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"IN SITU" CHARACTERIZATION OF GUANINE NUCLEOTIDE-BINDING PROPERTIES OF ERYTHROCYTE MEMBRANES

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SUMMARY: Unsealed membranes from human erythrocytes bind GTP and GTP analogs according to first order kinetics, a single rate constant being observed. With [^{35}S]GTPγS this is 0.15±0.2 min^{-1}. Treatment of the membranes with detergents decreases binding considerably. Scatchard plots reveal uncomplicated patterns of ligand association, with K_d values of 10.2±2.3 nM [^{35}S]GTPγS, of 18.2±4.3 nM [α-^{32}P]GTP and of 28.6±3.5 nM [α-^{32}P]GDP, respectively. The stoichiometry with the three ligands is strictly comparable, i.e. 65±7 picomoles/mg of membrane protein. Binding of each labeled nucleotide is competitively inhibited by the other two unlabeled ligands, the inhibition constants being very close to the corresponding K_d values. Metabolic depletion and subsequent repletion of intact erythrocytes result in membrane preparations still active in guanine nucleotide binding, with unmodified K_d values. However, the stoichiometry falls to 35 picomoles/mg protein with the "depleted" erythrocyte membranes and regains higher values (50 picomoles/mg protein) with the "repleted" cell membranes. Accordingly, the "in situ" characterization of guanine nucleotide-binding properties of erythrocyte membranes seems to represent a new tool for monitoring the metabolic state of intact erythrocytes.

Growing interest toward G proteins as transducers of receptor-mediated extracellular signals is justified by the importance of cellular functions that are under this type of transmembrane control (1-3). Although the erythrocyte is generally viewed as a simplified cell type, many of its biochemical properties, notably those which are not directly related to its main function of transporter of respiratory gases, are still incompletely known. Thus, a number of G proteins have been identified in the human erythrocyte and in part also purified, characterized and used in reconstructed systems for studies on signal transduction and on target functions in heterologous cell types (4-7). However, current knowledge on erythrocyte G proteins is limited by two facts: i) binding of guanine nucleotides may be impaired by extraction of G proteins from membranes; ii) the correlation between individual G proteins and specific properties

Abbreviations: GTPγS, guanosine-5'-(3-O-thio) triphosphate; G proteins, guanine nucleotide-binding regulatory proteins; G_p, a guanine nucleotide-binding regulatory protein originally purified from placenta membranes (ref. 17); NP40, Nonidet P-40.

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of the erythrocyte still remains elusive. In particular, no relationship has emerged so far between any known G protein and regulation of ion channels (notably for Ca^{2+}) in the erythrocyte, where the absence of classical, i.e. membrane-surrounded, intracellular stores makes the control of Ca^{2+} homeostasis to depend on direct exchange with the extracellular environment only (8).

This study is a first attempt to rationalize the G proteins present in the human erythrocyte membrane, by measuring the characteristics of binding of GTP and GTP analogs to the membrane itself. This conservative approach allows the "in situ" characterization of G proteins to be carried out, thus providing information on related functions of the intact erythrocyte.

MATERIALS AND METHODS

Materials. Unlabeled guanine nucleotides, detergents, buffers and reagents were the highest grade available commercially. The non-radioactive nucleotides were further purified on a FPLC system (Pharmacia, Uppsala, Sweden) using a Mono-Q HR 5/5 column. A linear gradient of 0.05-0.5 M NH_4HCO_3 pH 8.5, was used. [α-^{32}P]GTP (3,000 Ci/mmol), [γ-^{32}P]GTP (42 Ci/mmol), [α-^{32}P]GDP (5,000 Ci/mmol) and [^{35}S]GTPγS (1,320 Ci/mmol) were purchased from New England Nuclear, Dreieich, FRG.

Erythrocyte membranes. Unsealed, calmodulin-free membranes were prepared from human erythrocytes according to Niggli et al. (9) and stored at -80°C in 0.1 ml aliquots before being used in the binding experiments. Protein concentration was determined according to Lowry et al. (10).

Metabolic depletion and repletion of human erythrocytes. Erythrocytes freshly drawn from laboratory personnel were washed four times and resuspended under sterile conditions at a final 5% hematocrit in 130 mM KCL and 20 mM Tris-HCL, pH 7.4. After gentle rotation in a vertical plane at 37° for 8 hrs, the suspension was placed at 4°C overnight (metabolically depleted erythrocytes). Half volume of the suspension was then incubated at 37°C for 4 hrs with 0.1 vol of a solution (11) of 5 mM adenine, 100 mM inosine, 100 mM sodium pyruvate, 100 mM sodium phosphate, 100 mM glucose and 9% NaCl, pH 7.4 (repleted erythrocytes). Both erythrocyte suspensions were then processed for membrane preparations as described above. GSH was determined according to Beutler (12). Analyses of ATP, ADP, GTP and GDP were carried out by HPLC on perchloric acid extracts of erythrocyte suspensions, as described (11).

