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Binding of ^{14}C-furosemide to isolated human erythrocytes*

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Abstract: The incubation of ^{14}C-furosemide at high specific activity with intact red blood cells at 37°C, pH 7.4, has enabled the furosemide binding sites to be characterized with respect to time course, affinity and specificity. The binding reaction was rapid, reversible and close to thermodynamic equilibrium. Binding was dependent on cell and furosemide concentration and was saturable. At equilibrium, pharmacological doses of furosemide competitively inhibited ^{14}C-furosemide binding with 50% inhibition at 3×10^{-5} M. The Na^+/K^+ pump inhibitor ouabain had no effect on the ^{14}C-furosemide binding. Bumetanide, which is more potent than furosemide as inhibitor of Na^+/K^+ co-transport system and equally effective in inhibiting anion transport, was less effective than furosemide in displacing ^{14}C-furosemide from its binding sites, suggesting a different mechanism of action for the two drugs in the red blood cell. The preincubation of erythrocytes with 4,4′-diisothiocyano-stilbene-2,2′-disulphonic acid (DIDS), the potent and specific inhibitor of anion permeability, reduced specific furosemide binding by more than 80% at a furosemide concentration of 0.1 μM, while it had little effect on the non-specific furosemide binding. Taken together, these data suggest that furosemide interacts with specific binding sites in the human red blood cell, whose nature has not been clarified, but whose location is probably on (or near) the protein in band 3, i.e. the membrane macromolecule-mediating anion transport.

Keywords: *Furosemide; red blood cell; sodium–potassium co-transport; anion exchange.*

Introduction

The principal action of loop diuretics is the inhibition of chloride transport in the thick ascending portion of Henle's loop [1, 2].

Human red blood cells are widely used as a simple model system to study the activity of loop diuretics [3–5]. In fact, ion fluxes catalysed by the Na^+/K^+ pump, the Na^+/K^+ co-transport system and the anion carrier are precisely and easily measured.

Furosemide, one of the loop diuretics, has been shown to exert an inhibitory effect on various ion transport systems of the human erythrocyte (Cl^--dependent, Na^+/K^+ co-transport system, $Cl^-/NaCO_3^-$ anion exchanger, and, to a lower extent, Na^+/K^+ pump and Na^+-dependent glycine uptake) [3–5]. The first mentioned effect correlates well

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with the saliuretic activity of the loop diuretics [6], whereas the inhibitory activity on the anion exchanger seems correlated with the potency in inhibiting astroglial swelling [5].

Studying furosemide inhibition of chloride self-exchange flux across red blood cells, Brazy and Gunn [7] suggested that furosemide acts as a mixed-type inhibitor (competitive and non-competitive) of the anion carrier by reacting with the chloride transport unit at two sites. One is identical with the site at which chloride can cause self-inhibition (the modifier Cl_2-site introduced by Dalmark [8]). At the second site (discrete from the Cl^--transport site) furosemide non-competitively blocks chloride transport and alters chloride affinity for the transport site.

A competitive antagonism between chloride and loop diuretics has been reported by other authors in various cellular models [4, 9, 10], supporting the hypothesis that these diuretics compete with chloride for an anion binding site on the cell membrane.

Using ^{14}C-furosemide at high specific activity, we studied directly the interaction of furosemide with human red blood cells, and characterized furosemide binding sites with respect to time course, affinity and specificity. Moreover, in order to determine whether the membrane macromolecule-mediating anion exchange (known as protein in band 3 or capnophorin [11]) was involved in furosemide binding, we studied ^{14}C-furosemide binding to red blood cells preincubated with DIDS, the potent, specific and irreversible inhibitor of anion permeability [12–14].

