Effect of norepinephrine and hypertonicity on K influx and cyclic AMP in duck erythrocytes

FLOYD M. KREGENOW, DIANNE E. ROBBIE, AND JACK ORLOFF
Laboratory of Kidney and Electrolyte Metabolism, National Heart and Lung Institute, Bethesda, Maryland 20014

KREGENOW, FLOYD M., DIANNE E. ROBBIE, AND JACK ORLOFF. Effect of norepinephrine and hypertonicity on K influx and cyclic AMP in duck erythrocytes. Am. J. Physiol. 231(2): 306-312. 1976.—Cyclic adenosine 3',5'-monophosphate (cAMP) accumulation and cation transport were measured in duck erythrocytes after stimulation by norepinephrine (NE) or shrinkage induced by exposure to hypertonic media (S). Previously both NE and S were shown to initiate a similar transport process in this cell. NE elicited a rapid rise in cellular cAMP and 42K influx. Both effects were eliminated by propranolol. At concentrations of NE below 3 × 10⁻⁸ M (the concentration at which 42K influx saturates), there was good correlation between the magnitude of the permeability change and the increment in cAMP. In contrast, medium hypertonicity, at a level which stimulated K influx to the same extent as a near-maximal norepinephrine response, did not alter cAMP content. The data are discussed in terms of a model in which S and NE activate a final common transport pathway by different mechanisms, which in the case of S does not involve cAMP.

NOREPINEPHRINE, when added to an isotonic medium, initiates a rapid bidirectional movement of Na and K in duck erythrocytes (11). A similar change in transport is noted when these cells are incubated in a hypertonic medium in the absence of hormone (10). The mechanism responsible for the cation movements in both cases differs functionally and operationally from that of the classical cation pump.

Duck erythrocytes can vary their volume dynamically (9-11). A volume-controlling mechanism has been described that enables these cells to reestablish their initial volume after shrinkage induced by exposure to hypertonic media (10). The addition of norepinephrine to isotonic media also causes these cells to enlarge by a process resembling that initiated by hypertonicity (11, 17). The rapid bidirectional movement of Na and K mentioned above appears to be a basic component of this volume-regulatory response. The extracellular K concentration determines whether this rapid translocation serves as a simple cation exchanger, or, when cells enlarge, as the effector portion of the volume-controlling mechanism (9, 10). Raising the K concentration above 2.5 mM causes the transport process to increase asymmetrically; influx exceeds efflux, leading to a net accumulation of cations accompanied by anions and osmotically obligated water. The gain in water produces cell enlargement.

In view of the general hypothesis of Sutherland and co-workers (24) that a variety of hormones, including norepinephrine, exert their characteristic effects in receptor cells via intermediacy of cyclic adenosine 3',5'-monophosphate (cAMP), it was reasonable to inquire whether the norepinephrine-dependent transport process may also be regulated, in part, by catecholamine-induced changes in the cellular concentration of cAMP. Several lines of evidence implicate cAMP in the genesis of the norepinephrine-induced transport process. Avian erythrocyte membranes contain adenylate cyclase (9, 16, 20), and catecholamines raise the level of cAMP in avian erythrocytes (3, 7, 21). In addition, dibutyryl cAMP, when added to the bathing medium, mimics norepinephrine in causing cells to accumulate cations and enlarge, presumably by initiating the same transport process (17).

Marked similarities between the transport processes activated by norepinephrine and hypertonicity led us (11) to consider the possibility that cAMP might also serve as an intracellular mediator in the response to hypertonicity. Cyclic AMP has been implicated in another hypertonicity-induced transport process. Ripoche and co-workers (18, 19) have presented indirect evidence that the level of cAMP is important in the enhanced water movement across amphibian bladders that develops upon the addition of a hypertonic concentration of an impermeable solute to the serosal bathing medium (1, 5, 18, 25). Orloff and Handler (15) have shown previously that vasopressin induces water movement via cAMP in this tissue. To date, however, direct measurements of cAMP in shrunken hypertonicity-stimulated cells have not been reported. The present studies compare the relationship between cAMP levels and cation transport in duck erythrocytes stimulated by norepinephrine or hypertonicity. We have also inquired as to whether several incubation conditions that result in inhibition of the transport process also influence the cAMP content of the cells.

