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Brilliant Blue G Selectively Blocks ATP-Gated Rat $P2X_7$ Receptors

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ABSTRACT

There are few antagonists selective for subtypes of the several P2X receptors, but these are needed to identify the receptors expressed on native cells and tissues. In particular, $P2X_4$ and $P2X_7$ receptor subunits are colocalized on immune, epithelial, and exocrine gland cells, but both are relatively insensitive to suramin and pyridoxal-5-phosphate-6-azo-2′,4′-disulfonic acid derivative. In this article, we show that Coomassie Brilliant Blue G selectively inhibits $P2X_7$ receptors with nanomolar affinity. We measured currents in response to P2X receptor activation in HEK293 cells heterologously expressing human or rat $P2X_1$, $P2X_2$, $P2X_3$, $P2X_{2/3}$, $P2X_4$, $P2X_{1/5}$, and $P2X_7$ receptors. Brilliant Blue G produced a noncompetitive inhibition of rat and human $P2X_7$ receptors with IC_{50} values of 10 and 200 nM, respectively. IC_{50} values for inhibition of the other receptors ranged from 2 to >30 μM; the rat and human $P2X_4$ receptors showed IC_{50} values of >10 and 3.2 μM. Coomassie Blue G also blocked YO-PRO1 uptake and membrane blebbing, which are uniquely associated with activation of $P2X_7$ receptors. Thus, Brilliant Blue G is at least 1000-fold more potent at rat $P2X_7$ receptors than at rat $P2X_4$ receptors.

P2X receptors are ATP-gated ion channels that are present in both excitable and nonexcitable cells. Their activation by extracellular ATP opens a cation-selective channel that also allows significant calcium influx. P2X receptors mediate fast excitatory transmission at sympathetic neuromuscular synapses as well as at some neuroneuronal synapses in the spinal cord and brain. There is also good evidence to suggest that they may be involved in other physiological and pathophysiological functions including pain perception, endocrine and exocrine gland secretions, and release of interleukin-1β from immune cells (see reviews by North and Barnard, 1997; Ralevic and Burnstock, 1998; Burnstock, 1999; Di Virgilio et al., 1999; MacKenzie et al., 1999). Much of this physiological diversity can be attributed to differential tissue localization of multiple P2X receptor subunits.

Seven P2X subunits have been cloned. All except $P2X_6$ readily form cation channels in heterologous expression systems, and these homomeric receptors can be distinguished by a combination of distinctive kinetics and pharmacological (agonist and antagonist sensitivity) profile (North and Barnard, 1997; Ralevic and Burnstock, 1998; MacKenzie et al., 1999; North and Surprenant, 2000). Moreover, phenotypically distinct heteromeric receptors have been described after coexpression of pairs of subunits ($P2X_{2/3}$, $P2X_{1/5}$, and $P2X_{4/6}$) (Lewis et al., 1995; Lê et al., 1998, 1999; Torres et al., 1998; Khakh et al., 1999). In some cases, the correspondence between tissue localization of P2X subunits and phenotypic similarity of heterologously expressed and native receptors has allowed conclusions to be drawn concerning the composition of native P2X subunits underlying function. For example, these comparisons for homomeric $P2X_1$ receptor subunits suggest that ATP-mediated and nerve-evoked smooth muscle contractions are caused by $P2X_1$ receptor activation (Evans et al., 1997; Ralevic and Burnstock, 1998), a conclusion that has been substantiated further by recent studies on $P2X_1$ receptor knockout mice (Mulryan et al., 2000). However, functional and immunohistochemical localization studies have revealed that a single cell can express multiple P2X receptor subtypes (Collo et al., 1996; Vulchanova et al., 1997; Ralevic and Burnstock, 1998; Thomas et al., 1998; Groschel-Stewart et al., 1999) (see also Burnstock, 1999). In such cases, adequate dissection of the receptor subtype responsible for a specific functional effect relies on selective agonists and antagonists. There are few subtype-specific ligands currently available for P2X receptors, although homomeric and heteromeric receptors expressing α,β-methylene-ATP ($\alpha\beta$meATP)-sensitive subunits ($P2X_1$, $P2X_{1/5}$, $P2X_3$, and $P2X_{2/3}$) can be fairly well distinguished from each other and other P2X receptors by using the agonists D- and L-$\beta\gamma$meATP, and/or the antagonists TNP-ATP and di-inosine pentaphosphate (Trezise et al., 1995; Virginio et al., 1998; King et al., 1999; North and Surprenant, 2000).

