Activation of the Na-K-Cl Cotransporter NKCC1 Detected with a Phospho-specific Antibody*

Received for publication, June 24, 2002, and in revised form, July 16, 2002
Published, JBC Papers in Press, July 26, 2002, DOI 10.1074/jbc.M206294200

Andreas W. Flemmer‡, Ignacio Giménez, Brian F. X. Dowd, Rachel B. Darman§, and Biff Forbush¶

From the Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06520 and the Mount Desert Island Biological Laboratory, Salisbury Cove, Maine 04672

The Na-K-Cl cotransporter NKCC1 is activated by phosphorylation of a regulatory domain in its N terminus. In the accompanying paper (Darman, R. B., and Forbush, B. (2002) J. Biol. Chem. 277, 37542–37550), we identify three phosphothreonines important in this process. Using a phospho-specific antibody (anti-phospho-NKCC1 antibody R5) raised against a diphasophopeptide containing Thr212 and Thr217 of human NKCC1, we were readily able to monitor the cotransporter activation state. In 32P phosphorylation experiments with rectal gland tubules, we show that the R5 antibody signal is proportional to the amount of 32P incorporated into NKCC1; and in experiments with NKCC1-transfected HEK-293 cells, we demonstrate that R5-detected phosphorylation directly mirrors functional activation. Immunofluorescence analysis of shark rectal gland shows activation-dependent R5 antibody staining along the basolateral membrane. In perfused rat parotid glands, isoproterenol induced staining of both acinar and ductal cells along the basolateral membrane. Isoproterenol also induced basolateral staining of the epithelial cells in rat trachea, whereas basal cells in the subepithelial tissue displayed heavy, non-polarized staining of the cell membrane. In rat colon, agonist stimulation induced staining along the basolateral membrane of crypt cells. These data provide direct evidence that NKCC1 regulation in these tissues, and they further link phosphorylation of NKCC1 with its activation in transfected cells and native tissue. The high conservation of the regulatory threonine residues among NKCC1, NKCC2, and NCC family members, together with the fact the tissues from divergent vertebrate species exhibit similar R5-binding profiles, lends further support to the role of this regulatory locus in vivo.

NKCC1, the secretory or housekeeping isoform of the Na-K-Cl cotransporter, is expressed in most cell types, aiding in the regulation of cell volume. In polarized cells of secretory epithelia, NKCC1 is heavily expressed along the basolateral membrane, activated in response to secretagogues, and paramount for the transepithelial secretion of Cl− and water (2). NKCC1-mediated Cl− secretion has been well documented in rat parotid gland (3, 4), shark rectal gland (5), rat colon (6), and dog trachea (7). At least in shark rectal gland, the evidence is consistent with an indirect activation of NKCC1 upon agonist stimulation: secretagogues cause a drop in intracellular [Cl−] and volume via protein kinase A-mediated phosphorylation of apical chloride channels; in turn, low intracellular [Cl−] and low cell volume provide activation stimuli for the currently unidentified NKCC1 kinase(s) (2, 8).

Our laboratory (9) and others (10) have linked the phosphorylation of the intracellular N-terminal domain with NKCC1 activation. In the accompanying paper, Darman and Forbush (1) describe the phosphorylation of three residues in this regulatory domain, of which Thr184 and Thr189 are necessary for maximal sNKCC11 activation. Thr189 in particular is demonstrated as being essential for sNKCC1 up-regulation. The three phosphoacceptor sites reside in a 30-amino acid region of the N terminus that exhibits 80% homology between sodium-coupled cation chloride cotransporter isoforms and 97% homology within NKCC1 proteins in species ranging from shark to human (shark Thr184/Thr189 correspond to human Thr212/Thr217). This region of the N terminus also contains a protein phosphatase-1-binding site (RVXP), which is conserved across divergent species and thought to mediate regulated dephosphorylation (11).

To date, most studies of NKCC regulation have been conducted in isolated tissue preparations, cell cultures, or cell-free systems utilizing [H]benzmetanide binding, 32P incorporation, or isotopic uptake. Although these studies have provided a wealth of information at the cellular and molecular levels, little is known about the phosphorylation state of NKCC in native tissue. To address this issue, we have developed a phospho-specific polyclonal antibody (anti-phospho-NKCC1 antibody R5) raised against the in vitro phosphorylated peptide corresponding to the regulatory domain.

