THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 46, Issue of November 15, pp. 29080–29087, 1996

Printed in U.S.A.

A Guanine Nucleotide-independent Inwardly Rectifying Cation Permeability Is Associated with $P2Y_1$ Receptor Expression in *Xenopus* Oocytes*

(Received for publication, July 24, 1996, and in revised form, August 27, 1996)

Scott M. O'Grady‡§, Eric Elmquist‡, Theresa M. Filtz¶, Robert A. Nicholas¶, and T. Kendall Harden¶

From the ‡Department of Physiology and Animal Sciences, University of Minnesota, St. Paul, Minnesota 55108 and the ¶Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

The functional properties of the G protein-coupled $P2Y_1$ receptor were investigated in *Xenopus* oocytes. Incubation of oocytes expressing either the human or turkey $P2Y_1$ receptor with adenine nucleotide agonists resulted in an increase in Cl^- current and activation of a novel cation current with an inwardly rectifying current-voltage relationship. Activation of either the human $P2Y_2$ (P_{2U}-purinergic) or M1 muscarinic receptor expressed in oocytes resulted in an increase in Cl^- current similar to that observed in $P2Y_1$ receptor-expressing oocytes but had no effect on cation current. P2 receptor agonists stimulated both the cation current and Cl^- current in $P2Y_1$ receptor-expressing oocytes with EC_{50} values and an order of potency (2-methylthioadenosine diphosphate > 2-methylthioadenosine triphosphate (2MeSATP) > ATP > UTP) that were similar to those previously observed for activation of phospholipase C in 1321N1 human astrocytoma cells stably expressing the human or turkey $P2Y_1$ receptor. The P2Y receptor antagonists suramin and pyridoxal phosphate 6-azophenyl-2′-4′-disulfonic acid both shifted to the right the concentration-response relationship for 2MeSATP for stimulation of oocyte currents. Although injection of oocytes with either GDPβS (guanyl-5′-yl thiophosphate) or GTPγS (guanosine 5′-3-*O*-(thio)triphosphate) resulted in loss of adenine nucleotide-promoted Cl^- channel activation, neither guanine nucleotide altered the 2MeSATP-stimulated cation current. These data are consistent with the view that activation of the novel cation current by the $P2Y_1$ receptor does not involve a G protein. Tail current analysis of the novel $P2Y_1$ receptor-associated cation conductance revealed that the open channel current-voltage relationship was outwardly rectifying with a reversal potential of −38 mV for the turkey $P2Y_1$ receptor and −36 mV for the human $P2Y_1$ receptor. Replacement of Na^+ with K^+ ions in the bathing solution produced a shift in reversal potential to near zero mV, but significant outward rectification remained. The cation current was not permeable to either Ca^{2+} or Ba^{2+} and exhibited steady-state inactivation at holding potentials below −60 mV. These results indicate that the $P2Y_1$ receptor exhibits both metabotropic properties and a novel G protein-independent ionotropic response when expressed in *Xenopus* oocytes.

Extracellular adenine nucleotides regulate a variety of cellular events through interaction with P2 receptors (1–4). This receptor family was originally subclassified into the P2X and P2Y receptor subtypes (5), and additional subtypes subsequently were proposed on the basis of differences in pharmacological and signaling properties (1, 2, 6).

P2X receptors possess ionotropic properties, promote contraction of a variety of smooth muscle preparations, and are widely distributed in the central nervous system (1, 7–9). These receptors function as non-selective cation channels and produce membrane depolarization upon activation by ATP. The P2Y receptor was originally characterized as a receptor(s) that mediated ATP-induced smooth muscle relaxation in rabbit portal vein and guinea pig taenia coli. The P2Y receptor also was found to be activated by 2MeSATP[1] but not by P2X selective agonists such as α,β-methylene ATP or β,γ-methylene ATP (5). Multiple P2Y receptors are now known to exist and most of these couple to the G_q family of G proteins and promote inositol lipid hydrolysis (2, 3, 10).

A phospholipase C-activating receptor ($P2Y_1$ receptor) has been cloned from chick (11), turkey (12), and several mammalian species (13), including human (14). Stable expression of the $P2Y_1$ receptor in a null cell line confers pharmacological properties similar to those originally ascribed to P2Y receptors in many tissues. The primary sequence of this receptor is typical of members of the superfamily of G protein-coupled receptors and predicts the presence of seven α-helical transmembrane domains with an extracellular N terminus and an intracellular C terminus. Three additional P2Y receptor family members have been cloned. The $P2Y_2$ receptor is analogous to the originally described P_{2U}-purinergic receptor and is activated by ATP and UTP (1, 15). The two most recently cloned members of this family are selectively activated by uridine nucleotides (16–19).

In a previous study, stable expression of the cloned turkey (12) or human (14) $P2Y_1$ receptors in 1321N1 human astrocytoma cells conferred an inositol lipid signaling response upon addition of purinergic agonists. Here we describe the functional properties of the turkey and human $P2Y_1$ receptors expressed in *Xenopus* oocytes. We report that incubation of $P2Y_1$ receptor-expressing oocytes with adenine nucleotides not only results in activation of a Ca^{2+}-dependent Cl^- current as expected for a phospholipase C-coupled receptor (20, 21), but also activates an inwardly rectifying non-selective cation current that has not been described previously. The novel P2Y receptor-associated

* This work was supported by a grant from the 3M Company, United States Public Health Service Grants GM38213 and HL32322, and by Agricultural Experiment Station Grant MIN-16-82. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Physiology and Animal Sciences, 495 Animal Science/Veterinary Medicine Bldg., University of Minnesota, St. Paul, MN 55108.

[1] The abbreviations used are: 2MeSATP, 2-methylthioadenosine triphosphate; 2MeSADP, 2-methylthioadenosine diphosphate; PPADS, pyridoxal phosphate 6-azophenyl-2′-4′-disulfonic acid; GDPβS, guanyl-5′-yl thiophosphate; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

current is not dependent on G protein activation and functional characterization suggests that at least one member of the superfamily of G protein-coupled receptors can produce ionotropic responses that are dependent on receptor expression alone.

EXPERIMENTAL PROCEDURES

Materials—Xenopus laevis frogs were purchased from *Xenopus* I (Ann Arbor, MI) and maintained in aquaria as suggested by the supplier. Collagenase, penicillin, and gentamicin were obtained from Life Technologies, Inc. 2MeSATP, 2-methylthioadenosine diphosphate (2MeSADP), pyridoxal phosphate 6-azophenyl-2′-4′-disulfonic acid (PPADS) and suramin were obtained from Research Biochemicals Inc. (Natick, MA). Adenosine triphosphate (ATP) and uridine triphosphate (UTP) were obtained from Sigma.