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ABBREVIATIONS: $\alpha\beta$meATP, α,β-methylene-ATP; BzATP, 2′3′-*O*-(4-benzoyl)benzoyl-ATP; YOPRO-1, quinolinium,4-[(3-methyl-2-(3H)-benzoxazolylidene)methyl]-1-[3-(triethylammonio)propyl]di-iodide; PPADS, pyridoxal-5-phosphate-6-azo-2′,4′-disulfonic acid derivative.

P2X$_4$ and P2X$_7$ receptors pose a particular problem, because they are commonly expressed in the same tissues or cells, especially in immune, epithelial, and gland cells (Buell et al., 1996; Surprenant et al., 1996; Collo et al., 1997; Ralevic and Burnstock, 1998). However, biochemical and functional studies on heterologously coexpressed P2X$_4$ and P2X$_7$ receptors show that they do not form heteromeric assemblies (Cario-Toumaniantz et al., 1998; Torres et al., 1999). It has not been possible to adequately separate P2X$_4$ and P2X$_7$ receptor activation when they are expressed in a single cell. Thus, the ATP analog 2′,3′-(4-benzoyl)-benzoyl ATP (BzATP) is more potent than ATP at the P2X$_7$ receptor, but it acts as a partial agonist at other P2X receptors over the same concentration range (Evans et al., 1995, 1997; Surprenant et al., 1996; Rassendren et al., 1997; Ralevic and Burnstock, 1998; Di Virgilio et al., 1999). ATP itself is 10-fold more potent at P2X$_4$ than at P2X$_7$ receptors, but this difference cannot be readily exploited because all concentrations of ATP that activate P2X$_7$ receptors also activate P2X$_4$ receptors (Surprenant et al., 1996; Rassendren et al., 1997; Chessell et al., 1998). The two receptors are insensitive to activation by $\alpha\beta$meATP and are also insensitive to inhibition by the low micromolar concentrations of suramin, pyridoxal-5-phosphate-6-azo-2′,4′-disulfonic acid (PPADS), and reactive blue 2, which block other homomeric and heteromeric P2X receptors (Buell et al., 1996; Surprenant et al., 1996; Garcia-Guzman et al., 1997; Rassendren et al., 1997; Ralevic and Burnstock, 1999). Very high concentrations (>30–300 μM) of these antagonists are required to inhibit either P2X$_7$ or P2X$_4$ receptors, and these have several nonspecific effects (see Ralevic and Burnstock, 1998). Finally, P2X$_7$ receptors are functionally different from other P2X receptors, because their activation leads to the formation of a large pore that allows passage of molecules up to 900 Da and subsequently rapid cell death (Surprenant et al., 1996; Di Virgilio et al., 1999; MacKenzie et al., 1999; Virginio et al., 1999). However, there is also uptake of the large cationic dyes such as quinolinium,4-[(3-methyl-2-(3H)-benzoxazolylidene)methyl]-1-[3-(triethylammonio)propyl]di-iodide (YOPRO-1) by cells expressing P2X$_4$ receptors when the agonist application is prolonged, and this reduces the value of the measure as a way of distinguishing the two receptors.

Talamo and colleagues have performed calcium influx studies on rat isolated parotid acinar cells that have provided strong evidence for functional expression of both P2X$_4$ and P2X$_7$ receptors in an individual acinar cell (McMillian et al., 1993; Tenneti and Talamo, 1993; Tenneti et al., 1998). They have suggested further that Brilliant Blue G can be used as a selective antagonist to the P2X$_7$/P2Z response in these cells (Soltoff et al., 1989; Tenneti et al., 1998). However, no detailed pharmacological profile of Brilliant Blue G was presented, and its actions at other P2X receptors have not been examined. Therefore, we studied the activity of Brilliant Blue G on rat and human P2X receptors heterologously expressed in HEK293 cells by measuring agonist-evoked currents, YOPRO-1 uptake, and membrane blebbing.

Materials and Methods

Cell Cultures. HEK293 cells stably expressing human P2X$_1$, P2X$_3$, P2X$_4$, and P2X$_7$, and rat P2X$_2$, P2X$_{2/3}$, P2X$_{1/5}$, and P2X$_7$ receptors were used. Rat P2X$_1$, P2X$_3$ and P2X$_4$ receptors were transiently expressed in HEK293 cells by lipofection. Generation of stable cell lines and protocols of transient transfection have been described previously (Evans et al., 1995; Buell et al., 1996; Kawashima et al., 1997). HEK293 cells stably expressing the human P2X$_4$ receptor were provided by Prof. W. Stuhmer, Max-Planck Institute, Gottingen, Germany. Cells were plated onto 13-mm glass coverslips and maintained in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine at 37°C in a humidified 5% CO_2 incubator.

