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# EFFECTS OF OUABAIN AND ISOPROTERENOL ON POTASSIUM INFLUX IN THE TURKEY ERYTHROCYTE

## QUANTITATIVE RELATION TO LIGAND BINDING AND CYCLIC AMP GENERATION

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## Summary

Studies have been carried out in the turkey erythrocyte to examine: (1) the influence of external $K^+$ concentration on both [$^3$H]ouabain binding and the sensitivity of potassium influx to inhibition by ouabain and (2) the quantitative relation between β-adrenergic receptor site occupancy, agonist-directed cyclic AMP generation and potassium influx rate. Both [$^3$H]ouabain binding and the ability of ouabain to inhibit potassium influx are markedly reduced at increasing external $K^+$ concentrations, and at each $K^+$ concentration the concentrations of ouabain required for half-maximal binding to the erythrocyte membrane and for half-maximal inhibition of potassium influx are identical. Both basal and isoproterenol-stimulated potassium influx rise with increasing external $K^+$ concentrations. In contrast to basal potassium influx, which is 50—70% inhibitable by ouabain, the isoproterenol-stimulated component of potassium influx is entirely insensitive to ouabain. At all concentrations of $K^+$, inhibition of basal potassium influx by ouabain is linear with ouabain binding, indicating that the rate of transport per unoccupied ouabain binding site is unaffected by simultaneous occupancy of other sites by ouabain. Similarly, the rate of isoproterenol-stimulated cyclic AMP synthesis is directly proportional to β-adrenergic receptor occupancy over the entire concentration-response relationship for isoproterenol, showing that at all levels of occupancy β-adrenergic receptor sites function independently of each other.

Analysis of the relation of catecholamine-dependent potassium transport to the number of β-adrenergic receptor sites occupied indicates an extremely sensitive physiological system, in which 50%-maximal stimulation of potassium

transport is achieved at less than 3% receptor occupancy, corresponding to fewer than ten occupied receptors per cell.

## Introduction

Numerous studies over the past 5 years have utilized the turkey erythrocyte to examine mechanisms of monovalent cation transport stimulated by the β-adrenergic catecholamines [1—5]. These cells actively accumulate $K^+$ [4] and extrude $Na^+$ [1] against considerable concentration gradients: under normal conditions, 50—70% of this basal transport can be inhibited by the cardiac glycoside, ouabain, and thus appears to be mediated by a membrane-associated ($Na^+ + K^+$)-dependent ATPase [2]. Earlier studies have shown that isoproterenol and other β-agonists cause a rapid rise in intracellular cyclic AMP levels and an associated marked stimulation of potassium fluxes [4], and that the magnitude of these induced fluxes is strikingly dependent upon the concentration of $K^+$ in the external medium [5]. The present studies were undertaken in order to: (1) examine the influence of the external $K^+$ concentration on both ouabain binding to the turkey erythrocyte membrane and the ability of ouabain to inhibit potassium influx and (2) determine the quantitative relation between occupancy of β-adrenergic receptor sites by agonists, cyclic AMP generation and associated physiological changes in $K^+$ transport. The results indicate that inhibition of basal potassium influx is linear with ouabain binding at all concentration of $K^+$, that the rate of isoproterenol-stimulated cyclic AMP synthesis is directly proportional to β-adrenergic receptor occupancy over the entire concentration-response relationship for isoproterenol and that half-maximal stimulation of potassium transport occurs when fewer than ten receptors per cell are occupied.

## Materials and Methods

*Materials.* White female turkeys weighing 12—15 lb were obtained from local sources (The Butcher, Bronx, New York). The birds were fed a regular poultry diet (Layena Complete Animal Feed, Ralston Purina Co., St. Louis, MO) and were provided with water ad libitum. Ouabain was obtained from the Sigma Chemical Co. (St. Louis, MO). [$^3$H]Ouabain (12—20 Ci/mmol) and $^{42}$KCl (0.11—0.19 Ci/g $K^+$) were purchased from New England Nuclear (Boston, MA). (—)-Isoproterenol and (—)-propranolol were the generous gifts of Sterling-Winthrop Pharmaceuticals (Rensselaer, NY) and Ayerst Pharmaceuticals (New York, NY), respectively.

*Preparation of washed erythrocytes.* Heparinized blood (generally a 5-ml sample) was obtained by syringe from a wing vein. After centrifugation at 400 × *g* for 10 min, the plasma was removed and the erythrocytes were resuspended in incubation buffer containing 150 mM NaCl, 10 mM KCl, 11.1 mM glucose and 10 mM Tris at pH 7.4. After resedimentation of the erythrocytes and two further washes, the cells were resuspended in incubation buffer and kept on ice unless otherwise indicated. KCl was omitted in the washings in experiments in which effects of varying concentrations of $K^+$ were subsequently to be studied.

