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Identification of an ion channel activity of the Vpu transmembrane domain and its involvement in the regulation of virus release from HIV-1-infected cells

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Abstract HIV-1 Vpu catalyzes two independent functions, degradation of the virus receptor CD4 in the endoplasmic reticulum and enhancement of virus release from the cell surface. These activities are confined to distinct structural domains of Vpu, the cytoplasmic tail and the transmembrane (TM) anchor, respectively. It was recently reported that Vpu forms cation-selective ion channels in lipid bilayers. Here we report that this property of Vpu is a characteristic of its TM anchor. Expression of full-length Vpu in *Xenopus* oocytes increases membrane conductance. The Vpu-induced conductance is selective to monovalent cations over anions, does not discriminate Na^+ over K^+ and shows marginal permeability to divalent cations. Notably, introduction of the scrambled TM sequence into full-length Vpu abrogates its capacity to increase membrane conductance in oocytes and to promote virus release from infected cells. Reconstitution of synthetic Vpu fragments in lipid bilayers identified an ion channel activity for a sequence corresponding to the TM domain of Vpu. In contrast, a peptide with the same amino acid composition but with a scrambled sequence does not form ion channels. Our findings therefore suggest that the ability of Vpu to increase virus release from infected cells may be correlated with an ion channel activity of the TM domain, thereby providing a potential target for drug intervention based on the development of Vpu-specific channel blockers.

Key words: Channel protein; Lipid bilayer; Transmembrane helix; AIDS; Reconstitution

1. Introduction

The HIV-1 specific Vpu is an 81 residue oligomeric type I transmembrane phosphoprotein [1–4]. Vpu has two distinct biological activities, induction of degradation of the CD4 receptor in the endoplasmic reticulum (ER) [5], and augmentation of virus particle release from the plasma membrane [1,3,6]. Based on the fact that Vpu-induced CD4 degradation and regulation of virus secretion occur in different cellular compartments [7], and that they are controlled by separable structural domains of Vpu [8], we concluded that the two activities of Vpu are functionally independent. While the Vpu-mediated degradation is specific for CD4 [5,9], involves cytoplasmic sequences of Vpu [7,10], and is dependent on the phosphorylation of two conserved serine residues [7,11,12], Vpu's function on virus release depends on the integrity of the transmembrane (TM) domain [8]. A mutant of Vpu, Vpu_{RD}, encoding a TM domain with wild type amino acid composition but scrambled primary sequence, was unable to enhance the release of virus particles from infected or transfected cells, and virus encoding Vpu_{RD} had replication characteristics in T cells indistinguishable from that of a Vpu-deficient HIV-1 isolate [8]. Interestingly, Vpu_{RD} was integrated normally into membranes, was able to form homo-oligomers, and exhibited expression levels, protein stability, and intracellular transport similar to that of wild type Vpu. Vpu_{RD} binds to CD4 and induces its degradation with wild type efficiency in accordance with proper membrane topology and indicating that the alteration of the Vpu TM domain did not interfere with this function of Vpu.

Despite the finding that the Vpu TM domain is involved in the regulation of virus release, the molecular basis for this activity of Vpu remains unclear. In analogy to the observed interaction between the Vpu and CD4 cytoplasmic domains, which is a prerequisite for CD4 degradation [10], Vpu may interact with other cellular membrane-associated factors in performing its function on virus release. Alternatively, it is conceivable that Vpu exerts its effect indirectly, for example by altering the intracellular milieu near the membranes of the ER or the exocytic pathway. We postulated that Vpu could function as an ion channel [3,4,8,11] and, in fact, it was recently shown that recombinant Vpu forms cation channels after reconstitution in lipid bilayers [13].

Here, we report that expression of full-length Vpu in amphibian oocytes induces a cation-selective conductance with marginal permeability to divalent cations. We further show that a peptide with a sequence corresponding to the Vpu TM domain forms ion channels in lipid bilayers. Randomization of the Vpu TM sequence abolished ion channel activity in lipid bilayers and, when introduced into the *vpu* gene, abrogated the induction of membrane conductance by the expressed protein in oocytes. Together, our data are consistent with the reported ion channel activity of Vpu and demonstrate that the channel activity is stringently dependent on the structure of the TM domain. These findings are compatible with a model that envisions Vpu with two distinct structural modules that operate by two independent molecular mechanisms: release of progeny virions may be regulated by an ion channel activity of the Vpu TM domain occurring in a

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post ER compartment, while CD4 degradation in the ER is mediated by the phosphorylated cytoplasmic tail of Vpu [8]. A preliminary account of this research appeared elsewhere [14].

2. Materials and methods

2.1. Plasmid constructs

All plasmids containing HIV-1 sequences are derivatives of the infectious molecular clone pNL4-3 [15] (Fig. 1B). The mutant vpu_{R26}, carrying a randomized TM sequence based on the synthetic peptide Vpu^{rd} (Fig. 1A), was constructed by PCR mutagenesis [8]. Synthesis of cRNAs was performed using the following plasmids: pSP-9 [3], pSP-9/U_{DEL1} [8], pSP-9/$U_{2/6}$ [11], and pSP-9/U_{R26} [8].

