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Calcium influx through annexin V ion channels into large unilamellar vesicles measured with fura-2

Robert Berendes, Alexander Burger, Dieter Voges, Pascal Demange and Robert Huber

Max-Planck-Institut für Biochemie, Am Klopferspitz, 8033 Martinsried, Germany

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A new assay for the calcium channel activity of annexin V was developed. The calcium-sensitive fluorescence indicator, fura-2, was incorporated into large unilamellar vesicles (LUV). After establishing a calcium gradient across the liposomal membranes, native or mutated annexin V was added. The resulting calcium influx into the LUV detected through the fluorescence changes of fura-2 was used as a qualitative test for the electrophysiological properties of annexin V.

Annexin V; Calcium channel; Liposome; Fura-2

1. INTRODUCTION

The annexins are proteins that bind to negatively charged phospholipids in a calcium-dependent manner. They are found in a variety of cell types of higher and lower eukaryotes. The annexins form a family of calcium-binding proteins distinct from the 'EF-hand' family [1,2]. They show features of both soluble and membrane proteins. The primary sequences of the 13 members of the annexin family known so far are mostly composed of four repeats; the exception is annexin VI, which has eight repeats. These repeats are homologous between each other as well as between all the annexins. In contrast, the N termini of the annexins are different in length and diverse in sequence [3,4].

The in vivo role of the annexins is still unknown. Most convincing seems an involvement in membrane–membrane fusion and exocytosis [5]. Interaction with cytoskeletal proteins [6], anticoagulant activities [7], inhibition of phospholipase A_2 to regulate inflammation [8], and the formation of calcium selective ion channels in phospholipid bilayers by annexin V and VII [9–11] are just a few of the in vitro properties of the annexins described so far. Some members of the annexin family are expressed in a growth-dependent manner [12] and are targets for cellular kinases in vivo [1,13].

Correspondence address: Robert Berendes, Max-Planck-Institut für Biochemie, Am Klopferspitz, 8033 Martinsried, Germany. Fax: (49) (89) 8578 3516

Abbreviations: EGTA, ethylene glycol (bis(2-aminoethyl ether)) *N,N,N',N'*-tetraacetic acid; EDTA, ethylene diamine tetraacetic acid; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; TMA, tetramethylammonium hydroxide.

In 1990 Huber et al. determined the three-dimensional structure of annexin V to a resolution of 2.0 Å [14–16]. Based on this structural analysis, we investigate the ion channel activity of the native annexin V and a variety of mutants prepared by recombinant DNA techniques.

In recent years the patch-clamp technique has proven to be a powerful and sensitive method to investigate single ion channels [17]. However, a problem with patch-clamp experiments arises in the case where a putative ion channel fails to show activity; it is then difficult to establish whether the number of single-channel experiments is statistically significant enough to conclude that the protein is indeed inactive as an ion channel.

To address this point, we present a new assay using the fluorescence indicator fura-2 [18] incorporated into large unilamellar vesicles (LUV). We report that native annexin V leads to a strong calcium influx into the liposomes. We also show that native annexin V and the annexin V mutants behave in single-channel experiments as expected from the results of the calcium influx assay.

2. MATERIALS AND METHODS

LUV were prepared from a mixture of phosphatidylserine and phosphatidylethanolamine (Avanti Polar Lipids, Birmingham, AL, USA; molar ratio 9:1) in a modified procedure of the dehydration/rehydration technique [19]. After drying down the phospholipids from chloroform/methanol (v/v, 2:1), the lipid film was rehydrated for 20 min with nitrogen, which was saturated with water. The rehydrated phospholipids were gently overlayed with 100 μM fura-2 (pentapotassium salt, Calbiochem, San Diego, CA, USA), 180 μM EGTA, 162 mM sucrose, 5 mM HEPES, pH 7.4 (TMA). The suspension was kept for 2 h at 37°C without shaking. Finally, the LUV were separated

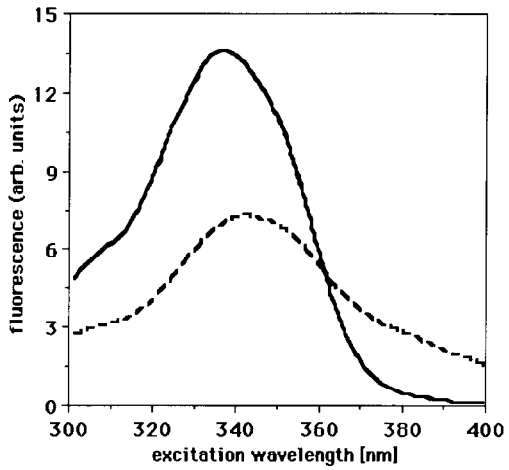

Fig. 1. Excitation spectra of LUV entrapping fura-2 (---) and after disruption with 0.2% Triton X-100 (—). 'Free' $[Ca^{2+}]_o$ was 250 μM.

from excess fura-2 by centrifugation at 12,000 × g for 10 min, removing the supernatant completely, and resuspending the LUV in 200 μM EGTA, 180 mM sucrose, 10 mM HEPES, pH 7.4 (TMA). This procedure was repeated four times. The last centrifugation step was carried out immediately before starting the influx assay. The side-product MLV float on the buffer solutions and are removed by the centrifugation. The LUV were used within 48 hours after the preparation.

