Nardo and Dr. Edmund W. Svastits for assembling the computer-based spectroscopic data acquisition and manipulation system.

Registry No. L-Trp, 73-22-3; indoleamine 2,3-dioxygenase, 9014-51-1.

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Nonionic Detergents Increase the Stoichiometry of Ligand Binding to the Rat Hepatic Galactosyl Receptor†

Darryl A. Ray, Janet A. Oka, and Paul H. Weigel*

Division of Biochemistry, Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, Texas 77550

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ABSTRACT: When digitonin is used to expose intracellular galactosyl (Gal) receptors in isolated rat hepatocytes, only about half of the binding activity for ^{125}I-asialoorosomucoid (ASOR) is found as compared to cells solubilized with Triton X-100. The increased ligand binding in the presence of detergent is not due to a decrease in K_d but could be due either to an increase in the number of ASORs bound per receptor or to exposure of additional receptors. Several experiments support the former explanation. (i) No additional activity is exposed even when 80% of the total cell protein is solubilized with 0.4% digitonin. It is, therefore, unlikely that receptors are in intracellular compartments not permeabilized by digitonin and inaccessible to ^{125}I-ASOR. (ii) Digitonin-treated cells are not solubilized by Triton X-100 if they are first treated with glutaraldehyde under conditions that retain specific binding activity. ^{125}I-ASOR binding to these permeabilized/fixed cells increases about 2-fold in the presence of Triton X-100 and a variety of other detergents (e.g., Triton X-114, Nonidet P-40, Brij-58, and octyl glucoside) but not with the Tween series, saponin, or other detergents. When these fixed cells are washed to remove detergent, ^{125}I-ASOR binding decreases almost to the initial level. (iii) Affinity-purified Gal receptor linked to Sepharose 4B binds approximately twice as much ^{125}I-ASOR in the presence of Triton X-100 as in its absence. The results suggest that the increase in Gal receptor activity in the presence of nonionic detergents is due to an increase in the valency of the receptor rather than to exposure of additional receptors.

The use of nonionic detergents has enabled the solubilization, purification, and characterization of a wide variety of membrane-bound proteins (Helenius & Simons, 1975; Lichtenberg et al., 1983). The nonionic detergent substitutes for phospholipids and membrane proteins and dilutes them during the process of membrane solubilization. In the final isolated protein preparations, detergent substitutes for the phospholipid

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* Author to whom correspondence should be addressed.

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naturally found in bilayers. Determination of the native molecular weight of an isolated membrane protein must correct for the presence of bound detergent (Davis, 1984). The effect of the detergent on the structure and function of solubilized membrane proteins is a relevant consideration but one which is difficult to investigate, since such proteins often cannot be studied in the absence of detergents (Furth, 1980). For many membrane receptors, which contain more than one subunit, there is no consensus on the number of different subunits in the native receptor or the stoichiometry of ligand binding. Notable exceptions include the extensively studied acetylcholine (Conti-Tronconi & Raftery, 1982) and insulin receptors (Czech, 1985; Pang & Shafer, 1984). The human and rabbit Gal[1] receptors have, respectively, one and two types of polypeptides (Ashwell & Harford, 1982). The rat Gal receptor has been shown by protein chemistry (Drickamer et al., 1984) and by gene cloning (Holland et al., 1984; Leung et al., 1985; Spiess & Lodish, 1985) to consist of at least two genes and three polypeptides. It is not yet known whether there is one kind of receptor that is a heterooligomer or if there are different receptor populations. We have recently suggested that there are two subpopulations of functionally distinct Gal receptors and two different parallel pathways for ligand uptake, dissociation, and degradation (Weigel, 1986).

In order to understand how the Gal receptor functions during receptor-mediated endocytosis and recycling (Ashwell & Harford, 1982), it is necessary to know the distribution of receptors within the cell and on its surface. The development of kinetic models describing the function of the system (Bridges et al., 1982; Schwartz et al., 1982) and the interpretation of morphological studies at the electron microscopic level (Wall et al., 1980; Kc'h-Bachofen, 1981; Matsuura et al., 1982; Geuze et al., 19' also require knowledge of the distribution of active (and i ive) receptors within the cell. Total cell receptor activi this and a variety of other membrane receptor syste s been determined by (i) cell disruption using homogeni or sonication, (ii) cell solubilization using nonionic deterg and (iii) cell permeabilization using digitonin or sapo. We have concentrated on the latter technique (Weigel et al., 1983) because it allows retention of gross morphology, can be used to follow the release of internalized ligand that has dissociated from receptor (Oka and Weigel, 1983), and provides access to intracellular receptors (Weigel & Oka, 1983b). For example, one can occupy surface receptors at 4 °C with nonradiolabeled ligand, wash and then permeabilize the cells, and subsequently only measure the binding characteristics of radiolabeled ligand to intracellular receptors. It is important to know, however, that all internal cellular receptors are being detected. Attempts to account for total cell receptor activity in digitonin-permeabilized cells, by reference to the amount of detergent-solubilized receptor activity, usually only account for about half of the activity. Here we report that a variety of nonionic detergents can increase the binding stoichiometry of the Gal receptor either in fixed cells or after affinity purification. A preliminary report of this work has been published (Ray et al., 1984).