In this report, we demonstrate R5 specificity and sensitivity in recognizing the phosphorylated conserved threonines. We determine a positive correlation between NKCC1 activation and Thr184/Thr189 phosphorylation in sNKCC1-transfected HEK-293 cells using the R5 antibody. We also investigate in vivo NKCC1 activity and address the universality of this regulatory domain by examining R5 immunohistographs of shark rectal gland and several rat secretory tissues. These data provide a critical link between molecular regulation studies and the activation profile of NKCC1 in vivo.

* This work was supported by National Institutes of Health Grant DK47661. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.
‡ Present address: University Children’s Hospital Munich, Lindwurmstrasse 4, 80337 Munich, Germany.
§ Present address: Dept. of Molecular Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215.
¶ Present address: Dept. of Cellular and Molecular Physiology, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06520-0826. Tel.: 203-785-4068; Fax: 203-785-6834; E-mail: biff.forbush@yale.edu.

1 The abbreviations used are: sNKCC1, shark NKCC1; hNKCC1, human NKCC1; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine.
phosphorylated Tyr 208/c141 non-phosphorylated (nicity of the Tyrh208 procedures. Fig. 1 shows that the R5 antibody had a slightly higher peptide was subsequently synthesized for analytic and purification produced, the second aspartate in this peptide was found to have been purified antibody was used for immunofluorescence analyses.

ELISA—For determination of antibody affinity and phospho-specificity by ELISA, phosphorylated and non-phosphorylated peptides were covalently coupled to N-oxysuccinimide binding plates (DNA-Bind, Costar Corp.). Plates were blocked with 20 μl ethanolamine, pH 8.2; washed twice with wash buffer (PBS, 1% bovine serum albumin, and 0.1% Triton X-100); and blocked with 7% milk in PBS in 0.1% Triton X-100. After washing once with wash buffer and sequential incubations with serially diluted sera and horseradish peroxidase-conjugated anti-rabbit IgG antibody, the optical density was measured with a spectrophotometer using o-phenylenediamine as a substrate.

For Western blotting, samples were subjected to Tricine/SDS gel electrophoresis (7.5 or 10%) and transferred to polyvinylidene difluoride membrane. Rabbit Farms, Canadensis, PA) was raised against this peptide coupled to maleimidoaniline acid-N-hydroxysuccinimide-activated keyhole limpet hemocyanin (Sigma) using standard procedures. The anti-phospho-NKCC1 antibody will be referred to as R5 for the remainder of the paper. For immunofluorescence studies, R5 was reactivity enables a sensitive analysis of the phosphorylation state of the material and diluted into SDS sample buffer. (b) Alternatively, cells were lysed in 1% SDS and subsequently diluted into sample buffer for Western blotting or dot blotting. For reasons that are yet unclear, we consistently saw a higher level of background R5 signal on dot blots (see Figs. 5 and 6) and gels (data not shown) of samples stopped with H3PO4/SDS stop medium compared with that seen when cells were harvested in 1% Triton X-100 (see Figs. 3 and 4).

**NKKC1** 32P Incorporation in Shark Rectal Gland Tubules—Shark rectal gland tubule isolation and 32P incorporation were conducted as previously described (1, 15). At specific time points following appropriate incubations, cells were solubilized by one of two procedures. (a) Cells were lysed either in 20 mM HEPES, pH 7.2 (see Fig. 2), or in phosphate buffer (PBS, 1% bovine serum albumin, and 0.1% Triton X-100) and subsequently diluted into sample buffer for Western blotting or dot blotting.

**Tissue Perfusion and Fixation**—Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were anesthetized via intraperitoneal injection of sodium pentobarbital (50 mg/kg of body weight). Each animal was kept warm using a heated plate, and body temperature was monitored with a rectal thermometer. In most experiments, a polyvinyl catheter was introduced into the appropriate artery (common carotid for the parotid gland, thoracic for the trachea, and mesenteric for the colon), and the animals were infused with prewarmed Krebs-Henseleit bicarbonate solution (140 mM NaCl, 5.4 mM KCl, 2.4 mM CaCl2, 124 mM NaHCO3, 21 mM HCO3, 2.4 mM HPO4, 0.6 mM H3PO4, pH 7.4; 300 mosmol/liter adjusted with mannitol). Agonists were added to the perfusate to stimulate transport activity as described in the figure legends for each particular tissue. For Western blot analysis of parotid tissue, the gland was removed and snap-frozen in liquid nitrogen. Frozen, pestle-ground tissue was placed in boiling SDS sample buffer.

