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Atrial Natriuretic Peptide Inhibits a Cation Channel in Renal Inner Medullary Collecting Duct Cells

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The patch-clamp technique was used to examine the effects of atrial natriuretic peptide (ANP) and its second messenger guanosine 3′,5′-monophosphate (cGMP) on an amiloride-sensitive cation channel in the apical membrane of renal inner medullary collecting duct cells. Both ANP ($10^{-11}M$) and dibutyryl guanosine 3′,5′-monophosphate ($10^{-4}M$) inhibited the channel in cell-attached patches, and cGMP ($10^{-5}M$) inhibited the channel in inside-out patches. The inner medullary collecting duct is the first tissue in which ANP, via its second messenger cGMP, has been shown to regulate single ion channels. The results suggest that the natriuretic action of ANP is related in part to cGMP-mediated inhibition of electrogenic Na^+ absorption by the inner medullary collecting duct.

ATRIAL NATRIURETIC PEPTIDE (ANP), a circulating hormone released from the atria of mammalian hearts in response to volume expansion, acts on the kidneys, adrenal glands, and vasculature to regulate fluid and electrolyte homeostasis (*1, 2*). The natriuretic and diuretic effects of ANP are mediated in part by inhibiting Na^+ and water absorption by renal inner medullary collecting ducts (IMCD) (*1–5*). Although this action of ANP has been linked to the intracellular second messenger cGMP (*1–4*), relatively little is known about the cellular mechanisms whereby ANP and cGMP reduce Na^+ absorption by the IMCD.

Electrogenic sodium absorption by the IMCD is a two-step process: passive diffusion across the apical cell membrane through an amiloride-sensitive conductive pathway and active extrusion across the basolateral membrane by a Na^+- and K^+-dependent adenosine triphosphatase (*6, 7*). In a recent patch-clamp study, we reported that electrogenic Na^+ uptake across the apical membrane of IMCD cells in primary culture and in the native epithelium is mediated by a cation channel (*6*). The channel was selective for monovalent cations (P_{Na^+}:P_{Cl^-} = 13:1), inhibited by micromolar concentrations of amiloride, and had a single channel conductance of 28 pS; however, the channel was not regulated by voltage. The present study on IMCD cells was conducted to determine whether ANP reduces Na^+ absorption by inhibiting the cation channel in the apical membrane.

Patch-clamp studies were conducted on rat IMCD cells grown in primary culture as described (*6*). The patch current was measured with a current-to-voltage converter (Yale Mark V design), low-pass filtered at 300 Hz, and digitized at 1 kHz with an IBM AT computer. Data were recorded for at least three 20-s periods (with a 30-s delay between each period) before and after each experimental condition to determine the probability of a single channel being open (P_o). P_o was estimated from the total time spent in the open state divided by the total time of the record (*6*). All experiments were conducted in a paired fashion. Data are reported as the mean ± SEM.

We first examined the effects of ANP on the cation channel in cell-attached patches of the apical membrane. Channels were spontaneously active in 10% of our experiments. We usually observed two or three channels in each patch; occasionally we saw as many as six active channels (Fig. 1A). Addition of