2.2. Antisera and antibodies

Vpu was detected by immunoblots using serum from an asymptomatic HIV-1 seropositive patient, a polyclonal anti-Vpu antiserum (sheep) against a synthetic peptide comprising residues 41–58 of Vpu [12], and a polyclonal anti-Vpu serum (rabbit), directed against the hydrophilic C-terminal domain of Vpu expressed in *E. coli* [4].

2.3. Peptide synthesis and purification

Sequences of the peptides Vpu^{1-27} (M^1EPIQIAIVALVVAIIIAIVVWSIVII^{27}), Vpu^{rd} (M^1IPIVIAVILAVAVQAIVIVIVSWIIE^{27}), and Vpu^{28-81} (E^{28}YRKILRQRKIDRLIDRLIERAEDSGN ESEGEISALVEMGVEMGHHAPWDIDDL^{81}) [11] are derived from the HIV-1 clone $HTLVIII_B$ [16] (Fig. 1A). Synthesis was performed on an ABI 433A peptide synthesizer using a research resin TentaGel R RAM-Fmoc. Peptides were purified by preparative HPLC on a VYDAC 218 TBP 1520 column and analyzed by analytical HPLC. Electrospray mass spectrometry was performed on a triple stage quadropole mass spectrometer TSQ 700 (Finnigan MAT, Bremen, Germany) with an electrospray (API) ion source. The accumulated distribution of multiple charged ions were deconvoluted to molecular mass spectra using the BIOMASS program (Finnigan). The exact molecular masses of peptide Vpu^{28-81} at 6293.1 Da, peptides Vpu^{1-27} and Vpu^{rd} at 2899.8 Da were established. Automated amino acid sequence determination of the first 12 amino acid residues from Vpu^{1-27} and Vpu^{rd} was performed on an Applied Biosystem 475 A gas-phase sequencing system and a 473 A pulsed-liquid phase sequencer (Applied Biosystems, Foster City, CA, USA). Total amino acid analysis was carried out on an Applied Biosystems 420 A Hydrolyzer/Derivatizer after peptides were completely hydrolyzed in 6 N HCl at 110°C for 48 h.

Table 1
Reversal potential of the ionic permeability induced by $Vpu_{2/6}$ in *Xenopus* oocytes

	V_R (mV)
125Na/2Ba	−21 ± 3.7
115K/10Na/2Ba	−26 ± 3.0
10Na/2Ba	−32 ± 4.5
10Na/20Ba	−35 ± 3.1
10Na/20Ca	−34 ± 2.5
NaCl	−32 ± 4.7
NaF	−36 ± 1.1
NaBr	−33 ± 5.9
NaI	−28 ± 3.6

Ionic conditions were (in mM): 125Na/2Ba: 125 NaCl, 1.8 $BaCl_2$, 2.8 KCl; 115K/10Na/2Ba: 115 KCl, 1.8 $BaCl_2$, 10 NaCl, 2.8 *N*-methyl-glucamine (NMG); 10Na/2Ba: 10 NaCl, 115 NMG, 1.8 $BaCl_2$, 2.8 KCl; 10Na/20Ba: 10 NaCl, 80 NMG, 20 $BaCl_2$, 2.8 KCl; 10Na/20Ca: 10 NaCl, 80 NMG, 20 $CaCl_2$, 2.8 KCl; all supplemented with 10 mM TES pH 7.4. NaCl is normal Ba^{2+}-Ringer's solution; NaF, NaBr and NaI refer to Ba^{2+}-Ringer's where 50 mM NaCl was substituted by an equimolar concentration of NaF, NaBr, or NaI, respectively. Reversal potentials were obtained from tail current analysis. 0.3 mM niflumic and flufenamic acids were used to minimize the contribution of the endogenous Ca^{2+}-activated voltage-dependent chloride channel [23]. Currents were elicited by hyperpolarizing to −30 mV from a holding potential of −40 mV, and applying test voltages between −60 mV to +40 mV in steps of 10 mV. Data are given as means ± S.E.M. with $n = 4$.

Table 2
Dependence of the Vpu-induced conductance on the extracellular pH

	Differential current ($Vpu_x - Vpu_{DEL1}$)	
	pH 6.5	pH 6.0
Vpu_{DEL1}	0.00	0.00
Vpu_{RD}	−0.01 ± 0.01	−0.12 ± 0.03
Vpu_{wt}	0.11 ± 0.06	0.21 ± 0.10
$Vpu_{2/6}$	−0.31 ± 0.06	−0.46 ± 0.09

Injected oocytes were incubated with Ba^{2+}-Ringer at the indicated pH. Ionic currents were elicited by stepping to −130 mV from a holding potential of −40 mV. Ionic currents were normalized with respect to that obtained at pH 7.4. Differential current (Δi) denotes the normalized current of oocytes expressing Vpu_x (where x refers to RD, wt (wild type) and 2/6) minus that from Vpu_{DEL1} injected oocytes. For a pH-dependent ion channel, Δi is expected to be ≥2 or ≤2, i.e., at least two-fold larger or smaller than that measured in control oocytes expressing endogenous channels. Values $-2 \leq \Delta i \leq 2$ signify that the pH-dependent currents arise primarily from endogenous channels. Values are given as means ± S.D. with numbers of oocytes ≥6.

