Refractoriness of Cation Transport in Turkey Erythrocytes to Stimulation by Cyclic Adenosine 3'-5'-Monophosphate

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SUMMARY

In turkey erythrocytes bidirectional fluxes of sodium and potassium develop a time-dependent refractoriness to stimulation by endogenous cyclic adenosine 3'-5'-monophosphate (cyclic AMP). The refractoriness of potassium influx and potassium outflux (both of which require extracellular sodium and potassium for stimulation by cyclic AMP) depends on the extracellular concentrations of sodium and potassium. In contrast, the refractoriness developed by sodium outflux (which does not require extracellular sodium or potassium for stimulation by cyclic AMP) does not depend on the extracellular concentrations of sodium or potassium. The refractoriness of these fluxes to cellular cyclic AMP reflects a decrease in the amount by which they can be maximally stimulated and appears to be proportional to the extent to which the transport system is utilized during the course of the incubation. Ouabain significantly reduces the rate at which cation transport in turkey erythrocytes becomes refractory to endogenous cyclic AMP. This effect of the glycoside is independent of the extracellular concentrations of sodium or potassium and does not correlate with how it alters the initial response of the transport systems to cyclic AMP.

We showed previously (1) that in turkey erythrocytes incubated with isoproterenol, stimulation of bidirectional potassium fluxes and sodium influx (but not sodium outflux) by endogenous cyclic adenosine 3'-5'-monophosphate depended intimately on the extracellular concentrations of sodium and potassium. We have also found that after adding isoproterenol, cellular cyclic AMP increases steadily during the 2-hour incubation period while bidirectional cation fluxes increase rapidly, reach a maximum at 20 to 30 min and then decrease steadily toward control values during the subsequent 90 min of incubation (2, 3). This time course can be reproduced by exogenous cyclic AMP, but not attributable to inactivation of catecholamine, changes in cellular ATP or an effect of incubation per se, and indicates that cation transport in turkey erythrocytes becomes refractory to stimulation by cyclic AMP (2-4). Adding ouabain to the incubation solution does not alter cellular concentration of cyclic AMP but partially relieves the refractoriness developed by the transport systems (2, 4).

In the present experiments using intact turkey erythrocytes we have explored the effects of varying the cation composition of the incubation solution on the time course of isoproterenol-stimulated cation transport, the ability of ouabain to alter isoproterenol-stimulated cation transport, and the cellular content of sodium and potassium.

METHODS

Erythrocytes from adult male turkeys were obtained and processed as described previously (2, 3). The cells were washed four times with ice, isosmotic choline chloride (pH 7.4) and placed in the preincubation solution (37°C) at an hematocrit of 0.1 to 0.5%. The low hematocrit was used to minimize changes in the extracellular cation concentrations during the preincubation period. After preincubation erythrocytes were separated by centrifugation at 3000 × g for 3 min and the supernatant was aspirated. Cells which were preincubated with 42K or 32PNa were washed three times with at least 60 volumes of ice, isosmotic choline chloride and resuspended (hematocrit 4 to 8%) at 37°C in the appropriate solutions to determine fractional outflux of potassium or sodium using a 20-min incubation period. Erythrocytes which were preincubated without radioactivity were washed once with 60 volumes of ice, isosmotic choline chloride and resuspended (hematocrit 4 to 8%) at 37°C in solutions containing 42K to determine potassium influx.

We have described the techniques used to measure potassium influx, fractional potassium outflux, fractional sodium outflux, and erythrocyte content of sodium and potassium in preceding papers (2, 3). Unless otherwise specified the standard incubation and preincubation solutions contained (mM): choline Cl, 160; Tris-HCl (pH 7.4), 10; glucose, 11.1. When other cations were added an equimolar amount of choline was removed.

RESULTS

Turkey erythrocytes were preincubated for 10 or 120 min with different concentrations of isoproterenol. Cells in each preincubation tube were washed once, and potassium influx was determined during a 10-min incubation in a solution identical with that used for preincubation. In cells preincubated for 10 min, stimulation of potassium influx could be detected at 5 × 10^-9 m isoproterenol and maximal stimulation was observed at 10^-7 m (Fig. 1). Ouabain (10^-3 m) inhibited potassium influx but did not alter the shape of the dose-response curve or the
Results obtained from the IO-min preincubation period (results not shown). There was a curvilinear relation between potassium influx and extracellular potassium, and ouabain reduced control potassium influx by 50 to 70% but did not alter the shape of the curve relating concentrations. Ouabain (10^{-3} M) reduced control potassium influx by 50 to 70%, but did not alter the magnitude by which maximally effective concentrations of isoproterenol stimulated potassium influx. In cells preincubated for 120 min stimulation of potassium influx could also be detected at 5 \times 10^{-7} M; however, higher concentrations of isoproterenol produced no further stimulation. Moreover, the cells preincubated for 120 min showed maximal responses to isoproterenol which were 10% of the responses observed in cells preincubated for 10 min. In cells preincubated for 120 min with ouabain (10^{-4} M), stimulation of potassium influx could be detected at 5 \times 10^{-7} M isoproterenol and maximal stimulation was observed at 5 \times 10^{-8} M. Maximal isoproterenol-stimulated potassium influx in cells preincubated for 120 min with ouabain was 50% of that observed in cells preincubated for only 10 min with ouabain but was 4 times that observed in cells preincubated for 120 min without ouabain. Results obtained from similar experiments performed using preincubation times progressively less than 120 min fell on curves which progressively approached the results obtained from the 10-min preincubation period (results not shown).

