New Cationic Lipids Form Channel-Like Pores in Phospholipid Bilayers

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ABSTRACT Two representatives of a new class of cationic lipids were found to have high pore-forming activity in planar bilayer membranes. These molecules, called BHHD-TADC and BHTD-TADC, have qualitatively similar effects on phospholipid membranes. Addition of 2.5–5 μM of either of them to the membrane bathing solutions resulted in formation of long-lived anion-selective pores with conductance in the range 0.1–2 nS in 0.1 M KCl. Pore formation was found to be dependent on the potential applied to the membrane. When negative potential was applied to membrane at the side of addition, the rate of pore formation was much lower compared to when the positive potential was applied. Dependence of pore formation on compound concentration was highly nonlinear, indicating that this process requires assembly of molecules in the membrane. Addition of any of these compounds on both sides of the membrane increased the efficiency of pore formation by one to two orders of magnitude. Pore formation was strongly pH dependent. Although pores were formed with high efficiency at pH 6.5, only occasional fluctuations of membrane conductance were observed at pH 7.5. Possible mechanisms of new compounds biological activity are discussed.

INTRODUCTION

Rapid advances in the field of functional genomics, expected in the near future, would identify a number of key genes and their products involved in various diseases (Collins and McKusick, 2001; Pandey and Mann, 2000). To benefit from this information and use these genes as therapeutic agents, efficient delivery systems need to be created. Nonviral vectors made of synthetic molecules are attractive for a number of reasons. Compared to viral vectors, nonviral vectors are more versatile and can be used to deliver different forms and sizes of nucleic acids, e.g., oligonucleotides, plasmids, linear DNA, RNA, etc. They are less immunogenic, nonintegrating and can be easily produced in large quantities (Porteous et al., 1997; Noone et al., 2000). Because they are built from synthetic molecules, they can easily be engineered to provide the multiple functions required for effective gene delivery and to incorporate many useful pharmacological properties for specific applications. However, current forms of synthetic vectors suffer from low efficiency of gene transfer. To be effective in therapeutics, the synthetic vector systems need to be improved considerably.

Among nonviral vectors, cationic lipid/DNA complexes are the most widely used (Katsel and Greenstein, 2000; Maurer et al., 1999; Chesnoy and Huang, 2000; Schwartz et al., 1995). Unlike natural lipids, cationic lipids have a positively charged polar head, which is responsible for neutralization of the negative charges of DNA and formation of a compact particle. In addition to this, the lipid component enables the nuclear delivery of DNA. The role of the cationic lipid in this multistep delivery process is not well understood. It has been proposed that cationic lipids facilitate the release of DNA from the endosomal compartment by mixing with the anionic lipids in the membrane (Xu and Szoka, 1996). A clear understanding of the mechanism is critical in the design of new drugs with improved transfection activity.

We have evaluated a new class of synthetic molecules for their gene delivery activity. These molecules are distinctly different from other cationic lipids. Structurally, they have three hydrophobic and two polar (hydrophilic) domains. One of the hydrophobic domains separates the two hydrophilic domains, and in an aqueous environment, this molecule may fold into a structure where the two hydrophilic domains are at the two ends and the hydrophobic domain at the center of the molecule. Electron microscopic studies indicate that they form micelles in aqueous medium. We have evaluated a number of these molecules with variations in the length and nature of the hydrophobic domains. Two of them, BHHD-TADC [5,18-Bis-(2-hydroxyhexadecyl)-1,5,18,22-tetraaza-docosane tetraoxalate] and BHTD-TADC [5,18-Bis-(2-hydroxytetradecyl)-1,5,18,22-tetraaza-docosane tetraoxalate] (for simplicity, we will use abbreviation X-TADC when talking about both of them), have been found to be excellent for transfection in vitro and in vivo. In vitro, transfection activity of BHTD-TADC and BHHD-TADC was up to 7.6-fold higher than lipofectamine, and the complexes were resistant to serum. Luciferase activity measured in mice lungs after intravenous injection of DNAO/DNA complexes was similar to that observed for DOTAP/Cholesterol/DNA (Yang et al., unpublished). Both BHTD-TADC (mol wt 1099) and BHHD-TADC (mol wt 1155) have very similar chemical structure, except for the length of hydrophobic chains (Fig. 1). The amphiphilic nature

