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Export of Cyclic AMP from Avian Red Cells

Independence from Major Membrane Transporters and Specific Inhibition by Prostaglandin A_1

L. E. HEASLEY, J. AZARI, AND L. L. BRUNTON[1]

Divisions of Pharmacology and Cardiology, School of Medicine, University of California, San Diego, La Jolla, California 92093

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SUMMARY

Prostaglandin A_1 (PGA_1) inhibits energy-dependent cyclic AMP export by pigeon red cells [Brunton and Mayer, *J. Biol. Chem.* **254**:9714 (1979)]. To assess the specificity of this action, we observed the effect of 10 μM PGA_1 (a concentration that inhibits cyclic AMP efflux greater than 95%) on a variety of membrane-protein-mediated processes that we could readily characterize and quantify in the pigeon red cell. Included in this study were isoproterenol-sensitive cyclic AMP production, ouabain-inhibitable $^{86}Rb^+$ influx, furosemide-sensitive NaCl-KCl symport, 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene (SITS)-sensitive sulfate exchange, Na^+-dependent α-aminoisobutyrate influx, and glucose and adenosine uptake. Remarkably, none of these membrane activities is significantly affected by PGA_1. Furthermore, SITS, nitrobenzylthioinosine, cytochalasin B, and Na^+-free extracellular medium (inhibitors of band 3, the nucleoside transporter, the hexose transporter, and amino acid uptake, respectively), failed to inhibit cyclic AMP export by pigeon red cells. On the basis of this data, we conclude that PGA_1 does not act via a generalized alteration of a basic property at the plasma membrane, such as its fluidity; rather, PGA_1 acts specifically to inhibit cyclic AMP extrusion. The data also imply that a transporter not relying on the Na^+ gradient and distinct from transporters of cations, anions, amino acids, sugars, and nucleosides mediates cyclic AMP export.

INTRODUCTION

In our studies of cyclic AMP export by pigeon erythrocytes and rat reticulocytes (1, 2), we have noted that prostaglandins inhibit active cyclic AMP export, PGA_1[2] most potently. From these early studies, we concluded that this effect of PGA_1 does not result from a depression of cellular ATP with consequent diminution of ATP-dependent processes (cyclic AMP production and export). We also argued, from order-of-potency data (PGA >> B = E > F), that the PGA_1-red cell interaction does not correspond to the order of lipophilicity of the prostaglandins and is thus more complex than simple partitioning between aqueous and membrane-lipid phases. Nevertheless, an amphipathic molecule such as a prostaglandin could conceivably perturb the membrane environment in a generalized manner and thereby affect activities of many membrane proteins. Indeed, several workers have proposed that prostaglandins alter the fluidity of the red cell membrane (3, 4). On the other hand, there are specific, apparently proteinaceous, binding sites that correspond to prostaglandin receptors linked to adenylate cyclase (5, 6). Our own bias is that the inhibition of cyclic AMP export by prostaglandin results from a relatively specific interaction of the prostaglandin with the cell membrane despite the amphipathic nature of PGA_1. We have addressed this question of specificity by observing the action of PGA_1 on a variety of readily measurable membrane protein-mediated activities in the pigeon red cell. Our data suggest that PGA_1 inhibits cyclic AMP efflux in a specific manner since seven other membrane activities are unaffected.

EXPERIMENTAL PROCEDURES

Red cell suspensions. Erythrocytes from male, white Carneux pigeons were collected and washed as previously described (1). Red cell suspensions (10% hematocrit, 5×10^8 cells/ml) in Earle's solution (with 50 μM Ro 20-1724 when cAMP accumulation was measured) were equilibrated at 37° for 30 min prior to experiments.

Cyclic AMP extrusion and accumulation. Extruded cyclic AMP was collected and analyzed as previously described (1) using the protein-

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[2] The abbreviations used are: PGA_1, prostaglandin A_1; Ro 20-1724, (±)4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; MIX, methylisobutylxanthine; AIB, α-aminoisobutyric acid; SITS, 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene; DIDS, 4,4′-bis(isothiocyano)stilbene-2,2′-disulfonate; NBTI, *S*-*p*-nitrobenzylthioinosine.

