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Effect of pH-variation on insertion and ion channel formation of human calcitonin into planar lipid bilayers

Silvia Micelli [1], Daniela Meleleo [1], Vittorio Picciarelli [2] and Enrico Gallucci [1]

[1] Dept. Farmaco-Biologico, and [2] Dept. Interateneo di Fisica, Università degli Studi di Bari, I-70126 Bari, Italy

TABLE OF CONTENTS

1. ABSTRACT

Human calcitonin is the physiological hormone involved in calcium-phosphorus homeostasis. However, its use is limited by its propensity to form aggregates. We find that the type of host lipid has a pronounced influence on human calcitonin fibrillation or incorporation, as assessed by channel formation, in planar lipid membranes at neutral pH. At pH 7, human calcitonin is able to interact and form channels with negatively charged dioleoyl-phosphatidylglycerol (DOPG) bilayers and with zwitterionic palmitoyl-oleoyl phosphatidylcholine (POPC) bilayers containing 15% negatively-charged DOPG, but not with POPC bilayers. At low pH (4.5 and 3.8), the conformational variation of the peptide enables it to insert into POPC and POPC:DOPG but not into DOPG bilayers. The model proposed for human calcitonin interaction and channel formation at acidic pH was based on theoretical predictions of the protonation-deprotonation state of some aminoacids, in particular in the fibrillating sequence of peptide molecules; the length of the α-helix, and the electrostatic and/or hydrophobic interaction also seem to be relevant. These results may suggest that human calcitonin at low local pH could be involved in osteoclasts' calcium-sensitive permeability through channel formation and/or receptor interaction.

2. INTRODUCTION

Calcitonin (Ct), a peptide hormone consisting of 32 amino acid residues plays a crucial role in the calcium-phosphorus homeostasis regulation. The hormone is highly preserved among species, the thyroidal hormone only differing from the ultimobranchial by 16 aminoacids. These peptides often show a variable conformation and a high degree of flexibility (1).

Though human calcitonin (hCt) is involved in many biological functions, calcium metabolism is the most relevant clinically. An increase in calcium concentration above its physiological value triggers calcium permeability in osteoclasts by a mechanism that is still matter of debate (2). However hCt shows a tendency to form fibrils in aqueous solution, thus limiting its clinical use. Electron microscopic studies have shown that the fibrils consist of fibres of 8 nm in diameter and that they often associate with one another (3). The mechanism of fibril formation is under intensive investigation, however it is critically influenced by solid-liquid interface and is also time-, pH- and concentration-dependent (4-7). In particular, it has been shown that in acidic aqueous solutions (8) an amphiphilic α-helical structure is formed in the central region of hCt. On the other hand, local conformational transitions from α-helix

to β-sheet at the C-terminus region were simultaneously induced during fibril formation in the acetic acid solution (pH 3.3) (6). The mechanism of fibril formation seems to consist in a first step in which the nucleus of fibrils forms by a homogeneous process followed by a second step in which the fibrils mature by a heterogeneous autocatalytic process (6). Besides, fibril formation depends on pH; in fact, at neutral pH fibril formation is much faster than in an acidic medium because the hCt monomer is more stable. The fibrils formed at pH 7.5 are composed of antiparallel β-sheets, whereas the mixture of antiparallel and parallel β-sheet structures is formed at pH 3.3. A model has been put forward to explain hCt fibrillation which takes into account the protonation of Lys 18 and the deprotonation of Asp 15 at different pH values (6). Recent work indicates a critical role of residues 18 and 19 for the oligomerization state and bioactivity of hCt. The ability of the short fragment (15-19) of hCt to form fibrils was investigated and a remarkable amyloidogenic potential of this pentapeptide (9) has been demonstrated.

Many diseases depend on the folding-unfolding properties of peptides such as HIV pg 41 (10,11), influenza hemagglutinin (12,13), sperm fusion protein PH-30 (14,15), Sendai virus (16), insulin (17), Alzheimer Aβ (18), prion protein (19), cystic fibrosis (20), and the α-synuclein involved in Parkinson's disease (21,22) that show fibrillating properties. hCt shares common properties with these molecules.