Oocyte Isolation and Injection—Ovarian lobes from adult *Xenopus* frogs were removed from anesthetized animals under sterile conditions. The tissue mass was dissociated with collagenase solution (in mM: 90 NaCl, 1 KCl, 0.82 $MgSO_4$, 10 HEPES (pH 7.4), 250 units/ml collagenase), and Stage V and VI oocytes were sorted, defolliculated, and maintained in standard amphibian Ringer solution (in mM: 90 NaCl, 1 KCl, 0.82 $MgSO_4$, 0.74 $CaCl_2$, 10 HEPES, pH 7.4, supplemented with 100 IU/ml penicillin and 50 μg/ml gentamicin) at 19–20 °C. After a 2–3-h recovery period, the eggs were injected with cRNA transcripts (25–50 ng/oocyte). Injections were performed with a Drummond Nanoject oocyte injection system, which electronically controls the volume (46 nl) delivered into the oocyte. Control eggs were injected with 46 nl of sterile water. Oocytes were stored for 2–7 days in amphibian Ringer solution supplemented with 100 IU/ml penicillin and 50 μg/ml gentamicin at 19–20 °C.

Preparation of RNA for Injection—cRNA was synthesized from linear cDNA encoding either the turkey $P2Y_1$ (12), the human $P2Y_1$ (14), the human $P2Y_2$ (22), or the human M1 muscarinic receptor (23) using Ambion Megascript.

Electrophysiological Measurements—Electrophysiological measurements were made using the two-electrode voltage clamp (Dagan TEV-200) technique at 20 °C. Recordings were made in amphibian Ringer solution containing (in mM) 90 NaCl, 1 KCl, 0.82 $MgCl_2$, 0.74 $CaCl_2$, 10 HEPES (pH 7.4) beginning 12–15 h after injection. Current- and voltage-measuring electrodes were pulled from borosilicate filament glass to resistances between 0.5 and 2 megohms, when filled with 0.5 M KCl. Data acquisition and analysis were performed with a Compaq 486 microcomputer using pCLAMP software (Axon Instruments).

Statistics—Data presented in the current-voltage relationships and concentration-response relationships represent the mean ± standard error. Student's *t* test for paired or unpaired means was used to determine statistical significance. A value of $p < 0.05$ was considered significant. Information regarding number of oocytes used for each experiment is given in the figure legends.

RESULTS

Expression of $P2Y_1$ and Other G Protein-coupled Receptors in Xenopus Oocytes—Voltage clamp analyses were carried out with oocytes expressing the human $P2Y_1$ receptor (Fig. 1). Oocytes were held at −20 mV in amphibian Ringer solution and sequentially stepped through a series of voltages from −140 to +140 mV in 20-mV increments. No ATP- or 2MeSATP-promoted inward currents were detected in water-injected control cells using this protocol (data not shown). Likewise, no inward currents were detected in $P2Y_1$ receptor-expressing oocytes in the absence of 2MeSATP (Fig. 1). However, addition of 500 nM 2MeSATP to $P2Y_1$ receptor-expressing cells resulted both in a slowly activating inward current in response to hyperpolarizing voltage steps and an increase in outward current in response to depolarizing voltage steps. The current was inwardly rectifying at negative voltages and outwardly rectifying at voltages above +60 mV with a reversal potential near −40 mV.

To determine if the inward current was uniquely associated with $P2Y_1$ receptors, two additional G_q/phospholipase C-coupled receptors were expressed in oocytes. $P2Y_2$ (P_{2U}) receptor-expressing oocytes were held at either −20 or −80 mV in amphibian Ringer solution (Fig. 2) and subjected to the same voltage step protocol described in Fig. 1. Control currents exhibited a near-linear current-voltage relationship with a reversal potential of −40 mV. Addition of 100 μM UTP elicited an outwardly rectifying whole cell current and shifted the reversal potential to −12 mV. Current activation was not altered over a range of holding potentials from −80 to −20 mV. The time course and current-voltage relationship of the UTP-activated current in these cells was nearly identical to the current response elicited by 100 μM carbachol in M1 muscarinic receptor-expressing oocytes under identical experimental conditions (data not shown) and to the 2MeSATP-promoted outward current described above (Fig. 1). These currents likely were due to opening of a Ca^{2+}-activated Cl^- channel secondary to elevation of intracellular Ca^{2+}. To test this hypothesis, Ca^{2+} levels were elevated independently of receptor activation by incubation of water-injected oocytes with thapsigargin or the calcium ionophore ionomycin. A Cl^- current was observed identical to that occurring in response to agonists in $P2Y_1$ receptor-, M1 receptor-, and $P2Y_2$ receptor-expressing oocytes (data not shown). However, in contrast to the result with $P2Y_1$ receptor-expressing oocytes, no inward cation current was detected in response to elevation of intracellular Ca^{2+} by thapsigargin or ionomycin.

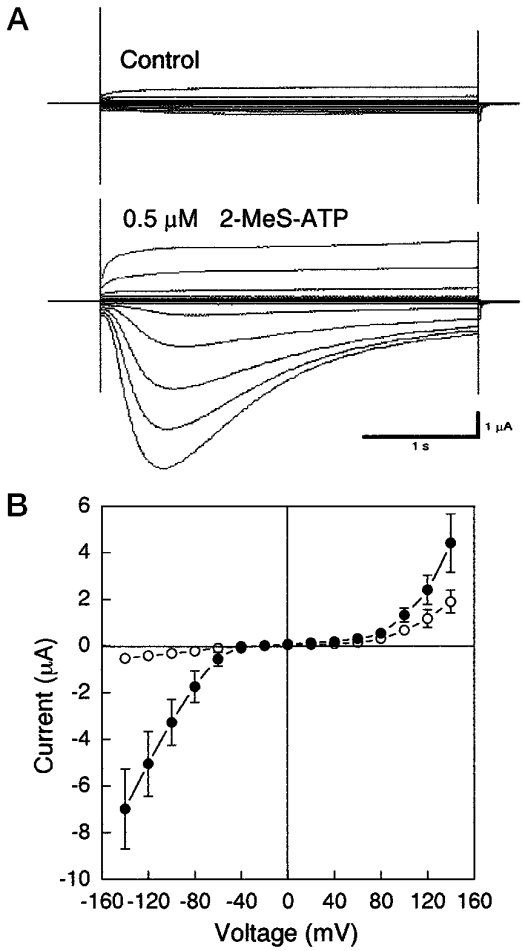


FIG. 1. **Expression of human $P2Y_1$ ($hP2Y_1$) receptor cRNA in *Xenopus* oocytes.** Oocytes were held at −20 mV in amphibian Ringer solution (in mM) 90 NaCl, 1 KCl, 0.82 $MgCl_2$, 0.74 $CaCl_2$, 10 HEPES (pH 7.4) and stepped through a series of voltages from −140 to +140 mV in 20-mV increments. *A*, representative tracing showing the effects of 0.5 μM 2MeSATP on the whole cell current. *B*, current-voltage relationship showing that both inwardly and outwardly rectifying currents are associated with 2MeSATP stimulation ($n = 7$). Data for cells incubated with vehicle (*open circles*) or 0.5 μM 2MeSATP (*filled circles*) human P2Y receptor are presented.

