Annexin II Modulates Volume-activated Chloride Currents in Vascular Endothelial Cells*

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The membrane-associated, microfilament-binding protein annexin II is abundantly expressed in endothelial cells from calf pulmonary artery (CPAE cells). We have analyzed its role in the regulation of volume-activated chloride currents (I_{Cl,vol}) by loading the cells via the patch pipette with a peptide comprising the N-terminal 14 residues of annexin II. This sequence harbors the binding site for the intracellular annexin II ligand, p11, and the peptide interferes with the annexin II-p11 complex formation. Loading of a CPAE cell with this peptide caused a gradual decrease in the amplitude of I_{Cl,vol} during repetitive stimulations with a 28% hypotonic extracellular solution. This run down of the current was virtually absent in untreated cells and in cells that were loaded with a mutated 14-amino acid peptide, which has a single amino acid replacement known to result in a more than 1000 times reduced affinity for binding to p11. We conclude that annexin II-p11 complex formation is either directly or indirectly involved in the activation of I_{Cl,vol} in endothelial cells.

Volume-activated Cl⁻ channels have been described in various non-excitable cell types, including endothelial cells. They are supposed to play a major role in cell volume regulation, the control of intracellular pH, and the transport of organic osmoles. Therefore, the presence of volume-sensitive Cl⁻ currents in endothelial cells may be involved in the organization of the membrane-associated cytoskeleton.

Annexin II forms a family of Ca⁺⁺-binding proteins and membrane phospholipid-binding proteins that are thought to participate in a variety of membrane-related events (for review, see Refs. 4–7). Several annexins bind to F-actin in vitro and may therefore be involved in the organization of the membrane-associated cytoskeleton (8, 9). The microfilm binding annexin II also binds to another cellular protein ligand, the S100 protein p11 (10). P11 forms dimers, and annexin II-p11 interaction thus leads to the formation of an annexin II-p11 heterotetramer. Monomeric annexin II is at least in part cytosolic, whereas the heterotetramer appears to be restricted to the cortical cytoskeleton (11, 12). The p11 binding site is contained within the 14 N-terminal residues of annexin II, which form an amphiphilic α-helix (13). A similar specific and high affinity binding to p11 is also observed for a synthetic peptide spanning the 14-residue sequence (Ac(1–14)) but not for a mutated peptide in which one of the crucial hydrophobic residues for p11 binding (Leu-7) has been replaced by glutamic acid (14).

In this report, we have investigated the effects of the synthetic annexin II peptide Ac(1–14) and the mutated Ac(1–14)-peptide (L7E) on volume-activated Cl⁻ currents. Disrupting the annexin II-p11 complex in the presence of Ac(1–14) significantly inhibits volume-activated Cl⁻ currents, whereas the mutated peptide does not affect these currents. It is concluded that annexin II-p11 complex formation directly or indirectly modulates mechano-sensitive Cl⁻ channels of pulmonary artery endothelial cells.

MATERIALS AND METHODS

Cells from a cultured bovine pulmonary artery endothelial cell line (CPAE, ATCC CCL 209) were used. The cells were grown in Dulbecco's modified Eagle's medium containing 10% human serum, 2 mmol/liter t-glutamine, 2 units/ml penicillin, and 2 mg/ml streptomycin. Cultures were maintained at 37°C in a fully humidified atmosphere of 10% CO₂ in air.

Whole-cell membrane currents were measured in nonconfluent single endothelial cells using ruptured patches. Currents were amplified with an EPC-9 (List Electronic, Germany) patch clamp amplifier and sampled at 4-ms intervals (2048 points per record, filtered at 100 Hz). The voltage protocol consisted of a step to −80 mV for 1.2 s from a holding potential of −150 mV for 0.4 s, and 5.2-s linear voltage ramp to +100 mV, before stepping back to the holding potential. This protocol was repeated every 15 s. Cells were superfused with normal Krebs' solution containing (in mmol/liter) 132.2 NaCl, 5.9 KC1, 1.2 MgCl₂, 1.5 CaCl₂, 11.5 glucose, 11.5 HEPES, adjusted to pH 7.3 with 1 M NaOH. Its osmolality, as measured with a Wescor 5900 osmometer (Schlag Instruments, Gladbach, Germany), was 290 mosm/kg H₂O. To induce cell swelling, we switched from a modified, isotonic solution containing (in mmol/liter) 94.6 NaCl, 4.2 CaCl₂, 0.9 MgCl₂, 1.1 CaCl₂, 8.2 glucose, 8.2 HEPES, 81 mmol/liter mannitol, adjusted to pH 7.3 with 1 M NaOH to a hypotonic solution with the same composition from which mannitol was omitted (HTS, 209 mosm/liter). CaCl₂ was used in these solutions to block the endothelial inwardly rectifying K⁺ currents. The internal pipette solution contained

