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Gln^5 Selectively Monodansylated Substance P as a Sensitive Tool for Interaction Studies with Membranes

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Substance P (SP) is a neuropeptide endowed with several important biological activities both in the central and peripheral nervous system. Taking advantage of the presence of glutamine residues in SP, the peptide was labelled with the fluorescent probe monodansylcadaverine using the transglutaminase (TGase)-reaction in order to study interactions between SP and model or natural membranes. Although it was verified that both adjacent glutamines of the peptide can act as substrate for TGase in a consecutive reaction, conditions were optimized to selectively label Gln^5. This fluorescent SP analogue was found to adopt environment-dependent conformations similar to those of the natural peptide and proved to be functionally active on guinea pig trachea. Fluorescence spectroscopy was used to demonstrate the potential use of dansylated SP in studies involving interactions with membranes.

Substance P (SP) (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), is a neuropeptide widely distributed in both the central and the peripheral systems [1]. Considerable work is currently being carried out on determining the role of membrane lipids in the mechanism of action of SP. It has been proposed that the lipid phase of the target cell membrane may be an important mediator of the peptide-receptor interaction [2,3], either by simply increasing peptide concentration at the membrane surface or by inducing the biologically active conformation [4,5]. Recent experiments using lipid-monolayer and ^{13}C-NMR spectroscopy have provided evidence for a partial insertion of

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Abbreviations: CD, circular dichroism; DNC, monodansylcadaverine; DNC-SP, monodansylcadaverine-substance P; $(DNC)_2$-SP, di(monodansylcadaverine)-substance P; EPC, L-α-phosphatidylcholine; NMR, nuclear magnetic resonance; PS, L-α-phosphatidyl-L-serine; RP-HPLC, reversed-phase high performance liquid chromatography; SP, substance P; SUV, small unilamellar vesicles; TFA, trifluoroacetic acid; TGase, transglutaminase EC 2.3.2.13; UV, ultraviolet.

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SP into membranes containing an appreciable amount of negatively charged lipids [6,7] and it has been suggested that different association states can occur depending on the degree of penetration in the lipidic bilayer, and may be relevant to the bioactive conformation [7].

Recent studies with surface active peptides [8] have addressed the advantages of using synthetic fluorescent derivatives, mainly non-tryptophan related probes, to monitor the binding of peptides to biological membranes. There are two inherent advantages to this approach, the possibility of performing studies at physiologically relevant concentrations, and the minimal interference from tryptophan present in membrane proteins. Thus, the sensitivity of the fluorescent label approach could be exploited to gain insight into the molecular mechanisms of SP-membrane interactions.

Since SP contains two adjacent glutamine residues (Gln 5 and 6), the possibility of incorporating a fluorescent probe (monodansylcadaverine-(DNC)) into the peptide was investigated using the transglutaminase (TGase)-reaction [9-12]. Experimental conditions for the enzymatic reaction were initially explored in order to obtain selective labelling of Gln^5. The myotropic potency on the guinea pig trachea of the DNC-labelled SP (DNC-SP), as well as its interaction with model phospholipid vesicles were then examined.

MATERIALS AND METHODS

Materials and chemicals. Substance P and DNC [N-(5-aminopentyl)-5-dimethylamino-1-naphtalene sulphonamide] were obtained from Serva (Heidelberg, Germany). Guinea pig liver transglutaminase (TGase, R-glutaminyl-peptide:amine γ-glutamyltransferase, EC 2.3.2.13) was from Sigma (St. Louis, MO). Fgg yolk L-α-phosphatidylcholine (EPC) was purchased from Merck (Darmstadt, Germany) and purified according to Singleton et al. [13]. Brain L-α-phosphatidyl-L-serine (PS) was from Sigma. Salts, buffers and reagents were of the highest purity available.

Transglutaminase-mediated chemical modification of substance P. Each 100 µl of reaction mixture contained 2 µM TGase, 20 mM dithiothreitol, 40 mM calcium chloride, 5 mM DNC and 40 nmoles of SP in 100 mM Tris-HCl (pH 8.0) buffer. Reaction mixtures were incubated at different times and temperatures (indicated in the Figures legends) and stopped by addition of 100 µl of 5% trifluoroacetic acid (TFA)-containing water:acetonitrile (7:3, v/v). The experimental conditions for the separation of modified and native peptide by reversed-phase high performance liquid chromatography (RP-HPLC) were similar to those described previously for the amphiphatic peptide melittin [10-11].

