Effect of Nanomolar Concentrations of Sodium Dodecyl Sulfate, a Catalytic Inductor of α-Helices, on Human Calcitonin Incorporation and Channel Formation in Planar Lipid Membranes

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ABSTRACT   Human Calcitonin (hCt) is a peptide hormone which has a regulatory action in calcium-phosphorus metabolism. It is currently used as a therapeutic tool in bone pathologies such as osteoporosis and Paget's disease. However, due to its amphiphilic property tends to form a gelatinous solution in water which consists of fibrils that limits its therapeutic use. Here we show that sodium dodecyl sulfate (SDS), an anionic detergent able to induce and stabilize α-helices in polypeptides, at a monomeric concentration ranging between 0.26 mM–5 pM (all concentrations are below the CMC), increases the rate and number of hCt channel formation in planar lipid membranes, at both high and low hCt concentrations, with a maximum increase at a molecular hCt/SDS ratio of 1000:1. This effect could be interpreted as a counteraction to the fibrillation process of hCt molecules by removing molecules available for aggregation from the fluid; furthermore, this action, independently of channel formation in the cell membrane, could improve the peptide-receptor interaction. The action of SDS could be attributable to the strength of the sulfate negative charge and the hydrophobic chain; in fact, a similar effect was obtained with lauryl sarcosine and not with a neutral detergent such as n-dodecyl-β-D-maltoside. The very low molecular ratio between SDS and peptide is suggestive of a possible catalytic action of SDS that could induce α-helices, the appropriate structures for interacting with the membrane. Moreover, in the experimental conditions investigated, the addition of SDS does not modify the membrane's electrical properties and most of the channel properties. This finding may contribute to the knowledge of environment-folding diseases due to protein and peptides.

INTRODUCTION

Calcitonin (Ct) is a 32-amino acid amphipathic peptide with carboxy-terminal proline and a 1–7 disulfide bridge between cysteine residues. Its structure is consistent with an N-terminal portion, specific for receptor activation, and a more variable central helical segment (Sexton et al., 1999). Segre and Goldring (1993) have proposed that Ct associates specifically with cells through a membrane-bound receptor of a superfamily of 7 transmembrane-spanning helices G-protein-coupled receptors that act via adenyl cyclase and/or phosphoinositide-specific phospholipase C pathways. However, G-protein stimulation by bovine Ct and β-amyloid (Aβ) has also been shown on cells where the receptors had been removed (Rymer and Good, 2001).

In humans, the main role of Ct is to supply the skeleton with calcium during lactation, growth, and pregnancy, although other roles have been found in different organs (Fisher et al., 1981; Rizzo and Goltzman, 1981). The hypocalcemic action of Ct is accomplished by inhibiting osteoclast resorption. Salmon Ct (sCt) and eel Ct (eCt) are widely utilized clinically for the treatment of bone disorders such as Paget's disease, osteoporosis, and hypercalcemia of malignancy (Wallach et al., 1999). However their use is limited by immunological reactions due to the nonspecies-specific peptide. Thus, human calcitonin (hCt) could be more appropriate for clinical use.

To ameliorate the potency of the molecule and to shed some light on the mechanism of amyloid formation, a correlation between the structure of the molecule and the biological function needs to be well understood.

On the other hand, hCt shows a fibrillating property in aqueous solution in which α-helices and β-sheets are present, and this phenomenon is critically influenced by solid-liquid interface and is also time-, pH-, and concentration-dependent (Arvinte et al., 1993; Bauer et al., 1994; Kamihira et al., 2000). A model has been put forward to explain the hCt fibrillation which takes into account the protonation of Lys-18 and the deprotonation of Asp-15 at different pH values (Kamihira et al., 2000).

In particular in this model the mechanism of action of fibril formation is described in two steps: the first, a rapid "nucleation process" consists in a homologous α-helical bundle formed through hydrophobic interaction between hCt monomers; the second, a slow "autocatalytic heterogeneous fibrillation" is characterized by the conformational variation of α-helical bundles into oligomeric β-sheet fibrillations.

hCt shares common properties with fusogenic molecules such as HIV pg 41 (Kliger et al., 1997; Pereira et al., 1997), influenza hemagglutinin (Rafalski et al., 1991; Gray et al., 1996), sperm fusion protein PH-30 (Muga et al., 1994; Niidome et al., 1997), and Sendai virus (Rapaport and Shai, 1994), and shows fibrillating properties similar to many peptides such as insulin (Nettleton et al., 2000), Alzheimer

Aβ (Harper and Lansbury, 1997; Terzi et al., 1997; Kremer et al., 2000; McLaurin et al., 2000), prion protein (Prusiner et al., 1984), cystic fibrosis (Thomas et al., 1992), and the α-synuclein involved in Parkinson's disease (Galvin et al., 1999; Shimura et al., 2001).

As the failure of peptides or proteins to assume their correct structure may be harmful to cell activity, studies devoted to understanding the molecular mechanism that subtends the peptide-peptide, lipid-peptide interaction, and aggregating/disaggregating mechanism can be beneficial from a biophysical and therapeutic point of view.

In a previous study (Stipani et al., 2001), we demonstrated that both sCt and hCt can form channels across black lipid membranes (BLMs) made up of POPC/DOPG (85:15).

