Published in final edited form as:

Biochim Biophys Acta. 2011 May ; 1810(5): 532–542. doi:10.1016/j.bbagen.2011.03.009.

P2X₇ Receptor Antagonists Display Agonist-like Effects on Cell Signaling Proteins

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Abstract

Background—The activation of various P2 receptors (P2R) by extracellular nucleotides promotes diverse cellular events, including the stimulation of cell signaling protein and increases in $[Ca^{2+}]_i$. We report that some agents that can block P2X₇R receptors also promote diverse P2X₇R-independent effects on cell signaling.

Methods—We exposed native rat parotid acinar cells, salivary gland cell lines (Par-C10, HSY, HSG), and PC12 cells to suramin, DIDS (4,4'-diisothiocyano stilbene-2,2'-disulfonic acid), Cibacron Blue 3GA, Brilliant Blue G, and the P2X₇R-selective antagonist A438079, and examined the activation/phosphorylation of ERK1/2, PKC δ , Src, CDCP1, and other signaling proteins.

Results—With the exception of suramin, these agents blocked the phosphorylation of ERK1/2 by BzATP in rat parotid acinar cells; but higher concentrations of suramin blocked ATP-stimulated $^{45}Ca^{2+}$ entry. Aside from A438079, these agents increased the phosphorylation of ERK1/2, Src, PKC δ , and other proteins (including Dok-1) within minutes in an agent- and cell type-specific manner in the absence of a P2X₇R ligand. The stimulatory effect of these compounds on the tyrosine phosphorylation of CDCP1 and its Src-dependent association with PKC δ was blocked by knockdown of CDCP1, which also blocked Src and PKC δ phosphorylation.

Conclusions—Several agents used as P2X₇R blockers promote the activation of various signaling proteins and thereby act more like receptor agonists than antagonists.

General significance—Some compounds used to block P2 receptors have complicated effects that may confound their use in blocking receptor activation and other biological processes for which they are employed, including their use as blockers of various ion transport proteins.

Keywords

parotid; ERK1/2; Src; CDCP1; P2 receptor; phosphorylation

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1. Introduction

Extracellular ATP and other nucleotides have effects on a wide variety of cell types by binding to P2 receptors (P2R) on the plasma membrane. These receptors can be classified into two large families consisting of G-protein coupled P2Y receptors and P2X receptors that are ligand-gated cation channels, and there are multiple members in each family (for review, see [1–3]). Physiological signals promoted by these receptors may be initiated by ATP released from neurons, chromaffin cells, and platelets; from cells in an autocrine or paracrine fashion; and upon cell death [4, 5]. Salivary glands have a variety of P2Rs, and the activation of P2X receptors produces an elevation of the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) due to Ca^{2+} influx via the ionotropic P2X₄ and P2X₇ receptors [6–9]. Human and rodent salivary gland acinar cells have P2X₄ and P2X₇ receptors, but display a very low population of P2Y receptors that respond poorly to extracellular nucleotides under normal conditions [9–11]. The stimulation of P2X₇ receptors produces large increases in the $[\text{Ca}^{2+}]_i$ in rat parotid acinar cells, and this acts similarly to muscarinic receptor activation to initiate events involved in the initiation of fluid secretion and saliva formation, including the opening of Ca^{2+} -sensitive K^+ and Cl^- channels [12]. Recent studies demonstrated that ATP promotes saliva secretion via the activation of P2X₇ receptors in mouse submandibular glands [11]. Activation of P2X₇ receptors in rat parotid acinar cells also stimulates many cell signaling proteins that are also downstream of the G-protein-coupled muscarinic type 3 receptor, including extracellular signal-related kinase 1/2 (ERK1/2), Protein Kinase C (PKC), and Protein Kinase D (PKD) [13, 14].

Suramin, DIDS (4,4'-diisothiocyano stilbene-2,2'-disulfonic acid), Cibacron Blue 3GA, and Brilliant Blue G are all polysulfonated compounds with multiple aromatic rings; and although they have been used as antagonists of multiple P2X and P2Y receptors [1–3, 15], they also are employed for other diverse inhibitory effects. Suramin is an anti-parasitic agent. It also binds to multiple types of growth factor receptors and blocks growth factor binding [16], thereby displaying anti-proliferative activity; and this was the rationale for its therapeutic use in multiple cancer trials in the past. Moreover, it binds to and inhibits protein tyrosine phosphatases at their active site [17], and this may explain observations by various groups that suramin increases tyrosine phosphorylation rather than reducing it via its action as a growth factor antagonist. DIDS blocks multiple ion transport proteins, including chloride channels and the cystic fibrosis transmembrane conductance regulator (CFTR) [18], the mitochondrial inner membrane anion channel (IMAC) [19], and multiple sodium bicarbonate cotransporters (NBC) [20] and SLC4 anion exchangers [21]. DIDS also blocks ATP uptake into isolated pancreatic zymogen granules, presumably by blocking the vesicular nucleotide transporter (VNUT) [22], which is a member of the SLC family. Cibacron Blue 3GA blocks IMAC at the same site to which DIDS binds [19]. Brilliant Blue G binds to many proteins, and is used as a general protein stain, including its use as a stain for proteins in polyacrylamide gels. It is highly selective in blocking P2X₇Rs over other P2XRs [23], and it blocks P2X₇R-mediated Ca^{2+} entry into rat parotid acinar cells much more potently than Cibacron Blue 3GA [24].