Cells preincubated for 10 min showed the same effects of isoproterenol and ouabain on potassium influx at varying extracellular potassium concentrations (Fig. 2, left) as found previously (1). There was a curvilinear relation between potassium influx and extracellular potassium, and potassium influx appeared to be approaching a plateau at higher extracellular potassium concentrations. Ouabain (10^{-3} M) reduced control potassium influx by 50 to 70%, but did not alter the shape of the curve relating potassium influx to extracellular potassium. With isoproterenol (10^{-5} M) potassium influx increased progressively and became maximal at 15 mM potassium. Ouabain did not alter the magnitude by which isoproterenol stimulated potassium influx at any of the extracellular potassium concentrations studied. Ouabain thus inhibited potassium influx at all of the potassium concentrations studied and the magnitude of this inhibition was the same with or without isoproterenol.

Erythrocytes preincubated for 120 min (Fig. 2, right) also showed a curvilinear relation between potassium influx and extracellular potassium, and ouabain reduced control potassium influx by 50 to 70% but did not alter the shape of the curve. With isoproterenol and increasing external potassium, potassium influx increased and reached a plateau at 4 mM extracellular potassium. With isoproterenol plus ouabain, potassium influx increased, reached a maximum when the external potassium was 5 mM, and then decreased. At external potassium concentrations from 3 to about 15 mM, preincubation for 120 min with isoproterenol plus ouabain resulted in greater potassium influx than with isoproterenol alone. At potassium concentrations above or below this range potassium influx was lower with isoproterenol plus ouabain than with isoproterenol alone.

The results in Fig. 2 show that at each concentration of potassium increasing the preincubation period from 10 to 120 min causes a 20 to 30% decrease in control potassium influx and no change in potassium influx in ouabain-containing solutions. The length of the preincubation period did not alter potassium influx at extracellular concentrations up to 4 mM, but increasing external potassium from 4 to 20 mM caused a progressive drop in potassium influx. Furthermore, at each potassium concentration above 1 mM isoproterenol-stimulated potassium influx was significantly increased when ouabain was present.
FIG. 2. Stimulation of potassium influx by isoproterenol in turkey erythrocytes after 10 min or 120 min of preincubation in solutions of varying potassium concentrations. Erythrocytes were preincubated (37°C; hematocrit 0.1 to 0.5%) in solutions containing 150 mM sodium and the indicated concentrations of potassium without (circles) or with 10⁻⁵ M ouabain (boxes); closed symbols represent preincubations containing 10⁻⁴ M dl-isoproterenol. At the end of the preincubation, cells were washed once with iced, isosmotic choline chloride and potassium influx was measured during a 10-min incubation (37°C; hematocrit 4 to 8%) in 4K-containing solutions identical in composition with those used for the preincubation. Results shown are the means of three experiments.

FIG. 3. Stimulation of potassium influx by isoproterenol in turkey erythrocytes after 10 min or 120 min of preincubation in solutions varying in sodium concentration. Erythrocytes were preincubated (37°C; hematocrit 0.1 to 0.5%) in solutions containing 10 mM potassium and the indicated concentration of sodium without (circles) or with 10⁻⁴ M ouabain (boxes); 10⁻⁴ M dl-isoproterenol was added to preincubations represented by closed symbols. At the end of the preincubation, cells were washed once with iced, isosmotic choline chloride and potassium influx was measured during a 10-min incubation (37°C; hematocrit 4 to 8%) in 4K-containing solutions identical in composition with those used for the preincubation. Results shown are the means of three experiments.

Inhibited potassium influx at each sodium concentration studied and the magnitude of this inhibition was the same with or without isoproterenol.

In erythrocytes preincubated for 120 min (Fig. 3, right) we also found that potassium influx increased slightly and reached a plateau at an extracellular sodium concentration of 60 mM. Ouabain reduced potassium influx by 50 to 60% and abolished the relatively small stimulation of potassium influx by extracellular sodium in control incubations. Isoproterenol caused a progressive increase in potassium influx with increasing extracellular sodium up to 30 mM. With isoproterenol plus ouabain, potassium influx increased, reached a maximum at 75 mM sodium, and then decreased. At external sodium concentrations above 15 mM, preincubating with isoproterenol plus ouabain resulted in greater potassium influx than with isoproterenol alone.
In erythrocytes preincubated for 120 min with increasing concentrations of extracellular sodium the magnitude of isoproterenol-stimulated potassium influx increased and reached a plateau at 30 mM sodium. With ouabain, isoproterenol-stimulated potassium influx increased and reached a maximum at an external sodium of 75 mM and then steadily decreased. At each sodium concentration studied isoproterenol-stimulated potassium influx was greater with than without ouabain.

It is evident from the results of Fig. 3 that increasing the preincubation period from 10 to 120 min produced a 25% decrease in potassium influx with or without ouabain. With isoproterenol, prolonging the preincubation period decreased potassium influx and the magnitude of this decrease became progressively greater the higher the extracellular sodium concentration. With isoproterenol plus ouabain, prolonging the preincubation period did not alter potassium influx at extracellular sodium concentrations up through 90 mM but increasing external sodium from 90 to 150 mM produced a progressively greater drop in potassium influx. Furthermore, at each sodium concentration the time-dependent decrease in potassium influx with isoproterenol was significantly less when ouabain was included in the preincubation solution. Results similar to those illustrated in Fig. 3 were obtained using exogenous cyclic AMP (10 mM) instead of isoproterenol.

