Biochimica et Biophysica Acta, 693 (1982) 497–502
Elsevier Biomedical Press

BBA Report

BBA 70074

DETERGENT EXTRACTION OF MEMBRANE PROTEINS RELATED TO THE ACTION OF ANTIDIURETIC HORMONE *

PIERRE RIPOCHE, MARIO PARISI, JACQUES CHEVALIER, EMILIO RIVAS and JACQUES BOURGUET

Département de Biologie, C.E.N. Saclay, 91191 Gif-sur-Yvette (France)

(Received June 11th, 1982)

Key words: Antidiuretic hormone; Oxytocin; Detergent extraction; Membrane protein; Polyoxyethylene nonylphenol; Water permeability; (Frog urinary bladder)

Antidiuretic hormone (ADH) induces, in the apical plasma membrane of target cells, the insertion of intramembranous particle aggregates that probably contain water channels. A mild attack of this membrane by a polyoxyethylene nonylphenyl detergent, which reversibly depressed ADH-induced water permeability, has been found to modify aggregate structure while extracting additional proteins. This simple procedure could be a valuable approach to the problem of aggregate isolation and characterization.

Freeze-fracture studies have made it possible to visualize some of the structural changes induced by ADH in epithelial barriers and strong experimental evidence indicates that the intramembranous particle aggregates observed in the apical border of granular cells, in amphibian urinary bladders, represent water channels [1–3]. These aggregates, related to the increase in water permeability [3] are probably transferred from cytoplasmic vesicles or tubules to the apical membrane during the hydrosmotic response [4]. Because the aggregates, after ADH action, are facing the incubation medium, it was possible to make a mild attack on the bladder apical surface employing various detergents, in an attempt to remove the aggregates while maintaining most of the cell integrity and function.

We now present results showing that a non-ionic surfactant (the six-oxyethylene nonylphenol) added to the apical medium (0.5–1.0 mg/ml during 5 min) induced reversible changes in membrane permeability and structure, accompanied by the extraction of 6–10 μg of protein per cm^2 of epithelial tissue. Different permeability modifications were observed if the detergent was added before or after ADH stimulation (oxytocin, in the present experiments, has been used instead, as a full equivalent of the natural antidiuretic hormone of the natural species). SDS-polyacrylamide gel electrophoresis of the surfactant extract also showed a different pattern if oxytocin was previously added to the solution bathing the serosal surface: under hormonal action one or two extra protein bands (molecular weight 50000 and 71000) could be observed. Furthermore, the extracted material was less phosphorylated in the presence of ADH than in control conditions.

Frog (*Rana esculenta*) urinary bladders were mounted as flat sheets between two lucite chambers containing a 2.5 mM bicarbonate Ringer solution, pH 8.1 [3]). In non-stimulated preparations it was observed (Fig. 1a) that, after 5 min incubation with the surfactant in the apical medium, neither the unidirectional nor the net water movement were modified, although the subsequent hydrosmotic response to oxytocin was in-

* Parts of this work were presented at the Symposium on 'Hormonal Control of Epithelial Transport', INSERM ed., Paris, 1979.