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binding assay of Gilman (7) to quantify cyclic AMP. To determine the effect of PGA_1 on hormone-sensitive adenylate cyclase *in vivo*, we equilibrated cells with 1 mM Ro 20–1724 and 1 mM MIX to minimize phosphodiesterase activity and cyclic AMP extrusion, added the β-agonist isoproterenol (1 μM) for 2 to 7 min, then acidified the suspension with trichloroacetic acid (10% final), and assayed total cAMP (cells plus medium).

Transport studies. All experiments were carried out at 37° with a 10% suspension of red cells unless otherwise noted. Studies of both uptake and efflux of radioactive solutes were terminated by placing duplicate 100-μl aliquots of cell suspensions into 400-μl Microfuge tubes containing a drop of inert phthalate/sebecate mixture ($p = 1.02$). Following a 20-sec centrifugation in a Beckman Microfuge B, the supernatants (above oil layer) were removed for scintillation counting of radioactive solute. For uptake studies, the bottoms of the tubes containing the packed cells were cut off and placed in scintillation vials. The cellular material was bleached with 200 μl of 30% hydrogen peroxide/60% perchloric acid (2:1) and then 100 μl of 15% ascorbic acid was added to each vial to reduce subsequent chemiluminescence. All values were corrected for extracellular space (~0.5% in 10 μl of packed red cells from a 100-μl aliquot of a 10% suspension) by measuring [^{14}C]sucrose trapping. All isotopes were counted in a liquid scintillation spectrometer: ^{3}H with an efficiency of 20–30% and ^{14}C, ^{35}S, and ^{86}Rb with an efficiency greater than 80%.

The extracellular concentration and specific activities of the radioactive solutes in the red cell suspensions were: $^{86}Rb^+$, 54 μM and 1.9×10^{11} cpm/mol of K^+ and Rb^+; $^{35}SO_4^{2-}$, intracellular, 3.4×10^{12} cpm/mol; [^{3}H]AIB, 100 μM and 1.0×10^{13} cpm/mol; [^{3}H]glucose, 0.5 μM and 2.45×10^{15} cpm/mol; [^{14}C]adenosine, 4 μM and 1.2×10^{14} cpm/mol.

Materials. D-[1-^{3}H]Glucose, [*methyl*-^{3}H]AIB, [8-^{14}C]adenosine, [^{14}C]sucrose, [^{35}S]sulfuric acid, and ^{86}RbCl (gift of Dr. Palmer Taylor, University of California, San Diego) were purchased commercially from New England Nuclear and Amersham. Prostaglandin A_1 (stored as a 10 mM stock in ethanol) was generously supplied by the Upjohn Company. *S-p*-Nitrobenzylthioinosine was a gift of A. R. P. Paterson (University of Alberta) and Ro 20-1724 was a gift from Hoffmann-LaRoche. Cytochalasin B and SITS were purchased from Calbiochem; ouabain and furosemide were purchased from Sigma.

RESULTS

We have previously established that PGA_1 is the most potent inhibitor of cyclic AMP extrusion among the common prostanoids (1). At 37°, 10 μM PGA_1 reduces cyclic AMP efflux to less than 5% of control. To test the specificity of this inhibitory action, we have assessed the effect of 10 μM PGA_1 on a variety of membrane processes in the pigeon red cell that are readily defined by simple pharmacologic manipulations.

Isoproterenol-stimulated cyclic AMP accumulation. Catecholamine-sensitive cyclic AMP production is an example of signal transduction across the membrane involving at least three membrane proteins (8). We tested the effect of PGA_1 on total cyclic AMP production in response to isoproterenol by pretreating a red cell suspension for 15 min with 1 mM Ro 20-1724 and 1 mM MIX (to eliminate cyclic AMP degradation) and then exposing portions of this suspension to 1 μM isoproterenol in the absence or presence of 10 μM PGA_1. Over a 7-min period, during which time the rate of cyclic AMP production was constant, both control cells and PGA_1-treated cells accumulated cyclic AMP with a rate of 6.6 pmol/min/10^7 cells. Thus, PGA_1 produces no significant effect on catecholamine-sensitive adenylate cyclase assessed *in vivo*.

