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[^{3}H]Bumetanide Binding to Avian Erythrocyte Membranes

CORRELATION WITH ACTIVATION AND DEACTIVATION OF Na/K/2Cl COTRANSPORT*

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The relationship between Na/K/2Cl cotransport activation in duck erythrocytes and binding of the diuretic [^{3}H]bumetanide to isolated membranes from stimulated cells has been assessed. Cotransport was activated by either cAMP-dependent (norepinephrine) or -independent (fluoride, hypertonicity) pathways. Membranes isolated from unstimulated cells possessed no specific bumetanide binding. In the presence of norepinephrine, cotransport and saturable binding rose in parallel, reaching a maximum after 5–7 min. In membranes from maximally stimulated cells the $K_{½}$ and B_{max} for bumetanide binding were 100 nM and 1.7 pmol/mg protein, respectively. The diuretic binding properties of these membranes were characteristic of interactions of ligands with the Na/K/2Cl cotransporter: specific binding required the presence of all three cotransported ions (Na, K, and Cl), and the rank order of potency for diuretic competition with bumetanide for binding sites was benzmetanide > bumetanide > furosemide. The appearance of specific bumetanide binding was also seen in membranes from erythrocytes activated by non-cAMP-dependent stimuli, with an excellent temporal correlation between cotransport activation and diuretic binding. On removal of all stimuli both cotransport and bumetanide binding declined in parallel. Duck erythrocytes treated with norepinephrine in a solution containing 15 mM K^+ swell to a new stable cell volume after 60 min, during which time cotransport becomes inoperative. Bumetanide binding to both whole cells and isolated membranes paralleled the decline in cotransport activity. It is concluded that bumetanide binding to isolated membranes faithfully reflects the state of activation of the Na/K/2Cl cotransporter in intact cells under a variety of conditions.

The Na/K/2Cl cotransport system plays a central role in salt and water transport in secretory and absorptive epithelia (for reviews see Greger, 1985; O'Grady *et al.*, 1987b; Haas, 1989). It is also involved in cell volume regulation in a variety of epithelial and non-epithelial cells (*e.g.* see Kregenow, 1973; Sands *et al.*, 1986). Although many physiological aspects of cotransport function have been well studied, little is known at the biochemical level about the molecular components of the cotransporter. One important feature of the system that has been exploited from both a physiological and a biochemical standpoint is its sensitivity to loop diuretics (O'Grady *et al.*, 1987b). Bumetanide is a representative of this class of compound that binds to and inhibits the Na/K/2Cl cotransporter. [^{3}H]Bumetanide has been used as a ligand for quantitation of Na/K/2Cl cotransporters in several tissues (*e.g.* Forbush and Palfrey, 1982, 1983; Haas and Forbush, 1986; O'Grady *et al.*, 1987a; Franklin *et al.*, 1989; O'Donnell, 1989; Wiener and van Os, 1989; Kort and Koch, 1989) and an analogous ligand, [^{3}H]piretanide, has been used in studies on Madin-Darby canine kidney cells (Giesen-Crouse and McRoberts, 1987) and on dog kidney membranes (Giesen-Crouse *et al.*, 1985).

Substantial evidence has accumulated that labeled diuretics bind directly to the cotransporter. For example, [^{3}H]bumetanide binding requires each of the cotransported ions (Na, K, and Cl), and these ions stimulate binding with K_m values similar to those found for ion transport itself (Forbush and Palfrey, 1983; Haas and Forbush, 1986). There is some evidence that bumetanide interacts with the second Cl site on the cotransporter (Forbush and Palfrey, 1983; Haas and McManus, 1983; O'Grady *et al.*, 1987a); in view of this, Haas and Forbush (1986) have suggested that the inhibited form of the Na/K/2Cl cotransporter has 1 mol each of Na, K, Cl, and bumetanide bound to it. Efforts have been made to extend these reversible binding studies by affinity labeling the Na/K/2Cl cotransporter with photoactivatable analogs of bumetanide (Haas and Forbush, 1987, 1988). In these studies, a protein of ~150 kDa (as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was labeled in dog kidney and duck erythrocytes that exhibited the affinity and ion dependence expected of binding to the cotransporter. Unfortunately, the low specific activity of this label has rendered further purification of the relevant protein difficult.

The regulation of the Na/K/2Cl cotransport system varies in different tissues, and a wide variety of agents affect its activity (for reviews see Palfrey and Rao, 1983; O'Grady, 1987b; Haas, 1989). In avian red cells Na/K/2Cl cotransport is accelerated by agents that raise the level of intracellular cAMP (*e.g.* β-adrenergic agonists, adenosine, cholera toxin, and cAMP analogs: see Riddick *et al.*, 1971; Palfrey and Greengard, 1981). Cotransport can also be stimulated independent of an increase in cAMP by fluoride, hypertonically induced cell shrinkage, and deoxygenation (Palfrey and Greengard, 1981). Stimulation by cAMP-dependent and -independent means is not additive suggesting that the pathways by which they activate the system converge on the same population of transporters. Further investigation of the processes underlying cotransport regulation have been hampered by the difficulty of studying the transport system in a cell-free environment. Thus far, efforts to reseal avian erythrocytes and retain cotransport activity have failed.