Experimental

Chemicals

Furosemide (4-chloro-*N*-furylmethyl-5-sulphamoyl-antranilic acid) and ^{14}C-furosemide were generous gifts from Hoechst Pharmaceuticals, FRG. Bumetanide (3-*n*-butylamino-4-phenoxy-5-sulphamylbenzoic acid) was provided by LEO Laboratories, Denmark. Ouabain, bovine serum albumin (BSA, fraction V), (±)-propranolol, DIDS, dimethyl sulphoxide (DMSO) and *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid (HEPES) were purchased from Sigma. Lumasolve and Lumagel were from Lumac, Italy. All other chemicals were of reagent grade and purchased from Merck. ^{14}C-furosemide with the carbon-14 label in the carboxylic group was kindly synthesized in the Radiochemical Laboratories of Hoechst Pharmaceuticals at a sp. act. 165 mCi g^{-1} (53.9 Ci mol^{-1}) and radiochemical purity >98%.

Erythrocyte isolation

Venous blood from normal fasted male volunteers was withdrawn in heparinized polypropylene tubes (143 U ml^{-1}). Erythrocytes were sedimented by centrifugation at 160***g*** for 10 min at 20°C and washed three times with physiological saline and twice with assay buffer (10 mM HEPES, 157.5 mM NaCl, 2.5 mM KCl, dextrose 0.2%, pH 7.4) by suspension and centrifugation at 400***g*** for 15 min at 4°C. Supernatant was aspirated carefully at each of these steps. The final erythrocyte pellet was suspended in assay buffer at 40% hematocrit (1:1, v/v). Cell viability, as determined by exclusion of Trypan Blue, was always >98%. For each binding assay, erythrocytes were counted in a Thoma counting chamber.

Binding assay

The binding assays were carried out in duplicate in siliconized glass tubes. Aliquots of erythrocytes (800 μl) were incubated at 37°C with 50 μl of 2 μM ^{14}C-furosemide and

10 μl of 0.1 M ouabain with or without 50 μl of competing ligands in a final volume of 1 ml of assay buffer. At the end of incubation, the unbound ligand was removed by centrifugation of the cell suspension at 400*g* for 15 min at 4°C. Erythrocyte pellets were washed twice with 3 ml of ice-cold assay buffer and then dissolved in 1 ml of Lumasolve–isopropanol (1:2), decolourized with 0.5 ml of H_2O_2, placed in scintillation vials with 15 ml of Lumagel–HCl 0.5 M (9:1) and finally counted in a Packard liquid scintillation spectrometer. All samples were quench-corrected. ^{14}C-Furosemide, furosemide and bumetanide dilutions were made up in assay buffer just before the assay from stock solutions in Tris (1 M), whereas ouabain was dissolved in DMSO. The final concentrations of solvents had no effect *per se* on binding.

Non-specific binding was assessed in the presence of 10 mM furosemide and was subtracted from the total binding to calculate the specific binding.

In experiments designed to study the effect of DIDS on the binding of ^{14}C-furosemide to human erythrocytes, cells at 25% hematocrit were preincubated for 30 min at 37°C in the dark with 10^{-4} M DIDS. The cells were then washed three times with assay buffer containing 0.5% BSA, in order to remove unreacted or non-covalently bonded DIDS, and then three times with assay buffer. By this procedure, the great majority of the inhibitor molecules were attached irreversibly to protein in band 3 [12, 13]. Anion fluxes were completely inhibited, without any significant alteration of the pH of the red cell suspension [13].

In control experiments, no evidence of red cell lysis during the incubation could be detected.

Unless stated otherwise, the experimental data reported in the text and figures are means ±SEM.

Results and Discussion

A steady-state for the specific binding of ^{14}C-furosemide to human erythrocytes was observed at 37°C, pH 7.4 (Fig. 1). With 0.11 μM ^{14}C-furosemide and 3.62×10^8 erythrocytes/ml, total binding reached a maximum of 45.2% within 30–40 min and remained constant thereafter. Non-specific binding accounted for 4.8% of the total

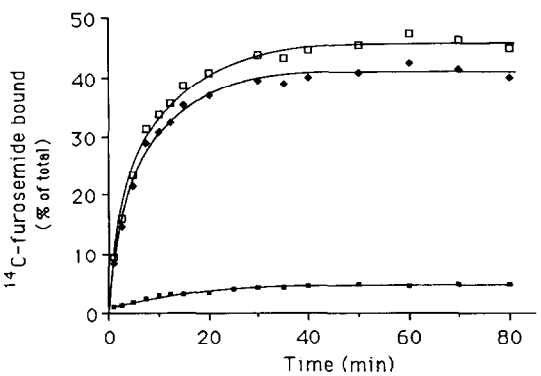

Figure 1
Binding kinetics of ^{14}C-furosemide (0.11 μM) to human erythrocytes (3.62×10^8/ml): □, total; ◆, specific; and ■, non-specific binding.

radioactivity, i.e. 10.5% of the maximum total binding. Specifically bound ^{14}C-furosemide plateaued at 40.8% of the total radioactivity.