*Binding of [$^3$H]ouabain.* Suspensions of washed erythrocytes were added to glass scintillation vials containing incubation medium and [$^3$H]ouabain either in the presence or absence of an additional displacing concentration of non-radioactive ouabain as indicated below. Stock [$^3$H]ouabain in ethanol/benzene (9 : 1) was evaporated to dryness under $N_2$ and redissolved in buffer prior to use. The vials were then capped, and the resulting suspensions, at a final hematocrit ranging between 3 and 5%, were incubated in a shaking water bath at 37°C. Ouabain binding to erythrocyte membranes was determined by transferring duplicate 100-$\mu$l aliquots of incubation mixture to 5 ml of distilled $H_2O$ at 0°C, vortexing the mixture and trapping the hemolyzed erythrocyte membranes on Gelman A/E glass filters (Gelman Instrument Co., Ann Arbor, MI). The filters were washed four times with additional 5-ml aliquots of iced, distilled $H_2O$ and transferred to glass scintillation vials, and radioactivity was determined in a Packard Model 3003 liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, IL) after the addition of 10 ml of Bray's solution [6]. Agreement between duplicate samples was excellent, differences rarely exceeding 5%.

*Hemoglobin and hematocrit determinations.* Hematocrits (percent packed red blood cell volume) were measured in capillary tubes by means of an Adams microhematocrit centrifuge (Clay-Adams Inc., New York). Hemoglobin was measured by the cyanmethemoglobin method [7]. Erythrocyte counts were performed under direct observation in a hemacytometer counting chamber.

$K^+$ concentrations in incubation media were checked at the end of each incubation in an Instrumentation Laboratory Model 143 flame photometer (Boston, MA).

*Measurement of cyclic AMP.* Intracellular cyclic AMP was determined according to a protocol previously described [3]. Intact, washed erythrocytes ($7.5 \cdot 10^8$/ml) were incubated at 37°C for 30 min in the presence of varying concentrations of (—)-isoproterenol as noted below. 1-ml samples were then iced and immediately centrifuged at $2500 \times g$ for 10 min. The supernatants were aspirated and the packed erythrocytes then hemolyzed in 2 ml of distilled $H_2O$. The resulting hemolysates were placed in a water-bath at 97°C for 5 min and then centrifuged at $2500 \times g$ for 10 min. The clear supernatant solutions were decanted into clean test tubes and stored at —20°C until the time of assay for cyclic AMP. Cyclic AMP was detected by radioimmunoassay according to the method of Steiner et al. [8]. The sensitivity of this assay was 1 pmol/ml. Specificity for cyclic AMP was demonstrated by the abolition of immunoreactivity following exposure to phosphodiesterase and by the fact that other nucleotides cross-reacted with the antiserum only when present at 1000-fold higher concentrations than that of cyclic AMP.

*Measurement of $^{42}K^+$ influx.* Potassium influx was determined by a minor modification of the method of Gardner et al. [4]. Washed turkey erythrocytes were preincubated for 2 h at 37°C in medium identical in composition with that to be used in the subsequent influx measurements, except for the absence of the radioactive tracer $^{42}K^+$. Following preincubation, centrifugation and washing of the cells, aliquots of washed packed cells were transferred to capped scintillation vials containing pre-warmed incubation medium (including $^{42}K^+$) in a shaking water bath at 37°C. Final hematocrits in the resulting incubation

mixtures ranged between 5 and 10%. At specified times, 100-μl aliquots of the incubation mixtures were transferred to plastic tubes containing 300 μl of chilled incubation medium and the erythrocytes were immediately centrifuged down in a Beckman Microfuge (Beckman Instruments, Palo Alto, CA). After aspiration of the supernatant fluid, the erythrocytes were resuspended in 300 μl of fresh cold medium and were re-sedimented. After two additional identical washings, each plastic tube containing the final washed erythrocyte pellet was placed in a 12 × 75 mm glass tube and the radioactivity determined directly in a Packard Auto-Gamma Model 578 scintillation spectrometer. Potassium influx both in the presence and absence of isoproterenol was shown to be linear with respect to time for at least 30 min; influx was therefore routinely determined by the difference between zero-time and 30-min samples in triplicate. The range of variation between triplicate samples by this method is extremely narrow, maximal variations rarely exceeding 2%, Radioactivity in zero-time samples was similarly low and generally less than 2% of that present after 30 min of incubation.

