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Trypanosome lytic factor, a subclass of high-density lipoprotein, forms cation-selective pores in membranes☆

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Abstract

Trypanosome lytic factor 1 (TLF1) is a subclass of human high-density lipoprotein that kills some African trypanosomes thereby protecting humans from infection. We have shown that TLF1 is a 500 kDa HDL complex composed of lipids and at least seven different proteins. Here we present evidence outlining a new paradigm for the mechanism of lysis; TLF1 forms cation-selective pores in membranes. We show that the replacement of external Na^+ (23 Da) with the larger tetramethylammonium$^+$, choline$^+$ and tetraethylammonium$^+$ ions (74 Da, 104 Da and 130 Da) ameliorates the osmotically driven swelling and lysis of trypanosomes by TLF1. Confirmation of cation pore-formation was obtained using small unilamellar vesicles incubated with TLF1; these showed the predicted change in membrane potential expected from an influx of sodium ions. Using planar lipid bilayer model membranes made from trypanosome lipids, which allow the detection of single channels, we found that TLF1 forms discrete ion-conducting channels (17 pS) that are selective for potassium ions over chloride ions. We propose that the initial influx of extracellular Na^+ down its concentration gradient promotes the passive entry of Cl^- through preexisting Cl^- channels. The net influx of both Na^+ and Cl^- create an osmotic imbalance that leads to passive water diffusion. This loss of osmoregulation results in cytoplasmic vacuolization, cell swelling and ultimately trypanosome lysis.

Keywords: Trypanosome lytic factor; Cation-selective pore; High-density lipoprotein; *T. b. brucei*; Haptoglobin-related protein; Apolipoprotein L-I

1. Introduction

Trypanosome lytic factors (TLFs) are circulating lipoprotein complexes in the blood of humans and some other primates that confer innate protection against infection by *Trypanosoma brucei brucei* [1–3]. Two structurally distinct TLF complexes have been identified. In humans TLF1 is a 500 kDa high-density lipoprotein complex composed of apolipoprotein A-I (apoA-I), haptoglobin-related protein (Hpr), apolipoprotein L-I (apoL-I), human cathelicidin antimicrobial peptide (hCAP18), glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD), apolipoprotein A-II and paraoxonase [4,5]. TLF2 is a 1000 kDa lipid-poor protein immunocomplex composed of apoA-I, Hpr, apoL-I, hCAP18, GPIPLD and IgM ([6] and unpublished data).

The correlation between trypanolytic activity and the presence of the *Hpr* and the *apoL-I* gene products in mammals makes them the two prime candidates for mediators of lytic activity. Based on data in this paper and other experimental observations outlined below, we and others [7] propose that Hpr and apoL-I do not primarily function independently but act in synergy with other components of TLF to mediate trypanosome lysis. We have detected the unique expression of Hpr in all primates that have trypanosome lytic activity in their sera and purified HDL, including humans, gorillas, baboons, mandrills, and sooty mangabeys [5,8]. Purified Hpr is lytic, although the specific activity is 800-fold less than native TLF1 [7]. However, transgenic mice expressing human Hpr do not have detectable trypanolytic activity in their serum or purified HDL [9]. Likewise, recombinant

Abbreviations: apoL-I, apolipoprotein L-I; HDL, high-density lipoprotein; Hpr, haptoglobin-related protein; TLF, trypanosome lytic factor

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human apoL-I has been shown to confer lytic activity to non-lytic foetal calf serum [10], but we have found that some trypanolytic primate sera/HDLs (baboon and sooty mangabey) do not contain apoL-I, while TLF1 from gorilla and human do. Moreover, lytic HDLs from all of these primates cause trypanosome swelling and lysis by a similar mechanism that requires endocytosis, temperature above 20 °C, and acidic pH [1–3]. Finally, when immunopurified HDL subfractions were analysed, 99% of lytic activity within total human HDL was accounted for by HDLs containing both apoL-I and Hpr, and the remaining 1% of lytic activity was distributed in HDLs that contained either apoL-I or Hpr [7].

In this paper we present evidence that the mechanism of TLF1-mediated lysis involves the formation of pores permeable to cations, which are capable of both dissipating the membrane potential and contributing to the osmolyte accumulation within the cell. This mechanism is consistent with the cytoplasmic vacuolization and cell swelling observed prior to lysis by normal human serum (NHS) [11] or TLF1 [2]. Throughout this study we use chimpanzee HDL as the most phylogenetically relevant control, because it has a similar composition to human HDL but does not contain Hpr or apoL-I, cannot form pores, and does not cause swelling and lysis of trypanosomes [5]. During revision of this manuscript it was shown that recombinant human apoL-I as a single component can form anion-selective pores in asolectin membranes [12]; these results will be addressed in the context of our data.

2. Materials and methods

2.1. Isolation of HDL (human and chimpanzee) and humanTLF1

Isolation of human lytic HDL and purification of TLF1 from normal human serum were performed as we have described [6]. One milligram of human lytic HDL contains ∼10 μg of TLF1. As a negative control, non-lytic HDL was isolated from chimpanzee serum as described [5]. Protein content was assessed using the BioRad DC protein assay.