Materials and Methods

Materials. The various detergents used in this study and their sources are listed in Table I. Orosomucoid was a gift from Dr. M. Wickerhauser of the American Red Cross Plasma Derivatives Laboratory and was desialylated as previously described (Weigel & Oka, 1982). Glutaraldehyde (25%, EM grade) was from Polysciences. ^{125}I-ASOR was prepared by using iodogen from Pierce Chemical Co. as described previously (Weigel & Oka, 1982). Sephadex G-75, superfine, was from Pharmacia. PMFS, CNBr-activated Sepharose 4B, and digitonin were from Sigma Chemical Co. Digitonin was used as a 1.4% (w/v) solution in absolute ethanol; to achieve final concentrations greater than 0.1% a 20% stock solution in Me_2SO was employed.

Cells. Isolated hepatocytes from male Sprague–Dawley rats (Harlan, Houston, TX) were prepared by using a modified (Weigel & Oka, 1982) collagenase perfusion procedure (Seglen, 1973). The cells were suspended on ice in medium 1/BSA and were >90% viable and single cells. Before use the cells were first incubated at 37 °C for 1 h to increase and stabilize the surface and total cell receptor content (Weigel & Oka, 1983a). Specific binding was determined by using a centrifugation assay at a saturating concentration of ^{125}I-ASOR (1.5 μg/mL) in the presence or absence of a 50-fold excess of ASOR (Weigel, 1980).

Media and Buffers. Medium 1/BSA is a modified Eagle's medium supplemented with 2.4 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.22 g/L $NaHCO_3$, and 0.1% (w/v) BSA, pH 7.4. Buffer 1 contains 143 mM NaCl, 6.8 mM KCl, and 10 mM HEPES, pH 7.4. Buffer A is buffer 1 with 10 mM $CaCl_2$ and 0.1% BSA. The extraction buffer contains 20 mM $CaCl_2$, 400 mM KCl, 1% Triton X-100, and 10 mM Tris-HCl, pH 7.8.

Digitonin-Permeabilized/Glutaraldehyde-Fixed Cells. Cells treated at 37 °C were washed and resuspended to 3×10^6 cells/mL in buffer 1. Digitonin was added to 0.055% (w/v) final concentration, and the cells were rotated gently at 4 °C for 20 min. Glutaraldehyde was added to a concentration of 0.025% (w/v), and the suspension was rotated for another 20 min. The cells were washed twice by centrifugation and resuspended in buffer A.

Gal Receptor Purification. We recently reported a modification of the procedure of Hudgin et al. (1974) for isolation of the Gal receptor from freshly isolated hepatocytes (Ray & Weigel, 1985). Briefly, cells were incubated at 37 °C for 1 h, chilled, centrifuged, and resuspended to 1×10^7/mL in buffer 1 containing 133 μM PMSF. All subsequent steps were at 4 °C in the presence of PMSF. Digitonin was added (0.2% final concentration), and the cells were rotated for 20 min, washed twice with buffer 1 containing 5 mM EGTA, and resuspended in half of the volume of extraction buffer. After being gently mixed for 20 min, the extract was centrifuged (12000*g* for 20 min) and the supernatant fluid was filtered and loaded onto an ASOR–Sepharose 4B column. The column was washed extensively with 50 mM Tris-HCl, pH 7.8, containing 50 mM $CaCl_2$, 500 mM KCl, 0.5% Triton X-100, and the receptor was eluted with 40 mM ammonium acetate, pH 5.5, 400 mM KCl, and 0.5% Triton X-100. A second ASOR–Sepharose column was used to separate the receptor from Triton X-100 (Hudgin et al., 1974). The purified receptor contained the typical three subunits ($M_r \sim$ 43000, 54000, and 63000) and was >95% pure by SDS–polyacrylamide gel electrophoresis. Purified receptor was coupled to CNBr-activated Sepharose 4B in 0.1 M sodium borate buffer, pH 8.5, in the presence of 0.5% Triton X-100 (150 μg of protein/mL of Sepharose) for 4 h at 22 °C. Residual active groups were blocked by incubation with 1 M ethanolamine on ice for 1 h, followed by three cycles of washing in alternate low and high pH buffers. Receptor–Sepharose was stored in buffer 1 containing 0.02% (w/v) azide at 4 °C.

