Volume-activated trimethylamine oxide efflux in red blood cells of spiny dogfish (Squalus acanthias)

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Koomoa, Dana-Lynn T., Mark W. Musch, Ainsley Vaz MacLean, and Leon Goldstein. Volume-activated trimethylamine oxide efflux in red blood cells of spiny dogfish (Squalus acanthias). Am J Physiol Regulatory Integrative Comp Physiol 281: R803–R810, 2001.—The aims of this study were to determine the pathway of swelling-activated trimethylamine oxide (TMAO) efflux and its regulation in spiny dogfish (Squalus acanthias) red blood cells and compare the characteristics of this efflux pathway with the volume-activated osmolyte (taurine) channel present in erythrocytes of fishes. The characteristics of the TMAO efflux pathway were similar to those of the taurine efflux pathway. The swelling-activated effluxes of both TMAO and taurine were significantly inhibited by known anion transport inhibitors (DIDS and niflumic acid) and by the general channel inhibitor quinidine. Volume expansion by hypotonicity, ethylene glycol, and diethyl urea activated both TMAO and taurine effluxes similarly. Volume expansion by hypotonicity, ethylene glycol, and diethyl urea also stimulated the activity of tyrosine kinases p72syk and p56lyn, although the stimulations by the latter two treatments were less than by hypotonicity. The volume activations of both TMAO and taurine effluxes were inhibited by tyrosine kinase inhibitors, suggesting that activation of tyrosine kinases may play a role in activating the osmolyte effuxes. These results indicate that the volume-activated TMAO efflux occurs via the organic osmolyte (taurine) channel and may be regulated by the volume activation of tyrosine kinases.

TRIMETHYLAMINE OXIDE (TMAO) is present in the tissues of most animals with high concentrations found in marine animals (13). Spiny dogfish (Squalus acanthias) maintain high concentrations of TMAO in both extracellular (>50 mM) and intracellular (up to 200 mM) fluids (12). Dogfish maintain osmotic balance with the marine environment by retaining extracellular TMAO along with urea and NaCl. The regulation of extracellular TMAO occurs mainly in the dogfish kidney (7). It is not clear, however, how intracellular TMAO is regulated. The high concentrations of intracellular TMAO suggest that the osmolyte functions in cell volume regulation in the dogfish.

There have been few studies on cell membrane transport of TMAO and its regulation in elasmobranchs or in other species. In skate red blood cells (RBCs), previous studies have shown that [14C]TMAO uptake occurs via a sodium-dependent pathway as well as a volume-activated sodium-independent pathway (24). Preliminary studies suggest that in dogfish RBCs, the efflux of TMAO is stimulated by hypotonic volume expansion (24). The purpose of the present study was to expand on the latter finding by doing a detailed investigation on the mechanism of TMAO efflux in dogfish RBCs and its activation by volume expansion. Our findings suggest that TMAO efflux in dogfish RBC occurs via a volume-activated pathway similar to that reported for taurine in other fish RBC (18). Volume activation of TMAO efflux occurs after hypotonic volume expansion, isotonic volume expansion with non-electrolytes, but not with isotonic expansion with electrolytes. Volume activation of tyrosine kinases accompanies, and possibly regulates, the activation of TMAO efflux under these conditions.

MATERIALS AND METHODS

Animals and facilities. Spiny dogfish (Squalus acanthias) were obtained from the Mount Desert Island Biological Laboratory at Salisbury, ME. The dogfish were maintained in seawater tanks at 15°C for no longer than 10 days.

Media. Isosmotic 940 mosmol/kgH2O elasmobranch incubation medium (EIM) consisted of (in mM) 300 NaCl, 5.2 KCl, 2.7 MgSO4, 5.0 CaCl2, 15.0 Tris, and 370 urea, pH 7.5. Hypotonic 460 mosmol/kgH2O EIM consisted of (in mM) 100 NaCl, 5.2 KCl, 2.7 MgSO4, 5.0 CaCl2, 15.0 Tris, and 250 urea, pH 7.5. Isosmotic nonelectrolyte media 940 EIM consisted of 400 mM ethylene glycol or 1,3-diethyl urea replaced 200 mM NaCl. Isosmotic electrolyte media 940 EIM consisted of 200 mM NH4Cl-replaced 200 mM NaCl.

