Biochimica et Biophysica Acta, 1109 (1992) 55–58

BBAMEM 75712

A new surfactant series, the *N*-alkylamino-1-deoxylactitols: application for extraction of 'op' opiate receptors from frog brain

R. Garelli-Calvet [a], P. Latgé [a], I. Rico [a], A. Lattes [a] and A. Puget [b]

[a] *Laboratoire des IMRCP, UA CNRS No. 470, Université Paul Sabatier, Toulouse (France) and* [b] *UPR CNRS No. 8221, Laboratoire de Pharmacologie et de Toxicologie Fondamentales, Toulouse (France)*

(Received 8 October 1991)
(Revised manuscript received 14 April 1992)

Key words: Opiate receptor; Surfactant; (Frog brain)

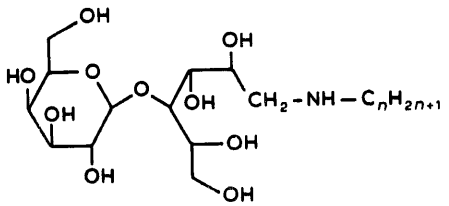
A new series of surfactants, the *N*-alkylamino-1-deoxylactitols, was prepared and employed to extract 'op' opiate receptors from frog brain. These surfactants are both cheap and convenient to prepare. Receptors were reproducibly extracted in a good yield using *N*-nonylamino-1-deoxylactitol. This derivative, which was not denaturing during the extraction process, could thus be used instead of the more costly digitonin, whose rather variable purity affects yield.

Introduction

Proteins can be extracted from membranes using amphiphilic molecules, although in practice rather variable results are obtained depending on the particular amphiphile and its state of purity. Extraction is a crucial step in the study of the mode of action of numerous membrane proteins, especially those of the receptor type.

A variety of surfactants have been employed, such as the widely used Triton series and SDS, although non-ionic sugar derivatives are now tending to be favored by biochemists. For example, various alkylglucosides [1–5], alkylmaltosides [2,3,6] and alkylthioglucosides [7,8] have been studied. However, the preparation of these derivatives requires initial protection of the sugar, and involves a lengthy and costly procedure.

We have recently synthesized a new series of sugar derivatives, the *N*-alkylamino-1-deoxylactitols (general formula, see Formula I).



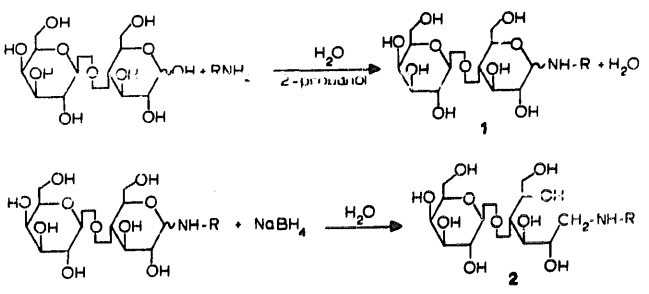
N-Alkylamino-1-deoxy-1-lactitol

Correspondence to: I. Rico, Laboratoire des IMRCP, UA CNRS No. 470, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse cedex, France.

The method of synthesis in two steps has been described elsewhere [9,10] and can be schematized as shown in Scheme I.

The *N*-alkyllactosylamines **1** cannot be used as surfactants since they hydrolyze spontaneously in aqueous solution to give the starting amine and lactose. Reduction of these derivatives stabilizes the open form, affording the *N*-alkylamino-1-deoxy-1-lactitols **2** in good yield (Table I). The compounds were characterized by ^{1}H-NMR, mass spectroscopy and elemental analysis [10]. The values of CMC of the compounds were determined from surface tension measurements in aqueous solution at 25°C (Table I). These derivatives are good surfactants and cost little [9,10]. We therefore examined their suitability for extraction of membrane proteins.

In a first set of experiments, we attempted to extract the 'op' opiate receptors from the brain of the frog *Rana ridibunda*. A variety of agents have been used for the extraction of these rather fragile receptors. Digitonin has been found to be the least denaturing, and to solubilize these receptors with least loss of activity. Up



Scheme I.