Specific binding increased linearly with increasing erythrocyte concentration up to a value of nearly 3×10^8 cells/ml and complete saturation was obtained at about 10^9 cells/ml, at which point approximately 59 nM ^{14}C-furosemide were specifically bound (data not shown). Most experiments were therefore carried out at a cell concentration of about 2.5×10^8 cells/ml and bound ^{14}C-furosemide was normalized to this cell concentration.

The binding reaction was close to its true equilibrium and fully reversible, as demonstrated by the data in Fig. 2. In fact, the addition of different amounts of furosemide from 0.05 to 10 mM induced quick tracer dissociation, at the new steady state, the fraction of ^{14}C-furosemide bound was identical with the fraction bound when the same amount of furosemide was present from time zero. In other words, at each

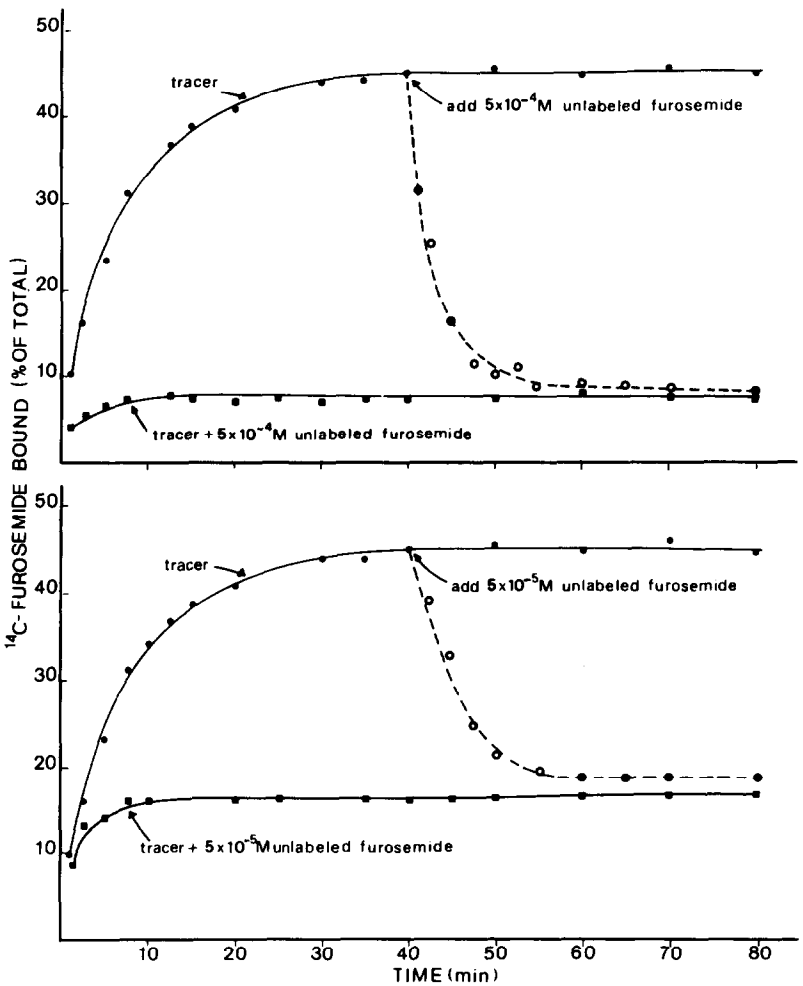

Figure 2
Kinetics and reversibility (dashed lines) of ^{14}C-furosemide (0.11 μM) binding to human erythrocytes (3.62×10^8/ml) at different concentrations of unlabelled furosemide.

concentration of furosemide, the same level of binding was achieved regardless of the time at which furosemide was added. The same experiment also demonstrates that the binding reaction was fully reversible (Fig. 2, dotted lines) and that the time required for ^{14}C-furosemide binding to reach its steady state decreased as the furosemide concentration increased.