[1] Abbreviations: ASOR, asialoorosomucoid; PMSF, phenylmethanesulfonyl fluoride; Gal, galactosyl; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

Table I: Effect of Detergents on ^{125}I-ASOR Binding to Permeable/Fixed Hepatocytes and Viability of Intact Cells[a]

detergent	% increase	% concn	% reversibility	solubilization	% viability
saponin[b]	0	1.0		no	0
digitonin[b]	0	1.0		no	0
sterox[e]	0	0.5		no	90
Tween 20[b]	10	0.5		no	80
Tween 40[b]	27	0.25		no	80
Tween 65[b]	33	0.5		no	80
Tween 80[b]	6	0.07		no	80
Tween 85[b]	17	0.5		no	80
hexyl β-D-glucopyranoside[c]	0	0.5		no	90
heptyl β-D-glucopyranoside[c]	0	0.5		no[d]	90
decyl β-D-glucopyranoside[c]	30	0.2		yes	0
SPAN 40[f]	26	0.25		no	70
SPAN 60[f]	36	0.25		no	80
SPAN 65[f]	21	0.1		no	90
SPAN 80[f]	32	0.25		no	80
SPAN 85[f]	9	0.25		no	90
glycerin-1-monooleate[f]	26	0.25		no	85
Brij 58[g]	40	0.5		no	0
Brij 35[g]	40	0.1		no	0
CHAPS[h]	40	0.1		no[d]	90
Triton N-101[i]	45	0.05	75	yes	0
dodecyl α-D-maltoside[c]	50	0.04	63	no[d]	0
Triton X-114[i]	65	0.05	75	yes	0
n-octyl β-D-glucopyranoside[b]	70	0.4	80	no[d]	50
Nonidet P-40[b]	90	0.07	80	no[d]	75
Lubrol PX[b]	100	0.1	65	no[d]	0
nonyl β-D-glucopyranoside[c]	130	0.2	65	no[d]	50
cholate[b]	110	0.25	75	no[d]	75
deoxycholate[j]	95	0.07	60	no[d]	50
Triton X-100[i]	102	0.05	75	yes	0
N,*N*-dimethyldodecylamine *N*-oxide[f]	140	0.07	80	yes	0
decanoyl-*N*-methylglucamide[k]	90	0.1	80	no[d]	90

[a] Other detergents were tested for their ability to stimulate ^{125}I-ASOR binding to permeable/fixed cells. Binding of ^{125}I-ASOR was performed in the presence of increasing amounts of the indicated detergents. The maximum stimulation observed is given as a percentage increase above the control value, and the detergent concentration (w/v) at this plateau (or the highest concentration tested) is indicated. The reversibility of the detergent-induced increase was determined for those detergents that caused greater than a 45% increase in binding. Cells were incubated at 4 °C for 20 min in the presence of the indicated concentration of detergent and washed three times by centrifugation, and ^{125}I-ASOR binding was determined. The degree of reversibility is expressed as the percent loss of the detergent-stimulated binding (100% reversibility would be equal to the original level of binding prior to detergent exposure). Cell viability was quantitated by determining the percentage of intact (nonpermeabilized, nonfixed) cells able to exclude trypan blue. The ability of detergents at different concentrations to solubilize intact cells was assessed qualitatively by light microscopy. [b] Sigma Chemical Co. [c] Calbiochem. [d] Cells were lysed at higher concentrations of detergent. [e] Perkin-Elmer. [f] Fluka. [g] Serva. [h] Pierce Chemical Co. [i] Research Products International. [j] Matheson Coleman and Bell Reagents. [k] A gift from Dr. Edmund Czerwinski.

General Methods. Protein was determined by a dye-binding method (Bradford, 1976), using BSA as the standard. Centrifugations of intact or permeable/fixed cell suspensions were, respectively, made at 800*g* or 1000*g* for 3 min in a Beckman Model TJ-6 refrigerated tabletop centrifuge. Permeable/fixed cells were also centrifuged for 20 s in a Beckman microfuge. ^{125}I radioactivity was determined by using a Packard Multiprias 2 γ counter.