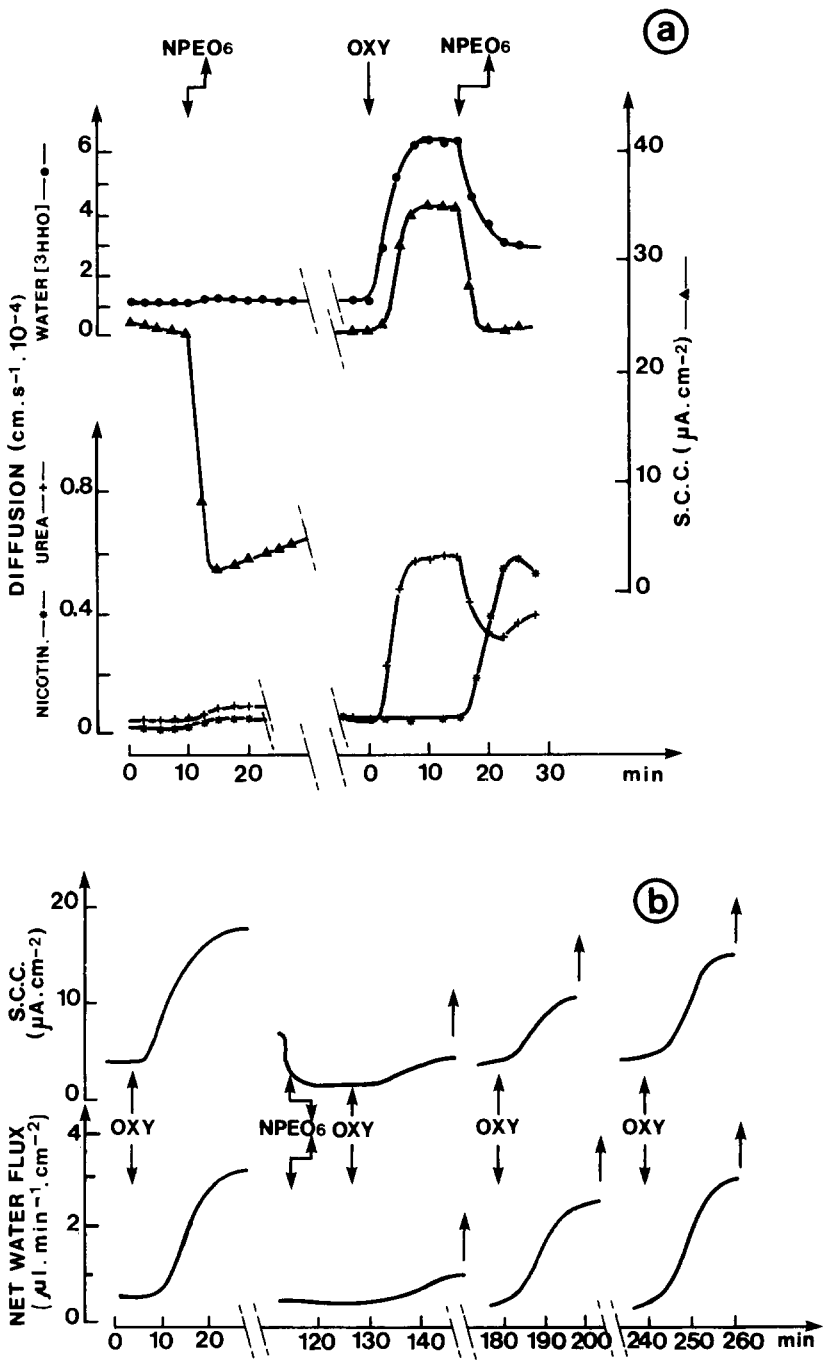
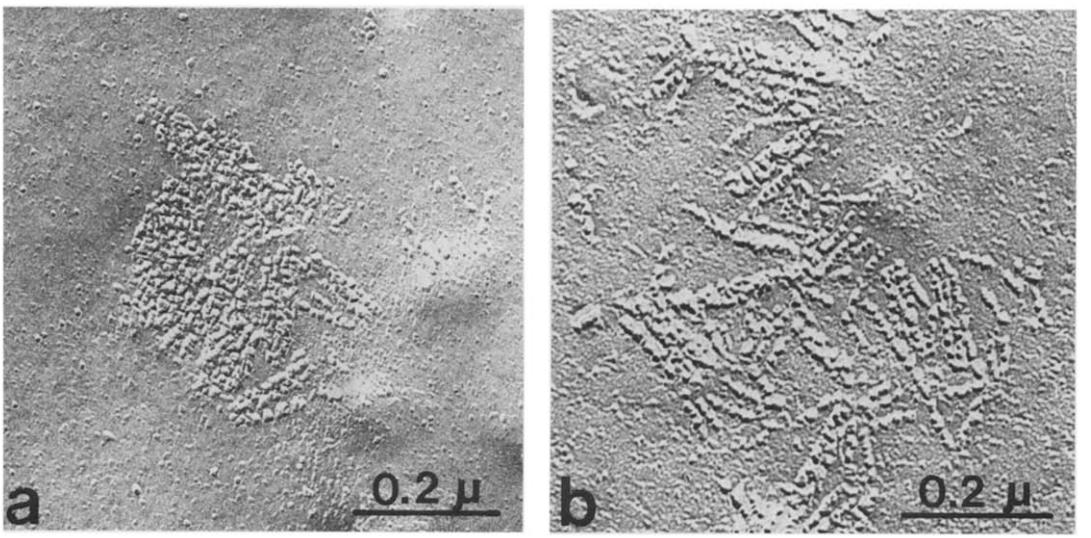