Others and we have used Coomassie Brilliant Blue G (BBG), Cibacron Blue 3GA, and DIDS to block P2X₇R-mediated Ca^{2+} entry and $[\text{Ca}^{2+}]_i$ elevation in parotid acinar cells [7, 24, 25]. Presently, genetic and molecular approaches are being used in many studies to define the specificity by which extracellular nucleotides exert their effects in salivary glands [11] and other tissues, and various compounds are now available for use as selective agonists and antagonists. Nonetheless, a number of nonselective agents used in earlier studies of P2Rs are also still being used to define P2R-mediated mechanisms as well as other biological processes. Thus, suramin was used recently to block intercellular Ca^{2+} waves generated by ATP release from mechanically stimulated HSG cells, a submandibular

gland cell line [26]; systemic administration of Brilliant Blue G was employed as a P2X₇R antagonist and had a protective effect against spinal cord injury [27]; Cibacron Blue 3A was used to study the P2X₇R ion conductances in mouse parotid glands [28]; and DIDS is frequently employed to block various ion transport systems [29, 30]. Although these compounds have been successfully employed as antagonists of P2Rs, which facilitated conclusions regarding the involvement of these receptors in multiple physiological and biochemical events, we have occasionally noted that these compounds also have off-target effects that result in the phosphorylation and activation of various cell signaling proteins, and here we decided to explore this in more detail.

In the present study, we examined four compounds still being used as P2R antagonists (DIDS, suramin, Brilliant Blue G, and Cibacron Blue 3GA) and/or as blockers of other processes, and we conducted studies to gain new information about their effects on both native cells and various cell lines. In some experiments we compared the effects of these compounds to A438079, a competitive antagonist for the P2X₇R [31]. We focused on the actions of these compounds on multiple signaling proteins, include ERK1/2, PKC δ , Src, and CDCP1; and using tandem mass spectrometry, we identified additional proteins that they activate. Our results demonstrate that these compounds have broad effects beyond their actions as ion transport inhibitors and P2R antagonists, but our findings also suggest that they may be useful tools for P2R-independent studies in some experimental settings.

2. Material and Methods

2.1 Reagents

2'-(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt (BzATP, B6396), Brilliant Blue G (BBG, 20139), and Cibacron Blue 3GA (C9534) were purchased from Sigma-Aldrich. DIDS (D-337) was from Invitrogen, and suramin was from Miles, Inc. A438079 (2972) was purchased from Tocris Bioscience. Polyclonal ERK 2 (SC-154), Dok-1 (SC-6277), Src (SC-18), and PKC δ (SC-213) and monoclonal ERK2 (SC-1647), were purchased from Santa Cruz Biotechnology. The following antibodies were from Cell Signaling Technology: Phospho-Thr202/Tyr204-ERK1/2 (9101), Phospho-Src Family (Tyr416, 2101), Phospho-PKC δ (Tyr311, 2055), Phospho-Tyrosine Mouse mAb (P-Tyr-100, 9411), and Phospho-CDCP1 (Tyr734). Anti-CDCP1 polyclonal antibody was obtained from rabbit immunization using the cytosolic domain of CDCP1 (Cocalico Biologicals). Secondary antibodies were as reported previously [14]. All other chemicals were reagent grade or better.

2.2 Salivary cell preparations and solutions

Parotid acinar cells were prepared from male Sprague-Dawley rats (Charles River Laboratories, Kingston, NY, ~150 g) as described previously [32]. The investigation conforms to the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). Our protocol was submitted to and approved by the Institutional Animal Care and Use Committee (Beth Israel Deaconess Medical Center). Cells were suspended at ~0.5–1 mg protein/ml in Solution A (116.4 mM NaCl, 5.4 mM KCl, 1 mM NaH₂PO₄, 25 mM Na HEPES, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 5 mM Na butyrate, 5.6 mM glucose, pH 7.4). All experiments were performed at 37 °C. Acinar cells were suspended using a magnetic flea to stir the cells in a water-jacketed chamber, treated as indicated, and lysed. Par-C10 cells were grown to near confluence at 37 °C in DMEM-F12 (1:1) medium containing 2.5% fetal bovine serum (FBS) and supplements similar to those specified elsewhere [33]. HSY and HSG cells were grown to near confluence at 37 °C in DMEM-F12 (1:1) medium containing 10% FBS.

Unless stated otherwise, cells were maintained overnight in DMEM-F12 containing 0.1% FBS, treated as indicated, and lysed.

2.3 Western blot analysis and LC/MS/MS Tandem mass spectrometry

Cell lysates were subjected to SDS-PAGE as described previously [14]. For mass spectrometry experiments to obtain Spectrum Counts of phosphorylated peptides, we used five 150 mm dishes of nearly confluent Par-C10 cells for each condition (vehicle, 100 μ M DIDS), and immunoprecipitated proteins using 45 μ g of anti-phosphotyrosine antibody collected with Protein G sepharose overnight. The collected beads were washed and subjected to SDS-PAGE. The gel was stained with Coomassie blue, destained, and the ~60 kDa band was excised. Samples were subsequently treated as outlined previously [34].

2.4 $^{45}\text{Ca}^{2+}$ Entry

The effect of several compounds on the ATP-promoted P2X₇R-mediated $^{45}\text{Ca}^{2+}$ entry into rat parotid acinar cells was measured using cells suspended in Mg^{2+} -free Solution A containing 1 mM Ca^{2+} , as reported previously [24, 25]. Agents were added ~10 min prior to 300 μ M ATP, which was added ~3 min after $^{45}\text{Ca}^{2+}$. The effects of the agents on the initial rate of ATP-promoted $^{45}\text{Ca}^{2+}$ entry were compared to that of control (vehicle) cells.

2.5 Quantification of protein phosphorylation

The phosphorylation status of proteins visualized on film was quantified by densitometry using the NIH Image J software program. For each sample, the phosphoproteins were normalized to total protein levels to account for gel loading/transfer variations. Blots were probed for phosphoproteins, stripped, and reprobed for total proteins. The phosphorylations for the various conditions were normalized to the phosphorylation under basal control (non-stimulated, no inhibitors) conditions as indicated. In other experiments using the Odyssey Imaging System, blots were simultaneously probed for phosphoproteins and total protein levels using polyclonal antibodies and mouse monoclonal antibodies, respectively, and fluorescent anti-rabbit and anti-mouse antibodies were used for visualization and subsequent quantification, as reported previously [14].

