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ORIGINAL PAPER

Acetyl-[Asn^{30},Tyr^{32}]-calcitonin fragment 8-32 forms channels in phospholipid planar lipid membranes

Daniela Meleleo · Enrico Gallucci · Vittorio Picciarelli · Silvia Micelli

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Abstract The N-terminally truncated derivative of salmon calcitonin (sCt) (acetyl-[Asn^{30},Tyr^{32}]-calcitonin fragment 8-32) (AC 187) lacks hormonal activity and is a potent and selective antagonist of the hormone and amylin receptor. It was investigated for its capability to interact and form channels in palmitoleoylphosphatidylcholine: dioleoylphosphatidylglycerol planar lipid membranes. Interestingly, AC 187 exhibits channel activity, whose parameters, i.e., central conductance (Λ_c), occurrence (number of channels/min), voltage-dependence and lifetime, are similar to those found for sCt although, in the same experimental conditions, it takes longer to incorporate into the membrane than sCt. This channel activity can be modulated by changing either the holding potential or the pH of the medium, or by adding picomolar concentrations of SDS. One evident difference between the two peptides is that sCt is unselective (1.03) while AC 187 displays a cationic selectivity (P_{K^+}/P_{Cl^-} = 2.7) at pH 7, increasing to 3.87 when the pH drops to 3.8. The present findings indicate that the 1-7 disulfide bridge is sufficient but not necessary for membrane interaction, in accordance with the observation reported on the interaction with membrane receptors. Furthermore, the remarkable pH dependence of the cationic channel could be taken into consideration for full biotechnological study.

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D. Meleleo · E. Gallucci · S. Micelli (✉)
Dipartimento Farmaco-Biologico,
Università degli Studi di Bari, 70126 Bari, Italy
e-mail: micelli@farmbiol.uniba.it

V. Picciarelli
Dipartimento Interateneo di Fisica,
Università degli Studi di Bari, 70126 Bari, Italy

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Introduction

The regulation of several biological processes, such as polypeptide translocation, the antimicrobial action of peptides, the insertion and folding of proteins and fibrillation properties, depends on peptide structure and peptide–lipid interaction. Furthermore, conformational studies on peptides demonstrate that a peptide segment inserts into a membrane when it reaches a threshold hydrophobicity necessary to associate it stably with the membrane surface and to assume an α-helix conformation (Deber and Li 1995). This process has demonstrated that anionic lipids, which make up about 20% of all biological membrane lipids, provide an electrostatic attraction for binding proteins/peptides into membranes.

In particular, the membrane surface imposes a new orientation and conformation to peptide hormones, thereby facilitating incorporation into the membrane, and later enhancing the interaction between the hormone and the receptor; the structural and energetic features of this interaction are reminiscent of the catalysis of bimolecular chemical reactions by detergent micelles (Sargent and Schwyzer 1986).

Calcitonin is a 32-aminoacid-long peptide present in all vertebrates whose structure consists of an N-terminal disulfide bridge and a C-terminal prolineamide residue. Calcitonin is implicated in bone remodelling and acts through specific receptors, a member of the seven transmembrane receptor family, present in large numbers in osteoclasts (Chambers and Magnus 1982; Nicholson et al. 1986). It is therefore used for the treatment of bone