Pharmacological Properties of the Expressed Human $P2Y_1$ Receptor—Concentration-response relationships for agonist-

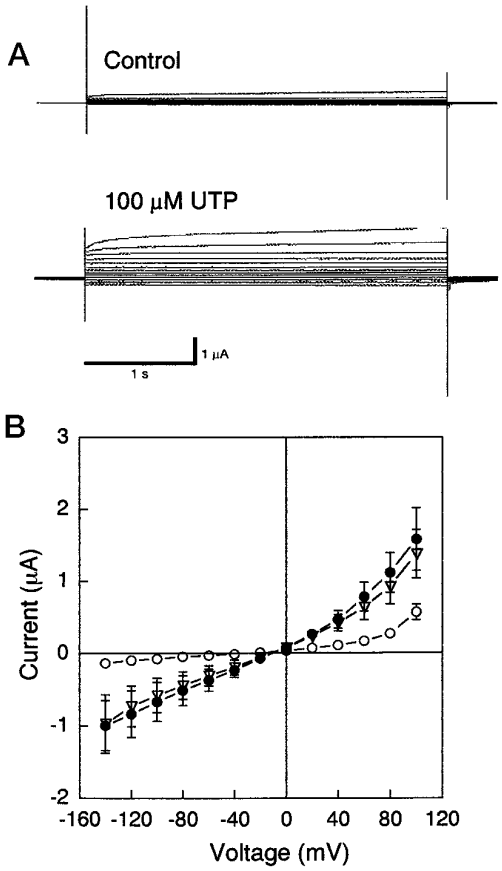


FIG. 2. **Expression of $P2Y_2$ receptor cRNA in *Xenopus* oocytes.** Oocytes were held at either −20 or −80 mV in amphibian Ringer solution and subjected to the same voltage step protocol described in Fig. 1. *A*, representative tracing showing the effects of 100 μM UTP on the whole cell current. *B*, current-voltage relationship showing that an outwardly rectifying current is associated with UTP stimulation. Control currents (*open circles*) exhibited a near-linear current-voltage relationship with a reversal potential of −40 mV. Addition of 100 μM UTP (*filled circles*) shifted the reversal potential to −12 mV (n = 5). Changing the holding potential from −80 to −20 mV (*inverted triangles*) did not significantly affect current activation.

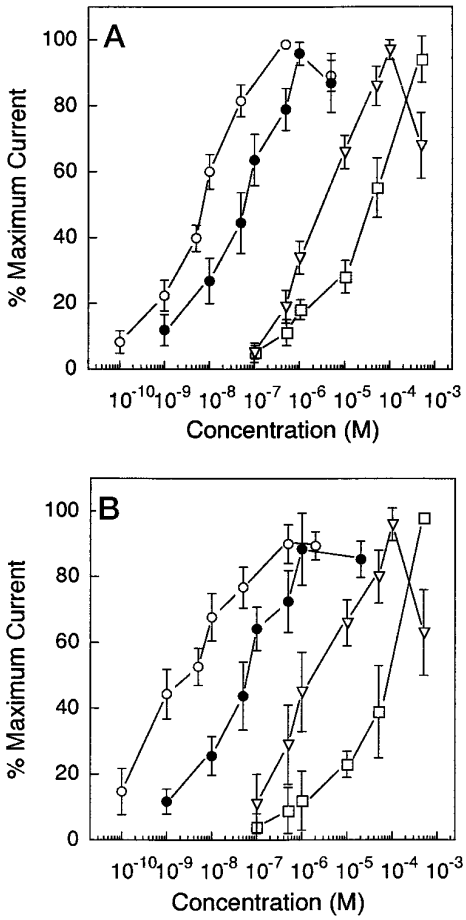


FIG. 3. **Pharmacological selectivity of adenine nucleotide-stimulated currents.** *A*, concentration-response relationship for the hP2Y1-promoted current showing the effects of 2-MeSADP (*open circles*, n = 6), 2MeSATP (*filled circles*, n = 6), ATP (*inverted triangles*, n = 6) and UTP (*squares*, n = 5) on inward current elicited by step hyperpolarization to −140 mV (holding potential (hp) = −20 mV). The EC_{50} values for each agonist were 0.008, 0.07, 3, and >70 μM, respectively. *B*, Concentration-response relationship for the hP2Y1 current showing the effects of 2MeSADP (*open circles*, n = 6), 2MeSATP (*filled circles*, n = 6), ATP (*inverted triangles*, n = 6), and UTP (*squares*, n = 5) on outward current produced by step depolarization to +120 mV (hp = −20 mV). The EC_{50} values for each agonist were 0.009, 0.08, 3, and >100 μM, respectively.

promoted currents in $P2Y_1$ receptor-expressing oocytes are presented in Fig. 3. The EC_{50} values for 2MeSADP, 2MeSATP, ATP, and UTP were 0.008, 0.07, 3, and >70 μM, respectively, for stimulation of the non-selective cation current (Fig. 4*A*), and were 0.009, 0.08, 3, and >100 μM, respectively, for stimulation of the outward Cl^- current (Fig. 3*B*). Therefore, the potencies of P2Y agonists for stimulation of the two currents were essentially identical and were very similar to those determined for stimulation of inositol phosphate accumulation in cells lines expressing recombinant $P2Y_1$ receptors (12, 14). The effects of two compounds previously shown to competitively antagonize $P2Y_1$ receptors also were examined (Fig. 4). Co-addition of 70 μM PPADS with various concentrations of 2MeSATP resulted in a 7.1-fold shift (from 0.07 to 0.5 μM) in the EC_{50} for 2MeSATP. Co-addition of suramin (10 μM) produced a 4.3-fold shift (from 0.07 to 0.3 μM) in the EC_{50} for 2MeSATP. The concentrations of PPADS and suramin used in these experiments did not significantly alter the maximum current response to 2MeSATP (data not shown).