MATERIALS AND METHODS

The procedures for obtaining and incubating erythrocytes from the common Muscovy duck have been described previously (9, 17). When preincubated for 90 min in a norepinephrine-free “standard synthetic medium,”
1 liter of freshly drawn erythrocytes lose approximately 8 mmol of K and 2% of their cell water (wt/wt) before reaching a steady-state condition [called lower steady state (LSS); see ref. 17]. LSS cells were used in most studies. They have the following composition: (K), - 110 mM, (Na), ~ 5 mM, (Cl), ~ 51 mM, and cell water (wt/wt) 59%, in which cellular concentrations represent millimoles per that number of cells which in the LSS occupied 1 liter. After removal of plasma and the buffy coat by brief centrifugation at 2,000 x g, the cells were washed at least twice in appropriate solutions by centrifugation both before and after the 90-min preincubation. Routinely the standard synthetic medium served as the washing solution except in some of the studies described in Tables 4 and 5. Solutions used for washing were kept at room temperature except those used in the Table 4 studies which were kept at 4°C.

The standard synthetic medium was isotonic (osmolality 323 mosM) and had the following composition (mM): MgCl₂, 2; CaCl₂, 1; NaHCO₃, 28; Na₂HPO₄, 3.8; NaH₂PO₄, 3.8; NaCl 115; KCl 2.5; dextrose 20; and albumin (2.5 g/100 ml). Procedures for increasing the toxicity of this solution by addition of NaCl have been described previously (10). The composition of the low K solution which was isosmotic with the standard synthetic medium has also been described previously (12). To lower the concentration of K in this solution further, we dialyzed albumin prior to its incorporation into the medium against 20 vol of ice-cold solution (1 mM CaCl₂ and 2 mM MgCl₂) for 24 h.

Influx and efflux measurements were performed according to procedures described previously (9); influx values were routinely calculated by using equation 1 (9). However, the consistency of packed cells in very hypertonic solutions (see Fig. 3) precluded the thorough mixing and accurate pipetting necessary for analysis; we therefore approximated changes in K influx by noting the loss of radioactivity from the various bathing media over a 4-min interval. Under these conditions ⁴²K uptake is rapid and roughly linear, whereas the K concentration of the medium remains constant, so that cellular ⁴²K uptake and therefore K influx is, to a first approximation, proportional to the loss of radioactivity from the bathing medium. However, this modified procedure is not as accurate since duplicates differed by as much as 7 compared to 1% for the routine method. This degree of inaccuracy, however, is not sufficient to alter any of the conclusions drawn for the studies described in Fig. 3.

For the measurement of cyclic AMP, 500-µl aliquots of cell suspensions (HCT 10-20%) were transferred with vigorous mixing from the incubation flasks into polystyrene tubes containing 1 ml of ice-cold 8% trichloroacetic acid (TCA)¹ and 1 pmol of [³H]AMP to monitor recovery. The samples were extracted at 0°C for a minimum of 30 min with occasional mixing. (No further cAMP was released from the sample upon extraction for 1 h in 8% ice-cold TCA.) They were then centrifuged at 1,800 x g for 20 min, and the supernatant fluid freed of TCA by passage over a cation-exchange column (Bio-Rad AG50W-X8, 100–200 mesh, equilibrated with 0.1 N HCl) as described elsewhere (14, 22). The aqueous eluates (5 ml) containing cAMP were lyophilized and the residue dissolved in a volume of 150 µl distilled H₂O; 30-µl aliquots were assayed in triplicate by a modification of the protein-binding method of Gilman (8).

Overall recovery of [³H]cAMP ranged from 50 to 70% with approximately 15% trapped in the TCA insoluble pellet; approximately 80% of that applied to the cation-exchange column appeared in the 5-ml aqueous eluate. Cell-free blanks prepared from aliquots of each synthetic medium used were processed in parallel with the samples, and the resulting values accordingly subtracted; these blanks were essentially unaffected by additions or changes in medium composition.