disorders such as Paget's disease, osteoporosis and hypercalcemia of malignancy. Fish calcitonin, such as salmon (sCt) and eel, differing in their structure by only three aminoacids, were about 40-fold more potent in their hypocalcemic action than mammalian type (human and porcine) (Azria 1989; Kapurniato and Taylor 1995; MacIntyre et al. 1967, 1988). Moreover, human calcitonin (hCt) has an enhanced potency, comparable to that of sCt, when fibrillation is avoided (Cudd et al. 1995; Micelli et al. 2004, 2006). Secondary and tertiary structural requirements are necessary for calcitonin to bind to the receptor; in fact, the helical portion of sCt (residues 8-22) is important for binding, whereas residues 2-6 are important for receptor activation of the transmembrane loop region, required for adenylate cyclase activation (Stroop et al. 1996). Truncated sCt 8-32 and AC 187 are considered potent and selective antagonists of amylin-induced hyperglycemia and hyperlactemia: AC 187 has been used to demonstrate that amylin receptors mediate the anorectic action of sCt (Silvestre et al. 1996; Lutz et al. 2000) and of the wild type hormone because they stabilize the receptors in an inactive state (Beaumont et al. 1995a, b; Pozvek et al. 1997; Disa et al. 1998; Hilton et al. 2000; Pham et al. 2005; Young et al. 1994; Young 1995; Harris et al. 1997). Truncated hCt 9-32 has been used in therapy in a spray formulation due to its cell-penetrating efficacy (Schmidt et al. 1998); furthermore, this fragment retains non-receptor-mediated carrier properties (Machova et al. 2002; Krauss et al. 2003, 2004, Boichot et al. 2004) and was used to internalize the green fluorescent protein, drugs and plasmid DNA. Cell-penetrating peptides represent a tool for polar molecules to overcome the hydrophobic plasma membrane barrier, in that their permeation could be facilitated by peptide cell penetration. The mechanism of the membrane-crossing process for hCt fragment 9-32 remains controversial. Many peptides require an α-helical conformation to penetrate the membrane. Furthermore, peptides with a β-sheet structure such as hCt and AβP undergo self-association to form fibrils.

The rationale of the present study was to evaluate: (a) the role played by the 1-7 disulfide bridge and the substitution of glycine^{30} for asparagine^{30} and of proline^{32} for thyrosine^{32} in the C-terminus of the sCt, which increases the hydrophobic nature of the molecule, in the incorporation and channel formation in planar lipid membranes (PLM) made up of palmitoleoylphosphatidylcholine (POPC): dioleoylphosphatidylglycerol (DOPG) (85:15, w:w). It has been shown that this membrane is suitable for Cts and their derivatives to incorporate, due to the presence of negatively charged DOPG which triggers interaction by an electrostatic driving force (Bradshaw 1997; Stipani et al. 2001; Wagner et al. 2004; Micelli et al. 2004, 2006; Meleleo et al. 2006; Herbig et al. 2005); (b) the influence of low pH as well as the effect of pre-incubating AC 187 with picomolar concentrations of SDS to enhance the affinity of the peptide toward the phospholipid bilayer, in order to facilitate channel formation, as previously found for hCt (Micelli et al. 2004). By comparison we also reported data on sCt channel incorporated in lipid membrane in the same experimental conditions. We demonstrate that AC 187 is able to interact and form channel in POPC:DOPG membrane that are not different in properties, such as conductance and voltage-dependence, from those found for sCt, demonstrating that the 1-7 disulfide bridge is sufficient but not necessary for interaction with the membrane to take place. Interestingly, the AC 187 channel could be considered a new weakly/strongly (pH = 7/3.8) programmable cationic channel.

Materials and methods

Chemicals

Salmon calcitonin, acetyl-[Asn^{30},Tyr^{32}]-calcitonin fragment 8-32 (AC 187) and SDS were purchased from Sigma (Munich, Germany) POPC and DOPG were purchased from Avanti Polar Lipids (Alabaster, AL, USA), KCl was from Merck (Analytical grade, Darmstadt, Germany) and bidistilled water was used. The salts used in the experiments were of analytical grade. The aqueous solutions were used unbuffered and had a pH of either 7 or 3.8.

Planar lipid membrane experiments

Planar lipid membranes were formed as described previously (Stipani et al. 2001) from a solution of POPC:DOPG (85:15, w:w) in 1% of *n*-decane across the hole (200 μm of diameter) of a teflon set dividing two 4 ml teflon chambers. After the membrane turned black, sCt or AC 187 or AC 187 incubated with SDS was added on the *cis*-side to a final concentration of 49 nM. Before addition, the basic conductance and capacitance of the bilayer was monitored for a long period. Both conductance and capacitance never exceeded 10 pS and 0.30 μF/cm^2 (up to a voltage of ±100 mV), respectively, and showed no channel-like activity.

