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External Location of Sites on Pig Erythrocyte Membranes that Bind Nitrobenzylthioinosine

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SUMMARY

Nucleoside transport in erythrocytes of various species is inhibited by the binding of nitrobenzylthioinosine (NBMPR) to high affinity sites associated with nucleoside transport elements of the plasma membrane. The present study examined binding of [^{3}H]NBMPR to unsealed ghosts and to sealed right-side-out vesicles (ROVs) and inside-out vesicles (IOVs) prepared from pig erythrocytes. K_d values for NBMPR dissociation from the ligand-site complex in unsealed ghosts, ROVs and IOVs were similar (1.6–2.4 nM), and B_{max} values (mean ± SD) were, respectively, 22.2 ± 5.5, 25.8 ± 6.4, and 37.3 ± 4.0 molecules/fg of protein, reflecting differences in the protein content of the membrane preparations. When temperatures were decreased from 22° to 4°, NBMPR binding to erythrocyte membrane preparations was reduced in IOVs relative to that in unsealed ghosts and ROVs. At 22°, the association of NBMPR molecules with IOVs was slower than with ROVs and unsealed ghosts, differences that were virtually eliminated by permeabilization of the membrane preparations with saponin. Thus, the binding sites were more accessible to external NBMPR in sealed ROVs and unsealed ghosts than in sealed IOVs, indicating that the NBMPR sites are located on the extracellular aspect of the membrane.

Various types of animal cells possess membrane transport systems that mediate the permeation of nucleoside molecules by passive, equilibrative (facilitated diffusion) processes of broad substrate specificity (1–3). Cells of diverse types, for example, human erythrocytes, HeLa cells and S49 mouse lymphoma cells, possess NT systems that are inhibited by NBMPR and several related S^6-derivatives of 6-thiopurine-9-β-D-pentofuranosides (4–6). These ligands are bound reversibly, but with high affinity (K_d 0.1–1 nM), to sites that are associated with NT elements of the plasma membrane (2, 3). NBMPR may be regarded as a permeant analog, and, because a direct correlation has been shown between (i) fractional occupancy by NBMPR of the high affinity sites and (ii) fractional inhibition of uridine transport in human erythrocytes (7), the NBMPR-binding site has been perceived as a component of functional NT systems of erythrocytes (8). Correlations have also been shown between the absence of NBMPR-binding sites and of NT activity in nucleoside-impermeable sheep erythrocytes (8) and in AE_1 cells, a transport-defective mutant clone of S49 cells (5). The foregoing observations, together with the finding that NBMPR is an apparently competitive inhibitor of uridine influx in sheep erythrocytes (9), have been interpreted to mean that permeating nucleoside molecules and NBMPR molecules compete at the same, or at overlapping, sites on the NT mechanism of erythrocytes (10).

Inhibitor interaction with erythrocytic NT systems has been investigated through kinetic analyses of uridine transport inhibition (9–11). In sheep erythrocytes, NBMPR inhibition of zero-*trans* influx of uridine was found to be competitive, whereas that of zero-*trans* efflux of uridine was noncompetitive, suggesting that NBMPR-binding sites are on the outer membrane surface (9, 10). Kinetic analyses of dipyridamole and NBMPR inhibition of uridine transport in guinea pig erythrocytes (11) indicated similar characteristics, and it was concluded that the two inhibitors interact at common or overlapping sites on the outer membrane surface. *p*CMBS inhibited binding of NBMPR to unsealed erythrocyte ghosts but had no effect on binding to intact sheep erythrocytes (10), indicating that free thiol groups, presumably on a transporter polypeptide accessible from the cytoplasmic face of the plasma membrane, are required for NBMPR binding. Similar results were obtained in a study that employed ROVs and IOVs from human erythrocytes to assess the sidedness (external or cytoplasmic orientation) of *p*CMBS-sensitive thiol groups (12). These studies

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ABBREVIATIONS: NBMPR (nitrobenzylthioinosine), [6-(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; IOVs, inside-out vesicles; ROVs, right side-out vesicles; NT, nucleoside transport; *p*CMBS, *p*-chloromercuribenzene sulphonate.

were interpreted as indicating that NBMPR and *p*CMBS interact preferentially with the outward- and inward-facing conformations, respectively, of the NT mechanism.

