Permeation Properties of a P2X Receptor in the Green Algae *Ostreococcus tauri*

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We have cloned a P2X receptor (*Ot*P2X) from the green algae *Ostreococcus tauri*. The 42-kDa receptor shares ~28% identity with human P2X receptors and 23% with the *Dictyostelium* P2X receptor. ATP application evoked flickery single channel openings in outside-out membrane patches from human embryonic kidney 293 cells expressing *Ot*P2X. Whole-cell recordings showed concentration-dependent cation currents reversing close to zero mV; ATP gave a half-maximal current at 250 μM. $\alpha\beta$-Methylene-ATP evoked only small currents in comparison to ATP (EC$_{50}$ > 5 mM). 2′,3′-*O*-(4-Benzoylbenzoyl)-ATP, $\beta\gamma$-imido-ATP, ADP, and several other nucleotide triphosphates did not activate any current. The currents evoked by 300 μM ATP were not inhibited by 100 μM suramin, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid, 2′,3′-*O*-(2,4,6-trinitrophenol)-ATP, or copper. Ion substitution experiments indicated permeabilities relative to sodium with the rank order calcium > choline > Tris > tetraethylammonium > *N*-methyl-D-glucosamine. However, *Ot*P2X had a low relative calcium permeability (P_{Ca}/P_{Na} = 0.4) in comparison with other P2X receptors. This was due at least in part to the presence of an asparagine residue (Asn^{353}) at a position in the second transmembrane domain in place of the aspartate that is completely conserved in all other P2X receptor subunits, because replacement of Asn^{353} with aspartate increased calcium permeability by ~50%. The results indicate that the ability of ATP to gate cation permeation across membranes exists in cells that diverged in evolutionary terms from animals about 1 billion years ago.

P2X receptors have a widespread distribution through vertebrate animals, where they fulfill diverse functional roles. These roles range from depolarization in excitable tissues such as pain-sensing neurons to stimulation of cytokine release in immune cells like macrophages (1, 2). The receptor family in vertebrates comprises seven subunits. These assemble to form membrane ion channels as homo- or heterotrimers (1). The receptors are integral ion channels that are opened by the binding of extracellular ATP; they are more permeable to cations than anions, and some of them are highly permeable to calcium (1). A P2X receptor with essentially similar functional properties has been described in the trematode worm *Schistosoma mansoni* (3). In contrast, they have not been identified in the genomes of other invertebrate multicellular organisms such as the nematode worm (*Caenorhabditis elegans*) or fruit fly (*Drosophila melanogaster*).

P2X receptors have also more recently been reported in the amoeba *Dictyostelium discoideum* (slime mold) that, for most of its life cycle, exists as a single-celled organism (4). One of these (*Dd*p2xA) was shown to function as a plasma membrane ATP-gated channel when expressed heterologously in HEK293[2] cells. This work has shown that it is preferentially activated by the non-hydrolyzable ATP analog $\beta\gamma$-imido-ATP, which it is blocked by copper at nanomolar concentrations, and that it is not blocked by more conventional P2X receptor antagonists (suramin, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP))(5). In *D. discoideum* the P2X receptor is found on the membrane of an intracellular organelle (the contractile vacuole) rather than the plasma membrane, and it plays a key role in volume regulation (4). When its function is blocked by copper, or when the gene is deleted from *D. discoideum*, the amoeba is unable to regulate cell volume and the cells lyse in hypotonic solutions. These findings in a single-celled organism have thus drawn attention to intracellular signaling roles for ATP acting at P2X receptors, which had previously been considered only in the context of synaptic, paracrine, or autocrine signaling between cells in multicellular animals.

The earlier sequencing of several genomes in the plant kingdom (for example, *Arabidopsis*, rice, and potato) provided no evidence for P2X receptors. However, the genome of *Ostreococcus tauri* (6) has four open reading frames that encode proteins distantly related to P2X receptors. *O. tauri* is the smallest free-living eukaryote known, a primitive green alga of Prasinophyceae that is close to the evolutionary origins of photosynthetic plants. Similar sequences are also apparent in the genome of *Ostreococcus lucimarinus* (7).