Preparation of cells. Blood (5–10 ml) was collected from a caudal vessel using a heparinized syringe fitted with a 20-gauge needle. The whole blood sample was centrifuged for 2
min at top speed in a DYNAC centrifuge (Clay Adams). The plasma and buffy coat were subsequently removed by aspiration. Erythrocytes were washed once in isosmotic (940 mosmol/kg H2O) EIM. The supernatant and any remaininguffy coat were aspirated, and the RBC pellet was resuspended as stated below.

Hematocrit determination. RBCs were resuspended and added at designated time points to test media in a 15°C shaking water bath. Samples were drawn into hematocrit tubes and sealed with Critoseal. Tubes were spun in a hematocrit centrifuge; then the ratio of RBC to total volume was determined. The relative cell volume was obtained by dividing the ratio of RBC to total volume at experimental time points by the ratio obtained at the zero time point.

Taurine efflux was measured as previously described by Goldstein and Brill (10). RBCs were resuspended to 20% hematocrit in EIM. Radioactively labeled taurine was added at a concentration of 2 μCi/ml to cell suspension and incubated for 3 h in a 15°C shaking water bath. After cells were loaded with radiolabeled taurine, they were washed successively in isosmotic 940 EIM with 0.2 M taurine to remove labeled taurine not taken up by cells. Cells were resuspended to 20% hematocrit in 940 EIM. A 0.3-mi aliquot was added to flasks containing isosmotic EIM, hyposmotic EIM, isosmotic nonelectrolyte, or isosmotic electrolyte media with and without inhibitors. After incubating in a 15°C shaking water bath for a designated time, 0.5 ml of sample, in duplicate, were removed from each flask, plated in microcentrifuge tubes, and centrifuged. Then, 0.4 ml of supernatant were removed and placed in liquid-scintillation vials containing scintillation cocktail. For measurement of radioactivity in cells after loading with labeled taurine, the pellet of the 0-min time point was lysed with 1 ml of 7% perchloric acid (PCA) for 10 min, centrifuged, and a 0.4-ml sample of supernatant was placed in a scintillation vial. For experiments testing piceatannol, after loading with radiolabeled taurine, piceatannol was added to 940 EIM at a final concentration of 0.1 mM, and cells were incubated for 10 min before beginning the experiment. Piceatannol (0.1 mM) was also added to incubation media during the timed experiment. TMAO efflux was expressed as \( t_0 \) TMAO released into the medium at time \( t \).

Electrolyte assays were performed as described by Davis-Amaral et al. (4). RBCs were resuspended to 20% hematocrit, then a 0.3-ml aliquot was added to flasks containing 3.0 ml of incubation media (940 EIM, 460 EIM, isosmotic nonelectrolyte, and isosmotic electrolyte) in a 15°C shaking water bath and sampled at designated time points. Samples (1.25 ml), in duplicate, were transferred to preweighed tubes and centrifuged. The supernatant was removed by aspirating, and the pellet was washed rapidly in isosmotic mannitol to remove extracellular electrolytes. Cells in hypsomotic media were washed in hypsomotic mannitol. Supernatant was removed after centrifugation, and the pellet weights were determined. The RBC pellets were lysed with distilled water and incubated overnight at 4°C. Then, a 0.1 vol of 70% PCA was added, and the sample mixture was incubated on ice for 15 min. The tubes were then centrifuged, and the supernatant was removed. The supernatant was used to determine sodium and potassium concentrations using an flame photometer (Instruments Laboratory) and to assay for chloride concentration by volumetric titration with mercuric nitrate (22). Electroletes were expressed as cellular concentrations (μeq/g RBC).