Both the relative rapidity and the full reversibility of the binding reaction at all the concentrations of furosemide studied, suggested that furosemide binds at a superficial site on the cell. This is in agreement with the previous reports on the inhibitory effect of this drug on anion self exchange [7] and with the difficulty for furosemide to rapidly cross the cell membranes because it is a large organic acid with a pK_a of 3.9.

To evaluate binding specificity, we studied the ability of furosemide, bumetanide, ouabain and propranolol to inhibit the binding of ^{14}C-furosemide to isolated human erythrocytes (Fig. 3). Bound ^{14}C-furosemide could be displaced by concentrations of furosemide as low as 1 μM and 50% inhibition occurred at 3×10^{-5} M furosemide (Fig. 3, open circles); bound radioactivity at 10 mM was considered non-specifically bound, because the binding remained constant beyond this concentration.

The Na^+/K^+ pump inhibitor ouabain at concentrations up to 10^{-3} M (Fig. 3, open diamonds), as well as the beta-antagonist propranolol (Fig. 3, open squares), were completely ineffective in displacing labelled furosemide from its binding sites.

Bumetanide was less effective than furosemide in inhibiting ^{14}C-furosemide binding to human erythrocytes (Fig. 3, solid circles). The inhibition occurred only at concentrations higher than 5×10^{-4} M. This result was unexpected, because bumetanide is more potent than furosemide as a diuretic [15] and as an inhibitor of Na^+/K^+ co-transport system [10, 16]. However, the two drugs displayed about the same IC_{50} in DIDS-sensitive, $LiCO_3^-$ influx in red blood cells [5]. For bumetanide, this value was almost three orders of magnitude higher than the IC_{50} for co-transport inhibition [5]. Taken together, these data suggest that the two drugs have different mechanisms and sites of action in the human erythrocyte.

Scatchard analysis of furosemide binding data (Fig. 3, inset) resulted in linear plots, suggesting one class of binding sites with a dissociation constant, K_D, of $1.14 \pm 0.53 \times 10^{-5}$ M ($n = 8$) and a number of sites, B_{max}, of $1.1 \pm 0.06 \times 10^7$ sites/cell. The K_D values were in agreement with the furosemide concentrations required for 50%

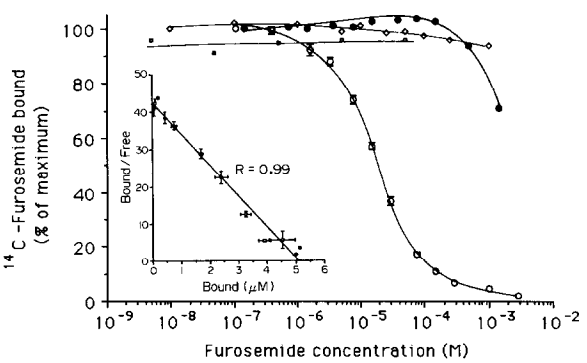

Figure 3
Inhibition of specific ^{14}C-furosemide binding to human erythrocytes by unlabelled ligands: ○, furosemide; ●, bumetanide; ◇, ouabain; □, (±)-propranolol. Inset: Scatchard analysis of specific furosemide binding data. Each value is the mean ± SEM of at least eight experiments.

inhibition of Na^+/K^+ co-transport in human and avian erythrocytes [4–6] and were one order of magnitude lower than the IC_{50} exhibited in the anion exchange ($2–5 \times 10^{-4}$ M) [5, 7, 17]. The concentration of ^{14}C-furosemide binding sites was about one order of magnitude higher than that found for DIDS binding sites, i.e. the sites related to anion transport [13].

