The food dye FD&C Blue No. 1 is a selective inhibitor of the ATP release channel Panx1

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The food dye FD&C Blue No. 1 (Brilliant Blue FCF [BB FCF]) is structurally similar to the purinergic receptor antagonist Brilliant Blue G (BBG), which is a well-known inhibitor of the ionotropic P2X7 receptor (P2X7R). The P2X7R functionally interacts with the membrane channel protein pannexin 1 (Panx1) in inflammasome signaling. Intriguingly, ligands to the P2X7R, regardless of whether they are acting as agonists or antagonists at the receptor, inhibit Panx1 channels. Thus, because both P2X7R and Panx1 are inhibited by BBG, the diagnostic value of the drug is limited. Here, we show that the food dye BB FCF is a selective inhibitor of Panx1 channels, with an IC_{50} of 0.27 µM. No significant effect was observed with concentrations as high as 100 µM of BB FCF on P2X7R. Differing by just one hydroxyl group from BB FCF, the food dye FD&C Green No. 3 exhibited similar selective inhibition of Panx1 channels. A reverse selectivity was observed for the P2X7R antagonist, oxidized ATP, which in contrast to other P2X7R antagonists had no significant inhibitory effect on Panx1 channels.

Based on its selective action, BB FCF can be added to the repertoire of drugs to study the physiology of Panx1 channels. Furthermore, because Panx1 channels appear to be involved directly or indirectly through P2X7Rs in several disorders, BB FCF and derivatives of this "safe" food dye should be given serious consideration for pharmacological intervention of conditions such as acute Crohn's disease, stroke, and injuries to the central nervous system.

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INTRODUCTION

Purinergic receptors, specifically the P2X7 receptor (P2X7R), have been recognized as a potential site of intervention for the treatment of several neurological disorders including spinal cord injury, Huntington's disease, and other neurodegenerative diseases involving neuroinflammation (Díaz-Hernández et al., 2009; Peng et al., 2009; Takenouchi et al., 2010; Lopatář et al., 2011; Traini et al., 2011; Arbeloa et al., 2012; Chu et al., 2012; Iriyama et al., 2012; Iwamaru et al., 2012; Kimbler et al., 2012). The P2X7R is a ligand-operated ion channel with high permeability to small cations (North and Barnard, 1997; North, 2002). In a second incarnation, the P2X7R also can form a large pore, which allows the flux of larger molecules such as the dye YoPro. Whether the large pore formation is an inherent property of the P2X7R protein or whether a pore-forming protein is associated with the P2X7R is a matter of debate (Pelegrin and Surprenant, 2006; Locovei et al., 2007; Chaumont and Khakh, 2008). Several drugs interact with the P2X7R and block its channel and large pore activity with high efficacy and good selectivity among purinergic receptors. This includes Brilliant Blue G (BBG), a dye widely used as a stain for protein assays. Depending on the species, BBG inhibits the P2X7R with an IC_{50} of 10 nM (rat) or 265 nM (human), while requiring 100–1,000 times higher concentrations to inhibit other P2X receptors (Jiang et al., 2000).

BBG exhibits some structural similarity to Brilliant Blue FCF (BB FCF), the "safe" food dye FD&C Blue No. 1. Many publications point to this similarity with the salient implication that BB FCF acts on the P2X7R in the same way as BBG. A Medline search with terms "P2X7" and "BB FCF" or other names of the dye such as "Erioglaucine" yields in excess of 100 references. Yet most (if not all) fail to contain data on effects of the dye on P2X7-mediated membrane currents. Instead, these papers describe effects of BBG and refer to the structural similarity between BB FCF and BBG. To our knowledge, the only study to actually test BB FCF for effects on any membrane channel is that of Jo and Bean (2011), who demonstrated that BBG with an IC_{50} of 2 µM inhibits voltage-gated sodium channels, and that BB FCF requires a considerably higher concentration to affect these channels.