Moreover, hCt at a high concentration in the medium facing the BLM displayed a lower channel activity that can be overcome by decreasing its concentration or by applying a potential as high as 150 mV to the membrane. This was tentatively attributed to the aggregating property of hCt molecules in solution or on the surface membrane. On the other hand, it is known that hCt contains numerous hydrophobic amino acids that could be involved in the fibrillation process, in fact, more recently hCt fragment (hCt$_{15-19}$) has been shown to form fibrillar structures in vitro (Reches et al., 2002). It is worth mentioning that the same phenylalanine structural motif is also present at position 15–19 of the Aβ(1–40) peptide.

On the other hand, sodium dodecyl sulfate (SDS) has proved to be useful for structural studies and purification of membrane proteins, in particular SDS shows ability to induce and stabilize α-helical structure in peptides (Reynolds and Tanford, 1970; Wu and Yang, 1978; Jorgensen, 1988; Papavoine et al., 1994; Pervushin et al., 1994; Young et al., 1997; Kragh-Hansen et al., 1998; Montserret et al., 2000). Like many ionic detergents, SDS can refold proteins. It is thought that the molecular modality by which SDS induces α-helical structure stabilization lies in the anionic polar head, which forms a bridge with a positively-charged group of amino acids, whereas the hydrophobic chain enwraps the hydrophobic part of the amphipathic peptides (Wu and Yang, 1978). On the other hand conformational variation (random-coil to β-sheet or to α-helix) has been found for Aβ(1–40) and Aβ(25–35) dissolved in organic solvents such as SDS and trifluoroethanol (Barrow and Zagorski, 1991; Sticht et al., 1995; Kohno et al., 1996; Coles et al, 1998).

This study aimed at understanding whether SDS at a concentration ranging between 0.69 mM–5 pM where the highest concentration corresponds to a monomeric concentration of 0.26 mM in a 1 M KCl medium (Quina et al., 1995), could be useful in inducing α-helix and preventing hCt aggregation either in solution or at the water-membrane interface during the kinetics of incorporation in model membranes made up of POPC/DOPG (85:15) as probed by channel formation.

METHODS

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. BLMs were formed from a 1% (w/v) solution of a mixture of POPC and DOPG (molar ratio of 85:15) or from POPC dissolved in n-decane as described previously (Benz et al., 1978). The salts used in the experiments were of analytical grade. The salt solutions had a pH of ~7 and the experimental temperature was ~22°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 hCt was added, has a positive polarity.

Data analysis

The single channel data were obtained from at least three experiments with more than 100 single events for each series performed on different days. The current transition directed upward preponderated with respect to terminating events. A histogram of the current amplitude distribution for each experiment was constructed and fitted by a Gaussian distribution function (GraphPad Prism^{TM} version 3.0; GraphPad Software, Inc, www.graphpad. com). From records extending over prolonged periods, the channel durations were measured considering the time between the opening/closing of the 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 ($A_2 = 0$). 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.

Chemicals

Salts and other basic chemicals were bought from Merck (Darmstadt, Germany, analytical grade) and biochemicals from Sigma (Munich, Germany). The phospholipids used in this study were purchased from Avanti Polar Lipids (Alabaster, AL). hCt was from Novartis Pharma AG (Basel, Switzerland).

RESULTS

Testing channel formation

Various experiments were performed preliminarily to test for channel formation by hCt in planar lipid membranes.

First of all, we tested the conductance and capacitance of each membrane by applying a voltage of ±200 mV for 10–15 min under stirring, to ensure that the membrane was stable.

Upon hormone addition, current jumps compatible with channel-type openings and closures with different conductance levels and lifetimes were observed. On the other hand, channel formation was inhibited when protease was added to

the medium before hCt addition. Control experiments with protease present in the medium facing the membrane failed to give discrete current fluctuations for several hours.

The concentration dependence of hCt channel conductance

In the first set of experiments, the concentration dependence of hCt conductance was studied at +150 mV of applied voltage, the minimal potential at which channels can be observed for the maximum hCt concentration of 125 nM used in this study; presumably this is a fibrillating concentration (Stipani et al., 2001).

Fig. 1, *a*, *c*, and *e*, show a typical example of single-channel recordings with associated distribution histograms at high (125 nM), low (24.5 nM), and very low (5 nM) peptide concentrations used in POPC/DOPG bilayers, when the medium was KCl 1M and the applied voltage +150 mV.