## Results

### *Ouabain binding to the turkey erythrocyte*

The turkey erythrocyte, unlike the human erythrocyte [1], possesses a complete catecholamine-responsive adenylate cyclase system which is linked physiologically to the mechanisms responsible for bi-directional ion fluxes [1,3,4]. Because of the desirability of studying the influence of $K^+$ concentration on ouabain-sensitive and ouabain-insensitive components of both basal and isoproterenol-stimulated ion fluxes in these cells, and because of the known potassium-dependence of ouabain binding in other systems [9—11], initial studies were carried out to define the characteristics of ouabain binding by the intact turkey erythrocyte with special reference to the effects of external $K^+$ concentration. Fig. 1 shows the kinetics of [$^3$H]ouabain binding by turkey erythro-

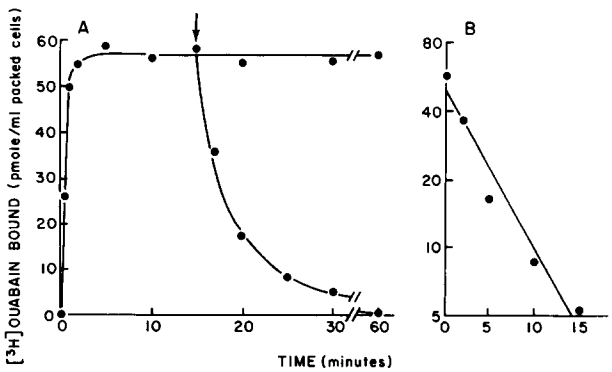



**Fig. 1. A. binding of [$^3$H]ouabain by turkey erythrocytes as a function of time in the presence of 0.4 mM $K^+$ and 0.8 μM [$^3$H]ouabain at 37°C. After 15 min of incubation, non-radioactive ouabain (1 mM) was added to one set of samples and the rate of loss of previously-bound radioactive ouabain was then followed as a function of time. The ordinate shows the amount of specifically bound [$^3$H]ouabain per ml of packed erythrocytes (see Methods). B. semilogarithmic plot of the loss of [$^3$H]ouabain binding after the addition of the non-radioactive ouabain. Dissociation follows first-order kinetics with a dissociation rate constant ($k_{-1}$) of 0.16 min$^{-1}$ at 37°C.**

cytes. At an external $K^+$ concentration of 0.4 mM, the uptake of [$^3$H]ouabain (0.8 $\mu$M) is extremely rapid and reaches an equilibrium value at 37°C within 5 min. After equilibrium has been achieved, addition of a 1250-fold excess of unlabelled ouabain results in a rapid loss of radioactive ligand binding with first-order kinetics and a dissociation rate constant ($k_{-1}$) of 0.16 $min^{-1}$.

Fig. 2A illustrates the equilibrium binding of [$^3$H]ouabain as a function of radioactive ligand concentration. Whilst the nonspecific component of binding (i.e., the amount of [$^3$H]ouabain bound in the presence of excess non-radioactive ouabain) rises approximately linearly with radioactive ligand concentration, specific [$^3$H]ouabain binding is saturable. Scatchard analysis [12] of the same data over a wide range of radioactive ligand concentrations indicates a single class of binding site with an affinity constant ($K_A$) of $4.8 \cdot 10^6$ $M^{-1}$ (Fig. 2B).

As in previous studies on the human erythrocyte [9,10], the affinity of ouabain binding was found to be strongly influenced by the external $K^+$ concentration, elevations in $K^+$ concentration leading to a marked fall in binding affinity. Scatchard analysis of ouabain binding over a wide range of external $K^+$ concentrations indicate that there is an approx. 10-fold decrease in the affinity of ouabain binding as the $K^+$ concentration increases from 0.5 to 5 mM (Fig. 3). The total number of available binding sites is not altered. Multiple determinations indicate a binding capacity for ouabain of 55 ± 2 pmol per ml of packed erythrocytes (mean ±1 S.E., 18 expts.). At a mean cell volume of 136 $\mu m^3$ and surface area of 205 $\mu m^2$ [13] this value corresponds to approx. 4500 ± 200 ouabain binding sites per cell and to 22 ± 1 sites per $\mu m^2$ surface area.