Cation transport. We tested the effect of PGA_1 on $^{86}Rb^+$ (tracer for K^+) uptake mediated by two cation transport systems, the (Na^+,K^+)-ATPase and furosemide-inhibitable, isoproterenol-stimulable NaCl-KCl symport. (Na^+, K^+)-ATPase activity was estimated as $^{86}Rb^+$ influx in the presence of 1 mM furosemide (to inhibit symport-mediated uptake) that was sensitive to 1 mM ouabain. Control activity (in the absence of 10 μM PGA_1) was 93 pmol of Rb^+ and K^+/min/10^7 cells; ouabain inhibited this influx by 80% (Fig. 1). Including 10 μM PGA_1 in the red cell suspension did not affect the rate of ouabain-sensitive $^{86}Rb^+$ influx (92 pmol/min/10^7 cells, 98.4% of control). These data indicate that cyclic AMP export is regulated independently of active cation transport, a conclusion suggested by the lack of effect of ouabain on cyclic AMP export itself (1).

NaCl-KCl symport was defined as $^{86}Rb^+$ influx [1 mM ouabain included to block the (Na^+,K^+)-ATPase] that was stimulated by 1 μM isoproterenol and inhibited by 1 mM furosemide(9). In the absence of isoproterenol, basal influx was 159 pmol of Rb^+ and K^+/min/10^7 cells (Fig. 2). Exposure of the cells to 1 μM isoproterenol for 15 min prior to $^{86}Rb^+$ addition stimulated influx to 244 pmol/min/10^7 cells, a 53% increase. While 1 mM furosemide inhibited greater than 98% of $^{86}Rb^+$ influx in the presence of isoproterenol, 10 μM PGA_1 exhibited no measurable effect (103% of control).

Anion equilibrium exchange. We tested the effect of PGA_1 on band 3-mediated anion transport, assayed as $^{35}SO_4^{2-}$ efflux from pigeon red cells equilibrated (37°, 60 min) in $^{35}SO_4^{2-}$-containing medium and resuspended in fresh, isotope-free buffer. Under equilibrium exchange conditions, $^{35}SO_4^{2-}$ exited the red cells with a rate of 3.9 pmol/min/10^7 cells (Fig. 3). The disulfonic stilbene SITS (10) effectively inhibited $^{35}SO_4^{2-}$ efflux (Fig. 3, inset; K_i = 11 μM), verifying that band 3-mediated exchange was being studied. However, exposure of cells to 10 μM PGA_1 failed to inhibit $^{35}SO_4^{2-}$ efflux (98.8% of control). We

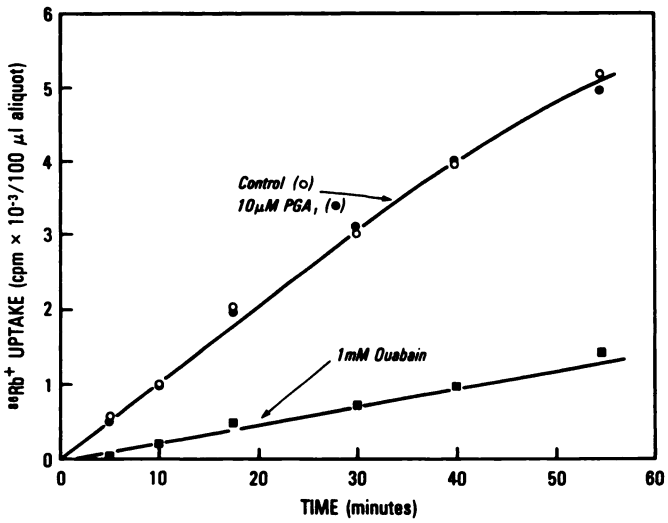

FIG. 1. *Ouabain-sensitive $^{86}Rb^+$ uptake*

Red cell suspensions (37°, 10% hematocrit) were exposed to ^{86}RbCl (~0.5 μCi/ml) and furosemide (1 mM) and either the appropriate diluent control, PGA_1 (10 μM), or ouabain (1 mM). Periodically, samples (100 μl, ~5×10^7 cells) were withdrawn at the indicated times and centrifuged, and cell pellet-associated radioactivity was determined.

