# Inhibition of Na/H Exchange in Avian Intestine by Atrial Natriuretic Factor

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## Abstract

**Effects of 8-bromo-cGMP (8-Br-cGMP) and synthetic rat atriopeptin III (APIII) on sodium absorption by isolated chicken villus enterocytes and intact chicken ileal mucosa were determined. In isolated cells, both agents significantly decreased initial rates of influx of $^{22}Na$ and caused a persistent decrease in intracellular pH ($pH_i$); effects that are not additive to those caused by amiloride ($10^{-3}$ M). The $ED_{50}$ for APIII was 0.3 nM. In intact mucosa, both 8-Br-cGMP ($10^{-4}$ M) and 5-(*N*-methyl-*N*-isobutyl)amiloride (MIBA) ($10^{-5}$ M) reduced $J^{Na}_{ms}$ and $J^{Na}_{net}$; their effects were not additive. APIII ($10^{-7}$ M) significantly increased cellular cGMP but not cAMP. Both 8-Br-cGMP ($10^{-4}$ M) and APIII ($10^{-7}$ M) stimulated a persistent increase in cytosolic calcium ($Ca_i$), which could be prevented by pretreating the cells with the cytosolic calcium buffering agent MAPTAM or with H-8, an inhibitor of cyclic nucleotide-dependent protein kinases. Furthermore, pretreatment of cells with H-8 or the calmodulin inhibitor, calmidazolium (CM), prevented the effects of 8-Br-cGMP and APIII on $pH_i$. However, the $pH_i$ response to subsequent addition of the calcium-ionophore ionomycin was blocked only by CM and not by H-8. These data suggest that APIII and 8-Br-cGMP inhibit amiloride-sensitive Na/H exchange by increasing $Ca_i$, an event requiring activation of cGMP-dependent protein kinase. (*J. Clin. Invest.* 1990. 86:585–591.) Key words: ANF • cyclic GMP • sodium transport • intestine • cytosolic calcium**

## Introduction

Atrial natriuretic factor (ANF),[1] a group of peptide hormones found in atrial cardiocytes, is believed to play a major role in volume and electrolyte homeostasis. When administered to man and other mammals, ANF stimulates natriuresis and diuresis, decreases blood pressure (1–3) and inhibits aldosterone secretion (3). The mechanism of action of ANF is still unfolding. Two distinct ANF receptors have been identified; a single polypeptide that has guanylate cyclase activity (4, 5), and a lower molecular weight peptide that lacks guanylate cyclase activity (6). The ratio of these two types of receptors varies in different tissues, which may be of importance in the modulation of ANF's physiologic effects. In a variety of diverse tissues, ANF has been shown to activate particulate guanylate cyclase with subsequent increases in cGMP concentration (7–10), implicating cGMP as an intracellular mediator of ANF.

Recent studies suggest that ANF may affect electrolyte transport in the intestinal tract. A direct inhibitory effect of synthetic rat atriopeptin III on Na/K/Cl cotransport has been demonstrated in marine teleost intestine (11, 12), an effect exactly mimicking that of 8-Br-cGMP. There are conflicting reports about ANF's effects on mammalian intestine. High affinity receptors for ANF have been identified along the epithelium of rat small intestine, most notably at the base of the mature columnar epithelium of villi (13, 14). In vivo studies in rat small intestine conflict, one showing an increase, another a decrease and yet another, no effect on sodium and water absorption in response to ANF or atrial extracts (15–17). ANF infusion in human subjects had no effect on jejunal water and electrolyte transport (18). In rat large intestine, ANF has been reported to increase short circuit current. This effect is blocked by tetrodotoxin and is not seen in T84 human colon cancer cell monolayers, suggesting it is mediated by neurons (19). More recently, a published abstract reports no effect of ANF on rat colon in vitro or in vivo (20).

To more directly study ANF's effect on the intestine, we utilized an isolated cell preparation from avian intestine. We report here that ANF inhibits amiloride-sensitive Na/H exchange in isolated chicken enterocytes by stimulating increases in intracellular calcium ($Ca_i$) activity: The data suggest that this event is initiated by an ANF-stimulated increase in cGMP concentration which, in turn, appears to increase $Ca_i$.

## Methods

*Materials.* Reagents were obtained from the following sources: Fura2 and MAPTAM from Calbiochem-Behring (San Diego, CA); 5,6-carboxyfluorescein diacetate from Molecular Probes (Junction City, OR); [$^{14}$C]*methyl*-α-glucopyranoside, $^{22}NaCl$, $^{125}$I-cyclic AMP and $^{125}$I-cyclic GMP radioimmunoassay kits from New England Nuclear (Boston, MA); amiloride HCl from Merck, Sharpe and Dohme (West Point, PA); 8-bromo-cGMP, phloridzin, bethanecol, and ionomycin from Sigma Chemical Co. (St. Louis, MO). Rat atriopeptin III (APIII) from Peninsula Labs (Beaumont, CA). Calmidazolium (CM) was a gift from Janssen Pharmaceuticals (Beerse, Belgium). *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide (H-8) was kindly provided by Hugo DeJonge (Erasmus University, Rotterdam, The Netherlands). 5-(*N*-methyl-*N*-isobutyl)amiloride (MIBA) was synthesized for this study using previously described methods (21).

