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Blockade of ATP binding site of P_2 purinoceptors in rat parotid acinar cells by isothiocyanate compounds*

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Abstract—Extracellular ATP activates a P_{2Z}-type purinergic receptor (purinoceptor) in rat parotid acinar cells that increases the intracellular free Ca^{2+} concentration via the entry of extracellular Ca^{2+} through an ATP-sensitive cation channel (Soltoff *et al.*, *Am J Physiol* **262**: C934–C940, 1992). To learn more about the ATP binding site of the purinoceptor, we examined the effects of several stilbene isothiocyanate analogs of DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid), which block the binding of [^{32}P]ATP to intact parotid cells (McMillian *et al.*, *Biochem J* **255**: 291–300, 1988) and blocked the activation of the P_{2Z} purinoceptor. The ATP-stimulated $^{45}Ca^{2+}$ uptake was blocked by DIDS, H_2DIDS (dihydro-DIDS; 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonic acid), and SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid), but not by DNDS (4,4′-dinitrostilbene-2,2′-disulfonic acid), a stilbene disulfonate compound lacking isothiocyanate (SCN-) groups, or by KSCN. The potency of the stilbene disulfonates was related to the number of isothiocyanate groups on each compound. Under the experimental conditions, the IC_{50} value of DIDS (~35 μM), which has two SCN- groups, was much lower than that of SITS (~125 μM), which has only one SCN- group. The inhibitory effects of DIDS appeared to be much more potent than those of SITS due to the kinetics of their binding to the purinoceptors. Eosin-5-isothiocyanate (EITC) and fluoroscein-5-isothiocyanate (FITC), non-stilbene isothiocyanate compounds with single SCN- groups, also blocked the response to ATP and were less potent than DIDS. Trinitrophenyl-ATP (TNP-ATP), an ATP derivative that is not an effective agonist of the parotid P_{2Z} receptor, blocked the covalent binding of DIDS to the plasma membrane, suggesting that ATP and DIDS bind to the same site. Reactive Blue 2 (Cibacron Blue 3GA), an anthraquinone-sulfonic acid derivative that is a noncovalent purinergic antagonist, also blocked the covalent binding of DIDS to the plasma membrane. These results suggest that isothiocyanate compounds interact with the ATP binding site of this P_2 purinoceptor, and that isothiocyanate groups make an important contribution in determining the effectiveness of the stilbene disulfonate compounds in blocking the binding of nucleotide agonists to this purinoceptor.

Extracellular nucleotides stimulate multiple physiological and biochemical processes in a variety of cells [1]. These effects are mediated by binding of nucleotides to purinoceptors on the plasma membrane. These receptors can be classified into subtypes based on the order of agonist potency [2, 3] and the types of effects that are initiated by the nucleotides [1]. Previously we reported that ATP activated a P_{2Z}-type purinoceptor, which is sensitive to ATP^{4-}, on rat parotid acinar cells [4–6]. ATP activates a non-selective cation channel that allows the entry of extracellular Ca^{2+} [7]. This produces a rapid elevation of the cytosolic free calcium concentration ($[Ca^{2+}]_i$), which in turn activates various Ca^{2+}-sensitive ion channels that are involved in fluid secretion and the initiation of saliva formation [5]. Many of the effects of ATP on the rat parotid cell are similar to those of the muscarinic agonist carbachol [5, 6], although the mechanisms of action of these two agents are different [4, 5]. Gallacher demonstrated that ATP produces effects similar to those of neurotransmitters on mouse parotid acinar cells [8], and recently Sasaki and Gallacher characterized the electrophysiological aspects of the ATP-gated nonselective cation channel in mouse lacrimal cells [9]. Thus, studies of exocrine cells, as well as many other cell types, suggest that ATP, which is costored and cosecreted with other neurotransmitters [10, 11], itself acts as a neurotransmitter.

In studies directed at identifying and characterizing these receptors, we found that the effects of ATP on $[Ca^{2+}]_i$ and Ca^{2+}-sensitive ion transport systems are blocked by the isothiocyanate stilbene disulfonate DIDS† [4, 5], while the response to muscarinic agonist is unaffected. DIDS reduces the binding of [^{32}P]ATP to intact parotid cells [4]. Reactive Blue 2 (Cibacron Blue 3A) also blocks the binding of ATP [12] as well as inhibiting the stimulation by ATP of Ca^{2+} entry and the elevation of $[Ca^{2+}]_i$ in parotid cells [13]. In the present paper, we examined structural aspects of stilbene and isothiocyanate compounds that are critical to their abilities to inhibit ATP binding, as judged by their abilities to block the activation of the ATP-gated nonselective cation channel. We also demonstrated that a noncovalent ATP analog can block the binding of DIDS, and that Reactive Blue 2, an anthraquinone-sulfonic acid derivative, can interact at the DIDS/ATP binding site. Our data indicate that isothiocyanate groups are critical in establishing the effectiveness of stilbene disulfonate compounds in blocking the binding of ATP to a P_{2Z} receptor on the parotid acinar cell and thus in preventing the subsequent activation of the cellular events initiated by this receptor.