Binding assay of labeled guanine nucleotides. Binding of [α-^{32}P]GTP, [α-^{32}P]GDP and [^{35}S]GTPγS to erythrocyte membranes was measured with a rapid filtration technique using HA 0.45 μM nitrocellulose filters (Millipore, Bedford, MA), according to Northup et al. (13), with some modifications. In the standard assay, samples of 200 μl containing 11 μg of membrane protein were incubated for 20 min at 37°C in 50 mM Tris-HCL, 5 mM NaF and 25 μM $MgCl_2$, pH 7.5 (Buffer A), containing non-radioactive GTP (or GDP, or GTPγS) at varying concentrations from 5 to 300 mM in the presence of a constant amount (10^5 cpm) of the corresponding labeled nucleotide. The reaction was terminated by diluting the samples with 4 ml of ice-cold Buffer A followed by rapid filtration through nitrocellulose filters. The filters were washed three times with 4 ml of cold Buffer A, placed in 5 ml of Picofluor 40 (Packard Instruments, Warrenville, IL) and the retained radioactivity determined in a scintillation counter. Non-specific binding, i.e. in the presence of 100 μM GTP, was less than 7% of the total radioactivity retained by the filters. Blank values (in the absence of erythrocyte membranes) were less than 0.5% of the total radioactivity applied.

GTPase activity. Membrane-associated GTPase activity was determined according to Cassel and Selinger (14), with modifications. The assay system (200 μl) contained 11 μg of membrane protein and 100 nM [γ-^{32}P]GTP in Buffer A. Samples were incubated for 20 min at 37°C and the reaction was terminated by addition of 1 ml of 20 mM sodium phosphate, pH 7.4, containing 5% (w/v) acid-washed

charcoal (Norit A, Sigma Chem. Co., St. Louis, MO). After centrifugation for 10 min at 4,200 rpm, aliquots (0.2 ml) of the supernatants were placed in 5 ml of Picofluor 40 and the liberation of $^{32}P_i$ was determined in a scintillation counter. Under these conditions, corresponding to those used in the binding assay of labeled guanine nucleotides, the extent of GTP hydrolysis was less than 20% irrespective of the membrane preparations used (i.e., from native, depleted, or repleted erythrocytes).

RESULTS

Binding of GTP and GTP analogs to intact erythrocytes membranes. The kinetics of association of [^{35}S]GTPγS to intact erythrocyte membranes are shown in Fig. 1. Equilibrium was reached after 20 min incubation at 37°C and no kinetic heterogeneity of [^{35}S]GTPγS binding to membranes was apparent. A first order rate constant of association of 0.15±0.02 min^{-1} was calculated and the half-life was 4.8±0.5 min. Binding of [α-^{32}P]GTP and of [α-^{32}P]GDP followed apparently similar kinetics. Under the conditions used in this study, binding of GTP was partially affected by concomitant GTPase-catalyzed conversion to GDP, which accounted for 10%-20% losses of GTP (see "Materials and Methods").

Treatment of membrane preparations with a number of detergents was found to affect the [^{35}S]GTPγS-binding capacity considerably. Thus, 0.02% Triton X--100 decreased the amount of bound GTPγS by 20%, while a 0.1% concentration reduced the binding activity by 74%. Similar figures were observed with NP40 from 0.02% to 0.1%. Sodium cholate at 0.1% decreased GTPγS binding by as little as 20% while a 0.5% concentration produced greater than 90% loss of binding. This inactivation contrasts with the behaviour of GTPγS-binding activity in bovine brain membranes, which was completely retained following extraction with 1% cholate (15).

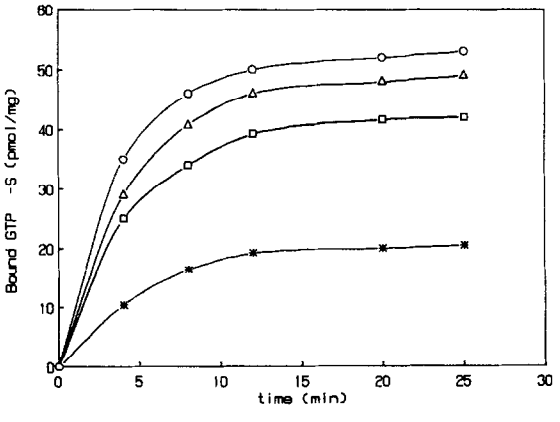

FIG. 1. Kinetics of association of [^{35}S]GTPγS to erythrocyte membranes. Incubations were performed at 37°C for the times indicated, as described under "Materials and Methods", in the presence of 5 (*), 20 (□), 40 (Δ) and 90 (○) nM [^{35}S]GTPγS.