2.6 CDCP1 downregulation

An siRNA directed against the 3'UTR sequence of the CDCP1 mRNA was used to suppress the expression of CDCP1. 27 mer oligonucleotides (MWG-Biotech) were used at 80 nM final concentrations. Transfection was performed using Dharmafect I (Dharmacon) according to the manufacturer's instruction. The siRNA sequence we used was: 5' UAA AGA AGA GGA AUU AUA CAG AAG GAA 3'. As control we used an siRNA matching the CDCP1 mRNA that lead to no knockdown, as determined by Western blotting.

2.7 Data analysis

Values were calculated as the mean $\pm$ S.E. of n number of independent experiments (each n from a different cell preparation or, for cell lines, a different experiment). The differences between the basal/control and the experimental samples were evaluated using a Student's t test. All Western blot experiments were performed at least 3 different times. Representative blots are shown in each figure. For each experiment to be analyzed using Western blotting techniques and/or the Odyssey system, multiple (duplicate or triplicate) cell samples were collected for each condition, and the average of the values obtained within each individual experiment were treated as $n=1$.

3. Results

3.1 Multiple P2R antagonists block P2X₇ receptor signaling

Initially, we examined whether agents used as P2R/P2X₇R antagonists themselves had any effect on basal ERK1/2 phosphorylation in rat parotid acinar cells. To activate the P2X₇R we used the ATP analog BzATP. Although this compound activates the P2X₇R, it also can activate P2X₁, P2Y₁₁ and P2Y₁₃ receptors [1, 2, 35, 36]. However, based on its potency [37] and the P2R population in rat parotid acinar cells (see Introduction), it activates primarily P2X₇Rs in rat parotid acinar cells. BzATP produced a rapid increase in ERK1/2 phosphorylation as noted previously [13, 14]. The P2R antagonists DIDS, suramin, Cibacron Blue 3GA, Brilliant Blue G (all at concentrations between 1–100 μ M), and the P2X₇R-selective agent A438079 (10 μ M) did not promote significant increases in the basal ERK1/2 phosphorylation (Fig. 1A). Except for suramin, all of these compounds were very effective in blocking the BzATP-promoted phosphorylation of ERK1/2 (Figure 1B, C). This is consistent with the BzATP effects on ERK1/2 being mediated via P2X₇R activation, and is also consistent with the effectiveness of these compounds in blocking other P2X₇R-initiated events, such as the entry of Ca²⁺ into rat parotid acinar cells and the subsequent activation of Ca²⁺-sensitive ion channels [24, 38, 39]. Although suramin is usually not considered to be a P2X₇R antagonist, it blocked the ATP-promoted ⁴⁵Ca²⁺ uptake into parotid acinar cells at very high concentrations (>100 μ M) (Figure 1D). Of interest, XAMR0721, a suramin analog, was ineffective at blocking the ATP-promoted ⁴⁵Ca²⁺ uptake at a concentration (1 mM) at which suramin completely blocked the uptake.

3.2 P2R antagonists increase PKC δ and Src phosphorylation in rat parotid acinar cells

We also examined the effects of P2X₇R agonist BzATP and the P2R antagonists on two other signaling proteins in rat parotid acinar cells. BzATP increased the phosphorylation of PKC δ on Tyr311 but did not significantly increase the phosphorylation of Src on Tyr416, its activation site (Figure 2A). In contrast to their lack of effect on basal ERK1/2 phosphorylation, DIDS, suramin, and Cibacron Blue 3GA (all at 100 μ M) produced significant increases in the phosphorylation of Src on Tyr416 and on Tyr311 of PKC δ . Thus, these compounds were precluded from blocking the BzATP-promoted increase in Tyr311-PKC δ , unlike A438079 which itself had no effect on Tyr311-PKC δ and blocked the effects of BzATP. Since BzATP did not significantly increase Y416-Src phosphorylation, the antagonists had similar effects on Src in the absence and presence of the P2X₇R agonist. Brilliant Blue G (10 μ M) had variable weaker effects on Src and PKC δ phosphorylation, and often appeared to block the larger action of BzATP on PKC δ (*e.g.*, Figure 2A).

The stimulatory effects of DIDS and suramin on Src and PKC δ phosphorylation were rapid, occurring within two minutes of exposure (Figure 2B). Cibacron Blue 3GA also produced similarly rapid increases in phosphorylation (not shown). Notably, pretreatment of cells with A438079, a P2X₇R-specific antagonist, did not block the increases in Src and PKC δ phosphorylation by DIDS and suramin, consistent with the effects of these agents being mediated via a P2X₇R-independent mechanism.

DIDS, suramin, and Cibacron Blue 3GA produced concentration-dependent increases in PKC δ and Src phosphorylation between 1–100 μ M (Figure 2C–E). These three agents produced relatively large increases in phosphorylation, while Brilliant Blue G had only modest effects. As noted above, BzATP increased PKC δ phosphorylation but did not increase Src phosphorylation, and A438079 did not affect the tyrosine phosphorylation of either signaling protein.

The data presented in Figures 1 and 2 demonstrate that although most of these antagonists block the P2X₇R receptor-mediated phosphorylation of ERK1/2, they also act independently of this receptor to activate important signaling proteins in rat parotid acinar cells.