Recording equipment

Voltage clamp conditions were employed, and contact with the aqueous phases was made using Ag/AgCl electrodes. Membrane formation was monitored through membrane capacitance and resistance. Membrane capacitance and single-channel recordings were monitored by means of a storage oscilloscope and a strip chart recorder. The single-

channel instrumentation had a time resolution of 1–10 ms depending on the magnitude of the single-channel conductance. The polarity of the voltage was defined with respect to the side where the Ct was added (the *cis*-side). A trans-negative potential (indicated by a minus sign) means that a negative potential was applied to the *trans*-side, the compartment opposite the one where Ct was added. Data were analyzed by hand. The single-channel data were obtained from at least three experiments (the tables report the number of individual channels—openings and closings—performed on different days). All single-channel events (more than 100 single-channel events for each experiment) were used to calculate the event amplitudes, irrespective of duration. A histogram of amplitude distribution for each experiment was constructed and fitted by a Gaussian distribution function (GraphPad Prism™ version 3.0; GraphPad Software, Inc., http://www.graphpad.com). Results are expressed as mean ± SE.

Selectivity was calculated from the reversal potential (ψ), determined in a 1/0.75(1/1.33 M) gradient of KCl at pH 7 (pH 3.8), by using the following equation:

$$\Psi = (RT/F) \times \ln\{(P_K[K]_t + P_{Cl}[Cl]_c)/(P_K[K]_c + P_{Cl}[Cl]_t)\} \quad (1)$$

where $[X]_t$ and $[X]_c$ are the concentrations of the ion species X in the *trans* and *cis* compartments, respectively; R, T and F have their usual meanings.

The average lifetime of the conductance unit was estimated by the formula:

$$N = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \quad (2)$$

where N is the number of channels that remain open for a time equal to or greater than a certain time t, and A_1 and A_2 are the zero time amplitudes. The τ_1 and τ_2 parameters are related to the fast and slow components of the time constant. The single-exponential distribution is included in the formula ($A_2 = 0$). In order to choose between the two models, we performed an appropriate statistical test (F-test GraphPad Prism 3).

Results

Electrical changes observed upon incorporation of AC 187 into POPC:DOPG planar lipid bilayer: comparison with sCt incorporation

The ability of AC 187 to induce ion conductance was tested in planar lipid bilayers at pH 7 and compared to that of sCt in the same experimental conditions. Insertion of the AC 187 molecule into the PLM about 100 min after addition on the *cis*-side of the medium causes a channel-like activity, i.e., a sudden appearance of discrete current fluctuations when a constant voltage of +80 mV was applied across the membrane (Fig. 1a). At lower potential, no significant changes in membrane conductance could be recorded compared with basal conditions (bare membrane). After channel activation, the applied voltage could be changed and the channel amplitude variation as a function of applied voltage could be monitored.

To exclude any unspecific artifact responsible for conductance variation, we performed experiments in which the peptide was pre-incubated with trypsin before it was added to the salt solution on one side of the membrane. In this case, we observed only an insignificant increase in membrane-specific conductance within 300 min.

In the same experimental conditions as AC 187, sCt addition caused the appearance of channel activity after

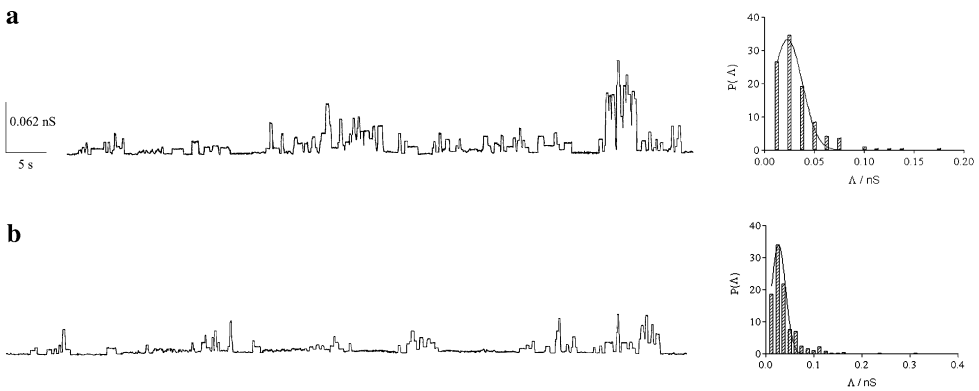

Fig. 1 Examples of chart recordings with associated amplitude histograms of AC 187 (**a**) and sCt (**b**) channel formation in POPC:DOPG PLMs. Each *trace* represents a fragment from the recording of the activity obtained in individual experiments. Experimental conditions: KCl 1 M, voltage 80 mV, pH = 7, T = 23 ± 1°C and Cts 49 nM present on the *cis*-side of the membrane. The histogram of the probability, $P(\Lambda)$, for the occurrence of a given conductivity unit was fitted by a Gaussian, which is shown as a *solid curve*

about 30 min, and this continued when the applied voltage was varied (Fig. 1b).