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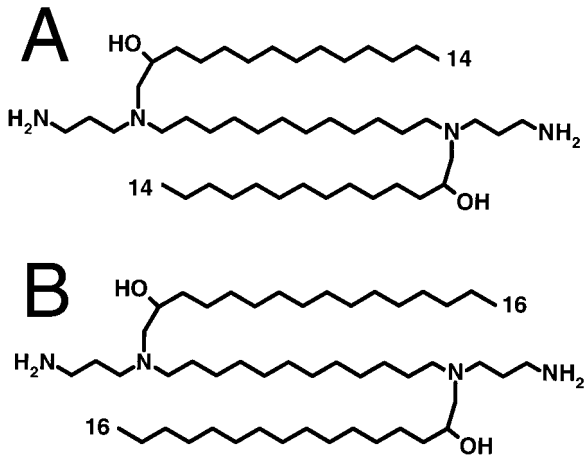

FIGURE 1 (*A*) Chemical structure of BHTD-TADC and (*B*) BHHD-TADC.

of X-TADC suggests that it may interact directly with the phospholipid bilayer. In the present work, we studied the effect of X-TADC on planar phospholipid bilayer membranes (BLM) to determine if there is any disruption of membrane barrier function that can be proposed as the basis for the biological activity of these compounds.

MATERIALS AND METHODS

Diphytanoyl-sn-glycerophosphocholine (DPhC), brain phosphatidylserine, dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylserine (DOPS), and cholesterol (Chol), were purchased from Avanti Polar Lipids (Alabaster, AL). BHTD-TADC, BHHD-TADC were synthesized in the Chemistry Research Laboratories, Therapeutic Area Oncology, Novartis Pharma AG, Basel, Switzerland.

BLMs were prepared by Montal technique (Montal and Mueller, 1972) with 10 mg/ml lipid solution of DPhC, phosphatidylserine, or different mixtures of DOPC, DOPE, DOPS, and Chol. The chamber, milled from Teflon, was similar to one described earlier (Chanturiya et al., 1999). It has two symmetrical compartments separated by Teflon partition and glass windows on both sides. The hole in 0.025 mm thick Teflon partition was 0.2 mm in diameter. A custom-made video microscope with 200 × magnification was used for visual control of BLM formation and quality. Both compartments were filled with 2 ml volumes of BLM bathing solution containing 100 mM KCl in 10 mM MES at pH 6.5 (standard buffer) or 100 mM KCl in 10 mM HEPES at pH 7.5, except when selectivity measurements were made. Pore selectivity was determined by measuring the current-reversal potential across the membrane using standard buffer in the *cis* compartment and 300 mM KCl in 10 mM MES at pH 6.5 in the *trans* compartment. Ag/AgCl electrodes (In Vivo Metric, Ukiah, CA) were connected with the membrane bathing solution through 200 μl pipette tips with long thin ends filled with 2% agarose in 0.2 M KCl. An electrode placed in the *cis* compartment was used for setting the potential across the membrane. The other electrode, in the *trans* compartment, was connected to a current/voltage converter based on a Burr-Brown OPA-111 operational amplifier with the gain of 1 mV/pA and frequency range 0–300 Hz or to the Axopatch 200B amplifier (Axon Instruments, Union City, CA). Data were recorded on a chart recorder, and in parallel on a computer disk using Axon Instruments Digidata 1322 A/D converter and Axoscope software. X-TADC stock solutions at 1mg/ml in distilled water were added to *cis* (or in some experiments to both) compartments of the BLM cell. Solution stirring was performed with 2 × 5 mm Teflon-coated stirring bars driven by miniature electronic stirrer (Eastern Scientific, Rockville, MD). All experiments were conducted at room temperature, 20–24°C.