Since the cation compositions of the preincubation solutions and the incubation solutions were identical in the experiments in Figs. 2 and 3, we wondered to what extent the relation between potassium influx and extracellular potassium was attributable to the cation composition of the preincubation solutions and to what extent it was determined by the cation composition of the incubation solution. To try to distinguish between these two determinants, turkey erythrocytes were preincubated for 120 min in solutions containing 150 mM sodium and varying concentrations of extracellular potassium. Cells from each preincubation tube were then separated by centrifugation, washed once with ice-cold isosmotic choline chloride, and divided into two portions. One portion of cells was incubated in a solution containing 42K and the same cation composition as the preincubation solution. The other portion was incubated in a solution containing 4K, 150 mM sodium, and 10 mM potassium. (Isoproterenol-stimulated potassium influx in cells incubated with 150 mM sodium and 10 mM potassium is 95% of the maximum value which we can achieve with this system (Ref. 1 and Fig. 2).) Cells preincubated 120 min with isoproterenol (10^{-5} M) and varying extracellular potassium and then incubated with 10 mM potassium plus isoproterenol showed a steady decrease in potassium influx which reached a minimum with 6 mM potassium in the preincubation phase (Fig. 4). Increasing the preincubation potassium concentration above 6 mM produced no further change in potassium influx. When these cells were incubated in solutions identical with those used for preincubation, potassium influx increased and reached a plateau with the cells exposed to 4 mM potassium. Furthermore, the maximum value of potassium influx in cells incubated with varying extracellular potassium was identical with the minimum value of potassium influx obtained when these same cells were incubated with 10 mM potassium. Cells preincubated for 120 min (without isoproterenol) with 10 mM potassium and 150 mM sodium showed a potassium influx of 6.4 ± 1.1 mmol per liter of cells per hour (mean ± S.D.; n = 5) determined in a medium containing 150 mM sodium and 10 mM potassium. As the preincubation potassium concentration was reduced to zero, potassium influx rose to 8.7 ± 1.3. On the other hand reducing the preincubation sodium concentration to zero did not significantly alter potassium influx (5.0 ± 0.9).

Cells preincubated for 120 min with isoproterenol (10^{-5} M), ouabain (10^{-5} M), and potassium concentrations of 3 mM or less maintained high potassium influx rates with 10 mM potassium, isoproterenol, and ouabain (Fig. 4). With preincubation concentrations of potassium greater than 3 mM, potassium influx decreased progressively and linearly with increasing extracellular potassium. Incubation in solutions identical with those used for preincubation caused increased potassium influx as extracellular potassium increased, became maximal at 7 mM potassium and then decreased. For cells preincubated with 8 mM potassium or higher, potassium influx was the same when later incubated with 10 mM potassium as when incubated in a solution identical with that used for preincubation. Erythrocytes preincubated for 120 min with ouabain, 10 mM potassium, and 150 mM sodium showed a potassium influx of 2.6 ± 0.4 mmol per liter of cells per hour (mean ± S.D.; n = 5) in a solution containing 150 mM sodium, 10 mM potassium, and ouabain. As the preincubation potassium concentration was reduced to zero, potassium influx increased slightly to 3.3 ± 0.2. Reducing the preincubation sodium concentration to zero did not significantly alter potassium influx (2.5 ± 0.2).

In cells preincubated with varying extracellular potassium plus isoproterenol and then incubated with 10 mM potassium plus isoproterenol, potassium influx was reduced by adding ouabain at an external potassium of 1 mM but at higher potassium concentrations ouabain significantly increased potassium influx. (If, however, the preincubation potassium concentration is increased to 15 mM or greater, potassium influx with isoproterenol plus ouabain will be less than that with isoproterenol alone. See Fig. 2.)

When the experiments in Fig. 4 were repeated, this time varying the preincubation sodium concentration, we observed a similar phenomenon (Fig. 5). That is, there was a time-dependent decrease in isoproterenol-stimulated potassium influx. The magnitude of this decrease became progressively greater with increasing extracellular sodium concentrations in the preincubation solution, and this decrease was significantly reduced when ouabain (10^{-3} M) was present in the medium.

In the experiments in Figs. 2 to 5, erythrocytes were preincubated in solutions containing 150 mM sodium and varying potassium concentrations or 10 mM potassium and varying sodium concentrations. The results in Fig. 6 illustrate the effects of varying the concentrations of both sodium and potassium in the preincubation solution containing isoproterenol with or without ouabain on potassium influx measured in an incubation solution containing 150 mM sodium, 10 mM potassium, and isoproterenol (10^{-5} M) with (right) or without (left) ouabain (10^{-3} M). In a sodium-free isoproterenol-containing preincubation solution, varying the potassium concentration did not alter isoproterenol-stimulated potassium influx (Fig. 6, left). With 50 mM sodium, there was a progressive, curvilinear decrease in isoproterenol-stimulated potassium influx with increasing preincubation potassium concentrations. Increasing the preincubation sodium concentration to 100 mM accentuated the decrease in isoproterenol-stimulated potassium influx which occurred with increasing preincubation potassium concentrations. Results obtained with 150 mM sodium were the same as those obtained with 100 mM sodium. In a sodium-free preincubation solution containing isoproterenol plus ouabain, varying the potassium concentration did not alter isoproterenol-stimulated potassium influx (Fig. 6, left). When the sodium concentration...
Fig. 4 (left). Effect of potassium in the preincubation phase on isoproterenol-stimulated potassium influx. Cells were preincubated (37°; hematocrit 0.1 to 0.5%) for 120 min in solutions containing 150 mM sodium, the indicated concentration of potassium and 10^-6 M dl-isoproterenol with (boxes) or without (circles) 10^-3 M ouabain. At the end of the preincubation, cells from each preincubation tube were washed once with iced, isosmotic choline chloride and divided into two portions. One portion was resuspended in a solution identical with that used for the preincubation (O, □) and the other portion was resuspended in a solution identical with that used for preincubation except the potassium concentration was 10 mM (O, ■). 85K was then added and potassium influx determined during a 10-min incubation period (37°; hematocrit 4 to 8%). Results shown are the means of four experiments.