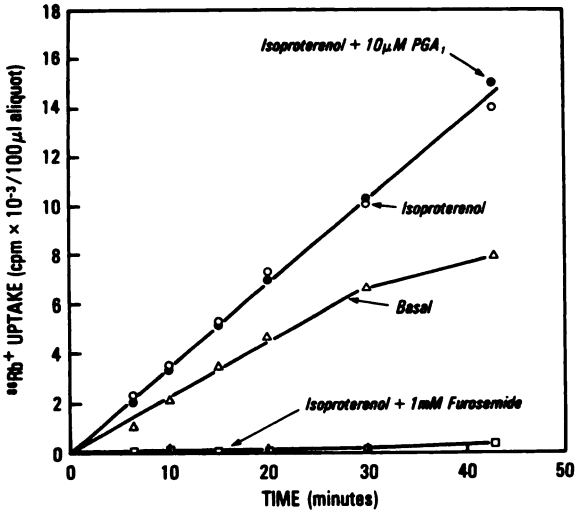

FIG. 2. *NaCl-KCl symport and inhibition by furosemide*

Red cell suspensions, containing diluent control, furosemide (1 mM), or PGA_1 (10 μM), were incubated (37°) for 15 min in the absence or presence of 1 μM isoproterenol (containing 100 μM ascorbate) and then exposed to $^{86}RbCl$ (~0.5 μCi/ml) and ouabain (1 mM). Samples were withdrawn at various intervals and centrifuged, and cell pellet-associated radioactivity was determined.

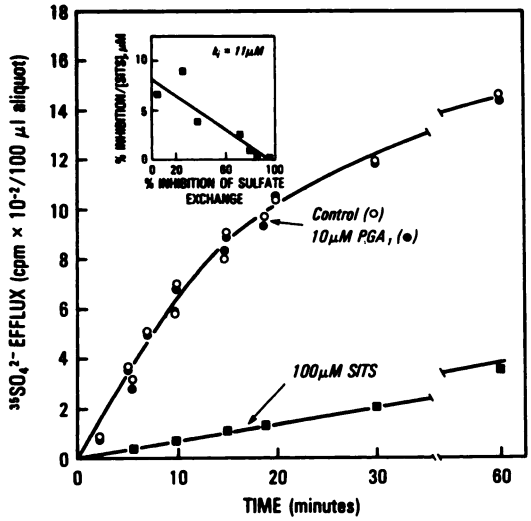

FIG. 3. *SITS-sensitive sulfate equilibrium exchange*

A cell suspension (37°) was incubated with $H_2^{35}SO_4$ (~1 μCi/ml) until isotopic equilibrium within the cells was achieved (60 min). The red cells were washed free of extracellular $^{35}SO_4^{2-}$, suspended in isotope-free ice-cold buffer, and aliquoted into polypropylene tubes (17 × 100 mm) containing diluent, SITS (100 μM), or PGA_1 (10 μM). The cells were rapidly warmed to 37° and at various times, samples were centrifuged and supernatant radioactivity was determined. Inset: Eadie-Hofstee plot of data where initial rates of sulfate efflux were determined in the presence of varying concentrations of SITS (0–1 mM).

obtained similar results with the more potent but light-sensitive compound DIDS (10). These data indicate the independence of band 3-mediated anion transport and PGA_1-sensitive export of cyclic AMP.

In addition, we tested SITS (100 μM) directly on cyclic AMP efflux. This concentration, which inhibits $^{35}SO_4^{2-}$ efflux by greater than 90%, reduces cyclic AMP export by only 12% (Table 1). From such data, we conclude that band 3 does not mediate cyclic AMP extrusion from

TABLE 1

Effects of pharmacologic modulators of various transport processes on cyclic AMP efflux

A 10% red cell suspension was equilibrated (37°, 30 min) with 50 μM Ro 20-1724, and then aliquots were exposed to 1 μM isoproterenol and the indicated treatments for 10, 20, and 30 min. In the case of KCN, cells were first loaded with cyclic AMP by a 15-min exposure to isoproterenol and then washed free of drug and allowed to extrude (±KCN) into fresh medium, facilitating the study of cyclic AMP efflux unhampered by the effect of KCN to depress adenylate cyclase activity via ATP depletion. For assessment of Na^+ dependence, cells were treated as described in the legend to Fig. 4, comparing extrusion of cyclic AMP by cells in normal Earle's solution to extrusion from cells in K^+ and choline-containing buffer. Data are the average of results from two experiments. Control extrusion of cyclic AMP ranged from 0.83 to 0.97 pmol/min/10^7 cells.