As shown in Fig. 1, *a* and *c*, single-channel activity sometimes occurred in highly variable steps; yet the number
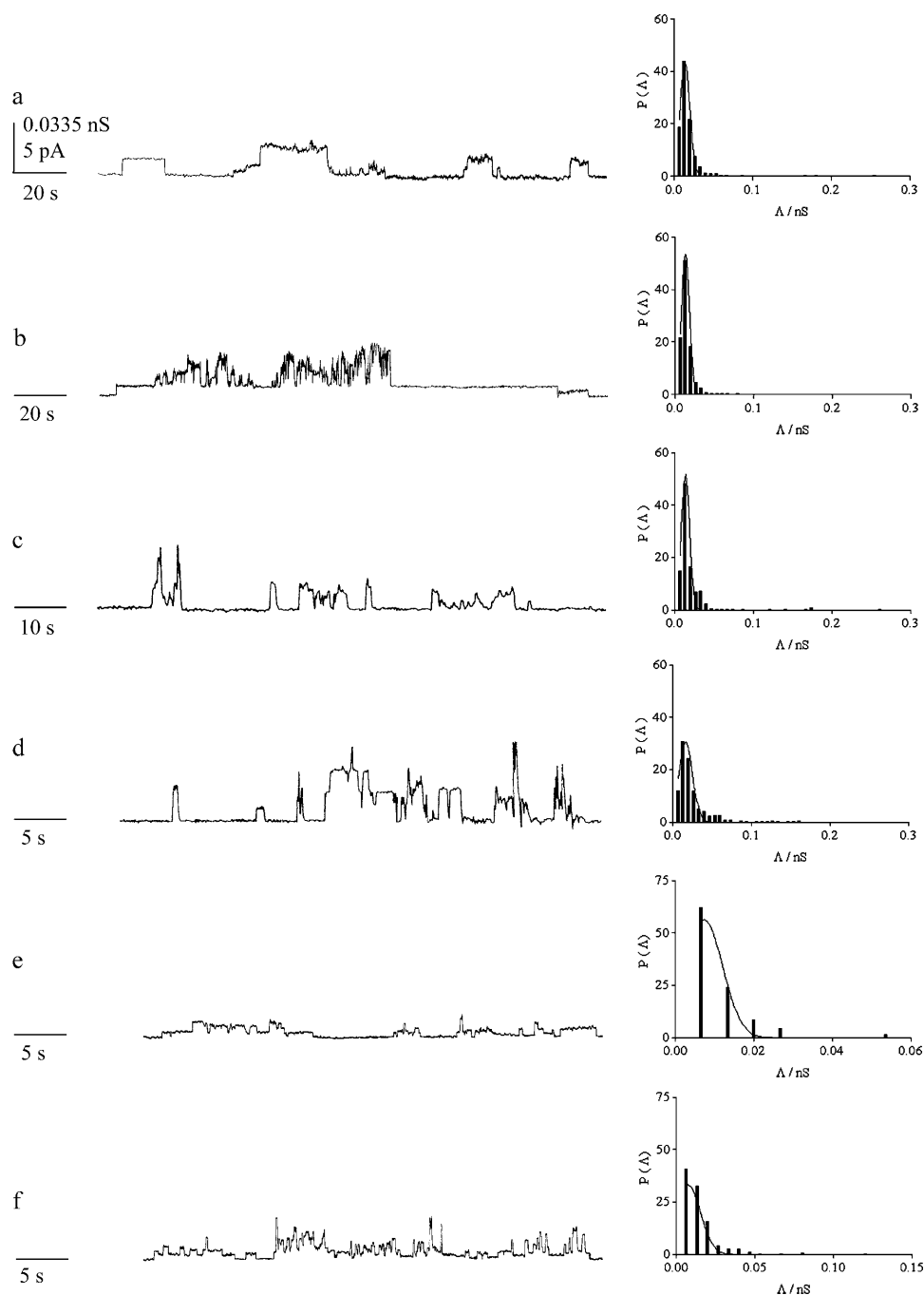

FIGURE 1 Single-channel features of hCt in the absence and in the presence of SDS with associated histograms of the conductance fluctuations. (*a*) hCt 125 nM; (*b*) hCt 125 nM + SDS (0.26 mM); (*c*) hCt 24.5 nM; (*d*) hCt 24.5 nM + SDS (0.26 mM); (*e*) hCt 5 nM; and (*f*) hCt 5 nM + SDS (5 pM). Experiments were performed on a POPC/DOPG (85:15) membrane in the presence of hCt and of hCt + SDS added to the *cis*-side; the voltage was set to +150 mV, the aqueous phase contained 1M KCl (pH 7), and $T = 22°C$. Note the increase in channel occurrence (channels/minute) when SDS was present in the medium.

of channels at 24.5 nM hCt concentration is almost twice that at 125 nM. Owing to hCt's fibrillating property, an inverse correlation seems to exist between peptide concentration and channel formation. We also observed alternating periods of paroxystic channel activity followed by quiescent periods, open times interrupted by brief closures, and occasionally conductance steps that were twice that of the central conductance, a clear indication that two channels were simultaneously incorporated. The results indicate that hCt molecules can aggregate to form channels in a concentration-dependent manner up to 85 nM. Above this concentration, channel conductance seems to be independent of hCt concentration, presumably due to the fact that the fibrillation process is driven by hydrophobic interaction between the peptide molecules, which reduces the number of monomers available to form channels (Table 1).

Effect of SDS on hCt channel activity

Since SDS is an α-helical inductor and a membrane mimicking agent, we wanted to test whether SDS could change the properties of hCt channels, such as mean conductance, voltage-dependence, ion selectivity, onset and occurrence of channel formation, and the rate of hCt channel formation.

First of all, we excluded any nonspecific and destabilizing effect of SDS per se on BLM by leaving SDS, at all concentrations used, in the medium facing the membrane for up to 24 hr. SDS caused no variations in membrane conductance and capacitance (Fig. 2, f and g).

Experiments were then performed by adding hCt to a medium containing SDS at a monomeric concentration of 0.26 mM. After addition of hCt, there was an early onset of step-like activity, indicative of channel formation, characterized by a continuous channel activity, with rare bursts, and with more frequent multiple levels of conductance (Fig. 1, b and d). Furthermore, these patterns were more evident when lower hCt concentrations were used (Fig. 1, e and f).