Haas and Forbush (1986) observed that activation of Na/K/2Cl cotransport in duck erythrocytes by norepinephrine

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(NE)[1] or cell shrinkage led to a parallel rise in specific [^{3}H] bumetanide binding, suggesting that bumetanide binds only to an activated state of the Na/K/2Cl cotransport system. In agreement with this notion, subsequent studies in other cell types by O'Donnell (1989) and Franklin *et al.* (1989) have shown that agents that modulate cotransport also change intact cell diuretic binding. However, whole-cell-binding studies have serious limitations. For example, in duck erythrocytes NE activation of cotransport reaches a maximum after 5 min at 41 °C (Kregenow, 1973), but specific bumetanide binding requires at least 10 min to attain equilibrium (Haas and Forbush, 1986) thus making it impossible to resolve the time dependence of the effect of NE on diuretic binding to the cotransporter. In addition, little new information regarding cotransport regulation in avian erythrocytes can come from this approach. We reasoned that if bumetanide binding is retained in isolated membranes from these cells it may be possible to probe modulatory influences on cotransport activity in the absence of a functional cell-free transport assay. In the present work, we show that stimulation of bumetanide binding induced in duck erythrocytes by both cAMP-dependent and -independent stimuli is retained in membranes isolated from these cells. The precise kinetic correlations between cotransport activation/deactivation and diuretic binding suggests that bumetanide binding accurately reflects the state of the cotransporter in the intact cell.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Bumetanide (specific activity 27 or 66 Ci/mmol) was the generous gift of Drs. Bliss Forbush, III (Yale University) or R. James Turner (National Institutes of Health). Unlabeled bumetanide and furosemide was the gift of Hoechst Pharmaceuticals (New Jersey). Other 3- and 4-substituted 5-sulfamoylbenzoic acid derivatives were provided by Dr. P. W. Feit (Leo Pharmaceuticals, Denmark).

Preparation of Cells—Blood was collected from white Pekin ducks at a local poultry house in heparinized HEPES-buffered saline (HBS, in mM: 155 NaCl, 2.5 KCl, and 10 HEPES, pH 7.4). The plasma and buffy coat were removed after centrifugation and the red cells washed three times with HBS. The cells were resuspended to 20% hematocrit in HBS 0.2% glucose, and penicillin (100 units/ml) plus streptomycin (100 μg/ml). The cells could be kept at 4 °C in this solution for up to 5 days without significant loss of function. Before use the cells were diluted to 5% hematocrit in fresh HBS, 0.2% glucose and allowed to equilibrate at 41 °C for 90 min.

Unidirectional ^{86}Rb Influx Measurements—^{86}Rb influx measurements were performed by minor modification of a previously described procedure (Palfrey *et al.*, 1980). Briefly, cells at 10% hematocrit were incubated at 41 °C in a solution that was either "low K" (HBS, 0.2% glucose) or "high K" (in mM, 142.5 NaCl, 15 KCl, 10 HEPES, pH 7.4, and 0.2% glucose). Cells were stimulated by 1:1 dilution in the appropriate solution containing norepinephrine (2×10^{-5} M), fluoride (20 mM, replacing chloride), or sucrose (140 mM). 0.09 ml of this suspension was transferred to 12 × 75-mm glass tubes and isotope influx initiated by the addition of 0.01 ml of HBS containing ^{86}Rb (2 μCi) and ouabain (10 mM) (final volume 0.1 ml). Influx was continued for 20–60 s at 41 °C and terminated by the addition of 4 ml of ice-cold stop solution (in mM, 160 choline Cl plus 10 HEPES, pH 7.4). The cells were centrifuged and washed successively with 4 and 2 ml of this solution. Packed cells were resuspended in 2 ml of stop solution, transferred to scintillation vials, and the Cerenkov radiation counted in a Packard scintillation counter with 20–30% efficiency. ^{86}Rb influx was expressed as mmoles of K^+ equivalents transported/liter of cells/hour.

Isolation of Membranes—A crude membrane fraction was prepared by minor modification of a previously described procedure (Beam *et al.*, 1979). Briefly, following appropriate treatments to activate cotransport, red cells (20% hematocrit) were immediately lysed in 20 volumes of ice-cold lysis buffer (LB, 10 mM HEPES, 5 mM $MgCl_2$, pH 8). Subsequent procedures were all at 0–4 °C. The ghosts were pelleted by centrifugation at 10,000 × *g* for 1 min (SS-34 rotor) and washed with an additional 20 volumes of LB, then resuspended in 0.3 volumes of LB and sonicated for 10 s with a probe sonifer (Branson). Nuclei and residual nucleated ghosts were removed by centrifugation (1500 × *g* for 10 min). Crude membranes were then pelleted from the supernatant by high speed centrifugation (100,000 × *g* for 50 min in a Beckman Ti-50.2 rotor). The pellets were resuspended in buffer A (in mM, 250 sucrose, 1 EGTA, 0.1 phenylmethylsulfonyl fluoride, and 10 HEPES brought to pH 7.4 with triethanolamine) and stored on ice or snap-frozen in liquid N_2 and stored at −70 °C.

Determination of [^{3}H]Bumetanide Binding to Membranes—[^{3}H] Bumetanide binding to membranes was measured by a filtration assay similar to that described by Forbush and Palfrey (1983). The binding reactions were performed in a final volume of 0.1 ml. The membranes were diluted with buffer A to a protein concentration of 2.86 mg/ml, as determined by the Folin reagent, and 0.07 ml (0.2 mg) added to each binding reaction containing 0.03 ml of a mixture of the appropriate amount of salts, HEPES buffer, and [^{3}H]bumetanide with or without unlabeled diuretic to give the desired final concentration of each. Unless stated otherwise the binding reaction contained the following: (in mM final concentration) 175 sucrose, 10 HEPES, pH 7.4, 20 Na^+, 40 K^+, 20 Cl^-, 20 SO_4^{2-}, 0.7 EGTA, 0.07 phenylmethylsulfonyl fluoride, and 120 nM [^{3}H]bumetanide (0.79 μCi) ± 10 μM unlabeled bumetanide. At various times the samples were diluted with 4 ml of ice-cold wash buffer (10 mM HEPES, pH 8.0) and filtered through Whatman GF-B filters on a 24-port filtration apparatus (Brandel) followed rapidly by three additional ice-cold 4-ml washes. Under these conditions dissociation of [^{3}H]bumetanide from membranes was negligible (*cf.* Fig. 3*b*). The filters were then placed in vials with 4 ml of Budgetsolve scintillation mixture (RPI), and counted with an efficiency of ~53%. The results are expressed in pmoles of [^{3}H]bumetanide bound/milligram of membrane protein.

