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P2Y Receptors Modulate Ion Channel Function through Interactions Involving The C-Terminal Domain

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ABSTRACT

Nucleotide stimulation of G_q-coupled P2Y receptors expressed in *Xenopus laevis* oocytes produces the activation of an endogenous voltage-gated ion channel, previously identified as the transient inward (T_{in}) channel. Expression of human $P2Y_1$, human $P2Y_2$, rat $P2Y_6$, human $P2Y_{11}$, or skate P2Y receptors in oocytes resulted in modulation of the voltage dependence and inactivation gating of the channel. Expression of the human $P2Y_4$ receptor, rat M_1-muscarinic receptor, and human B_1-bradykinin receptor did not alter the properties of the T_{in} channel. Replacement of the C-terminal domain of the human B_1-bradykinin receptor with the C-terminal domains of either the human $P2Y_1$ or human $P2Y_2$ receptor resulted in voltage dependence and inactivation-gating properties, respectively, of the T_{in} channel that were similar to those elicited by the respective native P2Y receptor. Systematic truncation of the C-terminal region of the human $P2Y_1$ receptor identified a short region responsible for modulation of the T_{in} channel. This region contains a conserved sequence motif found in all P2Y receptors that modulates the voltage dependence of the T_{in} channel. Synthetic 20-mer peptides from the C-terminal domains of human $P2Y_1$ and $P2Y_2$ receptors produced a shift in the voltage dependence and slowed inactivation gating, respectively, after injection into oocytes expressing human B_1-bradykinin or truncated human $P2Y_1$ receptors. These results indicate that certain P2Y receptors are capable of modulating the voltage sensitivity and inactivation gating of an endogenous oocyte ion channel through interactions involving the C-terminal region of the receptor. Such modulation of ion channel function could also exist in native mammalian cells that express P2Y receptors.

Extracellular nucleotides (ADP, ATP, UDP, and UTP) function as signaling molecules that mediate a variety of biological effects through a family of cell surface receptors known as P2 receptors. This family is divided into two groups: the ionotropic P2X receptors and the metabotropic P2Y receptors (Dubyak and el-Moatassim, 1993; Boarder et al., 1995; Boarder and Hourani, 1998; Ralevic and Burnstock, 1998). Currently, seven mammalian P2X receptors ($P2X_{1–7}$) and eight mammalian P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) have been cloned and functionally characterized (Ralevic and Burnstock, 1998; von Kugelgen and Wetter, 2000; Communi et al., 2001; Nicholas, 2001; Sak and Webb, 2002). P2X receptors function as nonselective cation channels with inwardly rectifying current-voltage relationships (Dubyak and el-Moatassim, 1993; Ralevic and Burnstock, 1998). Activation of these receptors with ATP or 2-methylthio-ATP (2MeS-ATP) produces membrane depolarization. $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors couple to $G_{q/11}$ and activate phospholipase C, resulting in increased inositol phosphate-3 formation and mobilization of intracellular Ca^{2+} (Parr et al., 1994; Communi et al., 1995; Schachter et al., 1996; Lazarowski et al., 2001). The $P2Y_{11}$ receptor activates both phospholipase C and adenylyl cyclase (Communi et al., 1997), whereas $P2Y_{12}$ and $P2Y_{13}$ receptors are coupled solely to G_i and inhibition of adenylyl cyclase (Daniel et al., 1998; Hollopeter et al., 2001). $P2Y_{14}$ receptors are orphan G protein-coupled receptors that are activated by UDP-glucose and couple to the $G_{i/o}$ class of G proteins (Chambers et al., 2000). The skate (s) P2Y receptor has 61 to 64% sequence similarity to the human (h) $P2Y_1$ receptor, is coupled to G_q/phospholipase C, and has a rank order of potency similar to the $P2Y_1$ receptor (Dranoff et al., 2000).

Previously, we showed that agonist activation of the $P2Y_1$ receptor expressed in *Xenopus laevis* oocytes stimulated a slowly activating inward current that inactivated within seconds after stimulation (O'Grady et al., 1996). The channel exhibits steady-state inactivation at strong hyperpolarizing potentials. This inward current was identified previously as the transient inward (T_{in}) current and was first observed

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ABBREVIATIONS: 2MeS, 2-methylthio-; T_{in}, transient inward; CaCC, Ca^{2+}-activated Cl^- channels; CFTR, cystic fibrosis transmembrane conductance regulator; PDZ, PSD-95, Disc-large, and ZO-1; MBS, modified Barth's saline; prefixes: s, skate; h, human; r, rat.

after injection of mRNA from rat brain (Parker et al., 1985) and subsequently observed when cloned 5-hydroxytryptamine-1a and 5-hydroxytryptamine-2c receptors were expressed in oocytes (Ni et al., 1997). The channel is expressed in stage V and VI oocytes but seems to be absent in earlier stages of oocyte maturation. It is reversibly blocked by polyvalent cations including Ba^{2+}, Mn^{2+}, and La^{3+}. T_{in} current activation requires membrane hyperpolarization and an increase in intracellular Ca^{2+} (Parker et al., 1985; Ni et al., 1997). Expression of $G\alpha_q$ in mature oocytes was found to be sufficient for activation of the T_{in} current (Guttridge et al., 1995). The channel responsible for the T_{in} current has not been cloned and seems to represent a new family of ion channels that has not been previously characterized.