2.4. Functional expression of Vpu proteins in Xenopus oocytes

Complementary capped RNAs (cRNA) were synthesized using SP6 promoter-driven in vitro transcription vectors of the pSP-9 type [3] using the mMESSAGE mMACHINE transcription kit (Ambion, Austin, TX, USA) [3,17]. Oocytes were harvested, dissociated, manipulated, microinjected with ~50 ng of cRNA and perfused (2–4 ml/min) with Ca^{2+}-Ringer solution (115 mM NaCl, 2.8 mM KCl, 1.8 mM $CaCl_2$, 10 mM tris[hydroxymethyl]-2-aminoethanesulfonic acid (TES), pH 7.4) as described [17]. Whole cell currents were recorded under voltage-clamp conditions (Turbo TEC 01C, NPI Electronics, Tamm, Germany) using two microelectrodes [17]. Oocytes were hyperpolarized from a holding potential (Vh) of −40 mV to −130 mV in steps of 10 mV. Voltage stimulation and data acquisition were carried out with a microcomputer equipped with pClamp 5.5 software (Axon Instruments, Burlingame, CA, USA). Capacitive currents were analog compensated. Data are given as mean ± S.E.M., with $n \geq 10$, where n denotes the number of oocytes. Only oocytes with membrane resting potentials ≤−30 mV were analyzed.

2.5. Quantitative immunoblotting analysis of Vpu expressed in Xenopus oocytes

Individual oocytes were lysed (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.5% Triton X-100, 10 mM iodoacetamide, 0.5 mM leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride) by gentle homogenization and three cycles of freeze thawing (−70°C/37°C). To remove yolk, lysates were centrifuged (10 min, 16000 × *g*, 4°C) and clarified supernatants were boiled in SDS-PAGE-sample buffer. Generally, 1–40 μl of lysates was analyzed on 12.5% SDS-PAGE, electrotransferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA). Membranes were blocked with 5% of bovine serum albumin and incubated with a 1:1000 dilution of Vpu specific polyclonal antibodies. Bound antibodies were identified by using ^{125}I-protein G (0.1 μCi/ml; Amersham Corp., Arlington Heights, IL, USA) followed by autoradiography. For standardization, five known concentrations (0.05 ng–0.5 mg) of the peptide Vpu^{28-81} [11] were analyzed. Vpu proteins were quantitated using a Fuji BAS 2000 Bio-Image Analyzer.

2.6. Single channel recordings in lipid bilayers

Lipid bilayers were assembled by apposition of two monolayers spread from a lipid solution in hexane [18,19]. The lipids were POPE/POPC [1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (Avanti Biochemicals, Alabaster, AL)], 4:1, in hexane (5 mg/ml). The aqueous subphase was composed of 0.5 M KCl or NaCl, 1 mM $CaCl_2$ and 5 mM HEPES, pH 7.4. Peptides were dissolved in trifluoroethanol (TFE) at 0.01 mg/ml and added to the aqueous subphase after bilayer formation to the final concentrations indicated in figure legends. Acquisition and analysis of single channel currents was as described [20,21]. Records were filtered at 1 kHz with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA, USA) and

digitized at 0.1 ms per point using an Axon TL-1 interface (Axon Instruments). pClamp 5.5 (Axon Instruments) was used for data processing. Conductance and lifetime values were calculated from continuous recordings lasting 30 s or longer and with $n \geq 300$ openings; openings with $\tau_o \leq 0.3$ ms were ignored. Values reported as mean ± S.E.M.; n = number of experiments. Bilayer experiments were performed at 24 ± 2°C.

3. Results

3.1. *Vpu increases the membrane conductance of Xenopus oocytes*

Wild type Vpu produced a small increase in membrane current (130 ± 60 nA at −130 mV and 96 h post-injection, $n \geq 10$; Fig. 2A, panel 1) relative to that measured in uninjected oocytes ($P < 0.05$, Mann-Whitney rank sum test [22], not shown). In contrast, expression of the phosphorylation mutant $Vpu_{2/6}$ (Fig. 1B), which contains the same TM anchor as wild type Vpu, but carries serine to asparagine substitutions at positions 52 and 56 within the cytoplasmic domain and thus lacks both phosphorylation sites [7,11], produced significantly larger currents (Fig. 2A, panel 2). No currents were recorded in oocytes injected with cRNA encoding a completely defective mutant Vpu_{DEL1} [8] (Fig. 1B, Fig. 2A, panel 3). Expression of Vpu proteins in oocytes was analyzed by immunoblotting with Vpu-specific antibodies (Fig. 2B). The phosphorylation mutant $Vpu_{2/6}$ was readily detectable whereas expression of wild type Vpu was below the sensitivity of the assay. Because of the low expression levels of wild type Vpu, all subsequent studies in oocytes were done with non-phosphorylated $Vpu_{2/6}$.

