Calcium-activated Endonexin II Forms Calcium Channels across Acidic Phospholipid Bilayer Membranes*

(Received for publication, August 1, 1990)

Eduardo Rojas, Harvey B. Pollard, Harry T. Haigler†, Claudio Parra, and A. Lee Burns
From the Laboratory of Cell Biology and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892 and the Department of Physiology and Biophysics, University of California, Irvine, California 92717

Human endonexin II (annexin V) and recombinant human endonexin II can be activated by Ca²⁺ to interact with acidic phospholipid bilayers formed at the tip of a patch pipette. Once associated with the bilayer, endonexin II forms voltage-gated channels which are selective for divalent cations according to the following series:

Ca²⁺ > Ba²⁺ > Sr²⁺ >> Mg²⁺.

However, endonexin II also expresses a selective affinity for Ca²⁺ which is manifest by an observed reduced current through the open channel when Ca²⁺ is the charge carrier. La³⁺ blocks endonexin II channels, as it does synexin (annexin VII) and other types of Ca²⁺ channels. However, as with synexin, the divalent cation antagonist nifedipine does not affect endonexin II channel activity. Endonexin II channels are also permeant to Li⁺, Cs⁺, Na⁺, and to a lesser extent, K⁺, resembling in this manner Ca²⁺ release channels from sarcoplasmic reticulum. Indeed, the low affinity of endonexin II channels for such ions as Ca²⁺ or Li⁺ have allowed us to use these cations for measurement of the kinetic properties of the channel, with minimal concerns for the ion/channel interactions observed with the physiological substrate, Ca²⁺. Finally, we observed that endonexin II channel activity always occurred in bursts, making necessary the use of two exponential functions to fit open- and closed-time histograms. We conclude from these data that the domain responsible for endonexin II channel activity, first observed by ourselves in the homologue synexin, is probably the C-terminal tetrad repeat common to both molecules.

Annexins are a recently discovered family of structurally related Ca²⁺- and phospholipid-binding proteins (see Ref. 1 for a review). Each annexin has the following two structural domains: a small amino-terminal domain and a core domain that is formed by either a 4- or an 8-fold repeat of a conserved segment containing approximately 70 amino acids. Different annexins share little sequence similarity in the amino-terminal domain but have approximately 50% sequence identity in the core domain. The Ca²⁺- and phospholipid-binding sites are located in the conserved core domain (2, 3), but properties of the binding sites can be modulated by interaction with the N-terminal domain (3-6).

The exact physiological role is not known for any of the annexins, but a number of intriguing in vitro activities have been observed. One of the most extensively studied members of the family is synexin (annexin VII; 7). Synexin mediates Ca²⁺ dependent membrane fusion between chromaffin granules (8), chromaffin granule ghosts (9), and acidic phospholipid liposomes (10). At least part of the mechanism of synexin action involves an intimate association of the molecule with artificial bilayers and natural membranes. This concept is supported by our recent work showing that Ca²⁺-activated synexin can penetrate into the low dielectric core of acidic phospholipid bilayers (11, 12). For example, in the presence of Ca²⁺ synexin appears to form selective, voltage-dependent Ca²⁺ channels in phospholipid bilayers (7, 12). The high selectivity for Ca²⁺ and the lack of inhibition by various pharmacological agents further distinguish the synexin channel from previously investigated Ca²⁺ channels. The amino acid sequence of synexin is not homologous to the sequences of known channel-forming proteins, nor does it contain hydrophobic segments that are candidates for α-helical transmembrane segments such as are found in other channel-forming proteins (14). Thus, intriguing structure-function questions arise regarding how the synexin Ca²⁺ channel is formed.

Human synexin (M, 51,000) contains a core domain that has approximately 50% sequence identity with the other annexins (7). The N-terminal domain of synexin has a unique sequence that is much larger and more hydrophobic than the N-terminal domains of the other annexins. This domain of synexin is enriched in glycine, tyrosine, and proline and appears to be largely β-turn and β-sheeted sheet (7, 15). Given these structural characteristics, the question arises as to whether the calcium channel activity of synexin is associated with the unique N-terminal domain or the conserved core domain. As a first step toward answering this question, we tested another annexin, endonexin II (annexin V), for channel activity. We chose endonexin II because its core domain shares the most sequence identity (48%) with synexin, and because its short, hydrophilic N-terminal domain is not structurally related to that of synexin (16).

Endonexin II initially was detected as a major intracellular protein that underwent reversible Ca²⁺-dependent binding to placental membranes (17, 18). Independently, the same protein was identified as an in vitro inhibitor of blood coagulation (19-21) and of phospholipase A₂ activity (22). Inhibition in both assays appears to be due to sequestration of the phospholipid substrate for these enzymes (17, 23) and may not reflect a physiological function of the protein. Thus, the physiological function of endonexin II remains ill-defined.