Effect of Guanine Nucleotides on $P2Y_1$ Receptor-promoted Responses—The deduced amino acid sequence of the $P2Y_1$ receptor predicts a seven-transmembrane spanning protein typical of that of members of the superfamily of G protein-coupled receptors. The signaling responses previously associated with the $P2Y_1$ receptor apparently occur secondarily to activation of members of the G_q family of G proteins. Therefore, the potential role of G proteins in the 2MeSATP-promoted currents of $P2Y_1$ receptor-expressing oocytes was examined (Fig. 5). Oocytes expressing the human $P2Y_1$ receptor were injected with 0.2 nmol (estimated intra-oocyte concentration = 400 μM) of either GDPβS or GTPγS. Voltage was held at −20 mV and stepped to either −140 mV or +120 mV to determine the effects of G protein modulators on 2MeSATP (20 μM)-evoked inward cation and outward Cl^- currents. Treatment with GDPβS inhibited the 2MeSATP-promoted Cl^- current but had no effect on agonist-stimulated inward cation current. GDPβS also had no effect on the action of thapsigargin (0.1 μM), indicating that GDPβS did not block the calcium-activated Cl^- current at a level distal to G protein activation. Injection of oocytes with GTPγS promoted an outward Cl^- current in the absence of 2MeSATP and ablated any further effect of 2MeSATP on the Cl^- current. In contrast, GTPγS alone did not induce the inward cation current in the absence of 2MeSATP and also had no effect on activation of the inward cation current by 2MeSATP. These results suggest that the $P2Y_1$ receptor-promoted inward cation current does not involve G protein activation.

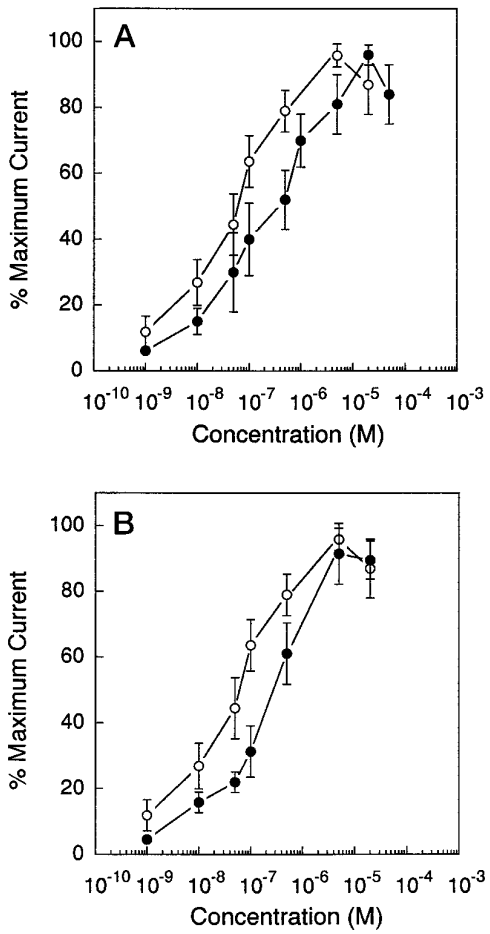


FIG. 4. **Effects of P2Y receptor antagonists on the 2MeSATP-stimulated inward current elicited by step hyperpolarization to −140 mV (hp = −20 mV).** *A*, effects of 70 μM PPADS (n = 6). PPADS produced a shift in the EC_{50} from 0.07 to 0.5 μM. Control, *open circles*; PPADS, *filled circles B*, effects of 10 μM suramin (n = 7). Suramin produced a shift in the EC_{50} from 0.07 to 0.3 μM. Control, *open circles*; suramin, *filled circles*.

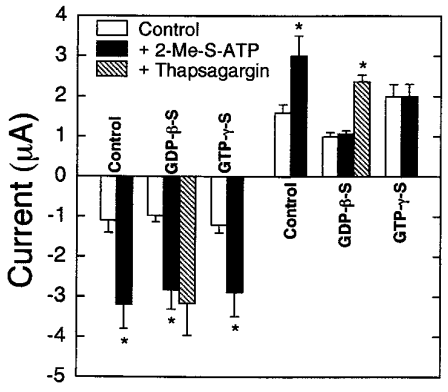


FIG. 5. **Effects of GDPβS and GTPγS on 2MeSATP-promoted currents in hP2Y receptor expressing *Xenopus* oocytes.** Oocytes expressing the hP2Y1 receptor were injected with 0.2 nmol (estimated intra-oocyte concentration = 400 μM) of either GDPβS (n = 12) or GTPγS (n = 7). Oocytes were held at −20 mV and stepped to either −140 mV or +120 mV to determine the effects of G protein modulators on 2MeSATP (20 μM) evoked inward and outward currents. Thapsigargin (0.1 μM) was used as a positive control in the GDPβS experiments to prove that GDPβS did not block the calcium-activated outward Cl^- current.

Holding Potential Sensitivity of the P2Y Current—The adenine nucleotide-promoted current in human $P2Y_1$ receptor-expressing oocytes exhibited holding potential sensitivity with respect to inward currents evoked by hyperpolarizing voltage steps (Fig. 6). Oocytes were voltage-clamped for 5 min at each of several holding potentials from −80 to 0 mV. The cells were stepped to −140 mV to activate the current, and peak inward current in responses to 0.5 μM 2MeSATP were plotted as a function of holding potential. Steady-state inactivation was observed at voltages below −60 mV. The increase in outward Cl^- current was not affected by holding potential. Analysis of the difference current derived from subtraction of whole cell currents obtained at holding voltages of −80 mV and −20 mV indicated that the agonist-stimulated cation current in $P2Y_1$ receptor-expressing oocytes is inwardly rectifying with a reversal potential of −40 mV (Fig. 7). Essentially identical results were obtained irrespective of whether the human or turkey homologues of the $P2Y_1$ receptor were studied (Fig. 6).