Interactions between expressed membrane proteins and endogenous ion channels that produce altered properties of these channels have been documented previously. For example, previous studies of Ca^{2+}-activated Cl^- channels (CaCC) in bovine artery endothelial cells showed that biophysical properties of the channel could be modulated by expression of cystic fibrosis transmembrane conductance regulator (CFTR) (Wei et al., 2001). Stimulation of the cells with forskolin and 3-isobutyl-1-methylxanthine produced activation of CFTR and simultaneously inhibited ATP-dependent activation of endogenous CaCC activity. This effect of CFTR on the regulation of CaCC function was independent of the PDZ domain located at the C terminus of CFTR, but was shown to involve sequences within the R domain. CFTR has also been shown to modulate the function of amiloride-sensitive Na^+ channels expressed in *X. laevis* oocytes (Boucherot et al., 2001). In this study, the first functional nucleotide-binding domain (NBF1) was proposed to be an interaction site between the two channels because mutations in the NBF1 region of CFTR resulted in a decrease in its ability to inhibit amiloride-sensitive Na^+ channel activity.

In this study, we examined the effects of native P2Y receptor subtypes on channel function and observed that several members of the P2Y receptor family modified the functional properties of the channel. To address the hypothesis that these receptors modulate the gating and voltage dependence of the T_{in} channel through membrane-delimited interactions involving specific structural domains of the receptor, we constructed truncation mutants and chimeric receptors involving the C-terminal regions of $hP2Y_1$ and $hP2Y_2$ receptors and determined the effects of these mutations on the biophysical properties of the T_{in} channel. Our results indicate that the C-terminal domains of these P2Y receptors are involved in regulating voltage dependence or inactivation gating of the T_{in} channel. An analysis of C-terminal sequences of P2Y receptors suggests that there are protein-protein interaction domains, distinct from their PDZ-binding motifs, which are involved in the coupling and modulation of channel function.

Materials and Methods

Materials. *X. laevis* frogs were purchased from Xenopus I (Ann Arbor, MI) and maintained in aquaria as suggested by the supplier. Collagenase and gentamicin were obtained from Invitrogen (Carlsbad, CA). 2MeS-ADP and 2MeS-ATP were obtained from Sigma/RBI (Natick, MA). UDP, UTP, carbachol, bradykinin, and isoproterenol were obtained from Sigma (St. Louis, MO). [D-Pen^2,D-Pen^5]-enkephalin was obtained from Bachem Biosciences (King of Prussia, PA).

Construction of Truncated and Chimeric Receptors. Truncated P2Y receptors were constructed by polymerase chain reaction using a 3′ primer with a stop codon at the desired location and an *Xho*I restriction site to aid in subcloning. Chimeric receptors were generated by overlap-extension polymerase chain reaction (Ho et al., 1989). All receptor constructs were verified by sequencing and were subcloned into pcDNA3.

Preparation of RNA for Injection. cRNA was synthesized from linear cDNA encoding either wild-type P2Y receptor or mutants using Megascript (Ambion, Austin, TX).

Oocyte Isolation and Injection. Ovarian lobes from adult *X. laevis* frogs were removed from anesthetized animals under sterile condition. The tissue mass was dissociated with collagenase solution (90 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 10 mM HEPES, pH 7.4, and 250 units/ml collagenase). Stage V and VI oocytes were sorted, defolliculated, and maintained in modified Barth's saline solution (MBS solution: 90 mM NaCl, 2 mM KCl, 0.82 mM $MgSO_4$, 0.74 mM $CaCl_2$, and 10 mM HEPES, pH 7.4, supplemented with 0.05 $\mu g/\mu l$ gentamicin) at 19 to 20°C. Oocytes were injected with cRNA transcripts (46 ng/oocyte) using a Nanoject oocyte injection system (Drummond Scientific (Broomall, PA). Control oocytes were injected with 46 nl of sterile water. Oocytes were stored for 2 to 7 days in MBS solution before analysis.

Peptide Synthesis and Purification. Peptides of 20 amino acids corresponding to the C-terminal sequence of the $hP2Y_1$ receptor (RKASRRSEANLQSKSEDMTL) or the $hP2Y_2$ receptor (RRSDRTDMQRIGDVLGSSED) were synthesized by the MicroChemical Facility at the University of Minnesota (St. Paul, MN). Peptides were purified by high-pressure liquid chromatography before injection, and the appropriate amino acid composition was confirmed by amino acid analysis.

Electrophysiological Measurements. Electrophysiological measurements were made using the two-electrode voltage-clamp technique at 20°C. Recordings were conducted in Cl^--free MBS solution (90 mM $NaMeSO_4$, 2 mM $KMeSO_4$, 0.82 mM $MgSO_4$, 0.74 mM calcium gluconate, and 10 mM HEPES, pH 7.4). Electrodes were placed in a separate Cl^--containing MBS solution and connected to the oocyte bathing solution with an agar bridge. Current- and voltage-measuring electrodes were pulled from borosilicate filament glass to resistances between 2 and 5 MΩ when filled with 0.5 M KCl. Data acquisition and analysis was performed with a Pentium PC using pCLAMP 8 software (Axon Instruments, Inc., Union City, CA).

Analysis and Statistics. Statistical significance was determined using Student's t test. Statistical significance was accepted at $p < 0.05$. Conductance-voltage relationships were analyzed using a Boltzmann function ($Y = 1/1 + \exp(V_{50} - X/\text{slope factor})$, where V_{50} represents the voltage at which the conductance is half-maximal, slope factor represents the relative degree of voltage dependence (steepness of the curve), Y represents the normalized conductance, $G/G_{-140\ mV}$, and X represents a specific voltage.