2.2. Trypanosome isolation and killing assay

T. b. brucei ILTat 1.25 were isolated from infected mouse blood [13]. Twenty-five microliters of parasites, 4×10^7 ml^{-1} in DMEM medium with 0.2% BSA, were added to 75 μl of either 150 mM NaCl or 150 mM tetramethylammonium chloride (TMACl), 150 mM choline chloride (cholineCl), 150 mM tetraethylammonium chloride (TEACl) such that the total Cl^- concentration was maintained at 141 mM while total Na^+ was reduced to 38 mM and replaced with the larger cations to a final concentration of 112 mM. For the incremental decrease of extracellular [Na^+], NaCl was isoosmotically replaced with increasing TMACl. For chloride replacement experiments parasites were re-suspended in 150 mM sodium gluconate, such that the total concentration of sodium was maintained at 151 mM while the total Cl^- was reduced to 29 mM.

For killing assays human lytic HDL (0.28–0.51 mg/ml) or Gramicidin D, a cation-specific pore-forming peptide (0.125 μg/ml), were re-suspended in either NaCl or TMACl, cholineCl, TEACl, or sodium gluconate as outlined above; the parasites 1×10^6 were then added ($T = 0$) and incubation was at 37 °C for 150 min or 60 min, respectively. Parasite lysis (%) was determined using a calcein–fluorescence assay [14] and verified by microscopic examination.

2.3. Preparation of liposomes

Chloroform solutions of lecithin, phosphatidyl-ethanolamine, phosphatidyl-serine, sphingomyelin and cholesterol were mixed to a molar ratio of 7:3:0.5:2:1 and, after flushing with nitrogen, the solvent was evaporated by high vacuum for 1 h. Small unilamellar vesicles (SUVs) were obtained as follows: dry lipids (5 mg) were re-suspended in 1 ml of 50 mM K_2SO_4, 25 mM HEPES–SO_4^{2-} (pH 7), sonicated (CP130 ultrasonic processor, Cole Palmer) at 40% power setting until clear (three pulses of 10 min) and centrifuged for 30 min at 100,000 × *g*. Texas Red-dextran-encapsulated large unilamellar vesicles (LUVs) were prepared as follows: dry lipids (5 mg) were hydrated with 1 ml of 0.3 M sucrose solution containing 5 mg/ml of Texas Red-dextran, 3000 Da. LUVs were allowed to form spontaneously for 2 h at 37 °C and then vortexed [15]. The free dye was removed by passing the liposome suspension through a NAP-10 column (Amersham-Pharmacia) equilibrated with TBS (50 mM Tris–HCl, 150 mM NaCl, pH 7.4). The eluted liposome suspension was washed three times with TBS and re-suspended in 200 μl of TBS. Phospholipid content of liposomes and lipoproteins was measured by Bartlett's method [16].

2.4. Dextran leakage assay

LUVs (5 μl, ∼125 μg lipid) were incubated in the presence of either human lytic HDL (300 μg/ml of protein; 175,000 Da, 55%, w/w, protein; 1.7×10^{-6} M), chimpanzee non-lytic HDL (300 μg/ml of protein, 175,000 Da, 55%, w/w, protein; 1.7×10^{-6} M) or the pore-forming peptides Gramicidin D (7.5 μg/ml, 4000 Da, 1.875×10^{-6} M) and Melittin (7.5 μg/ml, 2840 Da, 2.6×10^{-6} M). Incubations were done at 37 °C in 25 mM HEPES, 150 mM NaCl (pH 5.5) in a final volume of 200 μl. After 3 h the samples were centrifuged for 5 min at 1300 × *g* and the fluorescence of the supernatant was measured (Texas Red, excitation = 584 nm, emission = 612 nm) with a fluorometer (Labsystem Fluroskan II). The percentage leakage was calculated as % Release = $[(F_p - F_o)/(F_t - F_o)] \times 100$, where F_o and F_p are the fluorescence before and after the addition of HDLs or pore-forming peptides, respectively, and F_t is the maximum fluorescence in the presence of 0.5% Triton X-100.

2.5. Membrane potential measurements

Changes in membrane potential were measured using the fluorescent lipophilic ion 3,3′-dipropylthiocarbocyanine iodide

(diS-C_3(5)) (excitation = 620 nm and emission = 670 nm) [17]. The SUV suspension (2.5 μl, 50 μg phospholipid), which contains 50 mM K_2SO_4, 25 mM HEPES–SO_4^{2-} (pH 7), was diluted in 250 μl of 50 mM Na_2SO_4, 25 mM HEPES–SO_4^{2-} (pH 5–5.5). DiS-C_3(5) was added (2 μM, the dye distributes into the liposome membrane, the more positive the membrane potential the stronger the fluorescence signal) 3 min prior to the addition of the K^+ ionophore Valinomycin (0.4–1 nM). Valinomycin allowed K^+ to diffuse out of the vesicles creating a negative membrane potential that leads to the quenching of the dye's fluorescence. After 6 min, human lytic HDL or chimpanzee non-lytic HDL was added. Dissipation of the membrane potential resulting from Na^+ influx was detected as an increase in fluorescence (fluorescence recovery), monitored at 37 °C by a Fluoromax-3 spectrofluorometer (Jobin Yvon Inc., Edison, NJ). The percentage of fluorescence recovery (F%) was defined as $F\% = [(I_t - I_v)/(I_g - I_v)] \times 100$, where I_t is the fluorescence observed after adding HDLs, I_g the fluorescence in the presence of Gramicidin D, a cation-specific pore-forming peptide (3 μg/ml) alone and I_v is the fluorescence in the presence of only Valinomycin, a K^+-specific pore-forming peptide.

2.6. Lipid extraction

Total lipids from a pellet of 1×10^9 parasites were isolated using three cycles of extraction with 1 ml chloroform/methanol (2:1) [18]. The pooled organic phase was collected and dried under nitrogen, re-suspended in 100 μl of chloroform/methanol (2:1) and back-extracted with 400 μl of water/*n*-butanol (1:1) to remove the salts.