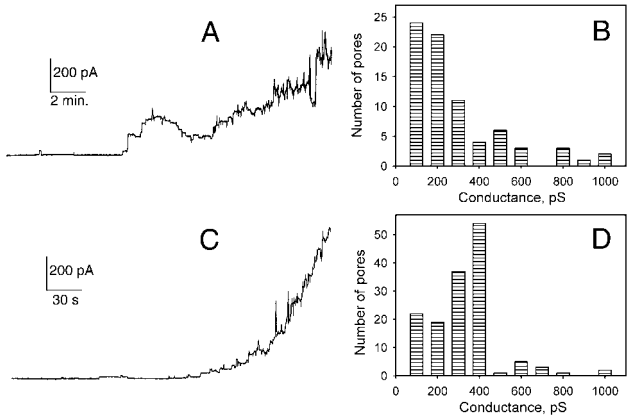

FIGURE 2 Pores formed by BHTD-TADC and BHHD-TADC in BLM. 12 μM (final concentration) BHTD-TADC (*A*) or 11 μM BHHD-TADC (*C*) was added to BLM 30–60 s before the beginning of the record. Membrane potential was +50 mV. Amplitude histograms of pore conductance at same experimental conditions are presented in *B* (BHTD-TADC, 80 pores total) and *D* (BHHD-TADC, 145 pores total). Only pore openings with more than 0.5 s lifetime were counted. BLM was formed from DPhC in standard buffer.

RESULTS

Fig. 2 shows the effect of X-TADCs on the conductance of bilayer membranes. Addition of 2.5–14 μM BHTD-TADC or BHHD-TADC (final concentration) to DPhC BLM bathed in standard buffer solution resulted in an increase in membrane conductance 5–10 min later. Although in most cases long-lasting conductance steps were observed before noisy conductance increases (Fig. 2 *A* and *C*), in some experiments only fast fluctuations of transmembrane conductance were observed after compound addition (not shown). An amplitude histogram of pores, calculated from conductance traces, is shown in Fig. 2 *B* and *D*. Small pores with conductance up to 100 pS were most common for BHTD-TADC, whereas BHHD-TADC histogram has clear peak in 300–400 pS region. Median pore conductance calculated is around 250 pS for BHTD-TADC and 350 pS for BHHD-TADC. Pore formation was observed at both positive and negative potentials on BLM, but required a higher concentration of X-TADCs when potential in the *cis* compartment was negative. At a positive potential and relatively low X-TADC concentration, the BLM conductance increased with time, first exponentially then linearly up to very high values (Fig. 3 *A*). When the potential was switched to negative, we typically observed a decrease in membrane conductance followed by a semistable conductance level. In some cases, conductance continued to increase at negative potential but at a significantly lower rate (Fig. 3 *B*). Conductance increase due to addition of these compounds was irreversible. Perfusion of the *cis* compart-