Determination of [^{3}H]Bumetanide Binding to Whole Cells—The method of Haas and Forbush (1986) was used to determine [^{3}H] bumetanide binding to whole cells.

RESULTS

Norepinephrine Stimulation of ^{86}Rb Influx and [^{3}H]Bumetanide Binding—We set out to correlate the time course of activation of ^{86}Rb influx by NE (10^{-5} M) in intact red cells with the activation of specific saturable [^{3}H]bumetanide binding in isolated plasma membranes (Fig. 1). After an initial lag of 0.5–1 min ^{86}Rb influx showed a first order increase reaching a maximum rate at 3–4 min (Fig. 1*a*). As previously described (Palfrey *et al.*, 1980), this increase is entirely due to stimulation of Na/K/2Cl cotransport and is completely inhibited by bumetanide (10^{-5} M). Aliquots of the same batch of erythrocytes were lysed at various times after addition of NE, membranes were isolated and a [^{3}H]bumetanide binding assay was performed (Fig. 1*b*). Membranes from untreated cells had no saturable [^{3}H]bumetanide binding, but in the presence of NE saturable binding rose in parallel with the stimulation of cotransport (no change in nonspecific binding occurred after NE stimulation). Both the rise in ^{86}Rb influx and in specific bumetanide binding could be fit as first order exponentials yielding time constants of 0.0251 ± 0.0039 (S.D.)s^{-1} and 0.0153 ± 0.0047 (S.D.)s^{-1}, respectively (Fig. 1*c*). The close similarity of these curves suggests that the same reaction that activates ^{86}Rb influx also activates specific bumetanide binding. Specific binding to membranes from stimulated cells was stable overnight at 0 °C and for extended periods if membranes were snap frozen and stored at −70 °C.

Equilibrium Binding and Kinetics of [^{3}H]Bumetanide Binding to Membranes Isolated from Norepinephrine-stimulated Cells—To establish that the specific bumetanide binding found in isolated membranes from stimulated cells was characteristic of interaction with the Na/K/2Cl cotransporter, several properties of this interaction were investigated. Specific binding was saturable (Fig. 2); a nonlinear least squares fit to the saturable component to a single binding site model

[1] The abbreviations used are: NE, norepinephrine; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; LB, lysis buffer.

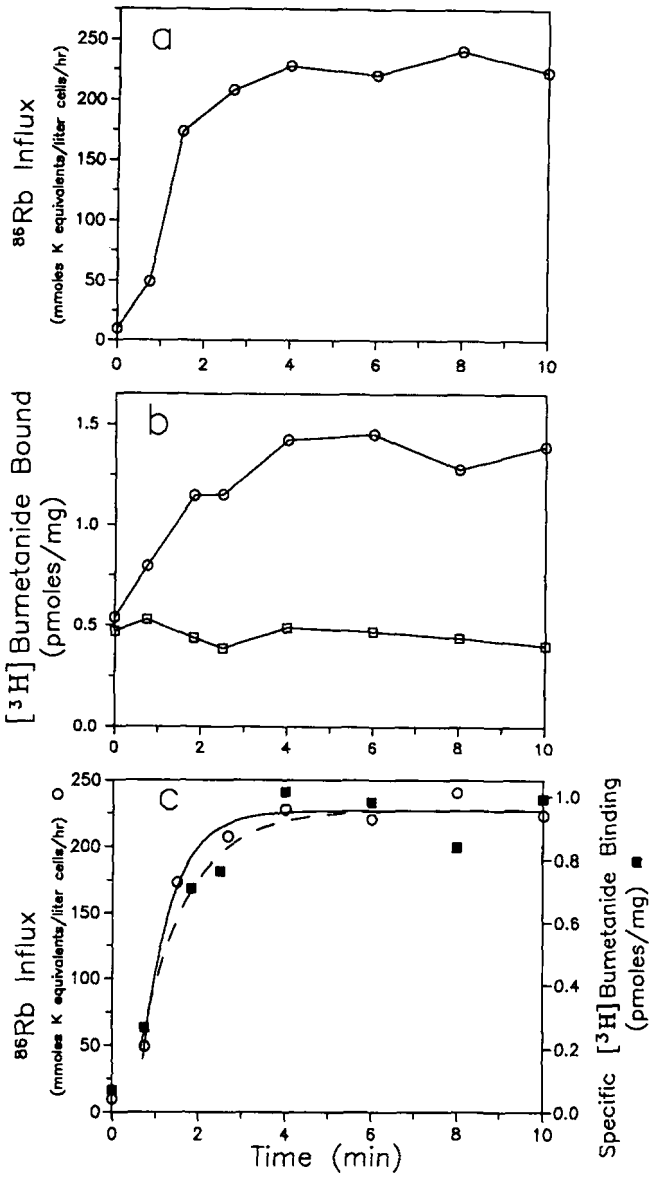

FIG. 1. **Norepinephrine stimulation of ^{86}Rb influx and [^{3}H] bumetanide binding.** ^{86}Rb influx (*a*) and [^{3}H]bumetanide binding to isolated membranes (*b*) were measured in parallel after addition of NE (10^{-5} M) to duck erythrocytes. Red cells were preincubated at 10% hematocrit in HBS, 0.2% glucose at 41 °C for 90 min. Following NE addition, ^{86}Rb influx was initiated in an aliquot of cells 30 s before, and terminated 30 s after the indicated time point. 1.5-ml aliquots from the same batch of cells were lysed in 30 ml of LB at the appropriate times then processed for membrane isolation and [^{3}H]bumetanide binding. Control values plotted at zero time) were determined in cells not exposed to NE. The binding reaction was allowed to equilibrate for 40 min at room temperature before filtration, and the average of duplicate samples for total [^{3}H]bumetanide binding (○), and binding in presence of 10 μM unlabeled bumetanide (□), was determined. In *c*, ^{86}Rb influx and specific bumetanide binding are replotted and each curve fit to a single exponential using a nonlinear least squares analysis program. The corresponding computer generated curves for influx (○——○) and specific binding (■---■) are shown. See text for time constants.