Results are usually expressed as nanomoles cAMP per that number of cells which originally occupied 1 liter in the LSS (nmol cAMP/L₄₀°C). WC (17) used hemoglobin measurements on aliquots of both the cell suspension and packed cells to determine the hematocrit and utilized the hematocrit and hemoglobin measurements, in turn, to convert values from nanomoles cAMP per liter of cell suspension to nanomoles cAMP per L₄₀°C. The following formula was applied to these experimental conditions using LSS cells and isotonic media

\[
nmol \text{ cAMP/L}_{40^\circ C} = \frac{nmol \text{ cAMP/liter cell suspension} \times 100}{Hct}
\]

In experiments utilizing hypertonic media, the hemoglobin measurements were utilized in addition to correct for the shrunken cellular state according to the formula

\[
nmol \text{ cAMP/L}_{40^\circ C} = nmol \text{ cAMP/liter cell suspension} \times \frac{100}{Hct} \times \frac{Hb, \text{unit vol LSS cells}}{Hb, \text{unit vol shrunken cells}}
\]

These calculations are based on the assumption that none of the cAMP assayed is present in the medium, but rather is associated with cells. Evidence that this was indeed the case, at least under the experimental conditions used in this study, was obtained from preliminary experiments in which cells and medium were first rapidly separated (~1 min) at 4°C by centrifugation in a Brinkmann microcentrifuge and the medium then analyzed separately for cAMP. Cyclic AMP was also assayed in uncentrifuged aliquots, simultaneously withdrawn from the same flasks. Less than 5% of the cAMP present before centrifugation in a suspension of control, hypertonicity-stimulated (427 mosM), or nor-epinephrine-stimulated (2.5 x 10⁻⁴ M) cells could be found in the medium after centrifugation. Further-

¹ Samples extracted in boiling TCA occasionally but inconsistently yielded slightly higher values in the cAMP assay than cold-extracted duplicates. Where such a discrepancy was observed, the apparent increments due to boiling were similar for control and experimental cells. Within the sensitivity limitations of the assay, we also observed the same increments due to experimental manipulation in boiled samples as in cold-extracted samples. Because of this variability, the reduced isotope recovery found in boiled samples and the possibility of complications related to hydrolysis, as cited by Maguire (19), we have used cold TCA instead of boiling to terminate the reactions and extract cyclic AMP.
more, a quantity of exogenously added cAMP calculated to be present before centrifugation could be quantitatively recovered in the medium after centrifugation, indicating that during the separation procedure there was no appreciable breakdown of extracellular cAMP. In contrast, cAMP levels associated with the cellular fraction were substantially less (20-50%) than the expected value. The cAMP lost from the cellular fraction during this separation procedure may be destroyed through the continued activity of phosphodiesterase, in view of its reported activity even at 0°C in avian erythrocytes (3). Previous reports, wherein cAMP has been assayed in centrifuged cells (3, 7), must therefore be cautiously interpreted from a quantitative standpoint.

In experiments in which the cell volume (hence number of cells per milliliter) differed from the volume of LSS cells, cAMP content has been expressed as nanomoles cAMP per liter of cell water (nmol cAMP/liter H2O) in order that all data be comparable. Measurements of percent cell water (vol/vol) (17) were used to convert values from nanomoles cAMP per liter of cells to nanomoles cAMP per liter of cell water according to the formula

\[
\text{nmol/liter of cell H}_2\text{O} = \frac{\text{nmol cAMP/liter of cells}}{\% \text{ cell H}_2\text{O (vol/vol)}} \times 100
\]