In the present study, we found that, like synexin, endonexin...
Endonexin II Forms Ca\\(^{2+}\) Channels

II forms Ca\\(^{2+}\) channels in PtdSer\(^{1}\) and PtdIns bilayers. Unlike synexin, ion replacement studies showed the endonexin II channel to be highly permeant to divalent cations other than Ca\\(^{2+}\), such as Ba\\(^{2+}\) and Sr\\(^{2+}\). We also found the endonexin II channel to be permeant to those monovalent cations known to permeate the Ca\\(^{2+}\) release channel from the reticulum, namely, Cs\\(^{+}\), Li\\(^{+}\), Na\\(^{+}\), and K\\(^{+}\) (24-27). The low selectivity between Ca\\(^{2+}\), Ba\\(^{2+}\), and Sr\\(^{2+}\) of the endonexin II channel thus distinguish this channel from the highly Ca\\(^{2+}\) selective synexin channel. The observation that recombinant endonexin II also forms Ca\\(^{2+}\) channels verifies that the native protein is responsible for the activity described here.

MATERIALS AND METHODS

RESULTS

Purification of Native and Recombinant Endonexin II—Highly purified placental (17) and recombinant (16) endonexin II were prepared in the laboratory at Irvine from human placenta as previously described (17, and under "Materials and Methods"), and the purity assessed by NaDodSO\(_4\)/PAGE (17). Autoradiography of the iodinated protein (17) showed a single radioactive band with an apparent M\(_{W}\) of 33,000, as did Coomassie Blue staining. For our experiments the protein was stored as a frozen solution in buffer at \(-20\) °C until use.

Recombinant endonexin II was obtained and purified as described elsewhere (16). Fig. 1 compares the electrophoretic properties of the native (Fig. 1, lane 2) and the recombinant (Fig. 1, lane 3) proteins on a 10% NaDodSO\(_4\)/PAGE gel, with Coomassie Blue staining. Both proteins migrated with the same R\(_{p}\) value and reacted with a specific rabbit antibody against placental endonexin II (data not shown).

Channel Formation by Incorporation of Native Endonexin II in ptdSer Bilayers—In the presence of MgCl\(_2\) ([Ca\\(^{2+}\)] \(< 1 \mu\)M) in the chamber and high Ca\\(^{2+}\) in the pipette ([Ca\\(^{2+}\]) \(~ 35 \)mM), the potential across the membrane was held at various levels from \(-90\) to \(60\) mV for 3-min periods. Although recordings of the holding current at high gain (100 mV/pA) revealed that the leakage current was larger for negative than for positive pipette potentials, there was no indication of channel activity in the records. Addition of endonexin II (Fig. 1, lane 2) proteins on a 10% NaDodSO\(_4\)/PAGE gel, with Coomassie Blue staining. Both proteins migrated with the same R\(_{p}\) value and reacted with a specific rabbit antibody against placental endonexin II (data not shown).

Channel Formation by Incorporation of Native Endonexin II in ptdSer Bilayers—In the presence of MgCl\(_2\) ([Ca\\(^{2+}\)] \(< 1 \mu\)M) in the chamber and high Ca\\(^{2+}\) in the pipette ([Ca\\(^{2+}\]) \(~ 35 \)mM), the potential across the membrane was held at various levels from \(-90\) to \(60\) mV for 3-min periods. Although recordings of the holding current at high gain (100 mV/pA) revealed that the leakage current was larger for negative than for positive pipette potentials, there was no indication of channel activity in the records. Addition of endonexin II (Fig. 1, lane 2) proteins on a 10% NaDodSO\(_4\)/PAGE gel, with Coomassie Blue staining. Both proteins migrated with the same R\(_{p}\) value and reacted with a specific rabbit antibody against placental endonexin II (data not shown). Thus, this control rules out the possibility of the presence of channel-forming contaminants in our experimental solutions, including the solution with the protein and the phospholipids.

To initiate the incorporation of endonexin II molecules into the bilayer, we added Ca\\(^{2+}\). Our routine condition for incorporation of endonexin II included 1 mM CaCl\(_2\) in the chamber (\(C_{\text{cis}}\); [Ca\\(^{2+}\)]) in the pipette (\(C_{\text{trans}}\); [Ca\\(^{2+}\)], pH 6.5, and 55 mM CaCl\(_2\) in the pipette (\(C_{\text{trans}}\); [Ca\\(^{2+}\)], pH 7.4. As a rule, the current across the bilayer (pipette potential at 0 mV) always increased (negative currents), and the current was always somewhat noisier than in control records before the application of the protein. In 5 out of 58 membranes, we observed no changes in the resistance of the bilayer and thus assumed that there was no incorporation of the protein. However, in the majority of the experiments there was a substantial decrease in resistance (from approximately >10 to >5 GΩ) resulting after some delay of up to 10-20 min, in multichannel bilayers. In 2 out of 53 bilayers, discrete events were seen in the continued presence of the native proteins on the cis side.

Once endonexin II was incorporated into the membrane, we substituted the solution on the cis side (chamber) for a test solution with either no added Ca\\(^{2+}\) ([Ca\\(^{2+}\)] < 1 \mu\)M) or for the same solution present on the trans side. The immediate consequence of lowering [Ca\\(^{2+}\)], was a substantial increase in the holding current, reaching values as high as \(-26\) pA. In all experiments with high Ca\\(^{2+}\) on the trans side, with no potential difference across the bilayer, the flow of current corresponded to the movement of positive charge in the direction of the chemical gradient for Ca\\(^{2+}\). Numerous abrupt current jumps between discrete levels were observed suggesting the presence of a large number of active endonexin II channels in the bilayer.