Results

Quantification of ^{125}I-ASOR Binding Activity in Permeable vs. Solubilized Hepatocytes. We have been unable to account for all of the cellular Gal receptor activity in digitonin-treated cells if we normalize results to the total activity in a Triton X-100 cell extract. ^{125}I-ASOR binding to permeabilized cells is measured by a centrifugation assay (Weigel, 1980). Binding activity in detergent extracts can be measured by an ammonium sulfate precipitation assay (Hudgin, et al., 1974) or a solid-phase competition assay (Ray & Weigel, 1985). Extracts consistently bind about twice the amount of ^{125}I-ASOR per cell equivalent than do permeabilized cells (Figure 1). These results could be explained by the presence of active receptors in intracellular compartments not permeabilized by digitonin or not accessible to exogenous ^{125}I-ASOR (M_r 40 000). Previous characterization of these digitonin-treated cells showed they are permeable to molecules of at least M_r 200 000 (Weigel et al., 1983). To rule out the inaccessibility explanation, ^{125}I-ASOR binding was determined in cells treated with increasing concentrations of digitonin, which solubilized successively more total cellular protein (Figure 2). Cells treated with 0.05% digitonin lose about 50% of their total protein, virtually all the soluble cytoplasmic contents, and show a maximal increase in the exposure of additional intracellular Gal receptor activity (Weigel et al., 1983). By light microscopy, permeabilized cells are ghostlike; they are more transparent and less refractile, although intracellular structures and organelles can still be seen. The residual protein in cells permeabilized with 0.05% digitonin is therefore predominantly membrane, nuclear, and cytoskeletal protein (black bars in Figure 2). The amount of ^{125}I-ASOR binding to the cell pellet remained nearly the same with increasing digitonin concentrations while the amount of associated protein decreased. Digitonin at 0.10% and 0.15% extracted, respectively, 40% and 55% of these residual proteins. Yet even when up to 80% of the total cell protein was solubilized, there was no further increase in the binding of ^{125}I-ASOR, indicating that no additional inaccessible, cryptic, or buried Gal receptors were exposed. In a separate control the 0.15% digitonin supernatant was mixed with ^{125}I-ASOR and chromatographed over Sephadex G-75. No radioactivity was observed in the region of receptor–ASOR complexes, showing that digitonin did not solubilize Gal receptors. These results indicate that the receptor, which is a transmembrane protein (Harford & Ashwell, 1981; Chiacchia & Drickamer, 1984), is anchored to the cy-

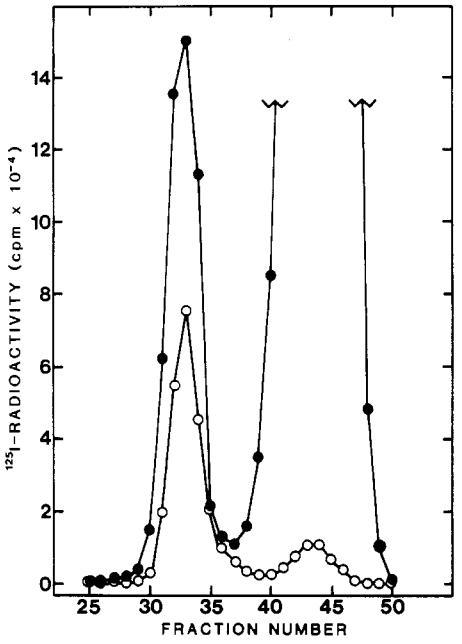

FIGURE 1: Comparison of ^{125}I-ASOR binding activity of digitonin-treated vs. Triton X-100-solubilized cells. Cells were treated with 0.055% digitonin for 10 min at 4 °C, washed, and allowed to bind ^{125}I-ASOR at 4 °C for 60 min in buffer A in the presence (●) or absence (○) of 0.5% Triton X-100. The permeabilized cells in the absence of detergent were then washed twice and solubilized in buffer A containing 0.5% Triton X-100. Both extracts were centrifuged, chromatographed over Sephadex G-75 columns (1 × 37 cm), and eluted with the same buffer. Radioactivity in the first peak, the void volume, represents ^{125}I-ASOR–receptor complexes; free ASOR elutes at fraction 43, the second peak. Specific binding assessed in the presence of a 50-fold excess of nonradioactive ASOR was 94%. Approximately 120% more ^{125}I-ASOR was eluted in the void volume when the digitonin-permeabilized cells were first solubilized with Triton X-100.

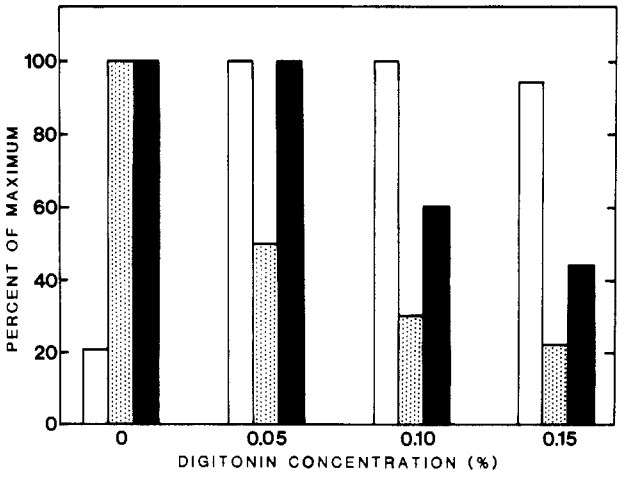

FIGURE 2: Effect of digitonin concentration on the exposure of Gal receptor activity. Cells were incubated at 4 °C for 90 min with buffer A containing 1.5 μg of ^{125}I-ASOR/mL in the presence or absence of excess ASOR and 0%, 0.05%, 0.10%, or 0.15% (w/v) digitonin. The cells were centrifuged, and the protein released into the supernatant was determined. The cell pellets were washed twice, and bound radioactivity was determined. The amount of specifically bound ^{125}I-ASOR (fmol; open bars), protein associated with the cell pellet (speckled bars), and membrane/cytoskeletal protein (solid bars) are normalized to the maximum value for each.

toskeleton. We conclude that the detergent effect on ligand binding is not due to inaccessible receptors in digitonin-permeabilized cells.