For organ fixation, periodate/lysine/paraformaldehyde fixative was perfused through the same catheter for 5–15 min (16). Tissues were removed from the animal and further fixed for 2–4 h at 4 °C. To achieve best fixation results, the trachea was also filled with fixative at 20 cm of H2O. Shark rectal gland isolates were frozen, and each

**Fig. 1.** ELISA analysis of R5 antibodies. Phosphorylated (●) and non-phosphorylated (▼) Tyr208–Arg223–Lys peptides, the modified phosphorylated Tyr208–Arg223–Lys peptide (○), and the water control (▲) were covalently coupled to N-oxysuccinimide plates and probed with various dilutions of R5 serum and affinity-purified R5 antibody as shown. Values represent means and range of duplicate determinations of the optical density of the ELISA reaction product.
sNKCC1 expression; lower panel middle panel tants and wild-type sNKCC1- and vector-transfected HEK-293 cells; panel the accompanying paper (1) (PKA-1 denotes186GHNT digitally corrected accordingly. The sNKCC1 mutants are described in correct for this variation, individual lanes in all three panels have been /H11011 showed identical volumes of solubilized protein representing 6 mm2 of tissue /H11002/Immunofluorescence—Small sections of fixed tissues were cryoprotected with 50% polyvinylpyrrolidone in 2.3 M sucrose overnight at 4 °C. Tissue sections were then mounted on aluminum nails and snap-frozen in liquid nitrogen. Semi-thin 0.5–1 μm sections were cut using a Reichert Ultracut E ultramicrotome fitted with a FC-4E cryotachment. Sections were placed on coated slides (Superfrost Plus, Erie Scientific, Portsmouth, NH) and washed with PBS prior to antigen retrieval by incubating the tissues with 1% SDS for 5 min. After quenching with NH4Cl for 15 min, sections were blocked with 0.1% bovine serum albumin and 10% goat serum in PBS, incubated for 1–2 h with the primary antibody, washed, and incubated with the appropriate secondary antibody conjugated to fluorescein isothiocyanate or Alexa dyes (Molecular Probes, Inc.). Sections were washed three times with PBS and mounted with Vectashield (Vector Laboratories, Inc., Burlingame, CA), and micrographs were taken with a Zeiss Axioshot mi-
croscope on Kodak Tmax 100 film (Eastman Kodak Co.) or using an Olympus Fluoview confocal microscope.

RESULTS AND DISCUSSION

In the accompanying paper (1), Darman and Forbush identify three phosphoacceptor threonines in an activation domain in the N terminus of shark NKCC1. In particular, phosphorylation of Thr184 and Thr189 is highlighted as a key element in sNKCC1- and hNKCC1-transfected HEK-293 cells. The specificity of R5 for phosphorylated NKCC is illus-
tration of whole cell lysate subjected to SDS gel electrophoresis and transferred to polyvi-
ylene difluoride membrane; R, phosphorymation of NKCC1 immunopurified with the J4 antibody; C, blot of whole cell lysate from A probed with the R5 antibody (Western blot); D–F, comparison of NKCC1 32P incorporation and R5 binding by quantitation of phosphorymages and Western blots. Closed symbols, activation in hypertonic media; open symbols, forskolin and calyculin A activation. D shows data from the images in A and C (analysis of whole cell lysates). E and F show combined data from three experiments (not including the one presented in D) in which the samples were analyzed together. Different symbols indicate different experiments. E shows the results from the analysis of whole cell lysates. F shows the results from the analysis of J4 immunoprecipitates.
were preincubated for 30 min in basic medium (135 mM NaCl, 5 mM RbCl, 1 mM CaCl₂/MgCl₂, 1 mM Na₂HPO₄/Na₂SO₄, and 15 mM Na-HEPES, pH 7.4) (open symbols) or low Cl⁻ hypertonic medium (closed symbols). A second preincubation, for the times plotted on the abscissa, was carried out in 0Na-0K-130Cl hypertonic medium (red dashed lines, ●), low Cl⁻ hypertonic medium (black solid lines, △), low Cl⁻ medium (purple dotted lines, ⬤), hypertonic medium (blue solid lines, ◦), or basic medium (green dashed lines, ▽). In alternate rows of the plate, a 2-min ⁸⁶Rb influx was then carried out in regular flux medium (basic medium with ~1 μCi/ml ⁸⁶Rb and 10⁻⁴ M ouabain) (upper panels); or the wells were sucked dry, rapidly solubilized in 70 μl of H₃PO₄/SDS, and dot-blotted with the R5 antibody (lower panels). Each point shows the value of the flux in a single well or, in the two curves for which error bars are shown, the mean and range in duplicate wells. Similar results were obtained in three other experiments.