Treatment	Rate of cyclic AMP efflux
	% control
SITS, 100 μM	88
Na^+-free buffer	96
Cytochalasin B, 1 μM	98
Cyanide, 100 μM	53
Nitrobenzylthioinosine, 5 μM	93

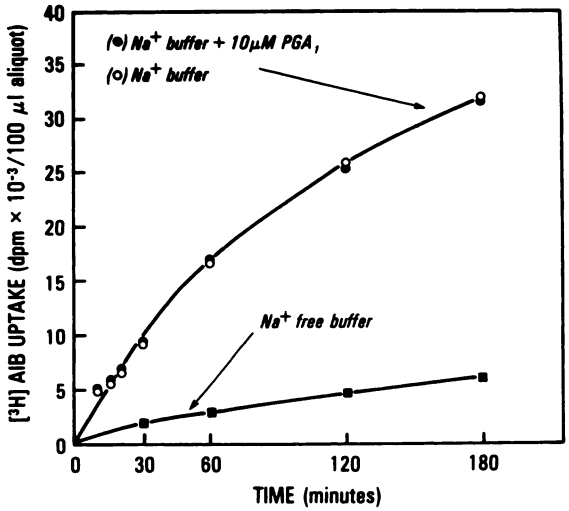

FIG. 4. *Na^+-dependent α-aminoisobutyric acid uptake*

Red cells suspended (37°, 10% suspension) in Na^+-containing buffer were exposed to [^{3}H]AIB (~2 μCi/ml) and incubated in the presence or absence of 10 μM PGA_1. In addition, a red cell suspension in Na^+-free buffer (Na^+ replaced by choline and K^+) was exposed to [^{3}H]AIB. Periodically, samples were withdrawn and cell pellet-associated radioactivity was determined.

pigeon red cells. In this regard, the flux of cyclic AMP from pigeon erythrocytes differs from cyclic AMP entry into human erythrocytes. According to Tsukamoto *et al.* (11), DIDS inhibits cyclic AMP entry into human red cells. However, the concentration dependence of this effect is shallow and the half-maximally effective concentration, 24 μM, is 100 times the concentration that half-maximally inhibits sulfate permeation (10).

Sodium-dependent amino acid transport. We used [^{3}H]AIB as a substrate to measure this transport activity. Red cells suspended in Earle's buffer accumulated 0.78 pmol of [^{3}H]AIB/min/10^7 cells (Fig. 4). Suspending red cells in a Na^+-free buffer (choline chloride replaced NaCl,

KH_2PO_4 replaced NaH_2PO_4, and $KHCO_3$ replaced $NaHCO_3$) depressed [^{3}H]AIB uptake to about 15% of that in Na^+-containing Earle's solution, demonstrating the Na^+ dependency of this transport system. Exposure of the cells to 10 μM PGA_1 had no inhibitory effect on [^{3}H]AIB uptake (110% of control). The independence of cyclic AMP and Na^+-dependent amino acid transport is additionally demonstrated by the failure of Na^+-free medium (choline replacement) to inhibit cyclic AMP export (Table 1).

Sugar transport. Hexose transport in avian red cells is sensitive to a number of experimental manipulations including stimulation by cyanide (12). In addition, we expected that carrier-mediated glucose uptake in these cells might be inhibited by cytochalasin B, similar to the hexose transport of mammalian erythrocytes (13). Using these criteria, we have assessed glucose uptake in pigeon red cells (Fig. 5). With 0.5 μM extracellular glucose, basal [^{3}H]glucose uptake was 1.4 fmol/min/10^7 cells and uptake in the presence of KCN (1 mM) was 2.7 fmol/min/10^7 cells. Cytochalasin B (1 μM) effectively inhibited labeled glucose import by 90% under both control and KCN-stimulated conditions. PGA_1 (10 μM) had essentially no effect on either basal or KCN-stimulated [^{3}H]glucose uptake, those rates being 87 and 114% of control, respectively. Similarly, cyanide (a stimulator of glucose uptake) inhibited cyclic AMP export (measured independently of ATP-dependent cyclic AMP synthesis; see Table 1). The effect of cytochalasin B further emphasizes the independence of glucose and cyclic AMP fluxes; 1 μM cytochalasin B did not inhibit cyclic AMP efflux (Table 1).

Nucleoside transport. Nucleosides and congeners of cyclic AMP such as methylisobutylxanthine are transported into cells by a system that is potently inhibited by NBTI, a nucleoside analogue that binds to membrane protein 4.5 of erythrocytes (14, 15). We have tested the effect of PGA_1 on uptake of [^{14}C]adenosine defined by this inhibitor. When pigeon red cells (5% suspension at 37°) were incubated with 4 μM [^{14}C]adenosine, they imported the labeled adenosine rapidly, with an initial rate of 56 pmol/min/10^7 cells (Fig. 6). The inhibition of this uptake by NBTI (K_i = 30 nM under our transport conditions; see inset, Fig. 6) indicated the carrier-mediated nature of the transport. Thus, 5 μM NBTI inhibited [^{14}C]adenosine uptake by more than 98%. Prostaglandin A_1 (10 μM) had no effect on this mediated uptake of labeled adenosine, the control and PGA_1-treated rates of uptake being virtually superposable (see Fig. 6 for a representative experiment). Conversely, an effective concentration of the inhibitor of the nucleoside transporter, NBTI (5 μM), did not reduce cyclic AMP export (Table 1).