Kinase activities. An immune complex assay protocol was used to determine the activities of various protein kinases in control and volume-expanded dogfish RBCs (25). RBCs were volume expanded for 5 min (unless indicated otherwise) in hypsomotic, isosmotic nonelectrolyte (ethylene glycol and diethyl urea), and isosmotic electrolyte (ammonium chloride) media. After the 5-min incubation, cells were rapidly pelleted and snap-frozen. Immediately before assay, cells were thawed on ice and lysed in 9 vol of Nonidet P-40 (NP-40) lysis buffer [immunoprecipitate (IP); 25 mM HEPES, pH 7.4, 225 mM NaCl, 1% (vol/vol) NP-40, 5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine with 10 μg/ml each of leupeptin, aprotinin, pepstatin A, and antipain]. Lysates were solubilized, then centrifuged. Supernatants were incubated with various antibodies specifically directed to the kinases prebound to protein A Sepharose. Kinases were allowed to bind for 120 min, washed twice with IP wash [25 mM HEPES, pH 7.4, 150 mM NaCl, and 0.1% (vol/vol) NP-40 with protease inhibitors as stated above], and then washed once with assay buffer (50 mM HEPES, pH 7.4, 10 mM MnCl₂, with protease inhibitors). The activity of the kinases attached to the beads was measured at 37°C for 20 min in 50-μl assay buffer with 5 mM p-nitrophenylphosphate and 1 mM [γ-32P]ATP. Reactions were terminated by the addition of 25-μl 3× SDS-PAGE stop solution, and samples were heated at 65°C for 10 min to elute the kinases. Beads were pelleted, and the samples were loaded onto a 10% SDS-PAGE, transferred to a polyvinylene difluoride membrane, and alkali treated before autoradiography. Images were quantitated using Image 1.54 software from National Institutes of Health.

Molecular dimensions. The molecular dimensions of TMAO were determined by D. D. Busath of Brigham Young University using the van der Waals diameters, bond lengths, and angles from CHARMM and Quanta (MSI, San Diego, CA) for the minimized structure. After the molecules were built, the potential energy was completely minimized using the CHARMM simulation program (3), and the molecular dimensions were calculated.
HPLC analyses of TMAO and taurine. These assays were kindly performed by P. H. Yancey of Whitman College as described previously (23, 26).

Materials. Taurine [specific activity = 1.12 TBq/mmol or 30.3 Ci/mmol] was purchased from New England Nuclear (Boston, MA); Piceatannol and AG 18 were from Sigma-Aldrich (St. Louis, MO); antibodies to p72syk, p56lyn (recognizing the α-, β-, and γ-isoforms) were from Upstate Biotechnology (Lake Placid, NY); protein A Sepharose was from Pharmacia (Piscataway, NJ); PVDF membrane was from Immobilion, Milipore (Medford, MA). All other reagents were of the highest grade available.

Statistical methods. Student’s t-test was performed by grouped data analysis.

RESULTS

Swelling in dogfish RBCs. The relative cell volumes of dogfish RBCs were determined after volume expansion of the cells with experimental solutions [hyposmotic, isosmotic electrolyte (ammonium chloride), and isosmotic nonelectrolyte (ethylene glycol and diethyl urea)] by comparing the hematocrits of the experimental solutions to the hematocrits of the isosmotic 940 EIM control. Cell volumes expanded to similar degrees in each of the experimental solutions ranging from 1.52 to 1.65 times the isosmotic control cell volume (Fig. 1A). As shown in Fig. 1B, treatment with hyposmotic and isosmotic nonelectrolyte media (ethylene glycol and diethyl urea) resulted in similar decreases in total electrolyte concentrations. Final concentrations were 121 ± 5 (n = 8), 124 ± 6 (n = 6), and 120 ± 6 (n = 6) μeq/g RBC (means ± SE), respectively, compared with the concentration in control cells, 177 ± 10 μeq/g RBC (n = 10). Treatment with isosmotic electrolyte medium (NH₄Cl), however, caused an increase in total electrolyte concentration, 222 ± 6 μeq/g RBC (n = 12). This increase in intracellular electrolyte concentration was due to an influx of chloride ions accompanying the NH₄⁺ entering the cells. The values shown for total electrolyte concentrations in NH₄Cl-treated cells do not include the increased NH₄⁺ levels (which were not measured in these cells).