The kinetic characteristics of nucleoside transport in erythrocytes from adult pigs are similar to those in erythrocytes of humans and of a number of other species (13–16). Transport of uridine by pig erythrocytes conforms to the simple carrier model with directional symmetry, but with differential mobility of permeant-loaded and -empty carriers (16). Pig erythrocytes possess between 5,000 (13, 15) and 10,000 (16) NBMPR-binding sites each, and the sensitivity of uridine transport to inhibition by NBMPR is similar in erythrocytes of humans and pigs (16). Molecular properties of the NBMPR-binding protein from adult pigs have been investigated recently (16, 17), and monoclonal antibodies directed against this protein have been prepared (18). In the present study, the external or cytoplasmic orientation of NBMPR-binding sites of pig erythrocytes was probed by examining the temperature dependence of [^{3}H]NBMPR association with these sites in unsealed ghosts and sealed vesicles (IOVs and ROVs). The results obtained indicate that the NBMPR-binding sites are located on the external aspect of the NT system in these cells. Portions of this work have been presented in a preliminary report (19).

Materials and Methods

Membrane preparations. Pig blood was collected at a local meat packing plant into 0.2 volume of anticoagulant solution (90 mM sodium citrate, 16 mM citric acid, 16 mM monosodium phosphate, 2 mM adenine, and 12 mM inosine) and used within 4 hr of collection for the preparation of membranes (ghosts) and membrane vesicles. In each set of experiments reported in this work, ghosts and vesicles were prepared from the same batch of erythrocytes. Unsealed ghosts were prepared as previously described (20). All procedures were performed at 4°. Erythrocytes were washed three times with buffered saline (150 mM NaCl, 5 mM sodium phosphate, pH 8.0) and hemolyzed by mixing 1 volume of packed cells with 39 volumes of 5 mM sodium phosphate (pH 8.0). Ghosts were collected by centrifugation (23,000 × *g*, 30 min) and washed three times with 5 mM sodium phosphate (pH 8.0).

Sealed IOVs were prepared by a modification of the method of Cohen and Solomon (21). Briefly, 4-ml portions of centrifugally sedimented unsealed ghosts were suspended in 39 volumes of 0.5 mM sodium phosphate (pH 8.0) and, after 30 min on ice, the suspensions were centrifuged (28,000 × *g*, 30 min) to obtain ghost pellets. After 16 hr at 4°, pellets were resuspended in 4 ml of 0.5 mM sodium phosphate (pH 8.0) and ghosts were disrupted (with the formation of vesicles) by five passes through a 27 gauge hypodermic needle. The suspensions were diluted 4-fold with 0.5 mM sodium phosphate (pH 8.0), layered on equal volumes of Dextran T-70 solution [4.46 g/100 ml of 0.5 mM sodium phosphate (pH 8.0)] in 13-ml plastic tubes, and centrifuged (154,000 × *g*, 4 hr) in a Beckman SW 41 rotor. IOVs were collected from the Dextran-medium interface and washed twice (28,000 × *g*, 30 min) with 5 mM sodium phosphate (pH 8.0); then, the final pellets were suspended in the same buffer to a protein concentration of 2–5 mg/ml. Protein was determined by the method of Lowry *et al.* (22), as modified by Peterson (23).

To prepare ROVs, 4-ml portions of centrifugally sedimented unsealed ghosts were suspended in 39 volumes of 0.5 mM sodium phosphate (pH 8.0) and kept at 4° for 30 min (21) before the addition of 100 mM $MgSO_4$ to a final concentration of 0.1 mM $MgSO_4$. Immediately after the $MgSO_4$ addition, suspensions were centrifuged (28,000 × *g*, 30 min) and the pellets were kept at 4° for 16 hr. ROVs were then isolated as outlined above for IOV preparations, except that the ROV suspending medium consisted of 0.5 mM sodium phosphate (pH 8.0) and 0.1 mM $MgSO_4$.