*Dose-response relationship of potassium influx to isoproterenol: effect of external $K^+$ concentration*

Fig. 4 shows dose-response relationships between isoproterenol concentration and potassium influx at both 1 and 10 mM external $K^+$. In the presence of

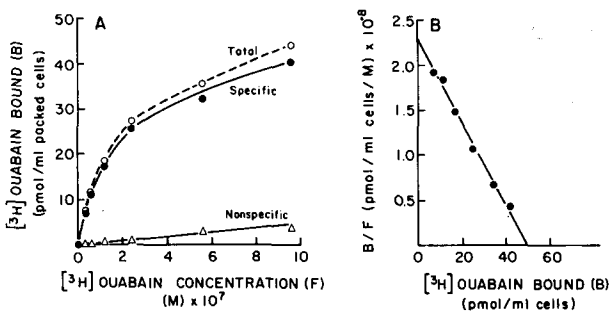
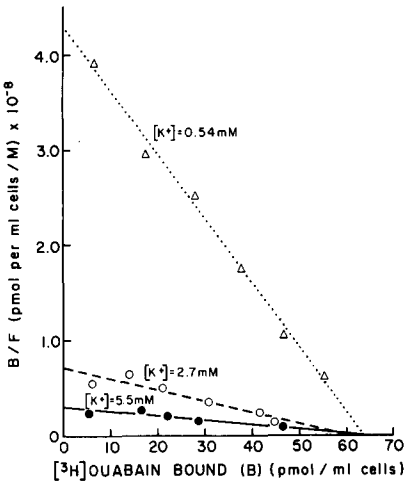



**Fig. 2. A. equilibrium binding of [$^3$H]ouabain as a function of radioactive ligand concentration in the presence of 0.5 mM $K^+$. Specific binding (●) was defined as the difference between radioactive ligand binding in the absence (○) and presence (△) of a displacing concentration (0.1 mM) of added non-radioactive ouabain (see text). B. Scatchard analysis [12] of the same data. The linear plot indicates a single class of ouabain binding sites with an affinity ($K_A$) of $4.8 \cdot 10^6$ $M^{-1}$ for ouabain ($K_D = 2.1 \cdot 10^{-7}$). Extrapolation of the line to infinite ouabain concentration indicates a total of 49 pmol of binding sites per ml of packed erythrocytes. B, pmol [$^3$H]ouabain bound per ml of packed erythrocytes; F, free [$^3$H]-ouabain concentration (M).**



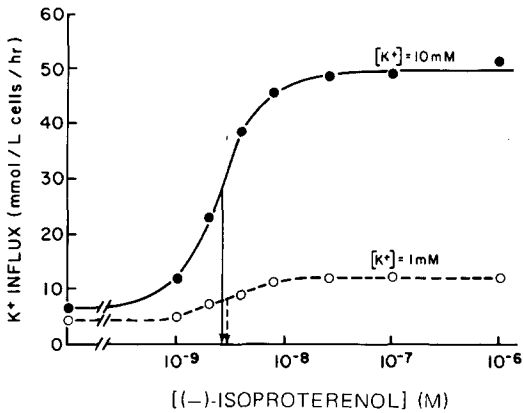




Fig. 3. Scatchard analyses [12] of [$^3$H]ouabain binding as a function of ambient $K^+$ concentration. $[K^+]$ = 0.54 mM (△), $[K^+]$ = 2.7 mM (○), $[K^+]$ = 5.5 mM (●). The $K_D$ values at these three $K^+$ concentrations are 0.15, 0.86 and 2.1 μM, respectively.

Fig. 4. Potassium influx as a function of (—)-isoproterenol concentration at two different external $K^+$ concentrations. The concentrations of isoproterenol required for half-maximal stimulation of potassium influx are indicated by the arrows.

10 mM $K^+$, the stimulation of ion flux by isoproterenol is 5- to 6-fold greater than at 1 mM $K^+$. At both $K^+$ concentrations, stimulation of potassium influx by isoproterenol is detectable at less than 2 nM isoproterenol and is nearly maximal at 10 nM. Despite the marked differences in magnitude of the isoproterenol-stimulated fluxes at these two external $K^+$-levels, the concentration of (—)-isoproterenol necessary for half-maximal stimulation of $K^+$ influx is the same in both instances (3 nM). This latter concentration of (—)-isoproterenol is consistent with the findings of Gardner et al. [4] who observed half-maximal stimulation of potassium influx at a (±)-racemate concentration of approx. 8 nM.

Propranolol entirely abolishes the isoproterenol-stimulated component of potassium influx (Fig. 5): in contrast, basal influx (i.e., influx in the absence of isoproterenol) is unaffected by the β-adrenergic antagonist. The latter observation indicates that β-adrenergic mechanisms make no detectable contribution to basal potassium influx under these conditions.