Electrophysiological Recordings. Whole-cell recordings were made 20 to 48 h after transient transfection and 24 to 72 h after passage of stable cells, using an EPC9 patch clamp amplifier (HEKA Elektronik, Lambrechet, Germany). Unless otherwise noted, membrane potential was held at −60 mV. Recording pipettes (4–7 MΩ) were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) and filled with an intracellular solution that consisted of (in mM): 145 NaF, 10 EGTA, 10 HEPES. The external solution contained (in mM): 147 NaCl, 10 HEPES, 13 glucose, 2 KCl, 2 $CaCl_2$, and 1 $MgCl_2$. Osmolarity and pH values of both solutions were 300 to 315 mOsm/l and 7.3, respectively. All the experiments presented here were performed at room temperature. Agonists and Brilliant Blue G were applied using an RSC 200 fast-flow delivery system (Biologic Science Instruments, Grenoble, France). Agonists were applied every 2 min except for experiments on P2X$_1$, P2X$_3$, and rat P2X$_4$, in which 3- to 8-min intervals were used because of the prolonged rundown of these responses (Evans et al., 1997; Virginio et al., 1998). The duration of agonist application was 1 s for the P2X$_1$ receptor, 2 s for the P2X$_2$, P2X$_3$, and P2X$_{2/3}$, and P2X$_4$ receptors, 3 s for the P2X$_{1/5}$ receptor, and 4 s for the P2X$_7$ receptor. For all except the P2X$_1$ receptor, repetitive stimuli at the frequencies noted above were applied in the absence of Brilliant Blue G until evoked currents were stable (±5%); the clamped cell was perfused with Brilliant Blue G (0.1–10 μM) for 4 min, and current evoked by agonist was measured. The peak current amplitude was expressed as a percentage of the amplitude obtained under the control conditions. Because of the marked rundown of currents at the P2X$_1$ receptor, Brilliant Blue G was added to the cells for 4 min after the second application of agonist. The ratios of the peak amplitude induced by the third application of agonist relative to the peak amplitude by the second application of agonist were calculated and compared in the absence and presence of Brilliant Blue G.

Cumulative concentration-current curves to BzATP were generated for the P2X$_7$ receptor in the absence and presence of Brilliant Blue G. BzATP was applied to the cells in increasing concentrations (3, 10, 30, 100, and 300 μM) first in the absence and then in the presence of Brilliant Blue G, with 4 min of preincubation. To determine whether the effect of Brilliant Blue G is voltage-dependent, ramps with a duration of 1 s from −120 to +40 mV were applied during the agonist application, both before and 4 min after the cells were exposed to Brilliant Blue G.

Single-Cell Imaging and Membrane Blebbing. YOPRO-1 uptake was measured using a Zeiss Axiovert 100 and oil immersion Fluar ×40 objective and the Photonics monochromator (TILLion VISION) system (Photonics, Planegg, Germany). YOPRO-1 (2 μM) was present in the extracellular solution throughout the experiment. Cells were perfused with normal extracellular solution (as control) or a given concentration of Brilliant Blue G for 5 min before and during a 3-min application of agonist (100 μM BzATP). Fluorescence was measured from individual cells and averaged after the background fluorescence in the absence of agonist was subtracted. Cell lysis characterized by membrane disruption and blebbing formation was monitored with a 100× Neofluar objective under transmitted light; digital images were taken at 0.5 to 2 Hz. Time from onset of agonist application to first image containing a fully disrupted edge was taken as time to membrane blebbing.

Data Analysis. The inhibition curves were constructed by plotting the current amplitude (I) as a fraction of its amplitude in the absence of Brilliant Blue G (I_0), as a function of the concentration of

Brilliant Blue G (B). The figures show mean ± S.E. for the number of cells tested with a given antagonist concentration. IC_{50} values were calculated by least-squares fitting of these mean values to $I/I_0 = 1/[1 + (IC_{50}/B])^n]$. The curve-fitting program (Kaleidagraph, Synergy Software, Reading, PA) reports the S.E. of the estimates for the number of antagonist applications applied (the number of cells tested is less than this, because several antagonist concentrations were applied in some cells). The dissociation equilibrium constant (K_B) for Brilliant Blue G was estimated on the assumption that the antagonism was insurmountable by fitting agonist concentrations (A) and antagonist concentrations (B) to $I/I_0 = 1/\{(1+(EC_{50}/A))\ (1+(B/K_B))\}$ (Kenakin, 1993). Comparisons between two groups (nonpaired) were made using Student's t test, and significance was given at the level of $P < .05$.

Chemicals. Culture media, sera, and other cell culture reagents were obtained from Life Technologies (Paisley, UK). YOPRO-1 iodide was obtained from Molecular Probes (Eugene, OR), and all other chemicals were obtained Sigma (St. Louis, MO).