3.3 Effects of P2R antagonists on ERK1/2, PKC δ , and Src phosphorylation in salivary cell lines

We also examined the effects of P2R antagonists on three salivary gland cell lines. Par-C10 cells are an SV40-immortalized line of rat parotid acinar cells, and these cells display many physiological properties of native rat parotid acinar cells [33]. HSY and HSG cells are cancer cell lines derived from human parotid and submandibular glands, respectively, and they also have been used in investigations of various salivary gland properties.

DIDS and Brilliant Blue G did not produce significant increases in ERK1/2 phosphorylation in Par-C10 cells (Figure 3A,D), similar to their lack of stimulatory effect on native rat parotid cells (Figure 1A,D); however, Cibacron Blue 3GA and suramin decreased the basal level of ERK1/2 phosphorylation. Quantitatively similar responses to these P2R antagonists were obtained if Par-C10 cells were pre-treated with A438079. In contrast, DIDS, Cibacron Blue 3GA, and Brilliant Blue G produced large increases in ERK1/2 phosphorylation in HSY cells; and these increases were not reduced by pre-treatment with A438079 (Figure 3B,E). DIDS and Brilliant Blue G also produced large increases in ERK1/2 phosphorylation in HSG cells (Figure 3C,F).

Although they did not increase ERK1/2 phosphorylation in Par-C10 cells, DIDS, Cibacron Blue 3GA, Brilliant Blue G, and suramin increased the tyrosine phosphorylation of PKC δ in these cells (Figure 3A). These increases were not reduced if cells were pretreated with the P2X₇R-specific antagonist A438079 (Figure 3A, G), suggesting that the stimulatory action of these agents on PKC δ was independent of the P2X₇R. DIDS and Cibacron Blue 3GA also increased the tyrosine phosphorylation of PKC δ in HSY cells in a P2X₇R-independent manner (Figure 3B, H). DIDS and Cibacron Blue 3GA also produce large increases in Src phosphorylation in both Par-C10 (Figure 3A, I) and HSY cells (Figure B, J).

A summary of the qualitative effects of the P2R antagonists on native rat parotid acinar cells and salivary gland cell lines is shown in Table 1. The P2X₇R-specific antagonist A438079 did not increase the tyrosine phosphorylation of any of the proteins that we examined. In contrast, the compounds that are used as P2X₇R antagonists and inhibitors of other proteins increased the phosphorylations of ERK1/2, Src, and PKC δ in a P2X₇R-independent manner.

3.4 P2R antagonists increase the tyrosine phosphorylation of multiple proteins in cell lines

Since the P2R antagonists produced increases in tyrosine phosphorylation of Src and PKC δ in multiple cells, we examined the changes that the antagonists produced in the overall tyrosine phosphorylation pattern of the three salivary gland cell lines. Initially, we immunoblotted cell lysates using a phosphotyrosine antibody. There were readily detectable increases in tyrosine phosphorylation in Par-C10 and HSY cells at ~135, ~75, and ~60 kDa (Figure 4A). Based on the previous observations, we stripped and reprobed the blot with phospho-specific and protein-specific antibodies to PKC δ and Src, and demonstrated that these proteins co-migrated with the 75 and 60 kDa proteins, respectively. Since we recently observed that CDCP1, a 135 kDa protein, binds to PKC δ and is phosphorylated on Tyr734 by Src [40], we examined whether CDCP1 might be the 135 kDa protein. Therefore, we reprobed the blot with phospho-Tyr734-CDCP1 and total CDCP1 antibodies, and found that CDCP1 migrated at an identical size to the 135 kDa tyrosine phosphorylated band. In addition, when we immunoprecipitated PKC δ using a PKC δ -specific antibody, we found that agents that produced large increases in the tyrosine phosphorylation of CDCP1, PKC δ ,

and Src in Par-C10 cells (Figure 4A) also promoted the association of tyrosine phosphorylated CDCP1 with tyrosine phosphorylated PKC δ (Figure 4B). Notably, suramin and DIDS also promoted the association of tyrosine phosphorylated CDCP1 with PKC δ in PC12 cells (Figure 4C), a rat cell line that is a neuronal cell model. These agents also promoted increases in the tyrosine phosphorylation of PKC δ and Src in PC12 cells (not shown), thus demonstrating that these effects of P2R antagonists are not selective for salivary gland cells (see Discussion).

In Par-C10 cells, Tyr416-Src did not appear to account for the heavily phosphorylated ~60 kDa band in cells exposed to DIDS, Cibacron Blue 3GA, and suramin, since Src appeared to migrate faster than the bulk of the band. To identify other proteins that might contribute to this phosphorylation signal, we used a phospho-tyrosine antibody to isolate phosphorylated proteins of this mass from DIDS-treated Par-C10 cells using SDS-PAGE, and subjected the proteins to identification by mass spectrometry. In one experiment, we identified the 62 kDa protein Dok-1 in the band that we excised, and found that it was phosphorylated on Tyr449. In another experiment, we isolated ~60 kDa proteins using a phosphotyrosine antibody, trypsinized the proteins, and identified the peptides via tandem mass spectrometry. We quantified the number of normalized spectral counts in control and DIDS-treated cells. Peptides from Dok-1, Src, Yes, and HSP7C were present in a larger number in samples from DIDS-treated cells compared to control (untreated) cells (Table 2). Peptides from several other proteins (STIP-1, PSMD3, HNRPK, and Shb) were present in fewer numbers in lysates from DIDS-treated cells compared to untreated cells, and one (PKM) was present in near-equal numbers.