Yet at all concentrations of hCt used, the channel occurrence frequency (i.e., the mean number of openings in a period of 60 s) increased and the activation time (i.e., when the first event appeared) decreased compared with the experiments in which only hCt was present (Table 1). However, a lower applied voltage (10 mV) is required to activate channel formation.

Furthermore, a t-test showed that, in the presence of SDS, the central channel conductance is statistically modified at each concentration of hCt used except for 49 nM. Another experimental protocol used was to mix peptide and SDS together and after 20 min the mixture was added to the medium-facing membrane. The results obtained in this case were comparable to those obtained with the previous experimental protocol.

The same results on central conductance, voltage-dependence, onset and occurrence of the channels were obtained if the experimental procedure was changed by adding hCt to the membrane before SDS, indicating that the aggregation phenomenon can be reversed.

On the other hand, if hCt was present on the *cis*-side while SDS was added to the *trans*-side of the membrane, SDS failed to produce the above-mentioned effects.

Optimal molecular ratio between hCt/SDS for hCt channel activity

To see the lower limit of SDS concentration at which the onset of incorporation and of the rate of channel formation were modified, we performed an experiment in which hCt was held constant at 125 nM and SDS was changed in the range of 0.0125–12.5 nM (Fig. 2 and Table 2). The results at the highest (0.26 mM) SDS concentration used are also reported. Fig. 2, f and g, as a control also reports the results obtained with the maximum (0.26 mM) and the minimum (0.0125 nM) SDS concentration respectively, on POPC/POPG membranes. It is interesting to note that there seems to be an optimum molecular ratio between hCt and SDS for obtaining

TABLE 1 Single-channel parameters for hCt channels in the absence or in the presence of SDS (0.26 mM) at different hCt concentrations

Mode	$\Lambda_c \pm$ SD (nS)	Occurrence $\pm$ SD	$\Delta\%$	Activation time (min)
[hCt] = 125nM	0.014 ± 0.006	2.73 ± 0.09	10.3	50
[hCt + SDS] = 125 nM + 0.26 mM	0.013 ± 0.005	3.01 ± 0.11		16
[hCt] = 85 nM	0.015 ± 0.006	4.26 ± 0.13	46.8	16
[hCt + SDS] = 85 nM + 0.26 mM	0.014 ± 0.008	6.38 ± 0.24		6
[hCt] = 49 nM	0.014 ± 0.006	1.25 ± 0.10	99.2	31
[hCt + SDS] = 49 nM + 0.26 mM	0.015 ± 0.008	2.49 ± 0.11		19
[hCt] = 24.5 nM	0.013 ± 0.005	1.10 ± 0.06	208.2	19
[hCt + SDS] = 24.5 nM + 0.26 mM	0.016 ± 0.009	3.39 ± 0.17		5
[hCt] = 5 nM	0.007 ± 0.005	1.26 ± 0.15	176.9	10
[hCt + SDS] = 5 nM + 5 pM	0.007 ± 0.008	3.49 ± 0.18		1