Time courses of TMAO and taurine effluxes. Figure 2 shows the time course for TMAO efflux from dogfish RBCs between 0 and 60 min. The time course for taurine efflux is also shown for comparison. Isosmotic treatment of RBCs resulted in little or no efflux of either TMAO or taurine. Cells treated with hypotonic media exhibited TMAO and taurine effluxes that were linear with time for taurine, but the rate of efflux for TMAO was significantly faster than for taurine. Over a period of 30 min, 47% TMAO and 17% taurine effluxed from the cells (Fig. 2). HPLC analyses showed that the initial TMAO and taurine concentrations were 152 ± 7 and 93 ± 4 mM (means ± SE, n = 3), respectively. In all subsequent experiments, TMAO and taurine effluxes were measured at 30 min. There was no hemolysis at any time during the experiment until cells were deliberately lysed at the end of the experiments.

Effects of pharmacological inhibitors. To determine whether TMAO efflux and its activation by volume expansion were occurring via the same osmolyte channel known to transport taurine in fish RBCs, a variety of inhibitors known to block osmolyte channels in fish RBCs was tested for their effects on volume-activated TMAO efflux and compared with the effects of the same inhibitors on taurine efflux. The anion transport inhibitors, DIDS and niflumic acid, and the channel blocker, quinine, have previously been shown to inhibit osmolyte channels in fish RBCs (4, 8, 19). Therefore, we tested the effects of these inhibitors on TMAO and taurine efflux in dogfish RBCs. As shown in Fig. 3, under hypotonic conditions, the efflux of TMAO and taurine was inhibited by 54 and 63%, respectively, when 0.1 mM DIDS was added to the incubation media and by 44 and 49%, respectively, when 0.1 mM niflumic acid was added to the incubation media. Quinine (1.0 mM) inhibited both TMAO and taurine effluxes by 87 and 64%, respectively.
Volume expansion with isosmotic media. Swelling with isosmotic nonelectrolytes has previously been shown to stimulate the efflux of taurine in trout RBCs (14). Therefore, the effects of isosmotic nonelectrolyte media (ethylene glycol and diethyl urea) on TMAO and taurine effluxes were tested in dogfish RBCs. Figure 4 shows that treatment with isosmotic nonelectrolyte media, ethylene glycol and diethyl urea, stimulated TMAO efflux to approximately the same levels achieved by hypotonic stimulation. A similar result was observed for taurine (Fig. 4) with effluxes stimulated approximately the same by media containing ethylene glycol, diethyl urea, or hypotonic media. The stimulation of TMAO and taurine effluxes by isotonic nonelectrolyte media was significantly inhibited ($P < 0.01$) by the addition of 0.1 mM DIDS in the incubation media (data not shown). TMAO efflux was inhibited by 66 and 63% for ethylene glycol and diethyl urea, respectively. Taurine efflux was inhibited by 64 and 38% for ethylene glycol and diethyl urea, respectively.

Previous studies have shown that both an increase in cellular volume and a decrease in intracellular electrolyte concentration were necessary for volume activation of taurine efflux in fish RBCs (14, 25). To test the importance of both swelling and intracellular electrolyte concentration in dogfish RBCs, TMAO and taurine effluxes were measured when cells were volume expanded in isosmotic electrolyte media (ammonium chloride). As shown in Fig. 4, there was no significant stimulation of TMAO or taurine effluxes in ammonium chloride medium. Thus, although isosmotic electrolyte medium caused a volume expansion similar to hypotonic medium, an elevated intracellular electrolyte concentration inhibited the stimulation of TMAO and taurine effluxes, indicating that volume expansion as well as a decrease in intracellular electrolyte concentrations are required for activation of this volume-sensitive pathway.