For pH and calcium measurements, cells were resuspended in a modified Hanks' buffered saline (HBS) containing 137 mM Na, 5 mM K, 142 mM Cl, 0.9 mM Mg, 0.4 mM $SO_4$, 0.3 mM $HPO_4$, 0.4 mM $H_2PO_4$, 1.1 mM Ca, 20 mM Hepes (pH 7.4). For $^{22}Na$ influx studies, cells were resuspended in a buffer containing 70 mM Na, 60 mM

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1. *Abbreviations used in this paper:* APIII, atriopeptin III; Beth, bethanecol; CM, calmidazolium; H-8, *N*-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide; I, ionomycin; MP, MAPTAM; MIBA, 5-(*N*-methyl-*N*-isobutyl)amiloride.

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*N*-methyl-*d*-glucamine (NMDG), 5 mM K, 1.65 mM $HPO_4$, 0.3 mM $H_2PO_4$, 1.0 mM Mg, 1.25 mM Ca, 0.2 mM $SO_4$, 25 mM Hepes (pH 7.4). Sodium-free stop buffer contained 119 mM NMDG, 5 mM K, 1.65 mM $H_2SO_4$, 0.3 mM $H_2PO_4$, 1.1 mM $MgCl_2$, 1.25 mM $CaCl_2$, 25 mM Hepes (pH titrated to 7.4 with HCl).

*Isolation of chicken enterocytes.* Enterocytes were isolated from the distal half of the small intestine of 10–12-wk old, white, leghorn chickens by enzymatic methods previously described (22, 23). The final cell concentration was $\sim 1 \times 10^{-7}$ cells/ml. The purity of our cell preparation was assessed by staining for cytokeratin, a marker of epithelial origin. Cytokeratin stain was performed by treating cells with the monoclonal antibody AE-3 followed by peroxidase staining. Cells containing cytokeratin stain brown. Cell viability was assessed by determining the uptake of [$^{14}$C]methyl-$\alpha$-glucopyranoside in the presence and absence of phloridzin (200 $\mu$M/liter) as previously described (23).

*$^{22}$Na influx studies.* Sodium uptake studies on isolated cells were performed as previously described (22). In all studies, cells were used within 30 min after isolation, except for the MAPTAM studies which required a 30-min incubation with MAPTAM to assure adequate loading of this agent. Paired control cells were included in all studies.

*Ion transport studies in intact mucosa.* The distal 10 cm of the ileum was removed, opened along its mesenteric border, and rinsed free of intestinal contents with iced bicarbonate-Ringer solution containing (in mmol/liter): Na 143; Cl 124; K 5; Mg 1.1; Ca 1.25; $HCO_3$ 25; $HPO_4$ 0.3; $H_3PO_4$ 1.65; at pH 7.4 and 37°C. Ileal segments were stripped of serosa and the two outer muscle layers by blunt dissection and mounted in Ussing chambers. Transepithelial electrical potential difference, total electrical conductance, resistance, and short-circuit current ($I_{sc}$) were measured as previously described (24). Transepithelial unidirectional fluxes ($J$) of sodium from mucosa (m) to serosa (s) and from s to m were measured under short-circuited conditions (24). For flux measurements 1.0 $\mu$Ci of $^{22}$Na was added to either mucosal or serosal reservoirs 20 min after the tissues were mounted. Tissue pairs that differed in electrical resistance by > 25% were discarded. All flux experiments were conducted in a modified bicarbonate-Ringer solution altered to contain 60 mM NaCl and isotonically balanced with mannitol, to reduce the passive paracellular flux component of sodium and chloride. Agents were added in sequential time periods in the same tissue following baseline Na flux determinations. Each time period was 12 min and the order in which agents were added was alternated. MIBA was also added serosally to determine its effects on unidirectional and net Na transport. Glucose (10 mM) was added at the end of each experiment to assess viability of the tissue.

*Intracellular calcium ($Ca_i$) measurements.* Isolated cells were suspended in calcium-free HBS and incubated with Fura2 (1 $\mu$M dissolved in DMSO) for 10 min at 39°C. The final DMSO concentration was < 0.1%. Omission of Ca minimized the activity of any carboxylesterases released from damaged cells. After 10 min, extracellular calcium was restored to 1 mM and the incubation was continued for another 15 min. After loading, the cell suspension was diluted threefold with warm HBS. Equal 5-ml aliquots were removed and diluted twofold with HBS and centrifuged at 200 *g* for 1 min. Pellets were resuspended in 2 ml HBS to yield $1–3 \times 10^6$ cells/ml and transferred to a cuvette. Fluorescence measurements were initiated 10 min later and completed within 20 min. Measurements were made with a Farand Mark I spectrofluorometer with a modified thermostated cuvette chamber which had a motor-driven rotary mixing unit that maintained a homogeneous cell suspension. Drugs and hormones were introduced into the cuvette with a Hamilton syringe through a guide mounted in the cuvette chamber lid. All measurements were made at 38°C in 1-cm square polystyrene cuvettes. Before each experiment an excitation spectrum was generated to confirm that intracellular Fura2 was in its free acid form. Fluorescence intensities were recorded at 510 nm emission (10-nm slits) using excitation wavelengths of 350, 363, and 380 nm (5-nm slits). Autofluorescence of unloaded cells was subtracted at each wavelength. Autofluorescence at all wavelengths represented < 20% of total signal. Ratios were determined by the fluorescence intensities obtained at 350 and 380 nm, relative to the isosbestic determination at 363 nm [F(350 nm − 363 nm)/F(363 nm − 380 nm)]. This effectively eliminates artifactual measurements resulting from changes in light scatter, contributions from autofluorescence, differences in intracellular indicator concentration and from photobleaching. Since emphasis in this study is placed on relative and not absolute changes in $Ca_i$, results are expressed in ratios. An increase in ratio represented an increase in $Ca_i$.