The P2X7R can act in concert with the ATP release channel pannexin 1 (Panx1; Pelegrin and Surprenant, 2006; Locovei et al., 2007). A plausible role for Panx1 in that collaboration is that of an amplifier, boosting the ligand concentration at the receptor. This potentially dangerous positive feedback loop is counteracted

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Abbreviations used in this paper: BB FCF, Brilliant Blue FCF; BBG, Brilliant Blue G; Cx46, connexin 46; oATP, oxidized ATP; P2X7R, P2X7 receptor; Panx1, pannexin 1.

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by a negative control mechanism in which ATP binds to a site on the extracellular surface of Panx1, inhibiting the channel activity of the protein (Qiu and Dahl, 2009; Qiu et al., 2012). The ATP-binding sites on Panx1 and P2X7R are similar in that positively charged amino acids are involved in forming the binding site and that several ligands to the receptor, including agonists such as BzATP and antagonists such as BBG, inhibit the Panx1 channel. The major difference between the binding sites is their affinity, with Panx1 having a considerably lower affinity to ATP and other ligands than P2X7R (Qiu et al., 2012).

In testing whether this relationship holds up also for other P2X7R ligands, we investigated the effect of BB FCF. Surprisingly, the food dye did not affect P2X7R-mediated currents. Instead, BB FCF inhibited Panx1 channel currents in submicromolar concentrations.

MATERIALS AND METHODS

Preparation of oocytes

Preparation of oocytes and electrophysiological recording were performed as described previously (Dahl, 1992; Dahl and Pfahnl, 2001). Mouse Panx1 was provided by R. Dermietzel (University of Bochum, Bochum, Germany), and connexin 46 (Cx46) was obtained from D.L. Paul (Harvard University, Cambridge, MA). Human P2X7R was provided by A. Surprenant (University of Sheffield, Sheffield, UK). The generation of $Cx32E_1 43$ was described previously (Pfahnl et al., 1997).

Synthesis of mRNA

The plasmid containing $Cx32E_1 43$ (pGEM 3Z; Promega) was linearized with Ssp1 and transcribed with SP6 polymerase. The plasmid containing Cx46 (rSP64T) was linearized with EcoR1 and transcribed with SP6 polymerase. Panx1, in pCS2, was linearized with NotI, and h-P2X7, in pcDNA3, was linearized with AvrII. In vitro transcription was performed with the polymerases T3, T7, or SP6, using the Message Machine kit (Ambion). mRNAs were quantified by absorbance (260 nm), and the proportion of full-length transcripts was checked by agarose gel electrophoresis. In vitro–transcribed mRNAs (~20 nl) were injected into *Xenopus laevis* oocytes.

Electrophysiology

Whole cell membrane current of single oocytes was measured using a two-electrode voltage clamp and recorded with a chart recorder. Both voltage-measuring and current-passing microelectrodes were pulled with a vertical puller (David Kopf Instruments) and filled with 3 M KCl. The recording chamber was perfused continuously with frog Ringer (OR2) solution (mM: 82.5 NaCl, 2.5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 1 Na_2HPO_4, and 5 HEPES, pH 7.5). Membrane conductance was determined using voltage pulses. Oocytes expressing Cx46 or $Cx32E_1 43$ were held at −50 mV, and depolarizing pulses of 5-s duration to 0 mV were applied. Oocytes expressing Panx1 were held at −60 mV, and pulses to +60 mV were applied to transiently open the channels.

ATP release assay

ATP flux was determined by luminometry. Oocytes, 2 d after injection of Panx1 messenger RNA, were pretreated in OR2 solution with and without BB FCF for 10 min and stimulated by incubation in OR2 solution (negative control), KGlu solutions (positive control), and KGlu solution with BB FCF, respectively, for 10 min. The supernatant was collected and assayed with luciferase/luciferin (Promega). As control, ATP at known concentrations was added