In a previous study (23), we demonstrated that salmon (sCt), eel (eCt), porcine (pCt) and hCt can form voltage-dependent channels across black lipid membranes (BLMs) made up of POPC:DOPG (85:15), moreover, at high concentrations, the lag time of hCt is the longest and the channel activity the lowest among these Cts. This low activity can be counteracted by decreasing the hCt concentration in the medium or by applying a potential as high as 150 mV to the membrane or by adding nanoconcentrations of SDS (24). This could roughly suggest that hCt molecules aggregate in solution or on the surface membrane. It has been demonstrated that hCt contains titratable side chain charged aminoacids and that their charges depend on the pH values (6). In fact, at acidic pH, Asp15 is protonated and Lys18 is deprotonated unlike at pH 7. Therefore the presence of two positive charges at acidic pH could lower the aggregation process in comparison to pH 7.

The aim of this study was to evaluate the effect of pH on the incorporation as proven by single-channel activity of hCt into PLMs of different composition as mechanisms to counteract peptide fibrillation. As it is known lipids, depending on their structure can either retard or enhance the complex process of incorporation. In fact, this process is driven by both peptide and lipid conformation, in order to match the length of both lipid acyl chain and peptide hydrophobic chain (25). In this contest the above mentioned investigation was performed with three kinds of PLM, namely POPC, or a mixture of POPC and DOPG or pure DOPG. Which were used for their physico-chemical characteristics, and because: POPC is the fundamental zwitterionic phospholipid component of plasma membrane and is considered determinant for peptide fibrillation process, such as hCt and Aβ 1-40 (26,27); POPC:DOPG (85:15) is reported an important mixture for the interaction of the amphipathic peptide such as Cts (23,28), it seems that the presence of 15% of anionic phospholipid is important for peptide interaction with membrane; DOPG as negatively charged membrane is appropriate to study the effect of this interface in the hCt interaction and channel formation.

3. MATERIALS AND METHODS

3.1. Chemicals

Salts and other basic chemicals were bought from Merck (Darmstadt, FRG, analytical grade) and biochemicals from Sigma (München, FRG). POPC was purchased from Avanti Polar Lipids, (Alabaster, AL), and DOPG from Sigma (München, FRG). hCt was from Novartis Pharma AG (Basel, Switzerland).

3.2. Planar membrane experiments

A Teflon chamber was used that had two aqueous compartments connected by a small circular hole with a surface area of 0.2 mm^2. Planar lipid membranes (PLMs) were composed of POPC, or a mixture of POPC:DOPG (85:15, w/w), or DOPG in 1% of n-decane. The salts used in the experiments were of analytical grade. The salt solutions had a pH of 7 or 3.8 and the experimental temperature was about 23 °C. The membrane current was monitored with an oscilloscope and recorded on a chart recorder for data analysis by hand. The *cis* and *trans* chambers were connected to the amplifier head stage by Ag/AgCl electrodes in series with a voltage source and a highly sensitive current amplifier. The single-channel instrumentation had a time resolution of 1-10 ms, depending on the magnitude of the single-channel conductance. The *cis*-side compartment, where the human calcitonin was added, has a positive polarity. In this study, an hCt concentration of 125 nM was used, one that seems to be a fibrillating concentration (23). In fact, at the same experimental conditions, an hCt concentration of 5-24.5 nM induces a channel activity that is higher than that observed at 125 nM (24). The phenomenology of the incorporation was studied as follow:

-To define the voltage-dependence characteristics of hCt we measured the amplitude of channel events at each membrane potential in the range of + 200 mV to + 10 mV, and constructed G-V curves.

- To identify the charge on the ion carrying the current, we measured the shift in the reversal potential induced by a change from a symmetrical to an asymmetrical KCl solution system. When the membrane conductance reached a virtually stable value, after hCt addition on the *cis*-side, the salt concentration on the *cis*-side of the membrane was raised by the addition of concentrated salt solution. A concentration gradient was set, with 0.9 M on one side (*cis*) and 0.5 M on the other (*trans*). The reversal potential was determined by changing the holding

potential step by step by ± 2 mV. The permeability ratio was calculated using the following equation:

$$V= (RT/F)*\ln\{ (P_K[K]_t + P_{Cl}[Cl]_c)/ (P_K[K]_c + P_{Cl}[Cl]_t)\}$$

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.

- To define the mean channel conductance (central conductance Λ_c),the single channel data were obtained from at least three experiments with more than 150 single events for each series performed on different days. Upward current transition (event) was more frequent than terminating events. A histogram of the current 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).