By the ratio method, at best, only two ratio measurements could be determined per minute. To achieve better time resolution of $Ca_i$ changes, separate studies were performed where changes in fluorescence were continuously monitored at 510 nm using a single excitation wavelength of 350 nm (isosbestic determinations at 363 nm remain essentially unchanged). Calcium values were calculated as previously described (25, 26).

*Intracellular pH ($pH_i$) measurements.* Isolated cells were loaded with 5,6-carboxyfluorescein (CF) (20 $\mu$M) as previously described (22). Fluorescence measurements were begun after a 5–10-min preincubation period. Changes in $pH_i$ were determined as changes in the ratio of fluorescence intensities ($R$ values) recorded at 530 nm emission (5-nm slits) using excitation wavelengths of 490 and 463 nm (5-nm slits). Autofluorescence of isolated cells that represented < 30% of the total fluorescence signal was subtracted in each case. None of the agents studied exhibited autofluorescence. A calibration curve of intracellular CF as a function of changes in intracellular pH in nigericin and valinomycin treated cells was generated, as previously described (22). CF ratio measurements accurately predict $pH_i$ between the range of 6.8 to 7.2. Because an estimated average of autofluorescence determined from unloaded cells was subtracted in each case, absolute $R$ values vary somewhat between batches of cells. Emphasis has been placed in this study on the relative changes in $R$ values from baseline after treatments. Cells with resting $pH_i$ > 7.2 were discarded because of the nonlinearity of the titration curve in this range and because high $pH_i$ correlated with decreased cell viability.

*Measurements of cGMP and cAMP.* Isolated cells were resuspended in HBS to a concentration of $\sim 1 \times 10^7$ cells/ml and warmed to 37°C in a rotary water bath. Cells were equally divided into experimental and control groups. APIII ($10^{-7}$ M) was added to one set of cells. Aliquots of 2 ml from both sets of cells were removed at 4 and 10 min, and centrifuged immediately at 200 *g* for 1 min. 5% TCA (2 ml) was added to the cell pellets on ice. This mixture was then homogenized by hand at room temperature with a glass pestle (12 strokes) and centrifuged at 2,500 *g* for 10 min. The supernates were transferred into glass tubes, extracted four times with 2 ml of water-saturated diethylether, and evaporated to dryness in a water bath at 90°C. The residues were dissolved in 0.5 ml of 0.05 M sodium acetate buffer (pH 6.2). For cGMP measurements, samples were acetylated by the method of Harper and Brooker (27) before assay. Cyclic nucleotides were determined in duplicate by radioimmunoassay as described by Steiner et al. (28). The TCA precipitates were dissolved in 1 N NaOH and assayed for protein by the method of Lowry. Results were calculated in picomoles cGMP or cAMP per milligram protein and expressed as percent paired control values.

*Statistical analysis.* Statistical comparisons of two means were performed by Student's $t$ test of paired variates. In studies where multiple means were compared, analysis of variance (ANOVA) was used for groups of equal or unequal sizes (29).

## Results

*$^{22}$Na influx.* We measured the effects of APIII ($10^{-7}$ M) and 8-Br-cGMP ($10^{-4}$ M) on initial $^{22}$Na influx into isolated chicken enterocytes (Fig. 1). Sodium uptake into chicken enterocytes had previously been shown to be linear for at least 1 min (30). Both APIII ($10^{-7}$ M) and 8-Br-cGMP ($10^{-4}$ M) significantly inhibited $^{22}$Na influx into our enterocytes, effects

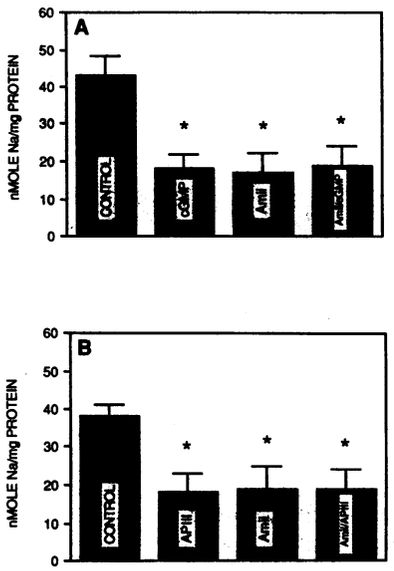


*Figure 1.* Effect of 8-Br-cGMP ($10^{-4}$ M), APIII ($10^{-7}$ M) and amiloride ($10^{-3}$ M) on initial $^{22}Na$ influx into isolated chicken enterocytes. Cells were incubated in 70 mM sodium HBS. All agents were added 5 min before measurements were made. 1 ml of buffer containing 1 $\mu$Ci $^{22}Na$ was added to 3 ml of cells to initiate Na uptake. Samples (300 $\mu$l) were taken at 0 and 45 s. Na uptake was terminated by dilution of samples in 1.2 ml of ice-cold sodium-free stop buffer. Results are expressed in nmoles Na/mg protein and each bar represents the mean±SEM of $n = 8$ (*A*) and $n = 4$ (*B*) experiments. * Different from control, $P < 0.05$ by ANOVA comparing multiple means.

similar in magnitude and not additive to that of amiloride ($10^{-3}$ M).