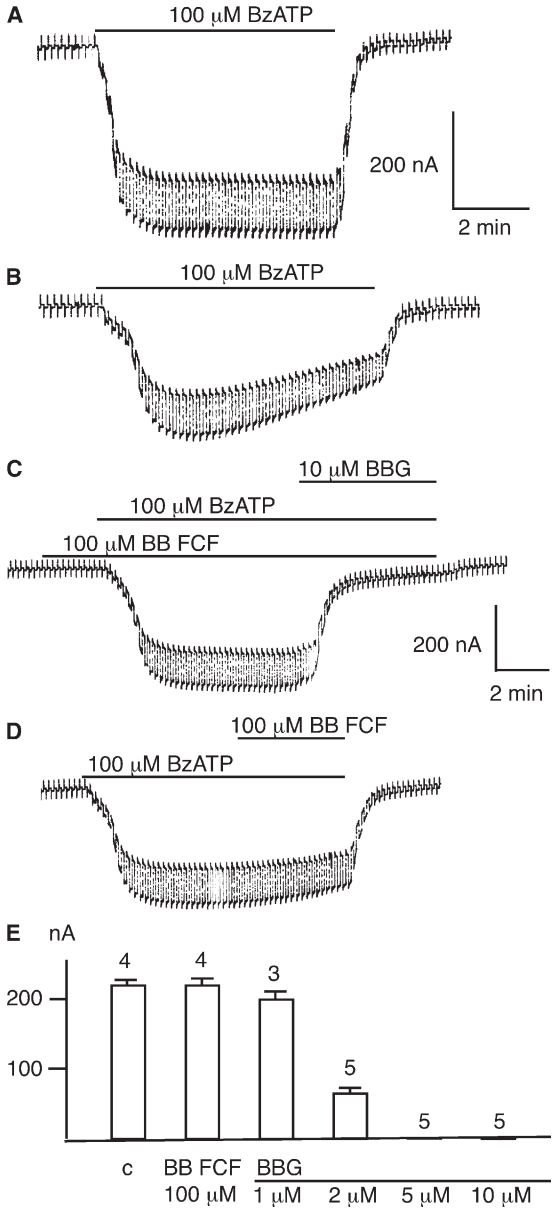

Figure 1. BzATP- induced currents in oocytes expressing the human P2X7R. The membrane potential was clamped at −60 mV, and brief 10-mV pulses were applied at a rate of 0.1 Hz for assessment of changes in membrane conductance. The currents and conductance changes induced by 100 μM BzATP were either sustained (A) or diminished (B) over time, despite the presence of the stimulant depending on the batch of oocytes. BzATP-induced currents in oocytes were inhibited by BBG but not by BB FCF (C–E). Experimental conditions were the same as in A, except the inhibitors BB FCF and BBG were included. (C) In the presence of 100 μM BB FCF, membrane currents of significant amplitude were induced by BzATP, which were attenuated by 10 μM BBG. (D) When applied in the presence of BzATP, the induced currents were minimally affected by 100 μM BB FCF. (E) Quantitative analysis of membrane currents induced by BzATP in the presence of 100 μM BB FCF or of various concentrations of BBG. Means ± SD are plotted, and *n* is indicated.