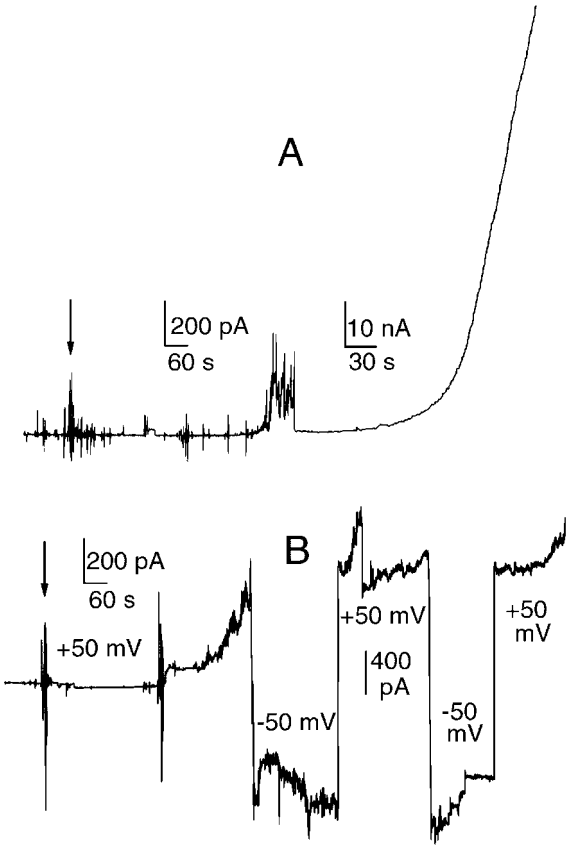

FIGURE 3 Potential-dependence of pore formation. (*A*) Time course of conductance increase at +50 mV positive potential. After the beginning of conductance increase, recorder was set to low sensitivity to get uninterrupted record of conductance growth. Visual observation confirmed the existence of the BLM even after recording went off scale. (*B*) Potential-dependence of BHTD-TADC induced conductance increase. Switching potential on the membrane from positive to negative after the beginning of conductance increase resulted in slowing down, complete termination, or reversal of conductance growth. BLMs were formed from DPhC in standard buffer. The arrows indicate addition of 3.5 μM BHTD-TADC.

ment with 3 vol of fresh buffer did not stop or slow down conductance increase (data not shown). Pore formation was observed when BLM was formed from DOPE/DOPC, DOPE/DOPC/Chol, or DOPE/DOPS. In the case of DOPE/DOPS, the rate of pore formation was ~10 times lower than that observed with electrically neutral BLMs.

It was difficult to stop conductance increase and get stable conductance level of X-TADCs-treated BLM, which is required for recording of current/voltage (I/V) dependencies of membranes. However, in presence of X-TADCs at low concentration, the rate of conductance increase was slow and it was possible to get almost undistorted I/V dependencies using fast recording protocol. Results are presented in Fig. 4. At low potentials, pores remain open and demonstrate ohmic behavior. Higher potentials (above ±60 mV) often cause pore closing (Fig. 4 *A*). Despite that, deviations from linearity seen in current/voltage plots (Fig. 4, *B–E*) are not sublinear but supralinear. There were no significant differences in single pore conductance and BLM selectivity between one- and two-sided addition of X-TADCs.

For quantitative measurements of the selectivity of membrane pores, BLMs were formed in asymmetrical conditions with buffers containing 100 and 300 mM KCl in *cis* and *trans* compartments respectively (see Methods). 12 μM BHTD-TADC was added to the *cis* compartment, and the potential at zero current (reversal potential) was measured several times at different conductance levels. Current reversal potential was always negative but changed significantly between measurements and between experiments (no dependence of reversal potential on BLM conductance was noticed). Reversal potential was found to be the same for both DPhC (-15.1 ± 4.4 mV, [mean ± SD, 10 measurements]) and DOPE/DOPS membranes (-15.1 ± 2.8 mV, 14 measurements) in the presence of BHTD-TADC. The value of the reversal potential obtained in similar experiments for BHHD-TADC in DPhC BLM was -19.3 ± 1.2 mV. The estimated mean Cl^-/K^+ permeability ratio is 3.8 for BHTD-TADC and 6.4 for BHHD-TADC, indicating significant anion selectivity of pores.

Dependence of the rate of pore formation on X-TADCs concentration was highly nonlinear. At concentrations below 3 μM, a lag period before the beginning of pore formation was long and conductance increase was slow. Above 15–20 μM, conductance rapidly increased to a very high level and the membrane ruptured a few minutes later. At an intermediate range of concentrations, the variability of the pore formation rate was significant, but this is typical in this kind of BLM experiments (Menestrina, 1983; Belmonte et al., 1987). Concentration dependencies of pore formation rates for both compounds with *cis* only and *cis*/*trans* additions are shown in Fig. 5. Although BHTD-TADC was significantly more active than BHHD-TADC, the slope of concentration dependence was roughly the same within the limit of experimental error. Two-sided addition of X-TADCs resulted in one to two orders faster conductance increase than one-sided addition.

Pore formation was also pH dependent. In contrast to high activity of compounds at slightly acidic pH (6.5), only short spikes of transmembrane current and occasional long-lasting pores were observed at pH 7.5. Acidification of *cis* or both compartments after addition of either BHTD-TADC or BHHD-TADC resulted in a significant increase of the rate of pore formation (Fig. 6).