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Multiple comparison test (level, pooled data. Significance was tested by means of the Turkey-Kramer II concentration in CPAE cells (see below).

0.1 EGTA, 10 HEPES, adjusted to pH 7.2 with 1M KOH. The time constant, obtained from a monoexponential fit, was 107 s.

For [Ca2+]i, measurements, cells were loaded with 2 µmol/liter Fura-II/AM or via the patch pipette with 200 µmol/liter penta-K+ salt of Fura-II (molecular mass of 832 Da). Changes in intracellular Ca2+ were described in

All experiments were performed at room temperature between 20 and 22 °C. Mean ± standard error of the mean were calculated from pooled data. Significance was tested by means of the Turkey-Kramer Multiple comparison test (level, p < 0.01).

Western Blots—Total cellular protein from 7.8 × 10⁶ CPAE or MDCK cells was extracted by boiling the washed cells in 1 ml of SDS sample buffer (17). About 25 µg of protein of each cell type were separated in SDS-15% polyacrylamide gels (17) and then analyzed by immunoblotting (18). Annexin II and p11 were stained using the mouse monoclonal antibodies H28 and H21, respectively (19). Peroxidase coupled antibodies (Dako) were employed as secondary antibodies, and immunoreactive bands were visualized using the ECL chemoluminescence system (Amersham-Buchler).

Peptides—The peptide comprising the N-terminal 14 amino acids of bovine annexin II (STVHEILCKLSLEG) (25) and the L7E mutant version (STVHEÍECKLSLEG) were synthesized on an automated sequencer (model 9050; Milligen). To verify the purity a small aliquot of the peptide was removed from the resin and deprotected by treatment with 95% trifluoroacetic acid, 2.5% ethanedithiol, and then purified by C18 reverse phase HPLC. The correctness of this reaction was monitored by the Kaiser test (20). The acetylated peptides were removed from the resin and deprotected by treatment with 95% trifluoroacetic acid, 2.5% ethanedithiol, and then purified by C18 reverse phase HPLC. The correctness of synthesis and acetylation was also verified by mass spectroscopy of the purified product.

RESULTS

Annexin II and p11 Are Expressed in Endothelial Cells—Western blotting was employed to analyze the expression of annexin II and p11 in CPAE cells. In these experiments epithelial MDCK cells were included for comparison as we had previously characterized the ratio of monomeric versus p11-complexed annexin II in this cell type (21). Fig. 1A reveals that annexin II and p11 are expressed at similar levels in both CPAE and MDCK cells. Since MDCK cells contain more than 90% of their annexin II in the (annexin II)p11 complex (21), this comparison indicates that the majority of CPAE annexin II is also found in the heterotetrameric complex. Semi-quantitative Western blots were employed to assess the total amount of annexin II in CPAE cells. Here the signal of a given amount of purified annexin II was compared with that of the annexin II band present in total protein extract from a known number of CPAE and MDCK cells. From this comparison we estimated the annexin II concentration in CPAE cells to be approximately 1 µM.

The competing annexin II peptide Ac-(1–14), which has a similar affinity for p11 binding, was loaded into the CPAE cell via the patch pipette at a concentration of 100 µM. This 100 times excess over the endogenous annexin II concentration will be sufficient for an efficient competition of the Ac-(1–14) with the endogenous annexin II for p11 binding and to disrupt the endothelial annexin II-p11 complexes.

