Avian Adenovirus Induces Ion Channels in Model Bilayer Lipid Membranes

Andrey A. Rosenkrantz,*,‡ Yury N. Antonenko,† Olga A. Smirnova,† Gleb K. Yurov,† Boris S. Naroditsky,† and Alexander S. Sobolev*,‡

*Department of Biophysics, Biological Faculty and ‡A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119899, Moscow, Russia; and †Russian Institute of Agricultural Biotechnology, 127550, Timiryazevskaya ul., 42, Moscow, Russia

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The action of duck egg drop syndrome 1976 (EDS-76) adenovirus on model bilayer lipid membranes (BLM) has been investigated on planar egg phosphatidylcholine membranes and small unilamellar vesicles. It was found that the adenovirus formed channels in planar BLM in a pH-dependent manner. The addition of EDS-76 to planar BLM at pH 5 induced voltage-independent channel activity of about 60 pS conductivity after a lag phase. At pH 3, EDS-76 induced irregular spikes of current across the planar BLM which disappeared after several minutes. The adenovirus also was able to induce pH-dependent leakage of calcein-loaded liposomes. EDS-76 did not induce channel activity in planar BLM or liposome leakage at neutral pH.

Materials and Methods

Isolation and purification of adenoviruses. CsCl purified Duck Egg Drop Syndrome 76 (EDS-76) virions were inoculated and grown in 9-12-day old duck embryos. After 6-day incubation, the virus-containing allantois fluid was harvested, centrifuged, homogenized, mixed with an equal volume of Freon 113 and then recentrifuged (20–21). The aqueous phase was then centrifuged through a discontinuous CsCl gradient (22), and the viral bands collected and centrifuged through a preformed linear CsCl gradient. The viral bands were again collected and stored at 4°C. Virion concentration was determined by spectrophotometric analysis (23).

Liposome leakage. Calcein (Serva)-loaded liposomes were prepared from egg yolk phosphatidylcholine (PC, Sigma) by reverse-phase evaporation (24), extensively dialyzed against 25 mM HEPES, pH 7.5, 150 mM NaCl and then stored under nitrogen at 4°C. The liposomes were purified by gel filtration on Sephadex G-25 in 25 mM citric buffer 150 mM NaCl, pH 5.0. The liposomes were incubated with adenoviruses (10 to 20 μl of CsCl solution) for 30 min at 37°C. The background leakage (0%) was measured after the incubation of liposomes (20 μl) in 200 μl of the same buffer adjusted to the required pH. Fluorescence (excitation at 493 nm, emission at 518 nm) was measured in 1 ml of 50 mM HEPES, pH 7.5, 150 mM NaCl using a Shimadzu 510 fluorescence spectrophotometer. Leakage kinetics were measured in 1 ml of citric-NaCl solution at the indicated pH. The background leakage (0%) was measured after the incubation of liposomes with identical volumes of CsCl solution lacking adenovirus. Maximal (100%) leakage was determined by adding 10 μl 10% Triton X-100 solution and results normalized according to this scale.
Bilayer lipid membranes (BLMs) were formed by conventional techniques (25) on a 0.3-mm diameter hole in Teflon partition separating two aqueous compartments of a cell, from a decane solution of 2% PC. The aqueous solution of the bath contained 25 mM citrate, 150 mM NaCl, with a pH between 3 and 7. Experiments were performed at room temperature, whereby the virus-containing CsCl solution was added to the 3-ml cell, and electric current recording performed under voltage-clamp conditions. The sign of the applied potential was such that the trans side was virtual ground. Currents were amplified using a patch-clamp amplifier (OPUS, Moscow) with a cutoff frequency of 500 Hz, and stored on a videotape after digitizing using Adarec (OPUS, Moscow).

RESULTS AND DISCUSSION

It is known that human and chicken adenoviruses are able to induce the release of substances from artificial PC liposomes (5, 15). We used a liposome leakage assay to measure dye release from small unilamellar liposomes induced by the action of EDS-76. The kinetics of the liposome leakage subsequent to the addition of adenoviruses was strongly nonlinear at acidic pH for all tested temperatures (2-37°C, Fig. 1 a, b, and c, upper curves). Calcein release from the liposomes was rapid, with the leakage rate dependent on the temperature. CsCl lacking adenovirus did not induce liposome leakage (fig. 1 a, b, c, lower curves).

The dye release from liposomes was dependent on the virus concentration up to 3×10^11 virions per ml (data not shown). In order to analyze the pH-sensitivity of the liposome leakage, all samples were adjusted to a pH of 7.5 after the 30 minute incubation to correct for the pH-dependent reduction in calcein fluorescence. We found that optimum conditions for egg PC liposome leakage was near pH 3 with half-maximal leakage at about pH 4 (Fig. 1d). Fig. 1 e compares the leakage kinetics at pH 5 (lower curve) and 3 (upper curve).