The integrity of IOVs and ROVs was assessed by determining the accessibility of (i) acetylcholinesterase, a marker of the external membrane surface, and (ii) NADH-cytochrome *c* oxidoreductase, a marker of the cytoplasmic membrane surface, before and after detergent treatment (20). The IOV and ROV preparations typically contained, respectively, 68–80% and 85–96% sealed vesicles with the desired orientation. IOVs apparently lost membrane proteins during preparation, since the specific activity of acetylcholinesterase in IOVs that were permeabilized by detergent treatment was consistently about 25% higher than that observed in unsealed ghosts.

NBMPR binding. Equilibrium binding of NBMPR to ghosts and vesicles was assayed by a filtration technique previously described (24). Before use, Durapore membrane filters (0.45 and 0.22 μm for ghosts and vesicles, respectively) were washed with a single 1-ml portion of 5 mM sodium phosphate (pH 7.4) containing 5 μM nonisotopic NBMPR and two 2-ml portions of cold 5 mM sodium phosphate (pH 7.4). Assay mixtures (final volume, 1.2 ml, and prepared in duplicate) contained graded concentrations of [^{3}H]NBMPR (0.25–8.0 nM) and a membrane preparation (unsealed ghosts, ROVs, or IOVs at 10 μg of protein/ml) in 5 mM sodium phosphate (pH 7.4). For determination of nonspecific binding of [^{3}H]NBMPR, 5 μM nonisotopic NBMPR was also included in assay mixtures. After 20 min at room temperature (22°), or 60 min at 4°, membrane material was collected by vacuum filtration from 1.0-ml portions of the assay mixtures on Durapore filters, which were then rapidly washed three times with 2-ml portions of ice-cold buffer. Filters were placed in scintillation vials with 8 ml of Triton X-100-based scintillation fluid (25) and, after 16 hr at room temperature, were assayed for ^{3}H-activity by liquid scintillation counting. For determination of free [^{3}H]NBMPR concentrations, the remaining assay mixtures were centrifuged (120,000 × *g*, 10 min) in a Beckman Airfuge, and 0.1-ml portions of the supernatants were assayed for radioactivity in 8 ml of scintillation fluid. Constants for NBMPR binding (K_d, B_{max}) were determined by mass law analysis of equilibrium binding data after subtracting nonspecific binding of [^{3}H]NBMPR, which was obtained by measuring retention of ^{3}H in the presence of 5 μM nonisotopic NBMPR. The nonspecific component of NBMPR binding accounted for 5–10% of total NBMPR bound in assay mixtures.

Rates of association of [^{3}H]NBMPR with membrane preparations were determined as follows. After 5 min at room temperature in the presence or absence of 5 μM nonisotopic NBMPR, 1-ml portions of membrane suspensions (10 μg of protein/ml) were collected on Durapore filters. Binding reactions were initiated while the filters were in the filtration apparatus by adding 0.5-ml portions of 2 nM [^{3}H]NBMPR in 5 mM sodium phosphate (pH 7.4) with or without 5 μM nonisotopic NBMPR to membrane samples. After graded intervals, binding reactions were terminated by filtration, and the filters were washed and assayed for radioactivity. Specific binding of NBMPR was determined as the difference between the [^{3}H]NBMPR content of samples acquired in the absence and presence of 5 μM nonisotopic NBMPR.

The effect of saponin on the specific binding of [^{3}H]NBMPR to membrane preparations was measured as follows. Assay mixtures that consisted of membrane preparations in 5 mM sodium phosphate (pH 7.4) with or without 0.01% (w/v) saponin were incubated at room temperature for 5 min before assay of [^{3}H]NBMPR binding under conditions described above, with correction for nonspecific binding of NBMPR. Stock solutions of saponin consisted of 0.1% saponin (w/v) in 5 mM sodium phosphate (pH 7.4).

Materials. [G-^{3}H]NBMPR (23 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA) and, after storage, was purified by high pressure liquid chromatography on a 10-cm Spheri-10 RP-18 column (Brownlee Laboratories, Santa Clara, CA) with methanol/water solutions. NBMPR was prepared in this laboratory (26). Durapore filters were obtained from Millipore (Canada), and Dextran T-70 and saponin were obtained, respectively, from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific (Fairlawn, NJ).