The non-polar upper phase was collected, clarified by centrifugation at 14,000 × *g*, dried under a stream of nitrogen and re-suspended in 50 μl of *n*-decane.

2.7. Single channel measurements in planar lipid bilayers

Planar lipid bilayers were formed as previously described [19]. The chamber contained two compartments (1 ml capacity each) separated by a partition with a 0.4 mm diameter hole where planar lipid bilayers were formed by painting across the partition a solution of total trypanosome lipids dissolved in *n*-decane as described in Section 2.6. The *trans* compartment was filled with 200 mM KCl, 10 mM HEPES, pH 7.4 and the *cis* compartment with 50 mM KCl, 10 mM HEPES, pH 5.5. Either human TLF1 (5 μg/ml) or chimpanzee non-lytic HDL (5–10 μg/ml), was added to the *cis* compartment. Experiments were conducted at room temperature (23–26 °C). Ag/AgCl electrodes were placed in direct contact with both aqueous solutions, and the reference electrode was in the *trans* compartment. Differences in electrode potential were corrected before forming the bilayer (in the presence of asymmetrical KCl solutions). The data were not corrected for junction potential (−0.7 mV). The current flowing through the bilayer was measured using a patch clamp amplifier (3900ADagan, Dagan Instruments, Minneapolis, MN). During acquisition, current traces were collected at 2 kHz and filtered at 500 Hz. During data analysis current traces were further filtered at 50 Hz. Data were acquired and analyzed by using Pclamp 6 and 8, respectively (Axon Instruments, Foster City, CA).

2.8. Reagents

Bovine serum albumin (BSA), tetramethylammonium chloride (TMACl), tetraethylammonium chloride (TEACl), choline chloride, sodium gluconate, Trizma base, HEPES, Gramicidin D, Melittin, Valinomycin, egg lecithin, sphingomyelin (from chicken egg yolk) and cholesterol, were purchased from Sigma (St. Louis, MO). Phosphatidyl-ethanolamine (from brain) and phosphatidyl-serine (from brain) were purchased from Avanti Polar Lipids (Alabaster, AL). Calcein AM, Texas Red-dextran (3000 Da, neutral) and 3,3′-dipropylthiocarbocyanine iodide (diS-C_3(5)) were obtained from Molecular Probes (Eugene, OR). Dulbecco's modified Eagle's medium (DMEM) was purchased from Cellgro (Mediatech, Inc.). Chloroform, methanol, *n*-decane and all other reagents used were of analytical grade.

3. Results

3.1. Human lytic HDL promotes the influx of sodium ions into T. b. brucei

The morphological changes observed upon exposure of *T. b. brucei* to human serum or TLF1 are cytoplasmic vacuolization, cell swelling and eventual lysis of the parasites [2,11]. The ability of an osmotically active molecule such as sucrose (0.25 M) to prevent swelling indicates that lysis involves an increase in membrane permeability leading to the influx of water [11]. To evaluate the cause of this water influx, we tested the effect of physiological concentrations of human lytic HDL that contains TLF1 on the Na^+ permeability of the trypanosome membranes. We focused on Na^+ because the extracellular milieu has ∼10-fold higher concentration compared to the intracellular [Na^+] (14 mM) [20]; this differential is maintained by low Na^+ permeability and ion pumps [21].

The isoosmotic replacement of extracellular Na^+ (23 Da) with the larger cations tetramethylammonium$^+$ (74 Da, TMA^+), choline$^+$ (104 Da) and tetraethylammonium$^+$ (130 Da, TEA^+) prior to the addition of lytic HDL ameliorates trypanosome swelling and thereby prevents cell lysis (Fig. 1A). Similarly, swelling and lysis caused by Gramicidin D, a pore-forming peptide that selectively allows the passage of small cations which depolarizes the trypanosome plasma membrane [22], was ameliorated by substitution of Na^+ by TMA^+, choline$^+$ or TEA^+ in the incubation buffer. A decrease in extracellular Na^+ (Fig. 1B) results in diminished swelling and lysis by both lytic HDL and Gramicidin D, which we attribute to a decreased influx of Na^+ and hence a lower osmotic imbalance. We could not reduce the extracellular [Na^+] below 38 mM because the parasites died within the 2–3 h assay. Overall, these data indicate that there is an increase in Na^+ permeability caused by lytic HDL.

As with all eukaryotic cells, a net increase in Na^+ influx will be followed by the passive entry of Cl^- via Cl^- channels. The increase in the movement of these 'osmolytes' creates an