Samples derived from control and norepinephrine- or hypertonicity-stimulated cells were assayed linearly with dilution over a twofold range and were degraded to approximately the same extent as known cAMP standards by bovine phosphodiesterase. Samples (20 \(\mu\)l) were treated in glass tubes with phosphodiesterase (1.33 mU) at 30°C for 1 h in a total volume of 80 \(\mu\)l containing 4 mM Tris-Cl (pH 8.0) and 1.5 mM MgCl₂. The reaction mixture was evaporated to dryness (1 h at 100°C) and the cAMP assay subsequently performed in the same tubes. However, it was necessary to use more concentrated suspensions of control and shrunken cells (HCT 50%) to determine reliably the effects of dilution and phosphodiesterase treatment because these treatments reduce the cAMP content in aliquots of standard (HCT 10% 20%) cell suspensions to levels which cannot be accurately assayed. The cAMP content of control and shrunken cells (nmol cAMP/LWCC) was unaffected by raising the hematocrit. The absence of interfering substances was further substantiated by the quantitative recovery of known amounts of cAMP added to cell extracts prior to the protein-binding assay.

\[^3\text{H}]\text{cAMP}\) (24.1 Ci/nmol) was purchased from New England Nuclear Corp. Norepinephrine was obtained from Calbiochem as \(\alpha\)-arterenol bitartrate hydrate, whereas furosemide and propranolol were a gift of Hoechst Pharmaceutical Company and Ayerst Laboratories, Inc., respectively. Norepinephrine and propranolol were added to each experimental flask after adding the cells, and propranolol preceded norepinephrine when the two were added together. Furosemide, on the other hand, was added as a solid before cell addition. The concentrations mentioned in the text have been calculated with the assumption that these agents remain in the extracellular phase.

**RESULTS**

Adding norepinephrine to LSS cells results in an immediate increase in cAMP content if the concentration of norepinephrine exceeds \(5 \times 10^{-9}\) M. Figure 1 shows the changes in cAMP content at two hormone concentrations over a 5-min interval. The higher concentration, as will be shown in Fig. 2, approaches the concentration at which the transport process saturates. Peak accumulation is already apparent at the beginning of the interval, the earliest time at which one can satisfactorily separate the cells for the initial determination in the flux measurement. Cyclic AMP levels change little throughout the remainder of the interval, although they fall slightly at the end.

Propranolol (\(10^{-4}\) M) completely inhibits the ability of norepinephrine to initiate the characteristic change in cation permeability (11), whereas it does not affect the response in hypertonic media (10). As shown in Table 1, \(10^{-4}\) M propranolol also blocks the immediate increase in cAMP content normally seen in the presence of norepinephrine. This finding is consistent with the view (23) that an elevated cAMP level is a requisite for hormone action.

To determine whether a correlation exists between the quantity of new cAMP accumulated and the magnitude of the increase in cation permeability, we examined the effect on each of increasing hormone concentrations. Figure 2 shows the results of experiments in which the hormone concentration was varied from 0 to \(5 \times 10^{-8}\) M. We chose to follow transport by measuring the norepinephrine-sensitive component of K influx.
The hormone-sensitive component of trans-
of the 3-min flux measurements and accurately re-
reflects, as shown in Fig. 1, cAMP levels throughout the
interval. The hormone-sensitive component of trans-
port saturates at a norepinephrine concentration of $3 \times 10^{-8}$ M. Below this concentration, the increments in
cAMP content and K transport correlate well, suggest-
ing that they are indeed causally related. In Fig. 2 this
correlation has been illustrated by representing the
norepinephrine-sensitive increment in both K trans-
port and cAMP as the percentage of that response
found with $3 \times 10^{-8}$ M norepinephrine, the concentra-
tion at which transport saturates. At lumwone concen-
trations that have a less than maximal effect on
permeability, the initial burst of cAMP seems to trig-
ger a proportionate permeability response.

The correlation between cAMP content and K trans-
port was not observed at higher hormone concentra-
tions. Hormone concentrations in excess of $3 \times 10^{-8}$ M
can further elevate cAMP content without causing an
additional increase in potassium permeability.