provides a K_d of 100 nM and a B_{max} value of 1.7 pmol/mg. The K_d value is in excellent agreement with values reported for the IC_{50} of bumetanide inhibition of Na/K/2Cl cotransport in avian erythrocytes and with the K_d values reported for bumetanide binding to intact duck red cells (Palfrey *et al.*, 1980; Haas and Forbush, 1986) as well as putative Na/K/2Cl cotransporters in other tissues (Forbush and Palfrey, 1983; Palfrey *et al.*, 1984; O'Grady *et al.*, 1987a, 1987b; O'Donnell,

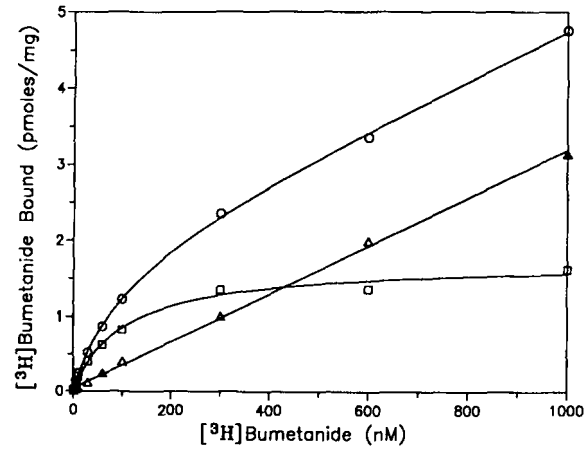

FIG. 2. **Equilibrium binding of [^{3}H]bumetanide to membranes.** The final binding reaction contained membranes isolated from maximally NE-stimulated duck red cells (2 mg/ml) and various concentrations of [^{3}H]bumetanide as shown. The samples were incubated at room temperature for 30 min before filtration. ○, total [^{3}H]bumetanide binding, △, nonspecific binding determined in the presence of 10 μM unlabeled bumetanide, and, □, saturable binding. The experiment was repeated several times with identical results. The results are the average of duplicate samples.

1989). A lower affinity site that has been found in certain other tissues (*e.g.* Wiener and van Os, 1989) was not observed in duck erythrocyte membranes. The B_{max} is slightly lower than the 2.5–5 pmol/mg membrane protein estimated from binding experiments on intact red cells (Haas and Forbush, 1986). This may be due to loss of binding activity during the membrane isolation procedure or contamination of the final crude membrane pellet with mitochondrial or other nonplasmalemmal membranes. It was found that the conditions of lysis had an effect on the extent of binding subsequently found in the membrane fraction. In particular, raising Mg^{2+} from 5 to 20 mM decreased the specific binding at 120 nM [^{3}H]bumetanide by 40%.[2]

The time courses of bumetanide binding to and dissociation from membranes isolated from NE stimulated cells are shown in Fig. 3. The association of bumetanide with the membrane, as reflected in the rise of saturable binding, was well fit by a single exponential (Fig. 3*a*; 1.38×10^{-3} s^{-1}). After equilibration of ligand binding and dilution into a large excess of unlabeled medium at 0 °C, the loss of specific binding was also fit by a single exponential yielding a time constant for dissociation of 1.97×10^{-4} s^{-1} (Fig. 3*b*).

The affinity of bumetanide for the saturable binding site can be estimated using the time constants for approach to equilibrium binding (Fig. 3*a*) and dissociation (Fig. 3*b*). Using a single binding site model,

$$R + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} R \cdot B$$

for the situation where the ligand (B) is in excess of the receptor (R), the time constant for approach to equilibrium binding is given by $K_a = (k_1 B + k_{-1})$, and the dissociation time constant is given by $K_d = k_{-1}$. From these equations and the values 1.38×10^{-3} s^{-1}, 1.97×10^{-4} s^{-1}, 120 nM for K_a, K_d, and B, respectively, an equilibrium binding constant for the saturable component of bumetanide binding, $K_{eq} = k_{-1}/k_1 =$ 20 nM, was determined. The difference between this value and the K_d value of 100 nM obtained from the equilibrium binding isotherm pictured in Fig. 2, probably arises because

[2] E. B. Pewitt, R. S. Hegde, and H. C. Palfrey, unpublished results.

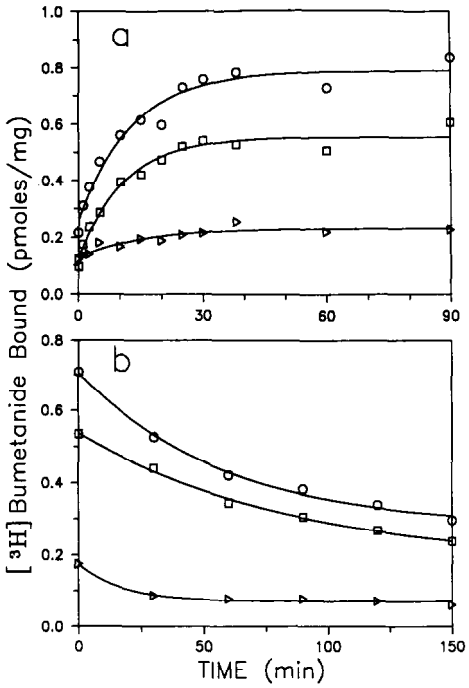

FIG. 3. **Kinetics of [^{3}H]bumetanide association and dissociation from stimulated membranes.** *a*, the time course of bumetanide association was determined by varying the time between addition of membranes to the binding reaction and filtration. The final binding medium contained membranes (2 mg/ml) and [^{3}H]bumetanide at a concentration of 120 nM (an excess of bumetanide over the number of sites). *b*, the time course of dissociation of [^{3}H]bumetanide bound to membranes was determined by preincubating membranes for 20 min at room temperature with [^{3}H]bumetanide (120 nM) then diluting with 4 ml of ice-cold 10 mM HEPES, pH 8. The diluted membranes were incubated on ice for the indicated times prior to filtration; ○, total bound, ▷, nonspecific binding, and □, specific binding. The curves drawn to the figure are single exponential computer fits to the data and the time constants are given in the text.