Spectroscopic measurements. The circular dichroism (CD) spectra were measured at 23°C with a Jobin Ivon CD-6 under constant nitrogen flush using a 0.5 mm optical pathlength cell. CD data were expressed in terms of mean residue ellipticity, in deg cm^2 $dmol^{-1}$. The reported spectra are the average of five scans. Fluorescence experiments were performed on a Perkin Elmer LS-50 fluorescence spectrophotometer (excitation 330 nm, 5 nm band-pass). All liposome binding assays were performed using small unilamellar vesicles (SUV) as previously described [14].

RESULTS AND DISCUSSION

The transglutaminase-mediated modification of SP was initially studied at 37°C and the reaction monitored by RP-HPLC. As illustrated in Fig. 1, three different peaks appeared in the chromatograms at retention times of 4.7, 18.0 and 20.5 min. These were found to correspond to free DNC, DNC-SP and $(DNC)_2$-SP respectively, when visualized by fluorescence detection. Simultaneous UV detection at 230 nm, however, showed the presence of an additional peptidic peak at a retention time of 7.5 min corresponding to native SP. It was observed that after 15 min incubation a gradual increase in the $(DNC)_2$-SP peak area occurred, with concurrent decreases in the DNC-SP and native SP peaks. The identity of the fluorescent SP peaks was confirmed by amino acid and sequence analysis, which showed that, in monolabelled SP, the probe was specifically attached to Gln^5, the first of the two adjacent glutamine residues, while both glutamines (Gln^5 and Gln^6) were modified in $(DNC)_2$-SP.

It is noteworthy that the two consecutive glutamine residues of SP acted as acyl donors, although the second only served as an effective substrate after incorporation of

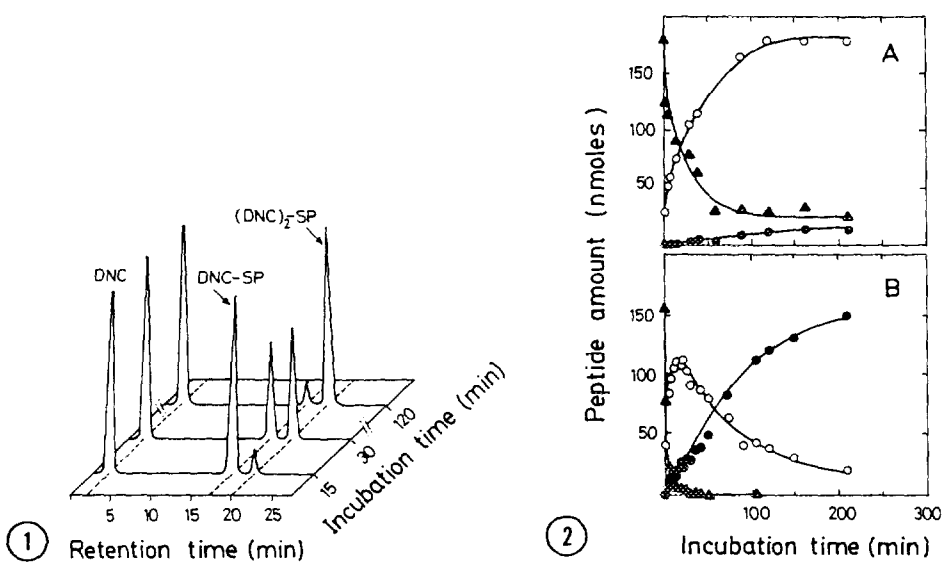

Fig. 1. RP-HPLC elution profiles, at different incubation times, corresponding to TGase-mediated modification of SP with DNC. Chromatographic conditions: column μBondapak C_{18}; flow rate 1 ml/min; injection volume 10 μl; detection by fluorescence emission at 520 nm on excitation at 330 nm; after a 10-min isocratic period, a 20-min linear gradient from 0.1% TFA-containing water-acetonitrile (75:25, v/v) to a final composition of 50:50 (v/v) was used.