The purpose of the present work was to determine whether the *O. tauri* sequence encodes a functioning P2X receptor and, if so, to investigate its properties. We did this by heterologous expression and recording of membrane currents. This approach allows one to examine sensitivity to various agonists and antagonists and to determine the properties of its ion permeation pathway. Such information might then be used, along with genetic approaches, to probe the function and distribution of P2X receptors in this single-celled eukaryote.

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[2] The abbreviations used are: HEK293, human embryonic kidney 293; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid.
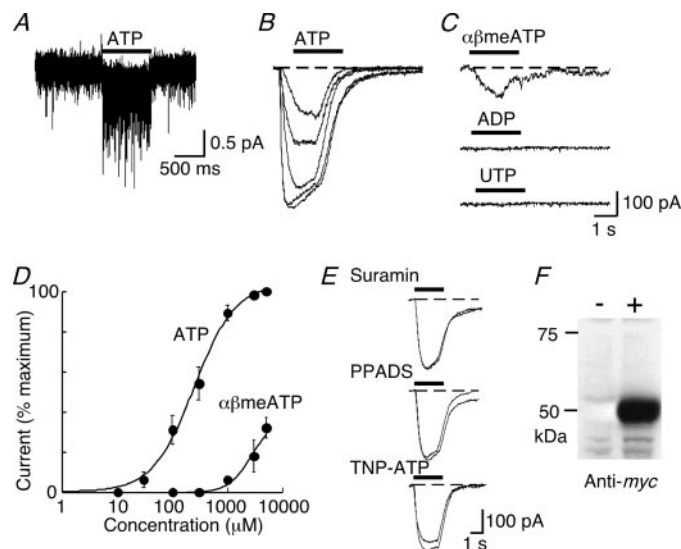
EXPERIMENTAL PROCEDURES

Cloning an Algae P2X Receptor—Total RNA was extracted from cultures of *O. tauri* (Roscoff culture collection) using TriReagent (Sigma). First strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and oligo(dT)$_{15}$ priming. A 1.2-kb cDNA encoding the open reading frame and Kozak sequence was amplified by PCR using oligonucleotide primers containing restriction linkers and a sequence encoding a 3′-Myc epitope. The cDNA was cloned into pcDNA3.1 for expression in HEK293 cells. The sequence corresponds to nucleotides 345177 to 346337 of chromosome 7 (GenBank[TM] CR954207, CAL54489).

Immunoblotting and Immunostaining—Total HEK293 cell lysate (50 mg) was subjected to SDS-PAGE (4–12% gradient; NuPAGE; Invitrogen). Transfer membranes were blotted using a mouse monoclonal anti-Myc IgG (1:5000; Sigma) followed by detection using horseradish peroxidase chemistry. For localization of the Myc-tagged receptor in HEK293 cells, cells were methanol-fixed and permeabilized with 0.1% Triton X-100. Cells blocked with 3% bovine serum albumin were incubated with a mouse monoclonal anti-Myc IgG (1:1000) for 1 h at room temperature, followed by washing and subsequent incubation with Cy3-conjugated donkey anti-mouse secondary antibody (Jackson Laboratories).

Electrophysiology—HEK293 cells were transiently transfected with 5 μg of P2X receptor construct and 0.1 μg of enhanced green fluorescent protein using Lipofectamine 2000. Whole-cell and outside-out patch recordings were made at room temperature (18–20 °C) 24–48 h following transfection. The extracellular solution contained (mM): 145 NaCl, 2 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 13 D-glucose, and 10 HEPES, pH 7.3. For whole-cell recordings the patch pipette contained (mM): 147 NaCl, 10 HEPES, and 10 EGTA, pH 7.3. NaF replaced NaCl in the pipette for outside-out recordings. Single channel recordings were sampled at 10 kHz and filtered at 3 kHz. For substitution of monovalent cations, P_{ion}/P_{Na} was calculated from $exp(\Delta E_{rev}F/RT)$, and for Ca^{2+} P_{Ca}/P_{Na} was calculated as $[Na]_i\{exp(E_{rev}F/RT)(1 + exp(E_{rev}F/RT))\}/4[Ca^{2+}]_o$, where E_{rev} is the reversal potential of the ATP-induced current, $[Na^+]_i$ is the cytosolic Na$^+$ concentration, F is Faraday's constant, R is the universal gas constant, T is the absolute temperature, and $[Ca^{2+}]_o$ is the extracellular Ca^{2+} concentration (8, 9). E_{rev} values were corrected for calculated liquid junction potentials. Numerical data are given as means ± S.E.