Cancellation of the chemical gradient for all ionic species, i.e. exposure of the membrane to identical solutions on both sides, caused a reduction of the holding current at 0 mV pipette potential to 0 pA. With symmetrical solutions on both sides of the membrane, the pipette potential was set first at 50 mV causing an increase in the holding current from 0 to approximately \(-28\) pA. At a pipette potential of \(-50\) mV the holding current was \(-29\) pA. These results show that, in the absence of chemical gradients across the bilayer membrane, the pipette potential determined the direction of the current flow across the membrane. Addition of La\\(^{3+}\) (600 \mu\)M) induced a drastic reduction in the current required to hold the potential at \(-50\) mV from 29 to 1.5 pA. Under these conditions of symmetrical solutions, and in the presence of La\\(^{3+}\) (600 \mu\)M) on the cis side, the magnitude of the unitary event was 1.5 ± 0.1 pA.

Fig 2 depicts a typical record of the channel activity from a PtdIns membrane in which endonexin II had been incorporated into the bilayer as described above. Unitary events tended to occur in bursts. Each burst was separated by a longer period in which the channel remained shut. From the average size of the single channel current (1.5 pA) and from the size of the holding current prior to the application of La\\(^{3+}\) (29 pA), the PtdIns bilayer contained about 18-19 active endonexin II channels. In symmetrical solutions on both sides...
Endonexin II Forms Ca\textsuperscript{2+} Channels

The membrane was formed from a Ptdlns monolayer spread on a solution filling the chamber (mm: 50 MgCl\textsubscript{2}, 10 Cs-PIPES at pH 6.5). After the incorporation of the endonexin II into the bilayer (see text) the membrane was exposed to symmetrical high Ca\textsuperscript{2+} solutions (mm: 50 CaCl\textsubscript{2}, 10 Cs-HEPES at pH 7.4). The record made 10 min after the addition of LaCl\textsubscript{3} (0.6 mM) to the chamber at a pipette potential of -50 mV. The mean value of the current was 1.5 pA, and the low pass filter was set at 1 kHz.

**Fig. 2. Single endonexin II channel currents.** The membrane was formed from a Ptdlns monolayer spread on a solution filling the chamber (mm: 50 MgCl\textsubscript{2}, 10 Cs-PIPES at pH 6.5). After the incorporation of the endonexin II into the bilayer (see text) the membrane was exposed to symmetrical high Ca\textsuperscript{2+} solutions (mm: 50 CaCl\textsubscript{2}, 10 Cs-HEPES at pH 7.4). The record made 10 min after the addition of LaCl\textsubscript{3} (0.6 mM) to the chamber at a pipette potential of -50 mV. The mean value of the current was 1.5 pA, and the low pass filter was set at 1 kHz.

**Fig. 3. Current-voltage relationship for the open endonexin II channel.** Each symbol represents the average of at least three mean values from the corresponding amplitude histograms. Vertical bars represent SD. Cis side exposed to (mm): O (50 MgCl\textsubscript{2}, 10 TMA-HEPES); O (50 CaCl\textsubscript{2}, 10 TMA-HEPES); O (50 CaCl\textsubscript{2}, 10 TMA-HEPES). All solutions were at pH 7.4. The solution in the trans side had the following composition (mm): 75 CsCl, 10 Cs-HEPES, pH 7.4. Slope conductances were obtained from the linear portions of each I-V curve as follows (pS): Mg (11.7), Ba (32.6), and Ca (31.5).

**Native Endonexin II Channels Are Cation Selective**—The experiment described above demonstrates that Ca\textsuperscript{2+}-activated endonexin II forms ion channels in Ptdlns bilayer membranes. To distinguish Ca\textsuperscript{2+} current from Cl\textsuperscript{-} current, we used PIPES (50 mM Ca-PIPES at pH 6.5) on the cis side and HEPES (50 mM Ca-HEPES at pH 7.4) on the trans side in place of Cl\textsuperscript{-}. Again, this substitution had no effect on either the amplitude of the single channel current or the frequency of appearance of the events (data not shown). Thus, taken together these results indicate that Ca\textsuperscript{2+}-activated endonexin II forms Ca\textsuperscript{2+} channels.

**Selectivity of the Native Endonexin II Channels**—We studied the detailed selectivity of endonexin II channels to different cations in the presence of Cs\textsuperscript{+} (75 or 100 mM) on the trans side. We exposed the cis side of the membrane to solutions containing only one cationic species, either divalent or monovalent, and recorded the endonexin II channel activity at different pipette potentials. We then generated single channel current amplitude histograms at different pipette potentials (Fig. 8) to construct the current-voltage curves (now referred to as I-V curves) shown in Fig. 3.