Results

Brilliant Blue G Strongly Inhibits Currents through $P2X_7$ But Not through $P2X_4$ Receptors. Because there is often colocalization of $P2X_4$ with $P2X_7$ receptors, we initially examined the actions of Brilliant Blue G in HEK293 cells expressing homomeric $P2X_4$ or $P2X_7$ receptors. At the rat receptors, Brilliant Blue G potently inhibited currents through $P2X_7$ receptors (IC_{50} concentration approximately 10 nM); in contrast, it inhibited $P2X_4$-mediated currents by <50% at a concentration of 10 μM (highest concentration examined) (Fig. 1, A and B; Fig. 2A; Table 1). Inhibition by Brilliant Blue G was concentration-dependent and only slowly reversible; reversal was incomplete after a 16- to 20-min wash (Fig. 1). At the human receptors, Brilliant Blue G also inhibited $P2X_7$-mediated currents to a greater degree than currents through $P2X_4$ receptors. However, in this case the potency difference was less, because Brilliant Blue G was relatively less effective at human than rat $P2X_7$ receptors and relatively more effective to block at the human $P2X_4$ than at the rat $P2X_4$ (Fig. 1, A and D; Fig. 2A; Table 1).

IC_{50} values for Brilliant Blue G inhibition of rat $P2X_7$ receptor-mediated currents were similar whether currents were evoked by half-maximal (30 μM) or near-maximal (100 μM) concentrations of the agonist (BzATP) (Table 1). This suggests a noncompetitive antagonism. Moreover, the inhibition by Brilliant Blue G showed no voltage dependence from −120 to 40 mV ($n = 4$). Concentration-response curves to BzATP were compared in the absence and presence of Brilliant Blue G. Consecutive control concentration response curves showed "run up," that is, EC_{50} values were significantly higher during initial agonist applications. Thus, BzATP EC_{50} values for first and second concentration-response curves were 36 ± 8 μM and 15 ± 6 μM ($n = 5$; $P < .001$, paired t test); however, there was no significant difference between the second and third set of runs ($n = 4$). Therefore, concentration-response curves in the presence of Brilliant Blue G were obtained after repetitive applications of BzATP (30 μM) showed that currents had stabilized. The antagonist caused a progressive inhibition of the currents and decrease in the maximum amplitude with no significant shift in the agonist EC_{50} value (Fig. 2B). This experiment provided an estimate of 9 nM for the dissociation equilibrium constant at the rat $P2X_7$ receptor; a similar experiment with the human receptor provided an estimate of 185 nM.

Brilliant Blue G Is Much Less Effective at Other P2X Receptors. Figure 3 and Table 1 summarize the effects of Brilliant Blue G at human and rat homomeric and/or heteromeric P2X receptors (i.e., $P2X_1$, $P2X_2$, $P2X_3$, $P2X_{2/3}$, and $P2X_{1/5}$). With the notable exception of the rat $P2X_2$ receptor, Brilliant Blue G produced <50% inhibition of ATP or αβmeATP-mediated currents at concentrations ≥5 μM (Table 1). However, Brilliant Blue G did inhibit currents through the rat $P2X_2$ receptor, although this was still approximately 150-fold less sensitive than the rat $P2X_7$ receptor. The inhibition at this receptor was concentration-dependent with an IC_{50} value of 1.5 μM (Fig. 3, A and B; Table 1), but in contrast to the slow reversibility observed at the rat $P2X_7$ receptor the inhibition was rapidly reversible and complete within 2 min (Fig. 3A). The inhibition by Brilliant Blue G at the $P2X_2$ receptor was similar when near-maximal (30 μM) or half-maximal (10 μM) ATP concentrations were used or whether the partial agonist BzATP (30 μM) was used. In the three cases, inhibition by 1.5 μM Brilliant Blue G was 56 ± 2% ($n = 4$), 50 ± 4% ($n = 4$), and 57 ± 5% ($n = 4$), respectively. These results show that inhibition by Brilliant Blue G at the $P2X_2$