TABLE I

Yields and CMC values of the N-alkylamino-1-deoxylactitols

C_n	Yield (%)	CMC (mol/l)
C_8	55	$1.5 \cdot 10^{-2}$
C_9	60	$5.6 \cdot 10^{-3}$
C_{10}	80	$2.6 \cdot 10^{-3}$
C_{12}	77	$6.0 \cdot 10^{-4}$

to 50% solubilization has been obtained in the best cases. However, there has been little success with the alkylglucoside or alkylmaltosides for this receptor [11]. For example, less than 10% of opiate receptors were solubilized with octylglucoside. Furthermore, with digitonin, the extent of solubilization ranges from 33% to 50% depending on the purity (50–80%) of commercially available product.

In the present study, we assayed extracted solubilized receptors from their specific binding to the tritiated ligand diprenorphine [11,12], and compared results obtained by solubilization with various *N*-alkylamino-1-deoxylactitols to those obtained with digitonin.

Materials and Methods

The frogs (*Rana ridibunda*) originally from Albania and Egypt were supplied by Arbona & Novier (Bourg-en-Bresse, France).

[15,16(*n*)-^{3}H]Diprenorphine (31 Ci/mmol) was supplied by Amersham, unlabeled diprenorphine was from Reckitt and Colman, and digitonin was from Sigma.

The *N*-alkylamino-1-deoxylactitols were prepared as follows according to a method described in detail elsewhere [9,10].

Synthesis of surfactants

N-Alkyllactosylamines 1

A solution of the alkylamine (50 mmol) in 2-propanol (100 ml) is added to a solution of lactose monohydrate (30 mmol, 10.8 g) in water (60 ml). The reaction mixture is agitated for 24 h at ambient temperature, and then heated at 60°C for 30 min. The solvent is evaporated under reduced pressure, the residue is taken up in ethanol and reevaporated in the presence of toluene to eliminate residual water. The solid product is recrystallized from ethanol, and then freeze-dried.

N-Alkylamino-1-deoxylactitols 2

A solution of alkyllactosylamine (0.01 mol) in water (70 ml) is cooled in an ice bath. A solution of $NaBH_4$ (0.013 mol) in water (15 ml) is then added dropwise. The mixture is agitated for 30 min, and then treated with active carbon. After filtration on Celite, the water is evaporated. The residue is taken up in methanol and evaporated five times in succession. A white flaky powder is obtained on freeze-drying.

Extraction of opioid receptors

Preparation of crude membrane fraction (CMF)

After decapitation, 2 g of frog brain are removed, weighed and homogenized by 20 up-and-down movements at 800–1000 rpm in a Potter-Elvehjem tube in Tris buffer (50 mM, pH 7.4). The homogenate is centrifuged at $100000 \times g$ for 35 min in a Polytron TGA 65 ultracentrifuge equipped with a type 30 rotor (Beckman). After removal of supernatant, the membrane pellet is resuspended in Tris buffer (50 mM, pH 7.4) and centrifuged as above. The pellet is homogenized again in a Potter-Elvehjem tube in 16 ml of Tris buffer (50 mM, pH 7.4). This constitutes the crude membrane fraction (CMF).

Preparation of soluble extract

Different amounts of alkylaminolactitols (0.5–4%, w/v) are added to 1 ml of crude membrane fraction. The highest solubilization was obtained after agitation for 1 h at 4°C. After centrifugation at $100000 \times g$, the supernatant constituted the soluble fraction (SF).

Opiate receptor binding assay

All the following experiments were carried out in triplicate.

(i) Unlabeling

Preparation of samples A for total binding assay. 50 μl of the soluble extract prepared with each concentration of surfactant is diluted with 750 μl of Tris buffer (50 mM, pH 7.4), 100 μl of an aqueous solution of NaCl (1.2 M) and 100 μl of an aqueous solution of tritiated diprenorphine (10 nM). The samples are incubated for 1 h at 25°C.