- To define the channel lifetime, from records extending over prolonged periods, the channel durations were measured considering the time between the opening and closing of each channel. The average lifetime of the conductance unit was estimated by the formula:

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

where N is the number of channels that remain open for a time equal to or greater than a certain time t, A_1 and A_2 are the zero time amplitudes, and τ_1 and τ_2 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). The *t*-test was performed using the Graphpad Prism 3 software.

In all experiments performed, the conductance and capacitance of each membrane were tested by applying a voltage of ± 200 mV for 10-15 min under stirring, to ensure that the membrane was stable. In all sets of experiments, the applied voltage was usually 150 mV, in order to compare the results in membranes of different composition and at different pH values. In fact, at this potential single channel insertion into POPC:DOPG PLMs can be easily observed for the maximum hCt concentration of 125 nM (24). However, in 25% of experiments, hCt single-channels were observed at 80 mV applied voltage. After the first channel insertion into PLMs, the applied voltage could be lowered to 10mV and the voltage dependence studied at different applied voltage.

3.3. Statistics

Results are expressed as means ±SE or SD. Statistical significance was assessed using the student's *t* tests.

4. RESULTS

Experiments were performed under different conditions:

I. In the first set of experiments, hCt conductance was studied at a pH value of 7 at which the fibrillation process seems to take place easily (6).

II. In the second set of experiments, hCt conductance was studied at a pH value of 3.8 in which the protonation of Asp is about the 90%

III. In a third set of experiments, hCt conductance was studied at a pH value which correspond to the value at which osteoclasts are active against bone resorption (29,30) and the protonation of Asp is about the 75% .Moreover acidic pH favors α-helix formation in the peptide (31).

IV. In a fourth set of experiments, hCt conductance was studied at a pH value of 7 which was then lowered to pH 4.5 and/or 3.8 (by adding a small quantity of concentrated HCl solution and controlling pH during and at the end of the experiment),

V. or *vice versa,* at the beginning hCt conductance was studied at a pH value of 3.8 and then the pH was increased at pH value of 4.5 and 7 (by adding a small quantity of concentrated KOH solution and controlling pH during and at the end of the experiment). Although we observed the bilayer with added hCt for many hours during the same day, and performed experiments in different months throughout the year, no hCt-induced channels were ever observed at pH 7 in POPC membranes, or at pH 3.8 or 4.5 in DOPG membranes.

Lowering/increasing the pH to 3.8/7, through the intermediate value of 4.5, in POPC/DOPG PLMs respectively, in the presence of hCt was usually followed by rapid step-like variations in the membrane current compatible with single channels, that can be activated by applied potentials as low as 50 mV. Figure 1 shows a typical example of single-channel recordings for POPC, POPC:DOPG and DOPG bilayers, respectively, when the medium was KCl 1M, the initial pH value 7 and the applied voltage 150 mV. However, the same pattern in the recordings is obtained in the range of 10-80 mV of applied voltages. It can be observed that single-channel activity appears when a 15% of the negatively- charged phospholipid in the bilayers is added to POPC and a further increase of activity is observed when pure DOPG membranes are used. We observed alternating periods of "paroxystic" channel activity followed by quiescent periods, open times interrupted by closures, and conductance steps that were twice that of the central conductance, a clear indication that two channels were simultaneously incorporated. On the other hand, when the pH value changes to 3.8, hCt forms single channels in zwitterionic POPC or in POPC:DOPG (85:15) bilayers, but fails to form channels in DOPG PLMs (Figure 1). In this set of experiments, we observed that the periods of "paroxystic" channel activity are more frequent than at pH 7 in POPC:DOPG PLMs. These periods are followed by quiescent periods and the open time interrupted by briefer closures than at pH 7. Moreover, conductance

Table 1. The mean conductance fitted by Gaussian distribution (Λ_c), the occurrence (channels/min), the fitted lifetimes (see text) of the single-channel events (s), the activation time and the total number (N) of channels in different PLMs at different pH.