Fig. 2. Time course of TGase-mediated modification of SP with DNC at 15°C (**A**) and 37°C (**B**), expressed as nmoles of (SP) (▲), DNC-SP (O) and $(DNC)_2$-SP (●).

a DNC molecule at Gln^5. This contrasts with the reported specificity of TGase where only the first Gln of an adjacent pair is recognized as acyl donor by the enzyme [10,15,16]. While Gln^5 of SP satisfies the minimal requirements for modification recently proposed by Coussons et al. [17], Gln^6 does not. This is presumably because of the positive charge at -5 position (Arg^1) which acts as a discouraging feature for transamidation. The incorporation of the DNC probe in Gln^5 of SP apparently converts Gln^6 into a good substrate for TGase. This observation is of interest regarding the factors that govern the specificity of transglutaminase-catalyzed reactions [17].

Experimental conditions for the TGase reaction were optimized in order to obtain the single fluorescently labelled SP as main product, which seems *a priori* a better candidate to mimic native peptide behavior. As observed in Fig. 2, the temperature of incubation appears to be a crucial parameter controlling the ratio of di- to mono-dansyl derivative. From the kinetic data it appears that a consecutive modification of the two SP glutamines occurs. DNC-SP was the main fluorescent product at 15°C (Fig. 2A) whereas $(DNC)_2$-SP predominated at 37°C (Fig. 2B). After testing the different experimental parameters involved in the enzymatic reaction (i.e., temperature, incubation time, concentration), the following standard assay conditions were chosen: incubation at 15°C for 3h using 200 nmoles of SP with 2 µM TGase in 500 µl of 100 mM Tris-HCl (pH 8.0) buffer containing 5 mM DNC, 20 mM dithiothreitol and 40 mM calcium chloride. These experimental conditions are very different to those previously described for other enzymatic modifications of short peptides [10,11,16]. However, the initial purpose of obtaining a mono-dansylated derivative, with quantitative suppression of molecular crosslinking and high reaction yields (>80%) was fulfilled.

DNC-SP was characterized spectroscopically by UV absorption, fluorescence and CD. Fig. 3A shows the UV spectrum in methanol of the mono-labelled peptide, which was similar to that obtained for an equimolar 1:1 mixture of SP and free DNC. Fig. 3A also shows the characteristic dansyl fluorescence emission spectra upon excitation at 330 nm, in methanol and in 50 mM Mops-NaOH (pH 7.0) buffer. Both emission maxima (530 nm in methanol and 570 nm in buffer) are indicative of a dansyl group exposed to the solvent [18]. Fig. 3B compares the CD spectra for DNC-SP measured in 95% methanol, 80% trifluoroethanol, pure water and in the presence of 15 mM SDS. These spectra were identical to those obtained for the parent compound in the same solvents (not shown) and similar to those reported for non-modified SP [2,6]. These results indicate that incorporation of the DNC probe does not significantly alter the peptide ability to undergo specific, environment-dependent, conformational changes.

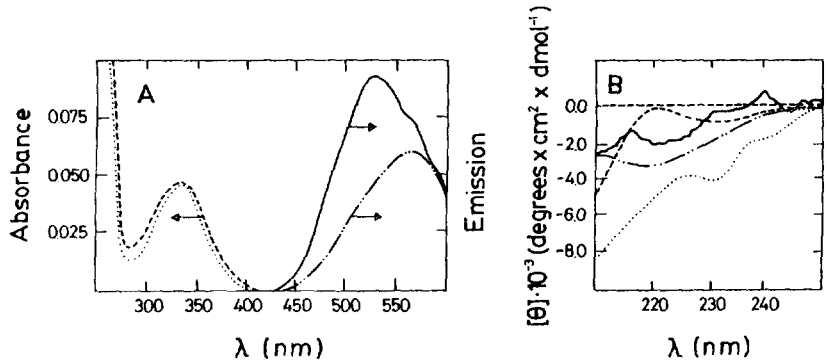

Fig. 3. (A) Left-hand axis: absorption spectra in methanol of DNC-SP (- - - -) and of an equimolar mixture of native SP and free DNC (• • •). Right-hand axis: fluorescence emission spectra of DNC-SP upon excitation at 330 nm in methanol (——) and in 50 mM Mops-NaOH (pH 7.0) buffer (— •• —). Peptide concentration 10 μM. **(B)** CD spectra of DNC-SP in 95% methanol (• • •), 80% trifluoroethanol (——), pure water (- - - -) and in the presence of 15 mM SDS (— •• —). Peptide concentration: 20 μM.