Results and Discussion

We previously reported that 2MeS-ADP stimulation of *X. laevis* oocytes expressing the $hP2Y_1$ receptor activated a voltage-dependent current with gating characteristics that were identical with the endogenous T_{in} channel in oocytes (O'Grady et al., 1996). The ability to measure these currents was dependent on receptor expression and on the presence of agonist. We followed up on these initial studies and report here that although all G_q-coupled P2Y receptors are capable of activating T_{in} channel currents in the presence of their cognate agonists, the electrophysiological properties of the channel vary markedly depending on the subtype of the receptor.

Bradykinin Activation of the hB_1-Bradykinin Receptor Elicits T_{in} Currents in *X. laevis* Oocytes. Figure 1A shows representative T_{in} current traces recorded from oo-

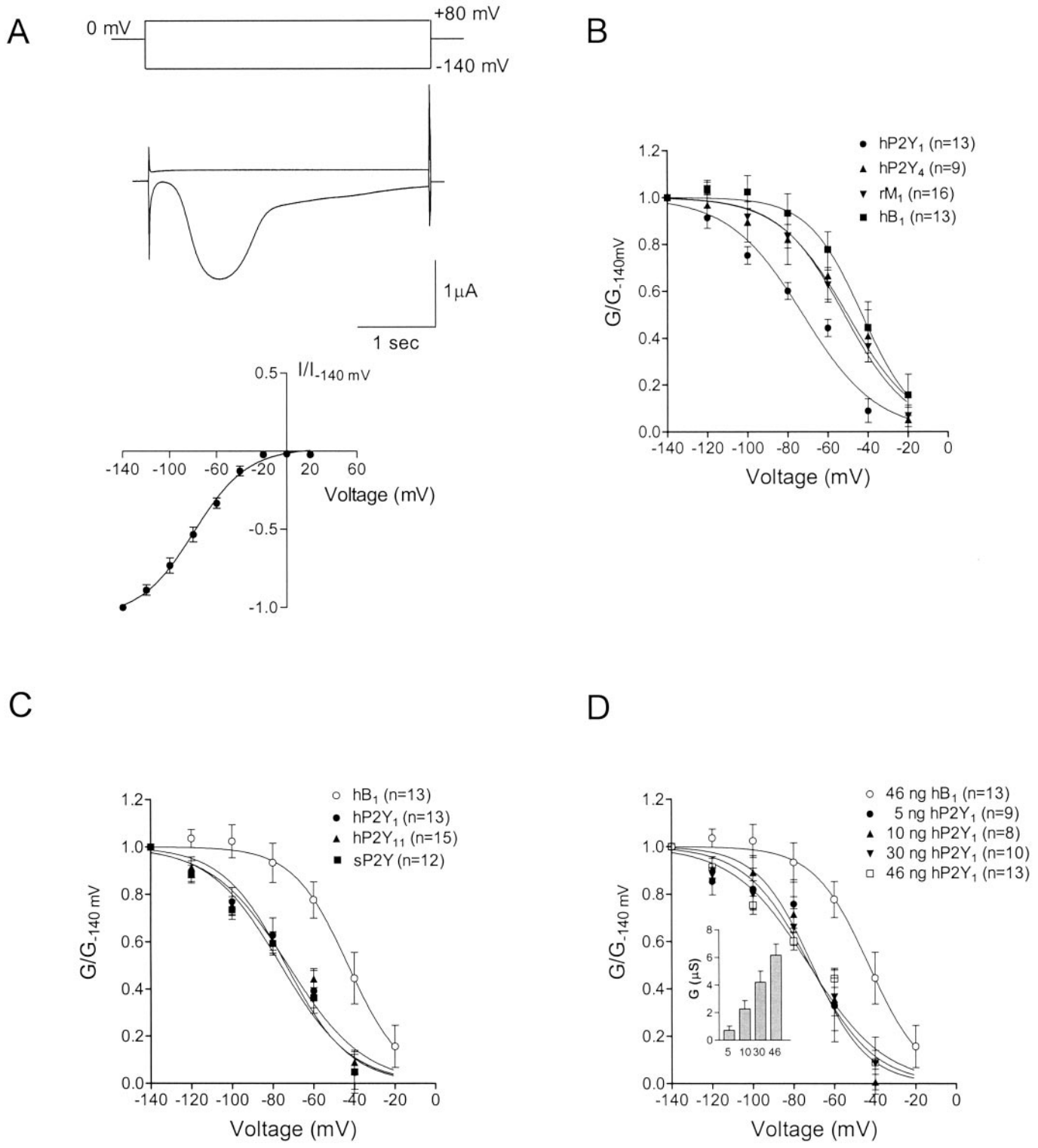

Fig. 1. Voltage dependence of the T_{in} current elicited by agonist-activated G_q-coupled receptors expressed in *X. laevis* oocytes. A, representative current traces recorded from oocytes expressing the hB_1-bradykinin receptor. Oocytes were held at 0 mV and then stepped from −140 mV to +80 mV in 20-mV increments. The data represent peak inward current at each voltage step. The I-V relationship was fit with a Boltzmann function. The reversal potential of hB_1-bradykinin receptor was determined from the fit and had a value of +13 mV. B, normalized conductance as a function of voltage for the T_{in} channel activated by $hP2Y_1$ ($n = 13$), $hP2Y_4$ ($n = 9$), rM_1-muscarinic ($n = 16$), and hB_1-bradykinin ($n = 13$) receptors. C, normalized conductance as a function of voltage for hB_1-bradykinin ($n = 13$), $hP2Y_1$ ($n = 13$), $hP2Y_{11}$ ($n = 15$), and the sP2Y ($n = 12$) receptors. The V_{50} values and slope factors for each conductance are listed in Table 1. D, the effects of $hP2Y_1$ receptor expression levels on the conductance-voltage relationship of the T_{in} channel; inset: conductance values obtained from oocytes injected with increasing amounts of $hP2Y_1$ receptor mRNA (nanograms).

