# Hormonal Modulation of Ionic Permeability in Human Red Blood Cells[1]

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## ABSTRACT

**It has previously been reported that both exogenous adenosine cAMP analogs and forskolin-induced elevations in intracellular cAMP concentrations selective increase relative ionic chloride permeability in normal human red blood cells (RBC). A similar selectively increase in relative ionic chloride permeability was observed in untreated uremic subjects in whom endogenous RBC cAMP concentrations are chronically elevated. To detect which hormones might modulate RBC cAMP and ionic permeabilities, RBC were exposed to norepinephrine, epinephrine, and parathyroid hormone. Thereafter, RBC cAMP concentrations were measured by RIA and relative ionic permeabilities were determined in human RBC ghosts with the potential sensitive fluorescent probe diS-$C_3$-(5). In ghosts prepared from normal RBC, norepinephrine and epinephrine significantly increased intracellular cAMP concentrations; in these ghosts, relative ionic chloride permeability (permeability of chloride/permeability of potassium (PCl/PK)), but not PNa/PK (permeability of sodium/permeability of potassium), was significantly increased. In contrast, exposure to parathyroid hormone did not affect either cAMP concentrations or relative ionic permeabilities. These results are consistent with the presence of adrenergic receptors and the absence of parathyroid hormone receptors in RBC. These studies demonstrate that hormonally induced changes in cAMP can modulate RBC relative ionic chloride permeability and suggest that, in uremic RBC, increased relative ionic chloride permeability could be consequent to elevated plasma levels of epinephrine or norepinephrine.**

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Previous studies have demonstrated that the intracellular second messenger cAMP can induce and/or regulate conductive chloride pathways in nonpolarized cells, such as the normal human red blood cell (RBC) (1) and the cultured lymphocyte (2), and in various epithelia such as kidney (3–6), trachea (7,8), gallbladder (9,10), and shark rectal gland (11). In chronic renal failure, intracellular cAMP and relative ionic chloride permeability are both increased in RBC of untreated humans; after treatment with peritoneal dialysis, both cAMP and chloride permeability return to normal levels (1). Of note, RBC cAMP concentrations and relative ionic chloride permeability are also significantly increased in a rat model of chronic renal failure (12). On the basis of the observation that renal failure is accompanied by elevated plasma concentrations of a number of hormones (such as norepinephrine, epinephrine, and parathyroid hormone [PTH]) that can activate adenylate cyclase in a variety of tissues (13–15), it was postulated that one or more of these hormones might mediate an increase in relative ionic chloride permeability of the RBC in uremia.

Because adrenergic receptors have been demonstrated on normal RBC (16) and norepinephrine and epinephrine levels are elevated in uremia and corrected by dialysis (17), these studies were designed to determine whether these hormones in pharmacologic doses could induce changes in RBC cAMP and thereby alter relative ionic chloride permeability in normal human RBC. PTH has been reported to increase RBC osmotic fragility (18), despite the inability to detect PTH receptors on these cells (19). Thus, PTH was evaluated to determine whether, despite a lack of receptors, PTH could alter cAMP and/or relative ionic permeability. These studies demonstrate that adrenergic receptor–mediated hormones induced elevations in intracellular cAMP concentration and altered relative ionic permeability in the normal human RBC, whereas PTH was without effect. These findings suggest that, in addition to the toxic effects of PTH, other hormones may play a significant role in the pathophysiology of the uremic state.

## METHODS

After informed consent was obtained from normal volunteers, 20 mL of blood was obtained by venipuncture into heparinized syringes and placed

into tubes containing 3-isobutyl-1-methylxanthine (IBMX; Sigma Chemical Co., St. Louis, MO), a phosphodiesterase inhibitor, at a final concentration of 1 mM. Blood was then divided into aliquots for preparation of RBC ghosts and the determination of RBC cAMP concentration. None of the subjects had received blood transfusions within 12 months before the study.