The membranes were formed from POPC/DOPG (85:15), the voltage was set to 150 mV, the aqueous phase contained 1M KCl (pH = 7), and T = 22°C. $\Delta\%$ = ((occurrence with SDS − occurrence without SDS)/occurrence without SDS)%. The number of events (N_t) considered for each series of experiments was 171 < N_t < 1015.
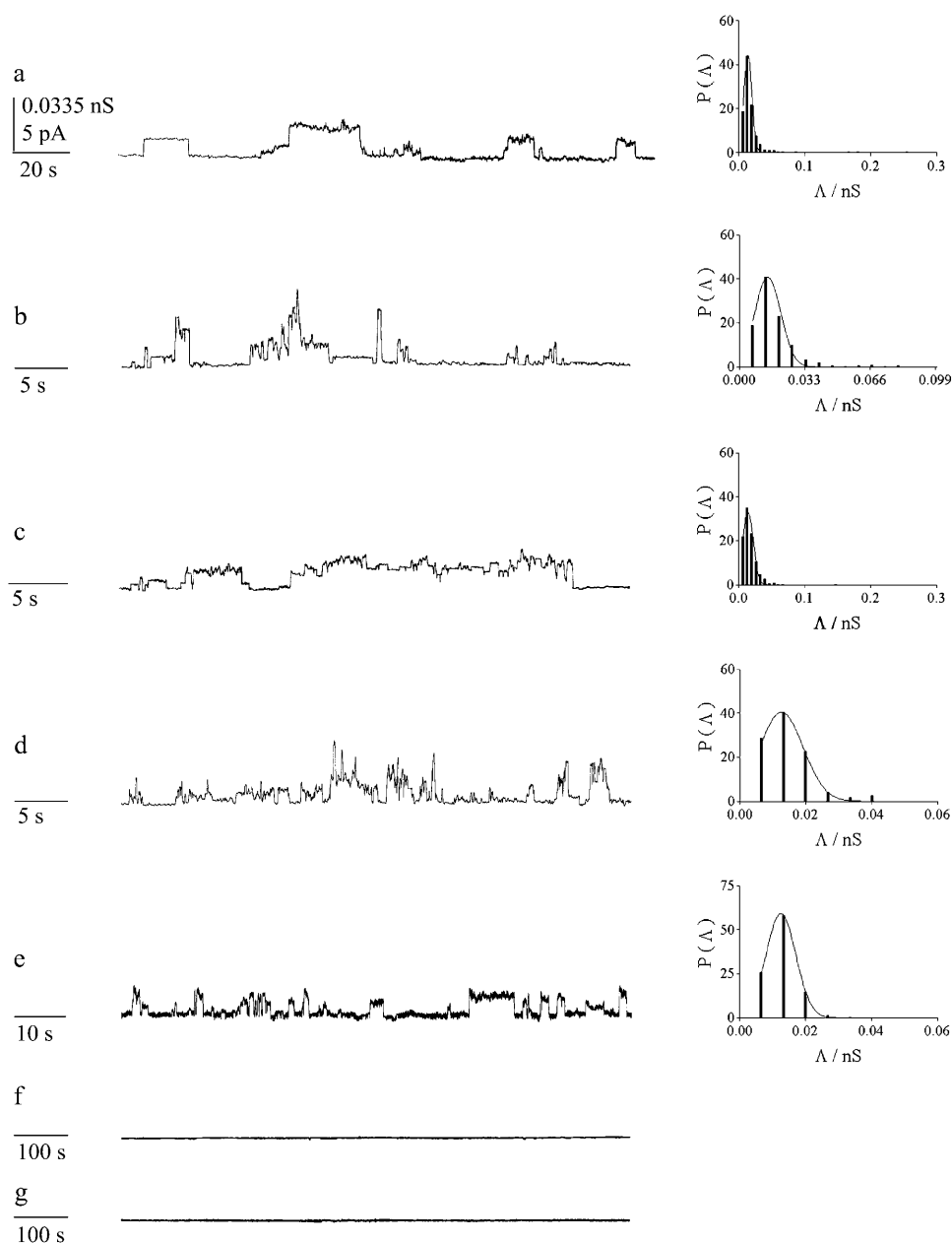
FIGURE 2 Single-channel features of hCt in the absence and in the presence of SDS at different concentrations with associated histograms of the conductance fluctuations. (*a*) hCt 125 nM; (*b*) hCt 125 nM + SDS (12.5 nM); (*c*) hCt 125 nM + SDS (1.25 nM); (*d*) hCt 125 nM + SDS (0.125 nM); (*e*) hCt 125 nM + SDS (0.0125 nM); (*f*) SDS (0.26 mM); and (*g*) SDS (0.125 nM). Experiments were performed on a POPC/DOPG (85:15) membrane in the presence of hCt and of hCt + SDS added to the *cis*-side; the voltage was set to +150 mV, the aqueous phase contained 1M KCl (pH 7), and $T = 22°C$.

the maximum increase in the occurrence of channel formation and the minimum time of the first channel appearance. The remarkable activity at a very low hCt-SDS molar ratio (1000:1 and 10000:1) could suggest a catalytic action of the detergent on the peptide.

Finally, experiments were performed in which hCt channel activity at a low peptide concentration (5 nM) maintaining the optimal molecular ratio between hCt/SDS (1000:1) (Fig. 1, *e* and *f*). In this case, the SDS-increased channel occurrence is remarkable, indicating that even at nonfibrillating hCt concentrations, quite near the physiological hormone concentration, the detergent is able to exert its action of α-helix induction.

Voltage-dependence and selectivity characteristics of hCt with and without SDS

The voltage-dependence and selectivity of hCt channels in the absence or in the presence of SDS is reported for an hCt concentration of 49 nM, where the central conductance of the channel was not changed.

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. As can be seen in Fig. 3, the conductance of the hCt channel is inversely correlated with membrane potential, as found with sCt (Stipani et al., 2001). The same behavior occurs in the presence of high and low SDS concentrations, indicating that the detergent does not

TABLE 2 Single-channel parameters for hCt channels in the absence or in the presence of SDS at different concentrations and at fixed hCt concentration

Mode	$\Lambda_c \pm$ SD (nS)	Occurrence $\pm$ SD	$\Delta\%$	Activation time (min)
[hCt] = 125 nM	0.014 $\pm$ 0.006	2.73 $\pm$ 0.09		50
[hCt + SDS] = 125 nM + 0.26 mM	0.013 $\pm$ 0.005	3.01 $\pm$ 0.11	10.3	16
[hCt + SDS] = 125 nM + 12.5 nM	0.014 $\pm$ 0.006	7.49 $\pm$ 0.32	174.4	12
[hCt + SDS] = 125 nM + 1.25 nM	0.014 $\pm$ 0.008	10.0 $\pm$ 0.54	266.3	6
[hCt + SDS] = 125 nM + 0.125 nM	0.013 $\pm$ 0.007	11.68 $\pm$ 1.07	327.8	5
[hCt + SDS] = 125 nM + 0.0125 nM	0.012 $\pm$ 0.004	5.82 $\pm$ 0.31	113.2	12

The membranes were formed of POPC/DOPG (85:15), the voltage was set to 150 mV, the aqueous phase contained 1M KCl (pH = 7), and T = 22°C. $\Delta\%$ = ((occurrence with SDS − occurrence without SDS)/occurrence without SDS)%. The number of events (N_t) considered for each series of experiments was 119 < N_t < 836.

modify the voltage dependence of the hCt channel. Furthermore, in the presence of SDS the channel occurrence increases by decreasing the applied voltage.