The total lipid concentration of the liposomes was estimated by phosphate analysis [20]. The trapped volume was calculated by measuring the fluorescence intensity at 510 nm when exciting at 340 nm of fura-2 in the presence of excess Ca on a Perkin-Elmer 650-40 fluorescence spectrophotometer. In all experiments, the excitation slit width was 2 nm. The emission wavelength was 510 nm with a slit width of 10 nm.

The internal volume of the LUV was calculated to be 12.1 l buffer/mol phospholipid.

The experiments were done in a fluorescence cuvette with a 1-cm pathlength. In the calcium influx assay, the LUV loaded with 100 μM fura-2 and EGTA were suspended in 220 μl 180 μM EGTA, 160 mM sucrose, 10 mM HEPES, pH 7.4 (TMA) at a lipid concentration of 16.5 mM. In the second step, calcium ions were added to the suspension to final concentration of 430 μM (corresponding to 250 μM 'free' Ca^{2+}). Calcium influx was initiated by adding annexin V wild type or one of the mutant proteins at nanomolar concentrations. The rate of calcium influx was monitored by measuring the fluorescence emission of fura-2 at 510 nm as a function of time. The calcium concentration in the liposomes ($[Ca^{2+}]_i$) could be estimated from the fluorescence intensity ratio calculated from the emission when using the two excitation wavelengths 340 and 380 nm [18]. Controls were done by adding the calcium ionophore Br-A23187 (Sigma, Deisenhofen, Germany).

3. RESULTS

The excitation spectrum of fura-2 entrapped in the LUV in the presence of 250 μM external 'free' calcium ions is shown in Fig. 1. Similar spectra were obtained for calcium concentrations between 0 and 500 μM. Incubation with the ionophore Br-A23187 drastically increased the fluorescence intensity at 340 nm, while the intensity at 380 nm decreased. After disrupting the liposomes by addition of Triton X-100, the typical fluorescence spectrum of the fura-2/Ca complex [18] was detected (see fig. 1). The fluorescence spectra of the LUV incubated for 30 min with annexin V (200 nM) and a concentration of 250 μM 'free' calcium ions outside the liposomes ($[Ca^{2+}]_o$) were similar in the presence and absence of EDTA in the external medium (data not shown). This result indicates that the great majority of the fura-2 complexes were present within the liposomes.

The presence of traces of fura-2 in the suspension medium leads to a small jump in the fluorescence ratio immediately after the addition of the calcium ions (see e.g. Fig. 2, at the position marked with 'Ca'). The addition of annexin V caused drastic changes in the fluorescence intensity identical to the effect of the ionophore. The rates of the calcium influx into the liposomes increased with increasing annexin V concentrations at a constant $[Ca^{2+}]_o$ (250 μM, see Fig. 2). The calcium influx through the ionophore Br-A23187 was about four times higher as compared to native annexin V at the same concentration. After denaturation by heating to 100°C for 10 min, annexin V did not mediate any calcium influx into the LUV (not shown).

Mutants of annexin V were prepared by site-directed mutagenesis (A. Burger et al., unpublished) and tested for their effect on the calcium influx into the fura-2 loaded LUV. The mutant N29, lacking the N-terminal 14 amino acids, did not facilitate any calcium influx into the liposomes, even in concentrations much higher than necessary for a significant influx by native annexin V (Fig. 3a). To confirm that the lack of calcium influx was not due to damaged LUV, native annexin V was added to the suspension after a sufficient incubation time with the mutant N29 (see Fig. 3a, at the time marked with

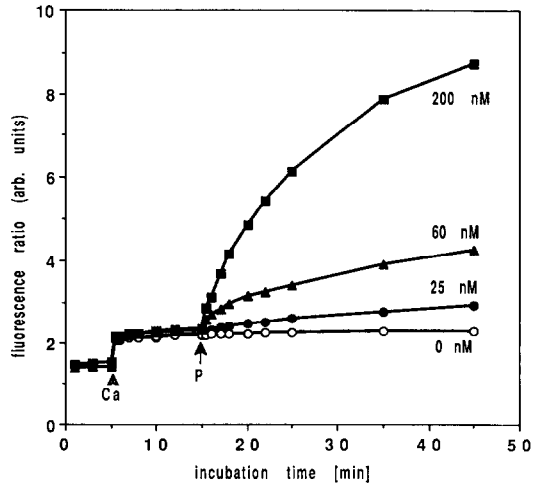

Fig. 2. Dependence of the fluorescence ratio on incubation time after the addition of different concentrations of native annexin V at a constant calcium concentration ('Free' $[Ca^{2+}]_o$ = 250 μM; calcium ions added at the time marked with 'Ca'). Annexin V was added at the time marked 'P' in concentrations of 25 nM (filled circles), 60 nM (filled triangles) and 200 nM (filled squares). Buffer with 0 nM annexin V was used as a control (open circles).