RBC, buffy coat, and plasma were separated by centrifugation at 4,500 × *g* for 10 min in a Sorvall Model RC-5B refrigerated centrifuge (Dupont, Wilmington, DE) with an SS-34 rotor. After removal of the plasma and buffy coat, the RBC pellet was diluted to five times the original blood volume with ice-cold 150 mM NaCl–1 mM IBMX (pH 7.4) and then centrifuged at 4,500 × *g* for 10 min. The uppermost layer of the pellet was discarded, and the procedure was repeated two additional times. Aliquots of the intact washed RBC were then incubated for 30 min at 37°C with 1 mM IBMX, without or with either 1 μM epinephrine (Sigma), 1 μM norepinephrine (Sigma), or bovine 1-34 PTH ($5 \times 10^{-7}$ M; Peninsula Laboratories, Belmont, CA). Ghosts were subsequently prepared without further additives by the methods detailed below and used for the study of relative ionic permeabilities.

### Preparation of RBC Ghosts

Ghosts were prepared by a previously reported (1,20) modification of the hypotonic lysis method of Bodemann and Passow (21) and Steck (22). Seven hundred fifty microliters of ghosts was resuspended in 40 mL of 100 mM KCl or 100 mM NaCl and 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), buffered to pH 6.5 with 5 mM KOH or NaOH, respectively, and incubated at 37°C for 90 min to reseal them. Thereafter, the resealed ghosts were centrifuged at 30,000 × *g* for 15 min. The pellet of ghosts was washed and centrifuged two additional times in ice-cold solutions identical to those in which they were sealed. The pelleted KCl or NaCl preloaded ghosts were stored on ice until study. The protein concentration of ghosts was determined by the method of Lowry *et al.* (23) with BSA as the standard.

### Determination of Intraghost (KCl) and Relative Ionic Permeabilities

Intraghost [KCl] ($[KCl]_{in}$) and relative ionic permeabilities of KCl preloaded ghosts were assessed with the positively charged potential sensitive fluorescent probe 3,3′-dipropylthiadicarbocyanine iodide [diS-$C_3$-(5)] by techniques identical to those previously described (1,24). In brief, initial fluorescence was recorded after the addition of aliquots (100 μg of protein) of ghosts to media containing 3 μM diS-$C_3$-(5), 0 to 100 mM KCl, and 50 mM K-HEPES (pH 6.5). The media [$K^+$] was varied from 0 to 100 mM by replacing KCl with equimolar concentrations of choline Cl. When fluorescence stabilized (40 s), valinomycin (3 μM) was added and fluorescence was monitored. Because valinomycin produces no change in fluorescence when the concentration of K in $[K^+]_{in}$ = the concentration of K out $[K^+]_{out}$, $[KCl]_{in}$ was calculated by determining the $[K^+]_{out}$ at the intersection of the regression lines of the fluorescence recorded at each media [$K^+$], in the absence and presence of valinomycin (1,24). The prevalinomycin regression line was fitted to the Goldman-Hodgkin-Katz constant field equation; the postvalinomycin line was fitted to the Nernst equation (1,24). After $[KCl]_{in}$ was determined, fluorescence was converted to membrane potential in millivolts. The ionic permeabilities of $Cl^-$ and choline$^+$ relative to potassium (PCl/PK and Pchol/PK) were then calculated by the constant field equation. After 100 mM NaCl was substituted for the 100 mM KCl in the media and initial fluorescence in these media was converted to membrane potential, PNa/PK was calculated by the constant field equation (1,24).

### Measurement of cAMP Levels

cAMP concentrations were determined in aliquots of RBC by use of a radioisotopic test kit (Amersham, Arlington Heights, IL). RBC cAMP was extracted by the addition of 0.5 mL of a 50% hematocrit of RBC to 1.5 mL of boiling $H_2O$. The mixture was boiled for an additional 3 min (16), cooled on ice, and centrifuged. An aliquot of the supernatant was lyophilized to dryness and then reconstituted to one-fifth its original volume. cAMP concentrations in RBC are expressed as picomoles of packed RBC per milliliter.

### Statistics

In each experiment, studies were performed and analyses were made on triplicate or quadruplicate samples of ghosts prepared on the day of the experiment. The mean of the triplicate or quadruplicate determinations provided one value in the calculation of the mean of all experiments. All data are expressed as the mean ± SE. Paired analysis and *t* test were used to determine statistical significance.