Membrane	pH	Λ_c(nS) ± SD	Occurrence ± SD	τ_1(s)	τ_2(s)	Mean activation time (min)	N° events
POPC	7.0	-----	-----	-----	-----	-----	-----
	3.8	0.015 ± 0.008	14.81 ± 0.41	1.40	-----	35	1315
POPC:DOPG	7.0	0.014 ± 0.008	3.30 ± 0.09	1.36	7.73	49	1234
	3.8	0.014 ± 0.001	10.16 ± 0.47	0.13	2.42	21	461
DOPG	7.0	0.010 ± 0.008*	25.28 ± 0.41	0.98	6.35	43	3402
	3.8	-----	-----	-----	-----	-----	-----

*P<0.0001

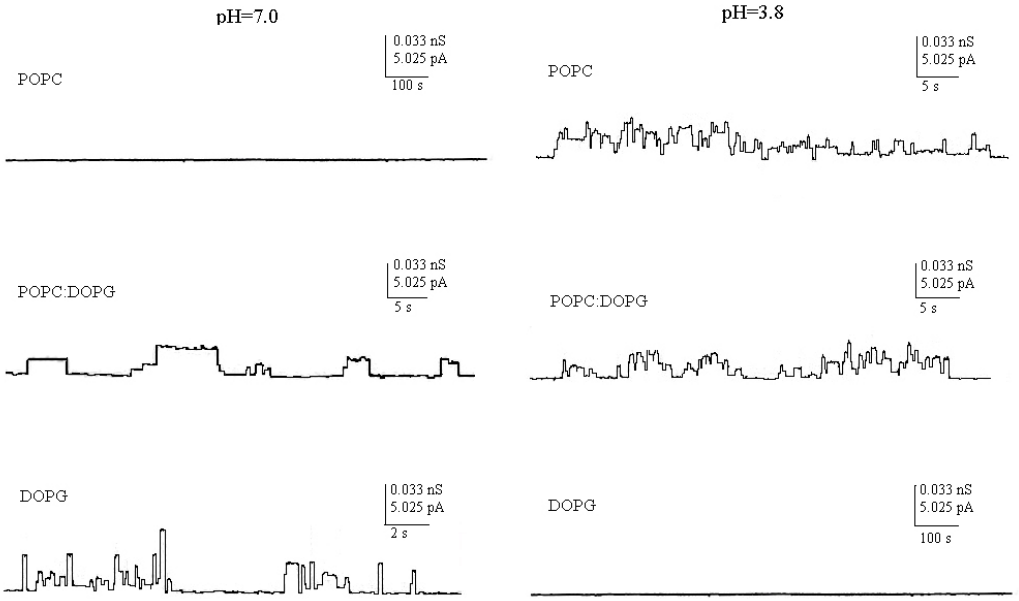

Figure 1. Recordings of transmembrane current of PLMs containing hCt channel in each PLM used at different medium pH values (pH=7 or 3.8, respectively) and 150mV applied voltage. Channel openings and closings are represented by upward and downward deflections, respectively. Each trace represents a fragment of the recording of the activity obtained in individual experiments. Experimental conditions: KCl 1M, hCt 125 nM was present on the *cis* side of the membrane.

steps were twice to three times that of the central conductance, for all PLMs. Finally single channel activity at a pH value of 3.8 is more frequent than at pH 7(see also table 1).

Figure 2 reports examples of single-channel traces for hCt in POPC bilayer at different pH and applied voltage. It can be seen that at pH 7 although a high voltage is applied no ion channel were observed; in contrast, by lowering the pH at 4.5 and subsequently at 3.8, the formation of well-defined ion channels at all applied voltage which present their characteristics voltage-dependence take place. Note that at pH 3.8 single channel activity (i.e. the number of events) is more frequent than at pH 4.5. In this contest the open probability (Po) is increased at low pH for POPC:DOPG at all examined voltage except at 10 mV(data not shown).

All single events were used to calculate the channel amplitudes. Current amplitudes analyzed in the different PLMs used revealed the existence of one main conductance level. A histogram of amplitude distribution was constructed and fitted by a Gaussian distribution function, giving the central value of the single-channel conductance ± SD (Figure 3). Table 1 summarizes the central conductance (Λc ± SD), channel occurrence frequency (i.e. the mean number of openings in a period of 60 s), hCt single channel life time, τ_1 and τ_2, and the activation time (i.e. the lag time between hCt addition and the first channel formation) at pH 7 and 3.8 for the responsive membranes. For the life time, no less than 300 individual channels (opening and closing) were utilized. Independently of the pH values, the distribution of the open times was found to follow a two-exponential function. τ_1 ranges between 0.13-1.40 s, whereas τ_2 ranges between 2.42 – 7.73 s, except for POPC at pH 3.8 where the open time follows a one-exponential function. At pH 7, hCt forms single channels with similar mean activation time and values of τ_1 and τ_2 in negatively charged DOPG and in mixed POPC:DOPG PLMs; on the other hand Λc values in DOPG are smaller than Λc