DISCUSSION

Delivery of DNA molecules into the cell nucleus is a complex, multistep process that includes DNA transfer across several different membranes (Perales et al., 1994). Here we report that two cationic amphiphilic molecules possessing high transfection activity in vitro and in vivo (Yang et al., unpublished) are capable of forming large pores

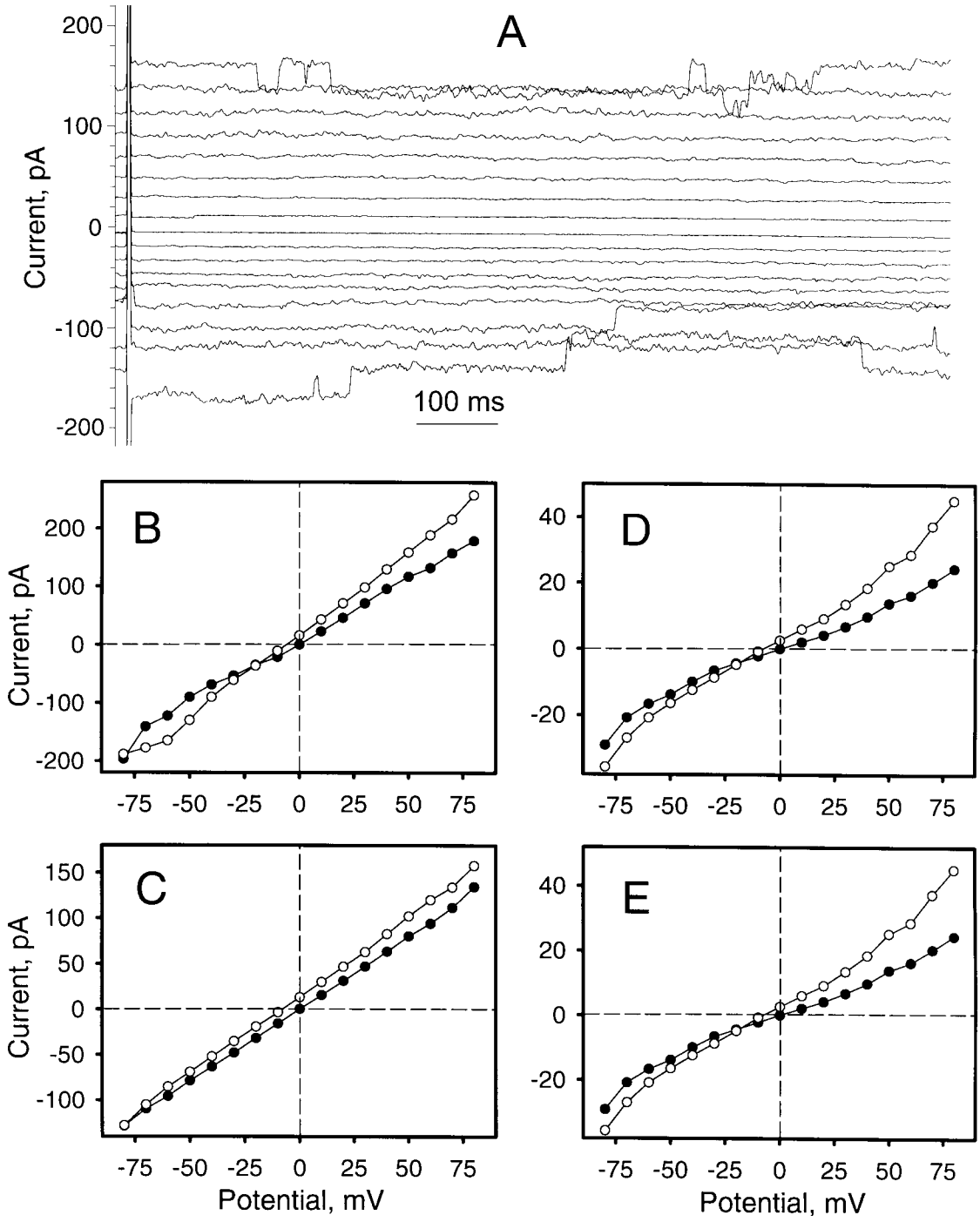

FIGURE 4 Current/voltage dependence and selectivity of pores. (*A*) Current recordings in symmetrical standard BLM bathing solution, in presence of 3.6 μM BHTD-TADC added *cis*, at different membrane potentials. Traces were recorded at potentials from −80 mV to +80 mV with 10-mV increment. (*B–E*) I/V dependencies of permeabilized membranes in symmetrical standard buffer solution (*filled circles*) and after addition of 50 mM KCl to *cis* compartment (*open circles*). (*B*) 3.6 μM BHTD-TADC added *cis*; (*C*) 2.9 μM BHTD-TADC added to both sides of BLM; (*D*) 3.5 μM BHHD-TADC added *cis*; (*E*) 2.6 μM BHHD-TADC added to both sides of BLM.