Materials and Methods

Reactive Blue 2 (Cibacron Blue 3GA) was obtained from Sigma; DIDS, dihydro-DIDS (H_2DIDS), 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), eosin-5-isothiocyanate (EITC), and trinitrophenyl-ATP (TNP-ATP) were from Molecular Probes; and 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) and fluoroscein-5-isothiocyanate (FITC) were from the Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were reagent

* A preliminary report of this work has been published in abstract form (Soltoff *et al.*, *Ann NY Acad Sci* **603**: 445, 1990).

† Abbreviations: DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; H_2DIDS, 4,4′-diisothiocyanato-dihydrostilbene-2,2′-disulfonic acid; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; SCN, isothiocyanate; FITC, fluoroscein-5-isothiocyanate; EITC, eosin-5-isothiocyanate; and TNP-ATP, 2′-(or-3′)-*O*-(trinitrophenyl)ATP.

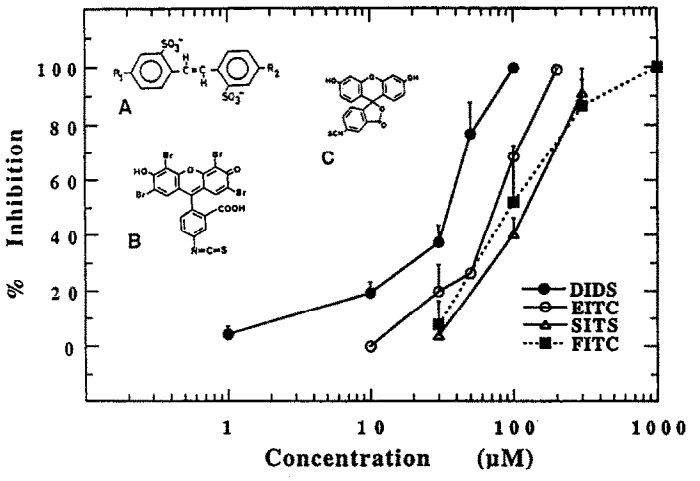

Fig. 1. Concentration dependence of DIDS, SITS, EITC, and FITC in inhibiting the ATP-stimulated uptake of $^{45}Ca^{2+}$ into rat parotid acinar cells. Cells were exposed to these inhibitors for 10–20 min before ATP (300 μM) was added. The uptake rates measured in the presence of these inhibitors were normalized to the rates measured in the absence of inhibitors. Values are the means ± SEM for 3–5 separate experiments, except as follows: EITC: 10 μM (2), 50 μM (2); FITC: 30 μM (1), 1000 μM (2). Inset: (A) Structures of the stilbene disulfonate compounds. DIDS: R_1 = R_2 = (—N=C=S); SITS: R_1 = (—N=C=S), R_2 = (CH_3CONH—); DNDS: R_1 = R_2 = ($—NO_2$). H_2DIDS has the same structure as DIDS, except that the C=C double bond is reduced. (B) Structure of EITC. (C) Structure of FITC.

grade or better. All isothiocyanate compounds and TNP-ATP were dissolved in dimethyl sulfoxide.

Rat parotid acinar cells were prepared as described previously [4]. Cells were suspended in a solution of the following composition, in mM: NaCl, 116.4; KCl, 5.4; NaH_2PO_4, 1; sodium HEPES, 25; $CaCl_2$, 1; β-hydroxybutyrate, 5; glucose, 5.6; pH 7.4.

$^{45}Ca^{2+}$ uptake measurements were performed on rat parotid acinar cells in suspension at 37° as previously described [5, 7]. Unless specified otherwise, stilbene and other compounds were added to the suspensions 10–20 min prior to the addition of 300 μM ATP.

In experiments in which the ability of Reactive Blue 2 to block DIDS binding was examined, cells were incubated at 37° for 15 min (t = 0 to t = 15). Some cells were exposed to DIDS (40 μM) for 5 min (from t = 10 to t = 15), or Reactive Blue 2 (200 μM) for 10 min (from t = 5 to t = 15). Other cells were exposed to Reactive Blue for 10 min (from t = 5 to t = 15) with DIDS added for 5 min (from t = 10 to t = 15). Control cells were not exposed to either agent. The cells were washed and resuspended, and the ATP (300 μM)-stimulated initial entry rate of $^{45}Ca^{2+}$ was measured. A similar protocol was employed using TNP-ATP (1000 μM) in place of Reactive Blue 2, except that a DIDS concentration of 100 μM was used.