Fluorescence Assay—*Ostreococcus* cultures were incubated with 5 mM sodium green indicator (Molecular Probes) and 0.2% (v/v) pluronic acid in artificial sea water medium at room temperature for 1 h. Cells were washed and resuspended in 1 ml of filtered sea water (Sigma) in a fluorescence cuvette. The suspension was continuously agitated by a magnetic flea, and sodium green fluorescence was measured with an integrating photomultiplier (Cairn Research, Faversham, UK). The suspension was excited by an ultraviolet xenon arc lamp with continuous acquisition. Drugs were administered manually and typically did not exceed 1% total volume.

FIGURE 1. **Functional expression of algae P2X receptor.** *A*, single channel recording from outside-out patch from HEK293 cells expressing *Ot*P2X. Currents were evoked by 100 μM ATP (*bar*); holding potential −100 mV. *B*, whole-cell currents evoked by ATP (100 and 300 μM, 1, 3, and 5 mM). *C*, currents were evoked by αβ-methylene-ATP (*αβmeATP*) (3 mM), but not by UTP or ADP (1 mM). Holding potential −60 mV. *D*, concentration-response curves for whole-cell currents evoked by ATP (*n* = 10) and αβ-methylene-ATP (*αβmeATP*) (*n* = 8). *E*, ATP-evoked currents in the presence and absence of suramin, PPADS, or 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (*TNP-ATP*) (each at 100 μM). Superimposed records show effects of ATP applied in control conditions and 5 min later in the presence of antagonist. *F*, anti-Myc Western blot of whole-cell lysates from untransfected and HEK293 cells (−) and cells transfected with Myc-tagged *Ot*P2X (+).
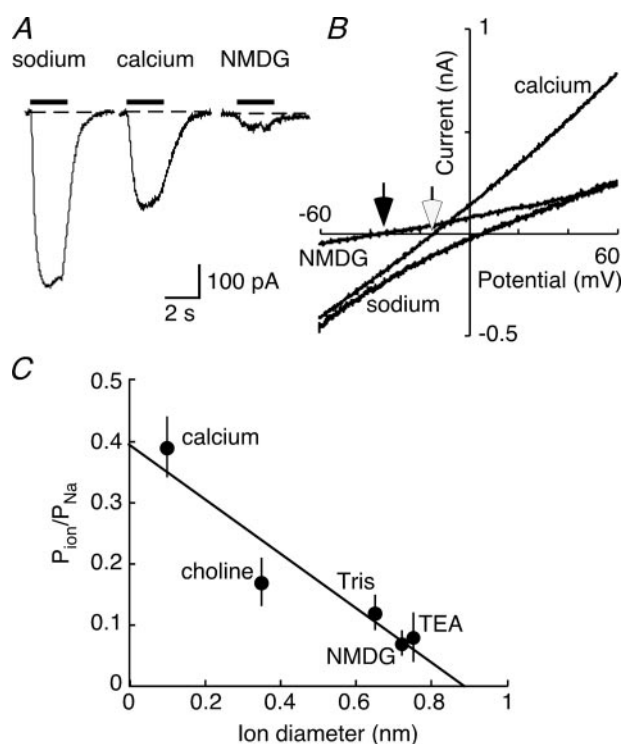
RESULTS

Cloning of a Functional P2X Receptor from Green Algae—We identified a putative P2X receptor gene in the *O. tauri* genome using *Dd*p2xA as a BLAST query (4). The gene encoded a protein of 387 amino acids (*Ot*P2X) that was 23% identical to the *Dictyostelium* receptor *Dd*p2xA (4) and ~28% identical to human receptors P2X$_1$ through P2X$_7$. Expression of the Myc-tagged construct produced a 50-kDa protein in HEK293 cells (Fig. 1*F*). Immunocytochemistry of the HEK293 cells revealed plasma membrane staining but also pronounced punctate intracellular staining of the algae protein. ATP (100 μM) evoked flickery openings of channels recorded in the outside-out configuration that were not observed in untransfected HEK293 cells (*n* = 6) (Fig. 1*A*).