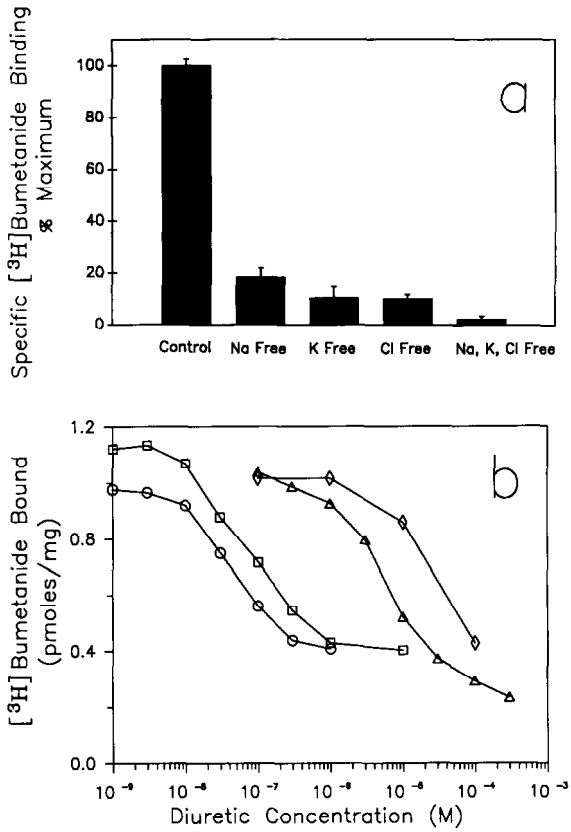

FIG. 4. **Characteristics of [^{3}H]bumetanide binding to stimulated membranes.** *a*, the ion dependence of [^{3}H]bumetanide binding was assessed by substituting the transported ions Na, K, and Cl in the binding reaction, using membranes isolated from maximally NE-stimulated erythrocytes. The control reaction contained the following ions (in mM) 20 Na^+, 40 K^+, 20 Cl^-, and 20 SO_4^{2-} (identical to the binding conditions used in Fig. 1). Na^+ was substituted with choline$^+$ (Na-free), K^+ with 20 mM choline$^+$ and 20 mM triethanolamine$^+$ (K-free), and Cl^- with 10 mM SO_4^{2-} (Cl-free). When all three transported ions were replaced, 30 mM triethanolamine sulfate was used (Na-, K-, Cl-free). The binding reactions all contained 120 nM [^{3}H]bumetanide ± 10 μM unlabeled bumetanide and were filtered after 20 min at room temperature. The results shown are the average ± S.E. of duplicate experiments on two spearate membrane preparations. *b*, competition for [^{3}H]bumetanide-binding sites by related compounds was determined. Membranes isolated from maximally NE-stimulated duck erythrocytes were incubated with [^{3}H]bumetanide (120 nM) and various concentrations of unlabeled diuretics. The compounds used were benzmetanide (○), bumetanide (□), furosemide (△), compound XII (◇) (Palfrey *et al.*, 1980). The plotted points are averages of triplicate samples. Apparent K_i values derived from these plots are 0.03, 0.07, 6, ~25 μM, respectively.

K_{eq} was determined at 0 °C while K_d was obtained at 24 °C. The fact that specifically bound [^{3}H]bumetanide dissociates with a $t_{1/2}$ of 1 h at 0 °C confirms that negligible dissociation of radioactivity occurs during the filter washing step of the binding assay.

Ion Dependence of [^{3}H]Bumetanide Binding—The cotransported ions, Na^+, K^+, and Cl^- have previously been found to be required for bumetanide to bind to the transporter and to affect the degree of inhibition of transport by the diuretic (Palfrey *et al.*, 1980; Forbush and Palfrey, 1983; Haas and Forbush, 1986). Thus, the ion dependence of [^{3}H]bumetanide binding to membranes isolated from NE-stimulated cells was examined to confirm that the specific component of binding is indeed to the cotransporter (Fig. 4*a*). Optimal [^{3}H]bumetanide binding was determined in a medium containing all three cotransported ions at concentrations designed to give a maximum level of binding. When any one, or all, of the cotransported ions was eliminated from the binding reaction, specific [^{3}H]bumetanide binding was greatly reduced or eliminated.

Competition for [^{3}H]Bumetanide-binding Sites by Other Loop Diuretics—An additional characteristic of loop diuretic inhibition of Na/K/2Cl cotransport in avian erythrocytes and other cells is the rank order of potency of members of this class of diuretics: benzmetanide > bumetanide > furosemide > 3-(5-1,3-dihydroisobenzofuranylmethylamino)-4-phenoxy-5-sulfamoylbenzoic acid(compound XII; Palfrey *et al.*, 1980). The same rank order of potency for competition with bumetanide binding was found in membranes from stimulated cells (Fig. 4*b*).

Trypsin Destruction of [^{3}H]Bumetanide Binding—To demonstrate that the specific [^{3}H]bumetanide-binding site is a protein, membranes isolated from maximally stimulated red cells were assayed for specific [^{3}H]bumetanide binding after incubation with trypsin for various times. Aliquots were removed and added to the binding reaction at 0 °C in the presence of an excess of soybean trypsin inhibitor to prevent further proteolysis during the binding assay. Specific [^{3}H]bumetanide binding in the trypsinized sample disappeared exponentially and was reduced >90% after 10 min; binding in control membranes not exposed to trypsin was reduced by only 27% over the same time period (Fig. 5).