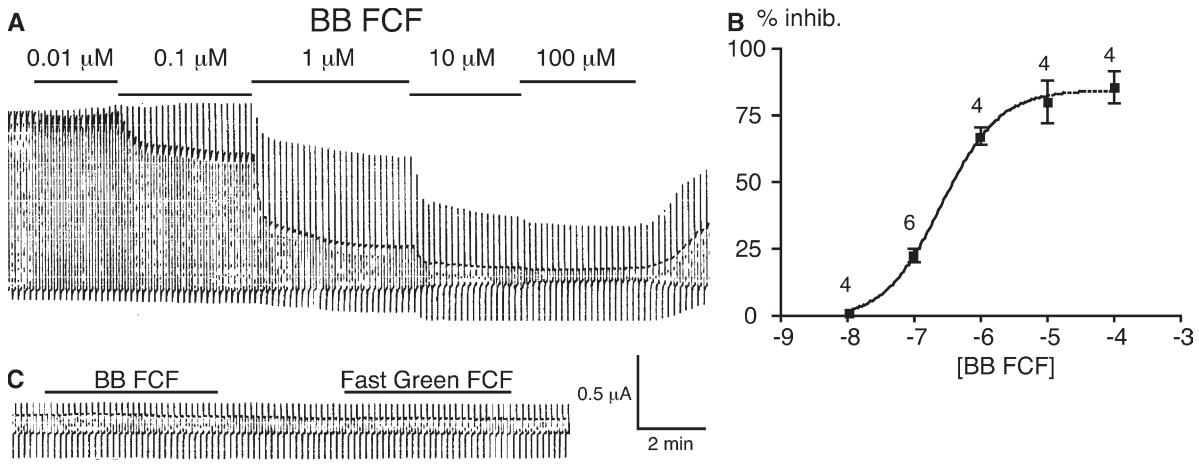

Figure 2. Dose-dependent inhibition of Panx1 currents by BB FCF. The membrane potential was clamped at −60 mV, and brief +60-mV pulses were applied at a rate of 0.1 Hz to open Panx1 channels. (A) BB FCF attenuated the Panx1-mediated currents in a dose-dependent fashion. (B) Dose–response relationship of inhibition of membrane currents by BB FCF. Means ± SD (n = 4) are plotted. (C) Uninjected control oocytes subjected to the same pulse protocol exhibited small currents unaffected by 10 μM BB FCF and by 10 μM Fast Green FCF.

to the assay solution to assess linearity and to determine the effect of the dye on the readout. BB FCF was found to interfere with the assay. Consequently, the assay was run without cells but with 10 nM ATP with and without 10 μM BB FCF to determine the correction factor necessary for determining the actual effect of BB FCF on ATP release from cells.

RESULTS

BB FCF in contrast to BBG does not affect the human P2X7R

The *Xenopus* oocyte expression system was used to test the effect of BB FCF on P2X7Rs. Fig. 1 shows BzATP-induced currents in oocytes expressing P2X7R that were absent in uninjected control oocytes. These currents were consistent in amplitude from oocyte to oocyte but variable in duration. In some oocytes, the response to BzATP was sustained throughout the presence of the ligand (Fig. 1 A), whereas in others, current inactivation set in within 2–3 min after the beginning of the BzATP application (Fig. 1 B). This apparent receptor desensitization typically correlated with batches of oocytes, suggesting that either some oocytes provide a desensitization factor or that other oocytes provide a factor abolishing desensitization. Subsequent experiments were restricted to batches of oocytes with little or no current inactivation in the presence of the agonist BzATP.

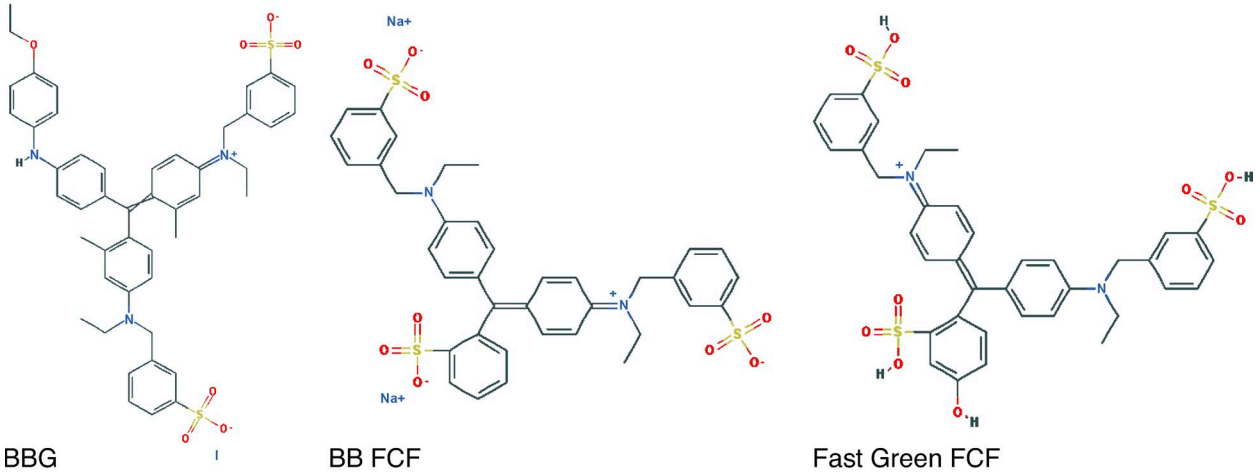

Figure 3. PubChem structures of dyes used in this study. The food dyes BB FCF and Fast Green FCF are structurally identical except for one OH group. BBG is structurally related to the food dyes but exhibits clear differences.

