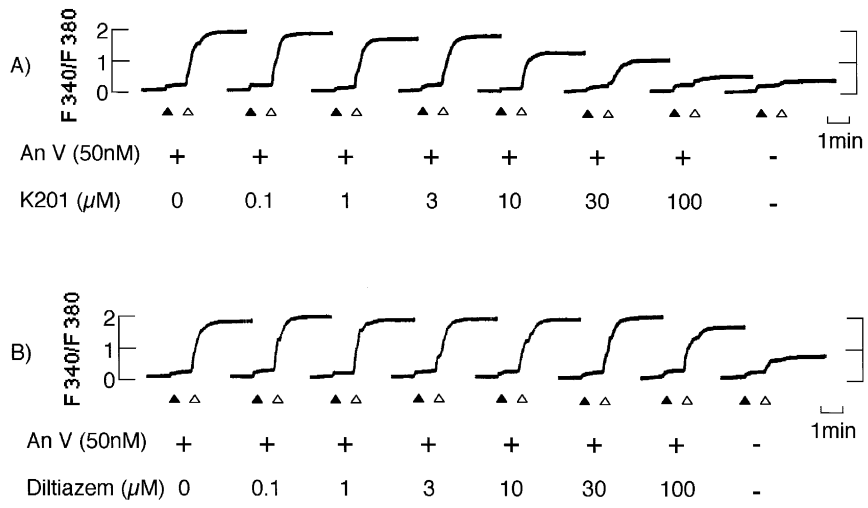

Fig. 6. (A) Effects of K201 on fluorescence intensity. (B) Effects of diltiazem on fluorescence intensity. LUV were incubated with annexin V (50 nM) and each reagent in the presence of Ca^{2+} (400 μM). Closed triangles: Time of resuspension of LUV. Open triangles: Time of addition of Triton X-100. An V: Annexin V.

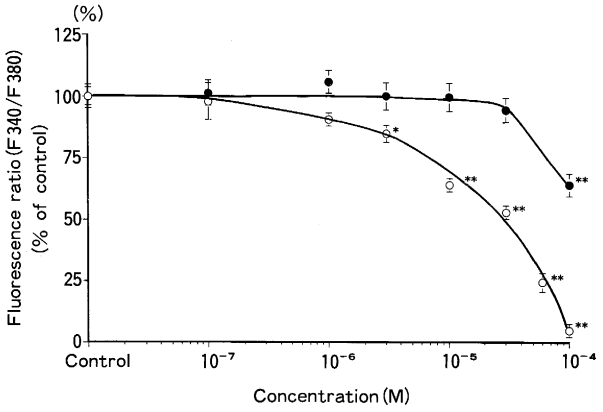

Fig. 7. Relationship between fluorescence intensity and concentrations of K201 and diltiazem. Open circles: K201, Closed circles: diltiazem. Horizontal bars show mean ± standard error. Each experimental group contained 4 or 5 cases ($n = 4$ or 5). $^{*}P < 0.05$, $^{**}P < 0.001$ (control vs. each group).

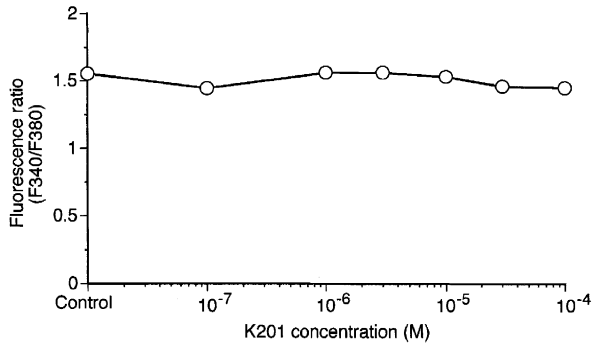

Fig. 8. Relationship between concentration of K201 and Ca^{2+} signal due to bursting of Ca^{2+}-incorporated LUV. Ca^{2+}-incorporated LUV were incubated with ca. $0–10^{-4}$ M K201 at 26°C for 10 min.