Detergent Stimulation of ^{125}I-ASOR Binding to Permeable/Fixed Cells. We have used digitonin-permeabilized, glutaraldehyde-fixed cells as a solid-phase source of receptors in a competition assay for soluble receptor activity. These cells retain at least 75% of their binding activity with about 90% specific binding, have a large number of binding sites, are not solubilized by detergents, and can be prepared in advance, frozen, and used as a reagent. When permeable/fixed cells were incubated in the presence of increasing concentrations of Triton X-100, the amount of specifically bound ^{125}I-ASOR increased by a maximum of about 100% (Figure 3). This stimulation of ^{125}I-ASOR binding occurred as a sharp threshold response above 0.03% detergent and was maximal at 0.05%. The mean stimulation at 0.05% Triton X-100 was 102 ± 35% (n = 22). The increase in apparent Gal receptor activity is reversed almost entirely by washing the permeable/fixed cells to remove the detergent. Permeable/fixed cells were treated with 0.05% Triton X-100 at 4 °C for 20 min and then washed by centrifugation 0–3 times with buffer A without Triton X-100. The final cell pellets were resuspended and incubated with 1.5 μg of ^{125}I-ASOR/mL for 60 min on ice (either in the presence or absence of Triton X-100), and ^{125}I-ASOR binding was determined. After a single washout, the mean loss of the detergent-stimulated binding activity was 75.6 ± 9.6% (n = 5); binding was close to the initial value prior to exposure to detergent. Additional washes of the permeable/fixed cells further decreased the amount of ^{125}I-ASOR binding by only 2–5%. Cells that were incubated with Triton X-100 after the washing steps bound the same amount of ^{125}I-ASOR as unwashed cells, indicating that no loss of cells or receptor binding activity occurred during the washes.

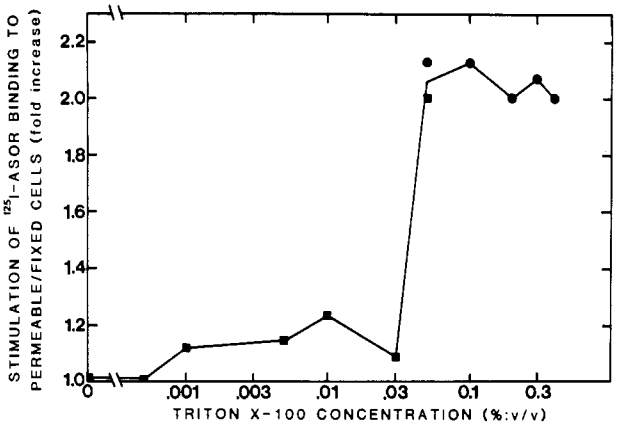

FIGURE 3: Effect of Triton X-100 on the specific binding of ^{125}I-ASOR to digitonin-permeabilized/glutaraldehyde-fixed cells. Specific binding of ^{125}I-ASOR was determined for permeable/fixed cells in buffer A with the indicated concentrations of Triton X-100. Two experiments are shown.

The reversible stimulation of ^{125}I-ASOR binding to permeable/fixed cells by Triton X-100 could reflect an increase in the number of specific binding sites or a decrease in the K_d of ^{125}I-ASOR–receptor complexes. Equilibrium binding studies with permeable/fixed cells showed that the affinity does not change in the presence of detergent but that the number of binding sites increases (Figure 4). The K_d values, calculated by the procedure of Scatchard (1949), in either the presence or absence of Triton X-100, were virtually identical (2.64×10^{-9} M in the presence and 2.88×10^{-9} M in the absence of Triton X-100). The number of femtomoles of ^{125}I-ASOR bound per 10^6 cells at saturation increased 120% in the presence of Triton X-100.