incubated NKCC1-transfected HEK-293 cells in low Cl⁻ hypertonic medium (3 mM Cl⁻ solution diluted 2-fold with water) or in high K⁺ medium (135 mM NaCl, 5 mM RbCl, 1 mM CaCl₂/MgCl₂, 1 mM Na₂HPO₄/Na₂SO₄, 15 mM Na-HEPES, pH 7.4, and 10 mM K⁺) to activate or deactivate the transporter, respectively. As illustrated in Fig. 2, the antibody signal was severalfold greater in a sNKCC1- and hNKCC1-transfected HEK-293 cells under activating conditions. As a further confirmation that the R5 signal is dependent upon phosphorylation of the cotransporter, the samples in this experiment were incubated at room temperature for 90 min following lysis of the cells to allow endogenous protein phosphatases to dephosphorylate NKCC1. As shown here, the R5 signal was almost completely eliminated by incubation in lysis buffer devoid of the protein phosphatase-1 inhibitor calyculin A.

We examined various NKCC phosphorylation site mutants to determine whether R5 recognizes the monophosphorylated as well as the diphosphorylated regulatory domains. Fig. 3 presents Western blots of transfected HEK-293 cells from such an experiment comparing vector alone, wild-type sNKCC1, and Thr¹⁸⁴/Thr¹⁸⁹ mutants under the activation and deactivation conditions described above. Total shark NKCC1 was indicated by J3 antibody binding (Fig. 3, middle panel); as shown here, the expression was similar for each of the constructs. For both Thr¹⁸⁴ and Thr¹⁸⁹ single mutants, the R5 signal was found to increase with activation (Fig. 3, upper panel), and although the level was clearly greater than in control HEK-293 cells, there was much less signal than for wild-type sNKCC1. The simplest explanation for this result is that the R5 antibody, although having the greatest affinity for the diphosphorylated regulatory domain, also has significant although lower affinity for the monophosphorylated forms.

The J3 antibody consistently recognized two bands in transfected cells (Fig. 3, middle panel), and we presume that this is due to different degrees of glycosylation, possibly reflecting different subcellular compartmentalization. Only the upper of these two bands was phosphorylated upon activation, as demonstrated by R5 recognition (the T189D mutant may exhibit a small amount of another lower band). The identity of the R5-positive band of lesser mobility in deactivated cells is a puzzle. Our interpretation is that this signal is from the endogenous HEK-293 cell cotransporter, based on two observations. (a) There was little or no J3 signal at this position (this R5 signal was from the region between the two strong J3 bands, better seen in superimposition of reblotting experiments (data not shown)); and (b) the upper J3 band exhibited only a very small upward mobility shift upon activation of the transporter. Surprisingly, and somewhat at odds with this interpretation, the native HEK-293 cell cotransporter appeared to undergo a mobility shift upon phosphorylation, despite the fact that the amount of HEK-293 cell R5 signal did not increase much upon activation (Figs. 2 and 3 and discussion below).

**Regulatory Phosphorylation of NKCC1 in Vivo**

**Fig. 5.** Time courses of phosphorylation of NKCC1 and of activation of NKCC-mediated ⁸⁶Rb influx. Transfected HEK-293 cells were preincubated for 30 min in basic medium (135 mM NaCl, 5 mM RbCl, 1 mM CaCl₂/MgCl₂, 1 mM Na₂HPO₄/Na₂SO₄, and 15 mM Na-HEPES, pH 7.4) (open symbols) or low Cl⁻ hypertonic medium (closed symbols). A second preincubation, for the times plotted on the abscissa, was carried out in 0Na-0K-130Cl hypertonic medium (red dashed lines, ●), 0Na-0K-130Cl hypertonic medium (black solid lines, △), low Cl⁻ medium (purple dotted lines, ◤), hypertonic medium (blue solid lines, ◦), or basic medium (green dashed lines, ▽). In alternate rows of the plate, a 2-min ⁸⁶Rb influx was then carried out in regular flux medium (basic medium with ~1 μCi/ml ⁸⁶Rb and 10⁻⁴ M ouabain) (upper panels); or the wells were sucked dry, rapidly solubilized in 70 μl of H₃PO₄/SDS, and dot-blotted with the R5 antibody (lower panels). Each point shows the value of the flux in a single well or, in the two curves for which error bars are shown, the mean and range in duplicate wells. Similar results were obtained in three other experiments.