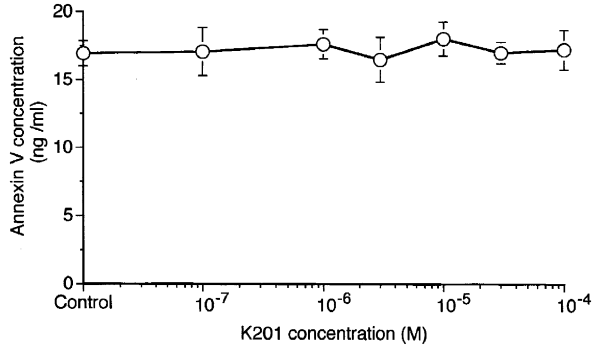

Fig. 9. Relationship between concentration of K201 and amount of annexin V binding to LUV. LUV were incubated with 50 nM annexin V and ca. $0–10^{-4}$ M K201 in the presence of 400 μM Ca^{2+} at 26°C for 10 min.

fluorescence ratio decreased significantly at 3 μM and higher (Fig. 7). The dose for 50% inhibition of inward Ca^{2+} movement was found to be 25 μM. Diltiazem did not show any effect at 30 μM, and showed an inhibitory effect only at very high concentrations (100 μM) (Fig. 6BFig. 7).

In the experiments using Ca^{2+}-incorporated LUV, no influence of K201 on the liposomes was recognized. Recollected Ca^{2+}-incorporated LUV incubated with $0–10^{-4}$ M K201 showed almost the same Ca^{2+} signal by burst method (Fig. 8). K201 also had no influence on the ability of annexin V to bind to liposomes. The amount of annexin V binding to LUV in the presence of Ca^{2+} did not change under the conditions of $0–10^{-4}$ M K201 (Fig. 9).

4. Discussion

In the annexin family, annexin I [18], II [19], V [6,7], VI [20,21], and VII [22] have been revealed to have voltage-dependent Ca^{2+} channel activity in vitro using patch clamp and dipping techniques. There have been some reports that suggest that annexin V has Ca^{2+} transport activity, using LUV and fura-2. Berendes et al. noted annexin V at 25 to 200 nM to exhibit calcium transport activity using fura-2-incorporated LUV, thereby promoting calcium influx in the presence of Ca^{2+} [15]. On the other hand, Goossens et al. could detect no Ca^{2+} channel activity even following the addition of 150 nM recombinant annexin V and native annexin V purified from human placenta to LUV in the presence of Ca^{2+} using fura-2-incorporated LUV, and concluded that annexin V in the presence of low concentrations of Ca^{2+} causes disruption of the lipid membrane with consequent leakage of fura-2, rather than inward Ca^{2+} transport [23]. Views on the functions of annexin V in experiments using fura-2-incorporated LUV are thus at variance.

Here, a study was conducted to determine whether rat native annexin V is capable of inducing inward Ca^{2+} movement across lipid membranes. Our preliminary study using fura-2-incorporated LUV indicated that annexin V induces an increase in the fluorescence ratio following centrifugation in the presence of a high concentration of Ca^{2+}. This result suggests

that a high concentration of Ca^{2+} makes LUV susceptible to disruption. However, addition of EGTA to the solution prevented this disruption by centrifugation. For the purpose of clarifying the effect of annexin V on inward Ca^{2+} movement into LUV, usage of fura-2-incorporated LUV as in the previous reports [15,23] would not be suitable, because it could not be determined whether an increase in fluorescence ratio in solution containing fura-2-incorporated LUV was due to an increase in inward Ca^{2+} movement into LUV or due to leakage of fura-2 from LUV. On the other hand, the burst method as shown in Fig. 2 reflects the Ca^{2+} content in LUV, which results from influx from the outside of LUV, so this method can avoid the artificial results described above.