Results

The intent of the experiments here described was to determine whether the accessibility of external NBMPR molecules to binding sites on the inner surface of the plasma membrane was different in ROVs and IOVs under conditions where diffusion of NBMPR across the membrane was minimized. If NBMPR-binding sites are located mainly on the external surface of the membrane [as others have argued (9–11)], then temperature-related reductions in rates of NBMPR binding might be greater in IOVs than in ROVs and unsealed ghosts, since access of NBMPR molecules to the binding sites in IOVs would require passage (presumably by diffusion) through the vesicle membrane. In experiments in which short incubations (0–5 min) were employed (data not shown), the specific binding of [^{3}H]NBMPR to all membrane preparations was drastically reduced at low temperatures; the specific binding levels of 4 nM [^{3}H]NBMPR to IOVs, ROVs, and unsealed ghosts in 5 min at 4° were, respectively, 18%, 30%, and 42% of that obtained at room temperature. Temperature effects on NBMPR binding are illustrated in the experiment of Fig. 1, which measured time courses of [^{3}H]NBMPR binding to high affinity sites on IOVs, ROVs, and unsealed ghosts during a 2-hr period. At 22°, the content of site-bound [^{3}H]NBMPR approached equilibrium values within 20 min in all three membrane preparations. At 4°, the approach to binding equilibrium was only slightly reduced in ROVs and unsealed ghosts whereas, in IOVs, the rate at which [^{3}H]NBMPR became associated with high affinity sites was much lower at 4° than at 22°, and equilibrium was not achieved within 2 hr.

Previous studies have demonstrated that pig erythrocytes possess plasma membrane sites of a single type that bind NBMPR with high affinity (K_d, 1.2–1.5 nM) (10, 13–16). Results of experiments that measured equilibrium binding of [^{3}H]NBMPR to vesicle preparations are presented in Fig. 2 and Table 1. These data demonstrate the saturable association of NBMPR with high affinity sites of a single type on unsealed ghosts, ROVs, and IOVs. Although the K_d values for site-bound NBMPR (approximately 2 nM) were similar in all membrane preparations, the B_{max} values differed in the three types of preparations, ranging from 22.2 molecules/fg of protein in unsealed ghosts to 37.3 molecules/fg of protein in IOVs. These differences were partly due to the loss of extrinsic proteins [e.g., spectrin (27)] when membranes were subjected to low ionic strength and alkaline pH during the preparation of IOVs. However, the content of NBMPR-binding sites in IOVs was significantly greater (about 70%) than in ROVs and unsealed ghosts, and cannot be attributed to differences in the protein content of the vesicle preparations.[1] Fig. 2 shows that, although reduction in temperature did not alter the accessibility of NBMPR-binding sites in ROVs and unsealed ghosts, NBMPR binding to IOVs at low temperature was greatly reduced. The latter result is consistent with the conclusion from Fig. 1 that the approach to NBMPR binding equilibrium was appreciably slower in IOVs than in ROVs and unsealed ghosts.[2]

The greater accessibility at 4° of high affinity sites to exogenous ligand in ROVs and unsealed ghosts, relative to that in IOVs, argues that the binding sites may be located on the external surface of the plasma membrane in pig erythrocytes. This interpretation was tested in the experiment of Fig. 3, which compared short (30-sec) time courses of NBMPR association with high affinity sites in the membrane preparations at 22°. In this experiment, specific binding of NBMPR has been expressed as the fraction of total sites occupied, since the site content (sites/fg of protein) differed among the three types of membrane preparations. The association of NBMPR with the high affinity sites of IOVs was clearly slower than with those of ROVs and unsealed ghosts, indicating that ligand