We examined directly the phosphorylation of Dok-1 by immunoprecipitating it from Par-C10 cells using a Dok-1 antibody and immunoblotting the immunoprecipitated protein using a phosphotyrosine antibody. DIDS increased the tyrosine phosphorylation of Dok-1, and the phosphorylated Dok-1 protein aligned with the bulk of the ~60 kDa phosphotyrosine band in cell lysates of DIDS-treated cells (Fig. 5A). As also shown in Figure 4, the two other major phosphorylated bands (~135 kDa and ~75 kDa) in the lysates of DIDS-treated cells co-migrated with Y734-CDCP1 and Y311-PKC δ when the blots were stripped and reprobed with these phospho-specific antibodies (Figure 5B). Dok-1 phosphorylation was also increased in suramin-treated cells but not in cells exposed to the PKC-activator PMA (Fig. 5C), indicating that PKC activation was not upstream of Dok-1 phosphorylation. Although DIDS increases the phosphorylation of both Dok-1 and Src, Dok-1 migrates a little more slowly than Src (Fig. 5D). These results are consistent with observations in which the phospho-Src band did not correspond to the major part of the heavily phosphorylated band in cells treated with P2R antagonists. Although we did not find a Dok-1 antibody that immunoblots Dok-1 in rat Par-C10 cells, these findings suggest that a substantial fraction of the 60 kDa band that increases in tyrosine phosphorylation by DIDS and other antagonists is Dok-1.

3.5 P2R antagonists do not transactivate the EGFR to increase ERK1/2 and Src phosphorylation

We explored several possibilities to explain how the P2R antagonists promoted increases in the phosphorylation of various signaling proteins. These agents act at the extracellular surface of the plasma membrane to block the binding of extracellular nucleotides and receptor ligands, and also bind to various membrane proteins (see Introduction). We hypothesized that the P2R antagonists bound to a transmembrane protein to exert their effects, and we examined if these agents promoted the transactivation of the EGF receptor (EGFR), which in some cells occurs downstream of P2R (P2Y₂R) activation [41]. To evaluate this, we analyzed whether AG1478, an EGFR kinase inhibitor, blocked the phosphorylation of ERK1/2 by these compounds. In previous studies we found that the basal

ERK1/2 phosphorylation and muscarinic receptor-mediated increases in ERK1/2 phosphorylation in these three cell lines are reduced by AG1478 [14]. AG1478 effectively blocked the activation of ERK1/2 by EGF in Par-C10 (Figure 6A), HSG (Figure 6B), and HSY (Figure 6C) cells. In Par-C10 cells, which did not display any enhancement of ERK1/2 by the antagonists, AG1478 significantly reduced the ERK1/2 phosphorylation to below basal levels in all conditions (Figure 6A,D). In HSY cells, inhibition of EGFR partially blocked the increases in ERK1/2 phosphorylation by DIDS and Cibacron Blue 3GA but not by Brilliant Blue G (Figure 6B,E). Inhibition of the EGFR in HSG cells also did not affect the increases in ERK1/2 phosphorylation by Brilliant Blue G, and had a partial inhibitory effect on the stimulation by DIDS (Figure 6C,F). At face value, these results suggest that the EGFR contributes to the stimulation of ERK1/2 phosphorylation by DIDS and Cibacron Blue 3GA but not by Brilliant Blue in HSY and HSG cells. We also examined the sensitivity of Src phosphorylation to EGFR inhibition. The basal and the P2R antagonist-promoted increases in Src phosphorylation were not reduced in Par-C10 and HSY cells treated with AG1478 (Figure 6F). These results suggest that the EGFR is not a global regulator of the changes in phosphorylation that we observe.

3.6 CDCP1 mediates the tyrosine phosphorylation of Src and PKC δ

Since many of the P2R antagonists produced large increases in the tyrosine phosphorylation of PKC δ and Src, we examined if they also were effective at increasing the tyrosine phosphorylation of CUB Domain Containing Protein 1 (CDCP1). Previously, we found that CDCP1, a ~135 kDa protein that is overexpressed in colon cancer cells and is also found in a variety of epithelia, binds to PKC δ via the C2 domain of PKC δ . This association also involves the tyrosine phosphorylation of CDCP1 by Src on Tyr734, which provides a binding site for Src on CDCP1 [40]. In fact, DIDS, Suramin, and Cibacron Blue 3GA increased the tyrosine phosphorylation of CDCP1 on Tyr734 in HSY cells (Figure 7).

Because CDCP1 is a Type I transmembrane protein, we considered the hypothesis that some of the P2R antagonist-initiated cellular signaling events were dependent on CDCP1. Therefore, we examined the effects of several of the antagonists on HSY cells in which we used siRNA to knock down CDPC1. In parallel, we also examined the effects of CUB1, a mouse monoclonal anti-CDCP1 antibody that binds to the extracellular side of CDPC1 and increases its tyrosine phosphorylation and signaling [42]. Not unexpectedly, the intensities of the DIDS-, suramin-, and Cibacron Blue 3GA-promoted increases in phosphorylated CDCP1, as well as those initiated by CUB1, were reduced as a consequence of a diminished level of CDCP1 protein (Figure 7). Of interest, the increases in Src and PKC δ tyrosine phosphorylations by all of these agents were reduced in cells with reduced amounts of CDCP1, consistent with the role of CDCP1 as a molecule critical for Src (and thereby PKC δ) activation in some cells [40, 43]. However, the increases in ERK1/2 phosphorylation by DIDS, suramin, and Cibacron Blue 3GA were not affected by the downregulation of CDCP1, suggesting that these phosphorylations were not dependent on CDCP1, Src, or PKC δ .