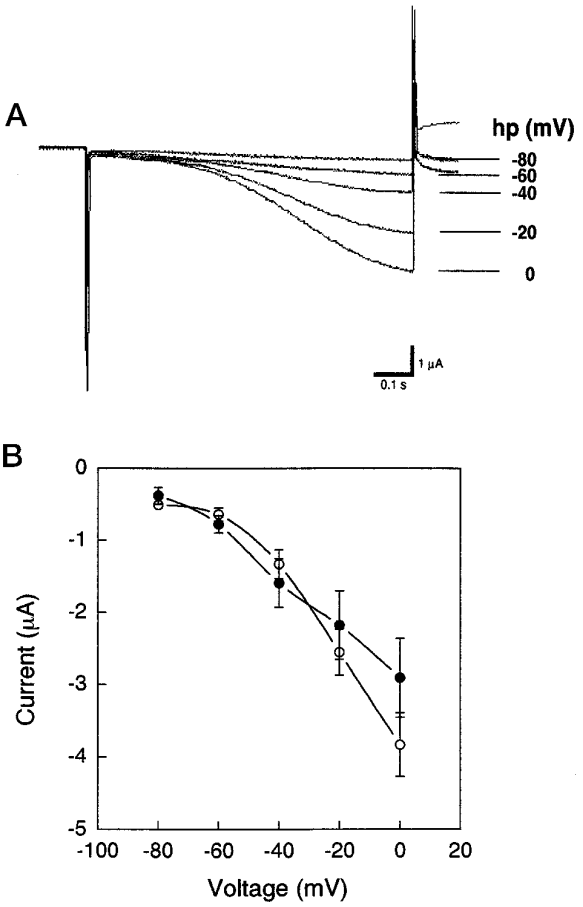


FIG. 6. **Holding potential sensitivity of the tP2Y1 and hP2Y1 currents.** Oocytes were voltage-clamped for 5 min at each of several holding potentials from −80 to 0 mV. The cells were then stepped to −140 mV to activate the current. *A*, representative tracing showing the effects of holding potential on the whole cell current. *B*, peak inward current responses were plotted as a function of holding potential (tP2Y1, *open circle*, (n = 12); hP2Y1, *filled circle* (n = 5)).

Analysis of Tail Currents and the Near-instantaneous Current-Voltage Relationship—Tail current analysis was carried out for both the human and turkey $P2Y_1$ receptor currents following activation with 100 μM ATP (Fig. 8). Oocytes were held at −20 mV in standard amphibian Ringer solution and stepped to −140 mV to activate the current. At the peak of activation the oocyte was stepped through a series of depolarizing voltages from +60 to −80 mV to determine the near-instantaneous (50 ms following the voltage step) current-voltage relationship and the deactivation time course. To control for differences in the degree of P2Y receptor expression between oocytes, the magnitude of the evoked current was refer-

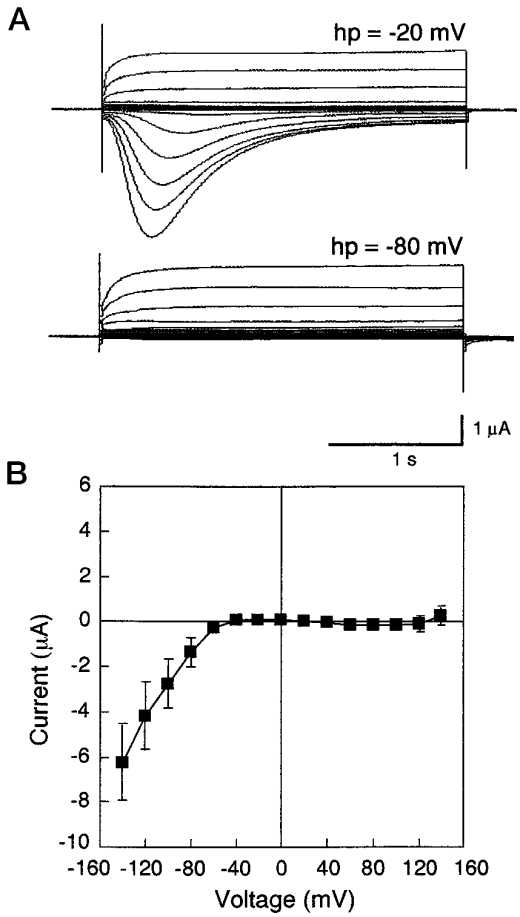


FIG. 7. **Difference current analysis of the hP2Y1 current.** *A*, representative tracings showing the effects of holding potential on the whole cell current in the presence of 0.5 μM 2MeSATP. *B*, current-voltage relationship of the difference current between −20 and −80 mV holding potentials (n = 6).

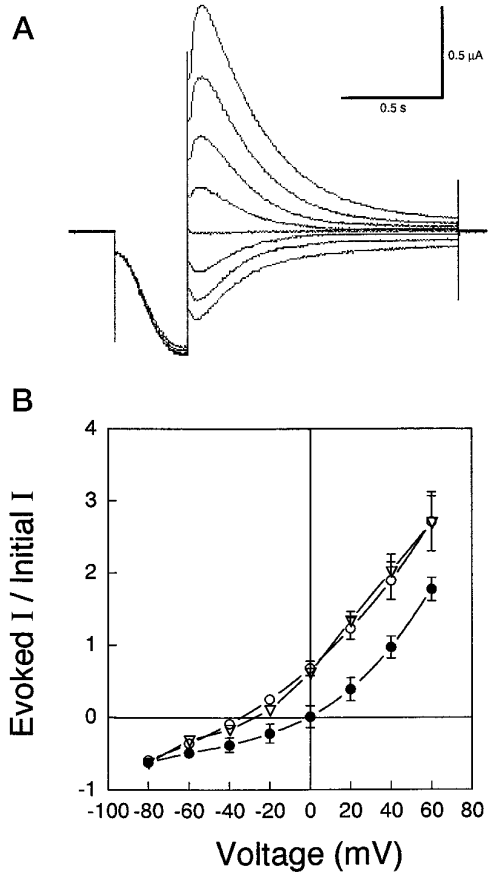


FIG. 8. **Tail current analysis of tP2Y1 and hP2Y1 currents following activation with ATP.** *A*, representative tracing showing tail currents from oocytes expressing the tP2Y receptor. Cells in the presence of 100 μM ATP were held at −20 mV and stepped to −140 mV to activate the current. At the peak of activation, the cell was stepped through a series of depolarizing voltages from +60 to −80 mV to determine the near-instantaneous (50 ms following the voltage step) current-voltage relationship and the deactivation time course. *B*, near-instantaneous current-voltage relationship for the tP2Y1 and hP2Y1 currents in amphibian Ringer solution and in high potassium Ringer solution (in amphibian Ringer: tP2Y, *open circle* (n = 10) and hP2Y1, *inverted triangle* (n = 8); high potassium Ringer: tP2Y1, *filled circle* (n = 6)). To control for differences in the degree of $P2Y_1$ receptor expression, the magnitude of the evoked current was referenced to the initial maximum activated current at −140 mV. The tP2Y1 and hP2Y1 I-V relationships were not significantly different from each other; both were outwardly rectifying with reversal potentials of −38 and −36 mV, respectively. Replacement of sodium in the bathing solution with potassium (high potassium conditions) produced a shift in the reversal potential to zero mV.

enced to the initial maximally activated current at −140 mV. The I-V relationships for the human and turkey $P2Y_1$ receptors were not significantly different from each other. Both were outwardly rectifying with reversal potentials of −38 and −36 mV, respectively. Replacement of sodium with potassium in the bathing solution produced a shift in the reversal potential to near zero mV, indicating a greater selectivity for potassium than sodium.