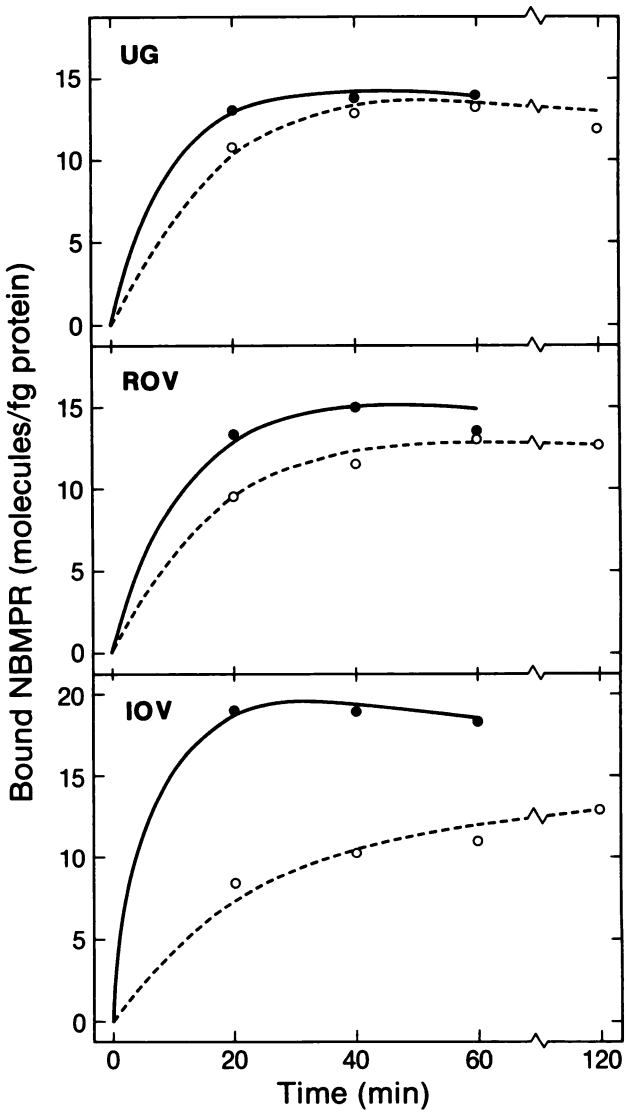

Fig. 1. Association of [^{3}H]NBMPR with erythrocyte membrane preparations. Assay mixtures, which contained 4.0 nM [^{3}H]NBMPR, and membrane preparations [unsealed ghosts (*UG*), sealed ROVs, or IOVs at a final concentration of 10 μg of protein/ml in 5 mM sodium phosphate (pH 7.4)] were incubated with and without 5 μM nonisotopic NBMPR at 22° (room temperature) (●) or 4° (○). After incubation for the intervals indicated, membrane material from duplicate 1.0-ml samples was collected by filtration on Durapore filters (0.45 and 0.22 μm for ghosts and vesicles, respectively), which were washed and assayed for radioactivity. Site-bound NBMPR was calculated as the difference between the [^{3}H]NBMPR content of the membrane samples incubated in the absence and presence of 5 μM NBMPR.

[1] The acetylcholinesterase activity of permeabilized IOVs was only about 25% greater than that of unsealed ghosts, raising the possibility that cryptic NBMPR-binding sites were "unmasked" during preparation of IOVs.

[2] The NBMPR binding activity observed in IOVs at 4°C can be attributed in part to the presence of ROVs and/or membrane fragments in the IOV preparations.