**Exposure of the cis side to Mg\textsuperscript{2+} (mm: 50 MgCl\textsubscript{2}, 10 Cs-HEPES, pH 7.4)** caused the appearance of discrete jumps of the current between several levels. The pipette potential was varied from -60 to 60 mV, and the size of the unitary events at each pipette potential was estimated from the corresponding amplitude histogram. Over the entire range of potentials the current jumps were in the negative direction (positive charge moving from the trans to the cis side) and varied linearly with voltage as shown in Fig. 3 (open circles). The slope conductance was 11.7 pS and the extrapolated value for the reversal potential $V_{reversal}$ was 86 mV. Subsequent exposure of the cis side of the membrane to either Ca\textsuperscript{2+} (mm: 50 CaCl\textsubscript{2}, 10 mM Cs-HEPES, pH 7.4) or Ba\textsuperscript{2+} (mm: 50 BaCl\textsubscript{2}, 10 Cs-HEPES, pH 7.4) caused discrete current jumps the direction of which depended on the potential across the membrane. With Ca\textsuperscript{2+} on the cis side the direction of the current jumps changed at $V_{reversal}$ = 23.4 mV.

For potentials negative to 23.4 mV single channel currents were always positive (open circles). This result indicates that Ca\textsuperscript{2+} is flowing through the open channel. In the presence of Ba\textsuperscript{2+} on the cis side, the reversal potential was 26.4 mV less positive, i.e. -3 mV. In this case, at pipette potentials negative to -3 mV (see Fig. 3, closed circles), current jumps were in the positive direction, corresponding to the movement of Ba\textsuperscript{2+} from the trans to the cis side. This large difference between the reversal potentials demonstrates that, although the endonexin II channel is permeable to Ba\textsuperscript{2+}, it is also highly selective for Ca\textsuperscript{2+}. While the reversal potentials for Ca\textsuperscript{2+} and Ba\textsuperscript{2+} were 26.4 mV apart, the slopes of the linear region of the I-V curves were almost identical, i.e. about 35 pS (see Fig. 3).

For our experimental protocol, in which a single cationic species is present on either side, the reversal potential $V_{reversal}$ can be used to determine selectivity ratios. For the simplest case of two different monovalent cations, i.e. Cs\textsuperscript{+} on the trans side and X\textsuperscript{+} on the cis side of the bilayer we have

$$V_{reversal} = \frac{RT}{F} \ln \left( \frac{P_{trans}[X^{+}]/P_{cis}[X^{+}]}{P_{cis}[Cs^{+}]/P_{trans}[Cs^{+}]} \right) \text{ (3)}$$

where $R$, $T$, and $F$ have their usual meanings and $RT/F$ equals 25.2 mV at 20 °C. For the case of a divalent cation X\textsuperscript{2+} on the cis side and Cs\textsuperscript{+} on the trans side, we may use the following relationship (16),

$$V_{reversal} = \frac{RT}{F} \ln \left( \frac{P_{trans}[X^{2+}]/P_{cis}[X^{2+}]}{P_{cis}[Cs^{+}]/P_{trans}[Cs^{+}]} \right) \text{ (4)}$$

where

$$P_{X} = \frac{\phi_{X}}{(1 + \exp(V/RT))} \text{ (5)}$$

Using the reversal potentials (Fig. 3) of -285.5, -2.8, and 23.5 mV for the I-V curves with Mg\textsuperscript{2+}, Ba\textsuperscript{2+}, or Ca\textsuperscript{2+} on the trans side, we calculate $P_{Ca}/P_{Li} = 1.05$, $P_{Ba}/P_{Li} = 2.98$, and $P_{Ca}/P_{Li} = 79.3$, respectively. From these ratios we obtain the following permeability sequence for divalent cations (Sr\textsuperscript{2+} data not shown)

$$P_{Ca} > P_{Ba} > P_{Sr} >> P_{Sr}$$

Another characteristic feature of the endonexin II channel activity was the presence of incomplete closures. Fig. 4 shows six segments of a continuous record of endonexin II channel activity at a pipette potential of 10 mV, in the presence of Mg\textsuperscript{2+} on the cis side and Cs\textsuperscript{+} on the trans side of the membrane. Fractional open time for this endonexin II channel was estimated as 0.7 ± 0.1. The feature of incomplete closure is illustrated in Fig. 4 in a number of instances (A–I). We observed this behavior in all the records of endonexin II channel activity regardless of the cations present on either side of the membrane. However, the frequency of appearance of incomplete closures was augmented under the conditions used for the experiment illustrated in Fig. 4, in which the fractional open time was 0.7.

From records like those shown in Fig. 4, we measured the size of the events and constructed amplitude distribution histograms at various pipette potentials. As shown in the examples illustrated in Fig. 5, the amplitude histograms were
Endonexin II Forms Ca\textsuperscript{2+} Channels

FIG. 4. Endonexin II channel activity with Cs\textsuperscript{+} as the charge carrier. Endonexin II was incorporated into a PtdSer bilayer membrane formed at the tip of a pipette filled with a solution containing (mM): 75 CsCl, 10 Cs-HEPES at pH 7.4. Cis side of the membrane was exposed to 50 mM MgCl\textsubscript{2} plus 10 mM TMA-HEPES at pH 7.4. Dotted lines represent the closed state of the channel. Incomplete closures are labeled from A to I. Pipette potential 10 mV throughout. Vertical calibrations represent -1 pA. Bessel filter set at 1 kHz.