To determine whether the channel was permeable to divalent cations, Na^+ was replaced with barium in the bathing solution of oocytes expressing the human $P2Y_1$ receptor (Fig. 9). Incubation with 2MeSATP resulted in an increase in inward current when sodium was present in the bathing solution. However, when sodium was replaced with barium, no 2MeSATP-promoted increase in inward current was detected. A decrease in basal inward current also was observed in each case. Similar results were obtained in experiments in which sodium was replaced with Ca^{2+} in the bathing solution (data not shown). Oocytes bathed in $BaCl_2$-containing or $CaCl_2$-containing Ringer solution exhibited a significantly greater outward current compared to oocytes in standard amphibian Ringer solution. This difference was presumably due to the higher Cl^- concentration in the $BaCl_2/CaCl_2$ Ringer solutions, which results in an increased driving force for Cl^- entry into the cell. Additional activation of the Ca^{2+}-activated Cl^- channel also may have resulted from Ca^{2+} or Ba^{2+} entry into the cell.

Analysis of the Activation, Inactivation, and Deactivation Gating Events—The voltage dependence of activation, inactivation, and deactivation was examined by measuring the time constants for these gating events obtained from the experiments described in Figs. 1, 7, and 8. Time constants for activation and inactivation were obtained by fitting the currents produced by the activation protocol from Fig. 1 with the sum of three exponentials at voltages between −60 and −140 mV ($y = C + a_1e^{(-t/\tau 1)} + a_2e^{(-t/\tau 2)} + a_3e^{(-t/\tau 3)}$). Two exponentials were required to fit the activation process, and a single exponential was sufficient to fit the process of inactivation (activation at −140 mV: $\tau_1 = 0.07 \pm 0.006$ s, $\tau_2 = 0.14 \pm 0.015$ s; inactivation at −140 mV: $\tau_3 = 0.67 \pm 0.1$ s). There was little voltage dependence associated with activation or inactivation. Deactivation time constants were obtained by fitting the current traces from tail current experiments described in Fig. 8. Deactivation was well described by a single exponential function from the current peak to the end of the voltage step and

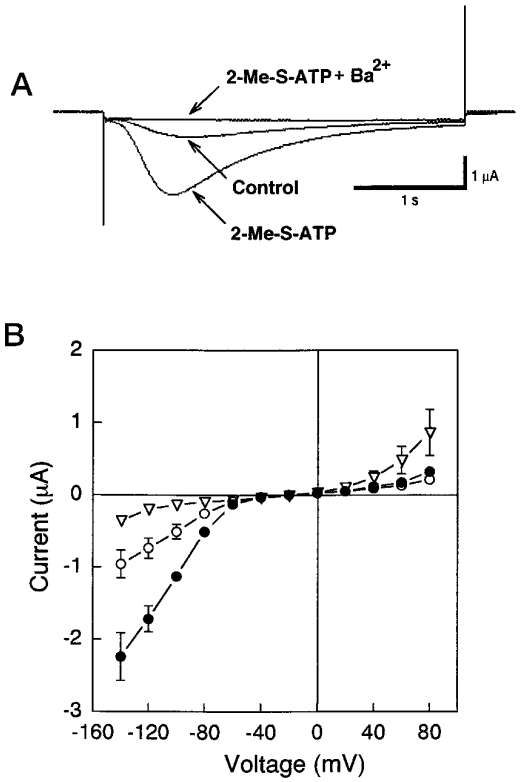


FIG. 9. **Effects of sodium replacement with barium in the bathing solution of oocytes expressing the hP2Y1 receptor.** *A*, representative tracing showing the effects of replacing extracellular sodium with barium. *B*, current-voltage relationship showing that barium replacement blocks inward current activation elicited by 0.5 μM 2MeSATP (n = 6; *open circle*, control conditions; *filled circle*, stimulation with 2MeSATP; *inverted triangle*, replacement with barium in the presence of 2MeSATP).

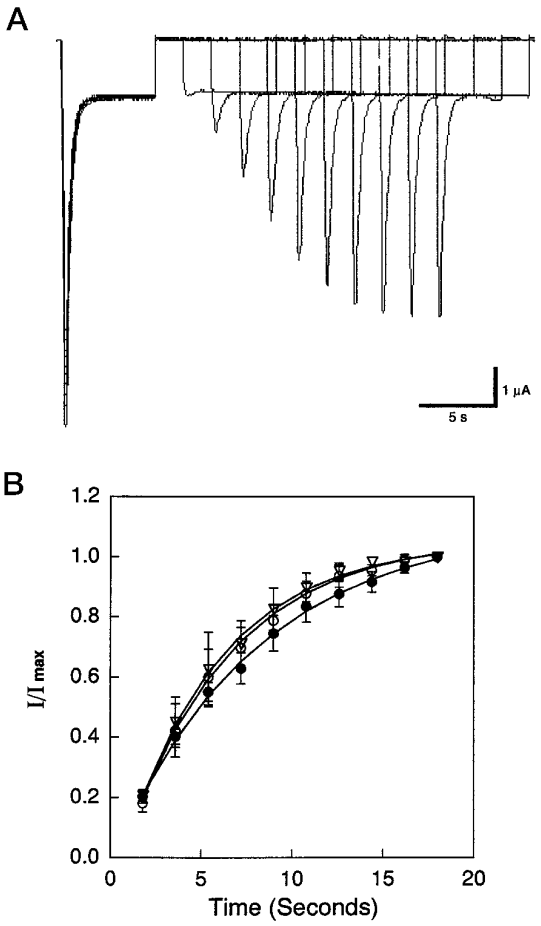


FIG. 10. **Voltage dependence of recovery from inactivation.** *A*, representative tracing showing a recovery from inactivation record obtained from a protocol where cells were held at either −40 mV, −20 mV, or 0 mV (hp = −20 mV) in the continuous presence of agonist. *B*, peak inward currents plotted as a function of time and fit with a single exponential function to determine the time constants for recovery from inactivation at each holding potential (hp = zero mV, *inverted triangles* (n = 5); hp = −20 mV, *open circles* (n = 5); hp = −40 mV, *filled circles* (n = 5)).

exhibited voltage dependence over the range of −40 to +60 mV (τ_d at +60 mV = 0.48 ± 0.09 s; τ_d at −80 mV = 0.2 ± 0.02 s).