Endothelial cells are very flat (approximate height 1 µm), and the diffusion time of the peptide may thus be considerably longer than that estimated for round cells (22). To obtain a rough idea of this diffusion time, we have added the pentapotassium salt of Fura-II to the pipette solution and monitored the time course of its emission (excitation wavelength of 360 nm) after breaking into the cell (Fig. 1B). The estimated mean time constant for eight cells was 112 ± 12 s. This value is in good agreement with the calculated value of 105 s for a molecular mass of 832 Da, an access resistance of 17 ± 2 MΩ, a capacitance 52 ± 4 pF, and a cell volume of 2600 ± 100 µm³ (n = 17), taking into account a cell thickness of approximately 1 µm and the membrane surface area calculated from the membrane capacitance. The diffusion time constant for the exchange of Ac-(1–14) (molecular mass of 1573 Da) between pipette and cell calculated with the same method is about 150 s.
is about 10 times longer than the diffusion time constant, the peptide will diffuse into the cell and compete with the endogenous annexin II for binding to p11. During this period the purpose is to follow the changes in amplitude of $I_{\text{Cl,vol}}$ during repeated stimulations with hypotonic solution. In order to attribute possible changes of the current to the peptide, we have to ascertain that no significant run down of the current occurs in the absence of the peptide.

**Stability of Volume-dependent Cl⁻ Currents**—Fig. 2 shows data from a typical experiment in which a cell was repetitively exposed to hypotonic challenges. The time course of the volume-activated current ($I_{\text{Cl,vol}}$) at $-80$ and $+100$ mV was reconstructed from the average currents during the initial voltage step to $-80$ mV or from a small window around $+100$ mV during the voltage ramp ($-150$ to $+100$ mV). Note that between a and b $K^+$ in the extracellular solution was replaced by Cs⁺ to block the inwardly rectifying $K^+$ current. B, current voltage relationships (IV curves) at the times indicated by the closed symbols in A. Note the block of the inwardly rectifying $K^+$ current after substitution of $K^+$ (a) by Cs⁺ (b). The HTS-activated current, $I_{\text{Cl,vol}}$, does not significantly decrease during subsequent hypotonic challenges.

**Fig. 2. Volume-activated currents during repetitive challenges with hypotonic solution (HTS).** A, time course of current activation and deactivation measured at $+100$ mV (circles) and $-80$ mV (squares). These values were obtained from the average current during the initial voltage step to $-80$ mV or from a small window around $+100$ mV during the voltage ramp ($-150$ to $+100$ mV). Note that between a and b $K^+$ in the extracellular solution was replaced by Cs⁺ to block the inwardly rectifying $K^+$ current. B, current voltage relationships (IV curves) at the times indicated by the closed symbols in A. Note the block of the inwardly rectifying $K^+$ current after substitution of $K^+$ (a) by Cs⁺ (b). The HTS-activated current, $I_{\text{Cl,vol}}$, does not significantly decrease during subsequent hypotonic challenges.

**Fig. 3. Inhibition of the volume-activated Cl⁻ current by the annexin II peptide, Ac-(1–14).** A, current amplitudes measured at $+100$ (circles) and $-80$ mV (squares) during repetitive hypotonic challenges. Note the gradual decline of the current amplitude during subsequent stimulations. B, time course of the 360-nm fluorescence after breaking into a cell (indicated by the arrow) with a pipette solution that contains the Fura-II-5 K⁺ salt. The time course of this fluorescence is a measure of the diffusion of Fura-II-5 K⁺ into the cell. $[\text{Ca}^{2+}]_i$ (not shown) only shows small fluctuations during this 40-min experiment. C, IV curves at the times indicated in panel A. Note the decline of the current amplitude during subsequent hypotonic stimuli without a significant shift in reversal potential.
the plasma membrane (trace a), the whole cell current shows a prominent inwardly rectifying K\(^+\) current at negative potentials, which was blocked by substituting external KCl by CsCl (trace b). The residual current in these cells is mainly a Cl\(^-\) current and has been extensively described elsewhere (1, 3, 23, 24). This limited run down of I\(_{\text{Cl, vol}}\) is consistent with our observations in a previous report where we showed that the volume-activated Cl\(^-\) current runs down very slowly by about 15% after 60 min (24). The currents activated by a hypotonic challenge (HTS) are slightly outwardly rectifying and reverse around \(-210\) mV. The discrepancy between this potential and the Cl\(^-\) equilibrium potential (\(-23\) mV) can be explained by the permeability of this anion-selective channel for the aspartate present in the patch pipette.\(^2\)