Figure 2 shows AC 187 channel activity at different applied voltages. The traces present transitions between open (conducting) and closed (non-conducting) states. The ion-channel activity is characterized by regular and discrete current steps of uniform amplitude and relatively long residence times (of the order of seconds) at all applied voltages. Openings are dominant over closings. For several current traces at different applied voltages, amplitude histograms were obtained which allowed us to estimate the mean conductance value in 1 M KCl. A summary of the single-channel central conductances and life times at all positive and negative applied voltages investigated in this study is given in Tables 1 and 2, respectively. Irrespective of the presence of one or two time constants, these high values agree with a good incorporation of peptides into the membrane and with their ability to form stable ion channels. In addition, the dispersion of the data collected could exclude the hypothesis that channel life time shows a voltage-dependence mechanism.

The occurrence frequency, i.e., the mean number of openings in a period of 1 min, obtained from the total number of records, as a function of the applied voltage, is reported in Fig. 3.

The most distinctive feature of the AC 187 channel is the slow frequency of current transition between the open and the closed states compared with sCt at all applied voltages (Fig. 3 a, b). Furthermore, it is worth mentioning that at an applied voltage of 80 mV, the channel activity of sCt first underwent the usual alternating period of opening and closure of channels, and then became convulsive and had a tendency for instability that often causes the membrane to break. This is indicative of high activity, although it is impossible to evaluate a true occurrence frequency. On the other hand, it should be noted that for AC 187 (pH = 7; pH = 3.8) and for AC 187-SDS (pH = 7), the occurrence frequency increased as the applied voltage at the membrane increased (Fig. 3b). This effect is more regular in the range of positive applied voltages.

One interesting result is that the single-channel conductance is inversely correlated with membrane voltage in both sCt and AC 187 (Table 1). We obtained almost symmetrical curves for trans-positive and trans-negative potentials (Fig. 4) for both peptides. This probably means that, in common with other Cts, AC 187 also has no dipole moment (Stipani et al. 2001; Micelli et al. 2004, 2006; Meleleo et al. 2006). Conductance was a non-linear function of voltage and was fitted by means of a one exponential equation:

$$Y = Ae^{(-kx)} + p \tag{3}$$

in which the parameter k can give an indication of the charges involved in the formation of the sCt and AC 187 channels.

Ion selectivity

The ion selectivity of the pore formed by AC 187 was investigated by establishing a KCl gradient across the