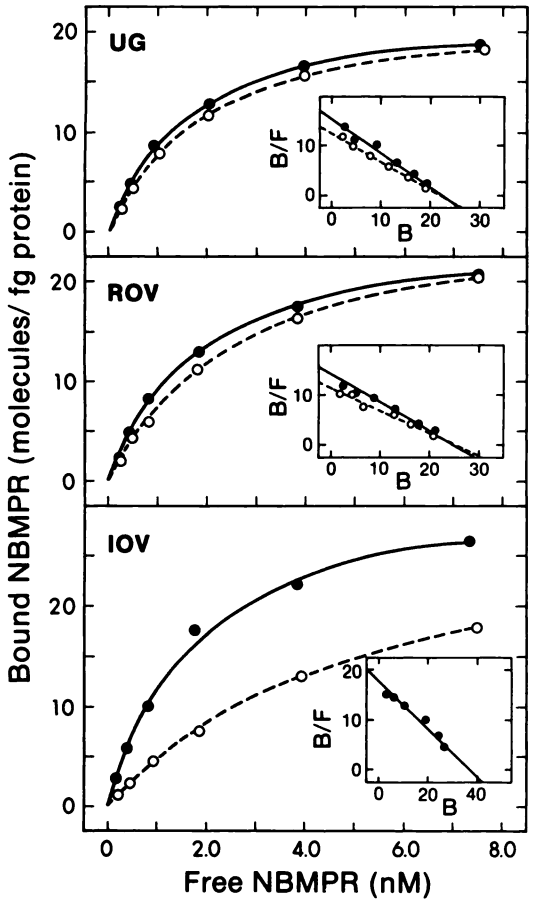

Fig. 2. Mass law analysis of equilibrium binding of NBMPR to erythrocyte membrane preparations. Binding of [^{3}H]NBMPR to unsealed ghosts (*UG*), ROVs, and IOVs, all prepared from the same blood sample, was measured under equilibrium conditions as described under Materials and Methods. Membrane preparations were incubated with graded concentrations of [^{3}H]NBMPR for 20 min at 22° (room temperature) (●) or for 1 hr at 4° (○) in assay mixtures with or without 5 μM nonisoptopic NBMPR. Site-bound NBMPR was determined as the difference between the [^{3}H] NBMPR content of the membrane preparations acquired in the absence and presence of 5 μM nonisotopic NBMPR. Mass law analysis of the binding data by the Scatchard method (*insets*) yielded constants included in the averaged data of Table 1. Analysis was not possible for IOVs incubated at 4° because binding equilibrium was not approached under these conditions (see Fig. 1). *B*, bound NBMPR (molecules/fg of protein); *F*, free NBMPR (nM).

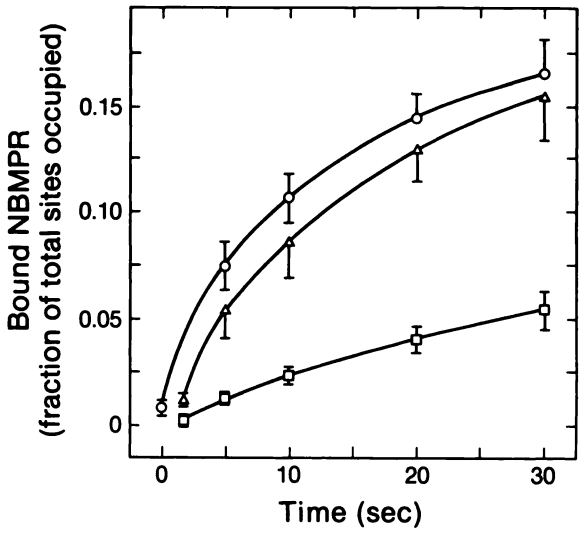

Fig. 3. Time course of specific binding of NBMPR to erythrocyte membranes. Membrane preparations [unsealed ghosts (○), ROVs (△), or IOVs (□), 10 μg of protein/ml] were suspended in 5 mM sodium phosphate (pH 7.4) with or without 5 μM nonisotopic NBMPR and were held at 22° (room temperature) for 5 min. Membrane samples from 1.0-ml portions of the suspensions were collected on 0.22-μm Durapore filters and, while still in the filter holder, were exposed to solutions containing 2 nM [^{3}H] NBMPR with or without 5 μM nonisotopic NBMPR for the intervals specified. Specific ligand association with the membrane preparations was determined as the difference between the [^{3}H]NBMPR content of membrane material acquired in the absence and presence of nonisotopic NBMPR. At 30 sec, the nonspecifically bound content of NBMPR was 16%, 17%, and 35% of the NBMPR content of unsealed ghosts, ROVs and IOVs, respectively. Because the NBMPR site content (sites/fg of protein) of the membrane preparations differed, site-bound NBMPR has been expressed as the fraction of total sites occupied, that is, as a fraction of the B_{max} value (sites/fg of protein) obtained from mass law analysis of equilibrium binding data (not shown) for each membrane preparation. Mean values (± standard deviations) from four separate experiments are shown.