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 mean reversal potential was 2.51 mV.

The permeability ratio was calculated using the following equation:

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

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.

The P_K^+/P_{Cl}^- was 0.71 for POPC/DOPG membranes, indicating a poorly anion-selective channel. Approximately the same result was obtained with the I-V curve, where the measured amplitude of the channel events at each potential was used; in fact the reversal potential was 2.65 mV.

However in the presence of SDS (1000:1) the P_K^+/P_{Cl}^- was 3.25, indicating that the presence of SDS shifts the selectivity of the channel toward cations.

The lifetime of the single hCt channel without and with SDS

The lifetime of the single-channel has been used to further characterize the channels. For this analysis, no less than 100 individual channels (opening and closing) were utilized. Independently of the calcitonin concentration or voltage used, the distribution of the open times was found to follow a two-exponential function, except for the calcitonin concentration of 85 nM in which the statistical test does

not distinguish between a one- or two-exponential function. τ_1 ranges between 0.1–1 s, where the lowest value is observed for low calcitonin concentrations, whereas τ_2 ranges between 3.3–7.5 s. Neither of the time constants is modified by SDS.

Effect of different detergents on hCt channel activity

To test the role of the detergent charge in this phenomenon, two more detergents, namely lauryl sarcosine and *n*-dodecyl-β-D-maltoside, were used at the same molecular ratio as the optimum activity found for SDS (1000:1). Table 3 reports the central channel conductance, their occurrence and the onset time as compared to the basal value of hCt alone. It can be noted that the occurrence frequency is very low for *n*-dodecyl-β-D-maltoside compared with SDS or lauryl sarcosine, indicating that a main role is played by the
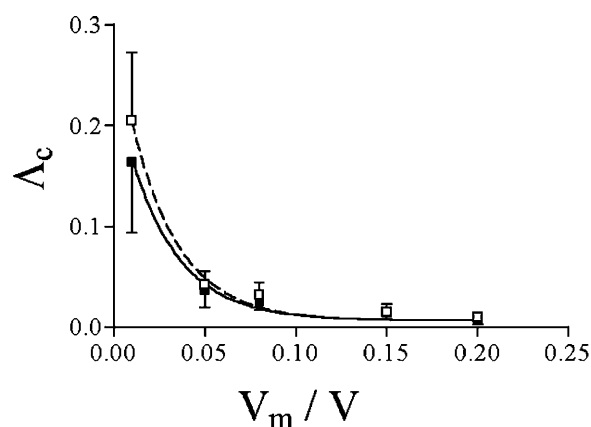

FIGURE 3 Conductance-voltage relationship for hCt channels in the absence (■) or presence of SDS (□). Experimental conditions: KCl 1M, hCt (49 nM) or hCt (49 nM) + SDS (0.26 mM) was present on the *cis* sides of the POPC/DOPG (85:15) membrane. The curves superimposed on the data are the results of the 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 ($n = KRT/F$). (■) $A = 0.234 \pm 0.004$ (nS); $p = 0.0067$ (nS); $K = 37.13 \pm 0.59$ (V^{-1}); $R^2 = 0.988$. (□) $A = 0.29 \pm 0.002$ (nS); $p = 0.0067$ (nS); $K = 38.3 \pm 0.48(V^{-1})$; $R^2 = 0.99$.

TABLE 3   Single-channel parameters for hCt channels in the presence of different detergents

Mode	$\Lambda_c \pm SD$ (nS)	Occurrence $\pm$ SD
[hCt + SDS] = 125 nM + 0.125 nM	0.013 ± 0.007	11.68 ± 1.07
[hCt + *N*-lauryl sarcosine] = 125 nM + 0.125 nM	0.016 ± 0.007	10.50 ± 1.07
[hCt + *n*-dodecyl β-D-maltoside] = 125 nM + 0.125 nM*	0.018 ± 0.012	2.74 ± 0.23

The membranes were formed from POPC/DOPG (85:15), the voltage was set to 150 mV, the aqueous phase contained 1M KCl (pH = 7), and $T = 22°C$. The number of events (N_t) considered for each series of experiments was $107 < N_t < 140$.
*Vs = 80 mV.

strength of the charged moiety as anchor to the peptide in facilitating the α-helix formation.

DISCUSSION

In this study, we show that hCt incorporates and forms single channels in PLMs of POPC/DOPG both at high (125 nM) and low (5 nM) peptide concentrations. This channel was found to be voltage-dependent and poorly anion selective; its lifetime generally follows a two-exponential function. However, the hCt α-helix between residues 9–16 is too short to span the bilayer, which suggests that hCt monomers must aggregate into oligomers to form channels. In fact, the number of molecules that act in concert to form a channel seems to be three, as found by the concentration-dependence of conductance (150 mV applied voltage) (Mueller and Rudin, 1968).

To accomplish its physiological activity, Ct must interact with receptors, and for this to take place an α-helical conformation is required (Epand et al., 1983; Keller et al., 1992). Moreover, an α-helix is required to penetrate the bilayer (Engelman and Steitz, 1981; Epand et al., 1983; Jacobs and White, 1989; Milik and Skolmick, 1993; White and Wimley, 1999).