Fig. 1. (a) Effect of oxyethylene nonylphenol (NPEO6, 1 mg/ml, 5 min pulse in the mucosal bath) on short-circuit current and unidirectional tritiated water, [^{14}C]urea and [^{14}C]nicotinamide fluxes, before and after oxytocin action, individual experiment. Mean values before and after 5-min detergent incubation, from non-stimulated bladders: short circuit current 11.4 ± 1.6 vs. 4.4 ± 1.0 $\mu A \cdot cm^{-2}$, $n = 10$; K_{trans} ($cm \cdot s^{-1} \cdot 10^{-4}$): tritiated water 1.23 ± 0.21 vs. 1.26 ± 0.22, $n = 6$); urea 0.016 ± 0.004 vs. 0.032 ± 0.003, $n = 5$; nicotinamide 0.020 ± 0.004 vs. 0.035 ± 0.003, $n = 10$. After oxytocin: short circuit current: 35 ± 7 vs. 8 ± 3 $\mu A \cdot cm^{-}$ 8U2, $n = 4$; K_{trans} ($cm \cdot s^{-1} \cdot 10^{-4}$): tritiated water 3.86 ± 0.6 vs. 1.05 ± 0.26, $n = 6$; urea 0.130 ± 0.048 vs. 0.080 ± 0.024, $n = 5$; nicotinamide 0.023 ± 0.003 vs. 0.095 ± 0.023, $n = 5$. (b) Recovery of the natriferic and hydrosmotic responses to oxytocin after inhibition by the detergent.

hibited (both parameters were simultaneously determined, minute by minute, in the presence of an osmotic gradient, as previously described [5]). Short-circuit current was simultaneously reduced in non-stimulated preparations, while nicotinamide and urea movements, estimated from fluxes of ^{14}C-tagged molecules, were increased (Fig. 1a). After oxytocin stimulation, the observed increases in water, urea and sodium fluxes were reduced by the detergent action, while nicotinamide transfer was strongly increased (Fig. 1a). All these effects were slowly but completely reversible (Fig. 1b).

Concomitant with the surfactant inhibition of the hydrosmotic response, the fraction of the surface area of the mucosal membrane occupied by the aggregates was significantly reduced, as estimated from freeze-fracture studies: 0.40 ± 0.10 $(n = 4)\%$ vs. 0.09 ± 0.03 $(n = 5)\%$ of the apical surface occupied by the aggregates, $P < 0.025$. (See Ref. 6 for details on freeze-fracture methodology). The few remaining aggregates sometimes appeared as splitted up and reduced to small alignments of particles (Fig. 2). Furthermore, the densities of intramembranous particles measured at rest both