TABLE 1

Binding constants for the interaction of NBMPR with erythrocyte membranes

Binding of [^{3}H]NBMPR to membrane preparations was measured under equilibrium conditions as described in Fig. 2. K_d and B_{max} values were calculated by linear regression analysis of mass law (Scatchard) plots. Values listed are means ± standard deviations of four separate experiments including that of Fig. 2.

Membrane preparation	B_{max}		K_d	
	22°	4°	22°	4°
	molecules/fg protein		*nM*	
Unsealed ghosts	22.2 ± 5.5	19.5 ± 3.9	1.6 ± 0.4	1.7 ± 0.6
ROV	25.6 ± 6.4	25.7 ± 6.6	2.0 ± 0.4	2.2 ± 0.6
IOV	37.3 ± 4.0	ND[a]	2.4 ± 0.4	ND

[a] ND, not determined.

permeation was rate-limiting for site-specific binding of NBMPR to IOVs.

Although both membrane surfaces of unsealed ghosts are accessible to nondiffusible probes, the inner membrane surface of sealed vesicles is not accessible to such probes as long as the permeability barrier is intact. The effects of a detergent, saponin, on accessibility of NBMPR-binding sites to exogenous ligand were investigated in the experiment of Fig. 4. Without saponin treatment, the specific association of NBMPR with IOVs was slower than with ROVs and unsealed ghosts. Saponin treatment had no effect on specific binding of NBMPR to ROVs and unsealed ghosts, but such treatment of IOVs increased NBMPR binding to levels comparable to those of saponin-treated ROVs.[3] Thus, sites that were otherwise sequestered in IOVs became accessible to external NBMPR following exposure to saponin.

Discussion

Earlier suggestions that NBMPR-binding sites are located at the external membrane surface of sheep erythrocytes arose from demonstrations that external NBMPR is an apparently

[3] In Fig. 4, the rate of NBMPR binding to IOVs in the absence of detergent was higher than that observed in Fig. 3, presumably because of differences in the relative amounts of contaminating ROVs in the two sets of experiments (30% and 24%, respectively) and slight variations in room temperature.