in lipid bilayers. Although further studies are required to determine the exact mechanism of the pharmacological activity of X-TADCs, it is clear that the disruption of the membrane barrier function can have profound effects on various stages of the DNA transport process.

How can these molecules form pores in phospholipid bilayer? Whereas detergents and lysolipids are known to increase membrane permeability by forming noisy fast flickering pores, we do not know any other small, lipid-like molecules capable of forming long-lived pores in model

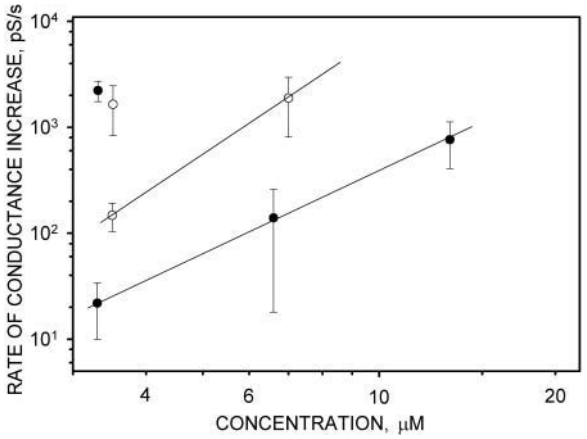

FIGURE 5 Concentration dependence of pore formation. Rate of BLM conductance increase at different concentrations of BHHD-TADC (*closed circles*) or BHTD-TADC (*open circles*) was measured. An average rate, measured at linear phase of conductance growth, ± SE of four to six experiments is plotted in logarithmic scale. Two data points in the left top side of the figure represent rates of conductance increase obtained with two-sided addition of 3.3 μM of BHHD-TADC and 3.5 μM BHTD-TADC. BLM was formed from DPhC in standard buffer.

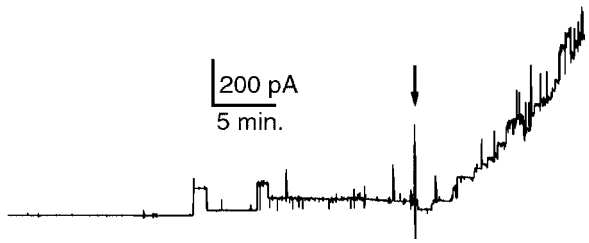

FIGURE 6 pH dependence of pore formation. 3.3 μM BHHD-TADC was added *cis* at neutral pH (100 mM KCl, 10 mM HEPES, pH 7.5) about 1 min before the beginning of the record. Very slow conductance growth was recorded up to the point when 10 μl of 0.1 M HCl was added *cis* at the moment indicated by an arrow (resulting pH 7.1 was measured later). BLM was formed from DPhC.

bilayers. Considering the simple chemical structure of both compounds, it is unlikely that a single molecule can form a large water-filled pore. More likely pore formation proceeds through the aggregation of several molecules in the membrane similar to that of membrane active peptides (Matsuzaki et al., 1998; Nir et al., 1999; Wyman et al., 1997). The nonlinear concentration dependence (Fig. 5), a significant lag time between compound addition and the beginning of pore formation (Fig. 3 *A*), and continued conductance increase after chamber perfusion indicate that aggregation of several X-TADC molecules in membrane is required for pore formation.

