Effect of Bradykinin on Na-K-2Cl Cotransport and Bumetanide Binding in Aortic Endothelial Cells*

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Janet D. Klein$ and W. Charles O'Neill$§

From the Renal Division, Departments §Medicine and §Physiology, Emory University School of Medicine, Atlanta, Georgia 30322

Simultaneous measurements of potassium influx and binding of [3H]bumetanide were performed in endothelial cells cultured from bovine aortas to determine how bradykinin regulates Na-K-2Cl cotransport. [3H]Bumetanide displayed saturable binding and was displaced by low concentrations of unlabeled bumetanide. All three transported ions were required for binding and high concentrations of chloride inhibited binding, consistent with binding of bumetanide to the second chloride site of the transporter. Scatchard analysis of binding under maximal conditions (100 mM sodium, 30 mM potassium, 30 mM chloride) revealed a single class of binding sites with a binding constant of 112 nM and a density of 22 fmol/cm² or approximately 122,000 sites/cell. Na-K-2Cl cotransport, measured as bumetanide-sensitive potassium influx, was stimulated 118 ± 30% by bradykinin (p < 0.01) at physiologic ion concentrations. Stimulation was inhibited by increased potassium or decreased external chloride concentrations and was not seen in conditions required for maximal binding of bumetanide. Simultaneous measurement of the binding of tracer [3H]bumetanide and its inhibition of potassium influx in medium containing 10 mM potassium and 130 mM chloride revealed a turnover number for the cotransporter of 293 ± 68 s⁻¹ which increased to 687 ± 105 s⁻¹ with bradykinin (p < 0.001). There was no change in cell volume and only a 5.6 mM increase in intracellular sodium concentration associated with this stimulation. Bradykinin also increased the affinity of the cotransporter for bumetanide as indicated by a decrease in the Kᵦ for potassium influx from 461 ± 46 nM to 219 ± 10 nM (p < 0.005). Our results show that [3H]bumetanide can be used to quantify Na-K-2Cl cotransporter sites in aortic endothelial cells and to determine the mechanism by which cotransport is regulated. The stimulation of cotransport in aortic endothelial cells by bradykinin is due to an increase in the activity of existing transporters rather than to an increase in the number of transporters. This, together with the increased affinity for bumetanide, strongly suggests that a change in cotransporter structure is occurring in response to bradykinin.

Na-K-2Cl cotransport, first characterized in erythrocytes and Ehrlich ascites tumor cells (1, 2), is now known to occur in a variety of cells. Its function may relate to its ability to mediate net transport of salt across the cell membrane since it is prominent in epithelia that absorb or secrete chloride (3). In other cells, net salt transport via Na-K-2Cl cotransport has been shown to mediate regulatory volume increase (2) and thus may be important in the regulation of cell volume. Despite the ubiquity of Na-K-2Cl cotransport and its prominent role in certain tissues, little is known about its structure and regulation. Cells enriched in cotransport are often either difficult to study or are not suitable for regulatory studies. One exception to this is aortic endothelial cells, which exhibit a high level of Na-K-2Cl cotransport in that is regulated by vasoactive hormones (4, 5). Since these cells are easily cultured in large quantities, they provide an excellent opportunity to study the regulation of Na-K-2Cl cotransport.

Specific inhibition by low concentrations of bumetanide has been shown to be very useful in studying Na-K-2Cl cotransport, not only in the identification of Na-K-2Cl-mediated ion fluxes but also in the quantification of cotransporters. [3H]Bumetanide has been shown to bind specifically and saturably with high affinity to membranes from canine kidney (6) and to intact duck red cells (7), vascular smooth muscle cells (8), colonic adenocarcinoma cells (9), and HeLa cells (10). Recently we (11) and others (12) have demonstrated saturable binding of bumetanide to bovine aortic endothelial cells in culture. In this report we have used simultaneous measurement of potassium influx and [3H]bumetanide binding to determine the turnover number for the Na-K-2Cl cotransporter in these cells and to determine how cotransport is stimulated by bradykinin. Preliminary accounts of this work have been presented in abstract form (11, 13).

EXPERIMENTAL PROCEDURES

Endothelial Cells—Endothelial cells were obtained from bovine aorta and grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a humidified, 5% CO₂ environment. Confluent cells were passaged at 3-5-day intervals using trypsin-EDTA and a 1:5 dilution of cells. Studies were performed on confluent cultures between passages 4 and 16, usually 4-6 days after passage. Medium was changed every 3 days and 12-24 h before study.