**Fig. 6.** ⁸⁶Rb influx and phosphorylation in the T202E mutant. sNKCC1-, T202E-, and vector-transfected cells were preincubated for two sequential 30-min preincubation periods as follows: black bars, basic/basic medium; dark gray bars, 0Na-0K-130Cl hypertonic medium; light gray bars, low Cl⁻ hypertonic/0Na-0K-130Cl hypertonic medium; white bars, basic/low Cl⁻ hypertonic medium. Following preincubation, ⁸⁶Rb influx was performed on one-fourth of the plate (upper panel, n = 6 on one plate); and the other wells were sucked dry, and H₃PO₄/SDS was added for dot blotting with the R5 antibody (lower panel, n = 15 on the same plate). A constant background value was subtracted from all dot-blot values; this represented 22, 50, and 54% of the maximum R5 dot signal in sNKCC1, T202E, and the vector, respectively. Data are plotted as means ± S.E. relative to the value with the last of the four preincubation protocols.

**Relationship between the R5 Antibody Signal and ³²P Incorporation in NKCC1**—To analyze the phosphorylation state of sNKCC1 residues Thr¹⁸⁴/Thr¹⁸⁹ in their native cellular environment, we examined ³²P incorporation and R5 binding in...
isolated shark rectal gland tubules preloaded with $^{32}$P$_i$ (1). Tubules were activated by incubation either in hypertonic media or in isotonic media containing forskolin (to activate apical Cl$^{-}$/H$^{+}$ channels and to lower intracellular [Cl$^{-}$] (8)) and calyculin A. Under both conditions, NKCC1 displayed a time-dependent increase of $^{32}$P incorporation in the 195-kDa band of NKCC1 as detected by $^{32}$P phosphorimaging of samples subjected to gel electrophoresis and transferred to polyvinylidene difluoride membranes (Fig. 4A); immunoprecipitation with the J4 antibody highlighted $^{32}$P phosphorylation of NKCC1 (Fig. 4B). Using the R5 antibody, NKCC1 phosphorylation was readily detected even in the crude lysates (Fig. 4C). Fig. 4 (D–F) illustrate the quantitative relationship between the R5 signal and $^{32}$P incorporation. It is clear from these data that R5 binding varies linearly with $^{32}$P incorporation within our measurements, both in crude lysates (Fig. 4, D and E) and in immunoprecipitates (Fig. 4F). Thus, Thr$^{184}$ and Thr$^{189}$ are indicators of overall sNKCC1 phosphorylation under these conditions, providing rational support for the use of R5 as a quantitative tool.

To further establish the relationship between phosphorylation and NKCC1 activation, we compared the effects of several activation media on the R5 signal and $^{86}$Rb influx. Fig. 5 illustrates the results of an experiment, similar to that of Fig. 8 of the accompanying paper (1), in which the time course of activation and deactivation was determined in various media. Following preincubation of cells in a single 96-well plate for each cell line, alternate rows were either lysed in H$_3$PO$_4$/SDS for dot-blot analysis (possible because only a single band was detected by R5; Fig. 1) or subjected to a 1-min $^{86}$Rb influx assay. It is readily seen in Fig. 5 that function mirrors phosphorylation, i.e. activating and deactivating conditions had similar effects on both measurements (a small discrepancy between phosphorylation and flux for hNKCC1 cells in low Cl$^{-}$/H$^{+}$ hypotonic medium transferred to 0Na-0K-130Cl hypertonic medium was seen in two of four such experiments). Human and shark NKCC1 exhibited similar behavior, except that hNKCC1 was less activated by the hypertonic sucrose medium.

We have used similar experiments to answer a question regarding the mechanism by which the T202E mutation produces a profound change in the pattern of cotransporter acti-

![Fig. 7. sNKCC1 activation in vivo.](image)

Shown are immunohistographs of semi-thin sections from shark rectal glands perfused with shark Ringer’s solution in the absence (left panels) or presence (middle panels) of forskolin (Forsk; 50 μM) and probed simultaneously with J4 and R5 antibodies. The superimposition of the two signals is also shown (right panels), demonstrating colocalization of the two antigens.