Although pK of primary amino group of X-TADCs is not known, the molecular structure indicates that at acidic pH, each X-TADC molecule can bear up to four positive charges that can interact with a transmembrane electric field. As we found, positive potential on the side of compound addition facilitates pore formation (Fig. 3 *B*). This indicates that transmembrane movement (flip-flop) of positively charged molecule from *cis* to *trans* monolayer of the membrane may be involved. (Lower pore-forming activity in negatively charged membranes compared to neutral membranes could be explained by stronger electrostatic binding of positively charged X-TADCs to negatively charged phospholipid headgroups). We propose that the X-TADC-formed pore consists of two half pores in each monolayer of the membrane, similar to the pores formed by polyene antibiotics (Cohen, 1992). This is supported by the finding that two-sided application of X-TADCs results in much higher rate of pore formation than one-sided application. Although exact calculation of the reaction molecularity from concentration dependence of pore-formation rate is not possible (Belmonte et al., 1987), significant nonlinearity of this process is a strong indication that pores are aggregates of several molecules. Taking into account the roughly cubic dependence of the rate of pore formation on the compound concentration when added to one side (Fig. 5), the minimal half pore is likely to consist of three X-TADC molecules, and thus a full pore will require at least six molecules. Larger pores are probably formed from a ring of four or more

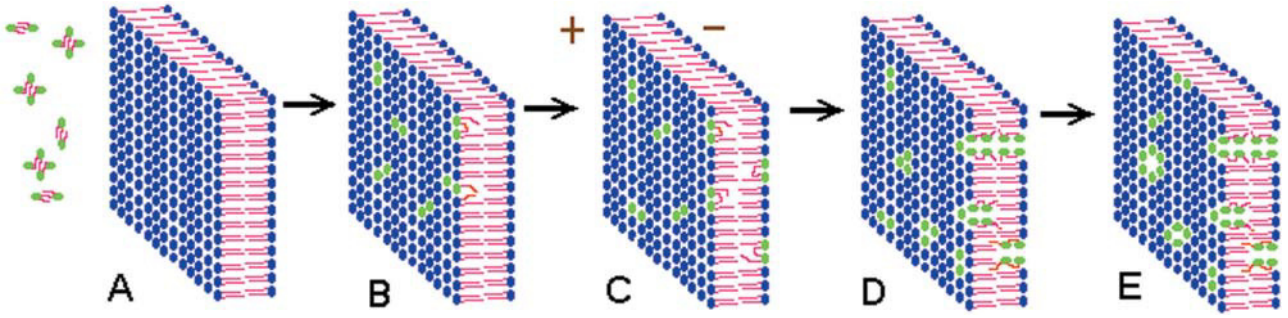

FIGURE 7 Proposed mechanism of pore formation by X-TADCs. Single molecules or micelles of X-TADC (*A*) become absorbed on the membrane surface and their hydrophobic tails anchor in the membrane hydrocarbon region whereas positively charged hydrophilic heads remain on the surface (*B*). Some of anchored molecules flip-flop from *cis* to *trans* monolayer and their concentration gradually increases in both monolayers (*C*). This process can be significantly enhanced by a positive potential on *cis* side of the membrane. Tri- (*D*) or higher order (*E*) oligomers are assembled in each monolayer and then unite into clusters that provide hydrophilic passage across membrane, i.e., pores with aqueous interior and positively charged walls.

molecules in each monolayer. Fig. 7 shows a proposed mechanism of interaction of X-TADCs with a phospholipid membrane.

Besides pore-forming ability, other properties of X-TADCs are also favorable for DNA delivery into the cell. Positive charges on these molecules allow them to form condensed complexes with negatively charged DNA. It cannot be ruled out that X-TADC/DNA complexes can deliver DNA directly into the cytoplasm by forming pores in the cell's outer membrane (van der Woude et al., 1995). Alternatively, these complexes may be internalized by cells via endocytosis and, upon acidification, in the endosome can become membrane active and release the DNA through pores formed in endosomal membrane (Zelphati and Szoka, 1996). The pH sensitivity of these molecules (Fig. 6) would help trigger the membrane activity and pore formation in the acidic environment of endosomes. The estimated size of 1 nS pore, in 100 mM KCl, is 2.8 nm. This is comparable to the diameter of a DNA double helix and positive charges on the pore wall are favorable for the ''priming'' of the DNA end into the pore (de Gennes, 1999; Zanta et al., 1999). Alternatively, DNA may be released from the endosome when endosome is exploded by the osmotic stress resulting from the formation of large number of pores in the membrane.

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