Potassium Influx—Potassium influx was determined by measuring the uptake of tracer ⁴²Rb over 10 min at 37 °C. Preliminary studies revealed that influx was linear for at least 15 min. Uptake was terminated by aspiration of the medium followed by three washes with ice-cold 110 mM MgCl₂. Radioactivity was extracted from the cells with 5% trichloroacetic acid and counted by Cerenkov radiation. Potassium influx was calculated by multiplying the fraction of ⁴²Rb taken up by the amount of potassium in the assay medium. All assays were performed in 12-well cluster dishes (22-mm diameter). For assays in physiologic ion conditions the medium consisted of the salts contained in Dulbecco's modified Eagle's medium with HEPES¹

¹The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
replacing HCO_3. The concentrations were as follows: Na_2HPO_4, 1 mm; KCl, 1.8 mm; KCl, 5.56 mm; MgSO_4, 0.5 mm; NaCl, 11.6 mm; Na-HEPES, 26 mm; pH 7.4. All other assays were performed in bumetanide-binding medium as described below.

**Bumetanide Binding** Cells were incubated in 120 mM sodium, 30 mM potassium, 30 mM chloride, 100 mM methanesulfonate, and 20 mM HEPES (pH 7.4) containing [3H]bumetanide, with increasing amounts of unlabeled bumetanide (0–735 nM, and 50 mM to determine nonspecific binding) for 20 min at 37 °C. Unless indicated otherwise, the concentration of [3H]bumetanide was 25 nM. Assays were terminated as described above, and radioactivity was determined by liquid scintillation counting.

**Sodium Content**—Cells were incubated for 15 min at 37 °C in HEPES-buffered Earle's salts without ouabain in the presence and absence of 10 μM bradykinin. After incubation, cells were thoroughly washed three times with 2-ml portions of ice-cold 110 mM MgCl_2. Cells were treated with 1.5 ml of 0.02% Acatonox for 0.5 h at room temperature, and the sodium concentration was determined by atomic absorption spectrophotometry.

**Cell Volume**—Cell water, as an index of cell volume, was determined by measuring the uptake of [14C]urea. Cells were incubated in HEPES-buffered Earle's salts with and without bradykinin for 10 min at 37 °C. Buffers were then replaced with identical buffers containing [14C]urea. After a 5-min further incubation, buffers were removed, and the cells were placed on ice and washed three times, rapidly, with 2-ml portions of HEPES-buffered Earle's salts solution. Counts were liberated with 5% trichloroacetic acid, and radioactivity was determined by liquid scintillation counting.

**Materials**—[3H]Bumetanide, synthesized in 1984 with a specific activity of 21 Ci/mmol (6), was obtained from Dr. J. Russell, University of Texas Medical Branch, Galveston. Unlabeled bumetanide was obtained from Dr. P. Sorter, Hoffman-La Roche, Nutley, NJ. 86Rb and [3H]bumetanide were purchased from Du Pont-New England Nuclear. Dulbecco's modified Eagle's medium was purchased from Hazelton Biologies, Lexena, KS, and fetal bovine serum was purchased from Gibco. All other reagents were obtained from Sigma.

**RESULTS**

Initial bumetanide binding studies were performed in a high potassium, low chloride medium (30 mM potassium, 30 mM chloride) as this was found to maximize binding in duck red cells (7). Preliminary studies indicated that specific binding of tracer [3H]bumetanide (25 nM) was rapid and complete by 20 min at 37 °C, and this incubation time was used for all assays. Specific binding of bumetanide was saturable with increasing concentrations of bumetanide and corresponded closely with the inhibition of potassium influx (Fig. 1A). Nonspecific binding, the binding of trace [3H]bumetanide in the presence of excess (50 μM) unlabeled bumetanide, was 59 ± 3% of total [3H]bumetanide bound in the absence of unlabeled bumetanide (n = 18). A Scatchard analysis of the binding in Fig. 1A was linear (Fig. 1B), indicating that only one class of binding sites was present. Data from five such experiments yielded a dissociation constant of 112 ± 23 nM and a maximal binding of 85 ± 15 fmol/well, corresponding to approximately 122 000 sites/cell.