*Intracellular pH measurements.* The nonadditivity of the inhibitory effect on sodium uptake of APIII or 8-Br-cGMP and that of amiloride suggested involvement of the Na/H exchanger which we had previously shown to be present in these cells (22). To determine whether APIII and 8-Br-cGMP had an effect on cell pH, we measured intracellular pH with CF. Fig. 2, *A* and *B* are representative tracings of fluorescence measurements over 10 min. As shown, both APIII and 8-Br-cGMP decrease CF fluorescence intensity measured at 490 nm excitation wavelength. Fig. 2, *C* and *D* show the ratios of these measurements averaged over 1- or 2-min time periods. 8-Br-cGMP ($10^{-4}$ M) decreased CF fluorescence ratios from 2.16 to 2.12 (Fig. 2 *C*). Likewise, APIII ($10^{-7}$ M) decreased ratios from 2.12 to 2.08 (Fig. 2 *D*). These ratio changes correspond to a decrease in $pH_i$ of ~ 0.10–0.15 pH units. The decrease in $pH_i$ persisted over 10 min. Addition of amiloride ($10^{-3}$ M) after 10 min produced no further decrease in fluorescence ratio (data not shown). No significant changes in resting $pH_i$ were observed in untreated paired control cells during these time periods. On average, resting $pH_i$ of cells was between 6.8 and 7.0.