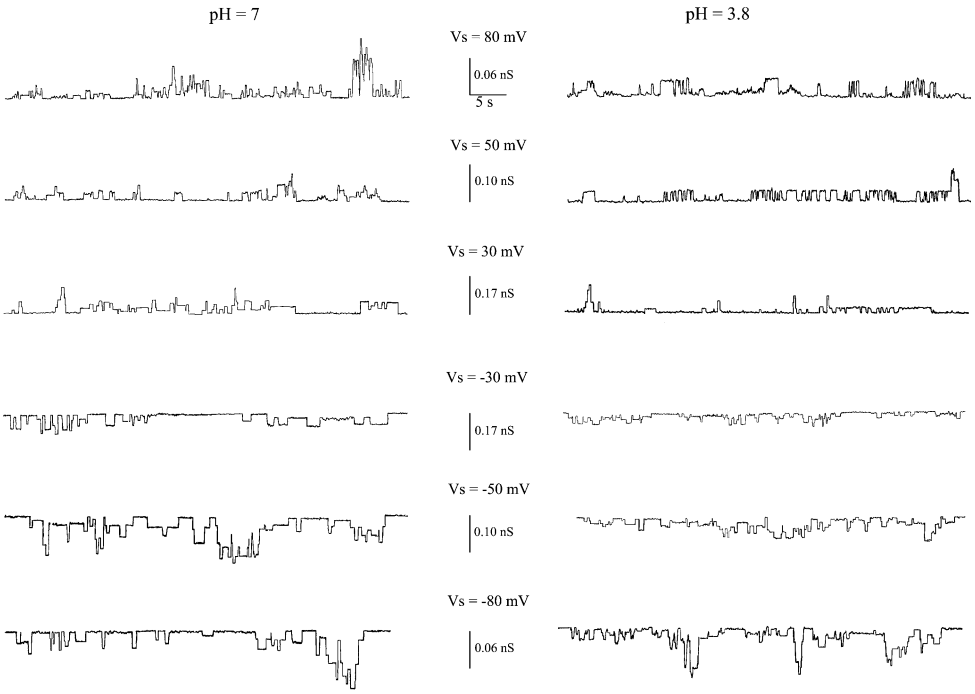

Fig. 2 Examples of chart recordings of AC 187 channel formation in POPC:DOPG PLMs at different medium pH values (pH = 7 or 3.8, respectively) and applied voltages. Each *trace* represents a fragment from the recording of the activity obtained in individual experiments. Experimental conditions: KCl 1 M, T = 23 ± 1°C, AC 187 (49 nM) was present on the *cis*-side of the membrane

Table 1 Values of central conductance for single channels of AC 187 in POPC:DOPG membrane under different experimental conditions

V (mV)	sCt (pH = 7) Λ_c(nS) ± SE $342 < N_t < 946$	AC 187 (pH = 7) Λ_c(nS) ± SE $209 < N_t < 507$	AC 187 (pH = 3.8) Λ_c (nS) ± SE $173 < N_t < 369$	AC 187-SDS Λ_c(nS) ± SE $185 < N_t < 472$
80	0.027 ± 0.001	0.023 ± 0.001	0.041 ± 0.001	0.025 ± 0.001
50	0.042 ± 0.001	0.043 ± 0.002	0.057 ± 0.001	0.040 ± 0.001
30	0.062 ± 0.002	0.054 ± 0.001	0.056 ± 0.002	0.051 ± 0.004
–30	0.059 ± 0.002	0.057 ± 0.001	0.058 ± 0.002	0.067 ± 0.001
–50	0.038 ± 0.001	0.040 ± 0.001	0.042 ± 0.001	0.045 ± 0.001
–80	0.025 ± 0.001	0.023 ± 0.001	0.025 ± 0.000	0.027 ± 0.001

For comparison, the sCt single-channel parameters are also reported. Λ_c is the central conductance fitted by a Gaussian distribution (nS). For each series of experiments, the mean values ± the standard error of central conductance, the minimum and maximum number of channels considered (N_t) are reported

Table 2 The lifetimes of the single-channel for AC 187 in POPC:DOPG membrane under different experimental conditions

V (mV)	sCt (pH = 7)		AC 187 (pH = 7)		AC 187 (pH = 3.8)		AC 187–SDS	
	τ_1(s)	τ_2 (s)	τ_1(s)	τ_2 (s)	τ_1(s)	τ_2 (s)	τ_1(s)	τ_2 (s)
80	0.1	2.6	0.1	2.7	1.2		0.1	2.3
50	0.5	10.1	0.2	3.4	1.2		0.1	2.4
30	1.4	6.3	1.8		2.3		2.2	
–30	0.9	5.8	3.6		0.1	2.4	1.7	
–50	1.7		2.7		0.2	4.3	0.1	2.9
–80	2.0		1.0	6.6	1.6		0.1	1.9

For comparison the sCt single-channel lifetimes are also reported. For the minimum and maximum number of channels considered (N_t) see Table 1

membrane and measuring the open-circuit voltage (reversal potential) across the bilayers. After the increase in membrane conductance induced by addition of the peptide, under stirring, a KCl gradient was established between the *cis*- and *trans*-sides, while keeping the solution level constant, so as to preserve membrane integrity. The reversal potential under conditions of KCl gradient of 1.0/0.75 M (*cis/trans*, pH 7) was –4.74 mV, revealing selectivity for K^+ over Cl^- of 2.70.

