D₁-like dopamine receptors downregulate Na⁺-K⁺-ATPase activity and increase cAMP production in the posterior gills of the blue crab Callinectes sapidus

Francis B. Arnaldo,1,2 Van Anthony M. Villar,2,3 Prasad R. Konkalmmatt,3 Shaun A. Owens,2 Laureano D. Asico,2,3 John E. Jones,2,3 Jian Yang,1 Donald L. Lovett,4 Ines Armando,2,3 Pedro A. Jose,2,3,5 and Gisela P. Concepcion1

1The Marine Science Institute, University of the Philippines, Diliman, Quezon City, Philippines; 2Department of Pediatrics, Georgetown University School of Medicine, Washington, District of Columbia; 3Division of Nephrology, Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland; 4Department of Biology, The College of New Jersey, Ewing, New Jersey; and 5Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland

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Arnaldo FB, Villar VM, Konkalmmatt PR, Owens SA, Asico LD, Jones JE, Yang J, Lovett DL, Armando I, Jose PA, Concepcion GP. D₁-like dopamine receptors downregulate Na⁺-K⁺-ATPase activity and increase cAMP production in the posterior gills of the blue crab Callinectes sapidus. Am J Physiol Regul Integr Comp Physiol 307: R634–R642, 2014. First published July 30, 2014; doi:10.1152/ajpregu.00555.2013.—Dopamine-mediated regulation of Na⁺-K⁺-ATPase activity in the posterior gills of some crustaceans has been reported to be involved in osmoregulation. The dopamine receptors of invertebrates are classified into three groups based on their structure and pharmacology: D₁- and D₂-like receptors and a distinct invertebrate receptor subtype (INDR). We tested the hypothesis that a D₁-like receptor is expressed in the blue crab Callinectes sapidus and regulates Na⁺-K⁺-ATPase activity. RT-PCR using degenerate primers, showed the presence of D₁βR mRNA in the posterior gill. The blue crab posterior gills showed positive immunostaining for a dopamine D₂ receptor (D₂R or D₁βR) antibody in the basolateral membrane and cytoplasm. Confocal microscopy showed colocalization of Na⁺-K⁺-ATPase and D₁βR in the basolateral membrane. To determine the effect of D₁-like receptor stimulation on Na⁺-K⁺-ATPase activity, intact crabs acclimated to low salinity for 6 days were given an intracardiac infusion of the D₁-like receptor agonist fenoldopam, with or without the D₁-like receptor antagonist SCH23390. Fenoldopam increased cAMP production twofold and decreased Na⁺-K⁺-ATPase activity by 50% in the posterior gills. This effect was blocked by coinfusion with SCH23390, which had no effect on Na⁺-K⁺-ATPase activity by itself. Fenoldopam minimally decreased D₁βR protein expression (10%) but did not affect Na⁺-K⁺-ATPase α-subunit protein expression. This study shows the presence of functional D₁βR in the posterior gills of euryhaline crabs chronically exposed to low salinity and highlights the evolutionarily conserved function of the dopamine receptors on sodium homeostasis. dopamine receptor; Na⁺-K⁺-ATPase; blue crab; cAMP; posterior gills

THE ATLANTIC BLUE CRAB, Callinectes sapidus, a euryhaline crustacean, must osmoregulate to survive in rapidly changing saline environments of estuarine habitats. It is considered to be a strong hyperosmoregulator that can maintain an almost constant hemolymph osmolality across a wide range of salinities (8). The change in Na⁺-K⁺-ATPase activity in the posterior gills, in response to changes in environmental osmolality, is one of several osmoregulatory mechanisms in euryhaline crustaceans (25, 33, 39, 43, 44). However, the regulatory pathways leading to modulation of Na⁺-K⁺-ATPase activity in posterior gills of euryhaline crustaceans are not fully understood. Dopamine has been shown to increase the Na⁺-K⁺-ATPase activity in posterior gills of crustaceans through a cAMP-dependent pathway (36). Invertebrates have three subfamilies of dopamine receptors, i.e., 1) the DOP1 subfamily, which is related to vertebrate D₁-like receptors; 2) the INDR subfamily, which is a distinct invertebrate group that functionally behaves like vertebrate D₁-like receptors; and 3) the invertebrate D₂-like receptor subfamily, which is related to vertebrate D₂-like receptors (38). Specifically in crustaceans, there are two types of D₁-like receptors, D₁αPAN and D₁βPAN (also termed D₁R and D₂R in humans, respectively). D₁-like receptors stimulate, while D₂-like receptors inhibit, adenylyl cyclase activity in vertebrates and invertebrates (6, 14). D₁-like receptors have been pharmacologically characterized in the crustacean Eriocheir sinensis (36); however, the role of this receptor subtype in the regulation of Na⁺-K⁺-ATPase in response to changes in environmental osmolality is still unclear.