Fig. 5 (right). Effect of sodium in the preincubation phase on isoproterenol-stimulated potassium influx. Cells were preincubated (37°; hematocrit 0.1 to 0.5%) for 120 min in solutions containing 10 mM potassium, the indicated concentration of sodium and 10^-6 M dl-isoproterenol with (boxes) or without (circles) 10^-3 M ouabain. At the end of the preincubation, cells from each preincubation tube were washed once with iced, isosmotic choline chloride and divided into two portions. One portion was resuspended in a solution identical with that used for the preincubation (O, □) and the other portion was resuspended in a solution identical with that used for preincubation except the sodium concentration was 150 mM (O, ■). 4K was then added and potassium influx determined during a 10-min incubation period (37°; hematocrit 4 to 8%). Results shown are the means of four experiments.

It is evident (Fig. 6) that in turkey erythrocytes preincubated for 120 min in a sodium-free solution containing isoproterenol and varying concentrations of potassium, influx of potassium is reduced by adding ouabain to the medium (both preincubation and incubation). As the preincubation potassium concentration was increased above 4 mM there was a progressive decrease in isoproterenol-stimulated potassium influx. Further increases in the extracellular potassium concentration above which isoproterenol-stimulated potassium influx begins to diminish and increased the potassium-dependent reduction in isoproterenol-stimulated potassium influx.

Fractional potassium outflux was determined in turkey erythrocytes preincubated for 120 min in sodium-free solutions containing 4K, 10 mM potassium, and varying concentrations of sodium. After the preincubation phase the cells were washed three times with iced, isosmotic choline chloride and incubated for 20 min.
Fig. 7 (left). Effect of extracellular sodium concentrations during preincubation on isoproterenol-stimulated potassium outflux. Cells were preincubated (37°C; hematocrit 0.1 to 0.5%) for 120 min in solutions containing 42K, 10 mM potassium, and the indicated concentrations of sodium. At the end of the preincubation, cells were washed three times with iced, isosmotic choline chloride and fractional potassium outflux determined during a 20-min incubation (37°C; hematocrit 4 to 8%) in solutions containing 150 mM sodium and 10 mM potassium without (△) or with (▲) 10^{-3} M dl-isoproterenol. ○, results when 10^{-5} M dl-isoproterenol was present in both the preincubation and incubation phases. □, results when 10^{-3} M dl-isoproterenol and 10^{-2} M ouabain were present in both the preincubation and incubation phases. Results shown are the means of four experiments.

Fig. 8 (right). Effect of extracellular potassium on isoproterenol-stimulated potassium outflux in turkey erythrocytes. Cells were preincubated (37°C; hematocrit 0.0%) for 120 min in solutions containing 34Na, 150 mM sodium, and the indicated concentrations of potassium. At the end of the preincubation cells were washed three times with iced, isosmotic choline chloride and fractional sodium outflux determined during a 20-min incubation (37°C; hematocrit 4 to 8%) in solutions containing 150 mM sodium and 10 mM potassium alone (△) or with 10^{-3} M ouabain (○), 10^{-2} M dl-isoproterenol (▲), or isoproterenol plus ouabain (●). Results illustrated by circles and boxes were obtained using the same experimental paradigm described in the legend to Fig. 3. That is, the preincubation solution contained 10^{-3} M dl-isoproterenol with (boxes) or without (circles) 10^{-2} M ouabain. After preincubation and washing, cells from each tube were divided into two portions. To determine fractional sodium outflux, one portion was resuspended in a solution identical with that used for preincubation (○, □), and the other portion was resuspended in a solution identical with that used for preincubation except that the potassium concentration was 10 mM (●, ■). The experiment shown is representative of four others.

with 150 mM sodium and 10 mM potassium to measure fractional potassium outflux. The concentration of sodium during preincubation did not affect fractional potassium outflux with or without isoproterenol (10^{-6} M) during the incubation phase (Fig. 7). However, increasing extracellular sodium during preincubation with isoproterenol produced a progressive, curvilinear decrease in fractional potassium outflux. Potassium outflux reached a minimum in cells preincubated with 60 mM sodium; higher concentrations caused no further effect on outflux. Adding ouabain (10^{-3} M) increased potassium outflux at each concentration of extracellular sodium and antagonized the sodium-dependent reduction in potassium outflux. Similar results were obtained using exogenous cyclic AMP (10 mM) instead of isoproterenol.

Further preincubation experiments were carried out for 120 min in solutions containing 34Na, 150 mM sodium, and varying concentrations of potassium to determine whether the time-dependent decrease in isoproterenol-stimulated fractional sodium outflux also depends on the cation composition of the medium. After preincubation the cells were washed three times with iced, isosmotic choline chloride and incubated for 20 min to determine fractional sodium outflux (Fig. 8). The concentration of extracellular potassium during the preincubation phase did not influence fractional sodium outflux during the incubation phase with 150 mM sodium and 10 mM potassium. Addition of isoproterenol (10^{-6} M), ouabain (10^{-2} M), or both to the incubation solution affected fractional sodium outflux in the same manner as described in a preceding paper (1). Fractional sodium outflux with either or both of these agents added was not influenced by the external potassium concentration during the preincubation phase.