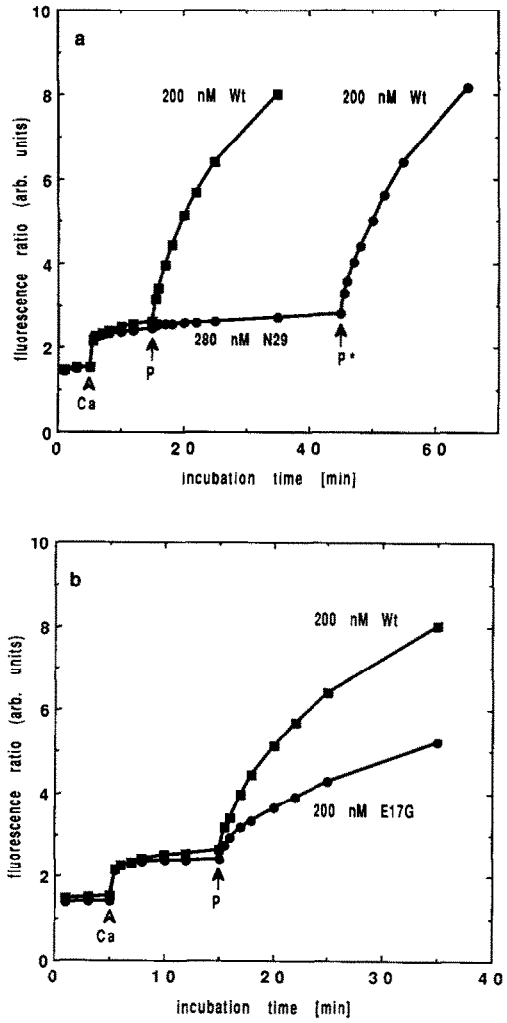

Fig. 3. Comparison of the mutants N29 (panel a; 280 nM; filled circles) and E17G (panel b; 200 nM; filled circles) with the native annexin V (panel a and b; 200 nM wild type (wt); filled squares) in the calcium influx assay. A 'free' $[Ca^{2+}]_o$ of 250 μM was established at the time marked with 'Ca'. The proteins were added at the position marked with 'P'. In panel a at the time marked with the 'P*' 200 nM native annexin V were added to the suspension containing 280 nM mutant N29.

'P'). The point mutant E17G, in which the glutamate residue 17 was substituted for a glycine, resulted in a significantly slower calcium influx compared to the native annexin V at similar protein concentrations when added to LUV (Fig. 3b).

4. DISCUSSION

Calcium ion fluxes mediated by a variety of channels and ionophores into liposomes loaded with indicator dyes like arsenazo III [21] and quin-2 [22] have been investigated. The much higher calcium sensitivity of the fluorescence indicator fura-2 [18] was a major advancement in the detection of calcium concentrations in small cells and liposomes [23,24]. Fura-2 also allows the investigation of the electrophysiological properties of annexin V. It has been shown that annexin V forms voltage-gated calcium channels after incorporation into acidic phospholipid bilayers in single channel experiments [10,11]. No macroscopic calcium currents of annexin V have been reported so far.

We report the measurement of macroscopic calcium currents into fura-2 containing LUV mediated by annexin V. Since annexin V is a soluble membrane-binding protein, it could easily be added in the suitable concentrations to the calcium containing liposome suspension after testing the stability and the leakage rates of the LUV. The fluorescence ratio of fura-2 changed due to the complex formation with the calcium ions flowing into the LUV. The rates of the calcium influx were proportional to the annexin V concentrations and the calcium concentrations in the liposome suspension. These influx rates allowed a qualitative determination of the calcium channel properties of annexin V.

The calcium influx assay proved to be a fast and powerful tool in predicting the single-channel characteristics of the mutants of annexin V. While the N-terminal deleted mutant N29 was inactive in the assay, the point mutant E17G had a significantly lower conductance than the native annexin V. These results are in accordance with single-channel experiments (Berendes et al., paper in preparation).

It has to be emphasized that differences in the calcium influx between the native annexin V and the mutants detected in the assay can have several reasons: changes in the conductance (see e.g. the two mutants described above) or drastic changes in the channel-opening probability could significantly influence the calcium influx. A special case involves the annexin mutants having amino acid substitutions within the calcium-binding sites. These proteins might not bind to the LUV under the assay conditions, but may still be active as ion channels under other conditions.

For these reasons, it is evident that the data from the calcium influx assay do not replace patch-clamp experiments and binding studies with annexin V and the mutants. The data rather simplify the detection and interpretation of the single-channel results. Furthermore, the large number of molecules tested in the assay statistically strengthens the conclusions with regard to inactivity of the protein in single-channel experiments.

The calcium influx assay can be applied to a variety of water soluble channel forming proteins and peptides besides annexin V. By using fluorescence dyes sensitive to other ions, e.g. sodium ions [25,26], the range of testable molecules could be further increased.

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