Fig. 2A, panel 2, depicts a family of whole cell inward

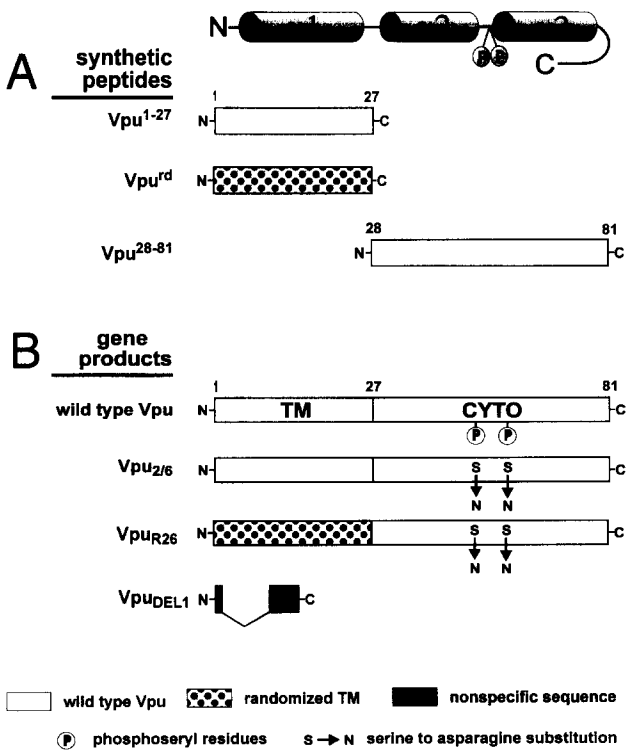

Fig. 1. Schematic structure of synthetic Vpu peptides and Vpu gene products. A: Synthetic Vpu fragments used for conductance analyses in lipid bilayers: Vpu^{1-27} = wild type TM peptide; Vpu^{rd} = randomized TM peptide; Vpu^{28-81} = cytoplasmic peptide. B: Vpu proteins used for voltage clamp analyses in *Xenopus* oocytes: $Vpu_{2/6}$ = phosphorylation mutant [7,11]; Vpu_{R26} = chimera between $Vpu_{2/6}$ and Vpu_{RD} [8] carrying a randomized TM sequence; Vpu_{DEL1} = deletion/frame shift mutant [8,36]. The secondary structure of Vpu which was determined previously by using a combination of CD and ^1H NMR spectroscopy ([8,32,37]; V. Wray, personal communication) is outlined at the top: the transmembrane (TM) anchor contains a hydrophobic α-helical structure denoted as α1; two amphipathic helices, α2 and α3, are located in the cytoplasmic domain (CYTO), and one single reverse turn is located at the very C-terminus. P = phosphoseryl residues in positions 52 and 56. N = NH_2-, C = COOH-terminus. Wild type sequences, randomized sequences, or non-specific sequences are coded as indicated at the bottom. Numbers refer to amino acid positions in Vpu.

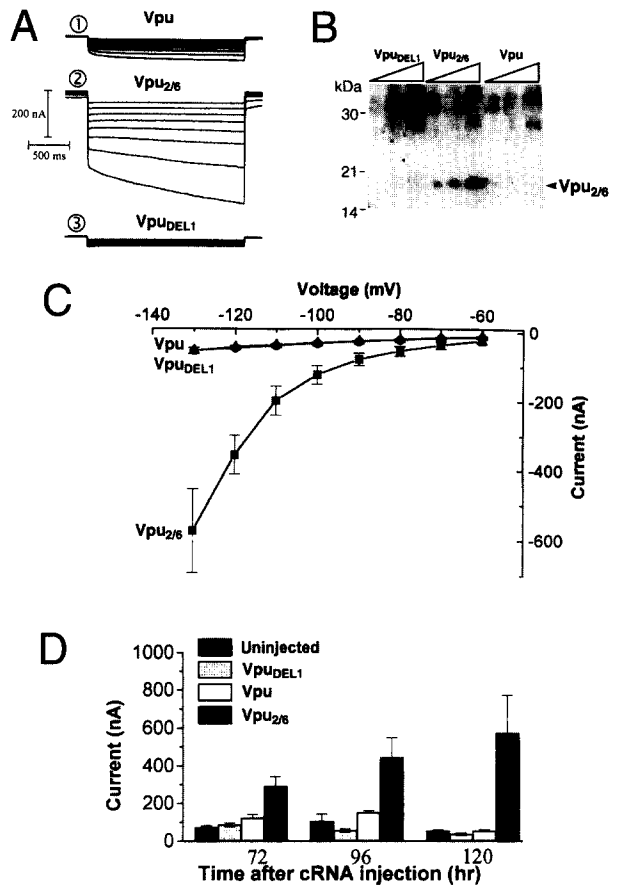

Fig. 2. Expression of $Vpu_{2/6}$ in *Xenopus* oocytes increases the membrane conductance. A: Family of whole cell ionic currents from injected oocytes evoked by hyperpolarizing test pulses of 2 s duration from −60 to −130 mV in 10 mV increments. The holding potential was −40 mV. Oocytes were injected with 50 ng of cRNA encoding Vpu (panel 1), $Vpu_{2/6}$ (panel 2) or Vpu_{DEL1} (panel 3). Downward deflections denote inward currents. Displayed currents were recorded 120 h post injection. Recordings were performed in Ca^{2+}-Ringer solution. B: Extent of expression of Vpu proteins in *Xenopus* oocytes. Increasing concentration of lysates (10%, 20%, and 40%) derived from oocytes evaluated by patch clamp were separated in 12.5% acryl aide gel and analyzed by immunoblotting using Vpu specific polyclonal antisera (sheep and rabbit) and a HIV-1-reactive human serum. C: Current-voltage relationships of $Vpu_{2/6}$-mediated ionic currents in oocytes analyzed 120 h post injection. Values are given as means ± S.E.M. with $n \geq 14$. D: Time course of the Vpu-induced changes in membrane conductance. Ionic currents elicited at −130 mV were measured at the end of the 2 s hyperpolarizing pulse and plotted as a function of time after cRNA injection. Values are given as means ± S.E.M. with $n \geq 14$. Only oocytes with membrane resting potentials ≤−30 mV were considered. Statistical analysis was performed using the nonparametric Mann-Whitney rank sum test [22]. The significance level for $Vpu_{2/6}$ with respect to Vpu_{DEL1} was $P < 0.001$.