Tyrosine kinase activities. Previous studies (17, 20, 25) have shown that the activities of the tyrosine kinases p72syk and p56lyn, phosphorylating tyrosine kinases of band 3, increased significantly when skate RBCs were volume expanded by hyposmotic media (460 EIM) and to a lesser extent by isosmotic nonelectrolytes (DEU and EG), but not by isosmotic electrolyte (ammonium chloride) solutions. Other phosphorylating kinases were not significantly stimulated by volume expansion. It has not been determined which protein kinases are present in dogfish erythrocytes because there are many. However, the stimulation of p72syk and p56lyn by volume expansion correlated with and was proposed to play a role in the volume stimulation of taurine efflux in skate RBCs. Therefore, the tyrosine kinase activities p72syk, p56lyn, and pp60src were measured in dogfish RBCs to determine whether these tyrosine kinases are involved in TMAO and taurine transport in dogfish RBCs. Figure 5 shows the time courses for activities of the tyrosine kinases p72syk, p56lyn, and pp60src in dogfish RBCs between 0 and 60 min in hypotonic media.
volume-stimulated activity of p72syk exhibited a dramatic increase between 0 and 5 min, reaching a peak at 5 min. The volume-stimulated activity of p56lyn followed a similar pattern as p72syk, with peak activity at 5 min. However, the volume-stimulated activity of p72syk was much greater (2 times) than that of p56lyn. The tyrosine kinases that are stimulated by volume expansion have been found to reach peak activity 5 min after volume expansion. However, the activity of pp60src was not stimulated by hypotonic stress and remained constant from 0 to 60 min, which agrees with previous experiments on skate erythrocytes. As shown in Fig. 6, the activities of tyrosine kinases, p72syk and p56lyn, were significantly stimulated in dogfish RBCs volume expanded with isotonic ethylene glycol and diethyl urea media in addition to hypotonic medium. The degree to which hyposmotic swelling stimulated p72syk was much greater (~2 times), however, than the stimulation by isosmotic ethylene glycol and diethyl urea. Swelling by isosmotic electrolyte media (ammonium chloride) did not stimulate the kinase activities of p72syk nor p56lyn. The activity of pp60src was not stimulated when cells were volume expanded with hyposmotic, isosmotic nonelectrolyte, or isosmotic electrolyte medium. Thus, as with volume stimulation of taurine efflux in skate RBCs, volume-induced increases in TMAO and taurine effluxes are accompanied by the stimulation of p72syk and p56lyn in dogfish RBCs.

In all subsequent kinase experiments, only p72syk and p56lyn kinase activities were measured at 5 min.

Tyrosine kinase inhibitors. Tyrosine kinase inhibitors are known to block the activation of the osmolyte channel in skate erythrocytes (17, 20). Therefore, the effects of piceatannol and AG18, two tyrosine kinase inhibitors known to block volume-activated taurine efflux in skate erythrocytes, were tested on volume-activated TMAO and taurine effluxes in dogfish RBCs as well as on the volume-stimulated activities of p72syk and p56lyn. Figure 7 shows that when piceatannol was added to hyposmotic incubation media, TMAO efflux was not significantly inhibited. In contrast, piceatannol inhibited taurine efflux significantly. Figure 8 shows that relatively high concentrations of piceatannol were required to inhibit the volume-stimulated activities of p72syk and p56lyn: 0.1–0.3 mM for 50% inhibition, respectively. Therefore, the effects of AG18, another tyrosine kinase inhibitor known to block taurine efflux in skate erythrocytes, were tested on TMAO efflux in dogfish RBCs. Figure 7 shows that AG18 significantly decreased TMAO efflux in dogfish RBCs, and, as shown in Fig. 8, the activities of p72syk and p56lyn were inhibited by more than 50% at AG18 concentrations of 0.01 and 0.03 mM, respectively.
lack of effect of piceatannol on TMAO efflux compared with AG18 may be related to the lower potency of piceatannol as an inhibitor of syk and lyn. As shown in Fig. 8, 0.1 mM piceatannol inhibited syk by only 55%, whereas the same concentration of AG18 inhibited syk by 90%.