cytes expressing the G_q-coupled hB_1-bradykinin receptor. Oocytes were held at 0 mV and then stepped to −140 mV and +80 mV in the presence of a 2 μM bradykinin. In the absence of either receptor mRNA or bradykinin, no time-dependent currents were observed upon hyperpolarization. In contrast, bradykinin elicited a characteristic T_{in} channel current in oocytes injected with hB_1-bradykinin receptor mRNA but not in noninjected oocytes (data not shown). Results for several G_q-coupled P2Y receptors were very similar, with the exception that a varying amount of T_{in} channel activation was observed under basal conditions in oocytes expressing P2Y receptors before agonist stimulation (O'Grady et al., 1996). This basal activation was probably caused by the accumulation in the bathing solution of nucleotides released from oocytes, a result similar to that observed in mammalian cells (Parr et al., 1994; Schachter et al., 1996).

Voltage Dependence of the T_{in} Current Elicited by Agonist-Activated G_q-Coupled Receptors Expressed in *X. laevis* Oocytes. Figure 1B shows the normalized conductance-voltage relationships for G_q-coupled receptors expressed in *X. laevis* oocytes. Only G_q-coupled receptors elicited T_{in} channel currents, because agonist-activated β_2-adrenergic (50 μM isoproterenol; G_s-coupled), δ-opioid (5 μM [D-Pen^2,D-Pen^5]-enkephalin; G_i-coupled), M_2-muscarinic (10 μM carbachol; G_i-coupled), and $P2Y_{12}$ (20 μM 2MeS-ADP; G_i-coupled) receptors were unable to activate T_{in} currents after hyperpolarization (data not shown). The conductance-voltage curves were analyzed using a Boltzmann function, where V_{50} represents the voltage at which the conductance was half-maximal (described under *Materials and Methods*). The V_{50} value for the conductance activated by the $hP2Y_1$ receptor, but not the rat (r) M_1-muscarinic or $hP2Y_4$ receptor, was shifted markedly to a more negative voltage compared with the conductance activated by the hB_1-bradykinin receptor and was significantly different from the V_{50} value elicited by the hB_1-bradykinin receptor (Table 1). Two other P2Y receptors, the $hP2Y_{11}$ and the sP2Y receptors, also activated currents with significantly more negative V_{50} values than the currents activated by the hB_1-bradykinin or the rM_1-muscarinic receptor (Fig. 1C) (Table 1).

To investigate the effect of receptor expression levels on the voltage sensitivity of the T_{in} channel, we injected increasing amounts of $hP2Y_1$ receptor mRNA into oocytes and monitored the conductance-voltage relationships after receptor activation with a maximum activating concentration (20 μM) of 2MeS-ADP (O'Grady et al., 1996) (Fig. 1D). Whereas increasing $hP2Y_1$ receptor mRNA elicited higher peak conductance levels, it had no effect on the voltage sensitivity of the T_{in} channel. These data strongly suggest that the modulation of T_{in} channel voltage sensitivity by the $hP2Y_1$ receptor is independent of the level of receptor expression.

Role of the $hP2Y_1$ Receptor C Terminus in Modulating T_{in} Channel Voltage Sensitivity. The data presented above suggest that the $hP2Y_1$ and $hP2Y_{11}$ receptors not only activate T_{in} channels, but also modulate channel properties, possibly through direct protein-protein interactions. To test this hypothesis, we examined the conductance-voltage relationship of the T_{in} current elicited by a chimeric hB_1-bradykinin receptor in which the C-terminal domain was replaced by the C-terminal region from the $hP2Y_1$ receptor (hB_1/Y_1). Figure 2A shows that the conductance-voltage relationship of the T_{in} channel elicited by the hB_1/Y_1 chimeric receptor was essentially identical with that elicited by the activated $hP2Y_1$ receptor. These data demonstrate a "gain in function" of the hB_1-bradykinin receptor containing the $hP2Y_1$ receptor C-terminal domain and strongly suggest that the C-terminal region of the $hP2Y_1$ receptor is involved in regulating the properties of the T_{in} channel.

To further characterize the region of the $hP2Y_1$ receptor C-terminal domain involved in modulating the biophysical properties of the T_{in} channel, we analyzed the conductance-voltage relationships of the T_{in} current elicited by a series of carboxyl terminal truncations (Fig. 2B). Removal of either the PDZ-binding motif ($hP2Y_1$369tr) or the last 13 amino acids ($hP2Y_1$360tr) from the C terminus of the $hP2Y_1$ receptor had no effect on the V_{50} values for the T_{in} channel compared with the full-length wild-type receptor (Fig. 2C) (Table 1). In contrast, deletion of either 31 ($hP2Y_1$342tr) or 39 ($hP2Y_1$334tr) amino acids from the C terminus resulted in V_{50} values that were very similar to those elicited by hB_1-bradykinin, rM_1-muscarinic, and $hP2Y_4$ receptors (Fig. 2D) (Table 1). Deletion of 24 amino acids ($hP2Y_1$349tr) resulted in an intermediate phenotype, with a V_{50} value in between the full-length $hP2Y_1$ receptor and the truncation mutant missing the entire C-terminal domain.