## RESULTS

### Effect of Norepinephrine on RBC of Normal Subjects

As depicted in Figure 1A, the incubation of normal RBC with 1 μM norepinephrine resulted in a significant increase in RBC cAMP concentration at 10 min (16.1 ± 4.3 versus 37.4 ± 10.2 pmol/mL of packed RBC; $P < 0.05$) and at 30 min (16.6 ± 5.8 versus 48.6 ± 18.5 pmol/mL of packed RBC; $P < 0.05$). Ionic permeability in RBC ghosts derived from these cells is depicted in Figure 1B. PCl/PK was significantly

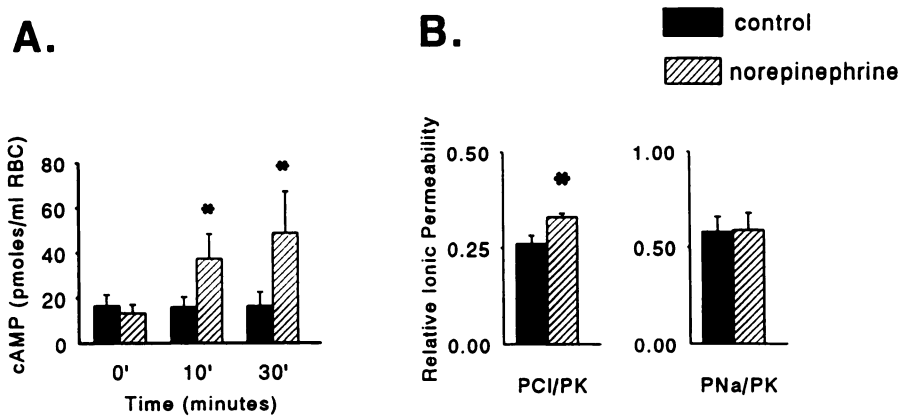


**Figure 1. (A) The cAMP concentrations (picomoles of packed RBC per milliliter) in paired control and norepinephrine (1 μM)-exposed RBC incubated for 30 min at 37°C. Data represent means ± SE of six experiments. *Indicates values significantly different from those of paired controls ($P < 0.05$). (B) Relative ionic permeabilities in paired control and norepinephrine-exposed RBC. PCl/PK and PNa/PK are ionic permeabilities of chloride and sodium, respectively, relative to potassium. Solid and hatched bars represent mean data from control and norepinephrine-treated RBC, respectively. *Indicates statistical significance ($P < 0.05$) between groups in three experiments.**

higher in ghosts prepared from RBC exposed to norepinephrine than in paired untreated ghosts (0.33 ± 0.01 versus 0.26 ± 0.02; $P < 0.05$). This increase in PCl/PK was selective because PNa/PK did not change (0.59 ± 0.09 versus 0.58 ± 0.08). As observed in our previous studies in normal RBC, $[KCl]_{in}$ was not altered by maneuvers that increased intracellular cAMP (90.2 ± 5.4 versus 88.7 ± 2.40 mM).

## Effect of Epinephrine on RBC of Normal Subjects

As depicted in Figure 2A, the incubation of normal RBC with 1 μM epinephrine also resulted in a significant increase in RBC cAMP concentration at 10 min (14.6 ± 6.9 versus 36.0 ± 9.8 pmol/mL of packed RBC; $P < 0.05$) and at 30 min (13.1 ± 5.9 versus 24.1 ± 8.0 pmol/mL of packed RBC; $P < 0.05$). Ionic permeability in RBC ghosts derived from these cells is depicted in Figure 2B. PCl/PK was significantly higher in ghosts prepared from normal RBC exposed to epinephrine than in paired ghosts from untreated RBC (0.42 ± 0.01 versus 0.30 ± 0.03; $P < 0.05$). The increase in PCl/PK was selective because PNa/PK was not affected (0.78 ± 0.07 versus 0.74 ± 0.04). $[KCl]_{in}$ was not altered by epinephrine (97.3 ± 4.7 versus 88.0 ± 3.50 mM).