Total binding. The previously prepared samples A are rapidly filtered through glass fiber disks (0.25 mm, Whatman GF/B) using a Millipore Model 1225 filtration ramp. The filters are soaked in an aqueous solution of polyethyleneimine (0.33%, v/v), and rinsed three times with 3 ml of 10 mM Tris buffer. 3 ml of Beckman MP scintillation fluid are added to each filter. The radioactivity determined in a Kontron MR 300 liquid scintillation counter is the total binding activity.

Preparation of samples B for non-specific binding assay. 50 μl of the soluble extract prepared with each concentration of surfactant is diluted with 650 μl of Tris buffer (50 mM, pH 7.4), 100 μl of an aqueous solution of NaCl (1.2 M) and 100 μl of an aqueous solution of tritiated diprenorphine (10 nM). Non-

specific binding is determined by addition of a large excess of cold diprenorphine.

Non-specific binding. The same procedure is used with samples B as described above for samples A.

Specific binding. For each concentration of surfactant, the specific binding is the difference between the total binding in samples A and the non-specific binding in samples B.

Percent solubilization of opiate receptors for each surfactant concentration. The specific binding of the crude membrane fraction (CMF) was obtained in the same way as described above for the soluble fraction. The percentage solubilization for each surfactant concentration is given by the ratio of the specific binding of soluble fraction over that of the crude fraction.

(ii) Prelabeling

Preparation of samples A for determination of total binding. 1 ml of a solution of NaCl (1.2 M) and 1 ml of a solution of tritiated diprenorphine (10 nM) are added to 8 ml of crude membrane fraction. The final concentration of NaCl and [^{3}H]diprenorphine are thus 120 mM and 1 nM, respectively.

Preparation of samples B for determination of non-specific binding. 1 ml of a solution of NaCl (1.2 M), 1 ml of a solution of tritiated diprenorphine (10 nM) and 1 ml of a solution of unlabeled diprenorphine (10 μM) are added to 7 ml of crude membrane fraction. The final concentrations of NaCl, [^{3}H]diprenorphine and unlabeled diprenorphine are thus 120 mM and 1 nM and 1 μM, respectively.

Total and non-specific binding. Samples A and B are incubated for 1 h at 25°C. The two labeled CMF are centrifuged at 100 000 $\times g$ for 35 min to remove excess radioactive ligand. After removal of supernatant, the residue is homogenized in 8.0 ml of Tris buffer (50 mM, pH 7.4). 1-ml aliquots of the two suspensions are then solubilized with different concentrations of surfactant for 1 h at 4°C, and then centrifuged (100 000 $\times g$, 35 min) to remove insoluble material. Binding is assayed on 100 μl aliquots as described above.

Specific binding and % solubilization of opiate receptors. Total binding is assayed in samples A for the different surfactant concentrations. Non-specific binding is assayed in samples B. Specific binding is represented by the difference between the two. The percentage solubilization of opiate receptors is calculated as described above for the un-labeling experiments.

Results

The results obtained with surfactants 2 of alkyl chain lengths of 8, 9, 10 and 12 carbon atoms are summarized in figs. 1 and 2. Un-labeling (Fig. 1) designates solubilization of the crude membrane extract by surfactant followed by labeling of the solubilized part.

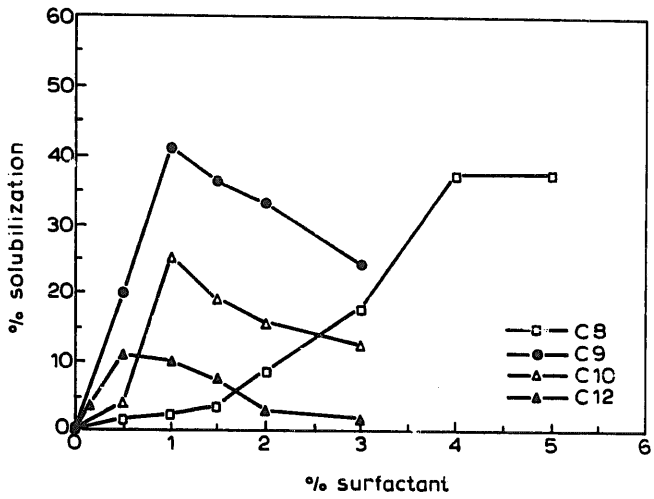


Fig. 1. Yields of solubilized opiate receptors: unlabeling. N.B. With digitonin, the maximum solubilization (2.06 mg/ml) was obtained at a concentration of 1%. It ranged from3 3% to 50% depending on the purity of the digitonin.