In mammals, there are currently two paradigms of the D₁-like dopamine receptor effect on ion transport that act in opposite manner, depending on the cell type. In human lung epithelia, dopamine via D₁-like dopamine receptors increases sodium transport by stimulating the rapid recruitment of Na⁺-K⁺-ATPase from cellular endosomes to the basolateral membrane (5). In the proximal and distal tubules of the mammalian kidney, however, dopamine decreases ion transport by acting on D₁-like dopamine receptors to increase cAMP, which leads to the phosphorylation of Na⁺-K⁺-ATPase, resulting in its internalization and inactivation (2, 4, 9, 11, 22, 26). Altered arachidonic metabolism may result in the failure of dopamine to inhibit Na⁺-K⁺-ATPase (28). The objective of the current study was to test the hypothesis that D₁-like receptors are expressed in the posterior gills of the euryhaline blue crab C. sapidus and function to increase cAMP production to ultimately regulate Na⁺-K⁺-ATPase activity.

MATERIALS AND METHODS

Animals. Male blue crabs in intermolt were collected from the Annapolis area and Hoopers Island, Chesapeake Bay, MD, between June-October and housed at 25°C in filtered recirculating tanks...
containing dilute (10 parts per thousand (ppt) salinity) or full-strength (32 ppt salinity) artificial seawater (Instant Ocean, Blacksburg, VA) (20). Crabs weighed between 110 and 230 g and had carapace widths from 11 to 15 cm. The crabs were fed once daily with a diet consisting of processed oysters and dried pellet food. The crabs were exposed to a 12:12-h light-dark photoperiod and, after exposure to dilute seawater for 6 days, examined before experimentation. This duration of exposure was adequate to stimulate the hypoosmotic response in the crabs and to upregulate expression of Na\(^+\)-K\(^+\)-ATPase in the epithelial cells of the gills (33).

**Drug infusion.** Crabs undergoing drug infusion were removed from the aerated tanks containing 10 ppt artificial seawater on day 5 of acclimation (32, 33), and a 2-mm hole was drilled through the carapace directly above the heart cavity, as described by Burnett et al. (7). The drill-bit was pressed onto the carapace to create a depression deep enough to allow needle-stick penetration but not cause any bleeding. Latex rubber and cyanoacrylate adhesive were used to cover the depression to prevent any hemolymph bleed out caused by the puncture. The crabs were allowed to recover for 24 h before the study. Subsequently, vehicle (137 mM NaCl, 3 mM KCl, 5 mM MgSO\(_4\), and 3 mM HEPES, pH 7.4) that is isosmotic with the crab’s hemolymph, with or without drugs (1 \(\mu\)M fenoldopam and 5 \(\mu\)M SCH23390), was infused directly (0.1 ml/min for 15 min) into the heart via an 18-gauge needle connected to an infusion pump. Initial experiments using lissamine green directly infused into the heart showed that the gills were fully perfused within 5 min. The fenoldopam (1 \(\mu\)M) and SCH23390 (5 \(\mu\)M) doses in our studies were based on studies in rats in which the drugs were infused directly into the renal artery (18, 53). These doses were lower than those used in the shore crab *Chasmagnathus granulatus* to avoid targeting other receptors, e.g., serotonin receptors (12, 35), which may occur when higher doses are used. The perfusion rate of 0.1 ml/min used was the same infusion rate used to perfuse the gills of *C. granulatus* (21). A drug infusion period of 15 min was chosen because the D\(_1\)-like receptor was phosphorylated and internalized into the cell cytoplasm following 15 min of exposure to dopamine in human embryonic kidney cells heterologously expressing the rat D\(_1\)R (41).