Choline uptake. Previous studies have shown that the uptake of organic cation, choline, is activated by hyposmotic media in trout RBCs (8) but not in skate RBCs (16). Therefore, we tested the ability of the swelling-activated osmolyte channel of dogfish RBCs to transport choline. There was no significant difference in choline uptake between cells treated in isosmotic and hyposmotic medium. After 60-min incubation, the uptake in RBCs was 9,068 ± 932 cpm [3H]choline for isosmotic medium and 8,800 ± 352 cpm [3H]choline (means ± SE, n = 4) for hyposmotic medium. Thus the substrate specificity of osmolyte channel in dogfish RBCs resembles that in the skate in this respect.

DISCUSSION

The results presented in this study show that the high concentrations of TMAO in dogfish (Squalus acanthias) are consistent with the role of this compound in cell volume regulation. In isosmotic medium, TMAO is retained in dogfish RBCs with a low rate of efflux in this condition. When the cells are volume expanded hypotonically or isotonically with nonelectrolytes, TMAO efflux rapidly increases. However, if cells are volume expanded isotonically with electrolytes, TMAO efflux was not observed under these conditions, suggesting that a decrease in electrolyte concentration as well as cell swelling are required for activation of the volume-sensitive organic osmolyte channel.

Despite the known importance of TMAO as an intracellular osmolyte, little is known about the cell membrane transport of this compound in these fishes, or that in other species for that matter. We have previously shown that TMAO is accumulated in skate RBC partly by an Na⁺-dependent, energy-requiring process and partly by a swelling-activated, Na⁺-independent pathway (24). Preliminary results obtained in the same study indicated that a swelling-activated TMAO efflux pathway existed in dogfish erythrocytes. It is this latter observation that led us to do a detailed study of the TMAO efflux pathway in the dogfish RBCs. Because we wanted to compare the characteristics of the TMAO efflux pathway with those of the well-known osmolyte channel pathway present in fish erythrocytes, we used taurine efflux as a marker for the osmolyte channel pathway.

We used a 50% reduction in medium osmolarity to maximally stimulate TMAO and taurine effluxes. Although the dogfish RBC is unlikely to ever encounter such reductions in extracellular fluid osmolarity, our previous studies done in other elasmobranch (e.g., skates) RBCs show that lower reductions in medium osmolarity (e.g., 30%) produce significant, albeit lower, stimulation of taurine efflux (11). The results showed that TMAO release from hypotonic volume-expanded dogfish RBCs was two to three times faster than taurine release. Two factors can account, at least in part, for the different rates. First, the diffusion gradient for TMAO (intracellular concentration ~150 mM) was greater than for taurine (intracellular concentration ~90 mM). Second, the molecular dimensions (length, width, thickness) for taurine are 7.9, 4.0, and 4.6 Å, respectively (16), whereas the same dimensions for TMAO have been determined (see MATERIALS AND METHODS) to be 6.5, 6.0, and 5.5 Å, respectively. Whereas the average molecular diameters are similar for TMAO (6.0 Å) and taurine (5.5 Å), the TMAO molecule is more...
ball-like, and the taurine molecule is more sticklike. Thus TMAO would be expected to diffuse through a pore or channel in the membrane with less physical restraint than taurine. Furthermore, TMAO, being less polar than taurine, could diffuse through hydrophobic regions of the membrane more easily. It should be noted, however, that the disparity between the rates of TMAO and taurine effluxes in vivo would not be as great as what was observed in the in vitro experiments in which TMAO and taurine were absent from the incubation medium. The extracellular concentrations of TMAO and taurine in dogfish are \(\sim 50–70\) and \(1\) mM, respectively (12, 21). Thus the RBC-to-extracellular fluid diffusion gradient for TMAO (150:60) is much less than for taurine (90:1), which would tend to reduce the efflux rate for TMAO much more than for taurine.