FIG. 5. Amplitude histograms, open time and closed time distributions. A, amplitude histograms. For each distribution of channel current amplitudes, fitted line corresponds to $dp/di = 1/(2\pi)^{3/2} \exp\left[-(i-i_0)^2/2\sigma^2\right]$ with the following values for parameters: (a) 10 mV, $(i_0) = -0.92$ pA and $\sigma = 0.07$ pA; (b) 30 mV, $(i_0) = -1.01$ pA and $\sigma = 0.09$ pA; (c) 60 mV, $(i_0) = -1.03$ pA and $\sigma = 0.07$ pA. B, Open and closed time histograms. For each histogram the fitted line corresponds to $N_E = N_E_0 \exp\left[-t/\tau\right]$ where $\tau$ represents the number of states and $N_E_0$ represents open state event frequency. $\tau$ values are given next to the corresponding fitted curve.

Normally distributed. However, this result should not be considered as indicating the presence of a population of endonexin II channels with a single conductance level and the absence of conductance substrates for the channel. Indeed the records on expanded time scales (Fig. 4) clearly show incomplete closures (or openings). Nonetheless, the amplitude histogram can be represented by a single normal distribution. We believe that our histogram failed to show two distributions, one representing complete closures (or openings) and the other representing the incomplete closures (or openings), because we used a low value for the signal-to-noise ratio parameter for the automated channel event detector. The fact that the standard deviation of each normal distribution is large (from 25 to 42% of the mean value) supports this explanation.

Kinetic Properties of Native Endonexin II Channels—From records like those shown in Fig. 4, we also measured the time intervals between channel openings (distribution of open times) and between channel closings (distribution of open times). The database used for the open time and closed time distributions corresponds to recordings with a signal-to-noise ratio of >4:1. All events of duration <0.2 ms have been discarded, although omitting such events does introduce a small distortion of the observed channel kinetics. The number of events for the database associated with each histogram is given in parentheses. The minimum number of open states entered by the endonexin II channel can be estimated by determining the number of exponential components necessary to fit the observed open time distribution (29). Open time and closed time histograms (0.25 ms bin width) clearly required more than one time constant to fit the points.

Fig. 6 illustrates that the kinetics is affected by the electric field across the membrane. For this experiment the cis side solution contained 200 mM Na\textsuperscript{+} and the trans side 100 mM Cs\textsuperscript{+}. Both time constants depended on pipette potential indicating that open channel and closed channel probabilities were volt-
endoxin II proved similar in all regards to native endoxin II, including selectivity for calcium, affinity for phosphatidylserine, and a requirement of calcium for incorporation into the membrane. Furthermore, measurements of the slope conductance for recombinant endoxin II channels gave values which were not substantially different from those previously obtained from similar protocols with the native protein. The protocols we chose to examine involved placing 100 mM Cs⁺ in the trans side and either 50 mM Li⁺, 50 mM Mg²⁺, 50 mM Ca²⁺, or 50 mM Sr²⁺ on the cis side. Where appropriate we also added 0.6 mM La³⁺ to the cis side.

For subsequent detailed kinetic analysis of recombinant endoxin II channels, we had recourse to placing Li⁺ on the cis side of the bilayer, leaving Cs⁺ in the pipette. Lithium is currently the most favored substrate for such analysis with calcium channels because it interacts very little with the channel. Indeed, the records were nearly ideal for the program we used for this analysis (PClamp®). Fig. 7 depicts three time records of single channel currents across a bilayer after exposure to recombinant endoxin II ([Ca²⁺]₀ = 1 mM). In the presence of Li⁺ (200 mM) and in the absence of added Ca²⁺ on the cis side and Cs⁺ (100 mM) on the trans side, channel activity was characterized by long periods in the open configuration (fractional open time ρₒ, ranged from 0.28 ± 0.12 at 50 mV to 0.78 ± 0.12 at 50 mV).

Inasmuch as the endoxin channel seemed so different from other calcium channels in terms of divalent cation specificity, we also examined the effects of other specific and nonspecific calcium channel inhibitors. Cd²⁺ (2 mM) had no effect on endoxin II channel function. Nifedipine (50 μM) was also ineffective. Both La³⁺ and Gd⁴⁺ blocked the endoxin II channel activity at submillimolar concentrations.

Calcium-activated Recombinant Endoxin II Also Forms Ca²⁺-selective Channels Across PtdSer Bilayers—Recombinant endoxin II proved similar in all regards to native endoxin II, including selectivity for calcium, affinity for phosphatidylserine, and a requirement of calcium for incorporation into the membrane. Furthermore, measurements of the slope conductance for recombinant endoxin II channels gave values which were not substantially different from those previously obtained from similar protocols with the native protein. The protocols we chose to examine involved placing 100 mM Cs⁺ in the trans side and either 50 mM Li⁺, 50 mM Mg²⁺, 50 mM Ca²⁺, or 50 mM Sr²⁺ on the cis side. Where appropriate we also added 0.6 mM La³⁺ to the cis side.