However, any of the other three monovalent cation
fluxes stimulated by norepinephrine (K efflux, Na
influx, and efflux) could also have been used. Cyclic
AMP was assayed in samples collected at the midpoint
of the 3-min flux measurements and accurately re-
fects, as shown in Fig. 1, cAMP levels throughout the
interval. The hormone-sensitive component of trans-
port was distributed between 2 groups of flasks: 1 group contained
tracer quantities of $^{88}$K and was used for flux studies, the other was
free of radioactivity. All flasks contained standard synthetic me-
edium. We added norepinephrine simultaneously, at concentrations
indicated on abscissa, to some of flasks from both groups. Samples
for influx measurements were removed at 1 and 4 min after addition
of norepinephrine; actual separation of cells by centrifugation was
complete 30 s later at 1.5 and 4.5 min. Samples for the cAMP assay
were removed from tracer-free flasks 3 min after addition of norepi-
ephrine, corresponding to midpoint of 3-min interval used to deter-
mine K influx. Hormone-dependent increments were obtained by
subtracting appropriate values for paired-control cells incubated
without norepinephrine. Mean control values (+ SE) were 0.6 ± 0.1
mmol K/$\text{L} \times \text{min}$ for K influx and 60 ± 5 nmol/L$_{\text{osc}}$ for cAMP.
Hormone-dependent increments have been normalized to those ob-
erved at $3 \times 10^{-8}$ M norepinephrine, the concentration at which K
influx is maximally stimulated. At this concentration, increments
were (+ SE): $3.10 \pm 0.73$ mmol K/$\text{L} \times \text{min}$ (K influx) and $214 \pm
34$ nmol/L$_{\text{osc}}$ (cAMP accumulation). Brackets represent SE ($n = 3$)
for both K influx and cAMP measurements.

### TABLE 1. Effect of propranolol on cAMP accumulation in norepinephrine-treated cells

<table>
<thead>
<tr>
<th></th>
<th>cAMP (nmol/L$_{\text{osc}}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No addition</td>
</tr>
<tr>
<td>Expt 1*</td>
<td>24</td>
</tr>
<tr>
<td>Expt 2</td>
<td>35</td>
</tr>
<tr>
<td>Expt 3*</td>
<td>163</td>
</tr>
</tbody>
</table>

Propranolol ($10^{-4}$ M) was added to the standard experimental
medium immediately after adding the cells and 10 s before the
addition of norepinephrine ($2.5 \times 10^{-8}$ M). Samples were removed for
analysis 3 min after the addition of norepinephrine. * The values
for control and hormone-stimulated cells illustrated by expt 1 and 3
represent respectively the lowest and highest found in all studies
reported in this paper involving this hormone concentration.

### TABLE 2. Effects of hypertonicity and norepinephrine on cAMP accumulation and K influx

<table>
<thead>
<tr>
<th>Medium ($[K]_{\text{osc}}$)</th>
<th>Δ cAMP, nM of cell H$_2$O</th>
<th>Δ K Influx, nmol K/L$_{\text{osc}}$ per 3 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotonic (323 mosM) + 2.5 x 10^{-8} M</td>
<td>341 ± 92</td>
<td>2.32 ± .40</td>
</tr>
<tr>
<td>Hypertonic (427 mosM)</td>
<td>3 ± 6*</td>
<td>2.45 ± .50</td>
</tr>
</tbody>
</table>