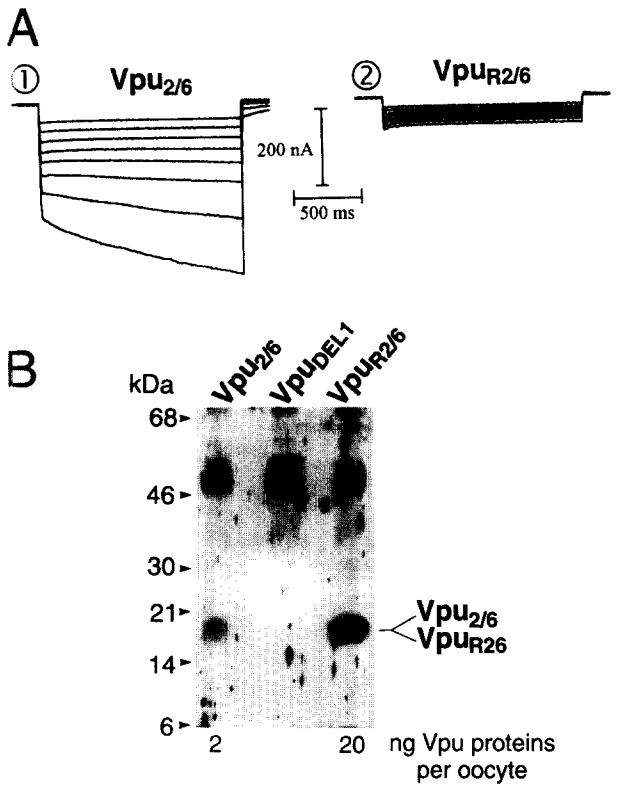

Fig. 3. Randomization of the TM anchor abolishes the Vpu-induced conductance. A: Family of whole cell inward membrane currents recorded from *Xenopus* oocytes injected with 50 ng of cRNAs encoding either $Vpu_{2/6}$ or Vpu_{R26}. Hyperpolarizing voltages steps, 2 s in duration (−60 mV to −130 mV), were performed as described for Fig. 2. The holding potential was −40 mV. Displayed currents were recorded 120 h post injection. B: Extent of expression of Vpu proteins in *Xenopus* oocytes. Lysates (10%) derived from oocytes evaluated by voltage clamp were separated in a 12.5% acryl aide gel and subjected to immunoblotting as described for Fig. 2B. Amount of Vpu proteins expressed per oocyte is indicated under the autoradiogram. Values were calculated based on a standard curve using the peptide Vpu^{28-81} [11].

currents in response to hyperpolarizing membrane voltages recorded from oocytes injected with cRNAs specific for $Vpu_{2/6}$. The inward currents elicited in response to a set of test potentials ranging from −60 to −130 mV from a holding potential (Vh) of −40 mV displayed a time course characterized by slow activation kinetics and the absence of inactivation during the 2 s hyperpolarizing pulse. For $Vpu_{2/6}$, membrane current increased as the potential was hyperpolarized, being conspicuous for voltages ≤−80 mV (Fig. 2C), and increased steadily up to 120 h post injection (Fig. 2D). In contrast, currents recorded in the presence of wild type Vpu were only slightly above the currents recorded from either uninjected or Vpu_{DEL1} injected oocytes (Fig. 2C,D).

3.2. The Vpu-induced conductance is cation-selective

To examine the ionic selectivity of the permeation pathway, tail current analysis under different extracellular ionic conditions was conducted ($n=4$ experiments) (Table 1). Replacement of external Na^+ by K^+ did not shift the reversal potentials (V_R) to any significant extent. A 10-fold increase of the $[Na^+]$ in the external bath displaced V_R ~10 mV towards more depolarizing potentials. In contrast, neither increasing 10 times the extracellular $[Ba^{2+}]$ and $[Ca^{2+}]$, nor replacement of Cl^- by F^-, Br^-, or I^- significantly change V_R (Table 1). Thus, the permeation pathway induced by expression of $Vpu_{2/6}$ is permeable to monovalent cations without discriminating Na^+ over K^+ and shows marginal permeability to divalent cations. Further, the $Vpu_{2/6}$-induced conductance appears to be largely pH-independent (Table 2). Together, these results suggest that $Vpu_{2/6}$ expressed in oocytes increases membrane cationic permeability.