Role of the C-Terminal Domain of the $hP2Y_2$ and $rP2Y_6$ Receptors in T_{in} Channel Inactivation Gating. We also observed a marked difference in the inactivation

TABLE 1
V_{50} and slope factor values of T_{in} currents elicited by agonist-activated receptors

Receptor Expressed in Oocytes	Agonist Concentration	Reference	V_{50}	Slope Factor
			mV	
hB_1 (n = 13)	2 μM Bradykinin	Simpson et al. (2000)	−43.0	−13.4
rM_1 (n = 16)	10 μM Carbachol	Wood et al. (1999)	−51.8	−16.2
$hP2Y_4$ (n = 9)	40 μM UTP	Communi et al. (1996)	−53.4	−18.8
$hP2Y_1$ (n = 13)	20 μM 2MeS-ADP	O'Grady et al. (1996)	−72.5*	−18.5
$hP2Y_{11}$ (n = 15)	80 μM 2MeS-ATP	Communi et al. (1999)	−73.5*	−15.6
sP2Y (n = 12)	20 μM 2MeS-ADP	Dranoff et al. (2000)	−76.0*	−17.3
$hP2Y_1$334tr (n = 9)	20 μM 2MeS-ADP	O'Grady et al. (1996)	−50.3	−7.6
$hP2Y_1$342tr (n = 18)	20 μM 2MeS-ADP	O'Grady et al. (1996)	−49.0	−10.8
$hP2Y_1$342tr + Y1 peptide (n = 8)	20 μM 2MeS-ADP	O'Grady et al. (1996)	−72.3*	−13.7
$hP2Y_1$349tr (n = 9)	20 μM 2MeS-ADP	O'Grady et al. (1996)	−56.7	−18.5
$hP2Y_1$360tr (n = 17)	20 μM 2MeS-ADP	O'Grady et al. (1996)	−64.4*	−23.0
$hP2Y_1$369tr (n = 10)	20 μM 2MeS-ADP	O'Grady et al. (1996)	−71.2*	−19.2
hB_1/Y_1 chimera (n = 6)	2 μM Bradykinin	Simpson et al. (2000)	−66.3*	−15.5
hB_1 + Y1 peptide (n = 7)	2 μM Bradykinin	Simpson et al. (2000)	−68.0*	−12.9

* Value was significantly different from the hB_1-bradykinin receptor value ($p < 0.05$).

gating of the T_{in} channel depending on which P2Y receptor was responsible for activating the current. Thus, whereas the current elicited by the $hP2Y_1$ receptor was almost completely inactivated within 3 s of the hyperpolarizing pulse, the current elicited by either the $hP2Y_2$ (stimulated with 40 μM UTP) or the $rP2Y_6$ receptor (stimulated with 40 μM UDP) showed significantly slower inactivation. (Fig. 3A). As observed with the voltage gating of the channel, the time course of inactivation was dependent on the identity of the C-terminal domain of the receptor. Thus, the inactivation time