The strong dependence of both basal and isoproterenol-stimulated $K^+$ influx upon external $K^+$ concentration made it of interest to examine these relationships in more detail. Potassium influx was accordingly examined as a function of external $K^+$ concentration under the influence of maximally effective concentrations of either isoproterenol (1 μM) or ouabain (1 mM) or both (Fig. 6). Both basal potassium influx and its ouabain-sensitive component rise until the external $K^+$ concentration reaches 2 mM: thereafter, the magnitude of the ouabain-sensitive component becomes fixed whilst total influx continues to rise. Isoproterenol-stimulated potassium influx continues to increase with rising external $K^+$ concentration well beyond 5 mM, and at all concentrations of $K^+$ is quantitatively accounted for by the ouabain-insensitive component alone [5].

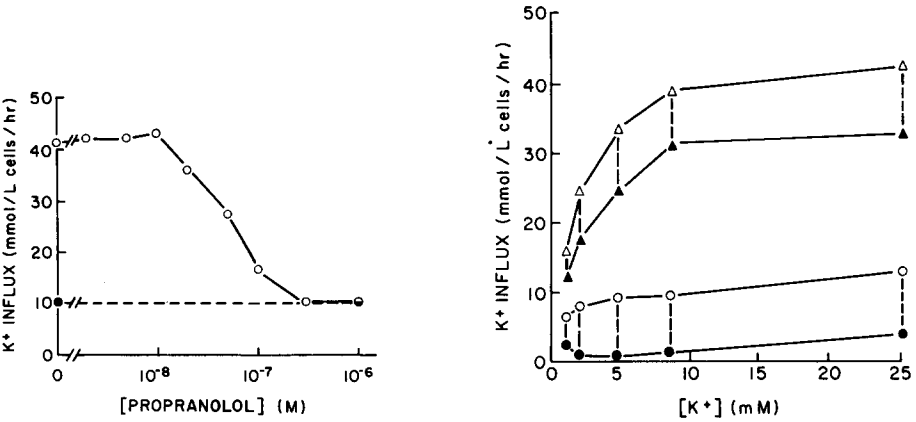



Fig. 5. Influence of propranolol on basal and isoproterenol-stimulated potassium influx rates. (○), $1 \cdot 10^{-7}$ M (—)-isoproterenol present; (●), isoproterenol absent.

Fig. 6. Potassium influx as a function of external $K^+$ concentration in the presence of maximally stimulatory and inhibitory concentrations of (—)-isoproterenol (1 μM) and ouabain (1 mM), respectively. (○), control; (●), ouabain; (△), isoproterenol; (▲), isoproterenol plus ouabain. Vertical lines in each instance represent decrements due to the presence of ouabain.

*Sensitivity of potassium influx to inhibition by ouabain: Relation to external $K^+$ concentration*

In order to examine a possible functional correlation of the marked dependence of ouabain binding upon external $K^+$ concentration, the sensitivity of potassium influx to inhibition by ouabain was also examined as a function of external $K^+$ concentration. Fig. 7 shows that, with increasing external $K^+$ concentrations, there is a striking shift in the ouabain concentration-inhibition curve such that the concentration of ouabain necessary for half-maximal inhibition of potassium influx rises markedly. The relation between potassium-induced changes in ouabain binding affinity and changes in this functional sensitivity of potassium influx to inhibition by ouabain is shown in Fig. 8. An identical relation is seen to exist between external $K^+$ concentration and the dissociation constant for ouabain binding and between external $K^+$ concentration and the concentration of ouabain necessary for half-maximal inhibition of potassium influx.

Fig. 9 presents an analysis of the data shown in Fig. 7, but this time by a method analogous to that of Scatchard [12]. The linearity of the data when plotted by this method indicates that the ouabain-inhibitable pump units function independently of each other, i.e., that when $X$% of the pumps are inhibited, the remaining $(100 - X)$% continue pumping at their original rate. The negative reciprocals of the slopes in Fig. 9 provide a functional measure of the dissociation constant ($K_D$) for the ouabain-($Na^+ + K^+$)-dependent ATPase complex as a function of the external $K^+$ concentration. As indicated in Fig. 8, the agreement between the $K_D$ values as operationally defined in this way, and the $K_D$ values for ouabain binding as determined directly by means of tritiated ligand, is excellent.