Specific binding of bumetanide required the presence of all three transported ions in the external medium (Fig. 2). There was essentially no binding when potassium was replaced with choline (with sodium and chloride concentrations maintained at 120 and 30 mM, respectively), and maximal binding was achieved at a concentration of 30 mM potassium with a K_m of approximately 4 mM. Substitution of sodium with choline (with potassium and chloride concentrations remaining at 30 mM) also indicated an absolute requirement for sodium with a K_m of approximately 50 mM. When chloride was replaced with CH_3SO_3 (potassium and sodium concentrations remaining at 30 and 120 mM, respectively) binding again was minimal. Binding was stimulated by low concentrations of chloride, reaching a maximum at approximately 30 mM chloride and declining with higher chloride concentrations. This requirement for sodium, potassium, and chloride ions with inhibition by high chloride concentrations is consistent with the original studies of bumetanide binding to kidney membranes (6) and duck red cells (7).

![Fig. 1. Bumetanide binding to cultured bovine aortic endothelial cells and inhibition of potassium influx by bumetanide.](image)

Exposure of endothelial cells to bradykinin at physiologic ion concentrations (Earle's salts) increased bumetanide-sensitive potassium influx 118 ± 30% (n = 4, p < 0.05) consistent with previous reports (4, 5). However, when these studies were repeated in 30 mM potassium, 30 mM chloride, and 120 mM

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8 J. Klein and W. C. O'Neill, manuscript in preparation.
Effect of Bradykinin on Cotransport

Table I

Determination of cotransporter turnover

| Addition          | [3H]Bumetanide bound | Potassium influx |
|-------------------|----------------------|-----------------|
|                   | fmol/well            | nmol/well/h     |
|                   |                      |                 |
| None              | 98 ± 4.7             |                 |
| [3H]Bumetanide    | 24.2 ± 1.31          | 87 ± 2.6        |
| [3H]Bumetanide + unlabeled bumetanide | 17.9 ± 0.3 | 22 ± 3.2 |
| +Bradykinin       |                      |                 |
| None              | 236 ± 15.4           |                 |
| [H]Bumetanide     | 38.7 ± 0.41          | 160 ± 2.4       |
| [H]Bumetanide + unlabeled bumetanide | 19.3 ± 0.59 | 19 ± 0.5 |

* Concentration of [3H]bumetanide was 25 nM.
* Concentration of unlabeled bumetanide was 50 pM.
* Concentration of bradykinin was 10 pM.

Table II

Effect of bradykinin on cotransporter turnover

Results are the mean ± S.E. of nine experiments, each performed in duplicate.

|                | Bumetanide-sensitive potassium influx | Potassium influx inhibited by [H]bumetanide | Turnover |
|----------------|-------------------------------------|-------------------------------------------|----------|
|                | [H]Bumetanide                       | %                                         | s⁻¹      |
|                | fmol/well/h                          | fmol/well/h                               |          |
| Bradykinin     | 104.8 ± 24                          | 12.9 ± 2.7                                | 11.1 ± 2.2 | 295 ± 69 |
|                 |                                    | %                                         | s⁻¹      |
|                 |                                    | 17.9 ± 2.7                                | 25.4 ± 2.8 | 687 ± 105 |
| Bradykinin*    | 171.7 ± 31                          | 12.9 ± 2.7                                | 11.1 ± 2.2 | 295 ± 69 |
|                 |                                    | %                                         | s⁻¹      |
|                 |                                    | 17.9 ± 2.7                                | 25.4 ± 2.8 | 687 ± 105 |
| p value        | <0.000                              | 0.02                                      | 0.001    | 0.001    |

* Concentration of [H]bumetanide was 25 nM.
* Significantly different from 0 (p < 0.001).

sodium to obtain maximal bumetanide binding, there was no stimulation of potassium influx by bradykinin. Subsequent studies were therefore performed in 120 mM sodium, 10 mM potassium, 130 mM chloride, a medium that produced measurable binding without completely inhibiting the effect of bradykinin. Scatchard analysis of the binding of [H]bumetanide under these conditions yielded errors large enough to prevent distinction between changes in $B_{max}$ and changes in $K_D$.