Consistent with its known effect on P2X7R, BBG attenuated the BzATP-induced membrane currents in oocytes injected with hP2X7R mRNA (Fig. 1 C). In contrast, despite its purported structural similarity to BBG, the food dye BB FCF did not prevent BzATP-induced currents (Fig. 1 C) and minimally attenuated sustained P2X7R-mediated currents when applied at concentrations as high as 100 μM (Fig. 1 D). For quantitative analysis and to be independent of the variable desensitization of BzATP-induced currents, the drugs were applied in a separate set of experiments before BzATP and continued to be present (Fig. 1 E). No significant inhibition of BzATP-induced currents was observed for 100 μM BB FCF, whereas BBG inhibited the currents in a dose-dependent fashion as described previously for this receptor (Jiang et al., 2000).

BB FCF inhibits Panx1 currents

Oocytes expressing Panx1 exhibit voltage-activated currents (Bruzzone et al., 2003; Bao et al., 2004). Although it is unlikely that under physiological conditions cells other than neurons and muscle experience positive membrane potentials, stepping the membrane potential transiently to positive potentials is a convenient way to demonstrate Panx1 activity experimentally. Fig. 2 A shows that the voltage-activated Panx1 channel currents were inhibited by BB FCF in a concentration-dependent fashion. The effect was rapid and was fully reversible within minutes. The IC_{50} was 0.27 μM (Fig. 2 B). The effect of BB FCF reached a plateau at 75% inhibition, leaving a residual conductance. At least part of this residual conductance is caused by channels endogenous to oocytes. The application of the same pulse protocol to uninjected oocytes induced small currents that were unaffected by BB FCF and the closely related food dye FD&C Green No. 3 (also known as Fast Green FCF; Fig. 2 C).

The Fast Green FCF differs in its structure from BB FCF by only one OH group (Fig. 3). Fast Green FCF had similar effects on Panx1 currents as BB FCF. The IC_{50} for the inhibition of Panx1 currents by Fast Green FCF was 0.27 μM and thus identical to that of BB FCF. Like BB FCF, Fast Green FCF did not inhibit ATP-induced P2X7 channel currents (not depicted).

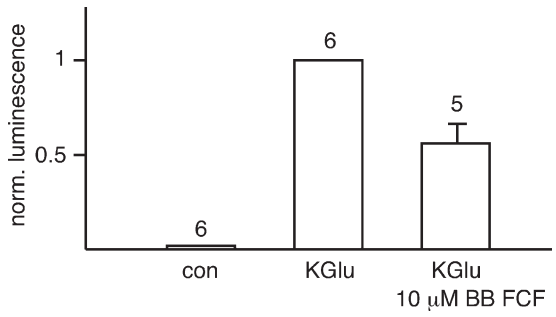

Figure 4. KGlu-induced ATP release from oocytes expressing Panx1 was inhibited by BB FCF. ATP release was determined with the luciferase assay, with which the dye interferes. A correction factor for this interference was determined and used for plotting the data.

BB FCF inhibits ATP release from Panx1-expressing oocytes

Because Panx1 serves as an ATP release channel, we tested the effect of BB FCF on ATP release. As shown in Fig. 4, ATP release was stimulated by increased extracellular K^+ concentration. The K^+-induced ATP release was attenuated by BB FCF. As shown previously (Bao et al., 2004), the Panx1-mediated ATP release from oocytes occurs against a background of vesicular, i.e., exocytotic ATP release, which is insensitive to Panx1 inhibitors but sensitive to brefeldin. Thus, no complete inhibition of ATP release by BB FCF can be expected.