Effect of Other Detergents on ^{125}I-ASOR Binding to Permeable/Fixed Cells. Triton X-100 is not unique in its ability to increase the specific binding of ^{125}I-ASOR to permeable/fixed hepatocytes. A large number of other non-

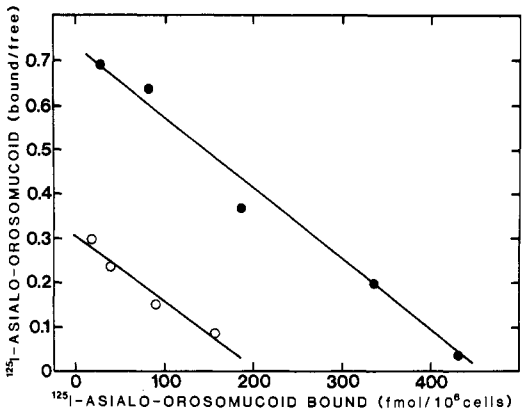

FIGURE 4: Scatchard plot of specific ^{125}I-ASOR binding to permeable/fixed cells in the presence or absence of Triton X-100. Permeable/fixed cells (4×10^6/mL) were incubated with concentrations of ^{125}I-ASOR ranging from 3 ng/mL to 10 μg/mL in either the presence (●) or absence (○) of 0.1% Triton X-100 for 75 min on ice. Cell-associated and free radioactivity in the medium were determined, and the data were analyzed by the method of Scatchard (1949). The lines calculated by a least-squares linear-regression analysis had correlation coefficients of −0.975 (●) and −0.988 (○).

ionic detergents were titrated for their ability to stimulate binding (Table I). Detergents such as Tweens, Spans, saponin, sterox, Brij 35 and 58, glycerin-1-monooleate, and CHAPS either had no effect or gave only a modest stimulation (<40%). In the Triton series, X-114 and N-101 were not as effective as X-100, although they did stimulate binding substantially. The greatest stimulation was also observed with detergents such as Lubrol PX, cholate, deoxycholate, Nonidet P-40, *N*,*N*-dimethyldodecylamine *N*-oxide, and decanoyl-*N*-methylglucamide (Hildreth, 1982). In all cases the shape of the titration curve for the stimulation of ^{125}I-ASOR binding was the same as shown in Figure 3. The *n*-alkyl β-D-glucopyranoside series showed an interesting trend. No stimulation was seen when the alkyl chain was six or seven carbons long. The eight-carbon alkyl glycoside gave a maximal increase of about 70% and the nine-carbon derivative stimulated binding 130%. Surprisingly the 10-carbon glucoside was less effective (30% stimulation) as was the analogous 12-carbon maltoside (50% stimulation). This result suggests that the hydrophobic portion of the detergent may have an optimal size for causing the increase in ^{125}I-ASOR binding to permeable/fixed cells.

Surprisingly, there was no correlation between the ability of a detergent to stimulate binding and its ability to permeabilize or solubilize intact cells (Table I). Not all of the detergents that gave the most stimulation were able to lyse intact cells or even to decrease their viability. For example, cells in the presence of cholate or decanoyl-*N*-methylglucamide showed little or no loss of viability but bound twice as much ^{125}I-ASOR. The detergents that stimulated binding ≥50% were tested further in permeable/fixed cells to determine if their effects were reversible. In all cases the detergent stimulation of ^{125}I-ASOR binding was reversed 60–80% by washing the cells.

Effect of Triton X-100 on Purified Gal Receptor. The detergent stimulation of ^{125}I-ASOR binding was also observed when affinity-purified Gal receptor was coupled to CNBr-activated Sepharose 4B. The receptor was immobilized in order to minimize the possibility of aggregate formation and to facilitate the quantitation of ^{125}I-ASOR binding. The coupling reaction was performed immediately after elution from the ASOR-Sepharose affinity column and was performed in the presence of 0.5% Trition X-100 so that soluble receptor molecules, rather than oligomers or aggregates (Kawasaki & Ashwell, 1976; Andersen et al., 1982), would be coupled to the matrix. The receptor–Sepharose was washed extensively to remove detergent and unbound receptor as described in Materials and Methods. Identical minicolumns containing 100 μL of receptor–Sepharose were washed extensively with buffer A with or without 0.5% Triton X-100, incubated with 200 μL of ^{125}I-ASOR (2 μg/mL) for 30 min, and washed with 5 mL of buffer A. Receptor-bound ^{125}I-ASOR was eluted with 10 mM EGTA, and radioactivity was determined. In two separate experiments, the immobilized purified receptor was stimulated to bind 121% and 162% more ^{125}I-ASOR by the presence of Triton X-100. In the latter experiment, if the receptor–Sepharose was washed with EGTA prior to binding ^{125}I-ASOR, the observed stimulation by Triton X-100 was 105%. Similar results were obtained even when the coupling reaction was performed in the absence of detergent.

Discussion

It is assumed by most investigators that one Gal receptor can bind one asialoglycoprotein molecule. This is reasonable since there is presently no consensus on a model for the subunit composition and stoichiometry of an individual native receptor. In the rat, there are at least three structurally different, although related, polypeptide subunits in affinity-purified receptor preparations (Drickamer et al., 1984; Harford et al., 1982). It is not yet known if there is only one type of receptor that is a heterooligomer containing each of these different subunits or if there are structurally distinct receptor subpopulations composed of these different subunits. Consistent with the latter possibility, we have characterized two functionally distinct populations of Gal receptors in isolated hepatocytes and have proposed that two parallel pathways for ligand uptake, dissociation, and degradation operate in this receptor system (Weigel, 1986; Weigel, et al., 1986).