The effect of bradykinin on cotransporter turnover was therefore determined directly by measuring potassium influx and bumetanide binding simultaneously in the same cells. The data from a typical experiment are shown in Table I. Cells were preincubated for 20 min with no bumetanide, with tracer [H]bumetanide alone, or with tracer [H]bumetanide and an excess of unlabeled bumetanide. 86Rb was then added to each well for 10 min, and the amounts of 86Rb taken up and [H]bumetanide bound were determined by dual channel scintillation counting. The inhibition of potassium influx by tracer [H]bumetanide, although small, was highly significant and was seen in each experiment. Use of higher concentrations of [H]bumetanide was precluded by the proportionate increases in nonspecific binding which introduced substantial error in the determination of specific binding. The potassium influx that was inhibited by the tracer [H]bumetanide was divided by the amount of bound [H]bumetanide that was displaced by excess unlabeled bumetanide to derive the turnover number for Na-K-2Cl cotransport. The combined results of nine experiments are shown in Table II. For control cells incubated without bradykinin this value was 293 ± 69 s⁻¹. This compares favorably with the turnover number of 344 ± 79 s⁻¹ obtained by dividing the total bumetanide-sensitive rubidium influx from turnover experiments (Table II) by the maximum number of binding sites determined by Scatchard analysis of bumetanide binding under maximal binding conditions (Fig. 1B).

Bumetanide-sensitive potassium influx was 75 ± 21% higher in the cells exposed to bradykinin, and this was associated with a 134 ± 46% increase in cotransporter turnover. A small but significant increase in the specific binding of tracer [H]bumetanide resulted from exposure of the cells to bradykinin. An additional finding was that in the presence of bradykinin, tracer [H]bumetanide inhibited a greater proportion of the total bumetanide-sensitive potassium influx (25 ± 3% versus 11 ± 2% in control cells). This apparent increase in the affinity of the cotransporter for bumetanide was confirmed by subsequent measurements of potassium influx over a range of bumetanide concentrations (Fig. 3). A
Kidney membranes (6), duck red cells (7), and endothelial concentrations of chloride were inhibitory. This indicates that consistent with results obtained by other investigators in bumetanide is binding to the second chloride site of the cotransporters was provided by the ionic requirements for cotransport by bumetanide and which is similar to values reported for binding to other cells (7-10). Further cotransport was measured under identical conditions. Simultaneous measurement of $[^3H]$bumetanide binding and potassium influx in this study yielded turnover for bumetanide doubled yet binding of tracer $[^3H]$bumetanide to intracellular cotransporters. Furthermore, the normally low intracellular sodium concentration should lower the affinity of cotransporters for bumetanide yet there was no evidence on Scatchard analysis for two binding sites for bumetanide. Lastly, our results are comparable to those obtained previously in aortic endothelial cells that were hypoosmotically lysed prior to measurement of bound $[^3H]$bumetanide (12).

The high affinity and ionic conditions of binding and the linearity of the Scatchard analyses provide strong evidence for 1:1 stoichiometric binding of bumetanide to the cotransporter, indicating that binding can be used to quantitate cotransporters. Such quantification in conjunction with ion transport measurements has yielded turnover numbers for Na-K-2Cl cotransport of 300 s$^{-1}$ in aortic endothelial cells (12), 70 s$^{-1}$ in vascular smooth muscle cells (8), 60 s$^{-1}$ in HT29 colonic adenocarcinoma cells (9), 700 s$^{-1}$ in HeLa cells (10), and 4000 s$^{-1}$ in duck red cells (7). In none of these studies, however, were ion transport and bumetanide binding measured simultaneously, and only in duck red cells were they measured under identical conditions. Simultaneous measurement of $[^3H]$bumetanide binding and potassium influx in this study yielded a turnover of 293 s$^{-1}$. Although there can be large errors associated with the method used to determine turnover, the error in determining $B_{max}$ from Scatchard analyses can be equally large. The actual error in determining turnover was small, and further evidence for the validity of this method was its close agreement with the value for turnover calculated using the $B_{max}$ derived from the Scatchard analyses of binding under maximal conditions, and its close agreement with the value of 300 s$^{-1}$ obtained previously in aortic endothelial cells (12).

Our data demonstrate that stimulation of Na-K-2Cl cotransport by bradykinin is associated with an increase in turnover number. Although cotransporter number could not be measured directly, the fact that the increase in turnover exceeded the increase in overall cotransport implies that cotransporter number did not increase and may have actually declined. The latter is also suggested by the fact that affinity for bumetanide doubled yet binding of tracer $[^3H]$bumetanide increased only 65%. One possible explanation for our results could be consistent with translocation of intracellular cotransporters.