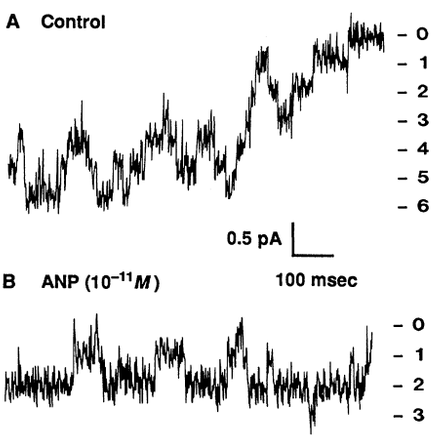

Fig. 1. Current records from a cell-attached patch of the apical membrane. (**A**) Control. Six channels were present in the patch; the single channel P_o was 0.70. (**B**) Three minutes after addition of ANP ($10^{-11}M$) to the bath [rat synthetic ANP, amino acids 1 to 28 (Sigma), the chief circulating form of ANP in rats (*32*)]. Simultaneous openings of more than three channels was not observed in the patch in the presence of ANP. The P_o decreased from 0.70 to 0.54. Vehicle had no effect on channel activity (n = 10). The command potential, Vc, in both records was +20 mV; the voltage across the membrane was the sum of Vc and the resting membrane voltage (~−60 mV). Voltage refers to the cytoplasmic side of the membrane referenced to the interior of the patch pipette. For patches with multichannel events, individual channels were found to be identical and independent by methods described by Palmer and Frindt (*33*). In addition, the number of channels and P_o were determined as described (*6, 33*). The numbers to the right of each current record indicate the number of open channels. Solutions: pipette (cell-attached and inside-out patches) and bath (cell-attached patches): NaCl, 140 m*M*; KCl, 5 m*M*; $CaCl_2$, 1 m*M*; $MgCl_2$, 1 m*M*; and Hepes, 10 m*M*; *p*H 7.4; bath (inside-out patches): NaCl, 5 m*M*; KCl, 140 m*M*; $CaCl_2$, 1 m*M*; $MgCl_2$, 1 m*M*; and Hepes, 10 m*M*; *p*H 7.4.

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ANP ($10^{-11}M$) to the bath solution of cell-attached patches reduced the P_o of the channel from 0.64 ± 0.08 to 0.44 ± 0.05 ($n = 4$, $P < 0.05$; Fig. 1B). A decline in channel activity was observed in every experiment, and the effect was evident within 2 to 4 min. The inhibition achieved a maximal level after 3 to 5 min and was independent of the command voltage (−80 to +60 mV). Maximum inhibition of the channel by ANP was achieved with $10^{-7}M$; P_o fell from 0.50 ± 0.17 to 0.05 ± 0.02 ($n = 4$; $P < 0.06$). The concentration for half-maximal inhibition of the channel by ANP was $1.4 \times 10^{-11}M$ (Lineweaver-Burk plot).

Cyclic GMP is a second messenger of ANP in a variety of cell types (*8, 9*). Because ANP increases intracellular levels of cGMP in the IMCD (*4, 10*), we conducted experiments to determine whether exogenous cGMP inhibited the cation channel. N^2,O^2-dibutyryl-cGMP (dB-cGMP; $10^{-4}M$), the membrane-soluble derivative of cGMP, rapidly (<30 s) inhibited channel activity when added to the bath. The P_o in cell-attached patches decreased from 0.58 ± 0.18 to 0.20 ± 0.15 ($n = 4$, $P < 0.05$). Thus, both ANP and cGMP inhibit the cation channel.

Because an increase in cGMP inhibited the cation channel, it follows that a reduction of intracellular cGMP should activate the channel. Accordingly, in our next experiment we added 6-anilino-5,8-quinolinedione (LY83583, $10^{-6}M$, Eli Lilly and Co.), a compound that lowers basal cGMP levels (*11–13*), to the bath solution of cell-attached patches (Fig. 2, A and B). This compound activated quiescent channels and increased the P_o from 0.00 to 0.26 ± 0.11 ($n = 7$, $P < 0.05$). Addition of dB-cGMP ($10^{-4}M$) to the bath completely reversed the activation of the channel by LY83583 ($n = 3$; Fig. 2C). These experiments indicate that ANP inhibits the cation channel in part by increasing intracellular levels of cGMP.

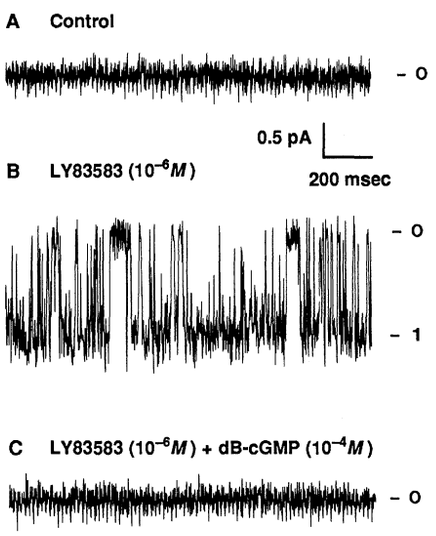

Fig. 2. Current records from a cell-attached patch of the apical membrane. (**A**) Control. Although there was a channel in the patch, it was inactive in control solutions. The P_o was 0.00. (**B**) Seven minutes after the addition of LY83583 ($10^{-6}M$) to the bath. This compound activated a quiescent channel. The P_o increased from 0.00 to 0.62. (**C**) Two minutes after the addition of dB-cGMP ($10^{-4}M$), in the presence of LY83583, to the bath. The channel was inhibited by dB-cGMP; P_o fell to 0.00. The command potential, Vc, was −60 mV in all records (voltage convention as described in Fig. 1). The numbers to the right of each record indicate the number of open channels.