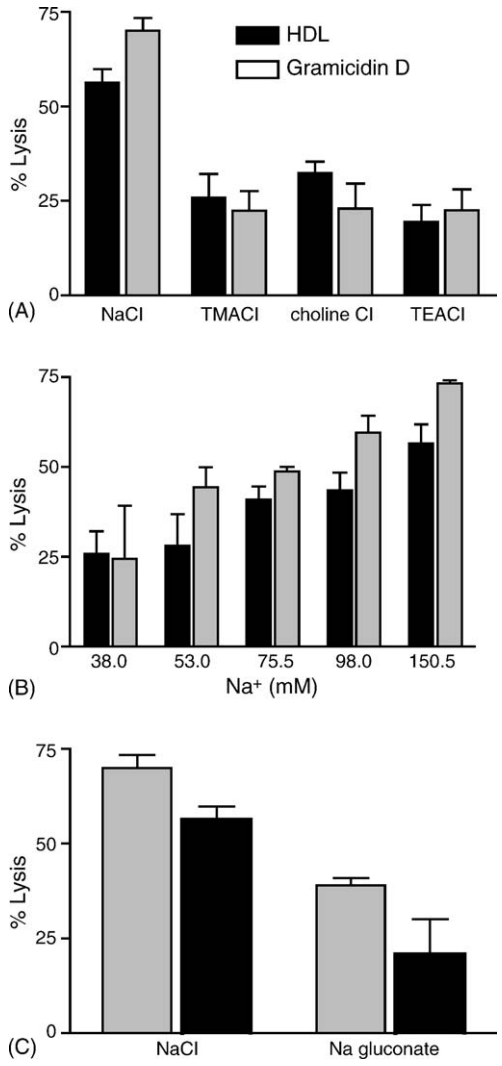

Fig. 1. Human lytic HDL promotes the influx of sodium into *T. b. brucei*. Trypanosomes were incubated at 37 °C in the presence of human lytic HDL (black bars) (0.28–0.51 mg/ml), 2.5 h or Gramicidin D (0.125 μg/ml) (grey bars), 1 h. Parasite lysis was measured (calcein–fluorescence) and expressed as a percentage of cells lysed. Results are the mean ± S.E.M. (A) Trypanosome lysis measured in medium where the sodium content was reduced from 151 mM (NaCl) to 38 mM, and isosmotically replaced with either tetramethylchloride (TMACl), choline chloride (cholineCl) or tetraethylammonium chloride (TEACl), $n = 6$. (B) Trypanosome lysis as a function of the sodium concentration in the incubation medium. Sodium concentration of the medium was reduced and replaced isosmotically with different amounts of TMACl. HDL, $n = 5$; Gramicidin D, $n = 3$. (C) Trypanosome lysis measured in a medium where NaCl was reduced and isoosmotically replaced with sodium gluconate (final concentration of 29 mM chloride). HDL, $n = 8$; Gramicidin D, $n = 6$.

osmotic imbalance leading to the influx of water. The increase in Na^+ permeability also changes the membrane potential and would also account for the previously observed efflux of K^+ [11]. If the Cl^- entry is prevented, the influx of Na^+ will be reduced because the negative charges outside the cell will retain the Na^+ (electroneutrality is always maintained). Accordingly, the partial replacement of Cl^- with $gluconate^-$, which cannot pass through chloride channels, ameliorates swelling and lysis by lytic HDL (Fig. 1C). Notably, reduction of the extracellular $[Cl^-]$ equally ameliorated swelling and lysis by both lytic HDL

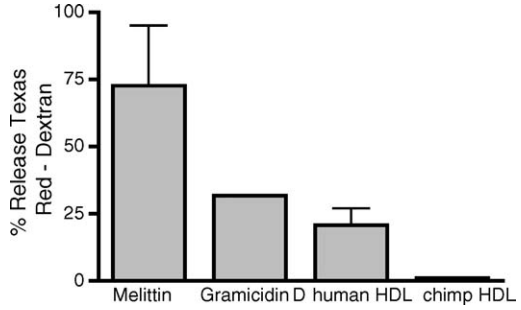

Fig. 2. Membrane destabilization. LUVs loaded with Texas Red-dextran (MW 3000) were incubated in the presence of Melittin (7.5 μg/ml, 2.6×10^{-6} M), Gramicidin D (7.5 μg/ml, 1.8×10^{-6} M), human lytic HDL (300 μg/ml, 1.7×10^{-6} M) and non-lytic chimpanzee HDL (300 μg/ml, 1.7×10^{-6} M) for 3 h at 37 °C in 25 mM HEPES, 150 mM NaCl, pH 5.5. Data are expressed as the percentage of fluorescence release and represent the mean of two independent experiments done in triplicate.

(31%) and Gramicidin D (35%), which only allows the passage of Na^+, K^+ and H^+ (Fig. 1C).

3.2. Human lytic HDL and Gramicidin D do not disrupt large unilamellar vesicles

Parasites exposed to purified TLF1 or lytic HDL are still able to exclude trypan blue (961 Da), propidium diiodide (668 Da) and ethidium bromide (394 Da) throughout the entire swelling process until lysis occurs (data not shown), which indicates that the plasma membrane is "intact". To prove that human lytic HDL does not generate large pores within the time frame of the trypanosome lytic assay (∼3 h), we evaluated over the same period of time, the effect of human lytic HDL on the release of Texas Red-dextran (3000 Da) from large unilamellar vesicles, which have a lipid composition similar to total bloodstream trypanosome lipids [23,24]. Non-lytic chimpanzee HDL was used as the most appropriate and phylogenetically relevant control. Measurements were performed at a low external pH to mimic the endosomal/lysosomal environment of the trypanosomes, since acidic conditions are critical for TLF-mediated trypanosome killing [25,26]. Fig. 2 shows that Melittin, a pore-forming peptide, which allows the passage of 3000 Da molecules [27] caused maximal release of Texas Red-dextran, whereas the cation-selective pore-forming peptide Gramicidin D, caused only partial release of Texas Red-dextran. Similarly maximal physiological concentrations of human lytic HDL (1 mg/ml) caused only partial release of Texas Red-dextran. We attribute the partial release of Texas Red-dextran to the slow influx of water into the LUVs due to the formation of small pores within the bilayer that allow the net influx of osmolytes. The influx of water will ultimately disrupt the LUVs. Noticeably, chimpanzee non-lytic HDL (1 mg/ml) did not cause release of Texas Red-dextran.