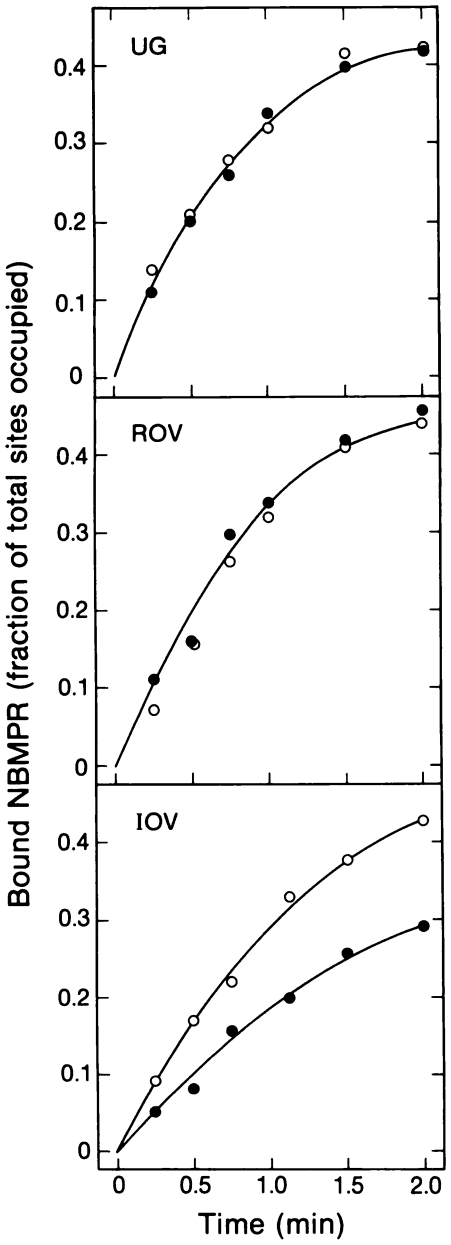

Fig. 4. Effect of saponin on NBMPR binding to erythrocytic membrane vesicles. ROVs, IOVs, and unsealed ghosts (*UG*) (10 μg of protein/ml) were incubated for 5 min at room temperature with (○) or without (●) 0.01% saponin in 5 mM sodium phosphate (pH 7.4) in the presence or absence of 5 μM nonisotopic NBMPR. Membranes from 1.0-ml portions of the suspensions were collected on 0.22-μm Durapore filters and washed twice with 2-ml portions of ice-cold buffer. The association of 2 nM [^{3}H]NBMPR with high affinity sites in the filter-mounted membrane samples during the intervals indicated was determined as in Fig. 3. Site-bound NBMPR (the difference between [^{3}H]NBMPR bound in the absence and presence of 5 μM nonisotopic NBMPR) is expressed as the fraction of total sites occupied. At 2 min, nonspecific binding was 15%, 14%, and 27% of total binding for unsealed ghosts, ROVs, and IOVs, respectively. The means of duplicate determinations are shown; a separate experiment yielded similar results (not shown).

competitive inhibitor of inward fluxes of uridine and a noncompetitive inhibitor of outward fluxes of uridine (9, 10). Similar findings were obtained for the inhibition by dipyridamole of uridine fluxes in guinea pig erythrocytes (11), and the authors concluded that dipyridamole and NBMPR interact with the same external site, which was considered to be "totally or partially within the permeation site." These interpretations presume that NBMPR competes with uridine at the permeation site and that the NBMPR-binding site and the permeation site are "coincident," that is, that they share a common domain. While the NBMPR binding and NT activities are both properties of band 4.5 proteins, as shown in reconstitution experiments with preparations of band 4.5 from human erythrocytes (28), direct evidence for coincidence of the sites for NBMPR binding and nucleoside permeation is lacking. The apparently competitive pattern of NBMPR inhibition of inward nucleoside fluxes could have an allosteric basis, deriving perhaps from NBMPR-induced conformational changes in transporter polypeptides that could alter permeation site affinity for nucleoside substrates. Indirect support for the coincident site model is found (i) in the direct correlation between fractional inhibition of uridine transport and fractional occupancy of NBMPR sites in human erythrocytes (7) and (ii) in the proportionality reported by Jarvis *et al.* (15) between NBMPR site abundance and transport capacity (V_{max}) in erythrocytes that differed substantially in these activities from one species to another.

A number of recent observations (summarized in Ref. 29) indicate that NBMPR binding and NT activities are distinct and separate in various lines of cultured cells, as the following examples suggest: (i) an equilibrative NT system of low NBMPR sensitivity ($IC_{50} > 1$ μM) is present in cultured Walker 256 carcinoma cells (30, 31); (ii) sites that bind NBMPR with high affinity are present in several lines of cultured hepatoma cells that possess NT systems of low NBMPR sensitivity ($IC_{50} > 1$ μM), suggesting that the NBMPR-binding protein may be "uncoupled" from nucleoside transporter elements in these cells (29–31); and (iii) as demonstrated by Cohen *et al.* (32) and Aronow *et al.* (33), NBMPR-binding activity may be altered independently of NT activity by mutagenic procedures in cultured S49 mouse lymphoma cells. From these observations, it would appear that the coincident site model may not be appropriate for the NBMPR-sensitive, equilibrative NT systems of nucleated cells. The applicability of these arguments to erythrocytic NT systems is presently uncertain.

In conclusion, we have noted that the inference by Jarvis and co-workers (9–11) (from the inhibitory effects of NBMPR on uridine fluxes in erythrocytes), that NBMPR-binding sites are located on the external aspect of the plasma membrane, requires a transporter model in which sites for uridine permeation and NBMPR binding have common or coincident regions. Evidence has accumulated that NT activities and NBMPR-binding activities are functionally and genetically distinct in several cultured cell types. The present study has provided evidence pertaining to the orientation of NBMPR-binding sites in pig erythrocytes that is interpretable directly from properties of membrane preparations, rather than from assumptions based on transporter models. At 4°, rates of high affinity binding of NBMPR to IOVs were greatly reduced relative to ROVs and unsealed ghosts. Furthermore, permeabilization of IOVs with saponin increased rates of association of NBMPR with the high affinity binding sites, but similar treatment of ROVs was without effect. These results provide direct evidence that the NBMPR-binding sites are located on the extracellular surface of pig erythrocytes.

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