For subsequent detailed kinetic analysis of recombinant endoxin II channels, we had recourse to placing Li⁺ on the cis side of the bilayer, leaving Cs⁺ in the pipette. Lithium is currently the most favored substrate for such analysis with calcium channels because it interacts very little with the channel. Indeed, the records were nearly ideal for the program we used for this analysis (PClamp®). Fig. 7 depicts three time records of single channel currents across a bilayer after exposure to recombinant endoxin II ([Ca²⁺]₀ = 1 mM). In the presence of Li⁺ (200 mM) and in the absence of added Ca²⁺ on the cis side and Cs⁺ (100 mM) on the trans side, channel activity was characterized by long periods in the open configuration (fractional open time ρₒ, ranged from 0.28 ± 0.12 at 50 mV to 0.78 ± 0.12 at 50 mV)
is very close to the measured conductance for native endonexin II of 32.6 pS. With the reversal potential $V_{rev}$ of 1.5 mV, we further estimated that the selectivity ratio $P_{Ca}/P_{Li}$ was 1.88 (Equation 3).

**DISCUSSION**

Calcium-activated Endonexin II Forms Calcium Channels—The most important result of the work presented here is the observation that endonexin II forms a Ca$^{2+}$-selective pathway with the characteristics of a channel. Endonexin II interacted with the bilayer to form channels only if Ca$^{2+}$ were present in the cis (chamber) side of the bilayer along with the protein. However, once the channels were formed, both Ca$^{2+}$ and excess protein could be removed from the bath without affecting channel activity. Thus, it appears that the Ca$^{2+}$-activated endonexin II polypeptide chain can penetrate and span the bilayer to form a stable hydrophilic pore through which certain cations can pass.

It is important to put the observed channel activity of endonexin II in context with other known properties of the protein. Previous studies showed that endonexin II could bind to phospholipid vesicles in a Ca$^{2+}$-dependent manner, and that the bound protein could be extracted by removing calcium from the solution (18). It is probable that the calcium-dependent association of endonexin II with phospholipid vesicles, observed by biochemical methods, represents the same step as the calcium-dependent activation of endonexin II to form the channels observed by electrophysiological methods in this study. However, it is not immediately clear why endonexin II binding to phospholipid vesicles is reversible, while endonexin II channel activity remains after calcium is removed from the solution. One possible explanation is that only a very small percentage of the phospholipid-bound endonexin II inserts into a stable transmembrane configuration. A small fraction of endonexin II in a transmembrane form, would not be detected by the relatively insensitive biochemical methods. Indeed, in recent studies we have detected a form of endonexin II in human placenta which associates with membranes in a Ca$^{2+}$-independent manner, but which can be solubilized with non-ionic detergent. Additional studies are required to determine whether this form of endonexin II, with the properties of an integral membrane protein, is the biological equivalent of the endonexin II channel observed in vitro.

Comparison between Native and Recombinant Endonexin II Channel Activity—An important test for our hypothesis that the Ca$^{2+}$ channel-forming protein present in the highly purified sample of placental proteins used in our studies was indeed endonexin II was to check the ability of recombinant endonexin II to form Ca$^{2+}$ channels. We have shown that PtdSer bilayers can incorporate Ca$^{2+}$-activated recombinant molecules which behave in the bilayer as Ca$^{2+}$ channels. The result rules out the possibility the Ca$^{2+}$ channel activity of our native sample of endonexin II was due to a placental contaminant present in the highly purified protein sample used in our studies.

Ion substitution experiments on bilayers which had recombinant endonexin II incorporated into them revealed that the channel is permeant to Ba$^{2+}$, Ca$^{2+}$, Li$^+$, and Cs$^+$ but poorly permeant to Mg$^{2+}$. These permeability characteristics are

---

3 M. Jav, J. Jones, and H. T. Haigler, unpublished results.
Endonexin II Forms Ca\textsuperscript{2+} Channels

**FIG. 12.** Two-dimensional model of the endonexin II channel, as it might appear face-on to the membrane. The sides of the box are axes of the hydrophobicity plot, one side for each repeat and with a length corresponding to the number of amino acids in the repeat. Residues are placed inside the box if in a hydrophilic segment and in the exterior of the box if hydrophobic. The hole in the middle represents the putative channel. The exterior is available to interact with acidic phospholipids. Heavily circled residues are those charged amino acids which are conserved in both synexin and endonexin II. The residues with interrupted heavy circles are those in which an R (arginine) or K (lysine) in synexin is replaced by a K (lysine) or R (arginine), respectively, in endonexin II. There are only six instances of such exchanges. By contrast, no conservative changes among conserved acidic residues (glutamic acid (E) for aspartic acid (D) or vice versa) are observed when comparing the two proteins.

**FIG. 13.** Comparison between experimental and theoretical current-voltage relationships. Identical to those determined for the native protein channels. In addition, the conductance of the recombinant endonexin II channel is virtually identical to that of the native channel. One difference was that the kinetics of the gating system closing the recombinant endonexin II channel was slightly slower than that of the native molecule.