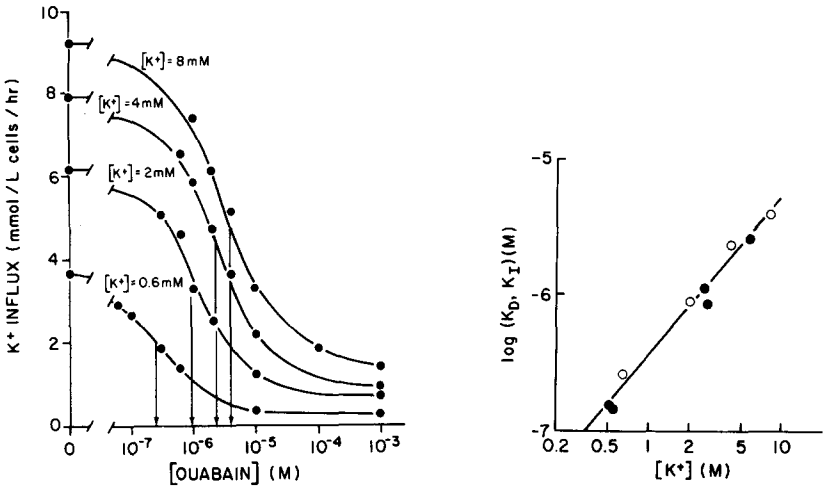



Fig. 7. Sensitivity of potassium influx to inhibition by ouabain as a function of external $K^+$ concentration. Vertical arrows indicate the concentrations of ouabain required for half-maximal inhibition of potassium influx at each different external $K^+$ concentration.

Fig. 8. Concentrations of ouabain required for half-maximal binding (•) and for half-maximal inhibition of potassium influx (○) as a function of external $K^+$ concentration. Note the logarithmic scale on both axes a straight line is also obtained when linear axes are employed since the $x$- and $y$-intercepts in the latter plot are both very nearly zero.

## *Relation between β-adrenergic receptor occupancy, cyclic AMP generation and potassium influx*

A plot of cyclic AMP accumulation rate as a function of isoproterenol concentration is shown in Fig. 10 (closed circles). The experimental points are seen to be virtually superimposable upon the theoretical isoproterenol binding curve

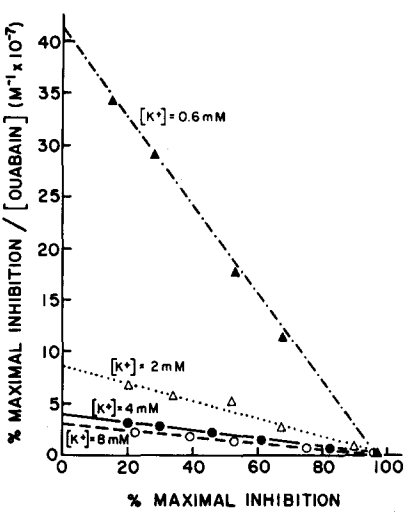



Fig. 9. Plot of (% maximal inhibition ÷ ouabain concentration) vs. (% maximal inhibition) as a function of external potassium ion concentration. The linearity of this relationship indicates that, at all concentrations of potassium ion, inhibition of ion transport is strictly proportional to ouabain binding, and hence that membrane ($Na^+ + K^+$)-dependent ATPase sites function independently of each other.

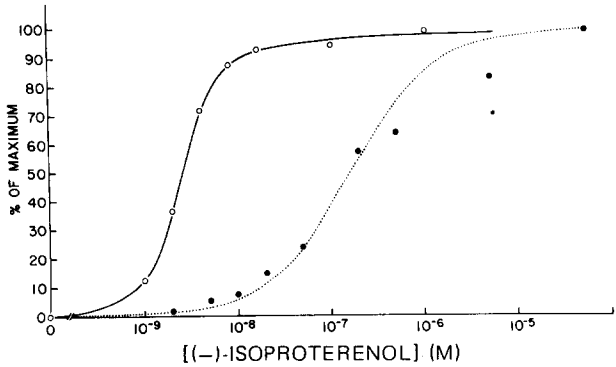



Fig. 10. Cyclic AMP generation rate (•) and stimulation of potassium influx (○) as a function of (—)-isoproterenol concentration. The dotted line corresponds to the binding of isoproterenol to membrane receptor sites with a dissociation constant ($K_D$) of $1.6 \cdot 10^{-7}$ M [14,15]. Results are expressed as per cent of maximal, the respective maximal values being as follows: cyclic AMP generation rate, 4100 pmol/$10^9$ cells per h; isoproterenol-stimulated potassium influx, 45 mmol/l cells per h; isoproterenol binding, 300 molecules per cell [15].