Values are given as means ± SEM. The number of determinations (N) represents separate cell preparations. In each preparation single or duplicate samples were collected for each condition.

Results

Isothiocyanate compounds—purinergic antagonists. Since previous studies indicated that DIDS blocks the effects of ATP by blocking the binding of [^{32}P]ATP to intact parotid cells [4], we examined the inhibitory effect of DIDS and related stilbene disulfonate compounds in more detail. To evaluate the specificity of the DIDS binding site, the abilities of several structurally related stilbene disulfonate compounds to block ATP-stimulated $^{45}Ca^{2+}$ entry were examined (Fig. 1). DIDS, which has two SCN- groups, and SITS, a stilbene disulfonate that has only one SCN- group, both blocked the effects of ATP, but the potency of DIDS (IC_{50} ~ 35 μM) was about 3 times greater than that of SITS (~125 μM). H_2DIDS (100 μM), the reduced form of DIDS, was nearly as effective as DIDS, and blocked the ATP-stimulated $^{45}Ca^{2+}$ entry by 93.9 ± 4.4% (N = 3). DNDS (1 mM), a stilbene disulfonate compound in which $-NO_2$ groups are substituted for SCN- groups, was completely ineffective (102.7 ± 7.1% control, N = 4), as was exposure of the cells to simple isothiocyanate groups in the form of KSCN (1 mM) (104.0 ± 7.3% control, N =

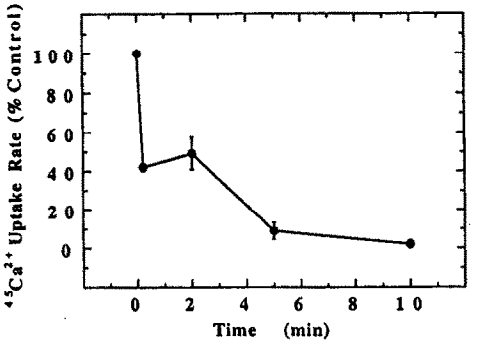

Fig. 2. Time course of inhibition by DIDS of the ATP-stimulated rate of $^{45}Ca^{2+}$ uptake. Cells were suspended at 37° and exposed to DIDS (100 μM) for 10 sec, or 2, 5, or 10 min prior to the addition of ATP (300 μM). The rates were normalized to the ATP-stimulated uptake measured in the absence of DIDS. Values are means ± SEM, N = 3–5.

4). The contrasting effects of DIDS, SITS, and DNDS indicate that the SCN- groups play an important role in determining the effectiveness of the stilbene disulfonate compounds in blocking ATP binding and preventing the activation of the cation entry pathway.

Under these conditions (10- to 15-min exposure at 37°), the effects of DIDS were not reversible by washing the cells, indicating that DIDS was a covalent ligand [4]. Since isothiocyanate compounds have been reported to be covalent inhibitors of a number of ATP binding sites, including that of the Na^+,K^+-ATPase [14], we examined whether the relatively lower potency of SITS was due to the kinetics of its binding to the P_{2Z} purinoceptor. When cells were exposed to 100 μM SITS for 60 min, instead of for 10–15 min as in Fig. 1 (in which SITS inhibited by 59.7 ± 6.0%, N = 3), SITS inhibited the ATP-stimulated $^{45}Ca^{2+}$ uptake rate by 94.8 ± 3.4% (N = 3). This degree of inhibition was comparable to the effect of a much shorter exposure (10–15 min) to 100 μM DIDS, which produced an inhibition of 99.8 ± 0.7% (N = 4) (Fig. 1).

The time course of DIDS inhibition was also examined. In these experiments, cells were exposed to 100 μM DIDS at 37° for various lengths of time up to 10 min, and then the ATP (300 μM)-stimulated rate of $^{45}Ca^{2+}$ entry was measured in the presence of DIDS (Fig. 2). The ATP-stimulated $^{45}Ca^{2+}$ uptake rate was diminished by 50–60% when DIDS was added either 10 sec or 2 min prior to ATP, and it was blocked nearly completely after a 5-min exposure.

To examine whether isothiocyanate compounds other than stilbene disulfonates antagonized ATP action, two monoisothiocyanate compounds, EITC and FITC, were examined for their effects on ATP-stimulated $^{45}Ca^{2+}$ entry. EITC inhibited with an IC_{50} of ~70 μM (Fig. 1), and was about half as potent as DIDS. A concentration of 200 μM EITC blocked the ATP-stimulated $^{45}Ca^{2+}$ entry nearly completely. FITC inhibited with an IC_{50} value of ~100 μM, and 300 μM blocked the stimulated entry by 87% (Fig. 1). Thus, both stilbene and nonstilbene isothiocyanate compounds inhibited the stimulatory effect of ATP on $^{45}Ca^{2+}$ uptake.