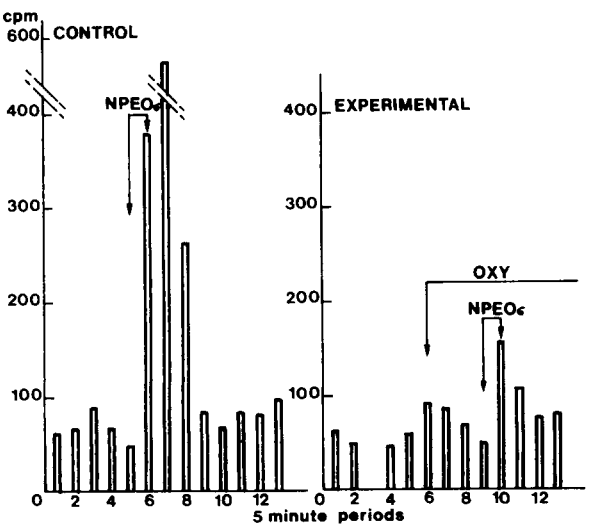


Fig. 2. Upper part: Effects of detergent extraction on the structure of particle aggregates induced by ADH in the apical border of frog urinary bladder. (a) Before surfactant action, typical particle aggregates are visible in the protoplasmic face. (b) After surfactant, some aggregates appear disintegrated. Linear arrays of particles are less densely packed than in normal aggregates whose typical organization remains still visible. Lower part: Phosphorylated protein release. A Ringer solution containing 100 μCi of ^{32}P/ml was added to the serosal side of bladders mounted in two channel lucite chambers. After 4 h incubation, the radioactive solution was repeatedly washed and oxytocin was added to one channel only. Twenty minutes later both sides were treated by the detergent (NPEO6) added in the apical bath (1 mg/ml, 5 min) and after that washed with cold Ringer solution. Fractions were collected at 5-min intervals, and proteins were precipitated, filtered and counted as described above. On the left: control channel. On the right: experimental channel, in the presence of oxytocin ($4.4 \cdot 10^{-8}$ M). Each bar corresponds to the labelled proteins released during 5 min.

on the cytoplasmic and external faces in the apical border of granular cells, were significantly reduced by the detergent action (particles per μm^2: cytoplasmic face 184 ± 12 vs. 0 ± 5, $n = 4$, external face 8518b + 26 vs. 413 ± 41, $n = 4$).

In other experiments, the enzymatic iodination of the apical border was carried out according to the technique described by Strum and Edelman [7]. After washing, the mucosal medium was replaced and collected every 5 min before and after a 5-min pulse with the nonylphenyl surfactant, 0.5 mg/ml (non-stimulated bladders). The samples were precipitated with trichloroacetic acid, passed through Millipore filters, washed, dried and counted for ^{125}I. A transient increase in the release of iodinated proteins was observed after the detergent action (first period before the detergent 1236 cpm/min, first period after 14254 cpm/min, mean difference 13018 ± 546 cpm/min, $n = 4$). There was some specificity in the surfactant-induced protein extraction: if the iodinated preparations were subjected to the action of trypsin (1 mg/ml, 30 min) or pronase (0.25 mg/ml, 30 min) a transient increase in the release of ^{125}I was observed, with no change in the hydrosmotic response to oxytocin. The further incubation with the nonylphenol induced further protein extraction, now accompanied by the inhibition of the hydrosmotic response to ADH.

Protein phosphorylation experiments were also performed, according to the technique described by De Lorenzo and Greengard [8]. It was observed that the surfactant also induced a transient release of ^{32}P linked to proteins, but this release was decreased in the oxytocin stimulated preparations (Fig. 2, lower part).

The extracted proteins were analyzed by polyacrylamide gel electrophoresis. Four batches (each representing 8 to 10 bladders) were extracted in each experiment: two under control conditions and two after oxytocin stimulation. The pattern of extracted proteins is shown in Fig. 3. Several peaks were regularly present in all preparations: number 7 was always the most important one (M_r 43400 $\pm$ 340, $n = 12$). In the oxytocin treated preparations (six experiments) one or two extra protein peaks appeared: peak 6 (M_r 50000) was observed in three experiments and peak 5 (M_r 71000) in four; both extra peaks were simultaneously present in one experiment. We have no explanation for this variability in the ADH-induced modification between different experimental series. Whether the 50 and 71 kDa peaks are part of independent or related molecular systems remains an open question.

Polyacrylamide gel electrophoresis of the phosphorylated protein extracted by the detergent were also made. The stained gels were dried and placed in close contact with Kodak Royal X-Omat films. After 3 or 10 days of exposure, stained gels and autoradiographs were read with a Vernon PIIS spectrophotometer. High radioactivity peaks were observed at 100, 56, 50 and 44 kDa. The radioactive level of both the 56 and 50 kDa bands were lower in the presence of oxytocin.