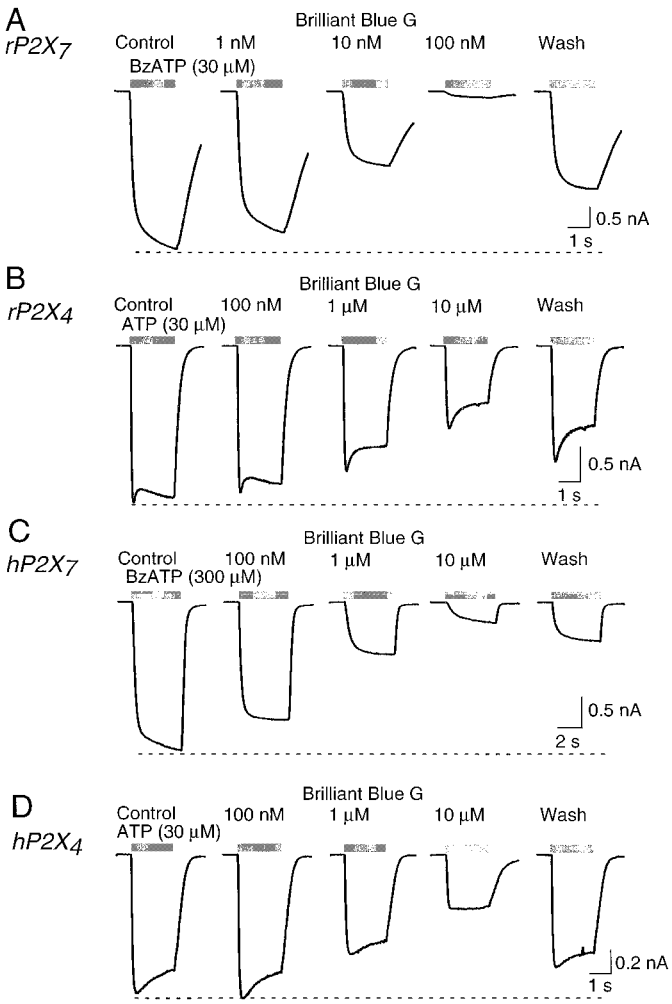

Fig. 1. Brilliant Blue G is a potent and selective antagonist at $P2X_7$ receptors. Each set of records show currents recorded from individual cells expressing rat $P2X_7$ (A), rat $P2X_4$ (B), human $P2X_7$ (C), and human $P2X_4$ (D) receptors. Currents are shown before, 4 min after applying Brilliant Blue G in the indicated concentration, and after 16- to 20-min wash.

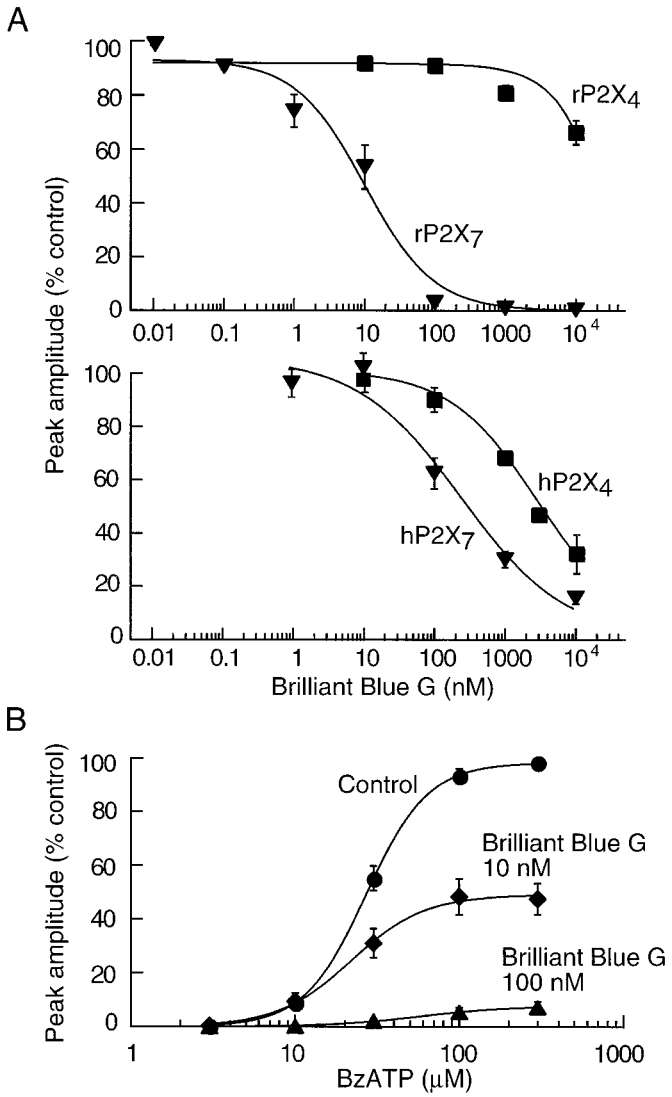

Fig. 2. Summary of actions of Brilliant Blue G at human and rat P2X$_7$ and P2X$_4$ receptors. A, inhibition curves for rat receptors (top panel) and for human receptors (bottom). Results are presented as percentage of current amplitudes before application of Brilliant Blue G. B, concentration-current curves for BzATP in the absence (Control) and presence of 10 and 100 nM Brilliant Blue G. Results are expressed as percentage of the maximal currents in the absence of Brilliant Blue G. Points are mean ± S.E. from four to eleven experiments. Lines are fitted by least squares (see *Materials and Methods*), except for rP2X$_4$ in A, which is drawn by eye.

receptor is largely independent of agonist and agonist concentrations used are consistent with noncompetitive block.