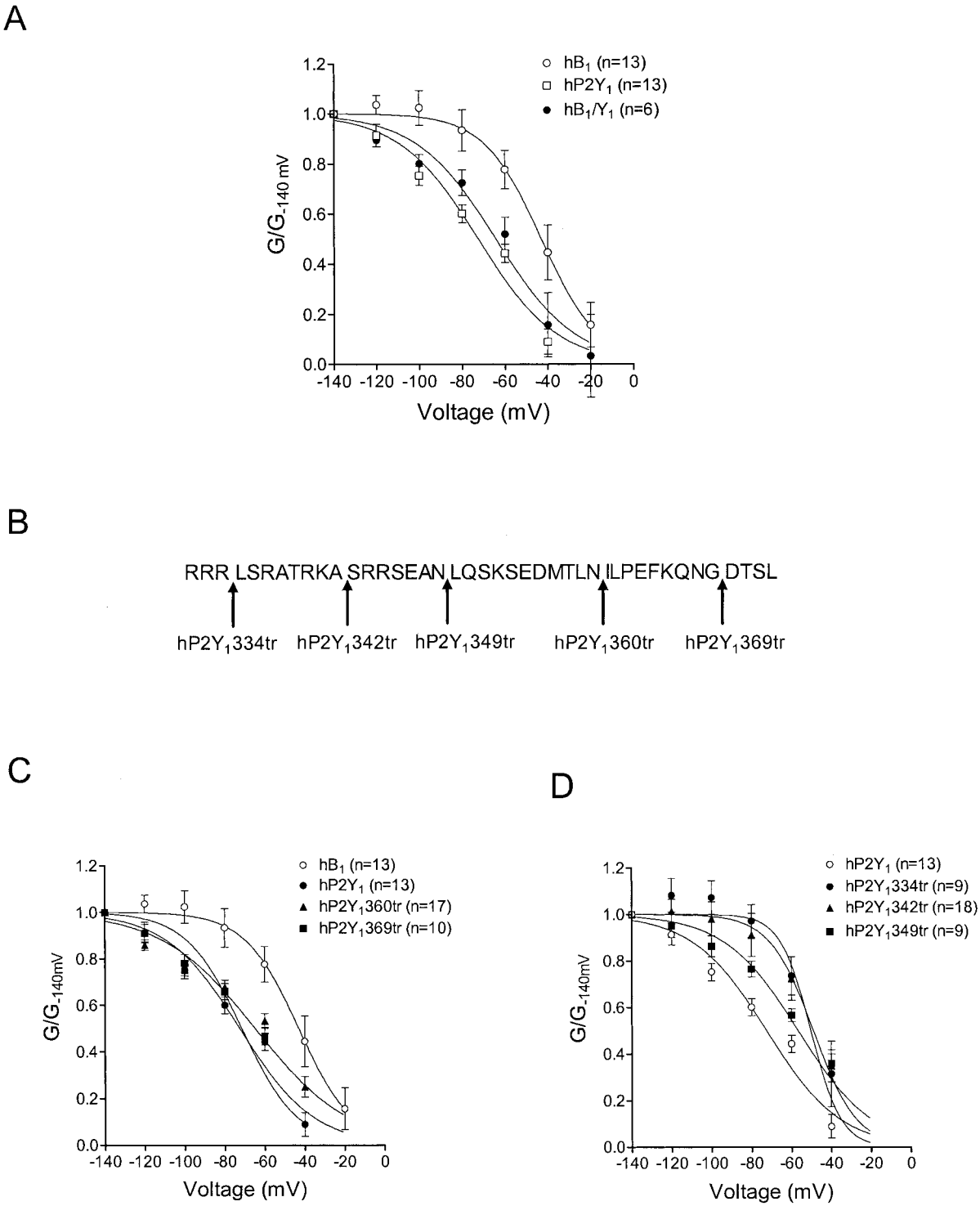

Fig. 2. Effect of C-terminal truncation on the conductance-voltage relationship. A, normalized conductance as a function of voltage for T_{in} currents elicited by activated hB_1-bradykinin (n = 13), $hP2Y_1$ (n = 13), and hB_1/Y_1 chimeric (n = 6) receptors. B, location of truncation mutations introduced into the $hP2Y_1$ receptor. The last four amino acids, DTSL, represent a consensus class 1 PDZ-binding motif. C, normalized conductance as a function of voltage for T_{in} currents elicited by activated hB_1-bradykinin (n = 13), $hP2Y_1$ (n = 13), $hP2Y_1$360tr (n = 17), and $hP2Y_1$369tr (n = 10) receptors. D, normalized conductance as a function of voltage for T_{in} currents elicited by activated $hP2Y_1$ (n = 13), $hP2Y_1$334tr (n = 9), $hP2Y_1$342tr (n = 18), and $hP2Y_1$349tr (n = 9) receptors. The V_{50} values and slope factors for each conductance are listed in Table 1.

courses of the $hP2Y_1$ receptor containing the $hP2Y_2$ C-terminal domain (hY_1/Y_2 chimera) (Fig. 3A) or the hB_1-bradykinin receptor containing the $hP2Y_2$ C-terminal domain (hB_1/Y_2) (Fig. 3B) were identical with that of the wild-type $hP2Y_2$ receptor (Fig. 3A).

Figure 3C shows the ratio of the current amplitude at the end of the voltage pulse (−140 mV, 3.8 s) to the maximum inward current elicited by a series of activated receptors. As suggested by the current traces in Fig. 3, A and B, the ratio