Additional experiments were performed to determine whether cGMP also inhibited the channel in inside-out patches of the apical membrane. Although channels were frequently inactive in cell-attached patches they spontaneously activated upon patch excision. Cation channels were seen in 30% of the inside-out patches. Cyclic GMP ($10^{-5}M$) in the solution bathing the cytoplasmic surface of inside-out patches immediately and reversibly inhibited the channel (Fig. 3, A and B). The P_o fell from 0.70 ± 0.08 to 0.53 ± 0.11 ($n = 6$, $P < 0.02$). This inhibition was independent of the voltage across the membrane patch (−60 to +60 mV). Maximal inhibition of the channel by cGMP was achieved with $10^{-4}M$; P_o decreased from 0.72 ± 0.05 to 0.46 ± 0.07 ($n = 12$, $P < 0.01$). The concentration for half-maximal inhibition of the channel by cGMP was $6.8 \times 10^{-6}M$ (Lineweaver-Burk plot). The inhibition of the channel by cGMP was not reproduced by adenosine 3′,5′-monophosphate [$10^{-3}M$; $n = 8$ (*6*)].

Finally, because hydrolysis of cGMP releases energy that may induce a conformational change in the channel, we examined the effect of 8-bromo-cGMP (8-bromo-cGMP: $10^{-4}M$), a poorly hydrolyzable analog of cGMP (*14*). 8-Bromo-cGMP reduced the P_o from 0.58 ± 0.17 to 0.36 ± 0.14 ($n = 6$, $P < 0.05$). The percent inhibition was indistinguishable from equimolar concentrations of cGMP (see above).

These data show directly that ANP, via its second messenger cGMP, inhibits a cation channel in the apical membrane of IMCD cells in culture. Inhibition of this channel by ANP and cGMP would reduce electrogenic Na^+ absorption by the IMCD and contribute to the natriuresis induced by elevated levels of ANP. Because in our experiments ANP inhibited channel activity at a concentration that was within the physiological range for rats [2×10^{-11} to $15 \times 10^{-11}M$; (*15, 16*)], we conclude that ANP modulates sodium absorption under basal conditions. Our results are consistent with previous studies demonstrating that ANP inhibits

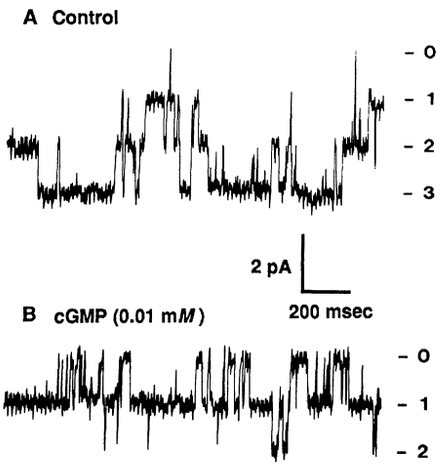

Fig. 3. Current records from an inside-out patch of the apical membrane. (**A**) Control. Three channels are evident; the P_o was 0.71. (**B**) Cyclic GMP ($10^{-5}M$) in the cytoplasmic solution. Although there were three channels in the patch, simultaneous openings were rarely observed. The P_o decreased to 0.34. The voltage across the patch was −60 mV; cytoplasmic side of the membrane negative referenced to the extracellular side of the membrane. The numbers to the right of each record indicate the number of open channels.

electrogenic Na^+ uptake by IMCD cells in suspension (*3*), and that ANP and cGMP inhibit electrogenic Na^+ absorption by LLC-PK1 renal epithelial cells (*17*). In addition, the data are in agreement with the reported half-maximal ($5 \times 10^{-11}M$) and maximal ($10^{-7}M$) inhibition by ANP of electrogenic $^{22}Na^+$ uptake in IMCD cells in suspension (*3*). Furthermore, our results are also consistent with reports demonstrating that ANP stimulates the intracellular production of cGMP in the IMCD (*4, 8, 10, 18*). Because LY83583 lowers intracellular cGMP and activates quiescent channels in cell-attached patches, we conclude that basal levels of cGMP inhibit channel activity.

The inhibition of the cation channel by cGMP in inside-out patches suggests that cGMP directly inhibits the channel protein. Cyclic GMP directly activates a nonselective cation channel in rod and cone photoreceptors and in olfactory cells (*19–24*). We cannot exclude the possibility, however, that additional cGMP-dependent mechanisms regulate the channel, especially in cell-attached patches. This possibility is supported by our finding that cGMP in cell-attached patches had a greater inhibitory effect on the channel than cGMP in inside-out patches. Cyclic GMP may also inhibit the channel by activating a cGMP-dependent protein kinase (*25, 26*), by modulating a G-protein that either directly or indirectly gates the channel (*27–31*) or by interacting with phospholipase C or protein kinase C (*29–31*).