Pre-labeling (Fig. 2) designates labeling of the crude membrane extract followed by solubilization.

The following results were obtained:

(i) For both un- and pre-labeling, the shorter the alkyl chain, the more surfactant was required for maximum solubilization.

(ii) Opiate binding in the solubilized extract was generally higher in the pre- than in the un-labeling conditions, especially for the long-chain surfactants ($C_{12} > C_{10} > C_9 > C_8$).

(iii) For un-labeling, the following conclusions were drawn about the behavior of the various surfactants. Low solubilization was observed with the C_{12} derivative at all concentrations tested. The C_9 and C_{10} derivatives at 1% gave maximum solubilizations of 41%

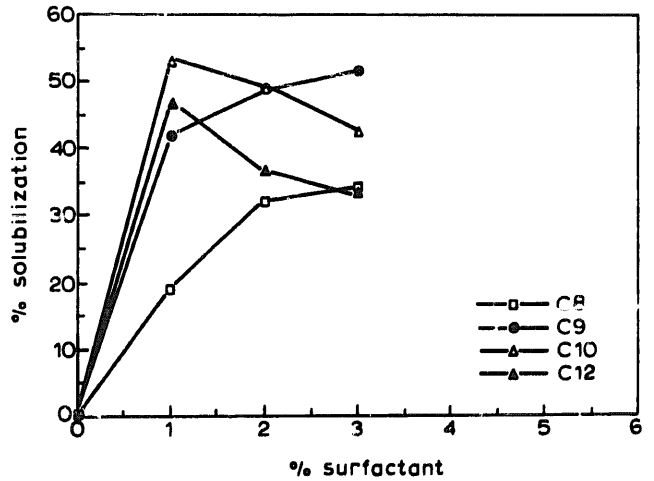


Fig. 2. Yields of solubilized opiate receptors: prelabeling. N.B. With digitonin, the maximum solubilization (61%) was obtained at a concentration of 1%.

and 25%, respectively. The C_8 derivative produced a 37% solubilization at a concentration of 4%.

(iiii) In the pre-labeling conditions, the C_{12} and C_{10} derivatives at a concentration of 1% gave maximum solubilizations of 47% and 53%, respectively. A higher concentration of the C_9 derivative (2%) was required for a comparable level of solubilization. The C_8 derivative only produced 30% solubilization at a concentration of 3%.

Discussion

(1) The observation that the shorter the alkyl chain, the more surfactant is required to obtain maximum solubilization can be accounted for in terms of hydrophobic interactions and critical micellar concentration (CMC). Proteins are thought to be solubilized by surfactants via micelle formation [13–15]. The shorter the carbon chain of the surfactant, the higher the critical micellar concentration (weaker hydrophobic interactions), and so the more surfactant will be required to reach the CMC and hence solubilize membrane proteins in both the un- and pre-labeling conditions. The C_8 derivative is thus too short for solubilization of membrane receptors.

(2) The fact that opiate binding in the solubilized extract was generally higher in the pre- than in the un-labeling experiments may be due to two processes:

(i) Surfactants especially at high concentrations tend to progressively denature opiate receptors. This has been observed for digitonin [12] and CHAPS [16], although it was observed for longer durations of incubation than those used in the present study (1 h at 4°C).

(ii) During un-labeling, the surfactant may itself bind to the receptor site. This inhibition of opiate binding has been invoked to account for variations in solubilization yield with digitonin under different conditions (un- or pre-labeling). This type of inhibition of opiate binding does not appear to be readily reversible without removal of the surfactant [11]. This inhibitory effect was less significant in the pre-labeling conditions as the receptor has more affinity for the radioactive ligand on initial exposure.