**Table III**

| Medium         | Cell water µl/well | [Na$^+$] mmol/well |
|----------------|--------------------|--------------------|
| −Bradykinin    | 0.401 ± 0.018      | 7.0 ± 0.5          |
| +Bradykinin    | 0.385 ± 0.017      | 8.9 ± 0.6          |

DISCUSSION

Aortic endothelial cells in culture exhibited saturable, high affinity binding of $[^3H]$bumetanide with a dissociation constant of 112 nM which corresponded well with the inhibition of Na-K-2Cl cotransport by bumetanide and which is similar to values reported for binding to other cells (7-10). Further evidence that bumetanide was binding directly to Na-K-2Cl cotransporters was provided by the ionic requirements for binding. All three transported ions were required, and high concentrations of chloride were inhibitory. This indicates that bumetanide is binding to the second chloride site of the transporter as indicated previously by kinetic data (14) and consistent with results obtained by other investigators in kidney membranes (6), duck red cells (7), and endothelial cells (12). Detailed Scatchard analyses revealed only one class of binding sites for bumetanide and indicated the presence of approximately 122,000 binding sites/cell, in the same range as values reported previously in these cells (12), vascular smooth muscle cells (8), and HeLa cells (10), but far greater than the number obtained in duck red cells (7). The significantly higher quantity of binding sites (2 X 10$^6$) reported in Ehrlich ascites cells (15) was associated with a much lower affinity and may represent binding to a Na-Cl cotransporter rather than the Na-K-2Cl cotransporter.

Because it is lipophilic, bumetanide could enter cells and bind to internal cotransporters that are not available for transporting ions. In previous studies, cells have been lysed after incubation with $[^3H]$bumetanide to release intracellular bumetanide so that only bumetanide bound to the plasma membrane was measured (7, 8, 12). To what extent intracellular contents are removed by this treatment, however, is unclear. Our results, with binding performed and measured in intact cells, do not indicate the presence of an intracellular pool of cotransporters. Intracellular receptors would be exposed to an ionic milieu that is not influenced directly by extracellular ion concentrations. The absence of specific bumetanide binding in low sodium, low potassium, or low chloride extracellular medium argues against significant binding of bumetanide to intracellular cotransporters.

**Fig. 3. The effect of bradykinin on cotransport of potassium influx.** Endothelial cells in 12-well cluster dishes were preincubated, in the presence and absence of 10 µM bradykinin with buffer (10 mM potassium, 120 mM sodium, 130 mM chloride, 20 mM choline, 20 mM HEPES, pH 7.4) containing bumetanide varied from 0 to 735 mM. Non-specific potassium influx was determined by incubating cells with buffer containing 50 µM bumetanide. All wells contained 0.1 mM ouabain. After preincubating for 5 min at 37°C, $^3$Rb was added, and cells were incubated for 10 more min. Influx was terminated by washing three times with iced 110 mM MgCl$_2$. Radioactivity was determined as described previously. Values are the means ± S.E. of nine separate determinations.

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The high affinity and ionic conditions of binding and the linearity of the Scatchard analyses provide strong evidence for 1:1 stoichiometric binding of bumetanide to the cotransporter, indicating that binding can be used to quantitate cotransporters. Such quantification in conjunction with ion transport measurements has yielded turnover numbers for Na-K-2Cl cotransport of 300 s$^{-1}$ in aortic endothelial cells (12), 70 s$^{-1}$ in vascular smooth muscle cells (8), 60 s$^{-1}$ in HT29 colonic adenocarcinoma cells (9), 700 s$^{-1}$ in HeLa cells (10), and 4000 s$^{-1}$ in duck red cells (7). In none of these studies, however, were ion transport and bumetanide binding measured simultaneously, and only in duck red cells were they measured under identical conditions. Simultaneous measurement of $[^3H]$bumetanide binding and potassium influx in this study yielded a turnover of 293 s$^{-1}$. Although there can be large errors associated with the method used to determine turnover, the error in determining $B_{max}$ from Scatchard analyses can be equally large. The actual error in determining turnover was small, and further evidence for the validity of this method was its close agreement with the value for turnover calculated using the $B_{max}$ derived from the Scatchard analyses of binding under maximal conditions, and its close agreement with the value of 300 s$^{-1}$ obtained previously in aortic endothelial cells (12).

Our data demonstrate that stimulation of Na-K-2Cl cotransport by bradykinin is associated with an increase in turnover number. Although cotransporter number could not be measured directly, the fact that the increase in turnover exceeded the increase in overall cotransport implies that cotransporter number did not increase and may have actually declined. The latter is also suggested by the fact that affinity for bumetanide doubled yet binding of tracer $[^3H]$bumetanide increased only 65%. One possible explanation for our results could be consistent with translocation of intracellular cotransporters.
porters that bind bumetanide but cannot transport ions, to
the plasma membrane where they can transport ions.