3.3. Pharmacology of the Vpu-induced conductance

The magnitude of the $Vpu_{2/6}$-induced currents in oocytes was unaffected by niflumic or flufenamic acids (300 μM), two well known, high affinity blockers of Cl^- channels [23]. This result indicates a negligible contribution of endogenous oocyte Cl^- channels to the Vpu-induced conductance. Further, the Vpu-induced conductance was insensitive to amantadine and memantine (100 μM), two specific blockers of the Influenza A virus M2 ion channel [24].

3.4. Randomization of the TM anchor abolishes the Vpu-induced conductance

To evaluate the sequence specificity of Vpu-induced conductance in oocytes two phosphorylation mutants were compared: $Vpu_{2/6}$ carrying the wild type membrane anchor, or Vpu_{R26} carrying the randomized TM sequence (Fig. 1B). $Vpu_{2/6}$ produced large currents with a characteristic slow activation (Fig. 3A, panel 1). In contrast, Vpu_{R26} was inactive and produced negligible currents (Fig. 3A, panel 2) comparable to those recorded from oocytes injected with control cRNA encoding the mutant Vpu_{DEL1} or uninjected oocytes (not shown).

To compare expression of $Vpu_{2/6}$ and Vpu_{R26} proteins, lysates of oocytes evaluated by voltage clamp (Fig. 3A) were subjected to immunoblotting (Fig. 3B). The extent of expression of the inactive randomized mutant Vpu_{R26} was approximately ten times higher than that of the active protein $Vpu_{2/6}$. These results, therefore, suggest that the conductance induced by $Vpu_{2/6}$ does not to arise from unspecific activation of endogenous oocyte channels consequent to the overexpression of a heterologous membrane protein [25,26].

3.5. The TM domain of Vpu forms ion channels in lipid bilayers

To identify the ion channel-forming motif, the activity of Vpu-specific peptides (Fig. 1A) reconstituted in lipid bilayers was investigated. As shown in Fig. 4, Vpu^{1-27}, a synthetic peptide comprising the entire Vpu TM domain, forms ion conductive channels in bilayers. The membrane current, in response to a constant applied voltage of 100 mV, fluctuated in discrete square steps, corresponding to the opening and closing of individual channels. Conductance events were heterogeneous in both amplitude and channel open and closed times. The most frequent openings had single channel conductances (γ) of 48 ± 2 pS and 12 ± 2 pS ($n=17$) in symmetric 0.5 M NaCl (Fig. 4A), and 28 ± 2 pS and 61 ± 3 pS ($n=37$) in 0.5 M KCl (Fig. 4B). The corresponding current histograms for the primary conductances are illustrated in Fig. 4C for γ = 48 pS in NaCl, and in Fig. 2D for γ = 28 pS in KCl. Discrete openings with smaller or larger conductances were also discerned: in NaCl, distinct openings with γ = 38 ± 2 pS and γ = 18 ± 2 pS were interspersed among the primary conductances (Fig. 4A), whereas in KCl, such openings displayed conductances of 10 ± 2 pS, 20 ± 2 pS, and 37 ± 2 pS (Fig.