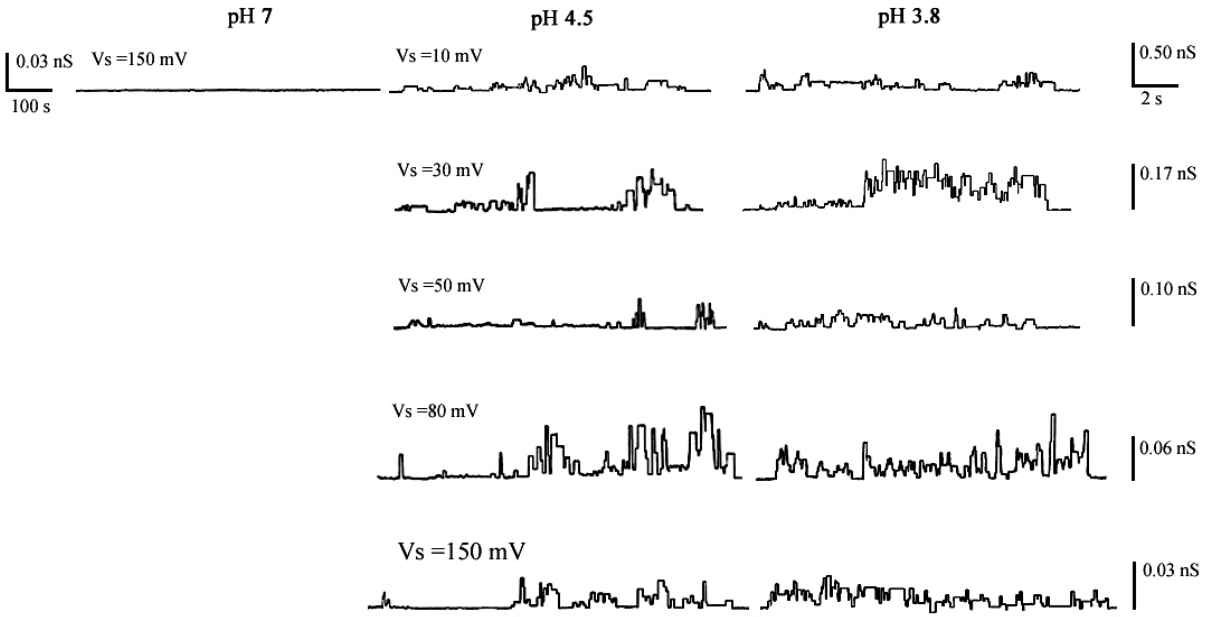

Figure 2. Examples of chart recordings of hCt channel formation in POPC PLM at different medium pH values and various holding potential (indicated on the top of the tracings). Channel openings and closings are represented by upward and downward deflections, respectively. Each trace represents a fragment of the recording of the activity obtained in individual experiment at different times. Experimental conditions: KCl 1M, hCt 125 nM was present on the *cis* side of the membrane.

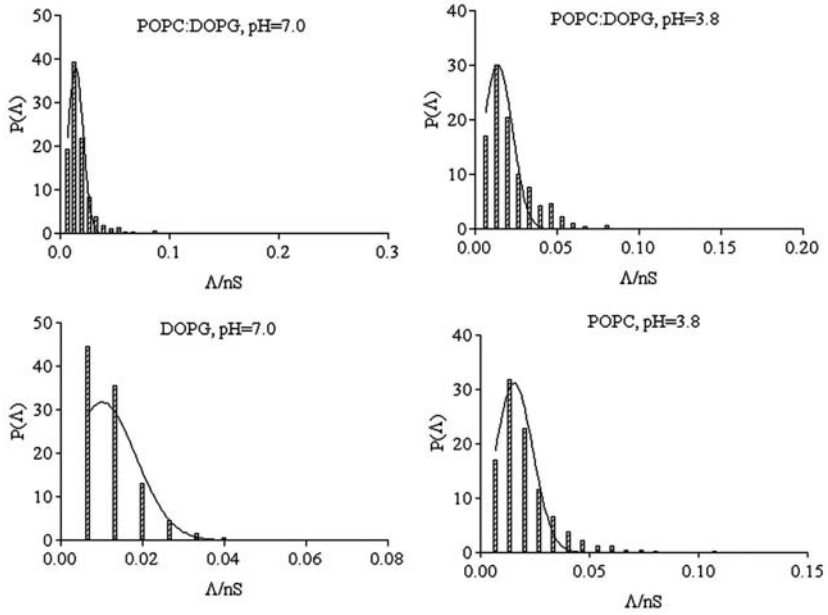

Figure 3. Amplitude histograms of hCt channel conductance relative to the examples reported in Figure 1. The histogram of the probability, P(Λ), for the occurrence of a given conductivity unit was fitted by a Gaussian, which is shown as a solid curve.

values in POPC:DOPG PLMs. Occurrence is 7.6 times higher in DOPG than in POPC:DOPG PLMs.