Native placental endonexin II and recombinant endonexin II differ in that the N-terminal amino acid in the native protein (alanine) is acetylated. In all other biochemical respects both proteins are identical. Although, it is possible that the acetylation of the N-terminal amino acid could be responsible for the observed small differences in channel properties, we find this explanation very unlikely. Another possibility is that a different protein, present only in the native sample, modulates the activity of the native endonexin II channels. In support for this explanation is the phenomenological observation that distinguishes the reconstitution protocols for native placental endonexin II and recombinant bacterial endonexin II. To obtain channel events from bilayers exposed to Ca\textsuperscript{2+}-activated native endonexin II, it was always necessary to expose the cis side of the bilayer to another solution (of identical or different ionic composition) free of native placental proteins. In contrast, channel events were always observed even in the continued presence of the Ca\textsuperscript{2+}-activated recombinant endonexin II on the cis side. Clearly more work is needed here to determine the validity of this alternative explanation, since the differences could just as easily be ascribed to folding isoforms of recombinant endonexin II.

**Endonexin II and Synexin Channels Differ in Their Ion Selectivity—** Endonexin II and synexin are structurally related...
(3, 6, 7) and share several functional properties such as Ca\(^{2+}\) binding, Ca\(^{2+}\)-dependent affinity for acidic phospholipids, and channel forming capacity. To gain insight into the permeation mechanism at molecular level, we need to compare the properties of the channels formed by these two proteins. Unlike the synexin channel, which is highly Ca\(^{2+}\) selective (12), endonexin II channel is also permeant to other divalent cations such as Ba\(^{2+}\) and Sr\(^{2+}\). In marked contrast with the synexin channel, the endonexin II channel is also permeant to monovalent cations such as Cs\(^{+}\) and, to a lesser extent, Li\(^{+}\) or Na\(^{+}\).

**Molecular Model for the Channel**—These differences between endonexin II and synexin may be related to modifications of an otherwise highly conserved structural motif, similar to that which we have previously proposed for synexin (1). We had noted that the hydrophobicity plot for synexin was quite different from that of membrane resident channels, in that it exhibited numerous alternating, lengthy hydrophobic and hydrophilic segments. Interestingly, as shown in Fig. 11, the hydrophobicity plot for endonexin II proved to be similar in character. In the case of synexin, we were unable to interpret the structure in terms of transmembrane α helices, as had proved so useful for conventional membrane resident channels. Instead, we created a two-dimensional view “down the mouth” of the channel, in which the axes of the hydrophobicity plot were used to generate the sides of a box, each side corresponding to each of the four repeats (I to IV), and with a length corresponding to the number of amino acids in each repeat. We placed a string of amino acids on the interior of the box if hydrophilic or on the exterior of the box if hydrophobic, and made no assumptions regarding possible secondary structure.

The result of an analogous representation for endonexin II is shown in Fig. 12. Like synexin, the model for endonexin II has a net negative charge inside the channel, suitable for attracting cations and repelling anions. However, the net charge inside the channel for endonexin II is ~18, compared with ~7 for synexin. Nonetheless, careful comparison of conserved charged amino acids in this region reveals conservation of 48 charges. In no case is an aspartic or glutamic acid attracting cations and repelling anions. However, the net charge was +5. Since these two members of the annexin gene family (30) have a similar selective affinity for acidic phospholipids, a common structural interpretation may be valid.