3.3. TLF1 dissipates the membrane potential of small unilamellar vesicles

To confirm that the movement of ions is a direct result of the exposure to TLF1 within human lytic HDL, we evaluated

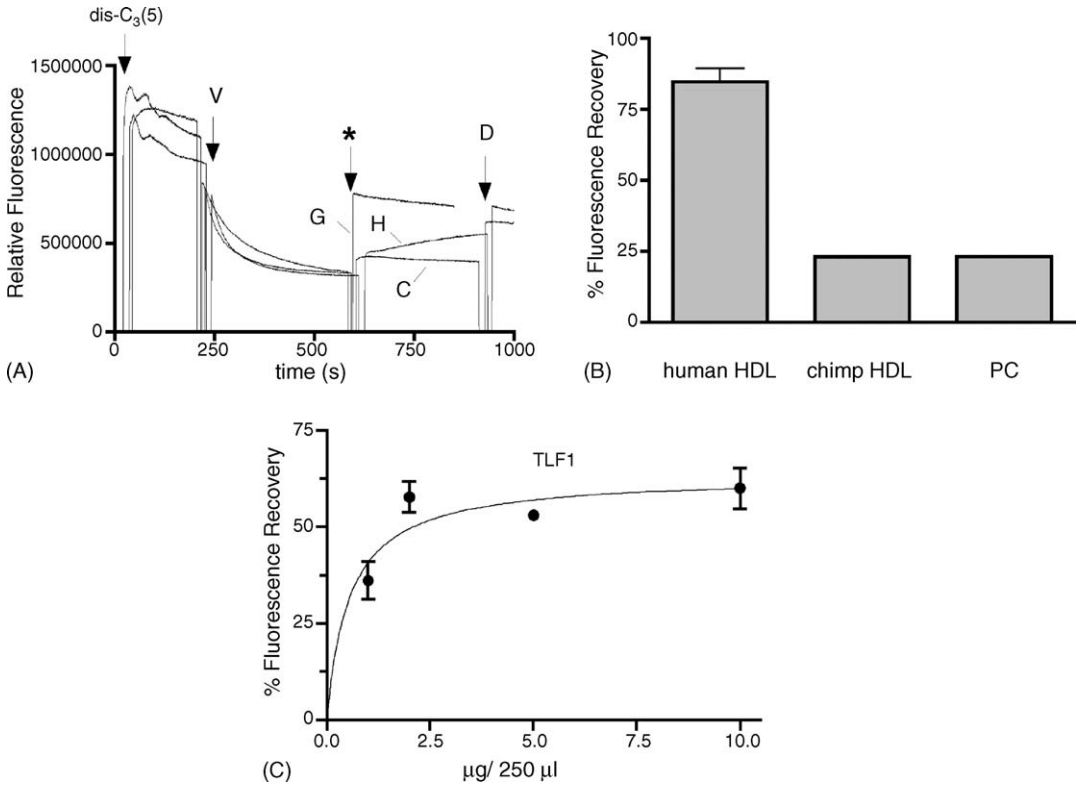

Fig. 3. TLF1 dissipates the membrane potential. (A) An increase in fluorescence caused by addition of the potential-sensitive dye, diS-$C_3(5)$, to potassium-loaded SUVs suspended in an isosmotic Na^+ medium, pH 5. Addition of Valinomycin (V) generated a negative membrane potential, resulting in quenching of the dye's fluorescence. After stabilization of the fluorescence the following additions were made (indicated by asterisk): human lytic HDL (H; 40 μg/ml), non-lytic chimpanzee HDL (C; 40 μg/ml) or Gramicidin D (G; 3 μg/ml). Final addition of Gramicidin D (D; 3 μg/ml) gave the maximal fluorescence recovery (dissipation of the membrane potential) achievable under each condition. (B) Dissipation of the membrane potential, expressed as fluorescence recovery after 5 min exposure to lytic human HDL (40 μg/ml), chimpanzee HDL (40 μg/ml) and to an equivalent lipid concentration of PC-liposomes (40 μg/ml). Results are mean ± S.E.M., $n = 3$. (C) Dissipation of the membrane potential, expressed as fluorescence recovery after 15 min exposure to increasing concentration of purified TLF1. Results are mean ± S.E.M., $n = 4$ using different TLF1 preparations.

the effect of lytic HDL and purified TLF1 on ion flux using a model system of small unilamellar vesicles, designed to represent inside out lysosomes. Potassium-loaded SUVs were suspended in an acidic sodium buffer (pH 5.5, as would be found inside the lysosome) that was isotonic with the intravesicular neutral buffer (pH 7, as would be found in the cytosol). The entire experimental scheme is outlined in Fig. 3A. First, the membrane potential-sensitive dye (diS-$C_3(5)$) was added to the SUVs, which in the absence of a membrane potential caused an increase in relative fluorescence units. Second, Valinomycin (V), a K^+ ionophore, was added to create a negative membrane potential inside the SUVs by allowing selective outward K^+ flow from the SUVs. The development of a negative potential quenches the diS-$C_3(5)$ fluorescence. Finally, addition of Gramicidin D (G), which facilitates the movement of Na^+ into the SUVs, dissipates the membrane potential and causes recovery of fluorescence, which is taken as 100%. Human lytic HDL (H) also causes recovery of fluorescence, although with slightly slower kinetics, presumably due to the release of pore-forming components from the lipids of the HDL prior to their insertion into the SUVs. In contrast, chimpanzee non-lytic HDL (C) has a negligible effect. To confirm that the SUVs are still fully responsive to ion movement in the presence of HDL, Gramicidin D (D) was added.