At pH 3.8 the Λc values in POPC and POPC:DOPG PLMs are not significantly different. The high values of occurrence frequency (higher in POPC than in POPC: DOPG PLMs) agree with a good incorporation of peptides through the membranes and their ability to form ion channels at low pH. Besides, hCt presents two life times in POPC:DOPG and one life time in POPC PLMs.

By comparing hCt channel parameters at the two different pH values for POPC:DOPG PLMs, it is

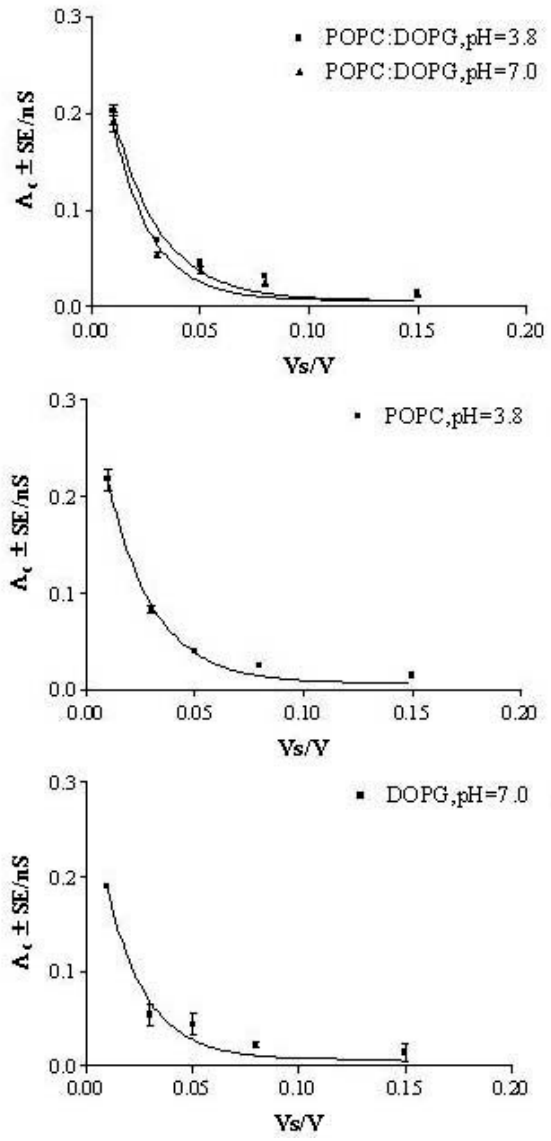

Figure 4. Conductance-voltage relationship for hCt channels in each PLM used at different medium pH values of 7 and/or 3.8, respectively. The data points were obtained from current histograms; at least 300 events were analyzed for each point. The curves superimposed on the data are the results of the 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) ; K is the constant correlated with the gating charge *n* (*n*=KRT/F). For POPC:DOPG membrane at pH 7.0/3.8:_A= 0.33± 0.05/0.31±0.04(nS); p=0.0067(nS); K= 57.7±10.0 (V^{-1}); *n*= 1.48/1.20;R^2=0.97/0.97.For POPC membrane at pH 7.0: A= 0.34± 0.02(nS); p=0.0067(nS); K= 47.4±4.2 (V^{-1}); *n*= 1.22;R^2=0.99. For DOPG membrane at pH 3.8:A= 0.31± 0.05(nS); p=0.0067(nS); K= 54.3±10.5 (V^{-1}); *n*= 1.39;R^2=0.97 Experimental conditions: KCl 1M, hCt 125 nM was present on the *cis* side of the membrane.

worth noting that the Λc values are not different and the occurrence frequency increases three times at low pH. This increase in occurrence frequency may be due to two factors: a) greater contents in hCt α-helices and b) titration pH-induced membrane surface. Moreover, the activation time is halved compared with pH 7, indicating that both peptide and membrane structural change play an important role in hCt channel formation.