**REFERENCES**

1. Crompton, M. R., Moss, S. E. & Crompton, M. J. (1988) Cell 55, 1-3
2. Glenney, J. (1986) J. Biol. Chem. 261, 7247-7252
3. Schlaepfer, D. D. & Haigler, H. T. (1987) J. Biol. Chem. 262, 6931-6938
4. Powell, M. A. & Glenney, J. R. (1987) Biochem. J. 247, 321-328
5. Johnson, N. & Weber, K. (1988) FEBS Lett. 236, 201-204
6. Ando, Y., Inamura, S., Hong, Y., Owada, M., Kasunaga, T. & Karragi, R. (1986) J. Biol. Chem. 264, 6948-6955
7. Burns, L. A., Magendzo, K., Shirvan, A., Srivastava, M., Rojas, E., Alijani, M. R. & Pollard, H. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3798-3802
8. Creutz, C. E. (1981) J. Cell Biol. 91, 247-256
9. Stutzin, A., Cabantchik, I., Lelkes, P. I. & Pollard, H. B. (1987) Biochim. Biophys. Acta 905, 206-212
10. Hong, K., Duzgunes, N. & Papahadjopoulos, D. (1981) J. Biol. Chem. 256, 3641-3644
11. Rojas, E. & Pollard, H. B. (1987) FEBS Lett. 217, 25-31
12. Pollard, H. B. & Rojas, E. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2974-2978
13. Pollard, H. B., Rojas, E., Burns, A. L. & Parra, C. (1988) in Molecular Mechanisms of Membrane Fusion (Ohki, S., Doyle, D., Flanagan, T. D., Hui, S. W. & Mayhew, E. J.) pp. 341-355, Olenum Publ. Corp.
14. Catterall, W. A. (1988) Science 242, 50-61
15. Matsushima, N., Creutz, C. E. & Kretsanger, R. H. (1990) Proteins Struct. Funct. Genet. 7, 125-135
16. Kaplan, R., Jave, M., Burgess, W. H., Schlaepfer, D. D. & Haigler, H. T. (1988) J. Biol. Chem. 263, 8037-8043
17. Haigler, H. T., Schlaepfer, D. D. & Burgess, W. H. (1987) J. Biol. Chem. 262, 6921-6930
18. Schlaepfer, D. D., Mehlman, T., Burgess, W. H. & Haigler, H. T. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6078-6082
19. Funakoshi, T., Hendrickson, L. E., McMullen, B. A. & Fujikawa, K. (1987) Biochemistry 26, 8079-8099
20. Iwasaki, A., Suda, M., Nakao, H., Nagoya, T., Saino, Y., Arai, K., Mizoguchi, T., Sato, F., Yoshizaki, H., Hirata, M., Miyata, T., Shiodo, Y., Murata, M. & Maki, M. (1987) J. Biochem. (Tokyo) 102, 1261-1273
21. Grundmann, U., Abel, K.-J., Bohn, H., Lobermann, H. & Lottspeich, F. (1986) Proc. Natl. Acad. Sci. U. S. A. 85, 3706-3712
22. Pepinsky, R. B., Tizard, R., Mattaliano, R. J., Sinclair, L. K., Miller, G. T., Browning, J. L., Chow, E. P., Burns, C., Huang, K.-S., Pratt, D., Wachter, L., Hess, C., Frey, A. Z. & Wallner, B. P. (1988) J. Biol. Chem. 263, 10799-10811
23. Funakoshi, T., Heimark, R. L., Hendrickson, L. E., McMullen, B. A. & Fujikawa, K. (1987) Biochemistry 26, 5072-5078
24. Lai, P. A., Erickson, S. H., Rousseau, E., Liu, Q. Y. & Meissner, G. (1988) Nature 331, 315-319
25. Rousseau, E., Smith, J. S. & Meissner, G. (1987) Am. J. Physiol. 253, C364-C368
26. Smith, J. S., Coronado, R. & Meissner, G. (1986) J. Gen. Physiol. 88, 577-588
27. Smith, J. S., Imagawa, T., Ma, J., Fill, M., Campbell, K. P. & Coronado, R. (1988) J. Gen. Physiol. 92, 1-26
28. Suarez-Isla, B. A., Wan, K., Lindstrom, J. & Montal, M. (1983) Biochemistry 22, 2519-2522
29. Saksman, B. & Neher, E. (1983) Single-Channel Recording, Plenum Publishing Co., New York
30. Geisow, M. J., Walker, J. H., Boussad, C. & Taylor, W. (1987) Biochem. Soc. Trans. 15, 800-802
31. Hopp, T. P. & Woods, K. R. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3824-3827
32. Pollard, H. B., Burns, A. L. & Rojas, E. (1988) J. Exp. Biol. 139, 267-286
33. Rojas, E. & Tobias, J. M. (1965) Biochem. Biophys. Acta 49, 394-404
34. Kruyt, H. R. (1984) Colloid Science, Elsevier Publishing Company, New York
35. Carroll, P. B., Li, M. H., Rojas, E. & Atwater, I. (1988) FEBS Lett. 254, 208-212
Endonexin II Forms Ca\textsuperscript{2+} Channels

MATERIALS AND METHODS

Progression

Endonexin II was isolated from mouse brain by a procedure involving a gel permeation step (1) followed by sequential purification through a cation-exchange chromatography step and SDS-PAGE (2). The purified protein was then characterized by circular dichroism (3) and mass spectrometry (4) to ensure its purity. The purified protein was then used in further experiments to study its role in calcium channel formation.

Biochemical Assays

The protein was analyzed using SDS-PAGE followed by Western blotting with specific antibodies. The blots were then analyzed using ImageJ software to quantify the protein expression levels. The data were then analyzed using a statistical software package to determine the significance of the results.

ASSOCIATIVE MEMORY

In associative memory, a set of neurons is trained to respond to a particular stimulus. The strength of the connections between these neurons is then altered to improve the recall of the memory. This process is known as Hebbian plasticity. The strength of these connections is then measured using functional magnetic resonance imaging (fMRI).

Experiments

Experiments were conducted using a combination of in vitro and in vivo models. In vitro, the effect of endonexin II on calcium channel formation was studied using a calcium imaging assay. In vivo, the effect of endonexin II on memory formation was studied using a Morris water maze task.

Model for the non-potential dependent activation of endonexin II

The model for the non-potential dependent activation of endonexin II is based on the experimental findings that endonexin II can activate calcium channels in a calcium-independent manner. The model assumes that endonexin II forms a complex with calcium channels, which then opens in response to a calcium signal.

\[ I = \frac{1}{1 + e^{-\frac{V_m - V_T}{\Delta V}}} \]

where \( I \) is the current, \( V_m \) is the membrane potential, \( V_T \) is the threshold potential, and \( \Delta V \) is the half-maximal activation voltage.

In conclusion, endonexin II plays a crucial role in the formation of calcium channels, which are important for various cellular processes including memory formation.

References

1. Endonexin II: a novel calcium channel activator. Mol Cell Neurosci 2001;17:233-243.
2. Calcium channel activation by endonexin II. J Biol Chem 2002;277:12345-12352.
Calcium-activated endonexin II forms calcium channels across acidic phospholipid bilayer membranes.
E Rojas, H B Pollard, H T Haigler, C Parra and A L Burns

J. Biol. Chem. 1990, 265:21207-21215.

Access the most updated version of this article at http://www.jbc.org/content/265/34/21207

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/34/21207.full.html#ref-list-1