There is plenty of evidence accounting for the folding of membrane-active peptides induced by phospholipid membranes (Sargent et al., 1988). Most of these peptides require negatively charged phospholipids (Jordi et al., 1989; Bradshaw, 1997; Terzi et al., 1997; McLaurin et al., 2000; Stipani et al., 2001; Yip and McLaurin, 2001); however, it has been found that some peptides do not require anionic lipids to fold or to induce channels (Martinez-Senac et al., 2002; Gallucci et al., 2003; Micelli et al., 2004).

It is important to consider that the process of incorporation is a complex phenomenon, driven by both peptide and lipid conformation, owing to the need to match peptide hydrophobic length and lipid acyl chain. Evidence of the importance of peptide-lipid matching has been substantiated in the principles of the "mattress model" (Mouritsen and Bloom, 1984; Bloom et al., 1991; Killian et al., 1996; Kremer et al., 2000; Rinia et al., 2000; Yip et al., 2002). Lipids can either retard or enhance the process, depending on their structure. It is worth mentioning that one of the sites at which Ct interacts with the receptor is located deep in the membrane (Sexton et al., 1999).

An important molecular feature of hCt is its high flexibility along the whole polypeptide chain (Amodeo et al., 1999); this structural feature may account for the different degree of its interaction with membranes (particularly when high concentrations of hCt are used) where the hydrophobic side of the helix can trigger intermolecular interaction, culminating in fibrillation, as indicated by the lower channel frequency at higher hCt concentrations.

In fact, single channels are formed more easily at low hCt concentrations and the channel central conductance is a linear function of the hCt concentration up to 85 nM, then the conductance decreases as the hCt concentration increases. Most probably, by increasing the hCt concentration, the monomeric-free concentration useful for incorporation into the membrane is reduced by the prevailing cooperative fibrillation process. These results, although indirect, can be appropriately correlated with the well-known fibrillating property of hCt (Arvinte et al., 1993). There is other indirect evidence corroborating this hypothesis: 1), the high potential needed to incorporate and form channels when hCt is used at high concentration; and 2), at the same hCt concentration the very low potential needed to induce channel formation in the presence of SDS.

In aqueous solution, Ct presents a random coil structure, and when SDS at CMC is added, an α-helical structure is promoted (Motta et al., 1998; Sexton et al., 1999), although hCt acquires the α-helix conformation more slowly (Arvinte et al., 1993) and shows a shorter helix (9–16) in SDS (Motta et al., 1991) than in a trifluoroethanol-water mixture (Doi et al., 1990) as compared to sCt.

The increased rate of channel formation using as low as pico-nano molar SDS concentrations (and to a lesser extent lauryl-sarcosine) is presumably due to the induction of a helical conformation in hCt that counteracts the transition from a monomeric to a fibrillar state by facilitating incorporation. In fact, SDS increases both the number and the frequency of hCt channel formation at all hCt concentrations used. It is worth recalling that at a concentration below the CMC SDS has been successfully used to isolate Na^+, K^+-ATPase (Jorgensen, 1988).

It could be hypothesized that SDS acts as a perturbing agent of the membrane, thus facilitating peptide incorporation; however, this hypothesis can be excluded as SDS has no effect on membrane conductance and capacitance when introduced on one side of the membrane with calcitonin on

the opposite side, nor when added to the medium-bathing the membranes and acting for several hours without hCt.

It is known that SDS incorporates very slowly into the outer leaflet of LUV liposomes (Doi et al., 1990; Kragh-Hansen et al., 1998; Tan et al., 2002) and flips to the other side, whereas higher energy is required to incorporate into planar lipid membranes. In fact, we observed no modification in the electrical properties of bare membranes upon SDS action, at high and low concentrations, over a period of 24 hr. Furthermore, hCt failed to assemble and form channels in POPC membranes, if SDS were to destabilize the membrane, we would expect a certain hCt incorporation; however, no incorporation was observed, even after a very long time. On the other hand, thermodynamic studies have shown that SDS incorporates in POPC membranes at the same rate as POPC/DOPG (Doi et al., 1990).

Although the exact modality of molecular interaction between peptide and SDS remains to be elucidated, it could be assumed that a bivalent contact takes place between the two molecules, with the cationic group of lysine and hydrophobic residues, similarly to that reported for lysozyme and for PF4(56–70) (Yonath et al., 1977; Montserret et al., 2000). In particular, by enwrapping the hydrophobic part (specifically the aromatic phenylalanines responsible for the process of self-assembly) of the peptide with its long acyl chain, SDS may induce both α-helix formation and the transmembrane position of the peptide that prevents interaction with other helices, thus not allowing the bundle to organize. In fact, due to its propensity to form β-sheet structures, the sequence from D^{15}–F^{19} of hCt has been implicated in the fibrillation process. Moreover, it has been found that the minimum length of polypeptide that will undergo fibrillation is four amino acids with a propensity for β-strand conformation (Reches et al., 2002; Tjernberg et al., 2002).

This property has also been found for human transthyretin, the major component of amyloid in senile heart. In fact, by using 2% SDS Altland and Winter (2003) found that dimer dissociation leads monomers to associate in a complex with SDS, thus losing their capacity to dimerize.