**Whole gill homogenate.** The crabs were anesthetized by being put in ice for 20 min and then killed by carapace removal. Gills 6 and 7 were excised to represent the anterior gills. The gills were homogenized in ice-cold buffer (250 mM sucrose, 2 mM EDTA, and 50 mM imidazole, pH 7.2) for Na\(^+\)-K\(^+\)-ATPase activity and cAMP production assays or lysis buffer (1% Triton X, 0.1% SDS, and 0.5% sodium deoxycholate) for immunoblotting studies. A protease inhibitor cocktail (10 mM AEBSF, 1 mM trypsin, and 10 mM PMSF) was added to prevent proteolysis. Saponin (20 \(\mu\)g/ml protein) was used to permeabilize the membranes to maximize substrate accessibility for the endogenous Na\(^+\)-K\(^+\)-ATPase. The crude homogenates were partially purified by centrifuging at 10,000 \(g\). The final protein concentration (BCA kit; Pierce, Rockford, IL) of each supernatant was adjusted to 1.0–1.5 mg/ml before storage at −80°C for subsequent studies.

**Immunoblotting.** Samples of uniform amounts of protein were resolved via 10% SDS-PAGE (Invitrogen, Carlsbad, CA). Proteins were electrophoresed onto nitrocellulose membranes (Bio-Rad, Hercules, CA) using a wet transfer apparatus (Invitrogen) and subjected to immunoblotting, as reported previously (19, 52, 54). The primary antibodies used were rabbit polyclonal anti-human D\(_1\)-R (Genex, San Antonio, TX), mouse monoclonal anti-chicken Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunit antibody and immunogen were kindly provided by Dr. Deborah J. Baro, Georgia State University. Donkey anti-mouse and goat anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used. The mouse monoclonal anti-chicken Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunit antibody has been used successfully for a range of arthropods, including *C. sapiens* (34, 48). The mouse monoclonal anti-actin antibody is reactive to many species, including *Drosophila*. The published immunogen for actin shares 71% sequence identity with that of *Drosophila* and 79% with that of *Callinectes*.

The bands were visualized using an enhanced chemiluminescence detection kit (Millipore, Billerica, MA) or by IR Western blot detection via the Odyssey Imager (Li-COR, Lincoln, NE) and quantified by densitometric scan (Scion Image, Frederick, MD). Actin was used as the housekeeping protein. To determine the D\(_3\)R epitope specificity, the immunoblots were incubated in antibody solution preincubated with or without the blocking peptide (cat. no. GTX77969; Genetex, San Antonio, TX). The company has not disclosed the immunogen sequence but revealed that a pairwise alignment of the sequence of the immunogen and that of *Panulirus interruptus* D\(_1\)R (the closest known homolog) showed 28% sequence identity. Total cell lysates from HEK-293 cells heterologously expressing the human D\(_3\)R and total gill homogenates from the spiny lobster *P. interruptus* that were supplemented with a protease inhibitor cocktail were used as positive controls.

**Degenerate primers and RT-PCR.** Forward degenerate primers were designed using the amino acid sequences YHIKDK (forward primer: 5′-tayachayathaaargay-3′) of the D\(_1\)-like receptors from the seven arthropods phylogenetically closest to the blue crab. Of these species, the crustacean *P. interruptus* is closest to the blue crab. Amino acid sequence alignment of D\(_3\)R from *Apis mellifera* (NP_001011595), *Bombbyx mori* (NP_001108459), *Anopheles darlingi* (XP_315207), *Ixodes scapularis* (XP_002409287), *Acrystohypnion pismum* (XP_001947683), *P. interruptus* (DQ295791), and *Peneaus monodon* (JQ901712) showed that the sequence YHIKDK is highly conserved in the D\(_3\)R of these seven species. Four reverse primers of 20–23 basepairs (23-bp reverse primer; 5′-GGGGTTTGTGGAGCT-TGAGGCGTG-3′) varying in their sizes at the 3′-end were derived from the 100-bp nucleotide sequence of the D\(_3\)R of the *Celula pagulator* (http://www.genome.ou.edu/FiddlerCrab_Illumina_seqs/A4_B1_4998828_100bp). RNA was isolated from the crab posterior gills using the RNeasy mini kit (Qiagen, Valencia, CA), reverse transcribed and amplified via PCR using four reverse primers mixed in equimolar concentration (1 \(\mu\)M) in combination with the forward primer (1 \(\mu\)M). The PCR products were resolved in 2% agarose gel and visualized under ultraviolet light after staining with ethidium bromide. The PCR amplicon was purified from the agarose gel and cloned into pcDNA3-TOPO cloning vector for further colony PCR and sequence analyses.