Present results show that the treatment of frog urinary bladder apical surface with the very hydrophobic oxyethylene nonylphenol reversibly and differently modified water, sodium, urea and nicotinamide permeation. The surfactant reduced sodium permeability both at rest and after oxytocin challenge. Water and urea permeabilities were reduced by the detergent only after ADH action. The fact that intramembrane aggregates are absent before hormonal stimulation [1,2] and that, after hormonal action, the detergent strongly reduced both both permeability and the number of aggregates indicates, as previously suggested [10], that these aggregates represent ADH-induced water channels. The increase in nicotinamide permeability, either in non-stimulated bladders or after ADH, probably also indicates an effect at the level of the lipid bilayer, the accepted pathway for this molecule.

These effects probably reflect detergent-protein and detergent-lipid interactions. Some of the extracted proteins were released from the apical plasma membrane, as indicated by iodination experiments. These proteins are very hydrophobic and we were unable to separate then from the detergent. Furthermore, a high decrease in the intramembranous particle density was observed in the apical membrane treated by the surfactant. It is difficult to establish the extent to which the observed permeability modifications are related to the presence of the detergent into the membrane. In some experiments using ^{14}C-labelled nonylphenol, the release of ^{14}C activity into the apical

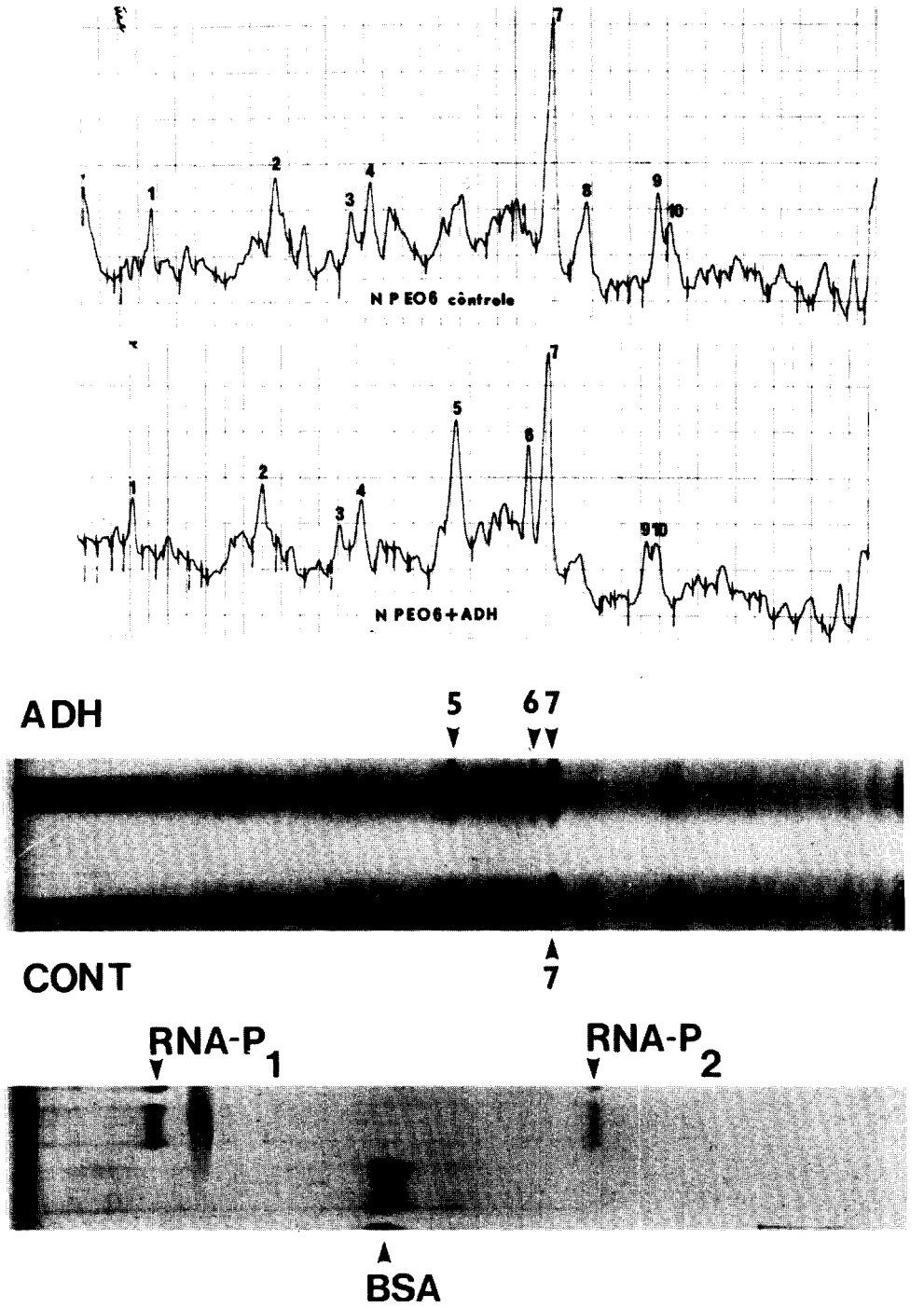


Fig. 3. Polyacrylamide gel electrophoresis of the surfactant extracted proteins in control conditions (CONT) or after ocytocin $4.4 \cdot 10^{-8}$ M (ADH). In order to concentrate the extracted proteins, batteries of 10 chambers were prepared, each having 3.14 cm^2 of exposed apical surface for a 3 ml mucosal bath. All the preparations were successively exposed, during 5 min, to the same 3-ml detergent solution (1 mg/ml; the mucosal solution was stirred with a teflon helix). Ice cold trichloroacetic acid (up to 10%) was added to the concentrated extract. After centrifugation at $1500 \times g$ for 10 min at 4°C, precipitated proteins were suspended in a large volume of cold distillated water and centrifuged again. This last