The data representing the percent fluorescence recovery (as described in Section 2.5) were collated and are shown in Fig. 3B. Human lytic HDL (40 μg/ml) caused a fluorescence recovery of ~70% (Fig. 3B), which indicates dissipation of the membrane potential resulting from Na^+ influx into the SUVs. In contrast, the maximum fluorescence recovery observed with chimpanzee non-lytic HDL (40 μg/ml) was 20% (Fig. 3B). Similar changes in fluorescence were observed with phosphatidylcholine liposomes (40 μg/ml; the major lipid component of HDL), indicating that the 20% change likely represents an interaction of the dye (diS-$C_3(5)$) with the added lipid components (Fig. 3B). Significantly, physiological concentrations of purified human TLF1 increased the fluorescence recovery (permeability of SUVs to Na^+) to 30% at 4 μg/ml and to 60% fluorescence recovery at 8–40 μg/ml (Fig. 3C).

3.4. TLF1 forms discrete cation-selective channels in planar lipid bilayers

We next used planar lipid bilayers as model membranes to examine pore-formation by TLF1 in real time. We found that TLF1 created discrete ion-permeable pores in lipid bilayers formed with purified lipids from *T. b. brucei* as described in Section 2. The *trans* compartment contained 200 mM KCl in 10 mM

HEPES at pH 7.4 to mimic the cytosol and the *cis* compartment contained 50 mM KCl in 10 mM HEPES at pH 5.5 to mimic the acidic environment of trypanosome endosomes/lysosomes. The KCl gradient was used to determine whether the pores were cation-selective (reversal equilibrium potential of potassium, $E_K = +34.9$ mV) or anion-selective (reversal equilibrium potential of chloride, $E_{Cl} = -34.9$ mV). In the absence of TLF1 no channel activity was observed in these membranes (Fig. 4A). Upon addition of TLF1 (5 μg/ml) to the *cis* compartment, single channel current events became detectable; their magnitude increased as the potential became more negative (Fig. 4B). Membranes displaying multiple channel currents were also observed (Fig. 4C, a section of the current record). The corresponding current amplitude histogram for the total current record of this