Values are means ± SE of 5 such experiments and represent
increments over paired control values determined in the isotonic
standard synthetic medium without norepinephrine. These mean
control values (+ SE) were: 122 ± 14 nM of cell H$_2$O for cyclic AMP,
and 45 ± .17 nmol K/L$_{\text{osc}}$ per 3 min for K influx. Experimental
protocol differs from that described in the legend to Fig. 2 only in
that: a) two experimental solutions were used, isotonic standard
synthetic medium and a hypertonic solution, and b) the addition of
cells to hypertonic solution coincided with addition of norepineph-
rine to isotonic solution. As described in legend of Fig. 2, aliquots for
flux measurements were withdrawn at 1 and 4 min, whereas aliquots
for cyclic AMP assay were taken at 3 min. * In a larger series of
experiments ($n = 12$), the mean ± SE was 2 ± 3.
content, it diminishes the accumulation of cAMP that develops in response to norepinephrine. The top half of Table 3 shows that at the osmolarity and norepinephrine concentration used in this study described in Table 2, we observed only 61% of the expected cAMP accumulation. The 61% increase, however, demonstrates that the enzyme system, or systems, responsible for accumulation of cAMP remains functional at this osmolarity, and that its inhibition is not the explanation for the lack of an effect of hypertonicity on basal cAMP levels. This reduction in norepinephrine-dependent cAMP accumulation may represent the initial appearance of a general inhibitory phenomenon which progresses as the medium tonicity increases and affects not only cAMP accumulation, but also norepinephrine- and hypertonicity-stimulated transport as well. Figure 3 shows that as the tonicity of the medium is increased beyond the levels employed in the previous study (Table 2), the permeability effects of both norepinephrine and hypertonicity are progressively inhibited. At high osmolarities, both permeability responses (Fig. 3) are almost completely blocked. And at these high osmolarities, as noted in the bottom half of Table 3, there is almost complete inhibition of cAMP accumulation as well.

Previous studies (10, 11) have demonstrated that both the norepinephrine- and hypertonicity-stimulated transport processes are identical. Moreover, additional studies (manuscript in preparation) provide more evidence for this similarity by showing that several changes in the composition of the bathing medium produce identical responses in each transport process. Replacing the extra- and intracellular anion (Cl with SO4 or adding the diuretic agent, furosemide, eliminates both responses. Similarly, omission of Na or K from the medium inhibits three of the four components of the rapid cation exchange. (The increases in K influx, K efflux, and Na influx are blocked, but the stimulation of Na efflux remains.) Table 4 demonstrates the inhibitory effect of two of the conditions (treatment with furosemide or removal of K).

> As expected, cells lose water and shrink when introduced to the various hypertonic media; the higher the osmolarity, the greater the initial shrinkage. In the most hypertonic solution used in this study, cells have an initial water content of 42% (wt/wt) compared with 59% in an isotonic medium.

### Table 3. Inhibitory effect of hypertonicity on norepinephrine-sensitive accumulation of cyclic AMP

<table>
<thead>
<tr>
<th>Medium</th>
<th>Norepinephrine-Sensitive Increment, nmol cAMP/liter cell H2O</th>
<th>Percent Isotonic Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotonic (323 mosM)</td>
<td>341 ± 76</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>Hypertonic (427 mosM)</td>
<td>307 ± 46</td>
<td>61 ± 4</td>
</tr>
<tr>
<td>Isotonic (323 mosM)</td>
<td>487 ± 91</td>
<td>12 ± 6</td>
</tr>
</tbody>
</table>

Values are means ± SE of four separate experiments, each of which duplicate determinations have been averaged. Cells from a single duck were added to flasks containing the isotonic standard synthetic medium and one of two hypertonic solutions. Shortly after addition of cells, norepinephrine (2.5 × 10^-6 M) was introduced to some of the flasks; 3 min later duplicate samples were removed for cAMP assay. Norepinephrine-dependent increments were obtained by subtracting values for paired control cells incubated in an otherwise identical medium, without norepinephrine.