operation was repeated 3 or 4 times. The pellet was dissolved in 1% SDS and 1% mercaptoethanol at pH 8.3 by incubation for 30 min at 90°C. Samples were centrifuged for 5 min at $12000 \times g$ and the extracted proteins separated in 9% polyacrylamide gels as described by Laemmli [9]. Heavy background in sample gels is due to some unavoidable detergent contamination, in our experimental conditions. BSA, bovine serum albumin; $RNA\text{-}P_1$ and $RNA\text{-}P_2$, RNA polymerase peaks 1 and 2.

bath showed the same time course as the recovery of the hydrosmotic response to oxytocin. The addition of egg yolk lecithin liposomes to the mucosal bath accelerated both the ^{14}C release and the recovery of the hydrosmotic response.

Two differences were observed after oxytocin action in the proteins extracted by the detergent: (1) the appearance of one or two extra protein bands. (2) A reduction in the phosphorylation of one of these bands. Further experiments will be necessary to associate these events with any one of the ADH-induced permeability modifications and specially with the intramembranous particle aggregates. Nevertheless, this detergent seems to be an usefull tool for the studies of the molecular modifications induced by ADH action.

M.P. and E.R. are established investigators from the 'Centre National de la Recherche Scientifique, France' and J.C. from the 'Institut National de la Santé et de la Recherche Médicale, France'.

References

1 Chevalier, J., Bourguet, J. and Hugon, J.S. (1974) Cell Tissue Res. 152, 129–140
2 Kachadorian, W.A., Wade, J.B. and DiScala, V.A. (1975) Science 190, 67–69
3 Bourguet, J., Chevalier, J. and Hugon, J.S. (1976) Biophys. J. 16, 627–639
4 Mueller, J., Kachadorian, W.A. and DiScala, V.A. (1980) J. Cell Biol. 85, 83–95
5 Parisi, M., Bourguet, J., Ripoche, P. and Chevalier, J. (1979) Biochim. Biophys. Acta 556, 509–523
6 Chevalier, J., Parisi, M. and Bourguet, J. (1979) Biol. Cell. 35, 207–210
7 Strum, J.M. and Edelman, I.S. (1973) J. Membrane Biol. 14, 17–32
8 De Lorenzo, R.J. and Greengard, O. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 880–884
9 Laemmli, U.K. (1970) Nature 227, 680–685
10 Bourguet, J., Chevalier, J. and Parisi, M. (1981) in Water Transport Across Epithelia (Ussing, H.H. et al., eds.), Alfred Benzon Symposium 15, pp. 404–421, Munksgaard, Copenhagen