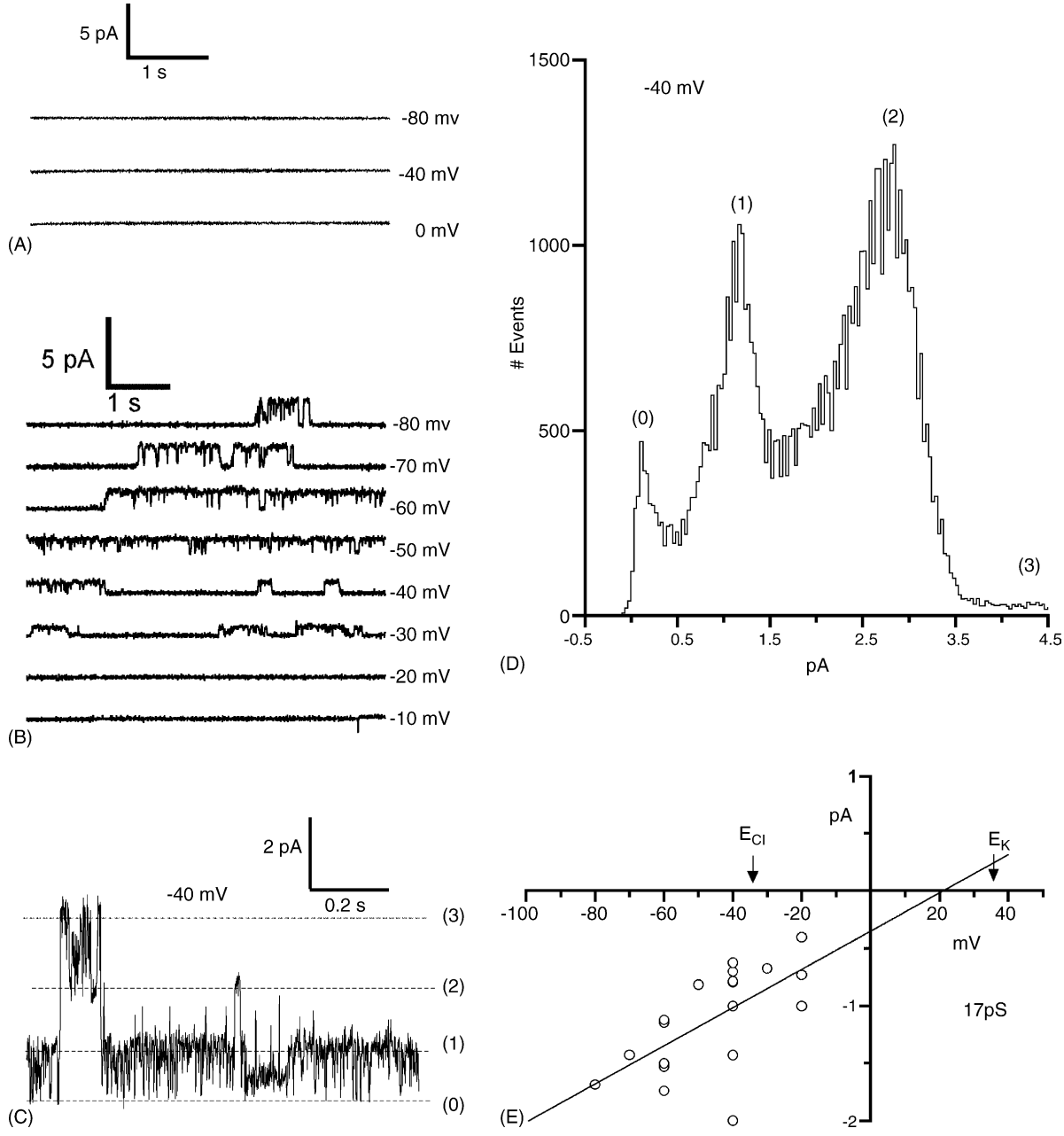

Fig. 4. Cation-selective channels generated by human lytic TLF1 in trypanosome lipid bilayers. Current traces recorded before (A) and after (B) TLF1 (5 μg/ml) was added to the *cis* compartment. (C) A trypanosome lipid bilayer displaying TLF1 generated multiple channel currents (section selected to show the various levels). The potential was held at −40 mV. Dotted lines indicate the closing level of the multiple channels, current levels when (1), (2) or (3) channels open simultaneously are indicated. (D) All point histogram for the entire data recorded at −40 mV in the experiment from which C was taken. The number of individual digitized data points (# Events) were plotted against their amplitude values (pA). The baseline was subtracted so that when the channels are closed (0) the amplitude value is 0 pA. The histogram shows that when one channel is open (1) the amplitude value is ∼1.2 pA, when two channels are open (2) the amplitude value is ∼2.6 pA (i.e. a mean of 1.3 pA/channel). In this recording, the time that three channels were open simultaneously was too brief to produce a clear peak in the histogram (3). (E) Current–voltage relationship of TLF1 (data generated from 11 channel positive membranes). Extrapolated regression intersects *x*-axis at ∼21 mV. E_K, reversal potential of $K^+ = +58 \log[K^+_{trans}]/[K^+_{cis}] = +34.9$ mV. E_{Cl}, reversal potential of $Cl^- = -58 \log[Cl^-_{trans}]/[Cl^-_{cis}] = -34.9$ mV.

membrane at −40 mV is shown in Fig. 4D. The peaks indicate the various current levels, when zero, one, two or three channels open simultaneously. The current magnitude difference between levels is similar to that observed in membranes containing only one channel at −40 mV (Fig. 4B); hence these current traces most likely represent the incorporation of multiple TLF1-generated channels. Fig. 4E shows the mean single channel current–voltage (*I–V*) relationship for TLF1-generated channels. The slope of the *I–V* plot indicates that the TLF1-generated channel has a conductance of approximately 17 ± 5 pS. The reversal potential, where the current is zero, extrapolates to a positive potential (approximately +21 mV) close to the equilibrium potential of K^+ (E_K = +34.9 mV). This type of single channel event was observed in 20% (11/47) of membranes to which TLF1 was added and in 0% (0/17) of membranes to which chimpanzee non-lytic HDL was added. Most of the TLF1-generated pores had a conductance of 17 pS. However, on two occasions we also observed a pore with a larger conductance (>800 pS) (not shown). The relative permeability of cations over anions was pK/pCl = 4.8. Taken together, these data demonstrate that the TLF1-generated pores that are selective for K^+ over Cl^- ions.

4. Discussion

In common with almost all eukaryotic cells, African trypanosomes actively maintain a low intracellular Na^+ level and a high intracellular K^+ level relative to their extracellular milieu [21]. This imbalance, along with a low Na^+ permeability, a low permeability of intracellular proteins (which are negatively charged), and a high K^+ permeability produces a negative membrane potential (−62 mV (±16 mV) [21], −82 mV [20]). It has been observed that prior to visually detectable swelling and cell lysis, the most rapidly (10–15 min) detectable effect of human serum (TLF) on susceptible trypanosomes is an increase in K^+ efflux [11] that is maximal by 60–90 min. This indicates an early disruption in the membrane potential. Furthermore, K^+ efflux is a mechanism utilized by cells to reduce the rate of water influx due to osmotic imbalance [28,29].

In eukaryotic cells, an increase in Na^+ permeability will lead to a net influx of Na^+ that is followed by the entry of Cl^- through preexisting Cl^- channels [30]. It is the intracellular increase of Na^+ and Cl^- that causes an osmotic imbalance, which is followed by passive water entry. If the entry of either Na^+ or Cl^- is prevented, the increase in water influx will be prevented. Therefore, in this study we propose that the observed TLF1-mediated influx of Na^+ is followed by Cl^- and water. First we show that reduction and replacement of Na^+ with a larger cations TMA^+, choline$^+$ and TMA^+, while maintaining a constant Cl^- concentration in the incubation medium can ameliorate lytic HDL-mediated trypanosome swelling, and therefore delay lysis (Fig. 1A). In addition, the reduction and replacement of extracellular Cl^- while maintaining a constant Na^+ concentration can ameliorate lysis by lytic HDL treated trypanosomes (Fig. 1C). Similarly, lysis by Gramicidin D, which is an exclusively cation-selective pore-forming peptide that does not allow the passage of Cl^-, is also prevented upon replacement of extracellular Cl^- (Fig. 1C) [31].