**Immunostaining and confocal microscopy.** Crabs were acclimated to 32 ppt salinity for 6 days before death and excision of gills 6 and 7. The gills were perfused with PBS then washed and stored in the histological fixative HistoChoice (AMRESCO, Solon, OH) for 3 days at 4°C. Four-millimeter-thick sections of the posterior gills were prepared for microscopy. For immunohistochemistry, the sections were incubated with either anti-D\(_3\)R antibody or anti-Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunit antibody for 2 h at room temperature. After being washed with PBS, the sections were incubated with donkey anti-mouse and goat anti-rabbit secondary antibodies for 1 h at room temperature. The biotinylated secondary antibodies were visualized using the ABC complex kit (Pierce) and Vector VIP (Vector Laboratories, Burlingame, CA), counterstained with hematoxylin (Sigma-Aldrich), and viewed with a Nikon E600 digital microscope.

For confocal microscopy, the slides were double immunostained with rabbit anti-D\(_3\)R antibody and mouse anti-Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunit antibody for 2 h at room temperature and probed with goat anti-rabbit (H+L)-Alexa Fluor 488 (Molecular Probes, Carlsbad, CA) and goat anti-mouse (H+L)-Alexa Fluor 568 (Molecular Probes) antibodies for 30 min. After being washed with PBS twice and distilled water once, the coverslips were mounted on glass slides using Vectashield mounting medium and sealed with nail polish. Negative
controls were likewise prepared but with the omission of the primary antibodies. Colocalization of D1βR and the Na+/K+-ATPase α-subunit was evaluated by laser scanning confocal microscopy, using an Olympus Fluoview FV300 inverted microscope using 450-nm excitation and 535-nm emission filters for Alexa Fluor 488- and 560-nm excitation and 645-nm emission filters for Alexa Fluor 568. Images were overlaid using Olympus Fluoview FV300 version 3C Acquisition Software to determine colocalization.

**cAMP assay.** Whole gill homogenates were pipetted into plate wells containing cAMP-specific rabbit antibody binding sites and competitive cAMP-acetylcholinesterase (AChe) conjugate (Cayman Chemical, Ann Arbor, MI). The plate was incubated overnight at 4°C in the dark for 2 h at room temperature. cAMP was quantified (412 nm) using a spectrophotometer (ThermoFisher Scientific). cAMP content of the samples was calculated with known standards and expressed as picomoles of cAMP per milligram of protein.

Measurement of Na+/K+·ATPase activity. Na+/K+·ATPase activity was measured (26) in an incubation buffer containing 140 mM NaCl, 5 mM KCl, 5 mM MgCl2, 30 mM Tris-HCl, 1 mM EGTA, 3 mM Na2ATP, and [γ-32P]ATP (2–5 Ci/mmol) in tracer amounts (5 nCi/μl), using a 15-min incubation period at 37°C in the presence or absence of 2 mM ouabain, a Na+/K+·ATPase inhibitor. When ouabain was present, NaCl and KCl were omitted from the incubation buffer. The reaction was initiated by the addition of 10 μl of homogenate to 90 μl of incubation buffer. The reaction was terminated by the addition of activated charcoal/trichloroacetic acid and rapid cooling on ice. After 1 h, the samples were centrifuged for 4 min at 14,000 rpm to separate the charcoal that contained the unhydrolyzed nucleotide. Radioactivity present in the supernatant containing the inorganic [γ-32P] produced by ATPase activity was measured in quadruplicate using a liquid scintillation spectrometer. Na+/K+·ATPase activity was calculated by subtracting the ouabain-insensitive ATPase activity from the total ATPase activity and expressed as millimoles of Pi per milligram of protein per hour.

**Chemicals.** Fenoldopam bromide and SCH23390 were purchased from Sigma-Aldrich. [γ-32P]ATP was purchased from Perkin-Elmer Life Sciences. All other chemicals were obtained from Sigma-Aldrich.

**Statistical analysis.** Numerical data are expressed as means ± SE. Data were analyzed using SigmaStat (Systat Software). Unpaired Student’s t-test was used to compare two different experimental groups. One-way ANOVA followed by Holm-Sidak post hoc test was used to compare more than two experimental groups. Results were considered significant when P < 0.05.