BB FCF does not affect connexin channels

Most drugs inhibitory to gap junction channels formed by innexins in invertebrates or by connexins in vertebrates also inhibit the membrane channels formed by Panx1. The reverse, however, is not true. Several inhibitors of Panx1 channels, such as ATP, BBG, or probenecid, do not affect gap junction channels. To test whether connexins are sensitive to BB FCF, two connexins that

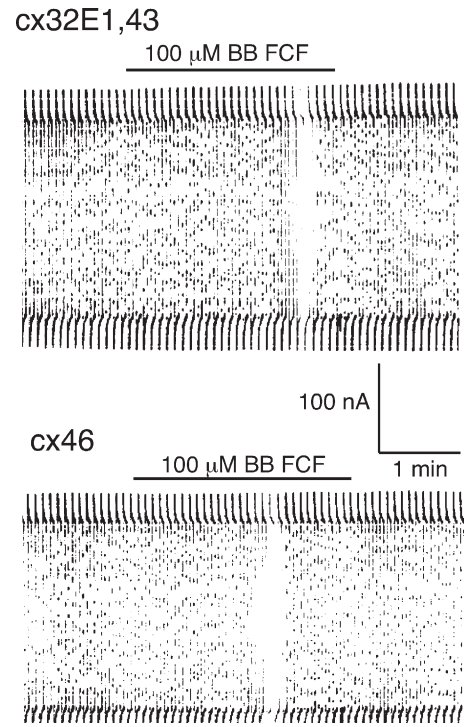

Figure 5. Failure of BB FCF to affect connexin channels. Two connexins, $Cx32E_1 43$ and Cx46, forming open "hemichannels" at regular calcium ion concentrations in oocytes expressing them, were tested. The membrane potential was clamped at −50 mV, and brief pulses to 0 mV were applied at a rate of 0.1 Hz to open the connexin channels. 100 μM BB FCF did not affect the currents carried by either connexin. The traces are representative of four trials for each connexin on different oocytes.

form open-membrane channels ("hemichannels"), wild-type Cx46 and $Cx32E_1 43$, were expressed in oocytes. As shown in Fig. 5, the currents carried by Cx46 or $Cx32E_1 43$ channels remained unaltered after the application of BB FCF.

Amino acids in Panx1 involved in binding/gating of the channel by the food dyes

An alanine scan of Panx1 has identified several amino acids in both extracellular loops of the protein that are essential for the inhibitory effect of ATP and BzATP on Panx1 channel currents (Qiu et al., 2012). To test whether the inhibition of the currents by BB FCF and Fast Green FCF involved similar positions of the protein, we tested the effects of the dyes on five alanine replacement mutants, which are not inhibited by BzATP. As shown in Fig. 6, the effect of BB FCF was attenuated in four of the five and that of Fast Green FCF in all five alanine mutants. Thus, it appears that the binding sites/gating structures for ATP and both food dyes overlap.

Exogenous expression of membrane channels in cells always occurs against a background of endogenous channels. Typically, this background in *Xenopus* oocytes is small and is responsible for ~1-µS conductance. However this "leak conductance" can vary between oocytes and thus may obscure the level of Panx1-mediated membrane conductance. Therefore, two established Panx1 inhibitors, carbenoxolone and probenecid, were tested in addition to the food dyes in the same cells. Although carbenoxolone was an effective inhibitor in all five mutants, the effect of probenecid was almost abolished in Panx1W74A and attenuated in Panx1S237A and Panx1L266A. Thus, probenecid either has an overlapping binding site with ATP and the dyes, or these amino acids are involved in the gating process triggered by probenecid.

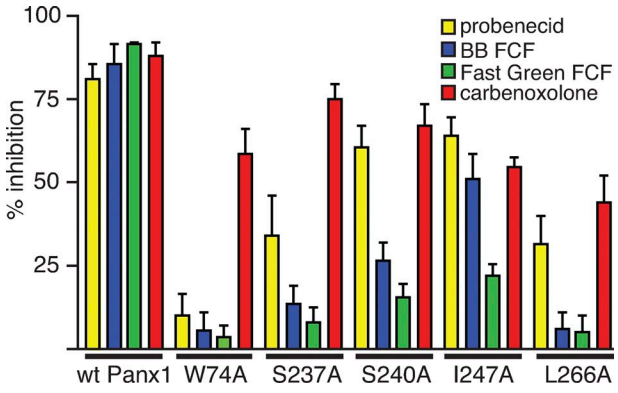

Figure 6. Effect of various Panx1 inhibitors in alanine replacement mutants lacking the feedback inhibition by ATP. The Panx1 inhibitors carbenoxolone, BB FCF, Fast Green FCF, and probenecid were tested in wild-type Panx1 and various alanine replacement mutants using the same protocol as in Fig. 2. The inhibition by probenecid was almost abolished in Panx1 W74A. Carbenoxolone and Fast Green FCF inhibited the currents effectively in all mutants. BB FCF inhibition was attenuated in all mutants except Panx1 I247A. Means ± SD are plotted. $n = 5$ for Panx1 I247A and L266A, and $n = 4$ for all other points.