corresponding to the dissociation constant for isoproterenol as determined in previous experiments by displacement of the radioactive ligand [$^{125}$I]-iodohydroxybenzylpindolol [14,15]. The close proportionality between isoproterenol binding and cyclic AMP generation rate throughout the entire concentration-response range indicates that the rate of cyclic AMP generation per occupied β-adrenergic receptor is constant and independent of overall percent receptor occupancy. In contrast to the concentration-response curve for cyclic AMP generation, which is superimposable upon the corresponding curve for receptor occupancy, the curve for isoproterenol-stimulated potassium influx is markedly shifted 'to the left' (Fig. 10). Whereas the concentrations of isoproterenol required for half-maximal isoproterenol binding and for half-maximal stimulation of cyclic AMP synthesis are both approx. 150 nM, the concentration of isoproterenol required for half-maximal stimulation of potassium influx (open circles) is over 50 times lower (2—3 nM). Half-maximal stimulation of potassium influx is thus seen to be achieved when only 3% of the total number of β-adrenergic receptor sites are occupied. Similarly, influx is 90% maximal at only 10 nM isoproterenol, when receptor occupancy and cyclic AMP generation rate are both less than 10% of their maximal values.

## Discussion

The binding of ouabain to the turkey erythrocyte, like that to the human erythrocyte [9,10], shows marked dependence upon external $K^+$ concentration. Binding affinity, but not the total number of binding sites per cell, is markedly reduced in the presence of increasing concentrations of $K^+$. Binding in the turkey erythrocyte, however, differs appreciably in a number of quantitative respects from binding to human cells. In the presence of 150 mM sodium ion and low external $K^+$ (0.4 mM), ouabain binds with an affinity constant ($K_A$) of the order of $5 \cdot 10^6$ $M^{-1}$. Under similar conditions, the affinity of the human red cell for ouabain is strikingly greater, approx. $1 \cdot 10^8$ $M^{-1}$ [9]. The

number of ouabain-specific binding sites in the turkey erythrocyte, whether expressed per cell, per unit of cell volume or per unit of cell membrane surface, is substantially greater than the corresponding number in the human erythrocyte, as is the basal ouabain-inhibitable rate of potassium influx (Table I). In contrast to the extremely low rate constant for the dissociation of tritiated ouabain from ($Na^+ + K^+$)-dependent ATPase sites in the human red cell ($k_{-1}$ approx. 0.001 $min^{-1}$ at 37°C [16]), the rate constant for the turkey cell is considerably higher (0.16 $min^{-1}$).

The relation between ouabain binding and inhibition of ion transport has been extensively examined in human erythrocytes in earlier studies and has been found to be linear [16]. The present studies on the turkey erythrocyte show a similar linear relationship. In all instances, and over the entire range of external $K^+$ concentrations tested, the concentrations of ouabain necessary for half-maximal ouabain binding to the turkey erythrocyte membrane ($K_D$) and for half-maximal inhibition of potassium transport ($K_I$) have been shown here to be identical. The mathematical form of the concentration dependence of the inhibition of potassium influx by ouabain moreover is such that, at external $K^+$ concentrations ranging from 0.6 to 8 mM (corresponding to a wide range of basal potassium influx rates), the rate of potassium influx per free ATPase site is uninfluenced by simultaneous occupancy of other sites by ouabain. To our knowledge, the identity between the $K_D$ for ouabain binding and the $K_I$ for ouabain-inhibitable potassium influx, extending over a more than 20-fold range of values, has not been previously demonstrated in other systems.

The respective effects of propranolol (1 μM) and of ouabain (1 mM) on various components of the potassium transport system in the turkey erythrocyte noted here confirm previous observations of others that: (1) 50—70% of basal potassium influx in the turkey erythrocyte is inhibitable by ouabain [2]; (2) isoproterenol-stimulated potassium fluxes are ouabain-insensitive [4,5]; (3) the isoproterenol-stimulated component of potassium influx is inhibitable by propranolol, whilst basal fluxes are not [4] and (4) the magnitude of both basal

TABLE I

COMPARISON OF HUMAN AND TURKEY ERYTHROCYTES: MORPHOLOGICAL CHARACTERISTICS, OUABAIN BINDING AND POTASSIUM INFLUX RATES

Numbers in brackets indicate references: rbc, red blood cells.