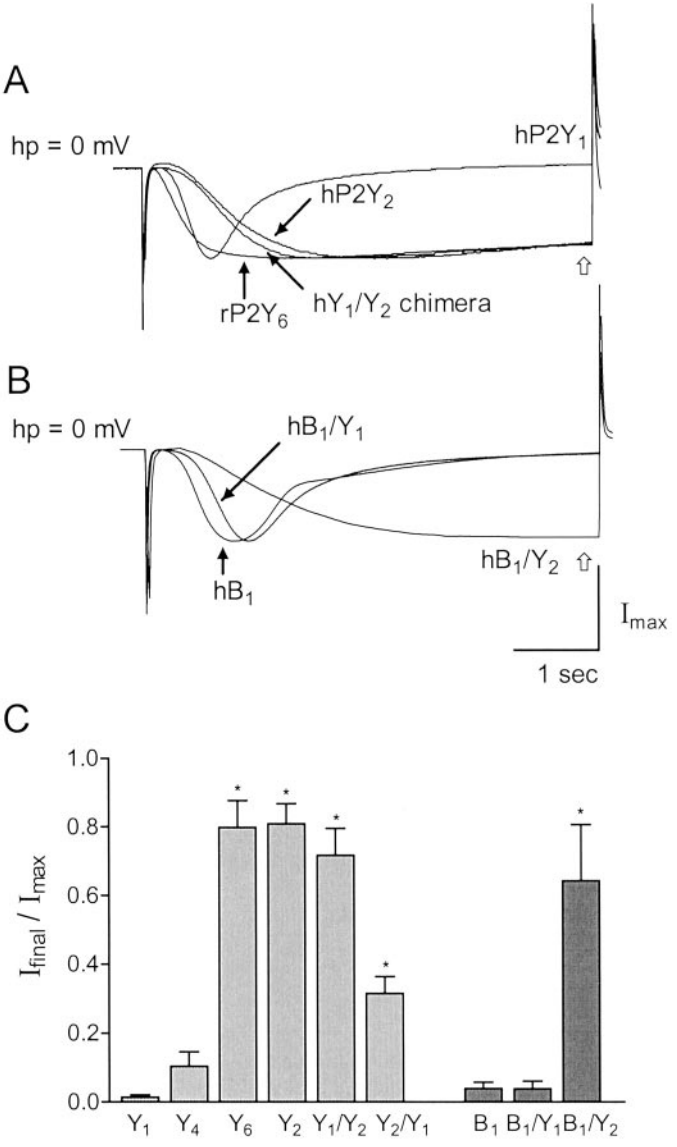

Fig. 3. Inactivation gating of receptor-activated T_{in} currents. A, representative traces of the T_{in} conductance elicited by activated $hP2Y_1$, $rP2Y_6$, $hP2Y_2$, and hY_1/Y_2 chimeric receptors. T_{in} channels were monitored for 5 s in the presence of the appropriate receptor agonist. UTP (40 μM) and UDP (40 μM) were used to stimulate the $hP2Y_2$ and $rP2Y_6$ receptors, respectively (Parr et al., 1994; Communi and Boeynaems, 1997). 2MeS-ADP (20 μM) was used to activate the $hP2Y_1$ receptor and hY_1/Y_2 chimeric receptors. B, representative traces of currents elicited by agonist-activated hB_1-bradykinin and hB_1/Y_1, and hB_1/Y_2 chimeric receptors after step hyperpolarization to −140 mV. C, I_{final}/I_{max} is the current amplitude at the end of the voltage pulse (as indicated by the open arrows in parts A and B) divided by the maximum inward current and represents the degree of T_{in} channel inactivation gating. This ratio was significantly increased in $hP2Y_2$ ($n = 10$), $rP2Y_6$ ($n = 11$), hY_1/Y_2 ($n = 7$), hY_2/Y_1 ($n = 7$), and hB_1/Y_2 ($n = 7$) chimeric receptors ($p < 0.05$).

derived from the $hP2Y_1$ receptor-activated currents was near zero. In contrast, the ratio was nearly 0.8 for the current elicited by the $hP2Y_2$ receptor, $rP2Y_6$ receptor, and the hY_1/Y_2 chimera, indicating slow inactivation. Although the ratio of the current elicited by the hY_2/Y_1 chimera was significantly decreased compared with wild-type $hP2Y_2$ receptor, it was still significantly greater than that observed after $hP2Y_1$ and $hP2Y_4$ receptor activation.

C-Terminal Sequence Comparisons of P2Y Receptors. Figure 4 compares the C-terminal sequences of all five of the G_q-coupled P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$). Within the C-terminal domains of the $hP2Y_1$, $hP2Y_{11}$, and sP2Y receptor, all of which modulate the voltage activation of the T_{in} channel, a common sequence motif was observed (RR**SE—QXK/RSE**) (bold letters identify conserved amino acids between P_2Y receptor subtypes). Importantly, this sequence motif falls within the narrow region of the $hP2Y_1$ receptor C-terminal domain shown to be involved in modulating voltage sensitivity (Fig. 2). Likewise, a different conserved sequence motif (**QRXG/R**) was observed in the C-terminal domains of the $hP2Y_2$ and $rP2Y_6$ receptor, both of which modulate T_{in} channel inactivation. This motif is not present in the other P2Y receptors.

Effects of Y1 and Y2 C-Terminal Peptides on the Conductance-Voltage Relationships and Inactivation Gating of the T_{in} Channel. To examine whether the unique sequence motif present in the C-terminal region of the $hP2Y_1$ receptor was able to modulate the conductance-voltage relationship of the T_{in} current, a synthetic peptide (Y1 peptide) (boxed region in Fig. 4) was injected into $hP2Y_1$342tr-expressing oocytes (final concentration ≈ 500 nM), and the conductance-voltage relationship of the T_{in} channel was determined (Fig. 5A). The V_{50} value of the conductance elicited by the $hP2Y_1$342tr receptor with 500 nM Y1 peptide (−72.3 mV) was significantly different from the value elicited by the $hP2Y_1$342tr receptor alone (V_{50} = −49.0 mV) and similar to that elicited by the wild-type $hP2Y_1$ receptor (V_{50} = −72.5 mV) (Table 1). The Y1 peptide produced a similar negative shift in the V_{50} value (−68.0 mV) in oocytes expressing the hB_1-bradykinin receptor compared with the hB_1-bradykinin receptor alone (−43.0 mV) (Fig. 5B).

To examine whether the proposed sequence motif present in the C-terminal region of the $hP2Y_2$ receptor was able to modulate the inactivation gating of the T_{in} current, a 20 amino acid peptide (Y2 peptide) (boxed region in Fig. 4) from the C terminus of the $hP2Y_2$ receptor encompassing this

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hP2Y1   RRRLSR-ATRKASRRSEANLQSKSEDMTLNILPEPKQNGDTSL
hP2Y11  LLYMAA-VPSLGCCCRHCPGYRDSWNPEDAKSTGQALPLNATAAPKPSEPQSRELSQ
Skate   RRRFTNAASRFMTRRSELSMQFRSEDSPLQPVSNISQNGDTSL

hP2Y4   RRQL-RQLCGGG-KPQP—RTAASSLALVSLPEDSSCRWAATPQDSSCSTPRADRL

hP2Y2   RRRLGLRRSDRTDMQRIGDVLGSSEDFRRTESTPAGSENTKDIRL
hP2Y6   RRRPHELLQKLTAKWQRQGR
rP2Y6   RRQPHDLLQKLTAKWQRQRV
mP2Y6   RRQPHDLLQRLTAKWQRQRV
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Fig. 4. C-terminal sequence comparisons between P2Y receptors. The underlined and bolded amino acids represent potential sequences involved in protein-protein interactions with the T_{in} channel. $hP2Y_1$, $hP2Y_{11}$, and sP2Y receptors modulate voltage dependence, whereas $hP2Y_2$ and $rP2Y_6$ receptors modulate inactivation gating. A similar sequence motif affecting inactivation gating of the channel is present in the mouse (m) $P2Y_6$ receptor. The boxed amino acids in the $hP2Y_1$ and $hP2Y_2$ receptor sequences indicate the peptide sequences used for experiments shown in Fig. 5.