Oxidized ATP (oATP) acting on P2X7R but not Panx1

Panx1 is an ATP release channel and serves as such in many cell types including astrocytes (Bao et al., 2004; Locovei et al., 2006; Iglesias et al., 2009; Ransford et al., 2009; Suadicani et al., 2012). Several ligands for the P2X7R, regardless whether they are agonists or antagonists of the receptor, also inhibit Panx1, albeit requiring higher concentrations of the ligands (Qiu and Dahl, 2009). Anderson et al. (2004), however, have shown that in astrocytes, ATP induces an ATP release that is not sensitive to the P2X7R antagonist oATP and is sensitive to some anion channel blockers. We, therefore, tested whether oATP, like other P2X7R ligands, inhibits Panx1 channels. As shown in Fig. 7, oATP did not inhibit Panx1 channels but attenuated P2X7R currents. Considering that Panx1 is inhibited by several chloride channel blockers, this is consistent with Panx1 serving as major ATP release channel in astrocytes.

DISCUSSION

This study shows that the food dye FD&C blue dye 1 (BB FCF, Erioglaucine) inhibits Panx1 channel currents with an IC_{50} of 0.27 µM. This is similar to the effect of BBG, which inhibits Panx1 channels with an IC_{50} of 3 µM (Qiu et al., 2012). Considering there is some structural similarity between the two drugs, this is not unexpected. However, at concentrations up to 100 µM, BB FCF did not alter BzATP-induced currents in oocytes expressing the human P2X7R, whereas BBG at much lower concentrations (2 µM) did. BB FCF at 100 µM had no effect on connexin channels and has been reported to only minimally affect voltage-gated sodium channels when tested at

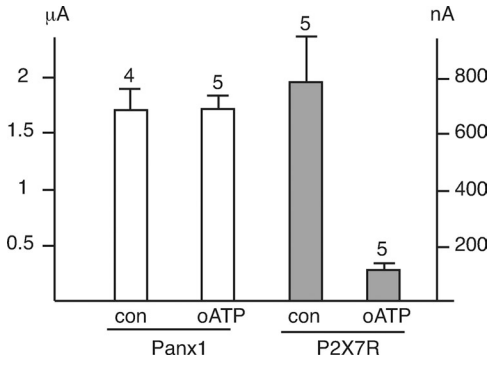

Figure 7. Effect of oATP on Panx1 currents and P2X7R-mediated currents induced by 100 µM BzATP. con, voltage-induced Pannx1 currents or BzATP-induced P2X7R currents in the absence of oATP. Oocytes expressing Panx1 or P2X7R were preincubated with 100 µM oATP for 2–3 h before measurements of membrane currents. oATP only attenuated BzATP-induced currents in P2X7R-expressing oocytes. Means ± SD are plotted, and n is indicated.

30 μM (Jo and Bean, 2011). Thus, BB FCF and Fast Green FCF, which differ by one OH group, are the most selective inhibitors of Panx1 channels presently known.

BB FCF is widely used as food dye and is generally considered to be safe (Borzelleca et al., 1990; Flury and Flühler, 1994). However, there was sufficient concern about this compound for it to have been banned by many European countries until after the establishment of the European Union, when those restrictions ceased. Because at one time there was a safety concern, there might be a target in the human body for BB FCF. Panx1 is now identified as a target. Whether it is the only one remains to be seen. The European Food and Safety Authority has set the acceptable daily intake for BB FCF at 6 mg/kg body weight/day (Aguilar et al., 2010). Because only ∼10% of the ingested dye is absorbed in the intestines, concentrations high enough to affect Panx1 channels are not to be expected if the acceptable daily intake value is adhered to. However, it is conceivable that by excessive ingestion of colored drinks and food, a cumulative effect may lead to BB FCF concentrations high enough to inhibit Panx1 channel activity in humans if human Panx1 has the same sensitivity to the dye as reported here for mouse Panx1.