DNC-SP was next assayed for retention of biological activity. This was verified using biological preparations of guinea pig trachea according to the protocol described by Mizrahi et al. [19]. The equimolecular response ratio (ERR) was calculated as described by Karagiannis et al. [20]. When using peptide concentrations around the IC_{50} determined for SP, i.e.10^{-7}M, we obtained an ERR(DNC-SP/SP)=0.9±0.2. Taken together, the above results suggest that incorporation of the fluorescent probe not only does not alter the peptide conformation in solution, nor does it interfere with the adoption of a putative bioactive conformation.

The interaction of DNC-SP with a model membrane was monitored by steady-state fluorescence. Fig. 4A shows the titration of the labelled peptide with negatively charged

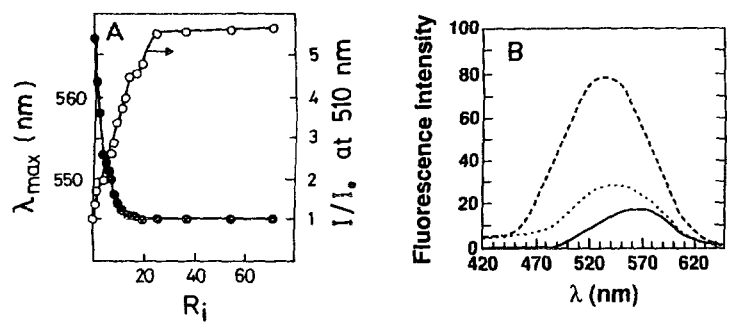

Fig. 4. (A) Titration of DNC-SP with EPC/PS (4:1, mol/mol) SUV. The fluorescence emission maximum, λ_{max}, (●) and the normalised fluorescence intensity ratio at 510 nm, I/I_0, (O) are expressed as a function of lipid/peptide molar ratio, R_i. λ_{exc} was 330 nm. **(B)** Fluorescence emission spectra of DNC-SP upon excitation at 330 nm in 50 mM Mops-NaOH (pH 7.0) buffer (——), in the presence of EPC/PS (4:1, mol/mol) SUV, R_i=60, before (- - - -) and after 4h of carboxypeptidase Y digestion (• • •).

phospholipid vesicles composed of EPC/PS (4:1, mol/mol). A pronounced blue-shift in the emission maximum (λ_{max}) occurred upon increasing the lipid/peptide molar ratio, R_i, with a sixfold increase in fluorescence intensity. The progressive burying of the probe in the bilayer was accompanied by a concomitant increase in fluorescence anisotropy, from 0.03 in buffer up to 0.07 when bound, indicative of a decrease in the mobility of the fluorophore in the lipidic phase when DNC-SP is incorporated into the membrane. In order to confirm that the main driving force in the binding is due to the peptide itself rather than to a hydrophobic contribution due to the presence of the probe, a solution containing membrane-bound DNC-SP was treated with carboxypeptidase Y. A decrease in fluorescence of the solution was observed as the labelled peptide was digested and released from the membrane (Fig. 4B).

Quantitative analysis of peptide-lipid interaction was carried out based on the above fluorescence intensity data. Binding to the vesicles could be described by either a partition model [21,22] or by a binding model [23,24]. The partition model yielded a partition coefficient of Γ=43000 M^{-1} and an effective charge number for the bounded peptide of υ=0.8±0.3 according to the Gouy-Chapman theory. Assuming a simple binding model, analysis of the data by Klotz's plots enabled an estimation of the number of lipid molecules needed to define an SP binding site, N=9, and of the apparent equilibrium dissociation constant, K_d=7.1x10^{-7}M.

Finally, the advantages of using this type of analogue in peptide-membrane interaction studies deserve some consideration. First, since it is known that dansyl emission is very sensitive to solvent environment [18,25] and particularly to the depth of membrane insertion [25], DNC-SP can be used to trace the degree of peptide penetration into a lipid bilayer. Second, the example shown in this paper demonstrates the potential of the dansyl-SP derivative for the quantitative analysis of the binding of the peptide to different model membranes. In addition, fluorescent analogues prepared in this manner should permit the obtention of dynamic parameters of SP-lipid interactions and may provide evidence for peptide aggregation by using fluorescence decay and energy transfer approaches. Finally, DNC-SP or similar fluorescent derivatives may be used for further investigation of SP-cell membrane interactions since the dansyl probe can be selectively excited without the interference of membrane proteins.

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