**RESULTS**

Expression of D1-like receptors in gills of the blue crab. Various in-house and commercially available antibodies against D1-like dopamine receptors were tested on blue crab gill homogenates. A rabbit polyclonal antibody generated against the human D3R (GeneTex antibody) was used on samples from HEK-293 cells stably expressing the human D3R (HEK-hD3R) and on gills from the spiny lobster (P. interruptus) and the blue crab (C. sapidus) (Fig. 1A). Three bands were visualized in the dark for 2 h at room temperature. cAMP was quantified (412 nm) using a spectrophotometer (ThermoFisher Scientific). cAMP content of the samples was calculated with known standards and expressed as picomoles of cAMP per milligram of protein.

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HEK-hD3R that correspond to glycosylated D3R (~125 kDa), native D3R (~55 kDa), and presumably degradation products (~30–35 kDa). In the lobster homogenate, three bands were visualized using the same antibody, i.e., ~125-kDa band, a faint 55-kDa band, and ~45-kDa band, and two bands were visualized in the blue crab homogenate, i.e., ~125- and 55-kDa band. An antibody raised against the lobster anti-D1βR (14) was able to visualize ~55- and ~30-kDa bands in the HEK-hD5R lysate, a single ~55-kDa band in the lobster homogenate, and ~120- and 55-kDa bands in the crab homogenate. All of these bands disappeared when the corresponding immunogen was added into the antibody solution. It is conceivable that the 55-kDa band in the crab homogenate, which was visualized using both the GeneTex and the anti-ligand antibodies, represents the crab D1βR, the homolog of the human D3R. We next evaluated the expression of D1βR in both anterior and posterior gills of the blue crab and found that it was expressed in both structures (Fig. 1B). The ~55-kDa band was not visualized when the immunizing peptide was added. RT-PCR of the cDNA prepared from the posterior gills amplified a predicted ~130-bp amplicon (Fig. 1C). Colony PCR and sequencing revealed that the cloned amplicon contained 125 base pairs (Fig. 1D). The amino acid sequence derived from the nucleotide sequence displayed 63 and 65% identity with corresponding regions of the D1βR from P. interruptus and P. monodon, respectively (Fig. 1E). These results suggest that the D1-like receptor is expressed in the gills of the blue crab and shares structural similarities with the arthropod and the rodent D1βR and the human D3R (6, 14).

The D1βR expression in the posterior gills was not different between crabs that were transferred to dilute seawater (10 ppt salinity) and crabs in full-strength seawater (32 ppt salinity) for 6 days (96 ± 6 vs. 100 ± 5%, respectively; Fig. 2). The same observation was made in the anterior gills (95 ± 0.2 vs. 100 ± 4%). Staining of the anterior gills was more pronounced closer to the tip of the lamellae. Scale bar = 50 μm.

ATPase α-subunit (Fig. 3A) and a D1βR (Fig. 3, B and C) in cells of the osmoregulatory patch along the central stem of the posterior gills. Staining for both proteins was more evident closer to the central stem compared with the outer edge of the lamellae. The Na+/K+-ATPase α-subunit staining was primarily in the basolateral membrane (Fig. 4A), while the D1-like dopamine receptor staining was in the basolateral membrane, as well as the cytoplasm (Fig. 4C). The normal morphology of the posterior gills is shown in the negative control images.

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**Fig. 2.** Effect of salinity on the expression of D1βR in posterior gills. Blue crabs (*C. sapidus*) were transferred to artificial seawater with either 32 parts per thousand (ppt) or 10 ppt salinity for 6 days. Gills 6 and 7 were excised and the whole homogenate was prepared, resolved via 10% SDSPAGE, and probed for D1βR and actin. D1-like dopamine receptor expression, normalized to actin, was not significantly different (P > 0.05) between 32 ppt and 10 ppt. Student’s t-test, n = 5/group. Immunoblots for each treatment are shown above the corresponding bar graphs. Numerical data are expressed as means ± SE.

**Fig. 3.** Immunohistochemistry of the posterior gills. Posterior gill sections of the blue crab *C. sapidus* in artificial seawater (10 ppt salinity) for 6 days were incubated with anti-chicken Na+/K+-ATPase α-subunit (A) or anti-human D3R dopamine receptor antibody (B and C), probed with donkey anti-mouse secondary antibody, and counterstained with hematoxylin. Both Na+/K+-ATPase α-subunit staining and D1βR staining were more pronounced closer to the central stem than towards the tip of the lamellae. Scale bar = 50 μm.