Here we used avian EDS-76 adenovirus which is able to induce calcein liposome leakage over a wide range of temperatures (up to physiological). This is in contrast to human adenovirus 2 (5) which only functions at acidic pH at 2°C due to virion self-aggregation (5). Our data show that the dye release from PC liposomes mediated by EDS-76 virus is maximal at about pH 3 at 37°C. In contrast, human adenovirus type 2-induced leakage of egg PC liposomes (5) and plasma membrane vesicles was at pH 5.5 (7), although liposome binding increased at pH's lower than 5 (5). PC liposome leakage was optimal at pH 5 or less for human adenovirus 5.

FIG. 1. Liposome leakage mediated by EDS-76. (a, b, c, and e) Time dependence of EDS-76-mediated release of calcein from liposomes. EDS-76 adenovirus (2.8×10^10 virions) in 50 μl of CsCl solution was added to the cell together with liposomes (3 nmol of PC) in 950 μl citric-NaCl solution at pH 3.0 (a-c, upper curves) at 2°C (a), 20°C (b) and 37°C (c). The lower curves in a - c represent liposome leakage after the addition of 50 ml of CsCl solution lacking adenovirus. The vertical bar in a-c corresponds to 200 nM released calcein. (d) pH dependence of EDS-76-induced liposomes leakage; liposomes (0.6 nmol of PC) were incubated for 30 min at 37°C in 200 μl of citric-NaCl solution with 5.6×10^6 EDS-76 virions; 800 μl of Hepes-NaCl, pH 7.5 solution was added before starting the measurement. (e) EDS-76-dependent liposome leakage at pH 3 (upper curve) and 5 (lower curve); data were adjusted to equivalent amounts of fluorescence (the fluorescence of calcein was 4.8 fold higher at pH 5 than at pH 3).
5.0. Curve A shows the opening of a single channel of 60 pS, while curves B and C show current fluctuations at + and −50 millivolts which were representative of several minutes of this particular recording. The amplitudes of the transitions only varied in the range of tens of pS from measurement to measurement. The channel activity was mostly non selective for cations or anions since formation of a NaCl gradient on the membrane produced only a small current asymmetry (the reverse potential was less than 15 mV for a ten-fold difference in NaCl concentration). The activity was voltage independent since the application of higher voltages resulted in proportional increases in amplitude of the current. The ability of the EDS-76 adenovirus to induce the channel activity appeared to be dependent on the nature of the BLM phospholipid used; e.g. when the membrane was formed using the synthetic diphytanoyl PC, EDS-76-mediated channel activity in the planar BLM was greatly reduced.

The entry of adenoviruses into cells has attracted considerable attention because of their capacity to penetrate the endosomal membrane (5–8, 10, 12–15, 17–19). Although the adenoviral penton base protein is involved in this (7), as well as being responsible for cell association via αv integrins and the permeabilization of membranes at acidic pH (31), the mechanism of membrane penetration is unclear. The results presented here suggest that an initial step of the interaction of adenovirus with membranes is formation of a channel of about 5 Å, calculated from the BLM data.

FIG. 3. Induction of channel activity by 2 μl (6·10¹⁰ virus particles) of EDS-76 at the cis side at pH 5.0 (+ 50 mV). (A) The addition of the EDS-76 is marked by an arrow; (B and C) current recordings at + and −50 mV.

and chicken CELO adenovirus (15), both of which have been used as components of receptor-mediated gene delivery complexes (13–15, 26–28); we have also recently used EDS-76 for this purpose (manuscript in preparation) together with an insulin-containing DNA-delivery construct with some success (29–30).

EDS-76 virus was found to induce ion channels at acidic pH in planar BLMs prepared from the same lipids. Figure 2 shows a typical trace of the current fluctuations in the BLM after the addition of 6·10¹⁰ EDS-76 virions on the cis side of the membrane (arrow on the Fig. 2) under the conditions of an applied voltage of +50 mV and a pH of 3.0. After a lag phase, EDS-76 induced irregular spikes of current across the BLM which disappeared after several minutes. Further addition of the EDS-76 led to second transient increase of the BLM current. The duration of the lag phase was dependent on the dose of EDS-76 and increased with decreasing amounts of virus added. EDS-76 did not affect the BLM current at neutral pH.

Figure 3 shows an example of the changes in current in the BLM induced by the addition of EDS-76 at pH 5.0. Curve A shows the opening of a single channel of 60 pS, while curves B and C show current fluctuations at + and −50 millivolts which were representative of several minutes of this particular recording. The amplitudes of the transitions only varied in the range of tens of pS from measurement to measurement. The channel activity was mostly non selective for cations or anions since formation of a NaCl gradient on the membrane produced only a small current asymmetry (the reverse potential was less than 15 mV for a ten-fold difference in NaCl concentration). The activity was voltage independent since the application of higher voltages resulted in proportional increases in amplitude of the current. The ability of the EDS-76 adenovirus to induce the channel activity appeared to be dependent on the nature of the BLM phospholipid used; e.g. when the membrane was formed using the synthetic diphytanoyl PC, EDS-76-mediated channel activity in the planar BLM was greatly reduced.

In summary, our results indicate that channel detection using the planar BLM system can be a useful tool to investigate mechanisms of adenovirus penetration through membranes. We show for the first time that the intact adenovirus (EDS-76) can form voltage-independent channels in planar BLM at acidic pH, and conclude that this may play a mechanistic role in adenovirus mediated endosomolysis.

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