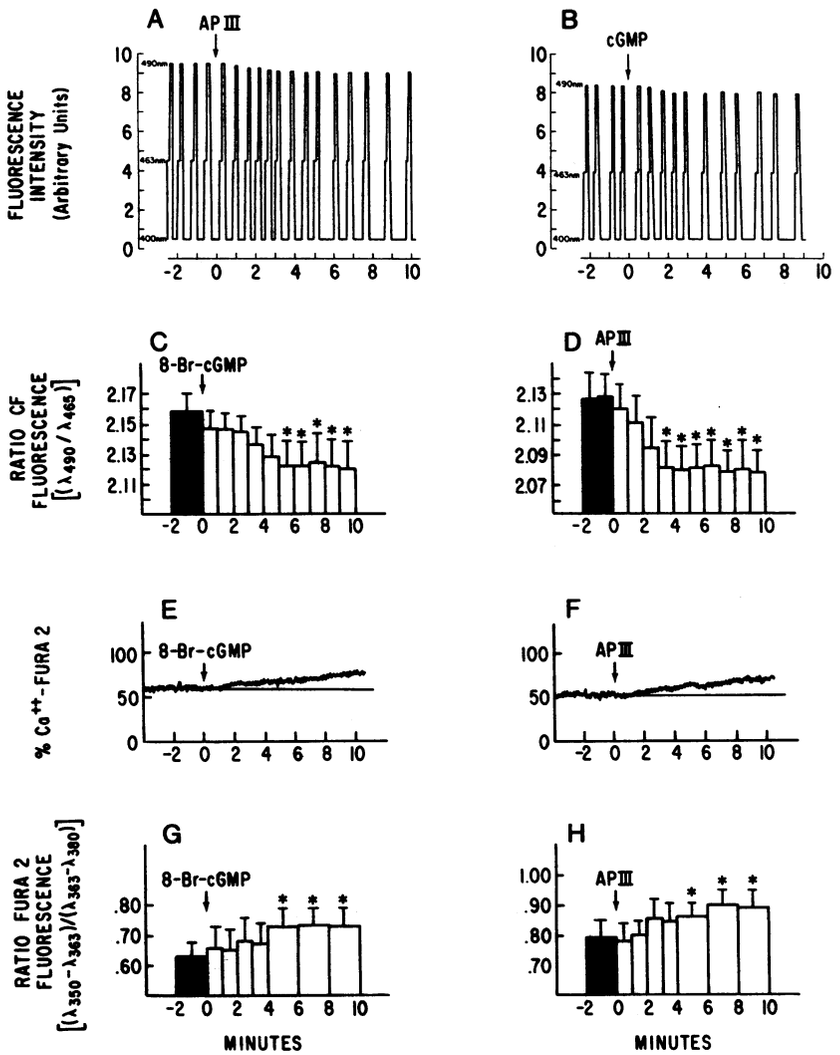


*Figure 2.* Effects and time course of 8-Br-cGMP ($10^{-4}$ M) and APIII ($10^{-7}$ M) on $pH_i$ and $Ca_i$. (*A* and *B*) Representative tracings of CF fluorescence intensity measured in isolated enterocytes before and after treatment with APIII and 8-Br-cGMP. Cells were suspended in HBS and loaded with CF (20 $\mu$M) over 15 min. For each pH determination equal aliquots of cells were washed free of extracellular dye and resuspended in 2 ml of HBS. Fluorescence measurements were made using excitation wavelengths of 400, 463, and 490 nm, respectively. At $t = 0$, cells were treated with APIII ($10^{-7}$ M) or 8-Br-cGMP ($10^{-4}$ M). (*C* and *D*) Derived CF fluorescence ratios from several experiments. The bars represent the mean±SEM of ratio determinations over the indicated time periods for $n = 6$ (*C*) and $n = 7$ (*D*) experiments. (*E* and *F*) Representative tracings of Fura2 fluorescence intensity before and after treatment with 8-Br-cGMP and APIII. Cells were loaded with Fura2 (1 $\mu$M) as described in the methods section. Equal cell aliquots were washed free of extracellular dye and suspended in 2 ml HBS. Fluorescence was measured at 510 nm using a single excitation wavelength of 350 nm. Measurements are expressed as a percent Ca-Fura2 in isolated enterocytes where 100% and 0% are defined as the fluorescence upon addition of 1 $\mu$M ionomycin and after quenching with 0.5 mM $MnCl_2$. (*G* and *H*) Measurements of $Ca_i$ by determination of the ratio of fluorescence intensity of Fura2. Fluorescence was measured at excitation wavelengths of 350, 363, and 380 nm. The bars represent the mean±SEM of ratios determined at 350 and 380 nm relative to the isosbestic point (363 nm), i.e., F350–363/F363–380, for $n = 5$ (*G*) and $n = 6$ (*H*) experiments. * Different from pretreatment values $P < 0.05$ by ANOVA comparing multiple means.

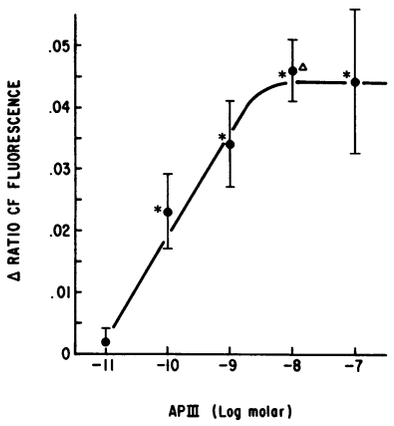


*Figure 3.* Effect of varying concentrations of APIII on $pH_i$. Cells were incubated in HBS. CF fluorescence measurements were made before and after treatment with the above doses of APIII. Changes in ratio were determined by subtracting the observed ratio (5 min after treatment with APIII) from the pretreatment value. Each point represents the mean±SEM of changes in ratio determinations for five separate experiments. * $P < 0.05$ compared to pretreatment values. $\Delta P < 0.05$ compared to responses at $10^{-10}$ M and $10^{-11}$ M by ANOVA.

The $ED_{50}$ of APIII's effect on fluorescence ratio changes was 0.3 nM (Fig. 3) with near maximal inhibition occurring at 10 nM. The results are consistent, therefore, with inhibition of amiloride-sensitive Na/H exchange by APIII and 8-Br-cGMP.

*Unidirectional transmural fluxes.* We measured the effects of MIBA ($10^{-5}$ M) and 8-Br-cGMP ($10^{-4}$ M) on unidirectional sodium fluxes across short-circuited chicken intestinal mucosa. MIBA was used because it is a more selective inhibitor of Na/H exchange than amiloride (21) and could be used at a smaller concentration. As shown in Table I, both mucosally added MIBA and serosally added 8-Br-cGMP decreased $J^{Na}_{ms}$, and hence net Na absorption, to the same extent. No significant effects on $J^{Na}_{sm}$ or $I_{sc}$ were observed. These effects were not additive, suggesting that both agents block neutral sodium transport by inhibiting brush-border Na/H exchange. MIBA ($10^{-5}$ M) was ineffective when added serosally (baseline $J^{Na}_{net}$ = 4.3±0.8; after MIBA, $J^{Na}_{net}$ = 4.1±0.9 μeq/cm² h, $n$ = 4). Since these agents inhibited sodium absorption by only 20–30%, one or more other sodium transport processes must have been present.

*Table I. Effects of 8-BR-cGMP ($10^{-4}$ M) and MIBA ($10^{-5}$ M) on Ileal Sodium Fluxes*

| Conditions | Unidirectional $^{22}Na$ fluxes (μeq/cm² · h) $J^{Na}_{ms}$ | $J^{Na}_{sm}$ |
|---|---|---|
| A | 13.8±0.9 | 5.4±0.4 |
| B +8-Br-cGMP | 11.9±0.8* | 5.4±0.5 |
| C +MIBA | 11.8±0.7* | 5.4±0.4 |
| A | 12.9±0.6 | 5.9±0.5 |
| B +MIBA | 10.7±0.7* | 5.4±0.6 |
| C +8-Br-cGMP | 10.3±0.6* | 6.0±1.0 |

MIBA and 8-Br-cGMP were added to the mucosal and serosal reservoirs, respectively. Periods *A*, *B*, and *C* represent 12-min collection intervals. *A* is a baseline flux period. Agents were added sequentially in the order listed. * $P < 0.05$ compared to *A* by ANOVA for $n$ = 5 experiments.

*cGMP and cAMP measurements.* The effects of APIII on the cGMP and cAMP contents of isolated enterocytes were determined by radioimmunoassay. As shown in Fig. 4, 10 min after stimulation with APIII ($10^{-7}$ M), cellular cGMP content increased 62% ($n$ = 6, $P < 0.01$) over levels found in paired control cells. In contrast, the cAMP content of APIII treated cells was unaffected.