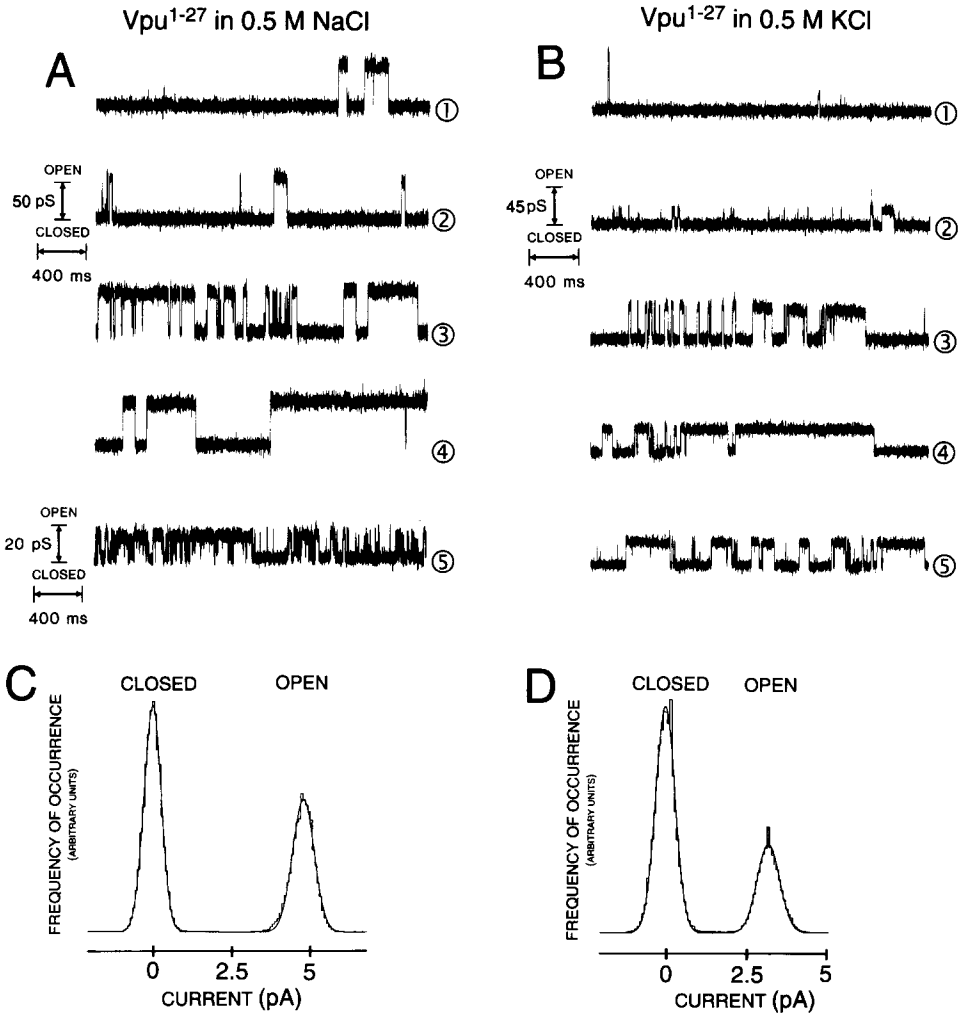

Fig. 4. Single channel currents from POPE/POPC bilayers containing the synthetic peptide Vpu^{1-27}. Currents were recorded at V = 100 mV in symmetric 0.5 M NaCl (A) or KCl (B), 1 mM $CaCl_2$, 5 mM HEPES pH 7.4. The final peptide concentrations were 10 μM (A) and 30 μM (B). In A, a segment of a continuous recording illustrates the occurrence of the 48 pS channel (traces 1–4) and the 12 pS channel (trace 5). In B, a segment of a continuous recording displays, from top to bottom, the occurrence of the 61 pS and 17 pS channels (trace 1), 17 pS channels (trace 2), 37 pS channel (trace 3), and 28 pS channel (traces 4 and 5). Current histograms for the primary conductances, generated from continuous segments of recordings lasting several minutes, are displayed in C for NaCl and in D for KCl. CLOSED and OPEN denote closed and open states. The probability of the channel being open, P_o, or closed, P_c, is calculated from the area under the corresponding Gaussian curve [19,20]. In NaCl, $P_o = 0.42$; in KCl, $P_o = 0.30$. The single channel current is calculated as the difference between the peaks associated with the closed and open states. The corresponding values for single channel conductances are 48 pS (NaCl) and 28 pS (KCl).

4B). Their occurrence was sporadic and the channel open times of the larger conductance events were significantly shorter than those of the primary conductances. In NaCl, both open (τ_o) and the closed (τ_c) times for the 48 pS channels are well fitted by a single exponential: $\tau_o = 5.1 \pm 0.5$ ms; $\tau_c = 14.4 \pm 2.0$ ms ($n = 3$). In contrast, in KCl the channels appear in bursts of activity: both open and closed times are best fitted by the sum of two exponentials with values for the 28 pS channels of $\tau_{o1} = 1.2 \pm 0.1$ ms and $\tau_{o2} = 27 \pm 2.0$ ms, and $\tau_{c1} = 2.9 \pm 0.4$ ms and $\tau_{c2} = 70 \pm 5$ ($n = 3$ experiments). The residence time of the channels in the open state was longer in NaCl than in KCl, as reflected in the conductance histograms (Fig. 4C,D). This result is representative for other channel conductances and tends to produce recordings in NaCl that appear more active than in KCl. These findings may arise from different structure or stability of the conductive oligomers of Vpu^{1-27} in Na^+ or K^+. The heterogeneity of channels recorded with Vpu^{1-27} presumably results from the self-assembly of non-covalently bonded oligomers, the conductance of which increases with the number of subunits [21]. These features of the Vpu TM channel are characteristic of amphipathic ion channel-forming peptides [21,27].

3.6. *The ion channel activity of the Vpu TM domain is sequence specific*

To evaluate the sequence specificity of the Vpu TM domain for its ability to form ion channels in bilayers, we designed a control peptide, Vpu^{rd} (Fig. 1A). Vpu^{rd} contains a scrambled amino acid sequence but the same amino acid composition as the wild type peptide Vpu^{1-27} (Fig. 1A). A predicted structural similarity between peptides Vpu^{1-27} and Vpu^{rd} was confirmed by circular dichroism (CD) spectroscopy ([8]; V. Wray, personal communication). As shown in Fig. 5A, well defined channel events were recorded for the wild type peptide Vpu^{1-27}. In contrast, peptide Vpu^{rd} did not form ion channels (Fig. 5B). The peptide incorporated into bilayers, as evidenced