Brilliant Blue G Prevents Membrane Blebbing and YOPRO-1 Uptake Associated with Activation of P2X$_7$ Receptor. Activation of rat or human P2X$_7$ receptors can also be followed by measuring dye uptake, membrane blebbing, or eventually cell lysis (Di Virgilio et al., 1999; MacKenzie et al., 1999; Virginio et al., 1999). In cells expressing the rat P2X$_7$ receptor, the time to initial membrane blebbing after application of BzATP was delayed significantly in the presence of 100 nM Brilliant Blue G and was prevented by 1 μM Brilliant Blue G (Fig. 4). YOPRO-1 uptake was 13 ± 2% (n = 27) of control cells (n = 18) in the presence of Brilliant Blue G. These results indicate that Brilliant Blue G inhibits not only the initial cationic current but also other downstream events associated with activation of the P2X$_7$ receptor.

Discussion

These results indicate that Brilliant Blue G is a selective P2X$_7$ receptor antagonist. Its primary significance is that this antagonist now provides a means for clearly differentiating P2X$_7$- from P2X$_4$-mediated responses. The IC$_{50}$ concentration for inhibition of rat P2X$_7$ receptors was 10 nM, whereas 10 μM Brilliant Blue G failed to produce even 50% inhibition of the rat P2X$_4$ receptor. Many in vitro studies on ATP-mediated responses are performed on rat tissues; given that suramin and PPADS are not discriminative (Buell et al., 1996; Surprenant et al., 1996; Garcia-Guzman et al., 1997), the >1000-fold difference for Brilliant Blue G may prove to be useful in tissues that express both P2X$_4$ and P2X$_7$ receptors. Brilliant Blue G was also 1000-fold less potent at most other receptors (P2X$_1$, P2X$_{1/5}$, P2X$_3$, P2X$_{2/3}$) compared with the rat P2X$_7$. The exception here was the rat P2X$_2$ receptor, where our finding of an IC$_{50}$ value of 3 μM was similar to that reported previously (King et al., 1997). This 150-fold selectivity between rat P2X$_7$ and P2X$_2$ that we observed would often be sufficient experimentally for differentiation of receptor subtypes. In any case, suramin and PPADS abolish P2X$_2$-mediated responses at concentrations (5–10 μM) that are ineffective at either rat P2X$_4$ or P2X$_7$ receptors (North and Barnard, 1997; Ralevic and Burnstock, 1998; Burnstock, 1999).

Prominent species differences in agonist and antagonist potencies are often observed among P2X$_7$ receptors. For

TABLE 1
Summary of actions of Brilliant Blue G recombinant P2X receptors expressed in HEK293 cells

Receptor	Agonist	IC$_{50}$	Slope	n
	μM	nM		
Rat P2X$_7$	BzATP (30)	10.1 ± 3.7	0.9 ± 0.3	52
Rat P2X$_7$	BzATP (100)	12.7 ± 2.2	1.0 ± 0.2	51
Human P2X$_7$	BzATP (300)	265 ± 192	0.6 ± 0.2	23
Rat P2X$_4$	ATP (100)	>10,000		31
Human P2X$_4$	ATP (30)	3,160 ± 745	0.7 ± 0.1	19
Rat P2X$_1$	ATP (0.5)	>5,000		12
Human P2X$_1$	ATP (0.5)	>5,000		19
Rat P2X$_2$	ATP (30)	1,370 ± 282	1.6 ± 0.5	37
Rat P2X$_3$	ATP (1)	>10,000		12
Human P2X$_3$	ATP (3)	>10,000		9
Rat P2X$_{2/3}$	αβmeATP (10)	>10,000		11
Rat P2X$_{1/5}$	ATP (1)	>10,000		20

n, number of antagonist applications to all cells tested.

example, at the $P2X_7$ receptor, the agonists ATP and BzATP have 10- to 30-fold lower EC_{50} values in rat than human, the antagonists suramin and PPADS are 10- to 50-fold less potent in rat, KN-62 blocks human receptors but is ineffective at rat receptors, and divalent cations are 5- to 20-fold more potent to block human receptors (Surprenant et al., 1996; Gargett and Wiley, 1997; Rassendren et al., 1997; Virginio et al., 1997; Chessell et al., 1998; Humphreys et al., 1998). Thus, it is not surprising to find similar differences for Brilliant Blue G. $P2X_7$ receptors show 80% amino acid identity between rat and human, which is less than the rat/human identity for the other receptors (North and Barnard, 1997).