The pipette solution in these experiments contained 200 \(\mu\)M Fura-II, which allowed us to monitor the diffusion of this dye with a molecular mass of 55% of that of Ac-(1–14) into the cell and to record the changes in intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) during cell swelling. In order to exclude effects that might be related to changes in [Ca\(^{2+}\)]\(_i\), such as the activation of Ca\(^{2+}\)-dependent Cl\(^-\) channels, we have restricted our analysis to cells in which no significant changes in [Ca\(^{2+}\)]\(_i\) could be observed. Results obtained in cells loaded with the Fura-II salt are comparable with those in cells loaded with Fura-II/AM or in the absence of the Ca\(^{2+}\) probe.

Some cells showed a “delayed” and incomplete recovery of the current induced by cell swelling. If, however, a slightly hypertonic solution was used to shrink the cells after an hypotonic challenge, the recovery was fast and complete (not shown). Apparently, some cells lose their ability to shrink to their initial volume during these extremely long lasting protocols. This phenomenon was observed in control cells and in cells loaded with either peptide. It can therefore not be attributed to an effect of the peptides.

**Suppression of I\(_{\text{Cl, vol}}\) by the Annexin II Peptide, Ac-(1–14).**—The same experimental procedure was used to analyze the effect of the annexin peptide Ac-(1–14) on I\(_{\text{Cl, vol}}\). Loading a CPAE cell via the patch pipette with 100 \(\mu\)M Ac-(1–14) resulted in a gradual decline of the amplitude of I\(_{\text{Cl, vol}}\) during repetitive hypotonic challenges (Fig. 3A). This run down was much more pronounced than in control cells that were not loaded with the peptide. The entry of Fura-II-5 K\(^+\) into the cell, as monitored by the changes in intensity of the emitted light of the dye after excitation at 360 nm, is represented in Fig. 3B. Some representative I–V curves are represented in Fig. 3C, from which it

\(^2\) T. Voets, G. Droogmans, V. Manalopoulos, and B. Nilius, unpublished observations.
is obvious that neither the reversal potential nor the shape of the IV curve was significantly different from those in control cells.

We have also made use of a mutated peptide (L7E Ac-(1–14)) to ascertain whether the effect of the peptide is due to a specific interference with the annexin II-p11 complex formation. This peptide contains a single glutamate for leucine replacement at position 7, and has a 1000 times lower affinity for p11. Fig. 4A shows the changes in membrane current measured at 0 mV during repetitive applications of hypotonic solution in a cell loaded with 100 μM of the mutated peptide. The sharp deflections in the current trace are caused by the voltage protocols that were applied every 15 s. It is obvious that the amplitude of the volume-activated current at this potential is not significantly different for the various stimulations with hypotonic solution. This cell was also pre-loaded with Fura-II/AM to monitor concomitantly the changes in [Ca$$^{2+}$$]$_i$, as shown in panel B. Except for the two fast Ca$$^{2+}$$ transients shortly after breaking into the cell and during the first stimulation with hypotonic solution, [Ca$$^{2+}$$]$_i$ was fairly constant during the whole course of this recording.

Panel C shows the time course of the current at 280 and 1100 mV as reconstructed from the voltage protocols. It is obvious from these data that the rundown of $I_{Cl,vol}$ in the L7E Ac-(1–14)-loaded cells is much less pronounced than in cells loaded with the Ac-(1–14)-peptide. The reversal potential of the volume-activated current as measured from the I–V relationships (Fig. 4D) was close to the Cl$$^{-}$$ equilibrium potential and was stable during the long-lasting experiment.

Fig. 5 summarizes the results obtained for the different experimental conditions. Except for a few cells, $I_{Cl,vol}$ did not significantly run down during loading of the cells with either Fura-II/AM, the K$$^+$$-penta salt of Fura-II, or the mutated peptide (Fig. 5, A–C). In contrast, all cells loaded with the p11 binding peptide, Ac-(1–14), show a marked rundown of $I_{Cl,vol}$ (Fig. 5D). To quantify these data, we have pooled the measurements between 3 and 10 min and those between 25 to 35 min after breaking into the cell (Fig. 5E). For Fura-II/AM, Fura-II-5 K$$^+$$, and L7E Ac-(1–14)-loaded cells, these data were not significantly different, while the 30-min current density of the cells loaded with the mutated peptide was significantly smaller than the 5-min value (Fig. 5, D and E).