Hypertonic and Fluoride Stimulation of Na/K/2Cl Cotransport and [^{3}H]Bumetanide Binding—Haas and Forbush (1986) showed that hypertonically induced shrinkage of duck eryth-

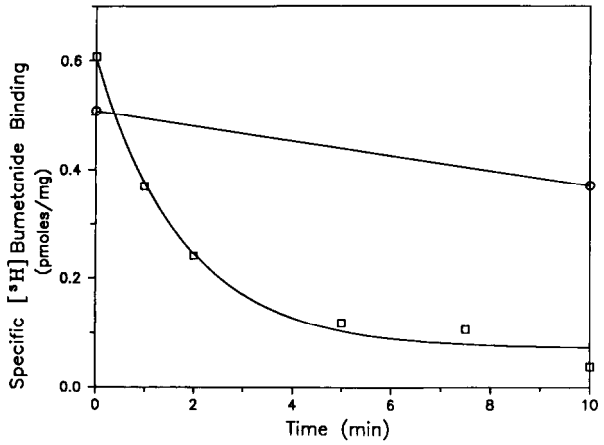

FIG. 5. **Trypsin destruction of [^{3}H]bumetanide binding.** Membranes (2 mg/ml) isolated from maximally NE-stimulated duck red cells were incubated with trypsin (50 μg/ml) at 30 °C in a solution consisting of sucrose (250 mM), HEPES, pH 7.4, (10 mM) and digitonin (0.01% w/v, included to allow trypsin access to the inside of any sealed vesicles in the membrane preparation). □, aliquots were removed at the appropriate times and added to a binding reaction mixture containing soybean trypsin inhibitor (1 mg/ml). O, a control sample of the same membranes was incubated in parallel without trypsin and aliquots were removed for a binding assay under identical conditions. The binding reactions were allowed to equilibrate on ice for 1 h before filtration.

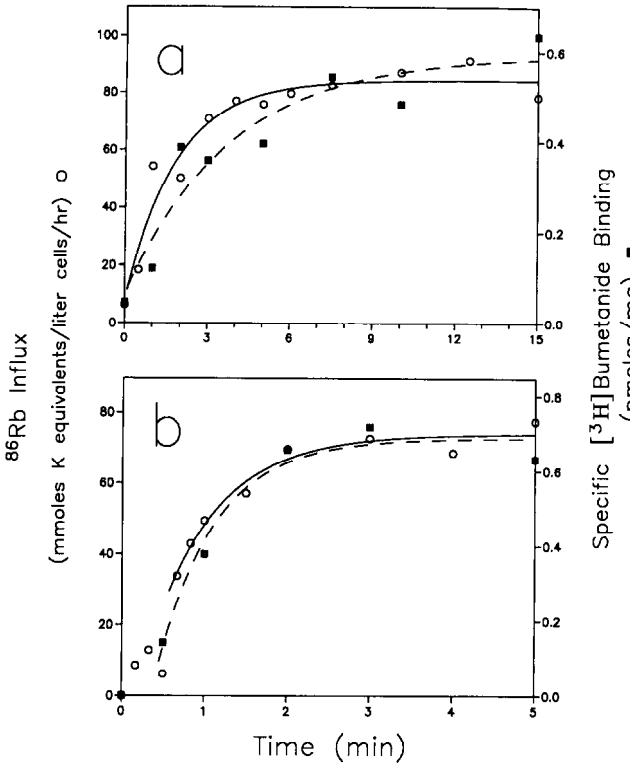

FIG. 6. **Hypertonic and fluoride stimulation of cotransport and [^{3}H]bumetanide binding.** The same general protocol as described in Fig. 1. was used to examine hypertonic and fluoride stimulation of cotransport and bumetanide binding. *a*, ^{86}Rb influx (O) (1 min uptake) and specific [^{3}H]bumetanide binding (■) were measured on cells stimulated by hypertonic treatment (1:1 dilution of the cells in HBS, 0.2% glucose buffer containing 140 mM sucrose). *b*, ^{86}Rb influx (O) (20 s uptake) and specific [^{3}H]bumetanide binding (■) was measured after stimulation of cells with NaF (10 mM). In both *a* and *b*, each set of points was fit to a single exponential using a non-linear least-squares analysis program and the computer generated curves are shown. See text for time constants.

rocytes led to an increase in binding of [^{3}H]bumetanide to intact cells similar to that induced by NE. In order to test whether stimulated [^{3}H]bumetanide binding would be retained in membranes after activation of transport by agents other than catecholamines, we examined specific binding in membranes from cells incubated with fluoride or in hypertonic media, conditions that activate Na/K/2Cl cotransport independent of a rise in intracellular cAMP (Palfrey and Greengard, 1981). Activation of ^{86}Rb influx was found to be half-maximally stimulated by raising the tonicity of the external medium by 75 mOsm in duck erythrocytes (data not shown). Exposure of cells to hypertonic media also stimulated specific [^{3}H]bumetanide binding in the isolated membranes in parallel with the stimulation of cotransport (Fig. 6*a*). The parameters showed a first order increase reaching a maximum 10 min after hypertonic shrinkage. When the rise in ^{86}Rb influx and in specific bumetanide binding were each fit to a single exponential, the time constants 0.0090 ± 0.0027 (S.D.)s^{-1} and 0.0044 ± 0.003 (S.D.)s^{-1}, respectively, were obtained. Activation of ^{86}Rb influx in duck erythrocytes was found to be half-maximally stimulated at 2.5 mM NaF and maximally at 10 mM NaF (data not shown). A comparison of the time course of fluoride (10 mM) stimulation of ^{86}Rb influx in duck erythrocytes with the parallel appearance of [^{3}H]bumetanide binding in isolated plasma membranes is shown in Fig. 6*b*. Following a lag of ~30 s, both cotransport activity and specific [^{3}H] bumetanide binding rise to a maximum 2–3 min after fluoride addition. A single exponential fit to the influx and to the specific binding curve yielded time constants of 0.0213 ± 0.0047 (S.D.)s^{-1} and 0.0237s^{-1} ± 0.0087 (S.D.)s^{-1}, respectively. The specific binding characteristics of membranes from cells maximally stimulated by hypertonicity or fluoride were similar to those described above for membranes from NE-stimulated cells. Such binding was also stable when membranes at 0 °C were stored overnight or at −70 °C for extended periods.