**Fig. 4.** Immunohistochemistry of the posterior gills. Posterior gill sections of the blue crabs *C. sapidus* transferred to artificial seawater (10 ppt salinity) for 6 days. A: sections incubated with anti-chicken Na+/K+-ATPase α-subunit. B: negative control (no primary antibody). C: sections incubated with anti-human D3R antibody. D: negative control (no primary antibody). All sections were probed with donkey anti-mouse secondary antibody and counterstained with hematoxylin. Both Na+/K+-ATPase α-subunit and D1βR were primarily localized in the basolateral membranes of the epithelial cells. Scale bar = 50 μm.
within the osmoregulatory patch and in cells near the tips of the lamellae. Scale bar from the central stem to the tip of the lamellae (Fig. 5).

membrane as well as the cellular junctions and was observed activity was due to D1-like dopamine receptors, the D1-like Pi·mg protein

bottom left). Differential interference contrast (DIC) images were also obtained to show the boundaries (bottom right). Colocalization was observed both in cells within the osmoregulatory patch and in cells near the tips of the lamellae. Scale bar = 50 um.

(FIG. 4, B and D). The cells found at the osmoregulatory patch are larger than the other cells in the posterior gills. These cells also have substantially more mitochondria and possess most of the Na⁺-K⁺-ATPase activity in response to D₁-like receptor stimulation (Fig. 5).

Effect of D₁-like receptor stimulation on D₁βR and cAMP levels. Posterior gills of crabs infused with fenoldopam (15 min) showed a slight decrease in D₁βR (55 kDa) compared with control (100 ± 1 vs. 87 ± 2%; P < 0.05; Fig. 6). Infusion with fenoldopam significantly increased cAMP production, relative to vehicle infusion (42.2 ± 2.5, n = 4, vs. 21.0 ± 3.2 pmol cAMP/mg protein). To show that the effect was due to D₁-like dopamine receptors, crabs were infused first with the D₁-like receptor antagonist SCH23390 for 15 min, followed by fenoldopam. SCH23390 abolished the stimulatory effect of fenoldopam (21.2 ± 5.5 pmol cAMP/mg protein). SCH23390 did not have a significant effect when infused alone (19.5 ± 1.9 pmol cAMP/mg protein; Fig. 7).

Na⁺-K⁺-ATPase activity in response to D₁-like receptor stimulation. Posterior gills of crabs infused with fenoldopam showed a significant decrease in Na⁺-K⁺-ATPase activity compared with vehicle infusion [fenoldopam = 13.3 ± 0.9 P₁·mg protein⁻¹·h⁻¹ vs. vehicle (control) = 24.6 ± 1.9 μmol P₁·mg protein⁻¹·h⁻¹; Fig. 8]. To show that the decrease in activity was due to D₁-like dopamine receptors, the D₁-like receptor antagonist SCH23390 was infused alone or with fenoldopam. The infusion of SCH23390 alone did not affect the activity of Na⁺-K⁺-ATPase (SCH23390 = 22.8 ± 1.2 μmol P₁·mg protein⁻¹·h⁻¹) but abolished the decrease in the activity elicited by fenoldopam infusion (SCH23390 + fenoldopam = 23.4 ± 2.0 μmol P₁·mg protein⁻¹·h⁻¹; Fig. 8).

There were no differences in the ouabain-insensitive ATPase activity among the groups, indicating that the activity of ATPases other than Na⁺-K⁺-ATPase was similar in all groups (data not shown). Fenoldopam infusion did not produce any change in the expression of the Na⁺-K⁺-ATPase α-subunit (Fig. 9), indicating that the decrease in Na⁺-K⁺-ATPase activity was not related to the decreased expression of the protein.

Fig. 5. Colocalization of Na⁺-K⁺-ATPase α-subunit and D₁βR in posterior gills. Posterior gill sections of the blue crabs C. sapidus were immunostained for Na⁺-K⁺-ATPase α-subunit (Alexa 488, green) and D₁βR (Alexa 568, red). Colocalization of both proteins is denoted by the yellow areas in the overlay image (bottom left). Differential interference contrast (DIC) images were also obtained to show the boundaries (bottom right). Colocalization was observed both in cells within the osmoregulatory patch and in cells near the tips of the lamellae. Scale bar = 50 um.