Our data show that ANP, by its second messenger cGMP, inhibits a cation channel

in the apical membrane of renal IMCD cells. These data suggest that the natriuretic activity of ANP is related in part to cGMP mediated-inhibition of electrogenic Na^+ absorption by the IMCD.

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Molecular Cloning of Genes Under Control of the Circadian Clock in *Neurospora*

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To investigate the regulation of messenger RNA abundance by circadian clocks, genomic and complementary DNA libraries were screened with complementary DNA probes enriched, by means of sequential rounds of subtractive hybridization, for sequences complementary to transcripts specific to either early morning or early evening cultures of *Neurospora*. Only two morning-specific genes were identified through this protocol. RNA blot analysis verified that the abundance of the transcripts arising from these genes oscillates with a period of 21.5 hours in a clock wild-type strain and 29 hours in the long-period clock mutant strain *frq*^{7}. Genetic mapping through the use of restriction fragment length polymorphisms shows the two genes, *ccg-1* and *ccg-2*, to be unlinked. These data provide a view of the extent of clock control of gene expression.

CIRCADIAN RHYTHMS ARE GENERALly thought to be the output of an intracellular metabolic network (*1*). However, these clocks must also act to regulate cellular metabolism, one salient aspect of which is clock control of mRNA abundance. Historically, a great deal of effort has gone into attempts to elucidate the molecular mechanism of the biological clock (*2*); however, in general, little is known about the nature of temporal information within cells (*3*). Thus, in order to begin to understand and identify the pathways and molecular components involved in the transfer of temporal information within a cell, we have undertaken the systematic isolation of clock-controlled genes (morning- and evening-specific genes) from the ascomycete *Neurospora crassa*. These studies have identified only two genes that are strongly regulated by the clock as measured by mRNA abundance.

The protocol we used for the isolation of genes under the control of the clock is based on a liquid culture system developed for biochemical studies on the *Neurospora* clock (Fig. 1A) (*4*). Disks are cut from a mycelial mat of a single cell type growing in a rich medium. The mycelia of the mat, and of each disk cut from it, contain identical and synchronous clocks. To prevent production of conidia (*5*) and subsequent clock desynchronization, the disks are transferred en masse to a nutritionally poor medium in which they will remain alive but not grow rapidly or form conidia. When such disks are transferred from light to constant darkness, the clocks are all reset to subjective dusk circadian time 12 (CT12) (*6*) and will run from that point at their endogenous rate. In wild-type *Neurospora* the circadian cycle is 21.5 hours. We also used a mutant of the *Neurospora* clock, *frq*^{7}, with an endogenous period of 29 hours at 25°C. The only known phenotypes associated with the semidominant mutations at the *frq* locus, including *frq*^{7}, are those affecting aspects of the circadian clock (*7, 8*).

Our initial isolation of clock-controlled genes relied on the use of subtractive hybridization (*9*). Polyadenylated [poly(A)$^+$] RNA (*10*) was isolated from cultures held 23 hours and 34 hours in the dark, representing subjective evening and morning, respectively. These RNAs were used as templates for the synthesis of radiolabeled cDNAs (*11*), which were then hybridized and subtracted twice with an excess of RNA from the opposite time to remove cDNAs complementary to RNAs present at both phases of the clock cycle (*12*). The subtracted cDNAs resulting from this protocol thus represented putative time-specific probes and were used to probe both a cDNA and a genomic library.

As a result of the culture and subtractive hybridization protocols, we expected to identify (i) developmentally regulated genes displaying activity changes resulting from the interruption in the natural progression toward conidiation (*5*), (ii) environmentally responsive genes with mRNAs regulated in response to either starvation or abrupt shifts in the ambient light level (*13*), and (iii) clock-controlled genes with activity that cycled according to circadian clock phase. These three types of genes were distinguished in the following way (Fig. 1): Total cellular RNA was isolated every 4 hours for 2 days from wild-type [*frq*$^+$ (*14*)] and *frq*^{7} mycelial disks held in liquid culture in total darkness. In wild-type *Neurospora* this time period corresponds to approximately 2.5 circadian cycles, whereas in the long-period

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