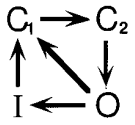
Recovery from Inactivation—Recovery from inactivation was studied in human $P2Y_1$ receptor-expressing cells held at either −40 mV, −20 mV, or 0 mV in the continuous presence of agonist (Fig. 10). Oocytes were subjected to a hyperpolarizing voltage pulse to −140 mV until complete inactivation was observed. The voltage was then stepped back to the initial holding potential for varying lengths of time, and reactivation was promoted by hyperpolarization to −140 mV. Peak inward currents were plotted as a function of time and fit with a single exponential function to determine the time constants for recovery from inactivation at each holding potential (estimated τ = 6 s). No significant effect of holding potential on recovery from inactivation was detected.

DISCUSSION

Expression of the $P2Y_1$ receptor in *Xenopus* oocytes confers an adenine nucleotide-activated cation current that has not been recognized previously. Although $P2Y_1$ receptor activation also resulted in an increase in Cl^- permeability that occurs with other G_q/phospholipase C-linked receptors (20, 21), holding potential sensitivity differentiated the effects of P2Y receptor agonists on cation and Cl^- permeabilities through an analysis of the difference currents at −80 and −20 mV. Oocyte expression of M1 muscarinic or $P2Y_2$ receptors under the same conditions conferred only the predicted Cl^- permeability in response to agonists. Therefore, the novel cation permeability associated with activation of the $P2Y_1$ receptor is neither a general property of G_q/phospholipase C-linked receptors nor of the P2 receptor subclass of the G protein-coupled receptor superfamily.

The unique adenine nucleotide-promoted cation current was inwardly rectifying and had very little open probability at holding voltages more negative than −40 mV. The open channel current-voltage responses associated with activation of both the human and turkey P2Y receptors were outwardly rectifying and reversed at voltages between −35 and −40 mV, suggesting a greater selectivity for potassium than sodium. Replacement of sodium with potassium in the bathing solution also resulted in a shift in reversal potential to near zero mV, which indicates that the cation channel was permeable to potassium. However, a permeability ratio could not be accurately determined from the tail current experiments since intracellular sodium and potassium activities were unknown. Rectification cannot be completely explained by the increased driving force for potassium efflux at voltages above 0 mV, since significant outward rectification was still apparent in high potassium Ringer solution. Thus, we suggest that outward rectification is an inherent property of the pore. The $P2Y_1$ receptor-associated cation current does not involve passage of divalent cations, and the inward current at voltages below −40 mV is carried by sodium ions.

A minimum state gating model for adenine nucleotide-promoted activation of a novel cation current is proposed (Gating



GATING MODEL

model). The time course of cation channel activation was slow, requiring nearly 500 ms to achieve a maximal inward current. Activation was well described by the sum of two exponentials, suggesting that the channel may transit through at least two closed states (C_1 and C_2) before it completely opens (O). Prolonged hyperpolarization in the continuous presence of agonist resulted in a decrease in inward current and indicates the existence of an inactivated state (I). Complete recovery following inactivation required approximately 20 s at holding potentials above −40 mV ($I \rightarrow C_1$) and exhibited no significant voltage dependence. Finally, the deactivation process ($O \rightarrow C_1$) as revealed in tail current experiments was well described by a single exponential function from the peak current to the end of the voltage step and appeared to be the only gating process to exhibit significant voltage dependence. The basis of the initial time-dependent increase in outward current following steps to positive voltages is not known. However, a similar phenomenon was observed in tail current studies with a cardiac delayed-rectifier potassium channel, where a time-dependent increase in inward current was observed following hyperpolarization from an initial depolarization step used for channel activation (24). The unique gating properties of the $P2Y_1$ receptor-activated cation current clearly distinguish it from previously characterized ion channels in the *Xenopus* oocyte.

The pharmacological selectivities of adenine nucleotide analogues for Cl^- and cation channel activation were similar to those we previously observed for activation of phospholipase C in 1321N1 cells expressing the human or turkey $P2Y_1$ receptors. In addition, 2MeSATP-promoted activation of the cation current was blocked by both suramin or PPADS, which previously were shown to be competitive antagonists at the $P2Y_1$ receptor. As predicted from previous studies of G protein-coupled receptors expressed in *Xenopus* oocytes, injection of guanine nucleotide analogs blocked the effects of $P2Y_1$ receptor activation on the Ca^{2+}-dependent Cl^- current. In contrast, no effect of guanine nucleotides on $P2Y_1$ receptor-promoted activation of the cation current was observed, which strongly suggests that $P2Y_1$ receptor-promoted increases in cation current are not dependent on receptor coupling to endogenous G proteins.

One interpretation of the results is that the expressed $P2Y_1$ receptor possesses both metabotropic and ionotropic properties. Since we are unaware of ion channel activity associated with other G protein coupled receptors, the $P2Y_1$ receptor may be a member of a novel subset of the superfamily of G protein-coupled receptors that exhibit ionotropic properties similar to those of P2X receptors. These similarities would include inwardly rectifying current-voltage relationships and corresponding selectivities for sodium and potassium. However, unlike P2X receptors, the $P2Y_1$ receptor exhibits voltage-dependent inactivation and is not permeable to calcium (9). Such discrepancies in functional properties may result from the significant structural differences that exist between these two purinergic receptor subtypes.

An alternative interpretation of the data in this study could be that $P2Y_1$ receptors possess a unique, G protein-independent mechanism for coupling to a previously uncharacterized cation channel in *Xenopus* oocytes. This interpretation would require that this cation channel not be activated by changes in membrane potential or by activation of the inositol lipid signaling cascade by M1 muscarinic or P2U receptors. In broad ranging studies with the natively expressed or cloned $P2Y_1$ receptors, we have seen no evidence for biochemical responses to purinergic agonists other than those associated with activation of phospholipase C (12, 14, 25–27). A less likely possibility is that expression of the $P2Y_1$ receptor in *Xenopus* oocytes also could promote transcription and subsequent expression of a novel cation channel, which then was activated in an agonist-dependent manner by a mechanism that does not require coupling to endogenous G proteins.