DISCUSSION

As extensions of our observation that PGA_1 inhibits energy-dependent cyclic AMP export by avian red cells (1), we have attempted to determine the specificity of the PGA_1 effect and the relationship of other common transport systems to cyclic AMP transport. To accomplish these aims, we have measured a variety of carrier-mediated transport and transduction events involving diverse solute structures and a range of flux rates (or activities) that spans 5 orders of magnitude: hormone-sensitive adenylate cyclase, (Na^+,K^+)-ATPase, NaCl-KCl symport, band 3-mediated sulfate exchange, Na^+-dependent amino acid transport, and adenosine and glucose uptake. All of these processes reflect the activities of membrane proteins and may be probed pharmacologically. Surprisingly, none of these processes is significantly affected by 10 μM PGA_1, a concentration that inhibits cyclic AMP efflux by more than 95% (1). Thus,

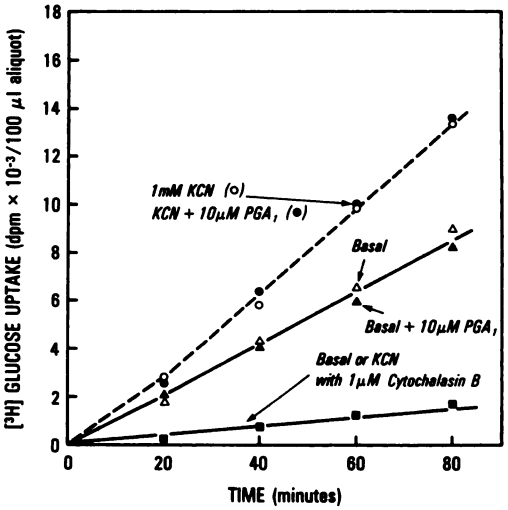

FIG. 5. *Cytochalasin B-sensitive, cyanide-stimulated glucose transport*

Red cells (37°, 10% hematocrit in Earle's solution with glucose omitted) were exposed in the absence or presence of 1 mM KCN to [^{3}H]glucose (~2.5 μCi/ml) and either a diluent control, cytochalasin B (1 μM) or PGA_1 (10 μM) and sampled at various intervals for cell pellet-associated tritium.

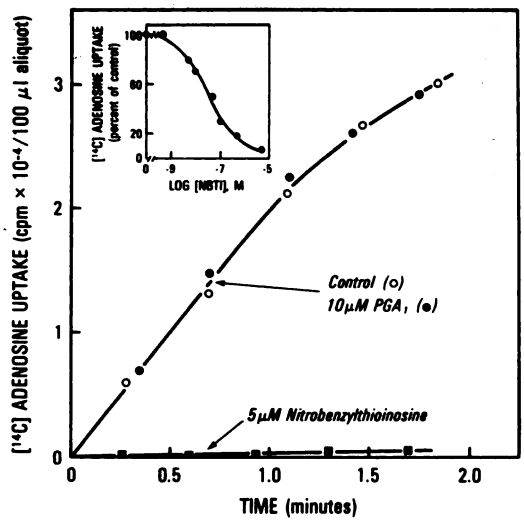

FIG. 6. *Adenosine uptake and inhibition by nitrobenzylthioinosine*

Pigeon red cells (5% suspension, 37°) were exposed to [^{14}C]adenosine (~5 × 10^5 cpm/ml) alone (control) or labeled adenosine and either *S*-*p*-nitrobenzylthioinosine (5 μM) or PGA_1 (10 μM). The cell suspensions were sampled rapidly and cell pellet-associated radioactivity was determined. Inset: inhibition of [^{14}C]adenosine uptake by nitrobenzylthioinosine. Data are from initial rates of uptake in the presence of varying concentrations of inhibitor and are expressed as a percentage of control uptake (~2 × 10^4 cpm/min/100-μl aliquot).

we conclude that PGA_1 acts quite specifically to inhibit cyclic AMP export by pigeon red cells and does not have, as a mechanism of this action, a general effect on the membrane such as an alteration of membrane fluidity. Effects of prostaglandins on membrane fluidity have been reported (3, 4) and invoked as mechanisms of prostaglandin action on rheologic properties of red cells (3). Our data, coupled with the specificity of the A-type prostaglandins to block cyclic AMP export (1), argue that this effect of PGA_1 results from the ligand's interaction with a membrane receptor.