| | Human | Turkey |
|---|---|---|
| Erythrocyte volume (μm³)[13] | 87 | 136 |
| Erythrocyte surface area (μm²)[13] | 163 | 205 |
| Ouabain binding sites: | | |
| pmol/ml rbc | 19 [16] | 55 |
| sites per cell | 990 | 4500 |
| sites per μm² membrane surface | 6.1 | 22 |
| Basal potassium influx (mmol/l rbc per h) | 2.3 * [16] | 8 ** |
| Ouabain-inhibitable component of basal influx (mmol/l rbc per h) | 0.8 * [16] | 6 ** |

* $[K^+]_{ext}$ = 8.3 mM.
** $[K^+]_{ext}$ = 10 mM.

and isoproterenol-stimulated potassium influx rates increases with increasing external $K^+$ concentration [5]. Additionally, the experiments described here have demonstrated that, although the absolute magnitude of isoproterenol-stimulated potassium influx is a strong function of the external $K^+$ concentration, the concentration of β-agonist required for half-maximal stimulation is entirely unaffected by the $K^+$ concentration of the medium. Finally, these experiments have shown that at external $K^+$ concentrations above 2 mM the magnitude of the ouabain-sensitive component is entirely unaffected by $K^+$ concentration, the rise in total potassium influx at increasing levels of $K^+$ being quantitatively accounted for by the rise in the ouabain-insensitive component alone. Throughout the entire range of external $K^+$ concentrations examined, stimulation of potassium influx was observed at concentrations of (—)-isoproterenol lower than 2 nM, with half-maximal stimulation occurring at 3.0 ± 0.2 nM.

The rate of cyclic AMP generation and the extent of β-adrenergic receptor occupancy by β-agonist were closely proportional over the entire range of isoproterenol concentrations examined, indicating functional independence of individual β-site-cyclase units. These observations confirm in a direct manner those previously made in isolated membrane preparations showing that site-site interactions among β-adrenergic receptors do not occur in the turkey erythrocyte [17,18]. Both agonist binding and cyclic AMP generation rate showed half-maximal values at isoproterenol concentrations far exceeding those present under any physiological conditions. In striking contrast, the effect of isoproterenol concentration upon potassium influx reached a half-maximal level at an isoproterenol concentration of only 2—3 nM (i.e., more than 50-fold lower) and was fully established (i.e., was over 90% maximal) at an isoproterenol concentration of 10 nM. This latter concentration corresponds to only 9% receptor occupancy, beyond which increased receptor occupancy and proportionately increased rates of cyclic AMP generation have no further effect upon potassium transport. Table II summarizes some of these relationships. It is of interest that, in a study of the interstitial cells of the testis, Mendelson et al. [19] similarly observed half-maximal stimulation of cyclic AMP-mediated

TABLE II

QUANTITATIVE RELATIONSHIPS BETWEEN β-ADRENERGIC RECEPTOR OCCUPANCY, CYCLIC AMP GENERATION AND STIMULATION OF POTASSIUM INFLUX IN THE TURKEY ERYTHROCYTE

| | Concentration of (—)-isoproterenol required for half-maximal effect (M) | Receptors occupied (%) | Number of occupied receptors per cell * |
|---|---|---|---|
| β-Adrenergic receptor occupancy | $1.6 \cdot 10^{-7}$ | 50 | 150 |
| Cyclic AMP generation | $1.5 \cdot 10^{-7}$ | 50 | 150 |
| Stimulation of $K^+$ influx | $2.5 \cdot 10^{-9}$ | 3 | 9 |

* Based upon estimate of a total of 300 isoproterenol binding sites per cell [15].

testosterone synthesis at a gonadotrophin concentration some 500-fold lower than that required for half-maximal saturation of gonadotrophin receptors.

The range of isoproterenol concentrations encompassing the responsive portion of the potassium-transport curve corresponds to the levels of total catecholamines circulating in the normal turkey [15]. At these levels, potassium influx is exceedingly sensitive to catecholamine concentrations and responses can be observed at a concentration of isoproterenol (1 nM) corresponding to occupation of fewer than two receptors per cell. What causes subsequent 'saturation' of the influx-stimulating mechanism at the levels of cyclic AMP generated at a concentration of only 10 nM isoproterenol is unknown. While it is possible that the enhanced levels of cyclic AMP generated at higher concentrations of isoproterenol are derived from different intracellular compartments mediating other cellular functions, the physiological significance of the capacity of these cells to generate additional cyclic AMP in response to concentrations of isoproterenol which vastly exceed those achievable under any conditions in vivo remains a puzzling question.

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