Pharmacological Properties—In whole-cell recordings at −60 mV, ATP evoked concentration-dependent inward currents (Fig. 1*B*). The activation threshold was ~30 μM, and half-maximal currents were elicited by 247 ± 1.2 μM (EC$_{50}$) (*n* = 10) (Fig. 1*D*). The Hill coefficient was 1.2 ± 0.2 (*n* = 10). The currents showed only modest desensitization during applications of 1–2 s, and ATP evoked reproducible currents when applied repeatedly at 5-min intervals (Fig. 1*B*). The ATP analog αβ-methylene-ATP evoked only small inward currents at millimolar concentrations (EC$_{50}$ > 5 mM) (Fig. 1*C*). ADP (Fig. 1*C*), UTP (Fig. 1*C*), GTP, ITP, CTP, 2′,3′-*O*-(4-benzoyl)benzoyl-ATP, βγ-imido-ATP, NAD, and FAD (up to 1 mM) did not evoke any currents in *Ot*P2X-transfected cells. Suramin and PPADS (10–100 μM), which block many mammalian P2X receptors (5, 10), did not alter the currents evoked by 300 μM
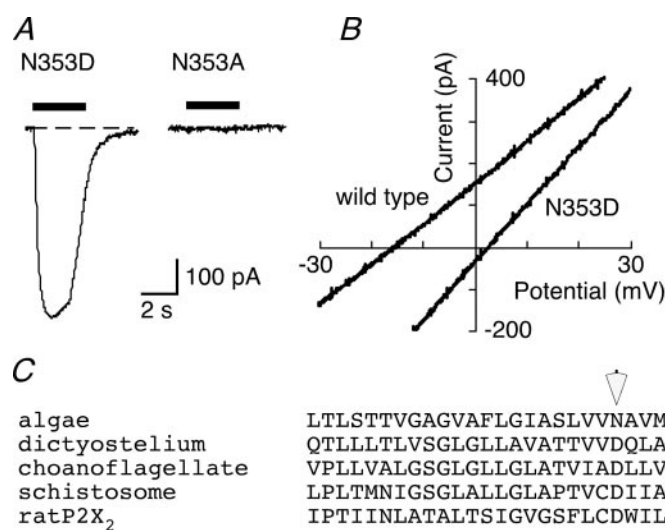
FIGURE 2. **Permeation properties of algae P2X receptor.** *A*, whole-cell currents evoked by ATP (1 mm); holding potential −60 mV. Currents were evoked in external solution containing 147 mM sodium chloride, 98 mM calcium chloride, and 148 mM *N*-methyl-D-glucosamine; chloride (*NMDG*). *B*, current-voltage plots for ATP-evoked currents in external sodium, calcium, or NMDG. Note left shift in reversal potential in calcium (*open arrowhead*) or NMDG (*filled arrowhead*). *C*, permeability of five cations relative to that of sodium as a function of their mean geometric diameter. Points are mean ± S.E. of the mean; *n* = 8 cells.

FIGURE 3. **Asparagine residue in second transmembrane domain accounts for low calcium permeability.** *A*, ATP (300 μM) evokes currents at *Ot*P2X[N353D] but not at *Ot*P2X[N353A] receptors. *B*, calcium permeability of *Ot*P2X[N353D] receptors is significantly higher than that of wild-type receptors. Voltage ramps are shown for whole-cell currents evoked by 1 mM ATP for wild-type and *Ot*P2X[N353D] mutant receptors, recorded in 98 mM external calcium. *C*, alignment of presumed second transmembrane domains of the *Ot*P2X receptor (*algae*), with those of *Dd*P2xA (*dictyostelium*, GenBank[TM] accession code EU047552), a flagellated protozoa (*choanoflagellate*),[4] a nematode worm (*schistome*, GenBank accession code AJ783803), and a mammalian receptor (*rat P2X₂*, GenBank accession NM_053656). *Open arrow* indicates aspartate conserved in all P2X receptors except *Ot*P2X.

ATP (Fig. 1*E*). The currents were also unaffected by copper (100 μM) and by the P2X₁- and P2X₃-selective antagonist 2′,3′-*O*-(2,4,6-trinitrophenol)-ATP (*TNP-ATP*) (100 μM) (Fig. 1*E*).