Propranolol Reversal of Norepinephrine Stimulated Cotransport and [^{3}H]Bumetanide Binding—If, on reaching a maximal transport rate in the presence of NE, avian erythrocytes are exposed to a 100-fold excess of the β-adrenergic antagonist propranolol, cotransport rapidly declines to a basal rate (Alper *et al.*, 1980). Similarly, [^{3}H]bumetanide binding to isolated membranes was also rapidly reversed by propranolol (Fig. 7, *a* and *b*). In this experiment, cotransport and [^{3}H] bumetanide binding to isolated membranes reached a maximum 7 min after addition of NE and returned to basal levels 15 min after addition of propranolol. Whole cell [^{3}H]bumetanide binding was also found to closely track NE stimulation and propranolol reversal of cotransport.

Relationship between Cotransport Activation and [^{3}H]Bumetanide Binding in Swollen Duck Red Cells—At $[K^+]_o$ = 2.5 mM, duck red cells maintain a constant volume after β-adrenergic stimulation. Under these conditions the Na/K/2Cl cotransporter remains active for extended periods of time. Elevation of external [K^+] to 15 mM establishes conditions favoring net salt uptake causing these cells to swell after NE treatment (*cf.* Kregenow, 1973). As the cells reach a maximum volume, the rate of cotransport progressively declines toward the basal level over the ensuing 60 min (Kregenow, 1973). We wished to ascertain if stimulated [^{3}H]bumetanide binding, both in whole cells and isolated membranes, would be reduced in parallel with cotransport in swollen cells. The time course of activation and inactivation of cotransport stimulated by NE in high K^+ medium is shown in Fig. 7*c*. Binding to whole cells and to isolated membranes measured in the identical batch of NE-stimulated cells followed the same time course as cotransport activity, peaking 5–10 min after stimulation

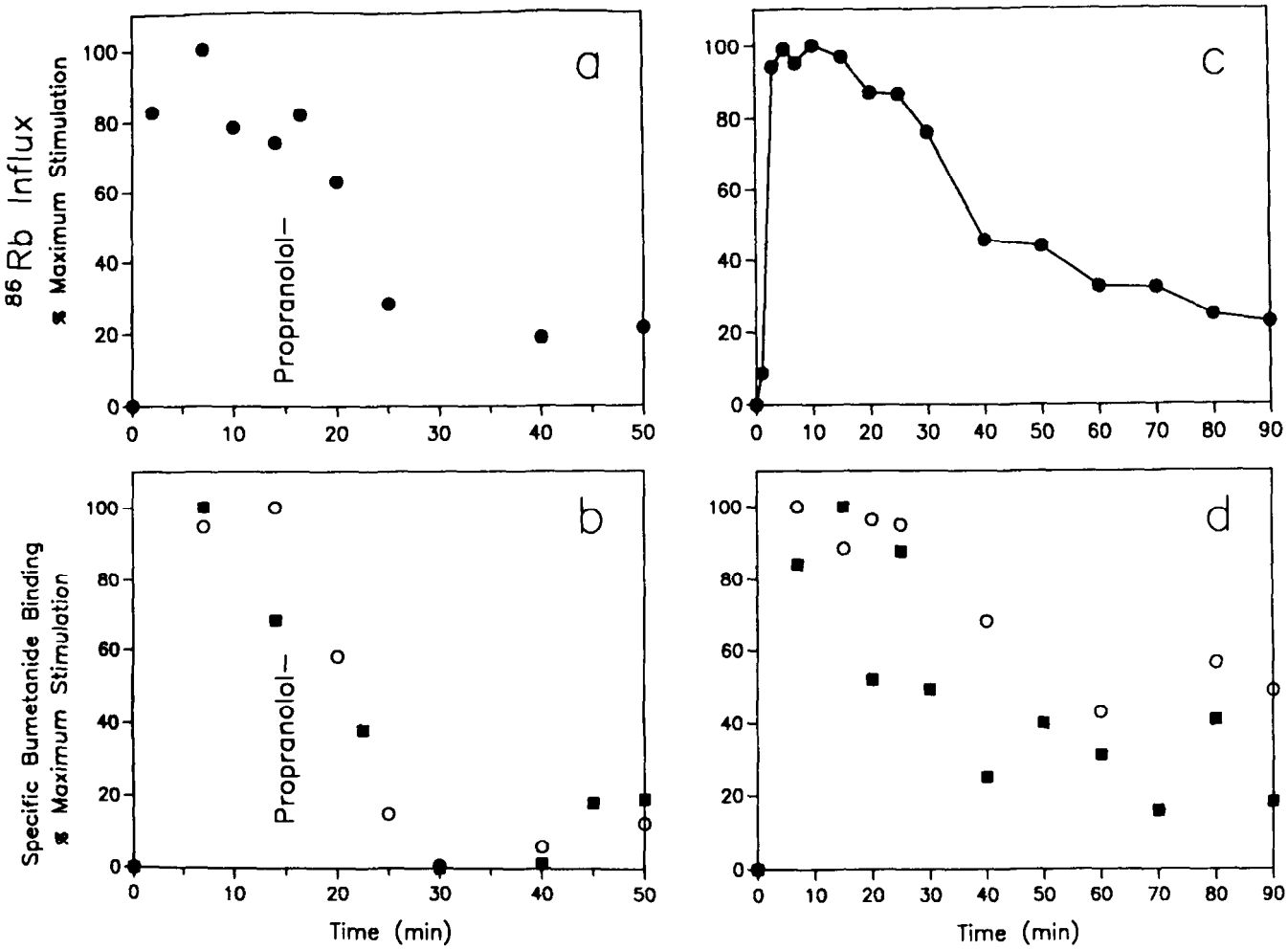

FIG. 7. **Reversal of NE activation of cotransport and [^{3}H]bumetanide binding by propranolol and cells swelling.** NE (5×10^{-6} M) was added to red cells (15% hematocrit) at time zero, and at various times aliquots of cells were removed for ^{86}Rb influx (1 min uptake), whole cell [^{3}H]bumetanide binding and [^{3}H] bumetanide binding to isolated membranes. Whole cell [^{3}H]bumetanide binding was determined by addition of cells to a binding reaction 10 min before filtration to allow equilibrium to be reached (the time point for the whole cell binding activity is plotted 5 min prior to filtration). The ordinates were normalized to % of maximum NE stimulation to facilitate comparison of ^{86}Rb influx (●), whole cell specific bumetanide binding (■), and specific bumetanide binding to isolated membranes (○). *a*, propanolol (1×10^{-4} M) was added 15 min after addition of NE. *b*, the cells were stimulated in high K medium to allow cell swelling resulting in cotransport deactivation (see text).

and declining toward base line over the ensuing 80 min (Fig. 7*d*).