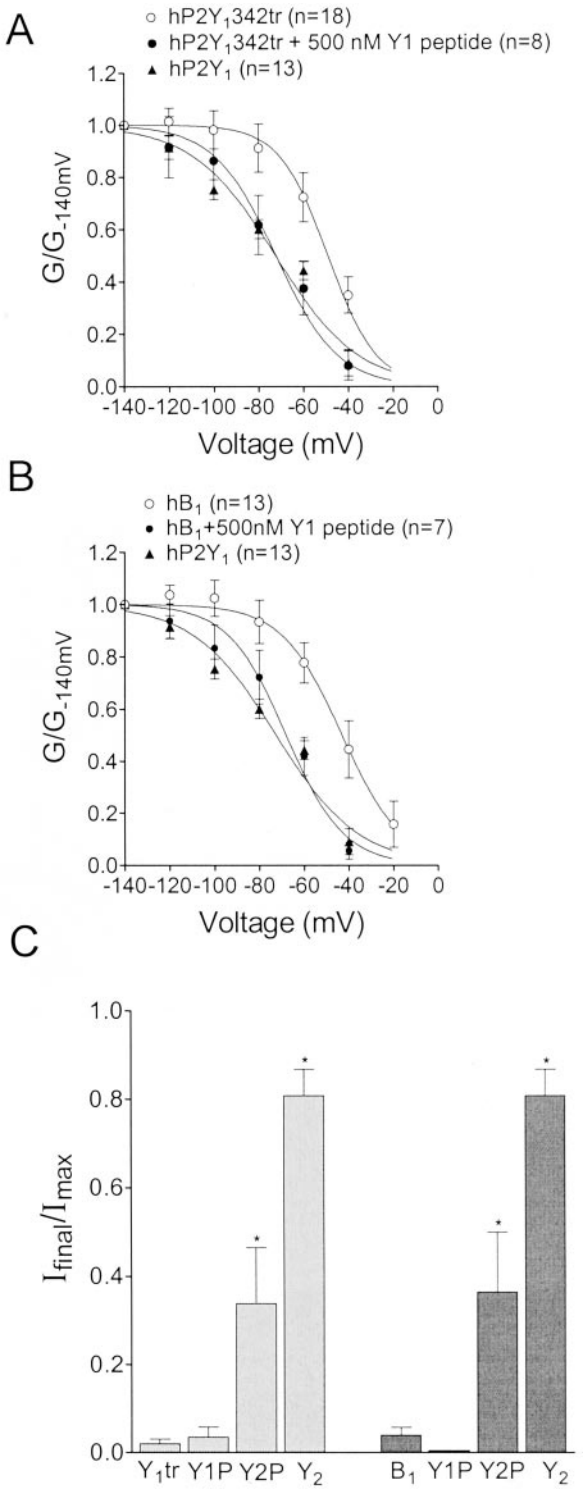

Fig. 5. Effect of Y1 and Y2 C-terminal peptides on the conductance-voltage relationship and inactivation gating of T_{in} currents. A, normalized conductance as a function of voltage of T_{in} currents elicited by the agonist-activated $hP2Y_1342tr$ receptor (Y_1tr; $n = 18$), $hP2Y_1342tr$ receptor with 500 nM Y1 peptide ($n = 8$), and $hP2Y_1$ receptor ($n = 13$). B, normalized conductance as a function of voltage of T_{in} currents elicited by the hB_1-bradykinin receptor ($n = 13$), the hB_1-bradykinin receptor with 500 nM Y1 peptide ($n = 7$), and the $hP2Y_1$ receptor ($n = 13$). C, I_{final}/I_{max} ratios of the T_{in} current elicited by the indicated receptors were calculated as described in the legend to Fig. 3. The ratios were significantly increased for the $hP2Y_1342tr$ receptor with 500 nM Y2 peptide ($n = 6$), the hB_1-bradykinin receptor with 500 nM Y2 peptide ($n = 6$), and the $hP2Y_2$ receptor ($n = 10$) ($p < 0.05$).

sequence was injected into $hP2Y_1342tr$- or hB_1-bradykinin receptor-injected oocytes (final concentration ≈ 500 nM), and the inactivation gating of the T_{in} channel was determined. Figure 5C shows the ratio of the current amplitude at the end of the voltage pulse (−140 mV) to the maximum inward current observed after activation. The ratio obtained from the $hP2Y_1342tr$ and hB_1-bradykinin receptor-activated currents was nearly zero, whereas the ratio for the current elicited by $hP2Y_1342tr$ or hB_1-bradykinin receptor with Y2 peptide was significantly increased (although not to the same level) compared with $hP2Y_1342tr$ and hB_1- bradykinin receptor-activated currents alone or $hP2Y_1342tr$ and hB_1-bradykin receptor-activated currents with Y1 peptide.

Taken together, these data strongly suggest that the C termini of the $hP2Y_1$ and $hP2Y_2$ receptors interact in some manner with the T_{in} channel to modulate its biophysical properties. We hypothesize that two sequence motifs, RR**SE—QXK/RSE** and **QRXG/R**, located in the C-termini of P2Y receptors, are important protein-protein interaction sites between P2Y receptors and the T_{in} channel, or alternatively an intermediate adapter protein. The presence of multiple protein-protein interaction domains within the C-terminal region of P2Y receptors suggests that these receptors can couple to a variety of membrane-associated proteins and potentially influence their function, independent of G-protein activation. Future studies directed toward identifying additional interacting protein partners should provide better insight into the role of protein-protein interactions in P2Y receptor signaling.

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