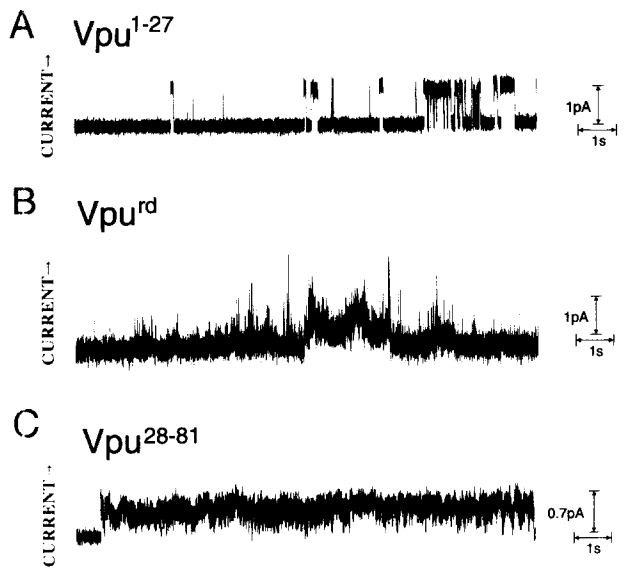

Fig. 5. The ion channel activity of the Vpu TM domain is sequence specific. Membrane currents recorded from lipid bilayers containing reconstituted peptides Vpu^{1-27} (A), Vpu^{rd} (B), or Vpu^{28-81} (C). The final peptide concentrations were 4.4 μM (A), 67 μM (B), and 15 μM (C). Currents were recorded at 100 mV in symmetric 0.5 M KCl, 1 mM $CaCl_2$, 5 mM HEPES, pH 7.4. The traces are representative segments of 12 s duration from continuous recordings lasting several minutes.

by erratic fluctuations in membrane current with variable amplitudes reflecting transient disruptions of the membrane core structure. However, no discrete conducting events were recorded ($n = 11$ experiments). Such disruptive activity consequent to the interaction of peptides with the membrane interface is indicative of surface activity and does not reflect the formation of ionic channels [19,27].

To assess the potential contribution of the cytoplasmic domain of Vpu to the ion channel-forming activity, the peptide Vpu^{28-81}, comprising the entire cytoplasmic domain (Fig. 1A) [11], was reconstituted in bilayers. As shown in Fig. 5C, peptide Vpu^{28-81} caused irregular stray fluctuations in membrane current but did not form discrete channels. Thus, the ion channel activity of the Vpu TM domain is specific for Vpu^{1-27} and is determined by the amino acid sequence of the Vpu membrane anchor.

4. Discussion

The present work aimed at the characterization of the molecular mechanism underlying the Vpu-facilitated virus release. The combined results obtained by two independent approaches, voltage clamp analyses in oocytes and single channel recordings in lipid bilayers, suggest that Vpu has ion channel activity which is linked to the structure of its TM domain. The results from voltage clamp analyses in oocytes expressing full-length Vpu (Figs. 2 and 3) are consistent with the planar lipid bilayer results (Figs. 4 and 5) and demonstrate that full-length $Vpu_{2/6}$, carrying a wild type TM domain but not a mutant, Vpu_{R26}, containing a scrambled TM anchor [8] increases membrane conductance. A peptide, Vpu^{1-27}, corresponding to the wild type Vpu TM domain reproducibly exhibited ion channel activity after reconstitution in lipid bilayers, while Vpu^{rd} containing a randomized sequence or a control peptide, Vpu^{28-81}, corresponding to the Vpu cytoplasmic domain consistently scored negative in such experiments. Together, these results support the notion that full-length Vpu has the inherent ability to form ion channels and is thus consistent with a recent report [13].

There is a remarkable correlation between the ability of Vpu to form ion channels and the capacity of this protein to enhance virus release. We reported that randomization of the TM sequence abrogated Vpu's ability to regulate virus release but not to induce CD4 degradation [8]. Based on these results we concluded that virus release is regulated by an activity of the Vpu TM domain while CD4 degradation is catalyzed by the Vpu cytoplasmic domain [8]. The Vpu TM mutants used in this earlier study are, in their transmembrane sequences, identical to Vpu_{R26} employed here in the oocytes studies. Similarly, the sequence of the peptide Vpu^{rd} used for the lipid bilayer studies corresponds to the TM sequences of these mutants [8]. We also established that wild type Vpu and Vpu_{RD} have similar protein stabilities and membrane topologies in HeLa cells [8], and that lack of phosphorylation does not abolish Vpu-mediated enhancement of virus release [7]. Last but not least, Vpu_{R26} is capable of forming homo-oligomers and exhibits similar subcellular distribution than wild type Vpu [8]. Thus, the principal difference between wild type Vpu and Vpu_{R26} with respect to their biochemical characteristics is their ability or inability, respectively, to form ion channels. It is thus likely that the observed loss of the virus release function of Vpu following randomization of the TM sequence is functionally related to the loss of ion channel activity.

How such a proposed ion channel activity of Vpu could actually modulate virus secretion is currently unclear. There is precedence that processes involving membrane fusion can be regulated by ion channel activities [28,29]: synexin, which forms a Ca^{2+}-sensitive channel, is involved in the fusion of synaptic vesicles and mediates the direct fusion of liposomes. The M2 protein of Influenza A viruses, which is structurally similar to Vpu [3,11,30–32], forms ion channels [24] and was implicated in regulating release of influenza A viruses [33], presumably by modulation the pH within the trans-Golgi network [33,34]. Amantadine blockade of the M2 channel converts HA1/HA2 to the low-pH form thereby inhibiting virus release [24,33,34]. While the parallels between M2 and Vpu on virus release are provocative, there is no evidence for similar mechanisms, because the Vpu-mediated particle release is largely independent of the Env glycoprotein [7,35]. It is conceivable that the channel activity of Vpu may regulate the transport and/or activity of hitherto unidentified cellular factors required for efficient virus secretion.

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