The experiments with known anion channel inhibitors (DIDS and niflumic acid) and the general channel inhibitor quinine indicate that both the osmolytes are released via the same (or a similar) pathway during hypotonic volume expansion. These three inhibitors had both qualitatively and quantitatively similar effects on the efflux of both osmolytes. We have previously shown that the tyrosine kinase inhibitors piceatannol and AG18 (also known as tyrophostin A23) inhibited hypotonic-activated taurine efflux in skate RBCs (17). As in the skate, piceatannol inhibited the taurine efflux significantly in dogfish RBCs. TMAO efflux, however, was only slightly inhibited by piceatannol. The second tyrosine kinase inhibitor AG18 did produce complete inhibition of TMAO efflux. Both tyrosine kinase inhibitors are known to inhibit both p72syk and p56lyn, two tyrosine kinases known to phosphorylate band 3 and to be involved in osmolyte transport in skate RBCs (17). The different effects of piceatannol and AG18 on TMAO efflux may be related to the greater inhibitory potency of AG18 on p72syk and p56lyn in dogfish RBCs (Fig. 8). It is possible that greater inhibition of syk and lyn is necessary to produce a significant decrease in TMAO efflux than is required to inhibit taurine efflux. Perhaps the osmolyte channel has to be more restricted to block TMAO compared with taurine.

Isotonic volume expansion with nonelectrolytes (ethylene glycol and diethyl urea) activated both taurine and TMAO effluxes. In addition, tyrosine kinases p72syk and p56lyn were also activated by isotonic volume but not as much as was observed with hypotonic expansion. The lower activation of the tyrosine kinases by isotonic expansion resembles similar findings in skate RBCs (25). Thus the relationship between tyrosine kinase activation and osmolyte efflux activation during isotonic volume expansion is different in dogfish RBCs than we previously observed in the skate RBCs where both parameters were lower during isotonic expansion compared with hypotonic expansion. Because activation of TMAO and taurine effluxes are inhibited by tyrosine kinase inhibitors in the dogfish RBCs (Fig. 7), tyrosine kinases do appear to be involved in osmolyte efflux activation in dogfish RBCs. The degree of osmolyte activation in dogfish RBCs, however, is not related in a quantitative manner to the level of activation of tyrosine kinases. It seems, therefore, that the tyrosine kinase activities may merely have to rise to a threshold level to play a role in the opening of the osmolyte channel in dogfish RBCs but that further enzyme activation has little effect on channel activity.

**Perspectives**

This study has defined the volume-activated efflux pathway of TMAO in dogfish RBCs as possessing the characteristics of the volume-activated osmolyte pathway previously shown to be present in the RBCs of both elasmobranch and teleost fishes. Because osmolyte pathways in the fish RBC have many similarities to the swelling-activated osmolyte pathway found in a variety of vertebrate cells (18), it is highly likely that TMAO accumulated in dogfish RBCs is modulated by a similar efflux pathway and possibly by tyrosine kinases acting in a manner similar to the enzymes reported in this study.

In addition to serving the purpose of reducing the intracellular osmolarity of elasmobranch cells exposed to a hypotonic environment, the efflux of TMAO from these cells also tends to keep the ratio of urea to TMAO inside the cells at a set point of about 2:1. This ratio is found under normal conditions in a variety of cells with high concentrations of urea and is important in maintaining normal cellular functions (27). Because urea rapidly diffuses across dogfish cell membranes (5), urea would leave the cells when the extracellular concentration is reduced by hypotonic dilution, and a mechanism for rapidly losing TMAO under these conditions is necessary to maintain the normal urea to TMAO ratio.

A response similar to that of the efflux of TMAO from dogfish RBCs occurs in renal papillary cells. The trimethylamines, betaine, and glycerophosphocholine rapidly efflux, along with urea, from rat papillary cells when the extracellular fluid is diluted during diuresis (1). Thus, as mentioned above, the osmotically induced efflux of cellular TMAO from dogfish RBCs is part of a more widespread phenomenon.

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**REFERENCES**

5. Fenstermacher J, Sheldon F, Ratner J, and Rootham A. The blood to tissue distribution of various polar materials in the