Equilibrium binding of [α-^{32}P]GTP, [α-^{32}P]GDP and [^{35}S]GTPγS to the intact erythrocyte membranes revealed a closely comparable number of binding sites for the three nucleotides, i.e. 65±7 picomoles/mg membrane protein (Fig. 2). No obvious heterogeneity of ligand association was detectable, and the K_d values were: 10.2± 2.3 nM for [^{35}S]GTPγS; 18.2±4.3 nM for [α-^{32}P]GTP, and 28.6±3.5 nM for [α-^{32}P]GDP.

Each of the three unlabeled nucleotides was found to inhibit binding of the other two labeled ligands to the erythrocyte membrane according to a competitive relationship. Fig. 3 shows for instance the effect of GTP on the equilibrium binding of [^{35}S]GTPγS. Table I summarizes the corresponding inhibition constants calculated on the basis of the effect of GTP, GTPγS and GDP, respectively, on equilibrium binding of each labeled nucleotide. The estimated Ki values were in close agreement with the direct K_d values obtained with each of the three guanine nucleotides (see above).

Effects of metabolic depletion of intact erythrocytes on binding of GTP and GTP analogs to membranes. Binding of GTP and GTP analogs was investigated with intact membranes prepared from native erythrocytes and, comparatively, also with membranes from metabolically depleted and from repleted erythrocytes, respectively. As shown in Table II, membranes from substrate-exhausted erythrocytes (retaining 33%, 28% and 14% of GSH, ATP and GTP, respectively) showed no apparent changes in the K_d values for the three ligands. However, the stoichiometry of binding was in all cases lower than with membranes from native erythrocytes. "Rejuvenation" of exhausted erythrocytes was found to enhance the concentra-

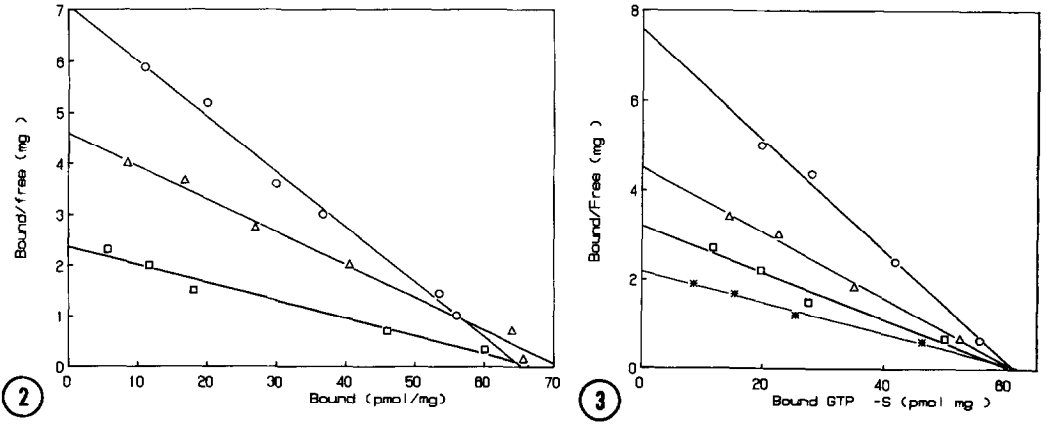

FIG. 2. Schatchard plot of guanine nucleotide binding to erythrocyte membranes. Results of a representative experiment are shown, which was performed in the presence of varying concentrations of [α-^{32}P]GTP (Δ), or of [α-^{32}P]GDP (□), or of [^{35}S]GTPγS (o), in the conditions described under "Materials and Methods".

FIG. 3. Competitive binding of GTP and [^{35}S]GTPγS to human erythrocyte membranes. Scatchard analysis of [^{35}S]GTPγS binding was carried out in the conditions reported under "Materials and Methods", in the absence (o) and in the presence of 40 (Δ), 80 (□) and 160 (*) nM unlabeled GTP.