Fig. 6. Effect of fenoldopam on the expression of D₁βR. Blue crabs (C. sapidus) in artificial seawater (10 ppt salinity) for 6 days were infused with vehicle plus fenoldopam or with vehicle alone (control). Posterior gills 6 and 7 were excised, and whole gill tissue homogenate was prepared, resolved via 10% SDS-PAGE, and probed with polyclonal D₁R antibody and with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase. Bands were visualized using chemiluminescence. Actin was used as housekeeping protein. Numerical data are expressed as means ± SE. Fenoldopam-infused crabs showed a small, but significant, decrease in expression compared with control (0.81 ± 0.02 vs. 0.74 ± 0.02, n = 5/treatment). *P < 0.05 vs. control, Student’s t-test. Immunoblots for each treatment are shown above the corresponding bar graphs.
two novel techniques to study the physiological response of the gills. Previous studies have indicated the presence of a dopamine receptor in crustaceans (14, 21, 23, 37, 46). However, among crustaceans, it is only in the spiny lobster *Panulirus interruptus* (SCH) a D₁DR antagonist, which by itself had no effect, blocked the stimulatory effect of fenoldopam (SCH + Fen) on cAMP levels, *P* < 0.05 vs. all other treatments, one-way ANOVA followed by Holm-Sidak posttest, *n* = 3/treatment.

**DISCUSSION**

This study reports the presence of a D₁-like dopamine receptor, which is most likely the D₁βR, in the posterior gills of the Atlantic blue crab *Callinectes sapidus*. When D₁-like dopamine receptors were stimulated with the D₁-like receptor agonist fenoldopam in intact crabs, the posterior gills responded with an increase in cAMP production and a decrease in Na⁺K⁺-ATPase activity without a change in expression of its α-subunit. Previous studies have indicated the presence of a dopamine receptor in crustaceans (14, 21, 23, 37, 46). However, among crustaceans, it is only in the spiny lobster *P. interruptus* that two D₁-like receptors, the D₁αR and D₁βR, have been described so far (14). In this study, an ~55-kDa band was visualized in the gills, which conceivably corresponds to the crab D₁βR. RT-PCR on cDNA prepared from the posterior gills also indicated the presence of D₁βR transcript.

Compared with previous studies, the present study employed two novel techniques to study the physiological response of the dopamine receptor in the posterior gills of blue crabs. First, the experiments in the current study were performed on living crabs and not on isolated gills. It is difficult to examine the in vivo effects of perfused drugs in isolated gills of *C. sapidus* since properly securing a closed circuit system necessitates clamping of the base of the gill where a large portion of the ion-transporting cells are located (1, 32). Second, this study used a continuous drug infusion technique that allows for continuous bioavailability of the drug and a more stable pharmacokinetic profile (17, 49). In contrast, previous studies have employed the technique of administering a single bolus of a pharmacological agent into the crabs (37, 44, 45).

The dopamine receptor and the sodium pump colocalized along the basolateral membrane, especially in cells within the osmoregulatory patch. In addition to showing a potential for the interaction between the sodium pump and the dopamine receptor, these results suggest that the dopamine receptor may also play an important role in the regulation of sodium uptake in the posterior gills that may be associated with the osmoregulatory response. Acute treatment with the D₁-like receptor agonist fenoldopam resulted in a slight decrease in D₁βR expression, which may be an acute compensatory mechanism to osmoregulate in lower salinities. In addition, the decrease in expression may indicate receptor degradation, similar to what occurs in the mammalian system (31).

Previous studies in crustaceans have shown that the D₁αR (D₁R in mammals) is coupled to the stimulatory G protein G₁α, while the D₁βR (D₁R in mammals) is coupled to stimulatory G₃α and G₅α, both of which increase cAMP production (14). In contrast, the D₂αPanR, a D₂-like dopamine receptor, is coupled to inhibitory G₁α/G₁β, which decreases cAMP production (13, 14). Thus changes in the level of intracellular cAMP can be used to monitor the physiological response of the dopamine receptors upon agonist activation. In the current study, the stimulation of the D₁-like receptors using fenoldopam led to an increase in cAMP production, indicating that the D₁-like receptor was functional and that its signal transduction pathway was intact in the posterior gills.