### Table 4. Inhibitory effect of 1 mM furosemide and low K medium on transport

<table>
<thead>
<tr>
<th>Inhibitory Condition</th>
<th>Transport Stimulated by</th>
<th>% Inhibition of Norepinephrine or Hypertonic Sensitive Component of Transport</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K Influx</td>
<td>K Efflux</td>
</tr>
<tr>
<td>1 mM Furosemide</td>
<td>Norepinephrine, 10^-5 M</td>
<td>97 ± 2</td>
</tr>
<tr>
<td></td>
<td>Hypertonicity, 427 mosM</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>Low K Medium*</td>
<td>Norepinephrine, 10^-5 M</td>
<td>83 ± 4</td>
</tr>
<tr>
<td></td>
<td>Hypertonicity, 427 mosM</td>
<td>85 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 3). Cells were added to the various isotonic and hypertonic media and norepinephrine (1 × 10^-6 M) to some of the flasks at time 0. The sampling routine was similar to that described in the Fig. 2 legend for the experiments with norepinephrine and to that described in the Table 2 legend for experiments in hypertonic media. Flux measurements were therefore calculated for a 3-min interval. Cells were rapidly washed twice with ice-cold isotonic solutions prior to the flux determinations. Cells which were to be incubated in the low K media were washed with the isotonic low K medium rather than the standard synthetic medium. The norepinephrine-sensitive flux components were obtained by subtracting values for paired cells incubated in an otherwise identical medium (standard synthetic medium or low K medium), but without norepinephrine. Hypertonicity-sensitive flux components were obtained by subtracting values for paired cells incubated in the isotonic standard synthetic medium or low K medium. * During the experimental period, the [K], in experiments using low K media was less than 2 mM.

These alterations, therefore, apparently affect cellular components common to both transport processes. Since the response to hypertonicity does not appear to depend on changing levels of cAMP, they may affect a
component of the norepinephrine-stimulated system independent of the generation of cAMP. To test this thesis, we examined the effect of treatment with furosemide or removal of K on cAMP levels in cells incubated with norepinephrine (2.5 × 10⁶ M). The results in Table 5 show the inhibitory effect of each manipulation on the norepinephrine-stimulated cAMP accumulation in comparison with the response of paired control cells stimulated with the same concentration of hormone but in the standard medium. Treatment with furosemide or removal of K did not significantly reduce the increment in cAMP accumulation, even though transport was markedly inhibited. (See Table 4.)

## DISCUSSION

Cation transport in duck erythrocytes activated by norepinephrine appears to depend on an elevation in the level of cAMP, detectable as early as 1.5 min after introducing the hormone. This initial increment in cAMP content seems to determine the magnitude of the resulting change in cation permeability. That increased cAMP accumulation is necessary for this response is indicated by 1) the ability of propranolol to simultaneously prevent norepinephrine-induced transport and the elevation in cAMP content, and 2) the correlation between the increment in cAMP content and the cation transport at submaximal concentrations of the hormone. We wish to emphasize, however, that a causal connection between the accumulation of cAMP and the regulation of cation permeability has not been unequivocally demonstrated.

Cyclic AMP concentration does not increase in the initial stages of the hypertonicity-induced transport process. The physical or biochemical changes associated with cell shrinkage in a hypertonic medium seem to imitate the action of cAMP in the hormone-stimulated cell. This finding and the many similarities between the transport process initiated by norepinephrine, on the one hand, and hypertonicity, on the other, suggest a model in which the two stimuli (norepinephrine and hypertonicity) initiate events which are first dissimilar but eventually impinge upon a common pathway leading to cation translocation. According to the generally accepted scheme (23), the first events in the response to norepinephrine involve an association between the hormone and membrane-receptor elements as well as the series of biochemical reactions which permit accumulation of cAMP; in contrast, the first events in the response to hypertonicity must differ. Following the formation of cAMP, some or all of the events in both responses are similar, including the actual cation translocation process itself.

In this model those perturbations which alter transport but not the gain in cAMP (1 mM furosemide and the virtual elimination of medium K, Table 4) would act somewhere along the common terminal pathway. In addition, the K-sensitive element which renders the system capable of net cation translocation must also intervene along the common terminal pathway. Since, as mentioned earlier, a net accumulation of cations brought about by an increase in the medium K concentration is similar in both the norepinephrine- and hypertonicity-stimulated process, the latter does not invoke changing levels of cAMP.

From the model, we can conclude that the response to hypertonicity could prove useful in illuminating the more distal events in a cAMP-mediated, hormonally induced transport process. It offers an opportunity to examine those events that develop subsequent to the point at which cAMP elicits its effect. The evidence that hypertonicity elicits the same response without changing cAMP levels also raises the possibility that a messenger other than cAMP may be involved in the hypertonicity response (24).

Received for publication 7 May 1975.

## REFERENCES