![Fig. 8. R5 analysis in rat parotid gland.](image)

(a, Western blot analysis of rat parotid gland perfused with Krebs-Henseleit solution in the presence (+) or absence (−) of isoproterenol (Iso; 5 μM). The membrane was probed with the T4 antibody (right panel), stripped, and probed with the R5 antibody (left panel). The 170-kDa band (but not the 140-kDa band) corresponds to the R5 band (+)-isoproterenol versus (−)-isoproterenol (n = 4); p < 0.05. (b) densitometric band analysis of the data in a, c, expression of results in b. Values represent the ratio of phosphorylated NKCC1 to total NKCC1 given by R5 signal/T4 signal. Data are represented as means ± S.D. and were tested by paired Student’s t test for significance at the 5% level. (d, immunohistographs of semi-thin sections of rat parotid gland perfused with shark Ringer’s solution in the absence (left and right panels) or presence (middle panel) of isoproterenol (5 μM). Tissues were probed with the R5 (left and middle panels) and N1c (right panel) antibodies, respectively. Ctrl, control.)
activation, abolishing the stimulation by hypertonic media (1). Fig. 6 presents the results of an experiment involving four activation conditions, each evaluated by R5 dot-blot analysis and by ⁸⁶Rb influx assays of samples from the same plate. For sNKCC1-transfected cells, the cotransporter was activated both by low Cl⁻ hypotonic medium and by 0Na-0K hypertonic medium; the results are similar to those in Fig. 5 in that the changes in ⁸⁶Rb influx mirrored the changes in the R5 signal. For the T202E mutant, ⁸⁶Rb influx was slightly increased over basal levels in 0Na-0K-130Cl hypertonic medium compared with low Cl⁻ hypotonic medium, and again this pattern was reproduced by the changes in the observed R5 signal. This result demonstrates that the effect of the Thr²⁰² mutation on cotransporter activation is the result of a change in the degree to which Thr¹⁸⁴/Thr¹⁸⁹ are phosphorylated by various stimuli.

Fig. 6 also illustrates an anomalous result with regard to the R5 signal of the endogenous HEK-293 cell cotransporter. Following preincubation in the normal flux medium, the R5 signal was high, although the flux was only ~20% activated (we suspect that a tendency of T202E in the same direction may be due to contamination by the endogenous HEK-293 cell signal). It is important to note that there was a relatively high level of R5 signal in HEK-293 cells (see the legend to Fig. 6 and “Experimental Procedures”) and that there was only a small fractional change on top of this. This phenomenon is also seen in the Western blots of Figs. 2 and 3, where the change in the HEK-293 cell R5 signal was very small upon activation. The nature of the HEK-293 cell cotransporter remains an enigma: although reverse transcription-PCR results are more consistent with its identity as NKCC1, its regulatory behavior is distinctly different from that of overexpressed human NKCC1 in several regards (1, 17).

**Fig. 9. NKCC1 activation in rat trachea.** Shown are immunohisto- graphs of semi-thin sections of rat trachea perfused with Krebs-Henseleit solution in the absence (a–c) or presence (d) of isoproterenol (Iso; 5 µM). Tissues were probed with the R5 (c and d) and N1c (a and b) antibodies, respectively. NKCC1 is seen in the epithelial cells, including the germinal layer, and in subepithelial glands (arrow in b). Ctrl, control.

**Fig. 10. NKCC1 activation in rat colon.** Semi-thin sections of rat colon perfused with Krebs-Henseleit solution containing either epinephrine (Epi; 10 µM) or isoproterenol (Iso; 5 µM) were immunostained with the R5 (left panels) and T4 (right panel) antibodies.

*NKCC1 in the Parotid Gland*—Numerous reports of divergent stimuli causing activation of NKCC1 in the parotid gland support a complex pattern of regulation in this tissue (3). In this study, we perfused the parotid gland in vivo in the absence or presence of the β-adrenergic receptor agonist isoproterenol; the subsequent rise in intracellular cAMP has been previously shown to increase NKCC1 activity in this tissue (19, 20). In Fig. 8 (a and b), tissue homogenate subjected to gel electrophoresis and probed with the R5 antibody displayed a 170-kDa band whose identity as NKCC1 was confirmed by the T4 antibody. Densitometric band analysis revealed a 2-fold increase in the
R5/T4 ratio under stimulatory conditions (Fig. 8c). In addition to the 170-kDa protein, the C-terminal T4 antibody detected a second band with an apparent molecular mass of 140 kDa. This band was not strongly recognized by the R5 antibody, and we presume it to represent immature, non-phosphorylated NKCC1 retained in intracellular compartments.