DISCUSSION

The results presented here show that specific bumetanide binding, characteristic of interaction with the Na/K/2Cl cotransporter, is exhibited in a membrane fraction isolated from stimulated duck red cells. Once isolated, such membranes retain their specific binding characteristics if stored overnight at 0 °C or for extended periods at −70 °C. The amount of specific binding recovered in the membranes is related to the activation state of the Na/K/2Cl cotransporter in the cells from which the membranes are isolated. Haas and Forbush (1986) showed that bumetanide binding to duck red cells could be stimulated by NE and hypertonic cell shrinkage, suggesting that bumetanide binds only to an activated state of the Na/K/2Cl cotransport system. The present work extends this finding and shows that this activated state survives the membrane isolation procedure irrespective of the initial stimulus used. The fact that specific bumetanide binding was not found in membranes from untreated cells argues against the possibility that stimulation of these cells results in translocation of transporters from some internal compartment (*e.g.* subplasmalemmal vesicles) to the plasma membrane. Such vesicles should appear in the high speed particulate fraction used here.

The [^{3}H]bumetanide binding properties of isolated avian erythrocyte membranes exhibited several properties characteristic of the Na/K/2Cl cotransport system. The K_d of 100 nM is in excellent agreement with the K_d reported for inhibition of transport by this diuretic in avian erythrocytes (Palfrey *et al.*, 1980; Haas and Forbush, 1986) and other systems (see O'Grady *et al.*, 1987b for review). The B_{max} of 1.7 pmol/mg protein is in reasonable agreement with the 2.5–5 pmol/mg estimated from whole cell binding experiments in duck erythrocytes (Haas and Forbush, 1986; Haas, 1989). The saturable component of [^{3}H]bumetanide binding required the simultaneous presence of all three cotransported ions, as shown in other systems (Forbush and Palfrey, 1981; Haas and Forbush, 1986; O'Grady *et al.*, 1987a; O'Donnell, 1989) and was inhibited with a characteristic rank order of potency of the 5-sulfamoylbenzoic acid class of "loop" diuretics. A protein

component of the membrane is responsible for saturable [^{3}H] bumetanide binding as demonstrated by the trypsin destruction of specific binding shown in Fig. 5.

Agents that activate the Na/K/2Cl cotransporter segregate into cAMP-dependent and -independent stimuli (Palfrey and Greengard, 1981) and, as shown here, members of both groups activate specific bumetanide binding in membranes isolated from cells exposed to them. In the case of cAMP-dependent stimuli, it is reasonable to suggest that cAMP-dependent protein phosphorylation of the Na/K/2Cl cotransporter itself or an associated regulatory protein may activate the system[3] (Alper *et al.*, 1980; Palfrey and Greengard, 1981). On the other hand, the mechanisms by which fluoride and cell shrinkage activate Na/K/2Cl cotransport are not understood, but it has been suggested they do not involve elevations in intracellular cAMP concentrations nor cAMP-dependent protein phosphorylation (Palfrey and Greengard, 1981). Stimulation of bumetanide binding by these agents also survives hypotonic lysis, removal of the cytoplasm and sonication. This finding argues against a hypothesis where cell shrinkage activates Na/K/2Cl cotransport through a rise in the cellular concentration of a cytoplasmic element due to a reduction in the cell water content. Activation by such a mechanism would not be expected to survive membrane isolation. However, if shrinkage serves to bring proteins together in the membrane, then the activation might well survive membrane isolation. In the case of fluoride stimulation, this anion is presumably removed during membrane isolation, since stimulation by fluoride can be easily reversed by simply washing the cells in fluoride-free media; yet the isolated membranes retain their ability to bind bumetanide. Stimulation of Na/K/2Cl cotransport by cAMP-dependent and -independent agents is not additive, suggesting these pathways of stimulation converge on the same population of Na/K/2Cl cotransport molecules. From the binding data presented here it appears that these two pathways effect the same conformational change in the Na/K/2Cl cotransporter that is responsible for its ability to bind bumetanide. This question can only be answered decisively at the level of the isolated Na/K/2Cl cotransport protein.

Measurement of bumetanide binding in isolated membranes rather than intact cells has several advantages. For example, in the present study it enabled an accurate assessment of the temporal relationship between transport activation and diuretic binding to be made. Lysing duck erythrocytes after activation appears to "freeze" the transporter in its activated state and subsequent bumetanide binding to the isolated membranes exactly parallels both the activation of cotransport (Figs. 1 and 6) and the deactivation of cotransport on removal of the stimulus (fig. 7). A further advantage of membrane *versus* whole cell binding experiments that is probably applicable to a variety of cell types is a reduction in nonspecific binding enabling a more accurate determination of kinetic constants. Moreover, we have shown that in membranes from stimulated cells bumetanide binding can be modulated by several agents including nucleotides and divalent cations.[2] It seems likely, therefore, that various conformational states of the Na/K/2Cl cotransporter can be successfully probed *in vitro* with labeled inhibitors. We are currently exploiting this paradigm to investigate various regulatory influences on the cotransporter in a cell-free system.

[3] E. B. Pewitt, R. S. Hegde, M. Haas, and H. C. Palfrey, submitted for publication.

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