Fractional sodium outflux measured during the subsequent incubation phase with 150 mM sodium, 10 mM potassium, and isoproterenol also was not affected by the concentration of potassium during preincubation and was only slightly greater than that in cells treated identically without isoproterenol in the medium (Fig. 8). Furthermore, fractional sodium outflux measured in these cells when they were incubated in solutions identical with those used for their preincubation was no different from that obtained when the incubation potassium concentration was increased to 10 mM (compare Figs. 3 and 8). The rate of outflux in solutions containing 150 mM sodium, 10 mM potassium, isoproterenol, and ouabain also was independent of the potassium concentrations during preincubation with 150 mM sodium, iso-
Ouabain, isoproterenol, and potassium. Under these conditions sodium ouabain flux was 4 to 5 times greater than that in cells preincubated without ouabain or isoproterenol and then incubated with 150 mM sodium, 10 mM potassium, and ouabain. Fractional sodium ouabain flux measured in these cells when they were incubated in solutions identical with those used for their preincubation was the same as that obtained when the incubation potassium concentration was increased to 10 mM (compare Figs. 3 and 8). Results similar to those illustrated in Fig. 8 were obtained using exogenous cyclic AMP (10 μM) instead of isoproterenol.

Cellular concentrations of sodium and potassium were measured in turkey erythrocytes incubated for 120 min at 37°C with isoproterenol (10^-4 M), ouabain (10^-3 M), or both in solutions varying in sodium and potassium content (Table I). Duck erythrocytes incubated for 90 min in solutions containing 154 mM sodium and 2.5 mM potassium lost potassium (9%), sodium (14%), and water (2.5%). These changes can be prevented by adding norepinephrine (10^-6 M) (5). Turkey erythrocytes incubated for 120 min with 150 mM sodium and 10 mM potassium lost potassium (11%) and sodium (22%) (Table I). Adding isoproterenol (10^-2 M) abolished the decrease in cell sodium and increased cell potassium by 6%. Ouabain did not prevent the loss of cellular potassium and increased cell sodium by 20%. With isoproterenol plus ouabain turkey erythrocytes gained sodium (350%) and lost less potassium (2%) than when they were incubated without these agents. In general, turkey erythrocytes incubated for 120 min under the various conditions indicated in Table I showed relatively small changes in cellular content of potassium. The greatest changes in cell potassium (15 to 20% decrease) tended to occur in potassium-free solutions with or without ouabain while the smallest changes were observed in solutions containing potassium and isoproterenol with or without ouabain. Many of the differences in cellular potassium listed in Table I are statistically significant as determined by a paired t test, but they are nevertheless small in degree compared to the differences in cation fluxes under the same conditions. Incubating turkey erythrocytes in sodium-free solutions for 120 min decreased cellular sodium by 60 to 80%. These changes tended to be greatest with isoproterenol and least with ouabain. In sodium-containing solutions, ouabain increased cell sodium by 200 to 300% and this increase was potentiated by isoproterenol in a potassium-containing solution and was reduced by isoproterenol in a potassium-free solution.

**DISCUSSION**

We found previously (2–4) that after addition of isoproterenol to turkey erythrocytes, influx and ouabain of both sodium and potassium increase rapidly, reach a maximum at 20 to 30 min and then decrease steadily so that by 120 min of incubation these fluxes are only slightly greater than those in cells incubated without isoproterenol. Since under identical conditions cellular cyclic AMP increases steadily during the 120-min incubation period, we concluded that cation transport in turkey erythrocytes incubated with β-adrenergic catecholamines becomes refractory to stimulation by cellular cyclic AMP (2–4). The present studies were designed to delineate further the relevant factors that might mediate this developing refractoriness.

One possible explanation is that since cellular cyclic AMP increases steadily during the course of a 2-hour incubation with isoproterenol, relatively low cellular concentrations of cyclic nucleotide might stimulate ion transport but sufficiently high concentrations might be inhibitory. Several of our observations, however, argue against this possibility. Maximal stimulation of cellular cyclic AMP requires 10^-7 M isoproterenol, and cellular cyclic AMP in cells incubated with 10^-4 M isoproterenol is 400% greater than that obtained with 10^-7 M isoproterenol (2, 3); however, maximal stimulation of cation fluxes in turkey erythrocytes is produced by 10^-7 M isoproterenol, and further increases in catecholamine concentration (up to 10^-4 M) do not alter cation fluxes (Fig. 1 and Refs. 2 and 3). In addition, removing extracellular sodium or potassium or both from the incubation solution reduces the amount of cellular cyclic AMP generated by turkey erythrocytes incubated with 10^-7 M isoproterenol by only 20% (1) but abolishes the decrease in isoproterenol-stimulated influx and ouabain of potassium which occurs during a 120-min incubation (see Figs. 4 to 8).