In situ analysis of rat parotid gland with the R5 antibody revealed pronounced staining along the basolateral membrane in ductal and acinar cells of glands perfused with isoproterenol (Fig. 8d). Basolateral staining with the Ni antibody confirmed that NKCC1 was expressed in both cell types. The finding that NKCC1 is abundantly expressed in the duct epithelium of rat parotid gland contrasts markedly with our previous finding of rabbit parotid gland NKCC1 expression only in the acini (12). This expression profile was confirmed (data not shown) and thus suggests a striking species-specific difference in the function of the duct epithelium.

**NKCC1 in the Trachea**—In the trachea, as in many other secretory tissues, NKCC1 works in concert with the cystic fibrosis transmembrane conductance regulator to aid in the vectorial secretion of fluid into the lumen (2). Haas et al. (7) have previously described NKCC1 activation in isolated dog secretory tissues, NKCC1 works in concert with the cystic fibrosis transmembrane conductance regulator to aid in the vectorial secretion of fluid into the lumen (2). Haas et al. (7) have previously described NKCC1 activation in isolated dog secretory tissues, NKCC1 working in concert with the cystic fibrosis transmembrane conductance regulator to aid in the vectorial secretion of fluid into the lumen (2).

**NKCC1 in the Distal Colon**—In this secretory tissue, NKCC1 activity has been linked to both K⁺ and Cl⁻ secretion (24, 25). This NKCC1-mediated secretory activity has been linked to both β- and α₂-adrenergic receptors, respectively, through increases in intracellular [cAMP], with a resultant fall in intracellular chloride, and through increases in intracellular [Ca²⁺], with the activation of basolateral K⁺ recycling channels (26).

In Fig. 10, the R5 antibody was used to address the activation state of NKCC1 in response to α- and β-adrenergic receptor agonists (epinephrine and isoproterenol, respectively). In tissue perfused with isoproterenol, strong staining was observed in the base of the crypts and decreased markedly along the crypt-villus axis. NKCC1 was expressed throughout the crypt, as demonstrated by T4 antibody binding and similar to the expression profile of the gastric gland. This segregation of activity further supports the hypothesis that the base of the crypts constitutes a region of cell proliferation and secretion (27, 28).

R5 staining was negative in epinephrine-perfused tissue (Fig. 10) and was similar to levels observed in control animals (data not shown). We believe that the absence of NKCC1 stimulation by epinephrine may be due to stimulation of Go₃ via α₁-adrenergic receptors in this colonic segment. This would correlate the response of NKCC1 to adrenergic stimuli with the local expression of receptor subtypes.

**Limitations on the Use of the R5 Antibody**—Although the R5 antibody has proven to be an outstanding tool for the investigation of cotransporter regulation, we have identified a number of limitations in its utilization. (a) It must be noted that, although R5 recognizes two phosphoacceptor sites in NKCC, available data imply that at least five phosphoacceptors are utilized in regulatory phosphorylation (see “Discussion” in the accompanying paper (1)). Also, although most experiments have supported the concept of phosphorylation by a single kinase, recent studies have raised the possibility of alternate modes of regulation (1, 3). (b) In the work presented here, we examined regulation of NKCC1 in secretory tissues in which it is very highly expressed. We have found that R5 is not suitable for immunofluorescence studies in tissues with lower levels of cotransporter expression due to background staining of cytoplasm and particularly of nuclei. (c) We are puzzled by a high level of R5 background signal when phosphorylation and dephosphorylation are “stopped” by acid and SDS compared with when they are stopped by Triton X-100, EDTA, and phosphatase inhibitors (see “Experimental Procedures”). (d) Although the R5 signal mirrors transport function both in shark rectal gland cells and in HEK-293 cells overexpressing shark or human NKCC1, in wild-type HEK-293 cells, there is an anomalously high R5 signal under basal conditions (Figs. 2, 3, and 6). We currently have no explanation for this observation, but appreciate that it may complicate the interpretation of future experiments in similar cell types.