Preincubation of cells with 10^{-4} M DIDS, reduced specific ^{14}C-furosemide binding to human erythrocytes by more than 80% and had little effect on the non-specific binding. Moreover, it increased the time required to reach the steady state (Fig. 4 versus Fig. 1). In fact, with a ^{14}C-furosemide concentration of 0.12 μM, only 15.5% of the total radioactivity was bound to 5.9×10^8 erythrocytes/ml after 5 h of incubation (Fig. 4, open squares). Non-specific binding (Fig. 4, solid squares) reached the value of about 7% of the total radioactivity, corresponding to 46.5% of the maximum total binding. Specific binding (Fig. 4, solid diamonds) plateaued at 7.2% of the total radioactivity in 3 h.

Binding reaction appeared slowly reversible, since <30% of radioactivity bound at equilibrium could be displaced by 10 mM furosemide (data not shown).

Inhibition of ^{14}C-furosemide binding by unlabelled furosemide (Fig. 5) occurred over the same concentration range observed with control cells (Fig. 5 versus Fig. 1). Scatchard

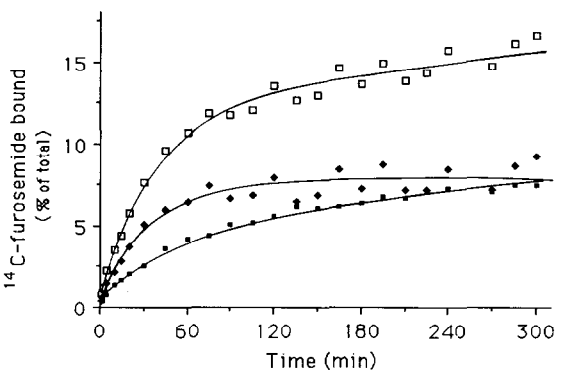

Figure 4
Binding kinetics of ^{14}C-furosemide (0.12 μM) to human erythrocytes (5.9×10^8/ml) preincubated with 10^{-4} M DIDS: □, total; ◆, specific; and ■, non-specific binding.

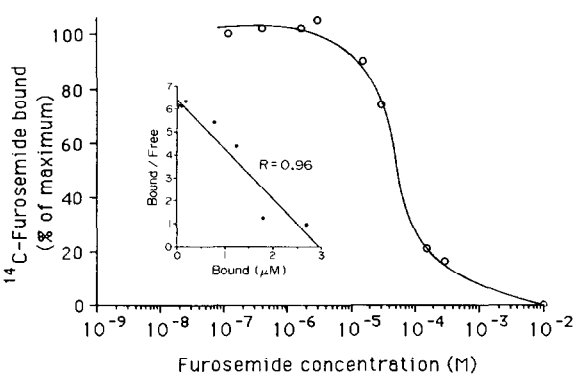

Figure 5
Inhibition of specific ^{14}C-furosemide binding to human erythrocytes preincubated with 10^{-4} M DIDS by unlabelled furosemide. Inset: Scatchard analysis of specific furosemide binding data. Each value is the mean of two experiments.

analysis (Fig. 5, inset) showed that the observed decrease of binding was accounted for by a decrease in the concentration of binding sites (B_{max} = 2.5 × 10^6 sites/cell as compared with 1.1 ± 0.06 × 10^7 sites/cell in control cells) rather than by significant alteration in affinity (K_D = 4.6 × 10^{-5} M as compared with 1.14 ± 0.53 × 10^{-5} M in control cells).

In conclusion, our experiments on ^{14}C-furosemide binding to intact human red blood cells showed that furosemide is bound avidly by erythrocytes. Binding was rapid, reversible, specific and saturable and was unaffected by ouabain, the specific inhibitor of the Na^+/K^+ pump. The large number of binding sites and the decrease of ^{14}C-furosemide binding observed when the protein in band 3, i.e. the membrane macromolecule-mediating anion transport, was irreversibly blocked by stilbene derivatives, suggested that furosemide binding sites are probably located on (or near) the protein in band 3. This seems to be in agreement with the conclusions of Brazy and Gunn [7] which were derived from measurements of anion fluxes. However, we cannot exclude that DIDS may merely be affecting the binding of furosemide at a site remote from the anion transport unit.

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