**DISCUSSION**

Volume-activated Cl$$^{-}$$ channels, $I_{Cl,vol}$, are involved in a variety of biologically important cell functions such as volume regulation, Ca$$^{2+}$$ signaling, cell proliferation, and pH regulation (see Refs. 1 and 2 for a review). The molecular identity and the gating mechanism of these channels are still unresolved. Volume-activated channels are likely to be modulated by cytoskeletal components (25). We have recently shown that in endothelial cells the general polymerization status of actin and tubulin does not play a prominent role for activation of $I_{Cl,vol}$ (25), but this analysis did not allow a precise evaluation of a possible contribution of the actin-based cortical cytoskeleton. Annexins are membrane-binding proteins that also bind to actin. Annexin IV has been shown to modulate the Ca$$^{2+}$$ sensitivity of Ca$$^{2+}$$-dependent Cl$$^{-}$$ channels (26). In this report, we have analyzed the possible involvement of a prominent annexin found in the cell cortex, annexin II, in the regulation of plasma membrane based mechano-sensitive anion channels. Annexin II is found in two physical states within cells, as a monomeric cytosolic protein and in a heterotetrameric complex with the S100 protein p11. While it is generally assumed that...
proteins of the S100 family act intracellularly by binding to and thereby regulating the activity of specific target proteins. Annexin II is among the few unambiguously identified targets of an S100 protein (for review see Ref. 27). P11 binding to annexin II alters its biochemical properties (28, 29) and also affects its intracellular distribution since only the (annexin II)$_2$-(p11)$_2$ complex but not the monomeric annexin II is tightly incorporated in the cortical cytoskeleton (30, 31). We show here that most of the annexin II in CPAE cells is found in the complex with p11, and we have used a synthetic peptide consisting of the 14 N-terminal amino acids of annexin II with an acetylated N-terminal serine (Ac-(1–14)) to specifically target the annexin II-p11 complex. This peptide efficiently competes with the endogenous annexin II for p11 binding and thus interferes with annexin II-p11 complex formation (13). This peptide was applied at a concentration that is about 100-fold higher than that of the endogenous annexin II. Taking into account that its affinity for p11 is similar to that of annexin II (13), the peptide excess should completely disrupt the endogenous annexin II-p11 complex. We have shown that this peptide, but not a mutant version with a strongly reduced affinity to p11, inhibits $I_{\text{Cl,vol}}$. It is therefore likely that annexin II-p11 complex formation is required for activation of $I_{\text{Cl,vol}}$: Co-activation of Ca$^{2+}$-dependent channels can be excluded in our experiments because we selected only those experiments in which no substantial Ca$^{2+}$ fluctuations could be observed.

Annexin II is present in caveolae and caveolae-like structures that might be important membrane regions involved in the unfolding of the cell surface during swelling (32, 33). Interestingly, we observed that in a caveolin-deficient rat thyroid cell line (FRT cells) hypotonic challenges elicit only small currents, which in addition occur with a long delay (time for half-maximal activation of 337 ± 37 s, n = 6, compared with 68 ± 12 s, n = 25 for CPAE cells in this study, or 106 ± 11, n = 4 for caveolin-positive MDCK cells as used in Ref. 33). This might be a further indication of the functional significance of annexin II for the activation of volume-sensitive currents. $I_{\text{Cl,vol}}$ is not regulated by [Ca$^{2+}$]$_i$ in vascular endothelium but needs a permissive submicromolar Ca$^{2+}$ level for activation by an increase in cell volume (34). Such Ca$^{2+}$ concentrations are also necessary for the Ca$^{2+}$-dependent binding of annexin II to the anionic inner surface of the membrane (6). It is tempting to speculate that the failure in the activation of $I_{\text{Cl,vol}}$ after intracellular Ca$^{2+}$ depletion beyond this threshold might be related to the annexin-membrane dissociation under such conditions.

In summary, our data provide the first evidence for the regulation of volume-sensitive Cl$^{-}$ channels by a member of the family of Ca$^{2+}$-dependent membrane binding annexins.

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