Ionic Permeability—The current through *Ot*P2X receptors reversed direction at a holding potential close to 0 mV (0.8 ± 0.2 mV, *n* = 12) and showed little rectification (current at −60/current at +60 mV was close to 1) (Fig. 2*B*). ATP also evoked inward currents when the main cation in the external solution was changed to choline, Tris, tetraethylammonium, or *N*-methyl-D-glucosamine (Fig. 2*A*). In each case the reversal potential shifted to negative potentials with respect to sodium: the calculated permeability ratios (P_{ion}/P_{Na}) were 0.17 ± 0.02, 0.12 ± 0.05, 0.08 ± 0.02, and 0.07 ± 0.02 for choline, Tris, tetraethylammonium, and *N*-methyl-D-glucosamine, respectively (*n* = 8). The permeability ratio was inversely correlated with the mean geometric ion diameter (*r* = 0.95) (Fig. 2*C*) (11).

Asn³⁵³ and Low Calcium Permeability—Isotonic replacement of sodium by calcium resulted in a substantial hyperpolarizing shift in the reversal potential of the ATP-evoked current (E_{rev} −11.4 ± 1.0 mV, *n* = 10; *p* < 0.01), which indicated that calcium was 2.5 times less permeable than sodium (P_{Ca}/P_{Na} = 0.39 ± 0.08, *n* = 10). This is unusual among P2X receptors, where P_{Ca}/P_{Na} typically ranges between 1.5 and 4 (1, 12). Amino acid residues within the second transmembrane domain have been shown to contribute to calcium permeability at P2X receptors (12, 13). Alignment of this region with other

sequences shows that the aspartate residue (Asp³⁴⁹ in rat P2X₂ receptor) that is completely conserved in all other P2X sequences is replaced by asparagine in *Ot*P2X (Asn³⁵³) (Fig. 3*C*). We found that the substitution of aspartate for this asparagine significantly increased the calcium permeability of *Ot*P2X (Fig. 3). The reversal potential was −3.0 ± 0.8 mV, *n* = 8; *p* < 0.01, corresponding to a calcium permeability (P_{Ca}/P_{Na}) of 0.64 ± 0.06 (*n* = 8). Alanine was not tolerated at this position; *Ot*P2X[N353A] did not produce a functional receptor when transfected into HEK293 cells (Fig. 3*A*).

ATP Responses in Intact Algae—We tested for the presence of functional cell surface P2X receptors by seeking ATP-activated sodium fluxes in cultures of *O. tauri*. Sodium green was taken up by the algae, as demonstrated by the large increase in fluorescence upon cell permeabilization with digitonin. Application of extracellular ATP (0.1–3 mM) failed to elicit any significant increase in sodium green fluorescence in successfully loaded cells (*n* = 8). However, the same cells responded with a clear increase in fluorescence when capsaicin (30 μM) was applied. Capsaicin is an agonist at TRPV1 receptors, and the *O. tauri* genome contains a TRPV homolog.[3] This result suggests that green algae express a cell membrane TRPV receptor but provides no evidence for a functional P2X receptor on the plasma membrane.

DISCUSSION

We have identified a P2X receptor in a single-celled photosynthetic alga. *O. tauri* is the smallest known living eukaryote, a single-celled protist containing a chloroplast, mitochondria,

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[3] S. J. Fountain, unpublished observations.

and one nuclear pore and comparable in size to most bacteria (6, 7). The existence of a P2X receptor in such a unicellular protist suggests that ATP-gated ionic flux is a conserved function, existing soon after if not before the evolutionary divergence of plants and animals. One of the early unicellular ancestors of animals is the protozoal choanoflagellate. We have also detected a P2X receptor in the genome of a choanoflagellate (*Monosiga brevicollis*) and shown by heterologous expression that it also encodes a functional receptor.[4] It is therefore intriguing that no P2X receptor genes have been identified in the genomes of higher plants, such as *Arabidopsis*, although extracellular ATP is a signaling molecule in plants (14). Identification and expression of further genes from simple organisms will help to clarify the molecular evolution of this major receptor class.