Another possibility is that during the 120-min preincubation of turkey erythrocytes with 150 mM sodium, 10 mM potassium, and 10^-5 M isoproterenol cellular concentrations of sodium and potassium might change and, by so doing, decrease the responsiveness of the cation transport mechanisms to stimulation by cellular cyclic AMP. However, as Kregenow observed (5) for

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**Table I**

*Effect of preincubation on sodium and potassium content of turkey erythrocytes*

Turkey erythrocytes were incubated for 2 hours at 37°C in the indicated solutions at an hematocrit of 0.3 to 0.6%. The cells were then washed four times with at least 100 volumes of iced choline chloride (100 mM, pH 7.4) and cellular sodium and potassium determined as described under "Methods." Each incubation solution contained Tris-HCl (pH 7.4), 10 mM, and glucose, 11.1 mM. When sodium or potassium was removed from the incubation solution, an equimolar amount of choline was added. Results are the means of six experiments ± 1 S.D. The sodium and potassium concentrations in turkey erythrocytes at the beginning of the 2-hour incubations were 3.19 ± 0.46 and 109.2 ± 8.3, respectively (mean ± 1 S.D.).

<table>
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<th>Preincubation cation concentration</th>
<th>Control</th>
<th>Isoproterenol (10^-5 M)</th>
<th>Ouabain (10^-5 M)</th>
<th>Ouabain + isoproterenol</th>
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<tr>
<td><strong>Cellular sodium</strong></td>
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<tr>
<td>Na = 150; K = 10</td>
<td>2.49 ± 0.22</td>
<td>3.07 ± 0.19</td>
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<td>Na = 150; K = 0</td>
<td>3.38 ± 0.34</td>
<td>3.95 ± 0.27</td>
<td>9.27 ± 1.72</td>
<td>4.43 ± 1.09</td>
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<td>Na = 0; K = 10</td>
<td>0.49 ± 0.14</td>
<td>0.39 ± 0.11</td>
<td>1.22 ± 0.15</td>
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<tr>
<td>Na = 0; K = 0</td>
<td>0.39 ± 0.05</td>
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<tr>
<td><strong>Cellular potassium</strong></td>
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<tr>
<td>Na = 150; K = 10</td>
<td>97.1 ± 7.1</td>
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</table>
duck erythrocytes treated with norepinephrine, turkey erythrocytes incubated for 120 min with isoproterenol, 150 mM sodium, and 10 mM potassium, show no change in cellular sodium and a slight (6%) increase in cellular potassium (see Table 1) while influx and fractional outflux of both sodium and potassium decreased by more than 80% (Figs. 1, 7, and 8 and Refs. 2–4). Omission of extracellular potassium reduced cellular potassium by 13%, increased cellular sodium by 28%, and abolished the decrease in potassium influx but did not alter the decrease in fractional sodium outflux. Thus, either the time-dependent decrease in cation fluxes stimulated by isoproterenol is not mediated by changes in cellular sodium or potassium or only the change in potassium influx (but not in sodium outflux) is attributable to changes in cellular cations. However, the decrease in isoproterenol-stimulated potassium influx does not appear to be attributable to a change in cellular sodium and is probably not due to a change in cellular potassium since preincubation of turkey erythrocytes for 120 min in a sodium-free solution reduced cellular sodium (by 90%) and reduced the fall in cellular potassium seen with a potassium-free solution by 50%, but was as effective in preventing this decrease in isoproterenol-stimulated potassium influx as was a potassium-free solution.

Since duck erythrocytes swell when incubated with 10^{-6} M norepinephrine (5), the possibility was considered that the increase in cell volume might alter or deform some membrane component critical for the membrane cation transport response to cyclic AMP. Additional support for this possibility is Krogrenov’s observation (6) that under conditions which prevent catecholamine-induced cell swelling (i.e. reducing extracellular potassium to 2.5 mM), ion fluxes (potassium influx, sodium influx, and sodium outflux) stimulated by norepinephrine do not decrease during the course of a 60-min incubation but under conditions which permit cell swelling (i.e. raising extracellular potassium to 15 to 17 mEq) catecholamine-stimulated ion fluxes decrease by more than 90% during the 60-min incubation. Although we have not measured changes in cell volume in turkey erythrocytes treated with isoproterenol, two types of experimental observations appear to exclude the possibility that the refractoriness to cyclic AMP developed by cation fluxes in turkey erythrocytes is mediated by changes in cell volume. Catecholamine-induced swelling of duck erythrocytes is observed only after the cells have been incubated for 90 min in a synthetic buffer solution free of catecholamine during which time they shrink and lose cellular sodium and potassium (5). Fresh duck erythrocytes incubated in a buffer solution containing norepinephrine (10^{-6} M) maintain cell volume, sodium, and potassium (5). Fresh turkey erythrocytes incubated with 150 mM sodium, 10 mM potassium, and 10^{-5} M isoproterenol maintain cellular sodium and potassium (see Table 1) but show an 80 to 90% decrease in catecholamine-stimulated ion fluxes at the end of a 120-min incubation period. Second, ouabain does not alter catecholamine-induced changes in cell volume (6); however, the glycoside significantly reduces the time-dependent decrease in cation fluxes stimulated by isoproterenol or exogenous cyclic AMP (Figs. 1, 7, and 8 and Refs. 2–4).