Treatment with fenoldopam led to a decrease in Na⁺K⁺-ATPase activity without a concomitant change in the expression of the Na⁺K⁺-ATPase-α-subunit expression. The Na⁺K⁺-ATPase expression data suggest that the decrease in activity was not due to the degradation of the transporter but rather to an alteration of the intrinsic conformation of the sodium pump, resulting in its internalization and inactivation. It is conceivable that the activation of Na⁺K⁺-ATPase observed in previous studies using high concentrations of dopamine, fenoldopam.
fenoldopam, and SCH23390 is not due to the activation of the D1-like dopamine receptors alone but to the concomitant activation of other receptors, e.g., serotonin receptors (vide infra). The role of D2-like receptors in the osmoregulation of euryhaline crustaceans should be also considered because it has been shown that the perfusion of their posterior gills with sipperone and domperidone, two potent D2-like dopamine receptor antagonists, blocked the ability of dopamine to increase the transepithelial potential difference (21). Previous studies have mostly used the natural ligand for the dopamine receptors, dopamine, as agonist; hence, the activation of the D2-like dopamine receptors is likely to occur in this context since dopamine is able to activate Gαs, Gαq, and Gαi in crustaceans (13, 14). Under certain circumstances, the mammalian D1R, a D2-like dopamine receptor, can be linked to Gαs (40). Thus it is possible that an increase or a decrease in cAMP content can ensue after dopamine activation, depending on which cascade dominates the response. However, what regulates the eventual response must be determined.

Previous studies have shown that an increase in cAMP production in the osmoregulatory gills of euryhaline crustaceans is associated with an increase in Na+-K+-ATPase activity and a transient activation followed by a subsequent inhibition of transepithelial potential difference (21, 23, 44). These authors suggested that the transient increase in cAMP content was responsible for Na+-K+-ATPase stimulation. However, direct proof of this hypothesis has not been forthcoming. It would be interesting to determine whether D1- and D2-like receptors interact in the regulation of Na+-K+-ATPase in crustacean gills. This occurs in mammalian renal tubule cells under conditions of moderate extracellular fluid expansion, where the D2-like receptors act synergistically with the D1-like receptors to increase sodium excretion by inhibiting Na+-K+-ATPase activity (3, 16, 29).

In mammals, higher concentrations of dopamine can activate nondopamine receptors, e.g., serotonin and adrenergic receptors. There are studies showing that D1-like receptor drugs, such as fenoldopam and SCH23390, have high affinities for serotonin receptors (12, 35), a possibility with the high concentrations (10 μM) used in previous reports. Several crustacean serotonin receptors, which also increase cAMP production upon stimulation, have been reported in the somatogastric ganglion but not in the gills; these crustacean serotonin receptors are not pharmacologically similar to vertebrate serotonin receptors (14, 46, 47). Octopamine (OA), a biogenic monoamine structurally related to norepinephrine, acts as a neurotransmitter, neuromodulator, and neurotransmitter in invertebrates, including the crustaceans. Activation of the OA receptors, which are similar to the mammalian adrenergic receptors, also invariably results in increased cAMP levels (42). These receptors, if proven to be present in the posterior gills, could conceivably account for the previous observations subsequent to the treatment with dopamine.

Perspectives and Significance

In summary, we have provided evidence for the presence of dopamine receptors, presumably the D1BR, which colocalize with the Na+-K+-ATPase at the basolateral membrane of the cells that populate the osmoregulatory patch of the posterior gills of the blue crab. While we have shown that agonist stimulation of these receptors resulted in diminished receptor abundance, increased cAMP production, and decreased sodium transport via the inhibition of Na+-K+-ATPase activity, the details of the molecular mechanisms involved in this process have yet to be established. The inhibition of Na+-K+-ATPase activity by the D1-like dopamine receptors appears to have been conserved in phylogenetically distinct organisms. In mammalian renal tubule cells under conditions of moderate sodium excess, the D1-like dopamine receptors are responsible for decreasing renal sodium transport by at least 50% (10, 18, 24, 50, 51). Mammalian renal tubule cells can adapt in an environment of low (50 mosmol/kg H2O) to high osmolality (1,200 mosmol/kg H2O) (30), similar to conditions to which euryhaline crustaceans are exposed during their migration from low- to high-salinity water. TonEBP (30), which plays an important role in the renal tubular response to hypertonicity, stimulates the renal proximal tubule production of dopamine (27). A blueprint for the regulation of sodium transport in specialized tissues is shared by various organisms belonging to various phyla; however, unique variations in terms of novel interacting proteins or novel functions for the same protein exist to allow the organisms to thrive in their own biological niche. It would be interesting to determine if the D1- and D2-like receptors interact in the regulation of Na+-K+-ATPase in crustacean gills and establish the cross talk between dopamine and other hormones in osmoregulation. Understanding the underlying mechanisms may shed light on the evolution of the pathways involved in ionic and osmotic regulation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

D₂-LIKE DA RECEPTORS INHIBIT THE BLUE CRAB SODIUM PUMP