Since Goossens et al. [23] reported that a very high concentration of Ca^{2+} (2.5 mM) and bovine serum albumin (5 g l^{-1} = about 80 μM) caused a significant increase in fluorescence with excitation and emission wavelengths of 340 and 500 nm using fura-2-loaded LUV, we measured the effect of BSA on inward Ca^{2+} movement into LUV by this burst method. BSA did not show any effect on fluorescence ratio up to 1 μM (Fig. 5). A high concentration of proteins such as BSA might cause nonspecific disturbance of LUV as described by Reeves and Dowben [14] and Goossens et al. [23]. Therefore, we used relatively low concentrations of annexin V (0 to 100 nM) and Ca^{2+} (0 to 450 μM) in this study to avoid nonspecific and/or artificial disturbance.

After disruption by Triton X-100, the fluorescence ratio of LUV solution was increased by annexin V and Ca^{2+} dose dependently as shown in Fig. 4. Maximum response was achieved at 50 nM annexin V in the presence of 400 μM Ca^{2+}, and at 400 μM Ca^{2+} in the presence of 50 nM annexin V. Thus, native rat annexin V induces Ca^{2+} movement into LUV and shows calcium influx activity in artificial phospholipid membrane in vitro.

Burger et al. [24] noted the X-ray crystal structure of annexin V to have features of channel-forming membrane protein. Recently, we found by protein X-crystallography that K201 binds with the central region of annexin V formed by domains II, III, and IV (unpublished observation: submitted for publication). K201, thus, shows not only ability to bind to annexin V but also inhibitory activity on annexin V-dependent inward Ca^{2+} movement into LUV in a dose-dependent manner (Fig. 7). K201 did not show effects to stabilize or destroy liposomes (Fig. 8) and there was no influence of K201 on the ability of annexin V to bind to liposomes (Fig. 9). Diltiazem, a 1,5-benzothiazepine, did not affect inward Ca^{2+} movement into LUV even at 30 μM and showed an inhibitory effect at 100 μM, and hardly bound to the same site in annexin V as K201 as shown by computer associated drug design method based on X-ray crystallography of annexin V (data not shown). Therefore, inhibition of diltiazem appears to be nonspecific, not being dependent on concentration.

Although K201 has been demonstrated to have a strong cardioprotective effect [8], the mechanisms of this action are unclear at the present time. Annexin V hardly associates with phosphatidylcholine and sphingomyelin, but it has a high affinity for the acidic phospholipids, such as phosphatidylserine [2]. Since phosphatidylserine is mainly located in the inner layer of the plasma membrane, annexin V, which is an intracellular protein, might reveal its function in the cytoplasmic site of cells under normal conditions. Recently, it has been reported that the translocation of phosphatidylserine from the inner side of the plasma membrane to the outer layer occurs in abnormal conditions, such as in the early stage of apoptosis and cell injury, and the annexin V binding site appeared in the outer membrane in these conditions [25–27]. On the other hand, we demonstrated that annexin V is displaced from its ordered distribution in the plasma membrane in the ischemic heart by immunohistochemical studies [12]. Furthermore, we also found that annexin V levels in plasma were substantially increased in patients with acute myocardial infarction [17]; that is, annexin V is released into the plasma in some forms of injury. These results suggest that intrinsic annexin V acts not only in the inner part of cells but also in the outer membrane in abnormal conditions. In this study, K201 inhibited the calcium channel activity of annexin V, and it acted as a cytoprotective agent in certain abnormal conditions and/or injuries, such as a myofibrillar overcontraction model of isolated rat heart [8], demonstrating myocardial preservation in the rat in vitro [9]. Although the role of annexin V channels in vivo is unclear at present, it is possible that annexin V causes deterioration of abnormal cellular functions

via accelerating inward Ca^{2+} current through its channel. In order to clarify this hypothesis, further studies, such as demonstrating the presence of annexin channels in apoptotic and injured cells, are necessary and K201 might be a good tool as an inhibitor of this channel.

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