BBG and most other P2X7 ligands, regardless of whether they are agonists or antagonists, inhibit Panx1 channel currents, Panx1-mediated ATP release, and dye uptake (Qiu and Dahl, 2009). Yet many papers using BBG still consider P2X7R as the exclusive target. Because the affinity of P2X7R for the drugs is higher than that of Panx1, the targets could in principle be discriminated by their dose–response curves. However, with the advent of specific drugs like BB FCF, the discrimination becomes considerably easier. For example, it should be possible now to test pharmacologically whether the P2X7R protein alone is capable of large pore formation without the contribution of another protein. If BBG but not BB FCF inhibits the large pore, a Panx1 contribution would be unlikely. On the other hand, inhibition of the large pore by BB FCF without an effect on the P2X7R-mediated cation current would suggest that Panx1 forms the large pore. Similarly, in the many pharmacologically (BBG) based links between P2X7R and diseases, it should be possible with the aid of BB FCF to determine whether P2X7R, Panx1, or both are the drug targets.

This study also indicates that a reverse selectivity exists. oATP was inhibitory to P2X7R-mediated currents in oocytes but failed to attenuate Panx1 currents in these cells. Thus, BB FCF in combination with oATP can be used to pharmacologically distinguish contributions of P2X7R and Panx1 in physiological and pathological settings.

Data obtained with alanine replacement mutants of Panx1 indicate that the binding/gating structures for BB FCF and ATP overlap but are distinct. For example, in one of the five alanine mutants tested, Panx1 I247A, BB FCF exhibited significant current inhibition, whereas BzATP inhibition was absent (Qiu et al., 2012). Intriguingly, in another mutant, Panx1 W74, the effect of probenecid was also highly attenuated. This amino acid position is close to or part of the channel pore (Wang and Dahl, 2010). Because there is no evidence that probenecid acts from the outside, a contribution of this amino acid to the gating process can be considered.

As pointed out recently (Dahl and Keane, 2012), there are several reasons why Panx1 should be targeted for pharmacological suppression of excessive inflammation and associated secondary cell death. (a) Panx1 mediates an early signaling event in the inflammatory process. (b) In conjunction with the P2X7R, Panx1 serves as amplifier in a positive feedback loop. (c) Several stimuli of the inflammasome and cell death, such as low oxygen, extracellular potassium ions, and glutamate, affect Panx1. (d) Panx1 as a membrane protein with extracellular exposure is easily accessible to drugs. With the discovery of BB FCF as a specific Panx1 inhibitory drug acting on the extracellular surface of the protein, the repertoire of drugs inhibiting excessive inflammasome activation is widened. For optimal treatment, a combination of a pannexin inhibitor with a specific P2X7R inhibitor has to be considered, because it is plausible that very high extracellular ATP concentrations will not require the amplification function of Panx1 for maximal inflammasome activation (Qu et al., 2011). Because of the difference in affinities between P2X7R and Panx1, such specificity can be obtained with low concentrations of BBG (Qiu et al., 2012). Another benefit of low concentrations of BBG is that the effects on sodium channels by the drug (Jo and Bean, 2011) also could be minimized.

It has been recognized that Panx1 is involved in several diverse diseases including Crohn's, AIDS, melanoma, epilepsy, neurotrauma, inflammation, and stroke (Thompson et al., 2006, 2008; Silverman et al., 2009; Santiago et al., 2011; Séror et al., 2011; Dahl and Keane, 2012; Gulbransen et al., 2012; Penuela et al., 2012). Thus, the demand for Panx1-specific drugs already exists and may continue to increase. BB FCF can serve as a "blueprint" for further development of more Panx1-specific drugs. The pun is intended because a predictable side effect of BB FCF treatment would be a transient blue coloration of the patient. That the structurally related food dye Fast Green FCF has almost identical effects on Panx1 as BB FCF indicates a potential for the development of Panx1 inhibitors without the coloration effects by slight modifications of these molecules.

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