Effect of pH-variation and of nanomolar concentrations of sodium dodecyl sulfate on incorporation and channel formation

AC 187 was tested for the effects of low pH on its pore-forming properties. When AC 187 (49 nM) was added on the *cis*-side of the bathing medium containing 1 M KCl at pH 3.8, incorporation of peptides into lipid bilayers occurred in about 17 min and gave rise to discrete, square current events that fluctuate between conductive and non-conductive states (Fig. 2). In this case, a transmembrane voltage of 50 mV was sufficient to initiate the process. Almost the same channel conductance, frequency and conductance-voltage characteristics were registered as at pH 7 (Table 1 and Fig. 3b). The only variation found regards the channel selectivity. In fact, the reversal potential under conditions of KCl gradient of 1.33/1.0 M (*cis/trans*, pH 3.8) was –4.3 mV, revealing selectivity for K^+ over Cl^- of 3.87.

In another series of experiments, AC 187 was incubated with picomolar concentrations of SDS for 20 min, and then added to the bathing solution of the PLM made up of 1 M KCl at pH 7. After about 30 min, channel-like activity was observed. Again, the characteristics of the channels seem to be unmodified.

Discussion

The aim of this research was to obtain insights into the role of peptide modification and its structural requirements for interaction with phospholipid membranes and peptide–peptide related to channel formation. To understand the role played by the N-terminal of sCt in the mechanism of interaction and translocation across the lipid membrane, we studied the interaction of AC 187 with POPC:DOPG (85:15, w:w).

Among the various calcitonins, sCt has been found to be the most biologically active. The hypocalcemic effect of all calcitonins has been correlated with the amphipathic structure of the molecule which drives the interaction with the membrane and/or receptor deeply located in the membrane. Stroop et al. (1996) showed that the helical structure binds to the receptor, whereas the N-terminal part is considered essential for receptor activation.

Silverman (1997) has demonstrated that hCt can penetrate the respiratory nasal epithelium, and a non-receptor-mediated internalization was proposed for the truncated 9-32 hCt (Schmidt et al. 1998). Two more aspects concerning the internalization of the C-terminal

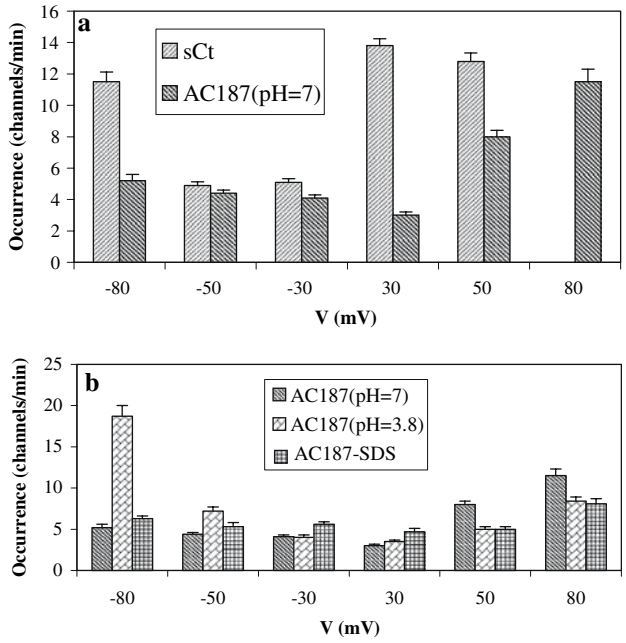

Fig. 3 The occurrence frequency as a function of applied voltage for sCt and AC 187 channels in POPC:DOPG PLMs at pH 7 (**a**), and for AC 187 channels at pH = 7, pH = 3.8 and AC 187 incubated with picomolar concentrations of SDS (**b**). The data points were the mean occurrence frequency ± SE