The P2X receptor in green algae contains many of the residues considered to be important for P2X receptor function, such as conserved lysine residues at positions equivalent to Lys^{69} and Lys^{308} of rat P2X$_2$. Two intracellular motifs are found in all known P2X receptors. The YXTXK/R motif at the N terminus is also present in *Ot*P2X, although the YXXXK motif in the C terminus tail that enhances membrane retention (15) is replaced by YESWL. The ectodomain as a whole is poorly conserved, although somewhat more related to the vertebrate and schistosome receptors than to the *Dd*p2xA. The second transmembrane domain (TM2) differs from previously known P2X receptors by the presence of an asparagine rather than aspartate at position 353 (see below). The pre-TM2 region is well conserved. In an alignment of *Ot*P2X with three other P2X receptors that have been shown to function as cation-permeable channels (*Dd*p2xA, *S. mansoni*, and human P2X$_4$), only 23 of its 387 amino acids are completely conserved. Despite these molecular differences, *Ot*P2X was selectively activated by adenosine triphosphates in the micromolar range. On the other hand, the poor conservation in the large ectodomain presumably has resulted in a loss of binding sites for the antagonists suramin, PPADS, and TNP-ATP, as was previously noted for the *D. discoideum* P2X receptor.

The estimated pore diameter derived from the relative permeability ratio (∼1 nm, Fig. 2) is of course very crude but nonetheless in broad agreement with estimates made for other mammalian and *Dictyostelium* P2X receptors (3, 4, 9). This would be consistent with architectural conservation of the selectivity filter between primitive and mammalian P2X receptors. On the other hand, *Ot*P2X exhibits the lowest calcium permeability of all known P2X receptors ($P_{Ca}/P_{Na} = 0.39$). Other estimates of P_{Ca}/P_{Na} are in the range of 1.2 to 4.2 (3, 4, 9, 16), although there are methodological differences in these studies. Permeability to calcium underlies many of the physiological effects of P2X receptor activation in mammalian cells, and hence understanding the molecular basis for calcium permeability is important.

With the exception of *Ot*P2X, an aspartate residue in the second transmembrane domain (position equivalent to position Asp^{349} in rat P2X$_2$) is conserved throughout all P2X receptors. We hypothesized that the absence of this aspartate may

underlie the low calcium permeability at *Ot*P2X receptors, because acidic moieties with a more extracellular position in the transmembrane domain have been shown to contribute to calcium permeability at other P2X receptors (12, 18). As for other P2X receptors, alanine substitution at this position renders receptors non-functional (4, 19). Reversion of the asparagine residue at this position to aspartate (*Ot*P2X[N353D]) significantly increased the calcium permeability of the receptor (by 53%, $P_{Ca}/P_{Na} = 0.64$). Despite this enhancement, the calcium permeability of *Ot*P2X[N353D] remains below the range of other P2X receptors, suggesting that additional residues may also contribute to enhanced permeability (7, 9, 12, 13). The converse substitution in P2X$_2$ receptors (P2X$_2$[D349N]) does not reduce calcium permeability (13). Thus, the increased calcium permeability may be due to the increased negativity provided by the aspartate side chain, but other residues are obviously also involved.

We have demonstrated previously that the P2X receptor from *D. discoideum* is localized inside the cell, where it controls organelle function (4). The lack of effect of extracellular ATP on sodium influx in *O. tauri* cells would be consistent with an intracellular rather than extracellular role, assuming that the organism makes a P2X receptor protein. The control experiment with capsaicin certainly suggested the presence of a plasma membrane TRPV receptor, and such a receptor is predicted in the genome (GenBank^{TM} accession code CAL54215). *O. tauri* is the smallest free-living eukaryote known, a primitive green alga of Prasinophyceae, that resides within the *Chlorophyta*. It remains to be determined whether its P2X receptors serve functional roles on intracellular membranes, whether analogous to volume regulation in *Dictyostelium* or perhaps related to energy metabolism.

This study provides evidence for the existence of cation-permeable P2X receptors in a simple protist. Unlike some other ligand-gated ion channels (glutamate, (20); proton, (21)), prokaryotic P2X receptors are yet to be identified. *O tauri* belongs to a green algae family that appeared more than 1 billion years ago, which makes it one of the oldest of the green lineage. Exploring P2X receptors in such organisms with limited sequence relatedness may offer new insight into the molecular operation of P2X receptors as well as an improved understanding of the cell physiology of ATP.

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[4] S. J. Fountain and R. A. North, unpublished observations.

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