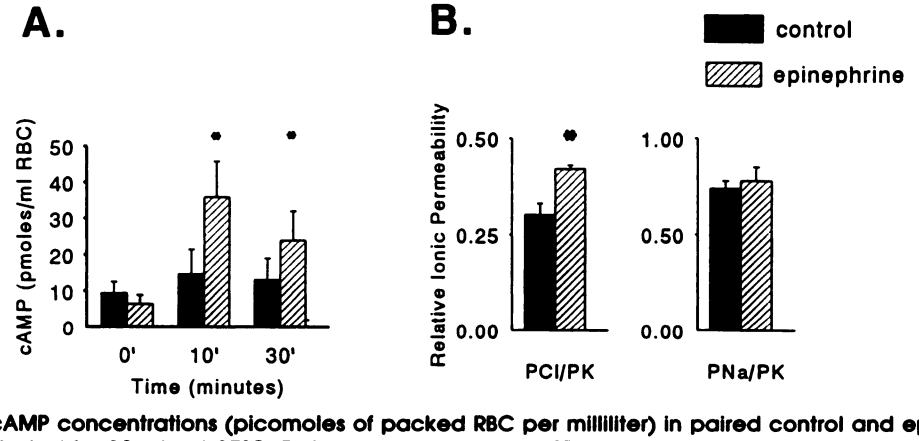


**Figure 2. (A) The cAMP concentrations (picomoles of packed RBC per milliliter) in paired control and epinephrine (1 μM)-exposed RBC incubated for 30 min at 37°C. Data represent means ± SE of five experiments. *Indicates values significantly different from those of paired controls ($P < 0.05$). (B) Relative ionic permeabilities in paired control and epinephrine-exposed RBC. PCl/PK and PNa/PK are ionic permeabilities of chloride and sodium, respectively, relative to potassium. Solid and hatched bars represent mean data from control and epinephrine-treated RBC, respectively. *Indicates statistical significance ($P < 0.05$) between groups in three experiments.**

### Effect of PTH on RBC of Normal Subjects

In contrast to the effects of adrenergic agonists, PTH did not affect RBC cAMP concentrations (Figure 3A). This is consistent with previous data that, despite PTH-induced effects on RBC osmotic fragility (18), RBC lack PTH receptors (19). Consistent with its failure to increase RBC cAMP, PTH did not affect the ionic permeability of RBC ghosts; neither relative ionic permeabilities (Figure 3B) nor $[KCl]_{in}$ (85.5 ± 1.9 versus 85.5 ± 1.0 mM) was altered in PTH-exposed RBC.

## DISCUSSION

These studies have provided evidence that the relative ionic permeability to chloride of human RBC membranes is selectively increased by cAMP-mediated hormones. Relative chloride permeability (PCl/PK) was consistently increased without a change in relative sodium permeability (PNa/PK) when cAMP was experimentally elevated in normal RBC by the exposure of cells to norepinephrine or epinephrine (Figures 1 and 2); relative permeabilities were unaffected in RBC of PTH-exposed cells, which had no increase in intracellular cAMP levels (Figure 3). These studies provide the first demonstration that the hormone-induced activation of cAMP can modulate the ionic permeability of $Cl^-$ relative to $K^+$ in erythrocyte membranes. RBC membranes contain outwardly rectifying $Cl^-$ channels (25); similar channels in other tissues are opened by phosphorylation subsequent to the cAMP-induced activation of protein kinase A (6). Because ionic permeabilities were determined in ghosts deprived of cytosolic metabolic and regulatory components, these studies also indicate that RBC membranes, like renal membranes (4, 26), have "memory": changes induced in intact cells are retained, presumably by separating the membranes from the initiating stimulus and counterregulatory mechanisms.

During these studies, RBC were stimulated by hormones in the presence of intact metabolic and regulatory components. Subsequently, during the ghosting process, membranes prepared from the control and hormone-stimulated intact RBC were depleted of ATP, thus depriving membrane Na/K ATPase of substrate and obviating any contribution of this primary active transport mechanism to the measurement of ionic permeabilities.