*Intracellular calcium.* To measure $Ca_i$ we used the Ca-sensitive fluorescent indicator Fura2. As shown in Fig. 2, *G* and *H*, 8-Br-cGMP ($10^{-4}$ M) and APIII ($10^{-7}$ M) significantly stimulated increases in $Ca_i$ (as determined by increases in ratio values) that persisted for at least 10 min. During this period, no significant changes in fluorescence ratio were observed in untreated paired control cells. Resting $Ca_i$ in these cells was calculated to be 240±25 nM ($n$ = 6). APIII ($10^{-7}$ M) and 8-Br-cGMP ($10^{-4}$ M) stimulated increases in $Ca_i$ of 252±15 nM and 285±28 nM ($n$ = 4, each) above resting levels, respectively.

Since by manual methods only about one ratio measurement could be generated per minute it was difficult to determine an accurate time course for the calcium changes when measured by the ratio method. Better time resolution of $Ca_i$ changes was achieved by measuring fluorescence using a single excitation wavelength of 350 nm. As shown in Fig. 2, *E* and *F*, ~ 2 min after their addition to cells loaded with Fura2, 8-Br-cGMP ($10^{-4}$ M) and APIII ($10^{-7}$ M) stimulate a slow increase in Fura2 fluorescence, hence an increase in $Ca_i$. No extracellular dye was fluorometrically detected from Fura2 loaded control cells for > 20 min. The $Ca_i$ changes in these tracings have a similar time course to those of $pH_i$ changes shown in Fig. 2, *A* and *B*.

*Calcium blocking studies.* To determine whether the increase in $Ca_i$ caused by APIII and 8-Br-cGMP mediates their inhibition of Na/H exchange, we tested agents that interfere with the intracellular action of calcium. First, $^{22}Na$ influx was measured in cells pretreated with MAPTAM (100 μM), an agent that buffers $Ca_i$. At this concentration of MAPTAM, 8-Br-cGMP does not stimulate an increase in $Ca_i$ (not shown). Fig. 5 shows that Na uptake was not inhibited when cells

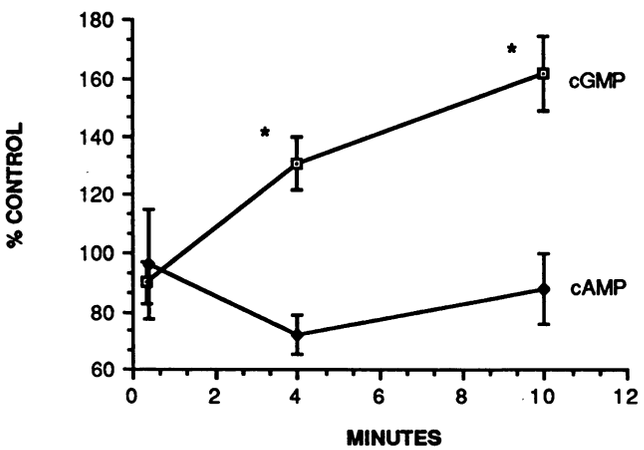


*Figure 4.* Effects of APIII ($10^{-7}$ M) on cellular cyclic nucleotide content. Results are expressed as a percent of paired unstimulated control cells. Each value represents the mean±SEM for six experiments performed in duplicate. 100% value for cAMP was 0.44±0.2 pmol/μg protein and 0.35±0.12 pmol/μg protein for cGMP. * $P < 0.01$ compared to paired control values.

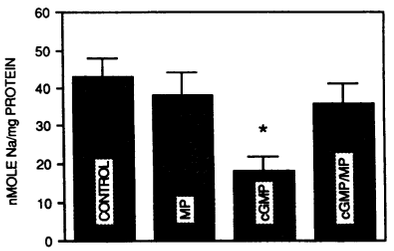


*Figure 5.* Effect of buffering $Ca_i$ with MAPTAM on 8-Br-cGMP inhibition of $^{22}Na$ influx into isolated chicken enterocytes. Cells were incubated in MAPTAM (50 μM) for 30 min and 8-Br-cGMP for 5 min before starting sodium uptake studies. Bars represent the experiments performed in duplicate. *Different from paired control $P < 0.05$ by ANOVA comparing differences between means.

loaded with MAPTAM were treated with 8-Br-cGMP ($10^{-4}$ M). MAPTAM by itself has no effect on Na uptake (Fig. 5).

We next measured $pH_i$ in cells pretreated with the calmodulin inhibitor calmidazolium (CM). This agent was chosen because of its greater potency and selectivity when compared to agents such as trifluoperazine (31). As shown in Table II, pretreatment of cells for 5 min with CM ($10^{-7}$ M) completely abolished the effects of APIII ($10^{-7}$ M) and 8-Br-cGMP ($10^{-4}$ M) on CF ratio fluorescence. CM has no apparent effect on resting $pH_i$. Subsequent addition of ionomycin ($10^{-6}$ M), a calcium ionophore, failed to decrease $pH_i$ in CM and APIII-treated cells. We also studied the effects of the isoquinolinesulfonamide, H-8, on $pH_i$. H-8, at low doses, is a potent and relatively selective inhibitor of cyclic nucleotide-dependent protein kinases (32, 33). H-8 (50 μM) had no effect on resting $pH_i$ but completely blocked the inhibitory effects of APIII and 8-Br-cGMP on $pH_i$ (Table II). However, when the Ca ionophore ionomycin ($10^{-6}$ M) was subsequently added, a decrease in CF fluorescence, hence $pH_i$, was observed (Table II) (Fig. 6 *A*).