4. Discussion

The results presented here demonstrate that various agents used to block multiple types of P2 receptors, including the BzATP-activated P2X₇R in rat parotid acinar cells, produce a variety of effects on native cells and cultured cell lines. In addition to blocking the BzATP-promoted increase in ERK1/2 phosphorylation in native rat parotid acinar cells, DIDS, Cibacron Blue 3GA, and to a lesser extent, Brilliant Blue G rapidly increased the tyrosine phosphorylation of Src and PKC δ . Suramin also increased Src and PKC δ phosphorylation, but was ineffective at blocking the BzATP-promoted ERK1/2 phosphorylation in native cells. These stimulatory effects were not mimicked or blocked by A438079, a P2X₇R-

selective antagonist. Three salivary gland cell lines, one of which (Par-C10) is an immortalized rat parotid acinar line and the others (HSY, HSG) are human salivary gland cancer cell lines, displayed partially overlapping (for Par-C10 and HSY) effects with native rat acinar cells on Src and PKC δ , but also responded uniquely (for HSY and HSG) with an increase in ERK1/2 phosphorylation by several of the P2R antagonists. At a minimum, these findings demonstrate that these antagonists can have cell-specific effects that do not appear to have anything to do with their actions as P2X₇R antagonists, as demonstrated by comparisons with A438079. Since several of these compounds are also used extensively as inhibitors of multiple ion transport proteins and antagonists of other receptors and protein-protein interactions (see Introduction), it is obvious that their multiple P2R-independent actions on cell signaling proteins could easily be improperly attributed to the particular tasks for which they are being employed as inhibitors of other processes. Notably, A438079, a P2X₇R-specific antagonist, did not have readily detectable effects on the phosphorylation of signaling proteins like the other multi-functional compounds.

The P2X₇ receptor is a nonselective ion channel permeable to Na⁺ and Ca²⁺. In parotid acinar cells, this receptor was initially characterized by observing extracellular nucleotide-initiated increases in [Ca²⁺]_i and ⁴⁵Ca²⁺ entry [37, 38]. The entry of ⁴⁵Ca²⁺ is blocked by a variety of P2R antagonists with differing potencies, including DIDS (IC₅₀ = ~ 35 μ M) and Brilliant Blue G (~0.4 μ M) [24, 25]. The relatively low potency (IC₅₀ ~ 250 μ M) of suramin (Figure 1D) in blocking the P2X₇R is consistent with its relative ineffectiveness in blocking the P2X₇R-initiated ERK1/2 phosphorylation at 100 μ M (Figure 1C). Brilliant Blue G is known to be a potent and selective antagonist of the P2X₇R [23], which was why we used it at a concentration (10 μ M) 10-fold less than most of the other blockers that we used in many of these studies.

The comparison of the P2R antagonists on the phosphorylation of multiple signaling proteins in four different cells (Table 1) demonstrated the lack of uniformity of their effect, although one can find some overlapping characteristics in selected cells. For example, DIDS and Cibacron Blue 3GA increased Src and PKC δ phosphorylation in native rat parotid acinar cells, Par-C10 cells, and HSY cells; however, their stimulatory effects on ERK1/2 were lacking in native cells and immortalized Par-C10 cells, but were robust in HSY cells. Although it is beyond the scope of this study, one reason for the differences between the effects of these agents on ERK1/2 phosphorylation in different cells might be due to the selective expression or activation of Dok-1 in native cells and Par-C10 cells. Dok-1 is phosphorylated by Src on multiple sites including Tyr449 [44], which we observed in DIDS-treated Par-C10 cells; and phosphorylated Dok-1 reduces ERK1/2 activation, in part by acting on Ras-GAP [45].

The rapidity with which signals are transduced in response to P2R antagonists along with their general membrane impermeability suggests that they bind rapidly to proteins at the plasma membrane to initiate the changes. Based on our studies of Src phosphorylation, we eliminated transactivation of the EGFR as a contributor to these effects. Since the only proteins that are known to bind all four of these compounds in common are P2Rs, these receptors are logical candidates to mediate these effects, and the fact that A438079 did not produce similar increases in phosphorylation and did not block effects of other antagonists could simply indicate that the P2X₇R can be excluded from mediating these effects. However, it seems unlikely that overlapping signals initiated by P2R agonists and antagonists are both mediated via P2Rs.

CDCP1 is a likely candidate for the plasma membrane mediator of at least some of the signaling effects of the antagonists. It is unknown whether DIDS, suramin, and Cibacron Blue 3GA can bind directly to the extracellular domain of CDCP1, which makes up the bulk

of the protein. The extracellular domain of CDCP1 does not have the consensus DIDS binding motif (Lys-X-X-Lys) found in anion exchangers [46], but there is evidence for DIDS binding to multiple other sequences, such as those found in the immunoglobulin-like domain of CD4 [47]. There are probably at least two pathways leading to the activation of signaling proteins, based on our observations that CDCP1 downregulation blocked Src and PKC δ but not ERK1/2 phosphorylation in cells exposed to P2R antagonists, and CUB1 increased CDCP1, PKC δ , and Src phosphorylation, but not ERK1/2 phosphorylation. In addition, these phosphorylations may involve an activation pathway different from that involved in the phosphorylation of Dok-1 and other proteins. It is likely that most of these phosphorylation events are P2R-independent events.

Brown et al. [48] reported that suramin, other P2R antagonists (PPADS, NF023), and Trypan blue increased the tyrosine phosphorylation of CDCP1, similar to our observations with suramin, DIDS, Cibacron Blue 3GA, and Brilliant Blue G. A common structural feature of all of these agents is that they are polysulfonated compounds. These investigators reported (as data not shown) that XAMR0721, a nonsymmetrical suramin analog, was ineffective at increasing CDCP1 phosphorylation; and other investigators found that suramin, but not XAMR0721, increased the tyrosine phosphorylation and dimerization of the EGFR [49]. Analogous to these observations, we observed that XAMR0721 did not mimic the inhibitory effect of suramin on the ATP-promoted entry of $^{45}\text{Ca}^{2+}$ into rat parotid acinar cells. These results are consistent with the hypothesis that polysulfonated compounds with defined structural characteristics can interact with a plasma membrane receptor/protein to promote the tyrosine phosphorylation of Src, PKC δ , and CDCP1. We cannot yet conclude that DIDS, suramin, Cibacron Blue 3GA, or Brilliant Blue bind directly to CDCP1 or whether they bind to a component of the plasma membrane, including a receptor tyrosine phosphatase that may play a role in the rapid phosphorylation changes.