Triton X-100 was the major detergent used in this study, since it is widely used to solubilize membrane proteins and is used to purify the Gal receptor (Hudgin et al., 1974). The 12 detergents that stimulated ^{125}I-ASOR binding by ≥45% do not have any common structural features, although they all have hydrophobic alkyl chains or rings of varying size. The critical micellar concentrations (cmc) for these detergents are in the millimolar range. For example, *n*-octyl β-D-glucopyranoside, deoxycholate, and Triton X-100 have cmc's, respectively, of 25, 5, and 0.24 mM (Helenius & Simons, 1975). In each case, the lowest concentration that caused a maximal stimulation of ^{125}I-ASOR binding to permeable/fixed cells was approximately equal to the cmc. For these three detergents the values were respectively 13.6, 1.6, and 0.77 mM. The detergent effect on the receptor apparently occurs only when the solubility limit of the detergent is reached and detergent molecules begin to aggregate and to form micelles.

The increase in ^{125}I-ASOR binding in the presence of detergent was not due to the exposure of additional Gal receptors. The titration of ^{125}I-ASOR binding activity with increasing concentrations of digitonin did not expose more binding activity than in the lightly permeabilized cells, even when as much as 60% of the membrane or cytoskeletal protein was solubilized. Under these conditions it is extremely unlikely that any intracellular compartments are still intact and could contain cryptic Gal receptors. The stimulation observed with permeable/fixed cells also suggests that the detergent effect is due to a perturbation of the receptor's structure or immediate environment. The detergents effective in eliciting a maximal response in the permeable/fixed cells were almost completely reversible, suggesting that the detergents did not expose additional receptor molecules in these cells. The covalently

immobilized receptor was also stimulated approximately 2-fold in the presence of Triton X-100. The purified rat receptor aggregates into an oligomeric series except in the presence of a nonionic detergent such as Triton X-100 (Kawasaki & Ashwell, 1976) or Brij 58 (Andersen et al., 1982). The stable monomeric size of the Brij 58 solubilized receptor, determined by sedimentation equilibrium analysis, is 264 ± 16 kDa (Andersen et al. 1982). This is presumably the form of the receptor covalently attached to Sepharose in the presence of Triton X-100. Our evidence supports the conclusion that the valency of the receptor in the presence of detergents is approximately twice that of the receptor when measured in intact or permeabilized cells.

We conclude that under normal native conditions in the intact cell, the receptor valency is half that of the isolated solubilized receptor in the presence of an appropriate detergent. This could be explained in several ways. One possibility is that in the cell the receptor is normally present as a dimer or is in a monomer–dimer (or x-mer–$2x$-mer) equilibrium in which dimer formation is favored. Solubilization by nonionic detergents or dissociation of the receptor dimer in the fixed/permeable cells would result in the exposure (activation) of a binding site in one of the monomers, which in the dimer was previously occluded or blocked sterically from interacting with the ligand. Such a model can explain why some detergents stimulate binding less than 2-fold. A detergent that does not drive the reaction to complete dissociation will stimulate binding only to the extent of dissociation (i.e. <100%). Greater than 100% stimulation, observed for several detergents, is difficult to explain with this model but could represent further dissociation of native monomeric receptor into active subunits.

It has been proposed in a number of other receptor systems that the formation of dimers and small aggregates or clusters of receptors is an important step either in driving receptors to coated pits for internalization or in the generation of subsequent transmembrane signals. The major 43-kDa subunit of the Gal receptor is a transmembrane protein, and there is evidence that the native Gal receptor is also a transmembrane protein (Chiacchia & Drickamer, 1984; Drickamer et al., 1984; Harford et al., 1982). Two native monomer receptors could interact as a dimer to generate a signal necessary for a subsequent step in the functional cycle. Detergents may perturb the normal interaction between these species. Regulation of the monomer–dimer equilibrium by either covalent modification (e.g., phosphorylation/dephosphorylation) or by the interaction of either one of these species with a particular cellular lipid that favored either state could be involved in the overall regulation and function of the recycling pathway.

An alternate possibility is that an individual receptor, whatever its makeup with respect to the number and type of subunits, potentially has available at least two binding sites, although only one is active. Radiation inactivation studies in membranes and in Triton X-100 solubilized preparations indicate that the binding domain for ASOR is respectively 110 and 148 kDa (Steer et al., 1981). The native molecular weight of detergent-solubilized receptor from rabbit and rat is about 250 000–260 000 (Kawasaki & Ashwell, 1976; Andersen et al., 1982). One would therefore deduce that the native receptor has two functional binding sites. Baenziger and Maynard (1980) also concluded that the human Gal receptor contains two oligosaccharide binding sites about 25–30 Å apart. Our results suggest that both binding sites can function under the perturbing influence of some detergents. In the presence of detergent the conformation and organization of the native receptor is changed so that both sites are able to bind ligand.