The effect of H-8 on APIII-stimulated $Ca_i$ changes was also studied. A representative tracing is shown in Fig. 6 *B*. H-8 (50 μM), by itself, had no effect on basal $Ca_i$ activity over a 7–15-min period (ratio values before and after H-8 incubation were 0.740±0.095 and 0.756±0.095, $n = 4$). To avoid prolonged exposure of cells to near ultraviolet light, cells were subsequently preincubated with H-8 for 10 min before initiating fluorescence measurements. The mean fluorescence ratio over the next 5 min was 0.786±0.052 ($n = 6$). After the addition of APIII ($10^{-7}$ M), the ratio did not change significantly (0.798±0.047). However, subsequent stimulation with the muscarinic cholinergic agonist bethanechol ($10^{-4}$ M), produced a transient increase in $Ca_i$ lasting 3–4 min with a peak ratio value of 0.860±0.065 ($n = 6$) ($P < 0.05$) occurring at 1 min (see Fig. 6 *B*). After this period, fluorescence ratios returned to pretreatment values.

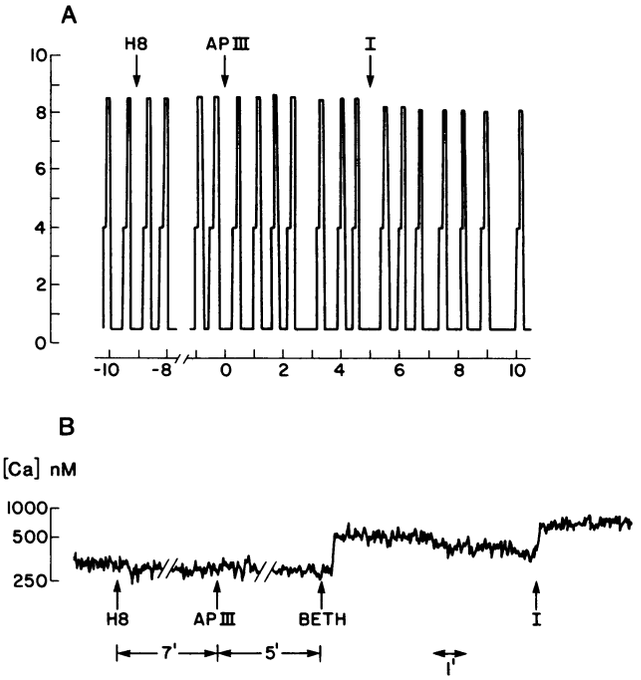


*Figure 6.* Representative tracings of H-8 effects on APIII-induced changes in $pH_i$ and $Ca_i$. (*A*) CF fluorescence intensity measured at excitation wavelengths 463 and 490 nm (see Fig. 2 for scale units). Cells were treated with H8 (50 μM) for 10 min, followed by APIII ($10^{-7}$ M) and ionomycin (I) ($10^{-6}$ M) for 5 min each. (*B*) Fura2 fluorescence intensity measured at a single excitation wavelength of 350 nm. Cells were treated with H8 (50 μM), followed by APIII ($10^{-7}$ M), bethanechol ($10^{-4}$ M), and ionomycin ($10^{-6}$ M).

*Table II. Effects of H-8 and Calmidazolium on 8-BR-cGMP and APIII-induced Changes in CF Ratio Fluorescence ($F_{490}/F_{463}$)*

| Basal | CM | H-8 | 8-Br-cGMP | I |
|---|---|---|---|---|
| 2.12±0.05 (6) | — | — | 2.07±0.04* | — |
| 2.03±0.02 (5) | 2.02±0.02 | — | 2.02±0.03 | — |
| 2.11±0.01 (3) | — | 2.11±0.01 | 2.11±0.01 | 2.06±0.01* |

| Basal | CM | H-8 | APIII | I |
|---|---|---|---|---|
| 2.05±0.03 (6) | — | — | 2.01±0.02* | — |
| 2.09±0.01 (3) | 2.11±0.01 | — | 2.11±0.01 | 2.12±0.01 |
| 2.09±0.02 (4) | — | 2.10±0.01 | 2.09±0.01 | 2.06±0.01* |

Cells were suspended in HBS. Basal ratios were determined from the average of measurements taken in the 2 min preceding treatments. Treatment ratios were determined from the average of measurements taken in the final 1 min of each treatment period. Treatment periods for various agents were as follows: CM ($10^{-7}$ M) for 5 min, H-8 (50 μM) for 10 min, 8-Br-cGMP ($10^{-4}$ M) for 5 min, APIII ($10^{-7}$ M) for 5 min, and ionomycin (I) ($10^{-6}$ M) for 4 min. Agents were added sequentially in the order above. $n$ = number of experiments.
* $P < 0.05$ compared to basal values by analysis of variance.

## Discussion

There have been few studies regarding the effects of ANF on intestinal epithelium. In teleost intestine, ANF has a direct inhibitory effect on salt absorption that appears to be mediated via cGMP (11, 12). However, in higher vertebrates, there are conflicting results as to ANF's effect on intestinal water and electrolyte transport and questions as to whether the effects reported are direct or mediated by neurons. Most of the studies on mammalian intestine were done in vivo or in vitro on intact tissue.