It has been suggested that the first step in the nucleation process is due to a hydrophobic interaction between hCt monomers in the aggregating phenomenon to form bundles of fibrils (Kanaori and Nasaka, 1995). On the other hand, it has been found that at pH 7 the positive charge of lysine and the negative charge of aspartate play a further role in the fibrillation process (Kamihira et al., 2000); by competing with the hCt molecules, SDS may disrupt the process by interacting with positively charged lysine, leaving the peptide negatively charged.

In our study, the novel aspect of SDS action is the high hCt/SDS (1000:1) molar ratio, at all hCt concentrations used, sufficient to trigger the phenomenon observed. This effect could be tentatively explained by a catalyst action exerted through a favorable electrostatic and hydrophobic interaction

that is worthy of further investigation. Thus the rapid induction of α-helices by SDS could be beneficial for lipid penetration and receptor interaction, as has been found for the ACTH hormone (Sargent et al., 1988). A competition can take place between hCt receptor and bilayer, depending on the receptor concentration and lipid structure of the membrane.

It is worth mentioning that many naturally occurring chaperons control protein/peptide folding. Examples are: Hsp-70 chaperone has been shown in a *Drosophila melanogaster* model to suppress the toxicity of α-synuclein, the main protein implicated in the pathogenesis of Parkinson's disease in man (Auluck et al., 2002); glycerol and methylamine for inducing the random-coil to the β-sheet structure of Aβ (Yang et al., 1999); and cholesterol to determine the conformational transition from random-coil to a structure designed to incorporate voltage-dependent anion channels into PLMs (Gallucci et al., 1996; Popp et al., 1996; Micelli et al., 2000).

It can be speculated that SDS could act as a chaperone, shielding the exposure of the hydrophobic groups of Ct and preventing their interaction with both polar and hydrophobic groups of congener molecules to form a bundle of helices. On the other hand, it has been estimated that α-helix (Δh_{helix}) formation accounts for 65% of the total enthalpy of magainin 2 incorporation in lipid membranes (Wieprecht et al., 1999). If we assume that the transition from a random-coil to an α-helix structure is rate limiting for incorporation (Jacobs and White, 1989; White and Wimley, 1999; Wieprecht et al., 1999), then α-helix formation will be a potent driving force for the incorporation of hCt, thus counteracting the natural tendency of hCt to form β-sheet structures which could be considered the prelude of fibrillar states.

Moreover, it is interesting to recall that another peptide, Aβ, thought to be responsible for the pathogenesis of Alzheimer's disease, does so by forming fibrils, and its incorporation into membranes reduces the potential formation of fibrils (Ji et al., 2002; Micelli et al., 2004). However, other similarities can be observed between hCt and Aβ(1–40); in fact:

a. In aqueous solution both peptides are in a random coil conformation.

b. Both peptides assume α-helix conformation in organic solvents such as trifluoroethanol and SDS micelles (Barrow and Zagorski, 1991; McLaurin and Chakrabartty, 1996; Terzi et al., 1997).

c. Both peptides easily incorporate in cholesterol-containing POPC membranes (data not shown) (Ji et al., 2002; Micelli et al., 2004).

d. Neither peptide incorporates into POPC or DMPC membranes (Epand et al., 1983; Terzi et al., 1994, 1995; Bradshaw, 1997; Stipani et al., 2001; Ji et al., 2002; Micelli et al., 2004), but the addition of the negatively charged DOPG leads to incorporation. In particular for

Aβ(1–40) the addition of negatively charged DOPG (25%) induced the transition to a β-structure; a further addition of POPG to vesicles determined an increase in α-helical structures, indicating that the lipid/peptide molar ratio is crucial for conformational variations to take place (Terzi et al., 1994, 1995).

It is conceivable that POPC induces a β-sheet conformation in hCt that is suitable for fibrillation to take place. A similar feature has been found for β-amyloid in DMPC vesicles (Ji et al., 2002).

We do not have direct evidence of channel formation by Ct in cell membranes, though this angle is currently being studied.

Although it has not yet been demonstrated that hCt forms channels in the target tissue, the action of SDS can be of valuable interest because SDS could facilitate hCt-receptor interaction sites in the membrane and counteract fibrils formation especially when therapeutically high concentrations of Cts are used. It is worth recalling that a large quantity of Ct is administered in Paget's disease, hypercalcemic malignancy, and osteoporosis; formulation of monomeric peptide, which is more accessible to the membrane, could improve its efficiency. On the other hand, SDS is commonly used in cosmetics, drug delivery, and to enhance intestinal absorption of poorly adsorbed drugs (Singer and Tjeerdema, 1993; Sirisattha et al., 2004), and it has been found that some formulations containing 5% SDS had only minimal collateral effects (Lee and Maibach, 1995). At the dose of 100 U/ml Ct only 0.017% of SDS would be present for a molar ratio hCt/SDS 1000:1. In this context, it may be worth considering that hCt fibrils is associated with thyroid carcinoma (Sletten et al., 1976) and with neurotoxicity (Schubert et al., 1995) and owing to the nonlysing activity of the mixture, a variety of applications can be sought.

This result would be useful in exploring whether such a simple molecule, or other similar molecules, could have a therapeutic interest besides technological applications, such as transmembrane transport of proteins or other compounds attached to the peptide-SDS complex notwithstanding the relevant biophysical interest.

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