Further proof of cation-specificity was assessed in lipid vesicle assays and planar lipid bilayers, which were made with the lipid composition of (or from) trypanosomes [23,24]; non-lytic chimpanzee HDL was used as a negative control. The partial release of Texas Red-dextran from LUVs by lytic human HDL was similar to that measured with Gramicidin D (Fig. 2), which suggests formation of equivalent discrete pores. We attribute the release to the slow (3 h) osmotically driven entrance of water following Na^+ as seen with whole parasites. Consistent with this observation, experiments with SUVs indicated that human lytic HDL and TLF1 promote an increase in Na^+ permeability, whereas chimpanzee non-lytic HDL does not (Fig. 3). Moreover, we found that lytic TLF1 forms pores in planar trypanosome lipid bilayers with a membrane conductance of 17 pS that are selective for K^+ over Cl^- (Fig. 4) and also allow the passage of Na^+ (data not shown). Under the same conditions non-lytic chimpanzee HDL did not generate pores.

The cation-selectivity of physiological lytic HDL or purified TLF1 generated pores described herein contrasts with the recently reported anion-selectivity of recombinant apoL-I generated pores [12]. Resolution of this apparent discrepancy could in part be explained by recognizing that different lipid compositions (asolectin versus trypanosome) were used to create the lipid bilayers. Moreover, although the pore-forming domain of apoL-I may have anion-selectivity as a single component, it is unknown how this protein would act in the context of the TLF1 protein-lipid complex. Significantly, the molar ratio of the main protein components of TLF1 (from normal human sera) is Hpr:ApoA-I:ApoL-I; 5:2:1 [7]. Thus, apoL-I represents a fraction of the total TLF1 composition, and its activity may in some way be modulated by interaction with Hpr and potentially other components so that the purely apoL-I lytic activity may constitute only a minor fraction of the total activity.

There are indications of synergistic activity between components within TLF1. Two protein components of TLF, Hpr and apoL-I, have been identified as candidates to account for TLF1 lytic activity [4,10]. Although, purified Hpr [32,7] and recombinant [10] or purified [7] apoL-I have both been shown to be lytic, their specific activity is never as robust as native TLF1 [6,7]. Recombinant apoL-I is also more lytic when reconstituted with human serum [12].

Taking into account everybody's data the most straightforward interpretation would be that TLF1 generates cation-selective pores (five-fold relative permeability) and that the apoL-I within TLF1 may contribute by increasing the already high anion-permeability. The intracellular [Cl^-] is unusually high in trypanosomes (106 mM) [33] and almost equal to the physiological extracellular milieu (108 mM). In contrast, the intracellular [Na^+] is low (14 mM) [33] and the extracellular [Na^+] is high (116–140 mM). Thus, a depolarization at the plasma membrane can only be mediated by an increase in the cationic permeability. It is clear that net influx of Na^+ will be the major osmolyte driving water accumulation in trypanosomes. The increase in the net Na^+ influx caused by TLF1 is sufficient to cause a net Cl^- influx via preexisting Cl^- channels and

hence osmolyte imbalance. The osmolyte imbalance could be exacerbated by an additional increase in Cl^- influx mediated by apoL-I.

In contrast to our findings, Perez-Morga et al. did not see protection from NHS-mediated lysis upon replacing extracellular Na^+ with choline$^+$, but this discrepancy can be explained by the timing of extracellular Na^+ replacement. To prevent the movement of Na^+ into the cell down its 10-fold concentration gradient, the extracellular Na^+ should be replaced prior to (this paper) the addition of TLF/lytic HDL/NHS as we did, and not 1 h later as they reported [12].

Potential cation-selective pore-forming candidates within TLF1 could include Hpr and cathelicidin (hCAP18) a cationic antimicrobial peptide. We have found that Hpr has a γ-core motif, which is present in pore-forming disulphide linked antimicrobial peptides [34]. In some cases the γ-core motif alone is sufficient for pore-forming activity. This motif can also serve as a scaffold, to which complementary antimicrobial determinants (e.g. α-helices or β-sheets) can attach and act in synergy [34].

Based on our data and that of others we propose that, within the trypanosome endosome/lysosome, the lytic protein components are either released from the 500 kDa lipoprotein complex and/or undergo a conformational change at acidic pH; thus, enabling the active component(s), alone [12] or in combination (this paper), to insert into and form a pore in the lipid bilayer of the endosomal/lysosomal compartment, and thus cause disregulation of ion gradients between lysosome and the cytoplasm. Collapse of the lysosome membrane potential, and therefore ion gradients has been shown to occur when trypanosomes are incubated with lytic normal human serum but not with non-lytic FCS [12]. Given the high turnover rate of trypanosome membranes in the endocytic/exocytic pathway [35,36], it is also feasible that a portion of the endosomal/lysosomal membrane with TLF-generated pores is recycled back to the cell membrane. This would cause disregulation of cation gradients between the cytoplasm and extracellular milleu, and would explain the amelioration of TLF-mediated trypanolysis observed by replacing in the extracellular milleu a small permeant cation, Na^+ (that can pass through the pore), for larger impermeant cations such as TMA^+, choline$^+$ and TEA^+, which cannot pass through the pore.

This pore-forming mechanism of TLF1 is reminiscent of a common innate immune defense strategy used widely in nature against pathogens. Many organisms, including humans, synthesize cytotoxic peptides that adopt an amphipathic α-helical conformation when bound to lipid membranes, and under acidic conditions these peptides can insert into the lipid bilayer and cause permeabilization or disruption of the membrane [37]. It is noteworthy that both trypanosomes and *Leishmania* are susceptible to several anti-microbial peptides that depolarize the membrane potential and dissipate ion and water gradients through non-selective cation channels [38–40]. Extrapolating beyond our data with trypanosomes, it is conceivable that TLF1 might have a general pore-forming activity against other microbial organisms and could constitute another potential effector of human innate immunity.

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