Parker and Scarpa (28) recently described a slowly activating current in guinea pig ventricular myocytes that was stimulated by ATP and 2MeSATP but not by α,β-methylene ATP or UTP. This current was inwardly rectifying with a reversal potential near zero mV. 2MeSATP-stimulated activation of this non-selective cation current apparently was not G protein-dependent, since internal perfusion would have significantly diluted any soluble constituents within the cell. A similar inwardly rectifying non-selective cation current also was identified in dissociated neurons from the tuberomammillary nucleus of the rat brain. 2MeSATP was found to be more potent than α,β-methylene in producing activation of the channel, and no effect of adenosine was observed (29). These results suggest that a P2Y receptor was responsible for channel activation. Thus, P2Y receptors that are natively expressed in at least one population of CNS neurons and in guinea pig cardiac myocytes activate a cation current that is similar to that observed in oocytes expressing the cloned human or turkey $P2Y_1$ receptor.

In summary, activation of a novel cation channel has been shown to be associated with activation of the $P2Y_1$ receptor expressed in *Xenopus* oocytes. The results suggest dual functionality of this G protein-coupled receptor. The functional and structural bases of the apparently G protein-independent ionotropic properties of the $P2Y_1$ receptor will need to be reconciled with those of classically defined ionotropic receptors of much different structure.

Acknowledgments—We thank Nancy Stewig for help with the oocyte expression experiments. We are indebted to Joel Schachter for the human $P2Y_1$ receptor clone, to Eduardo Lazarowski for the human $P2Y_2$ receptor clone, and to Neil Nathanson for the human M1 muscarinic receptor clone. We are also indebted to Brenda Lindsey Asam for typing the manuscript and to Drs. Kim Cooper, Barry Pallotta, Bob Rosenberg, José Boyer, and Joel Schachter for discussions and suggestions.

REFERENCES

1. Dubyak, G. R., and El-Moatassim, C. (1993) *Am. J. Physiol.* **265,** C577–C606
2. Harden, T. K., Boyer, J. L., and Nicholas, R. A. (1995) *Annu. Rev. Pharmacol. Toxicol.* **35,** 541–579
3. Boarder, M. R., Weisman, G. A., Turner, J. T., and Wilkinson, G. F. (1995) *Trends Pharmacol. Sci.* **16,** 133–139
4. Abbracchio, M. P., and Burnstock, G. (1994) *Pharmacol. Ther.* **64,** 445–475
5. Burnstock, G., and Kennedy, C. (1985) *Gen. Pharmacol.* **16,** 433–440
6. Fredholm, B. B., Abbracchio, M. P., Burnstock, G., Daly, J. W., Harden, T. K., Jacobson, K. A., Leff, P., and Williams, M. (1994) *Pharmacol. Rev.* **46,** 143–156
7. Valera, S, Hussy, N, Evans, R. J., Adami, N., North, R. A., Surprenant, A., and Buell, G. (1994) *Nature* **371,** 516–519
8. Brake, A. J., Wagenbach, M. J., and Julius, D. (1994) *Nature* **371,** 519–523
9. Surprenant, A., Buell, G., and North, R. A. (1995) *Trends Neurochem. Sci.* **18,** 224–229
10. Filtz, T. M., Harden, T. K., and Nicholas, R. A. (1996) in *Purinergic Approaches in Experimental Therapeutics* (Jacobson, K. A., ed) John Wiley & Sons, New York, in press
11. Webb, T. E., Simon, J., Krishek, B. J., Bateson, A. N., Smart, T. G., King, B. F., Bumstock, G., and Barnard, E. A. (1993) *FEBS Lett.* **324,** 219–225
12. Filtz, T. M., Qing, L., Boyer, J. L., Nicholas, R. A., and Harden, T. K. (1994) *Mol. Pharmacol.* **46,** 8–14
13. Henderson, D. J., Elliot, D. G., Smith, G. M., Webb, T. E., and Dainty, I. A. (1995) *Biochem. Biophys. Res. Commun.* **212,** 648–656
14. Schachter, J. L., Li, Q., Boyer, J. L., Nicholas, R. A., and Harden, T. K. (1996) *Br. J. Pharmacol.* **118,** 167–173
15. Lustig, K. D., Shiau, A. K., Brake, A. J., and Julius, D. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 5113–5117
16. Chang, K., Hanaoka, K., Kumada, M., and Takuwa, Y. (1995) *J. Biol. Chem.* **270,** 26152–26158

17. Communi, D., Pirotton, S., Parmentier, M., and Boeynaems, J.-M. (1995) *J. Biol. Chem.* **270,** 30849–30852
18. Nguyen, T., Erb, L., Weisman, G. A., Marchese, A., Heng, H. H. Q., Garrard, R. C., George, S. R., Turner, J. T., and O'Dowd, B. F. (1995) *J. Biol. Chem.* **270,** 30845–30848
19. Nicholas, R. A., Watt, W. C., Lazarowski, E. R., Li, Q., and Harden, T. K. (1996) *Mol. Pharmacol.*, **50,** 224–229
20. Wickman, K., and Clapham, D. E. (1995) *Physiol. Rev.* **75,** 865–885
21. Zuhlke, R. D., Zhang, H.-J., and Joho, R. H. (1995) *Methods Neurosci.* **25,** 67–89
22. Parr, C. E., Sullivan, D. M., Paradiso, A. M., Lazarowski, E. R., Burch, L. H., Olsen, J. C., Erb, L., Weisman, G. A., Boucher, R. C., and Turner, J. T. (1994) *Proc, Natl. Acad. Sci. U. S. A.* **91,** 3275–3279
23. Shapiro, R. A., Scherer, N. M., Habecker, B. A., Subers, E. M., and Nathanson, N. M. (1988) *J. Biol. Chem.* **263,** 18397–18403
24. Shibasaki, T. (1987) *J. Physiol.* **387,** 227–250
25. Harden, T. K., Stephens, L., Hawkins, P. T., and Downes, C. P. (1987) *J. Biol. Chem.* **262,** 9057–9061
26. Harden, T. K., Hawkins, P. T., Stephens, L., Boyer, J. L., and Downes, C. P. (1988) *Biochem J.* **252,** 583–593
27. Boyer, J. L., Downes, C. P., and Harden, T. K. (1989) *J. Biol. Chem.* **264,** 884–890
28. Parker, K. E., and Scarpa, A. (1995) *Am. J. Physiol.* **269,** H789–H797
29. Furukawa, K., Ishibashi, H., and Akaike, N. (1994) *J. Neurophysiol.* **71,** 868–873