We investigated the voltage-dependence of hCt channels in all PLMs used by performing experiments at various voltages (in the range 10 to 150mV) and measuring the amplitude of channel events. The data were parameterized with the exponential form:

$$\lambda_c = Ae^{(-KV_m)} + p$$

where A is the difference between the conductance at V_m =0 and at V_m= membrane black (p) ; K is the constant correlated with the gating charge *n* (*n*=KRT/F). hCt channel conductance remains inversely correlated with membrane potential no matter the pH value or the membrane composition (Figures 2 and 4). The results with POPC:DOPG PLMs corroborate the previous findings (23,24).

To gain information about the ion selectivity of channel at different pH, experiments in asymmetrical conditions, i.e. in the presence of a salt gradient, have be performed on POPC:DOPG PLM. The voltage difference at the zero current is the reversal potential. The selectivity of the channel has been quantified in terms of a permeability ratio P_{K+}/P_{Cl-}, from the reversal potential, using the Goldman-Hodgkin-Katz equation. Both hCt channels at pH 7 and pH 3.8 in POPC:DOPG membranes show poor anion selectivity in asymmetrical KCl conditions. For hCt channels at pH 7/3.8, the reversal potential was 2.1/3.88 mV in a 0.9/0.5 M ratio of KCl concentration giving a calculated K^+/Cl^- permeability ratio of 0.75/0.58, respectively.

5.DISCUSSION

Membrane-induced conformational variations of peptide could be one of the first steps promoting the α-helical structure, which is considered to be a prerequisite for incorporation into membranes and for receptor interaction, a concept expressed as a catalyst of membrane lipid phase as a prelude reaction to receptors binding (32). Furthermore, specific lipids may also participate as molecular chaperons in the folding of membrane proteins (33,34,35). Epand et al. (36) reported that POPC does not solubilize hCt, whereas hCt is fully solubilized by DMPG.

In this work we followed the strategy to overcome the amyloidal formation of hCt by modulating a pH-induced proto-deprotonation of amino acids responsible of the molecules aggregation. With this aim we concentrated on the role of the pH-induced charge variation of hCt and its ability to interact with a

membrane bilayer composed of zwitterionic, negatively-charged, and mixed phospholipid membranes. We considered channel activity as being any indication of an hCt fraction in an inserted state, even at a quantity too small for detection by less sensitive biochemical methods. At pH 3.8, we found that zwitterionic membranes are more active in incorporating hCt than acidic phospholipid ones.

In a previous paper we found that at pH 7 Ct's interact with DOPG containing POPC-membranes and assemble to form channels (23), but not with POPC membranes. This failure has been tentatively explained as being due to the fibrillation that occurs on the membrane surface, although this can be counteracted by applying a high potential to the membrane or by adding nanomolar amounts of SDS (23,24). Fragment 15-19 is considered the most crucial for fibril formation (9). It is worth remarking that a similar group is present in AβP(1-40), a peptide that undergoes fibrillation and - similarly to hCt - does not interact with POPC membranes (27). hCt fibrillation has been studied by various groups of researchers (6,31) who found that pH can play an important role in the assembly of hCt fibrils. In fact, at low pH (pH = 3.8) the side chains of Lys18, His20, and of the NH_3 terminus are positively charged, whereas the negatively-charged Asp15 is protonated. These variations in the molecular organization of hCt slow down the formation of fibrils which are composed of a mixture of antiparallel and parallel β-sheets up to the furthest C-terminal region (37).

The molecular conformation of both POPC membranes and hCt could be responsible for the lack of interaction at pH 7. This could be the result of the lack of POPC to form negative curvature in the presence of hCt at pH 7. In support of this is the result that a small amount of DOPG (15%) is needed to catalyse hCt incorporation and molecular assembly to occur in the POPC membrane at pH 7, or a reduction in the pH (3.8) of the medium that protonates the His20 group of peptide and increases one positive charge in the area of the peptide which is known to contain the fibrillating sequences (9). The increase in the number of positive charges in the peptide would cause repulsive force between congener molecules, thus not allowing the peptides to interact with each other to form the seed of nucleation preceding the fibrillation. Furthermore, in organic solvent or SDS micelles at low pH, the protonation of Asp15 allows hCt to form one more helix turn involving the amino acid segment from 15/16 to 20/21 (9). Moreover, at low pH, the positive lipid head-group charge dominates the equilibrium and although the peptide also shows a positive charge, it could be the protonated Asp group that is responsible for incorporation. This finding has been found for other proteins and peptides such as diphtheria toxin, annexin V and VI, vesicular stomatitis virus (VSV) and colicin E_1 that show maximum efficiency of incorporation at low pH (38-43). Similarly to hCt, these proteins possess His groups that are charged at low pH. Our results lend support to the notion that the bilayer interface determines the deprotonation of His which acts as an anchor for further protonation of negatively charged Asp (44).