TABLE I

K_i values of GTP, GTPγS and GDP toward binding of labeled guanine nucleotides to erythrocyte membranes

Unlabeled nucleotide	[α^{32}P]GTP	[α^{32}P]GDP	[^{35}S]GTPγS
		K_i (nM)	
GTP	-	18.4±3.2	16.0±2.1
GTPγS	9.0±0.9	11.2±2.6	-
GDP	28.2±2.0	-	30.7±4.1

K_i values ± SD were calculated (ref. 16) from 5 experiments performed at constant concentrations of each unlabeled nucleotide, with increasing concentrations of each labeled nucleotide.

tions of GSH, ATP and GTP consistently (Table II). The membranes from these repleted cells still bound [α-^{32}P]GTP, [α-^{32}P]GDP and [^{35}S]GTPγS with unchanged affinities with respect to the native and the exhausted erythrocytes. The number of binding sites, however, showed a clearcut trend to increase, up to intermediate values between native and metabolically depleted cell membrane preparations (Table II).

TABLE II

Stoichiometries (n) and K_d values of equilibrium binding of GTP and GTP analogs to membranes from native, depleted and repleted erythrocytes

Erythrocytes		Native	Exhausted	Repleted
Metabolites				
GSH (mM)		6.1 ± 0.9	2.0 ± 0.2	5.0 ± 0.3
ATP (mM)		0.90±10	0.25± 0.04	0.70± 0.09
ADP (mM)		0.25+ 0.04	0.32+ 0.06	0.21+ 0.02
GTP (μM)		42 ± 8	6 ± 1	34 ± 6
GDP (μM)		24 ± 5	32 ± 7	31 ± 6
Labeled Ligands				
[α-^{32}P]GTP	n	65 ± 6.0	32 ± 6.5	50 ± 4.0
	Kd (nM)	18 ± 4.3	21 ± 3.1	19 ± 3.9
[α-^{32}P]GDP	n	60 ± 4.2	38 ± 7.8	53 ± 4.3
	Kd (nM)	29 ± 3.5	31 ± 3.4	28 ± 3.9
[^{35}S]GTPγS	n	68 ± 2.9	36 ± 4.4	51 ± 2.8
	Kd (nM)	10 ± 2.3	9 ± 1.0	10 ± 1.1

Determinations of metabolites and analyses of equilibrium binding with labeled ligands were carried out in 4 different experiments, as described under "Materials and Methods". Values ± SD are given. n values indicate picomoles of labeled ligand/mg of membrane protein.

DISCUSSION

Binding of GTP and GTP analogs proves to be an intrinsic property of unsealed erythrocyte membranes. This property seems to be important in view of, a) the number of binding sites (around 18,000 copies per erythrocyte), b) the high affinity of guanine nucleotide binding, as revealed both by direct measurements and by competition experiments. The K_d values obtained in this study are remarkably lower than those reported for instance for G_s purified from rabbit liver (13), and of the same order as the K_d of G_p (17) purified from cholate extracts of bovine brain membranes (15). This fact and the loss of GTPγS binding following treatment of membranes with detergents including cholate clearly demonstrate that the native architecture of erythrocyte membranes is required for optimal association to occur.

Lack of apparent heterogeneity in the binding of each of the three ligands investigated over a reasonably wide range of concentrations does not necessarily contradict the known multiplicity of G proteins within erythrocyte membranes (1, 4-7). Rather, it seems to suggest comparable affinities of individual classes of G proteins for guanine nucleotides. In spite of this apparent uniformity, threshold-mediated correlations between individual G proteins and specific effector-linked functions of the erythrocyte may still hold, for instance through quantitatively different concentrations of each of the G proteins within the membrane or by a variety of alternative mechanisms recently discussed by Neer and Chapman (18).

The "in situ" characterization of guanine nucleotide binding to intact membranes might provide useful information on the metabolic state of erythrocytes, adding to morphological (19) and to other biochemical parameters that are currently investigated to this purpose. These include metabolic fluxes, determinations of key metabolites and of cation-outpumping activities, analysis of protein and lipid components of the membranes (11). The potential value of estimating the guanine nucleotide binding to unsealed membranes is suggested by the loss of binding sites occurring upon metabolic depletion of erythrocytes and by the subsequent partial regain of binding activity following repletion of metabolites. It may be relevant that in isolated hepatocytes loss of intracellular thiols inhibits the increase in inositol phosphates that follows phospholipase C stimulation by both hormonal and non-hormonal (including NaF and $AlCl_3$) stimuli (20). This inhibition is reversed by addition of dithiothreitol which also re-establishes responsiveness to extracellular stimuli. Whatever the underlying mechanism of the present findings may be (for instance, changes in SH groups), these reversible changes in guanine nucleotide-binding activity indicate flexibility in some erythrocyte properties and functions that are related to G proteins.

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