The agents in the present study have been used successfully in early studies as well as contemporary studies to block P2Rs and thereby demonstrate their involvement in biological events; additionally, some of these compounds are currently used to block other proteins. The studies presented here demonstrate that some of these blockers promote diverse effects that can confound conclusions about the involvement of P2 receptors in biology. However, some of these compounds may be useful experimental tools to increase the phosphorylation of particular signaling proteins. For example, although an increase in CDCP1 phosphorylation on tyrosine residues can be achieved with great specificity by a monoclonal antibody to the extracellular domain of human CDCP1 (see Fig. 7), this antibody does not recognize CDCP1 in rodent species, and thus cannot be used to activate CDCP1 in rodent tissues. Cibacron Blue 3GA, DIDS, and suramin are able to do this, and under some circumstances, this may provide an alternative approach to examining signaling mediated by CDCP1 and/or its activation by extracellular ligands.

Acknowledgments

We thank Dr. John Asara for advice and excellent assistance with the mass spectrometry experiments. This work was supported by National Institutes of Health Grant DE-10877 (SPS).

Abbreviations: The abbreviations used are

DIDS	4,4'-diisothiocyano stilbene-2,2'-disulfonic acid
CDCP1	CUB Domain Containing Protein 1
BzATP	2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate

P2R	purinergic 2 receptor
PMA	phorbol-12-myristate-13-acetate
ERK	extracellular signal-related kinase
[Ca²⁺]_i	intracellular Ca ²⁺ concentration

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Figure 1. Inhibition of P2X₇R-initiated ERK1/2 phosphorylation and ⁴⁵Ca²⁺ entry in rat parotid acinar cells by P2R antagonists

A. Quantification of ERK1/2 phosphorylation in cells exposed to various concentrations (1–100 μ M) of DIDS, suramin (Sur), Cibacron Blue 3GA (CB), and Brilliant Blue G (BBG) for 5 min; to A438079 (10 μ M) for 2–10 min; and to BzATP (10 μ M) for 2 min. N=3–4.

*P<0.05 versus basal. B. Western blot analysis of cells pretreated for 10 min with A438079 (A, 10 μ M), DIDS (D, 100 μ M), Cibacron Blue 3GA (CB, 100 μ M), Brilliant Blue G (BB, 10 μ M) and suramin (Sur, 100 μ M), followed by BzATP (10 μ M) or vehicle for 2 min. Cell lysates were subjected to SDS-PAGE and immunoblotted as indicated. C. Quantification of changes in ERK1/2 phosphorylation relative to basal control (no additions). N=4–5. *P<0.01

versus basal. D. The rate of ATP (300 μ M)-stimulated $^{45}\text{Ca}^{2+}$ entry into suramin- and XAMR0721-treated cells was compared to the control (no inhibitor) entry rate (6.48 ± 0.65 nmol Ca^{2+} /mg/min, n=6). *P<0.01, **P<0.05 *versus* control. N=3–6.

Figure 2. Effects of P2R antagonists on Src, PKCδ, and ERK1/2 phosphorylations in rat parotid acinar cells

A. Western blot analysis of cells pretreated for 10 min with A438079 (10 μM), DIDS (100 μM), Cibacron Blue 3GA (100 μM), Brilliant Blue G (10 μM) and suramin (100 μM), followed by BzATP (10 μM) or vehicle for 2 min. B. Western blot analysis of cells pretreated for 10 min with A438079 (10 μM) or vehicle, and subsequently treated with DIDS (100 μM) and suramin (100 μM) for 2 and 5 min. C. Western blot analysis of cells treated with DIDS, suramin, and Cibacron Blue 3GA for 5 min at 1–100 μM, and PMA (100 nM, 2 min). D,E. Quantifications of changes in phosphorylation of Tyr416-Src (D) and Tyr311-PKCδ (E) relative to basal (no additions) for cells treated with DIDS, Cibacron Blue 3GA, Brilliant Blue G, and suramin (all 1–100 μM, 5 min), A438079 (10 μM, 5,10 min), and BzATP (10 μM, 2 min). N=3–5. *P<0.01, **P<0.05 *versus* basal.

Figure 3. Effects of P2R antagonists on ERK1/2, PKCδ, and Src phosphorylation in salivary gland cell lines

A–C. Western blot analysis of Par-C10 (A), HSY (B), and HSG (C) cells exposed to DIDS (100 μ M, 5 min), Cibacron Blue 3GA (100 μ M, 5 min), Brilliant Blue G (10 μ M, 5 min), and suramin (100 μ M, 5 min). Where indicated, cells were pretreated for 30 min with A438079 (10 μ M) or vehicle. D–F. Quantification of changes in ERK1/2 phosphorylation relative to basal (no additions) for Par-10 (D, N=4–5), HSY (E, N=4–5), and HSG (F, N=3–5) cells treated as in A–C. G,H. Quantification of changes in Tyr311-PKCδ phosphorylation relative to basal (no additions) in Par-C10 (G, N=3) and HSY (H, N=3) cells treated as described in A and B. I,J. Quantification of changes in phosphorylation of Tyr416-Src in

Par-C10 (I, N=7–8) and HSY (J, N=6–7) cells relative to basal (no additions) for cells treated as in A and B. *P<0.01, **P<0.05 *versus* basal (no addition).