Differential Scanning Calorimetry studies on flanking His-hydrophobic peptide incorporation in the DPPC lipid at pH 5 have shown a decrease in relative enthalpy, indicative of an interaction between a positively charged peptide and a hydrophilic lipid headgroup (45), as well as a hydrophobic interaction. Moreover, it has also been found that positively charged peptides induce more disturbance on DPPC bilayers than on uncharged ones (45), and that this disturbance can facilitate peptide incorporation. On the contrary, at low pH, in membranes made up of DOPG, hCt does not form channels. At this low pH, the positively charged hCt electrostatically interacts with negatively charged lipids, forming an anchor and making translocation into the hydrophobic medium difficult. Based on the results shown here, we propose that, depending on the membrane surface and on the protonation/deprotonation of His and Asp residues, through a balance between electrostatic and hydrophobic interaction, hCt forms more or less easily amphipathic α-helices required for insertion. This result provides information on the lipid-peptide interaction involved in many function of the cell such as protein/peptide translocation and membrane-receptor binding, and evidentiates that a very low variation of electrostatic charge may lead to a wide effect.

As proposed in our previous papers (23,24) at least 4 inserted hCt molecules are assembled to form a transmembrane channel with a hydrophilic central pore. In other words, the increased rate of hCt incorporation and channel formation could be a valid mechanism for destabilizing the reaction between congener peptide molecules which form the seed of nucleation before fibril formation. The therapeutically marked activity of salmon and eel Ct may be the result of an ancestor channel activity owing to the need that these fish species have to fine-tune ion regulation during migration. In fact, both sCt and eCt show conspicuous channel activity *in vitro*. However, hCt has also shown vestiges of channel activity which could be exhumed by environmental conditions such as high potential, nanomolar concentrations of SDS (23,24) and - as found in this study - by low pH.

From the physiological point of view, osteoclasts have shown sensitivity to Cts both *in vivo* and *in vitro* (46). Ct inhibits bone resorption by inhibiting osteoclast activity. Bone resorption is accomplished by osteoclasts, whose membrane ruffles are the initial events in the process that transports acidifying vesicles along the microtubules and inserts them into the plasma membrane (47,48). In these contact areas, acidification of the environment by H^+-ATPase initiates bone demineralisation (29,30); consequently, high levels of extracellular Ca^{2+} are sensed by a receptor that will constrain the detachment of the osteoclasts from the bone surface (49-52). Preliminary results on PLMs show Ca^{2+}permeability through hCt channels at low pH. One

tempting possibility could be that when hCt senses low environmental pH, it undergoes a structural conformation promoting its incorporation and assembly into POPC-rich plasma membranes, to form channels that are sensitive and permeable to Ca^{2+}; this aspect is under investigation. These channels could be considered parallel pathways, complementing the known Ca^{2+}-sensing receptor, (53,54) and potentiating osteoclast detachment. Channel formation by Ct may thus be considered an additional mechanism to push Ca^{2+} across the membrane into the cytosol for signal transduction. Besides this aspect, pharmacological approaches in gene therapy are stimulating basic study into the uptake of peptides, oligonucleotides and DNA through plasma membranes, and physiological peptides could be promising tools.

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Abbreviations: palmitoyl-oleoyl phosphatidylcholine: POPC; dioleoyl-phosphatidylglycerol :DOPG; human calcitonin:hCt; salmon calcitonin:sCt; eel calcitonin:eCt; porcine calcitonin:pCt ; planar lipid membrane: PLM

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Send correspondence to: Dr Enrico Gallucci, Dipartimento Farmaco-Biologico, Università degli Studi di Bari, I-70126 Bari, Italy, Tel: 39 080 5442796, Fax: 39 080 5442796, E-mail: gallucci@farmbiol.uniba.it

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