While it is not unusual for amphipathic prostaglandins to interact with membrane receptors (see, for instance, Ref. 5), the specificity with which PGA_1 inhibits cyclic AMP transport seems partly explained by the selective internalization and metabolism of PGA_1 to a glutathione adduct.[3] This adduct, formed in less than 0.5 min under all conditions used in the experiments reported here, apparently interacts with the membrane at the intracellular surface to modulate cyclic AMP flux. In light of this metabolism to a hydrophilic compound, a property not shared by other prostaglandins, a specific interaction of the PGA_1 metabolite seems more readily understood than if the interaction of the ligand and the membrane were driven strictly by hydrophobic interactions.

Prostaglandins have long been known to alter cyclic AMP metabolism, chiefly as stimulators or inhibitors of adenylate cyclase (see Refs. 5 and 6, for instance). Pigeon red cells have no prostaglandin-sensitive adenylate cyclase (1). Thus, the inhibition of cyclic AMP efflux represents a second specific interaction by which prostaglandins can alter cellular cyclic AMP.

The characterization of multiple transmembrane phenomena permits some additional conclusions about the nature of cyclic AMP export. Sensitivity of cyclic AMP efflux but not a variety of other processes to inhibition following exposure to PGA_1 distinguishes cyclic AMP export from those other transport processes. Moreover, pharmacologic characterization of other membrane transport events demonstrates that cyclic AMP export is not affected by concentrations of SITS, NBTI, or cytochalasin B that inhibit specific transport activities, nor is cyclic AMP export stimulated by cyanide (as glucose uptake is) or inhibited by removal of extracellular Na^+ (as aminoisobutyrate flux is) (Table 1). Combining these data, we can exclude the participation of a Na^+ gradient, band 3, and band 4.5 in cyclic AMP transport.

Not all previous results agree with our findings. Tsukamoto *et al.* (11) found that cyclic AMP entry into human red cells can be inhibited by stilbene analogues, but at concentrations several orders of magnitude greater than required to block sulfate flux. Holman (16) has demonstrated blockade of exit transport of cyclic AMP from human erythrocyte ghosts by cytochalasin B, $K_i = 0.4\ \mu M$, a potency similar to that for inhibitors of glucose transport in red cell ghosts. Cyclic AMP transport in these cells follows the Haldane relationship, is *not* active transport, and is thus quite different from the pigeon red cell system. In many systems, cytochalasin B inhibits sugar and uridine transport (13, 17) and microfilament activity (18) such that interpreting a positive effect of cytochalasin B as implicating participation of a particular molecular and subcellular structure is difficult. However, the lack of effect of cytochalasin B on cyclic AMP efflux from avian red cells unambiguously distinguishes it from these various susceptible activities.

While admitting to anthropomorphizing, we do wonder whether it is reasonable for a red cell to have a membrane transport system specifically to rid itself of cyclic AMP. Some of the common characteristics of cyclic AMP export from kidney and the red cell suggest an answer to this query. A variety of organic anions and cations inhibit cyclic AMP efflux in an apparently competitive manner, including probenecid (19), phenol red and quinine (20), and indomethacin and ethacrynic acid.[4] A common feature of these compounds is their secretion by renal tubules (21, 22). The mammalian kidney secretes cyclic AMP and its phosphorothioate analogues by a means probenecid inhibits (23, 24), presumably by the organic ion transporter. In addition, PGA_1 inhibits the accumulation of *p*-aminohippurate by slices of rabbit renal cortex (25). Thus, renal handling of organic ions including cyclic AMP bears some superficial resemblance to cyclic AMP export from avian red cells. Perhaps cyclic AMP export from pigeon red cells is an example of a transporter with broad specificity, akin to the organic ion transporter of the kidney.

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Send reprint requests to: L. E. Heasley, Division of Pharmacology, M-013H, School of Medicine, University of California, San Diego, La Jolla, CA 92093.