**Conclusions**—As demonstrated above, the R5 antibody enables us to directly investigate the phosphorylation state of the two conserved thereonines in cells and tissues and to discern the subcellular localization of activated NKCC1 via immunofluorescence. We show the first reported data of NKCC1 activation in vivo using fixed sections of secretory tissue. Analyzing these data, we conclude that Thr(P)¹⁸⁴ and/or Thr(P)¹⁸⁹ in shark and the homologous mammalian residues parallel NKCC1 activation in vivo and that this phosphorylation profile is independent of the tissue type and vertebrate species tested. We propose that these regulatory thereonines are part of a universal regulatory locus necessary for NKCC1 activation.

**Acknowledgments**—We thank James Elliot (W. M. Keck Facility, Yale University) for phosphopeptide synthesis, Chris Lytle for the Ni antibody, Sue Ann Mentone for expert preparation of histological samples, and Grace Dillard for excellent technical assistance.

**REFERENCES**

1. Darman, R. B., and Forbush, B. (2002) J. Biol. Chem. 277, 37542-37550
2. Haas, M., and Forbush, B., III (2000) Annu. Rev. Physiol. 62, 515-534
3. Tanimura, A., Kuribara, K., Reshkin, S. J., and Turner, R. J. (1995) J. Biol. Chem. 270, 25282-25288
4. Evans, R. L., and Turner, R. J. (1997) J. Physiol. (Lond.) 499, 351-359
5. Forbush, B., III, Haas, M., and Lytle, C. (1992) Am. J. Physiol. 262, C1000-C1008
6. Payne, J. A., Xu, J. C., Haas, M., Lytle, C. Y., Ward, D., and Forbush, B., III (1995) J. Biol. Chem. 270, 17977-17985
7. Haas, M., Johnson, L. G., and Boucher, R. C. (1990) Am. J. Physiol. 258, C557-C569
8. Lytle, C., and Forbush, B., III (1996) Am. J. Physiol. 270, C457-C448
9. Lytle, C., and Forbush, B., III (1997) J. Biol. Chem. 272, 25438-25443
10. Kuribara, K., Moore-Hoon, M. L., Saitoh, M., and Turner, R. J. (1999) Am. J. Physiol. 277, C1184-C1193
11. Darman, R. B., Flemmer, A., and Forbush, B. (2001) J. Biol. Chem. 276, 34359-34362
12. Lytle, C., Xu, J. C., Biemesderfer, D., and Forbush, B., III (1995) Am. J. Physiol. 269, C1496-C1505
13. McDaniel, N., and Lytle, C. (1999) Am. J. Physiol. 267, G1273-G1278
14. Lytle, C., Xu, J. C., Biemesderfer, D., Haas, M., and Forbush, B., III (1992) J. Biol. Chem. 267, 25428-25437
15. Lytle, C., and Forbush, B., III (1992) Am. J. Physiol. 263, C1009-C1017
16. McClean, W., and Nakane, P. F. (1973) J. Histochem. Cytochem. 21, 1077-1083
Regulatory Phosphorylation of NKCC1 in Vivo

17. Isenring, P., Jacoby, S. C., Payne, J. A., and Forbush, B., III (1998) J. Biol. Chem. 273, 11295–11301
18. Flemmer, A. W., and Forbush, B., III (1999) FASEB J. 13, A399
19. Paulais, M., and Turner, R. J. (1992) J. Clin. Invest. 89, 1142–1147
20. Kurihara, K., Nakanishi, N., Moore-Hoon, M. L., and Turner, R. J. (2002) Am. J. Physiol. 282, C817–C823
21. Boers, J. E., Ambergen, A. W., and Thunnissen, F. B. (1998) Am. J. Respir. Crit. Care Med. 157, 2000–2006
22. Russell, J. M. (2000) Physiol. Rev. 80, 211–276
23. Panet, R., Marcus, M., and Atlan, H. (2000) J. Cell. Physiol. 182, 109–118
24. Halm, D. R., and Frizzell, R. A. (1986) Am. J. Physiol. 251, C252–C267
25. Dharmsathaphorn, K., Mandel, K. G., Masui, H., and McRoberts, J. A. (1985) J. Clin. Invest. 75, 462–471
26. Schulteiss, G., and Diener, M. (2000) Eur. J. Pharmacol. 403, 251–258
27. D’Andrea, L., Lytle, C., Matthews, J. B., Hofman, P., Forbush, B., III, and Madara, J. L. (1996) J. Biol. Chem. 271, 28969–28976
28. Matthews, J. B., Hassan, I., Meng, S., Archer, S. Y., Hrnjez, B. J., and Hodin, R. A. (1998) J. Clin. Invest. 101, 2072–2079