For the $P2X_4$ receptor, Brilliant Blue G also showed a 10-fold difference in potency between human (IC_{50} 3 μM) and rat $P2X_4$ (IC_{50} >10 μM) receptors, although it is important to emphasize that the difference was opposite in direction to that seen for the $P2X_7$ receptors. Because Brilliant Blue G was less potent to inhibit human $P2X_7$ receptors but more potent to inhibit human $P2X_4$ receptors, this means that there is only an approximately 15-fold selectivity between human $P2X_4$ and $P2X_7$ receptors, compared with >1000-fold for the rat. Some caution will be required in interpreting experiments with Brilliant Blue G inhibition of P2X responses in human tissues. Significant differences in inhibition by suramin and PPADS have been noted between the rat and human $P2X_4$ receptor, and studies of chimeric receptors identified a region in the extracellular domain that was responsible for suramin binding (Garcia-Guzman et al., 1997). It may be expected that studies of chimeric $P2X_7$ receptors will help to identify residues in the extracellular loop responsible for these differences found in this study. Given that Brilliant Blue G is a polysulfonate, like suramin and many other antagonist dyes, it would not be surprising if interactions with the

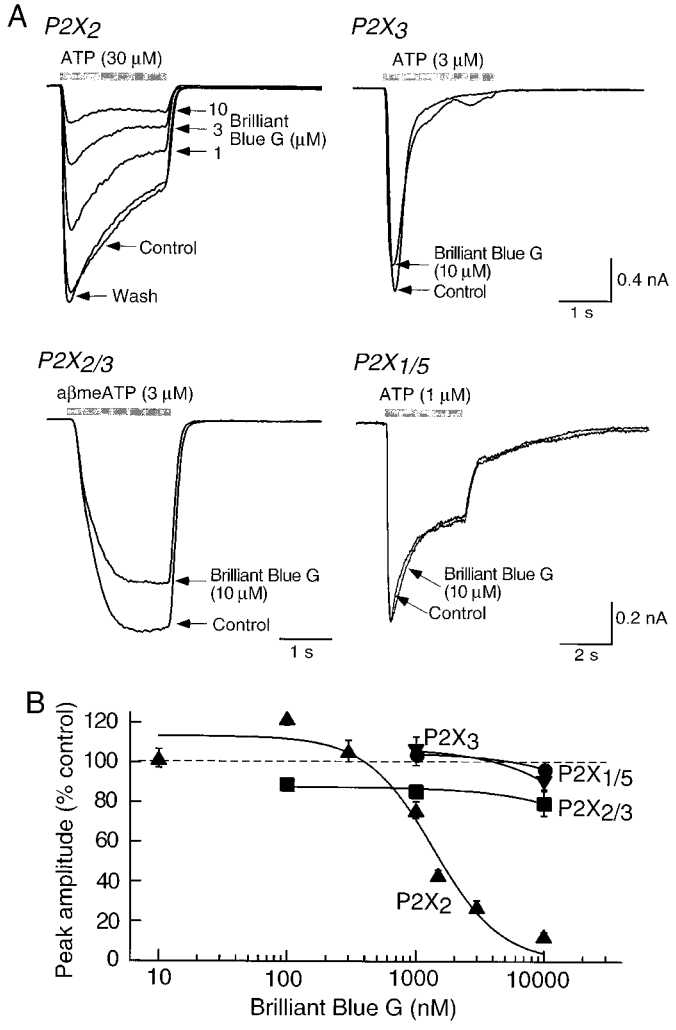

Fig. 3. Brilliant Blue G has little or no effect at most P2X receptors. A, superimposed currents recorded from individual cells expressing the indicated receptors before (Control) and after application of Brilliant Blue G at the concentrations indicated. B, concentration-response curves for rat $P2X_2$, human $P2X_3$, rat $P2X_{2/3}$, and rat $P2X_{1/5}$ receptors. Note that the lower concentrations of Brilliant Blue G slightly potentiated currents at $P2X_2$ receptors. Points are mean ± S.E. for three to eleven experiments. Line for $P2X_2$ is fitted by least squares; others are drawn by eye.