Figure 4. Changes in tyrosine phosphorylation of Par-C10, HSY, HSG, and PC12 cells promoted by P2R antagonists

A. Western blot analysis of cells treated for 5 min with DIDS (100 μ M), Cibacron Blue 3GA (100 μ M), Brilliant Blue G (10 μ M), and suramin (100 μ M). Cell lysates were subjected to SDS-PAGE and immunoblotted with a phosphotyrosine antibody. Asterisks designate major increases in tyrosine phosphorylation in the lysates. The blots were stripped and reprobed as indicated to identify the phosphorylated proteins marked by the asterisks (top, CDCP1; middle, PKC δ ; bottom, Src). B. Par-C10 cells were exposed P2R antagonists as in A, and PKC δ was immunoprecipitated from cell lysates using an anti-PKC δ antibody. The immunoprecipitated proteins were subjected to SDS-PAGE and immunoblotted as indicated. The blot was reprobed to demonstrate that the higher and lower tyrosine phosphorylated proteins are CDCP1 and PKC δ , respectively. C. PC12 cells were exposed to DIDS and suramin as in A, and CDCP1 and PKC δ were immunoprecipitated from cell lysates and immunoblotted as indicated.

Figure 5. DIDS and suramin increase the tyrosine phosphorylation of Dok-1 and Src in Par-C10 cells

A. Western blot analysis of cells treated with DIDS (100 μ M, 5 min) or vehicle, after which Dok-1 was immunoprecipitated using an anti-Dok-1 antibody. The immunoprecipitated proteins and cell lysate samples were subjected to SDS-PAGE, and immunoblotted using a phosphotyrosine antibody. Asterisks designate the major increases in phosphorylation in the lysates. B. The immunoblot in A was stripped and reprobed as indicated. The top two asterisks in A co-migrate with tyrosine phosphorylated CDCP1 and PKC δ , respectively. C. Cells were treated with PMA (100 nM, 5 min), DIDS (100 μ M, 5 min), and suramin (100 μ M, 5 min). Dok-1 was immunoprecipitated from the lysates using a Dok-1 antibody, and the immunoprecipitated proteins and a lysate sample were subjected to SDS-PAGE and immunoblotted with phosphotyrosine antibody. D. Src and Dok-1 were immunoprecipitated from lysates of cells treated with DIDS (100 μ M, 5 min) or vehicle, subjected to SDS-PAGE, and immunoblotted as indicated. Note that the Dok-1 protein migrated more slowly than Src.

Figure 6. Effect of EGFR inhibition on ERK1/2 and Src phosphorylation in salivary gland cell lines exposed to P2R antagonists

Cells were exposed to vehicle or the EGFR inhibitor AG1478 (300 nM, 30 min), and then treated with EGF (100 ng/ml, 5 min), DIDS (100 μ M, 5 min), Cibacron Blue 3GA (100 μ M, 5 min), Brilliant Blue G (10 μ M, 5 min), and suramin (100 μ M, 5 min). Par-C10 (A), HSY (B), and HSG (C) cell lysates were subjected to SDS-PAGE and immunoblotted with the indicated antibodies. Changes in ERK1/2 phosphorylation were quantified relative to basal control (no AG1478) for Par-C10 cells (D; N=4–6), HSY cells (E, N=4–6), and HSG cells (F, N=4–8). In G, changes in Src phosphorylation were quantified relative to basal control (no AG1478) for Par-C10 (N=3) and HSY cells (N=3). * $P < 0.01$, ** $P < 0.05$ versus basal (no addition). # $P < 0.01$, \$ $P < 0.05$ +AG1478 versus –AG1478.

Figure 7. Effect of CDCP1 downregulation on P2R antagonist- and CUB1-initiated signaling in HSY cells

CDCP1 was downregulated using siRNA techniques, and cells were treated with DIDS (100 μ M, 5 min), suramin (100 μ M, 5 min), Cibacron Blue 3GA (100 μ M, 5 min), and CUB1 mAb (0.5 mg/ml, 10 min). Lysates were subjected to SDS-PAGE and immunoblotted as indicated. Film exposures of different lengths of time (identified as short and long) are shown to evaluate the effects of CDCP1 downregulation on the phosphorylation of PKC δ , Src, and ERK1/2.

Table 1

Effects of P2R antagonists on ERK, Src, and PKC δ phosphorylation in rat parotid acinar cells and salivary cell lines.

		ERK	Src	PKC
Rat Parotid	BzATP	+	NC	+
	DIDS	NC	+	+
	Cibacron Blue	NC	+	+
	Brilliant Blue G	NC	+	+
	Suramin	NC	+	+
	A438079	NC	NC	NC
Par-C10	DIDS	NC	+	+
	Cibacron Blue	–	+	+
	Brilliant Blue G	NC	+	+
	Suramin	–	+	+
	A438079	NC		NC
HSY	DIDS	+	+	+
	Cibacron Blue	+	+	+
	Brilliant Blue G	+	+	NC
	Suramin	NC	NC	NC
	A438079	NC		NC
HSG	DIDS	+		
	Cibacron Blue	NC		
	Brilliant Blue G	+		
	Suramin	NC		

+, Increase above basal

–, Decrease below basal

NC, No significant change from basal

Table 2

Total number of peptide spectra per protein identified

Protein	Number of Phosphotyrosine Peptides	
	Basal	DIDS
STIP-1	22	9
Dok-1	9	22
HNRPK	18	7
HSP7C	3	12
PSMD3	10	2
PKM	14	16
Src	1	5
Yes	1	3
Shb	4	1

Stress-induced phosphoproteins-1 (STIP-1), Docking protein-1 (Dok-1), Heterogeneous nuclear ribonucleoprotein K (HNRPK), heat-shock cognate 71 kDa protein (HSP7C), 26S proteasome non-ATPase regulatory subunit 3 (PSMD3), Pyruvate kinase isozymes M1/M2 (PKM), Src protein tyrosine kinase (Src), Src homology 2 domain-containing adapter protein B (Shb)