(3) This inhibitory effect was most marked with the C_{12} derivative (Figs. 1 and 2). From the CMCs shown in Fig. 1, the inhibitory effect appears more to be significant than a denaturing action. At 16 times the CMC (comparable in water at 25°C and in Tris buffer at 4°C), the percent solubilization was much lower for the C_{12} derivative (10.7% at 0.5% surfactant) than for the C_{10} derivative (17% at 2% surfactant $\approx 16 \times$ CMC for C_{10}). There was a progressive increase in solubilization (1.8% at the CMC to 10.7% at $16 \times$ CMC) for concentrations of C_{12} ranging from 0.03% to 0.5% (CMC – $16 \times$ CMC). These observations indicate that the inhibitory effect could be due to the more flexible alkyl chain, which when extended may fit into the receptor binding site [13–15].

(4) The inhibitory effect fell with decreasing chain length (Figs. 1 and 2). It should be noted that at a concentration of 1% ($4 \times$ CMC), the C_9 derivative gave comparable yields in the pre- and un-labeling experiments. At this concentration it does not appear to inhibit ligand binding. Moreover, the C_9 derivative gave a 40% solubilization of receptors, which is comparable to that obtained with digitonin.

Conclusion

These preliminary results using *N*-alkylamino-1-deoxylactitols for extraction of opiate receptors are encouraging. Out of a series of derivatives (C_8 to C_{12}), *N*-nonylamino-1-deoxylactitol (C_9) gave similar levels of solubilization to those obtained with digitonin in both pre- and un-labeling experiments. This derivative has the advantage over digitonin that it does not inhibit ligand binding to opiate receptors when employed at a concentration of 1%. Furthermore, apart from being expensive, the rather variable purity of digitonin (50% to 80%) gives rise to non-reproducible results. *N*-Nonylamino-1-deoxylactitol has higher purity and is much cheaper than digitonin, and could thus replace it for extraction of 'op' receptors.

References

1 Stubbs, G.W., Smith, H.G. and Litman, B.J. (1976) Biochim. Biophys. Acta 425, 46.
2 De Grip, W.J. and Bouee-Geurts, P.H.M. (1979) Chem. Phys. Lipids 23, 321.
3 Rosevear, P., Vanaken, T., Baxter, J. and Ferguson-Miller, S. (1980) Biochemistry 19, 4108.
4 Gould, R.J., Ginsberg, B.H. and Spectok, A.A. (1981) Biochemistry 20, 6776.
5 Vanaken, T., Foxall-Vanaken, S., Castleman, S. and Ferguson-Miller, S. (1986) Methods Enzymol. 125, 27.
6 Knudsen, P. and Hubbell, W.L. (1978) Membr. Biochem. 1, 297.
7 Saito, S. and Tsuchiya, T. (1985) Chem. Pharm. Bull. 33, 503.
8 Shimamoto, T., Saito, S. and Tsuchiya, T. (1985) J. Biochem. 97, 1807.
9 Latgé, P., Rico, I., Lattes, A. and Godefroy, L. *N*-Lactylamines et leurs procédés de préparation, French Patent No. 9005338, 26-04-1990.
10 Latgé, P., Rico, I., Garelli, R. and Lattes, A. (1991) J. Disp. Sci. Technol. 12, 227.
11 Mollereau, C., Pascaud, A., Baillat, G., Mazarguil, H., Puget, A. and Menier, J.C. (1988) Eur. J. Pharm. 150, 75.
12 Baillat, G. (1988) Thèse de Docteur-Ingénieur, No. 985, Université Paul Sabatier, France.
13 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29.
14 Tanford, C. and Reynolds, J.A. (1976) Biochim. Biophys. Acta 457, 133.
15 Helenius, A., McCaslin, D.R., Fries, E. and Tanford, C. (1979) Methods Enzymol. 56, 734.
16 Simonds, W.F., Koskei, G., Streaty, R.A., Hjelmeland, L.M. and Klee, W.A. (1980) Proc. Natl. Acad. Sci. USA 77, 4623.