The 2-fold increase in ligand molecules bound per receptor may be related to the size of the ligand (M_r 40 000). With a smaller ligand one might see a larger increase that would suggest the native receptor was an oligomer composed of multiple active subunits. Each might be able to bind an oligosaccharide chain, and the number of chains bound per native receptor would depend on the size of those chains (Baenziger & Maynard, 1980). Larger ligands would exclude and prevent binding to the maximum number of sites, which would therefore be unoccupied. Hardy et al. (1985) have recently proposed a novel model involving a matrix of carbohydrate binding sites to explain how this receptor can accommodate a different number and arrangement of sugars, which would occur in different oligosaccharide structures and which would in turn give rise to different binding affinities.

Detergent effects have also been reported for other proteins. For example, it was proposed that Triton X-100 intercalates into sarcoplasmic reticulum vesicles and interacts at low concentrations with the Ca^{2+} ATPase to produce a functionally modified enzyme with low affinity for Ca^{2+} and no cooperativity between calcium ion binding sites (McIntosh & Davidson, 1984). Triton X-100 also affects ligand binding and autophosphorylation of insulin and epidermal growth factor receptors (Hwang et al., 1985). The conformational state of the mitochondrial adenine nucleotide carrier is dependent on the detergent used for its solubilization (Block & Vignais, 1986). These and our results indicate that one should not assume that the number of ligand binding sites seen in detergent extracts of a receptor accurately reflects the number of active sites available on native receptors in intact cells.

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Registry No. Tween 20, 9005-64-5; Tween 40, 9005-66-7; Tween 65, 9005-71-4; Tween 80, 9005-65-6; Tween 85, 9005-70-3; SPAN 40, 26266-57-9; SPAN 60, 1338-41-6; SPAN 65, 26658-19-5; SPAN 80, 1338-43-8; SPAN 85, 26266-58-0; Brij 58, 9004-95-9; Brij 35, 9002-92-0; CHAPS, 75621-03-3; Triton N-101, 9016-45-9; Triton X-114, 9036-19-5; Nonidet P-40, 39320-65-5; Lubrol PX, 9002-92-0; Triton X-100, 9002-93-1; decyl D-glucopyranoside, 58846-77-8; glycerin-1-monooleate, 111-03-5; *n*-octyl D-glucopyranoside, 29836-26-8; dodecyl D-maltoside, 69227-93-6; nonyl D-glucopyranoside, 69984-73-2; cholate, 81-25-4; deoxycholate, 83-44-3; *N*,*N*-dimethyldodecylamine *N*-oxide, 1643-20-5; decanoyl-*N*-methylglucamide, 85261-20-7; digitonin, 11024-24-1; galactose, 59-23-4.

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Investigation of the Interaction between Thallous Ions and Gramicidin A in Dimyristoylphosphatidylcholine Vesicles: A Thallium-205 NMR Equilibrium Study†

Dikoma C. Shungu, James F. Hinton,* Roger E. Koeppe II, and Francis S. Millett
Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas 72701
Received March 18, 1986; Revised Manuscript Received June 6, 1986

ABSTRACT: This study reports the first *direct observation* of multiple occupancy of the gramicidin A channel by Tl^+ ions. ^{205}Tl NMR has been used to study the equilibrium binding of Tl^+ by gramicidin A incorporated in sonicated dimyristoylphosphatidylcholine vesicles. It is shown that only multiple-channel occupancy can account for the ^{205}Tl chemical shifts measured. The data are analyzed to yield the equilibrium association constants of 450–600 and 5–20 M^{-1} for the binding of the first and the second ions at 34 °C, respectively.

Gramicidin A, a linear pentadecapeptide antibiotic isolated from *Bacillus brevis*, has a well-known amino acid sequence (Sarges & Witkop, 1965). In natural and artificial lipid membranes, it dimerizes to form ion-transporting transmembrane channels (Hladky & Haydon, 1970, 1972; Bamberg & Lauger, 1973; Urry, 1971; Veatch & Stryer, 1977; Krasne et al., 1971). The channels exhibit a number of properties that make them an ideal model for studying cation transport across biological membranes. They are selective to monovalent cations, impermeable to anions and blocked by divalent cations (Myers & Haydon, 1972; Bamberg & Lauger, 1977; Eisenman et al., 1977; Sandblom et al. 1977); they show high ionic flux rates, ca. 10^7 ions/s^{-1}, concentration dependence of permeability ratios, and single file transport (Finkelstein & Andersen, 1981; Neher et al., 1978; Urban et al., 1980; Andersen, 1984 and references therein). The structure of the membrane-bound, ion-permeable channel has now been established to consist of two left-hand helices, joined head-to-

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