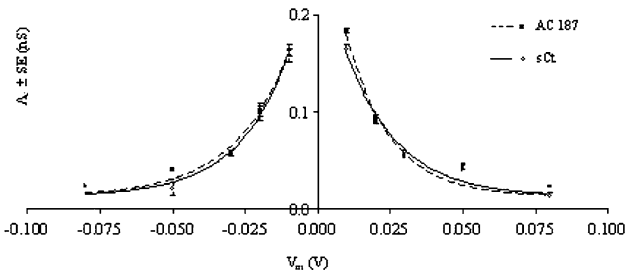

Fig. 4 Conductance–voltage relationship for AC 187 and sCt channels in POPC:DOPG PLMs at pH 7. The data points were the central conductance ± SE obtained from conductance histograms. The *curves* superimposed on the data are the results of the fit with the model: $\lambda_c = Ae^{-KV_m} + p$, where A is the difference between the conductance at $V_m = 0$ and at V_m = membrane black (p = 0.0125 nS); K is the constant correlated with the gating charge n (n = KRT/F). For the POPC:DOPG membrane, AC 187/sCt: $A = 0.33 \pm 0.05/0.24 \pm 0.029$(nS); $K = 66.72 \pm 11.33/50.54 \pm 7.12$($V^{-1}$); R^2 = 0.97/0.98, at positive applied voltage; $A = 0.25 \pm 0.02/0.24 \pm 0.02$(nS); $K = -52.40 \pm 5.93$/–49.84 ± 5.08(V^{-1}); R^2 = 0.98/0.99, at negative applied voltage. Experimental conditions: KCl 1 M, AC 187 49 nM was present on the *cis*-side of the membrane

fragment of hCt are the transport of fluorescent protein into bovine nasal mucosa (Machova et al. 2002) and the formation of channels through lipid bilayers (Harris et al. 1997; Stipani et al. 2001; Micelli et al. 2004, 2006; Meleleo et al. 2006).

Our results have shown that AC 187 shows a longer latency in incorporation as compared to sCt, this means that the 1-7 disulfide bridge is necessary for easier insertion into the lipid bilayer to take place (Stipani et al. 2001; Micelli et al. 2004, 2006; Meleleo et al. 2006)—this first step could be a prerequisite for interaction with the membrane receptor, considering that membrane lipids are far more concentrated than receptors which lie deep within the membrane. It is worth mentioning that hCt fragment 9-32 required a peptide concentration higher than CMC (CMC = 2.5×10^{-7} mol/l) for spontaneous insertion into the lipid bilayer (Wagner et al. 2004). The interaction may take place when the hydrophobic surface of the peptide is exposed to the membrane surface, stimulating a conformational change in the peptide and making it suitable for incorporation. This longer lag time is probably necessary to render the AC 187 conformation competent with the membrane. One positive aspect of AC 187 lies in its propensity to be activated by the potential, by picomolar concentrations of SDS, known α-helical inductor, or by acidic pH, that determines the protonation–deprotonation state of some aminoacids of peptide. Its channel properties, such as conductance and voltage-dependence, are no different from those found for sCt, except for its cation selectivity at pH 7.

The structure of sCt seems to be important as it contains flexible loops connecting the head and the helix and the helix and the tail region (Amodeo et al. 1999). In the case of AC 187, a loop connection is missing and the α-helix portion (from 8-22) is shorter because it lacks the two Cys1-Cys7 residues. The shorter helix could be responsible for the delay in peptide incorporation. This is in line with the requirement that peptides need a high conformational flexibility to penetrate membranes (Davies et al. 1998).

One clear difference between sCt and AC 187 is that the hormone is unselective (Stipani et al. 2001) and the truncated Ct displays a weakly cationic selectivity at pH 7. The fact that cation selectivity increases with a decrease in medium pH deserves particular consideration. This finding seems paradoxical, considering that low pH protonates the negative charges of glutamate, leaving the positive charges unaffected. It is worth considering that these characteristics have been found for other positively-charged peptides, such as the sodium channel polypeptide, magainin 2 (Gallucci et al. 2003). This result raises questions about the structure of the pore and makes the correlation between peptide charge and channel selectivity highly critical. The interesting finding emerging is that the protonation of a single glutamate aminoacid moves selectivity dramatically toward the cation (Kaneez and White 2004).

Since AC 187 has 24 aminoacid residues, its length would be expected to be 36 Å (1.5 Å per residue), i.e., long enough to span the PLM; moreover, the helical portion is short enough to provide a hydrophilic pathway. It thus appears obvious that such a large pore cannot be formed by one molecule; it must be rather assumed that an aggregate of peptide molecules is necessary to establish a hydrophilic pathway.

It is worth recalling that transmission electron microscopy has demonstrated that sCt forms annular oligomers compatible with a pore like structure (Diociaiuti et al. 2006). The N-terminally truncated derivative of sCt, lacking hormonal activity, is an antagonist of the hormone and amylin receptor and in the light of the present results is a novel weakly/strongly (pH = 7/3.8) cationic peptide able to interact and form channels in POPC:DOPG PLMs in a programmable manner. This latter aspect could be useful in exploring whether the peptide could have therapeutical interest.

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