In a preceding paper (1) we showed that in turkey erythrocytes the effect of β-adrenergic catecholamines on sodium influx, potassium influx, and potassium outflux depended intimately on the concentrations of sodium and potassium in the incubation solution. In contrast, isoproterenol stimulation of sodium outflux was independent of the cation composition of the incubation solution. Omission of extracellular sodium or potassium or both produced relatively minor changes in isoproterenol-stimulated cellular cyclic AMP indicating that extracellular cations alter the responsiveness of cation transport to cellular cyclic nucleotide. Furthermore, by using exogenous cyclic AMP instead of isoproterenol we observed the same effects of extracellular cations and ouabain on the time course of cation transport. In the present studies we found that the extent to which influx (Figs. 4 to 6) and outflux (Fig. 7) of potassium becomes refractory to stimulation by cellular cyclic AMP depends directly on the extracellular concentrations of sodium and potassium while the time-dependent decrease in sodium outflux was not dependent on the extracellular cation concentrations (Fig. 8). These effects of extracellular cations were not attributable to effects of the altered cation composition per se since preincubating cells for 120 min with varying sodium concentrations without isoproterenol did not alter the magnitude by which isoproterenol when added to the incubation solution was able to stimulate ion transport (Fig. 7). Instead, those fluxes which require extracellular sodium and potassium for catecholamine stimulation develop a refractoriness to cyclic AMP which is dependent on the extracellular sodium and potassium concentrations.

The time-dependent change in the relationship between isoproterenol-stimulated potassium influx and the extracellular concentrations of potassium or sodium (Figs. 2 and 3) appears to be attributable to a reduction in the magnitude by which influx can be maximally stimulated (Figs. 4 and 5). The potency with which extracellular cations enhance stimulation of potassium influx by cyclic AMP does not appear to change over time; therefore, maximal values for isoproterenol-stimulated potassium influx can be achieved with lower concentrations of potassium or sodium in the incubation medium (compare left and right panels in Figs. 2 and 3). This pattern of effects of extracellular cations on isoproterenol-stimulated cation fluxes suggests that the refractoriness to stimulation by cellular cyclic AMP is a function of the extent to which the transport system is utilized. That is, the more the isoproterenol-stimulated transport system is utilized the more rapidly it becomes refractory to cellular cyclic nucleotide. If the refractoriness of the transport system to cellular cyclic nucleotide were independent of the degree to which the system is stimulated, then the shape of the dose-response curve relating potassium influx to isoproterenol concentration should be independent of the duration of the preincubation time. However, the magnitude by which maximal concentrations of isoproterenol stimulate cation transport should decrease progressively with time. Fig. 1 illustrates that although the amplitude of the dose-response curve is reduced by 80% after 120 min of preincubation, the concentration of isoproterenol required to produce maximal stimulation of potassium influx appears to be reduced by at least 95% (from 10^{-7} M to 5 × 10^{-8} M).

The extent to which the cation transport system was utilized did not, however, appear to be the sole determinant of the refractoriness to cellular cyclic AMP. When the medium contained 150 mM sodium, 10 mM potassium, and isoproterenol during both the preincubation (for 120 min) and incubation phases, potassium influx fell to the same rate with isoproterenol concentrations ranging from 5 × 10^{-6} M through 10^{-6} M and was significantly greater than zero (Fig. 1). At none of the concentrations of isoproterenol tested did we find that preincubating at a relatively low concentration of catecholamine resulted in a greater value for isoproterenol-stimulated potassium influx than was observed with higher concentrations. A similar phenomenon was observed when the preincubation (120 min) and incubation phases contained 10 mM potassium, 10^{-5} M isoproterenol, and varying concentrations of extracellular sodium.
higher sodium concentrations. On the other hand, preincubation each of these fluxes with equal effectiveness.

isoproterenol-stimulated potassium flux. The results in Fig. 8 indicate that potassium concentration increased above 3 mM, isoproterenol-stimulated potassium influx decreased progressively. That is, at a relatively low concentration of extracellular potassium, the magnitude by which isoproterenol stimulates potassium influx is reduced; however, the stimulation which does occur lasts longer and after a sufficient preincubation time will exceed that observed with higher concentrations. Thus, it may be that although the degree of refractoriness of cation fluxes to stimulation by cellular cyclic AMP depends on the extent to which the system has been utilized, the rate at which the refractoriness develops may be independent of the extent to which the system is initially stimulated and instead, be determined by another factor such as the extracellular potassium concentration. Additional experiments will be necessary to evaluate fully this possibility.

There is a discrepancy between our finding that the decrease in fractional sodium outflux from turkey erythrocytes preincubated for 120 min is independent of the extracellular potassium concentration (Fig. 8) and that of Kregenow (6) who found that sodium outflux decreased in duck erythrocytes preincubated for 55 min with 154 mM sodium, 10^{-4} M isoproterenol, and 17.0 mM potassium but remained unchanged when the extracellular potassium concentration was reduced to 2.5 mM. Although the basis of this discrepancy is presently unclear, it may be that in duck erythrocytes catecholamine-stimulated sodium outflux depends on the extracellular potassium concentration in contrast to turkey erythrocytes in which catecholamine-stimulated fractional sodium outflux does not depend on the extracellular concentration of either sodium or potassium (1).

Previously we have shown that in turkey erythrocytes the cardiac glycoside ouabain partially relieves the refractoriness to stimulation by cellular cyclic AMP which develops in the cation transport mechanisms (2, 4). In solutions containing 150 mM sodium and 10 mM potassium, ouabain, which inhibits sodium influx and potassium influx, but does not alter sodium influx or potassium outflux (2, 4), prolonged the responsiveness to cellular cyclic AMP of influx and outflux of both sodium and potassium. In contrast, varying extracellular sodium or potassium could prolong the responsiveness of sodium influx, potassium influx and fractional potassium outflux but did not affect the refractoriness of fractional sodium outflux to stimulation by cellular cyclic AMP. In further contrast to the effects of extracellular cations where there was a close correlation between their ability to alter isoproterenol-stimulated ion transport and their ability to relieve the refractoriness of ion transport to cellular cyclic nucleotide, the effect of ouabain on the refractoriness to cyclic AMP developed by cation fluxes did not correlate with ouabain’s effect on the initial stimulation of cation transport by isoproterenol. That is, initially ouabain does not alter isoproterenol-stimulated potassium influx (1, 2), potentiates isoproterenol-stimulated sodium influx and fractional potassium outflux (1, 2, 4), and inhibits isoproterenol-stimulated fractional sodium outflux (2, 4); however, ouabain prolonged the responsiveness to cyclic AMP of each of these fluxes with equal effectiveness.