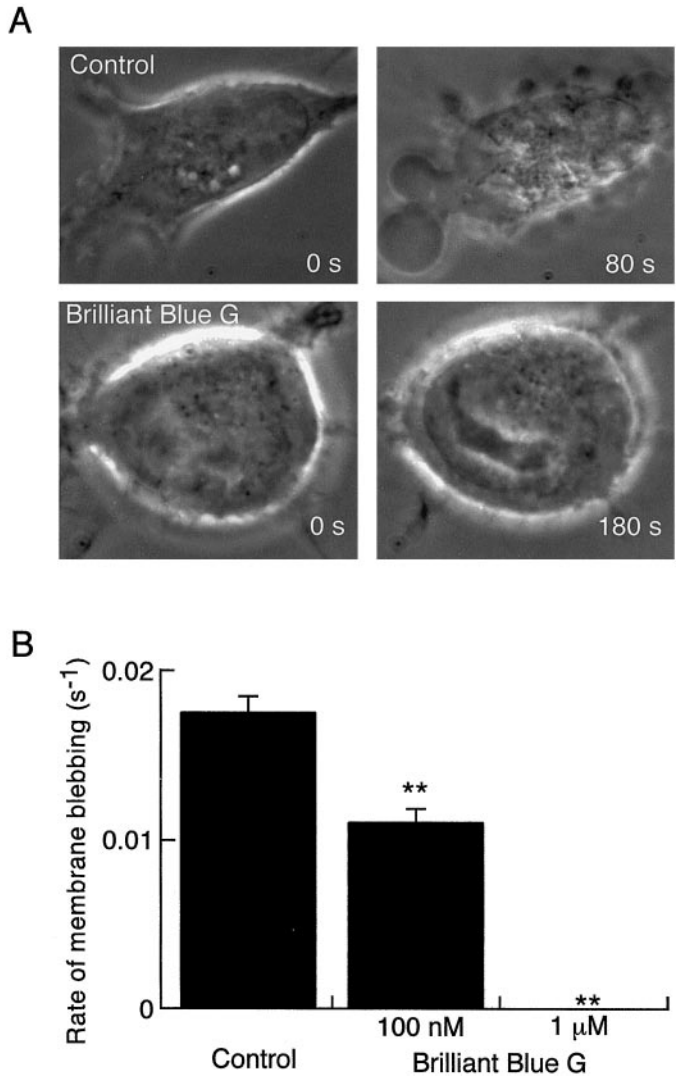

Fig. 4. Brilliant Blue G prevents membrane blebbing in cells expressing $P2X_7$ receptors. A, a single cell before (control) and in the presence of Brilliant Blue G (1 μM). Times indicated are the times after adding BzATP (100 μM). Membrane blebbing is clearly evident at 80 s in the control conditions but was not observed in the presence of Brilliant Blue G. B, summary of all experiments; $^{**}P < .05$ The time to membrane blebbing was measured in control cells ($n = 4$) and cells treated with Brilliant Blue G (100 nM) ($n = 4$) or 1 μM ($n = 3$). Ordinate is rate of blebbing (reciprocal of time elapsed before blebbing occurred).

many positively charged amino acid residues on the ectodomain contribute to its binding.

Inhibition of $P2X_7$-mediated currents by Brilliant Blue G was noncompetitive; it occurred without change in the BzATP EC_{50} concentration. It was also voltage-independent, very slowly reversible, and incomplete even at 20 min of washout. Furthermore, it inhibited not only the BzATP-evoked currents in $P2X_7$-expressing cells, but also other consequences of P2X receptor activation (uptake of YO-PRO-1 and the membrane blebbing). These results are most consistent with a simple allosteric regulation of the agonist binding site rather than a block of the ion channel. The inhibitory actions of Brilliant Blue G at the $P2X_7$ receptor are similar to those of divalent cations such as Cu^{2+} and Zn^{2+}, which exert their effects by allosteric modulation of $P2X_7$ receptors (Li et al., 1996, 1997; Chessell et al., 1997; Virginio et al., 1997).

$P2X_4$ receptor mRNA and protein are densely expressed throughout neurons of the brain as well as in immune cells and exocrine gland cells (Collo et al., 1996; Seguela et al., 1996, 1997). $P2X_7$ receptors, which have not been found in neurons, have often been found to colocalize with $P2X_4$ receptors in non-neuronal cells (Collo et al., 1997; Cario-Toumaniantz et al., 1998; Tenneti et al., 1998). This colocalization, along with calcium influx studies in gland cells (Christoffersen et al., 1998; Tenneti et al., 1998) and electrophysiological studies in B-lymphocytes (Markwardt et al., 1999), initially suggested that heteromeric $P2X_{4/7}$ receptors may underlie the functional responses observed in these tissues. However, it has been shown now that $P2X_4$ subunits do not heteropolymerize with $P2X_7$ subunits (Cario-Toumaniantz et al., 1998; Torres et al., 1998), and it appears most likely that functional responses in these cells result from the simultaneous activation by ATP or BzATP of homomeric $P2X_4$ and homomeric $P2X_7$ receptors. Our present characterization of Brilliant Blue G as a nanomolar affinity, highly selective antagonist at rat $P2X_7$ receptors provides a useful pharmacological tool for discriminating functional responses to P2X receptor activation in native tissues expressing both $P2X_4$ and $P2X_7$ receptors.

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