The finding that regulation of Na-K-2Cl cotransport can
affect its affinity for bumetanide in addition to altering its
activity must be taken into account when using the binding
of bumetanide to quantitate cotransporters. Unless concen-
trations of [3H]bumetanide substantially greater than satu-
rating concentrations are used, changes in binding measured
with just one concentration of [3H]bumetanide could reflect
a change in affinity and not necessarily a change in trans-
porter number. Thus, previous findings that cAMP, cGMP,
norepinephrine, phorbol ester, and atriopeptin decreased
binding to aortic endothelial cells (12), all determined with a
single concentration of [3H]bumetanide, may not necessarily
indicate changes in the number of cotransporters. The in-
crease in maximal binding of bumetanide to duck red cells
after exposure to hypertonicity or norepinephrine cannot be
explained by changes in affinity and indicate an increase in
cotransporter number (7). Scatchard analyses of bumetanide
binding were performed in HT29 colonic adenocarcinoma
cells and the decrease in Na-K-2Cl cotransport in response
to phorbol esters was ascribed to a decrease in the number of
cotransporters. This result, however, was accompanied by a
decrease in turnover number, particularly after prolonged
exposure to phorbol esters, suggesting a second mechanism
for phorbol ester-induced decrease in cotransport (10).

Although there was a small increase in intracellular sodium
after incubation with bradykinin, it is unlikely that the in-
creased turnover of Na-K-2Cl cotransport was due to a change
in internal substrate ions. Studies in red cells have not pro-
vided evidence for trans effects of substrate ions (10). In red
cells the cotransporter also mediates a potassium-potassium
exchange that is stimulated by internal sodium (16), but no
such exchange has been found in aortic endothelial cells, as
indicated by the lack of chloride-dependent potassium influx
in the absence of external sodium (4, 5), including cells treated
with bradykinin (4). Measurement of cell water revealed a
small decrease that, although not significant, may be of im-
portance since cell shrinkage is a potent stimulus for Na-K-
2Cl cotransport in endothelial cells.3

In summary, our results demonstrate that the stimulation
of Na-K-2Cl cotransport by bradykinin occurs through a
change in the turnover number of cotransporters which is
associated with an alteration in the affinity for bumetanide.
In the absence of evidence for substrate effects, this suggests
that bradykinin is inducing a structural change in the Na-K-
2Cl cotransporter. Whether this is the primary mechanism
for regulation of this transporter and how it occurs remain to
be determined.

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REFERENCES
1. Haas, M., Schmidt, W., and McManus, T. (1982) J. Gen. Physiol.
80, 125–147
2. Geck, P., and Heinz, E. (1986) J. Membr. Biol. 91, 97–106
3. Kinne, R. (1984) Ann. N. Y. Acad. Sci. 435, 39–47
4. Brock, T., Brugnara, C., Canessa, M., and Gimbrone, M. (1986)
Am. J. Physiol. 250, (Cell Physiol. 19) C88–C95
5. O’Donnell, M. (1983) Am. J. Physiol. 257, (Cell Physiol. 26)
C36–C44
6. Forbush, B., III, and Palfrey, H. C. (1983) J. Biol. Chem. 258,
11787–11792
7. Haas, M., and Forbush, B., III (1986) J. Biol. Chem. 261, 8434–
8441
8. O’Donnell, M., and Owen, N. (1988) Am. J. Physiol. 255, (Cell
Physiol. 24) C169–C180
9. Franklin, C. C., Turner, J. T., and Kim, H. D. (1989) J. Biol.
Chem. 264, 6667–6673
10. Kort, J., and Koch, G. (1989) J. Cell. Physiol. 141, 181–190
11. O’Neill, W. C., and Coker, S. (1989) FASEB J. 3, 304 (abstr.)
12. O’Donnell, M. E. (1989) J. Biol. Chem. 264, 20326–20330
13. Klein, J., and O’Neill, W. C. (1990) Clin. Res. 380, 442 (abstr.)
14. Haas, M., and McManus, T. (1983) Am. J. Physiol. 245, (Cell
Physiol. 14) C235–C240
15. Hoffmann, E., Schiodt, M., and Dunham, P. (1986) Am. J.
Physiol. 250, (Cell Physiol. 19) C688–C693
16. Duhm, J. (1987) J. Membr. Biol. 98, 15–32