We should also point out that the effects of ouabain on the time course of isoproterenol-stimulated cation transport require the simultaneous presence of both ouabain and isoproterenol. In agreement with Kregenow’s observations in duck erythrocytes (6), when turkey erythrocytes are preincubated for 120 min with isoproterenol and then incubated with isoproterenol plus ouabain, the glycoside does not potentiate or restore the responsiveness of cation transport to cellular cyclic AMP (4).^2

The ability of ouabain to prolong the duration of catecholamine-stimulated ion transport cannot be attributed to an effect on the production of cellular cyclic AMP since ouabain does not alter isoproterenol-stimulated cellular cyclic AMP (1, 5). Thus, ouabain must effect one or more of the steps through which cyclic AMP stimulates ion transport or act directly on the ion transport systems themselves to alter their responsiveness to cellular cyclic AMP. In addition, the effect of ouabain on the time course of isoproterenol-stimulated cation fluxes is not likely to reflect an effect on cell volume since Kregenow (6) has found that catecholamine-stimulated changes in the volume of duck erythrocytes are ouabain-insensitive. Finally, the effects of ouabain cannot be attributed to changes in cellular concentrations of ATP since cellular ATP in cells incubated for up to 120 min with 150 mM sodium, 10 mM potassium and ouabain, isoproterenol, or isoproterenol plus ouabain did not differ significantly from that in control incubations (4).

Turkey erythrocytes preincubated for 120 min with 150 mM sodium, 10 mM potassium, 10^{-4} M isoproterenol, and 10^{-3} M ouabain have a higher cellular sodium and a lower cellular potassium than erythrocytes preincubated in an identical solution but without ouabain (Table 1). This raises the possibility that maintenance by ouabain of the effect of cellular cyclic AMP on cation fluxes might be attributable to its effect on cellular ion concentrations. The clearest indication that increasing cellular sodium is not the mechanism through which ouabain prolongs isoproterenol-stimulated cation transport was found with 120-min preincubation experiments using potassium-free solutions containing 150 mM sodium and 10^{-3} M isoproterenol. Ouabain under these conditions increased cellular sodium and enhanced the fall in cellular potassium obtained with 10 mM potassium (Table 1); yet ouabain was as effective in prolonging isoproterenol-stimulated sodium outflux from cells preincubated without potassium as it was when using cells preincubated with potassium (Fig. 8). These results also argue against ouabain’s prolongation of isoproterenol-stimulated fractional sodium outflux being due to a reduction in cellular potassium since preincubating turkey erythrocytes in a potassium-free solution (containing sodium, isoproterenol, and ouabain) did not enhance the effect of ouabain on the time course of isoproterenol-stimulated fractional sodium outflux. Furthermore, as discussed in an earlier paragraph, the time-dependent changes in cation fluxes stimulated by isoproterenol do not appear to be attributable to changes in cellular sodium and potassium concentration. Therefore, it seems unlikely that the ability of ouabain to alter the time courses of isoproterenol-stimulated cation fluxes would be attributable to its effect on cellular cation concentrations. This question, however, cannot be answered with certainty until a detailed study is made of the relation between cellular cation concentrations and bidirectional fluxes of sodium and potassium. Some of our observations do indicate in a preliminary fashion that cation transport in turkey erythrocytes is a function of at least the cellular sodium concentration. The results in Fig. 6 indicate that potas-

^2 Unpublished results.
sium influx stimulated by isoproterenol with or without ouabain is higher in cells having a sodium content of approximately 4 mmol per liter of cells than in cells having a sodium content of 0.25 to 0.50 mmol per liter of cells. On the other hand fractional potassium outflux alone or with isoproterenol does not appear to be a function of cellular sodium (Fig. 7).

As discussed previously (1, 2, 4) we have not found a biochemical explanation for the relief by ouabain of the refractoriness developed to cyclic AMP. It is conceivable that this effect might reflect participation of an acyl phosphate intermediate in the ouabain-inhibitable Na+,K-dependent ATPase reaction (7–9) as a substrate for the cyclic AMP-dependent protein kinase reaction (10–12). Whatever the biochemical basis, the characteristic effect of ouabain on the time-dependent changes in cation fluxes stimulated by isoproterenol appears to be to reduce the rate at which each of the various fluxes becomes refractory to stimulation by cellular cyclic AMP. This effect of ouabain also appears to be independent of the effects (or lack thereof with fractional sodium outflux) of extracellular sodium and potassium since the glycoside does not alter the basic pattern of the changes produced by varying extracellular sodium or potassium. Instead, ouabain reduces the magnitude of the effect produced during a given period by a given change in extracellular sodium, potassium, or isoproterenol. This action of ouabain in reducing the refractoriness to stimulation by cyclic AMP is expressed, under certain conditions, as apparent potentiation by the glycoside of isoproterenol-stimulated ion fluxes (Figs. 1 to 3).

Acknowledgments—We thank Blanche Fors for preparing the manuscript and G. D. Aurbach for helpful discussions.

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