We utilized an isolated avian intestinal cell preparation to more directly study ANF's effects on sodium absorption in the intestine. Our method of cell isolation removes only the top layer of villus epithelial cells. Upon histologic examination of

the remaining tissue, the deeper villus and crypt cells are intact. Our cell preparation is composed of > 90–95% of epithelial cells when viewed under the microscope. No inflammatory cells such as neutrophils were seen. In addition, > 95% of the cells in our preparation stained positive for cytokeratin, a cytoskeletal protein found exclusively in epithelial cells (34), evidence for a nearly pure epithelial cell preparation.

Since we had previously shown that Na/H exchange is a major pathway for sodium entry into isolated chicken enterocytes (22), we first looked at whether ANF affects Na/H exchange. Both APIII and 8-Br-cGMP inhibited sodium uptake and decreased intracellular pH. Their inhibitory effects were nonadditive to those of amiloride, a known inhibitor of Na/H exchange. APIII has a dose dependent effect on changes in intracellular pH with an $ED_{50}$ of 0.3 nM, which is lower than that seen in other tissue systems (7), but in line with that determined in isolated and cultured cell systems (35, 36).

Others have reported that Na/H exchange is present in both brush border and basolateral membranes of rabbit ileum (37). Since only Na/H exchange at the intestinal brush border membrane contributes to transmural sodium transport, it was important to attempt to identify ANF's site of action in our isolated cells. To study this question we measured the effects of 8-Br-cGMP and MIBA on unidirectional sodium fluxes in intact chicken ileum. Mucosally applied MIBA and serosally applied 8-Br-cGMP inhibits only mucosal to serosal sodium fluxes, an effect that is not additive. Although the changes in MIBA-sensitive $J_{ms}^{Na}$ were small relative to total $J_{ms}^{Na}$, we hypothesize that the endogenous neurohumoral tone of the mucosal preparation may already account for a significant inhibition of Na/H exchange. Alternatively, it is possible that other MIBA-insensitive Na absorptive pathways also exist. No changes in short-circuit current were seen with either of these agents. Addition of glucose at the end of each experiment showed an increase in $I_{sc}$ which argues against these effects being due to inhibition of the sodium pump. Our flux results show that MIBA and 8-Br-cGMP inhibit Na/H exchange in the brush-border membrane of chicken intestine. Why there was not a greater inhibition of Na/H exchange by these agents in intact ileum is not clear.

ANF has been reported to affect sodium uptake pathways in other tissues. ANF directly inhibits sodium absorption in kidney medullary collecting duct (38) and cortical collecting duct (39). Cantiello and Ausiello (35) found that ANF inhibits an amiloride-sensitive conductive sodium channel but not Na/H exchange in the renal epithelial cell line LLC-$PK_1$. In vascular smooth muscle cells O'Donnell and Owen (40) showed that ANF stimulated Na/K/Cl cotransport. Whereas in rabbit aorta, ANF was found to stimulate Na/H exchange by a cGMP independent pathway (41). Recently a cGMP-gated cation channel has been reported in patch-clamp studies of the apical membrane of rat inner medullary collecting duct (42). These diverse findings suggest that ANF can affect more than one sodium transport pathway and the direction of its effect can vary.

APIII stimulated an increase in cGMP but not cAMP in our cells, consistent with reports by others that suggest ANF exerts its physiologic effects by increasing cGMP. We had independently observed that 8-Br-cGMP elevated intracellular calcium in chicken enterocytes and wondered whether calcium might also mediate the effects of ANF in our cells. Measurements of intracellular calcium show that both APIII and 8-Br-cGMP stimulate an identical slow increase in $Ca_i$ in our enterocytes. This increase in $Ca_i$ is not due to cell acidification. We have previously shown that transient acidification of our enterocytes with butyric acid does not increase $Ca_i$ (22). The best evidence that these slow calcium changes are by a unique pathway stimulated by cGMP is the fact that they can be completely blocked by H-8, yet subsequent addition of the muscarinic cholinergic agonist bethanechol to the same cell preparation produces a typical spiked increase in cell calcium. At low doses, H-8 selectively inhibits cGMP and cAMP-dependent protein kinase (32) and is nontoxic to tissue (33). Bethanechol transiently increases $Ca_i$ in chicken enterocytes through the activation of phosphatidylinositol-4,5-bisphosphate hydrolysis and the generation of inositol 1,4,5-trisphosphate (43). Calcium activation by cGMP in chicken enterocytes is likely to involve mechanisms distinct from those of phosphatidylinositol metabolism or increases in membrane calcium permeability, both of which cause a spike and plateau response.

The effects of APIII and cGMP on cytosol calcium concentration, along with the evidence that prevention of the rise in intracellular calcium blocks APIII and cGMP's inhibitory effects on Na/H exchange, implicates $Ca_i$ as the mediator for inhibition of Na/H exchange by these agents in chicken enterocytes. Although we can not completely exclude the possibility that the above effects are occurring in or secondary to contaminating cells, i.e., lymphocytes or mesenchymal cells, the percentage of nonepithelial cells in our preparation is very small, and it is doubtful that changes in pH or calcium occurring in these cells would be detected. Furthermore, increases in intracellular calcium in lymphocytes has been shown to stimulate Na/H exchange (44), which is opposite from what we observe.

In summary our results show that APIII and 8-Br-cGMP inhibit Na/H exchange in isolated chicken enterocytes by an increase in $Ca_i$, which appears to require cGMP-dependent phosphorylation. The mechanism by which this slow increase in intracellular calcium occurs is not known.

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