Although the PCl/PK of RBC ghost membranes is significantly lower than that observed in intact RBC (1,12), the mechanism of this reduction is not completely understood. Because ghosts maintain KCl gradients, this effect cannot be ascribed to a failure of the ghosts to reseal. Moreover, as ionic potassium permeability is increased in RBC ghosts, presumably as a consequence of the ghosting process (27), it is possible that the lower PCl/PK in ghosts represents a rise in PK, rather than a fall in PCl. However, studies in renal proximal tubule brush border membranes suggest that cAMP increases PCl (4,12). Independent of the mechanism, as RBC ghosts of control and hormone-treated RBC were prepared simultaneously and identically, it is assumed that the same factors that are responsible for the reduction in PCl/PK relative to that of the intact RBC membrane would be operative in both populations of ghosts. In this context, any differences in ionic permeabilities between control and hormone-exposed membranes should be consequent to factor(s) related to the hormone and not the ghosting process. Despite the acknowledged discrepancies between intact and isolated membranes, the latter remain useful tools to

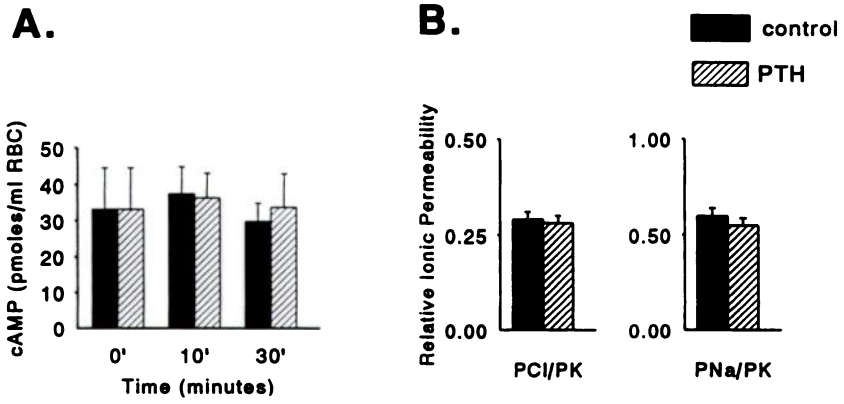


Figure 3. (A) The cAMP concentrations (picomoles of packed RBC per milliliter) in paired control and PTH ($5 \times 10^{-7}$ M)-exposed RBC incubated for 30 min at 37°C. Data represent means ± SE of eight experiments. (B) Relative ionic permeabilities in paired control and PTH-exposed RBC. PCl/PK and PNa/PK are ionic permeabilities of chloride and sodium, respectively, relative to potassium. Solid and hatched bars represent mean data from control and PTH-treated RBC, respectively, in three experiments.

qualitatively assess the effects of various perturbations on membrane properties.

An increasing body of evidence has linked elevated PTH levels in uremia to a variety of abnormalities characteristic of the uremic state. However, although PTH does alter RBC osmotic fragility (18), PTH receptors have not been detected on RBC membranes (19) and PTH does not elevate RBC cAMP ([19]; Figure 3A) or alter RBC ghost membrane permeability (Figure 3B). Thus, the increase in RBC cAMP and relative chloride permeability in uremic humans (1) and rats (12) must result from some other mediator. These studies suggest that norepinephrine and epinephrine, which are elevated in uremia (15), may be the mediators responsible for changes in RBC cAMP and permeability. We have previously demonstrated that PTH, which produces an increase in renal cortical cAMP, results in an increase in relative chloride permeability in renal proximal tubule brush border membranes (28) that is similar to the increase detected in brush border membranes from uremic animals (12). Of interest, $\alpha_2$-agonists such as norepinephrine and epinephrine decrease proximal tubule cell cAMP (29). Thus, in the kidney, these hormones would limit the increase in cAMP and membrane permeability caused by PTH. If the results of these studies can be generalized to other cells, these data suggest that the interactions of a variety of hormones may play an important role in the pathophysiology of the uremic state, and that, depending on the presence of hormone receptors and signal transduction mechanisms, the pathologic effects of elevations in the levels of individual hormones in uremia may be tissue specific.

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