Protection of the DIDS/ATP binding site by an ATP analog. To determine whether DIDS and ATP bound to the same site on the purinoceptor, we investigated the ability of a purinergic analog to block the covalent binding of DIDS. In preliminary experiments it was observed that it was difficult to fully reverse the stimulatory effects of a pre-exposure to ATP; therefore, an ineffective ATP analog, TNP-ATP, was used to compete with DIDS in place of ATP. TNP-ATP did not cause a measurable stimulation of $^{45}Ca^{2+}$ entry (data not shown), and blocked the effect of ATP; the stimulation of $^{45}Ca^{2+}$ entry promoted by ATP (300 μM) was reduced by 96.8% (N = 2) in the presence of TNP-ATP (300 μM, added 3 min prior to ATP). Cells were exposed to DIDS in the presence of TNP-ATP (added 5 min before DIDS) and washed, and the effectiveness of DIDS inhibition was evaluated by measuring the ATP-stimulated entry of $^{45}Ca^{2+}$. In cells that were pre-exposed to both TNP-ATP and DIDS (see Materials and Methods), the irreversible inhibitory effect of DIDS on the ATP-stimulated $^{45}Ca^{2+}$ uptake was diminished by 69% (Table 1A), suggesting that DIDS and ATP acted at the same site.

In these experiments DIDS was removed from the cells by washing after a 5-min exposure. The level of inhibition by 100 μM DIDS (56%, Table 1A) under these conditions was less than that observed (91%, Fig. 2) when ATP was added to cells in the continued presence of DIDS after an equivalent period of exposure. Cells washed after exposure to DIDS for an even shorter time (10–120 sec) recovered an even greater portion of their response to ATP (data not shown), indicating that the extent of the reversibility of DIDS inhibition was dependent in an inverse manner to the time of exposure to DIDS. This indicates that DIDS can inhibit both noncovalently and covalently, with the formation of covalent bonds requiring a longer time. This may also have been true for H_2DIDS. Inhibition of the ATP (300 μM)-stimulated $^{45}Ca^{2+}$ uptake by 100 μM H_2DIDS was complete (100%) when ATP was added in its presence after a 15-min exposure period, but the inhibition was somewhat less (84.4%, N = 2) if cells were washed after a 15-min exposure to H_2DIDS.

Protection of the DIDS/ATP binding site by Reactive Blue 2. Since Reactive Blue 2 also blocks the effects of ATP [13], we examined the possibility that the same site was involved in inhibition by Reactive Blue 2 and DIDS by investigating the interaction between the two compounds. The inhibitory effects of a maximally effective concentration (200 μM) of Reactive Blue 2 were fully reversible by washing the cells (Table 1B). In contrast, a submaximal concentration of DIDS (40 μM) produced irreversible inhibition of ATP-stimulated $^{45}Ca^{2+}$ uptake. However, when cells were exposed to DIDS in the presence of Reactive Blue 2 (see Materials and Methods), the irreversible inhibitory effect of DIDS was reduced by 66% (Table 1B), illustrating the protective effect of Reactive Blue 2 on the covalent modification of the purinoceptor binding site by DIDS.

Discussion

DIDS blocked ATP-stimulated $^{45}Ca^{2+}$ entry into rat parotid acinar cells, consistent with our previous findings that DIDS blocks the ATP-stimulated elevation of $[Ca^{2+}]_i$ [4] and blocks the activation by ATP of Ca^{2+}-sensitive ion channels and ion fluxes [5, 6]. In those studies, we showed that DIDS acts selectively on the parotid P_{2Z} purinoceptors, and does not block the activation of phospholipase C-linked (muscarinic, α-adrenergic, or substance P) receptors. The response mediated by phospholipase C-linked receptors consisted of an initial transient $[Ca^{2+}]_i$ elevation, which was due to the release of Ca^{2+} from intracellular stores, and a sustained $[Ca^{2+}]_i$ elevation which was due to the influx of Ca^{2+} across the plasma membrane. DIDS did not affect either of these phases of the responses to the muscarinic agonist carbachol. Thus, the effect of DIDS on the ATP-stimulated entry of Ca^{2+} was not due to a general inhibitory effect on Ca^{2+} entry, consistent with DIDS acting at the initial ATP binding step. DIDS also was effective in blocking the activation of $^{45}Ca^{2+}$ entry by other nucleotide analogs, including 3-*O*-(4′-benzoyl)benzoyl ATP (BzATP) (data not shown), which is the most potent and effective P_{2Z} agonist of the rat parotid acinar cell [7]. In other types of cells that appear to express the P_{2Z} subtype, DIDS also blocks the effects of ATP. The ATP-promoted stimulation of $^{45}Ca^{2+}$ entry into Friend erythroleukemia cells is blocked by LIDS [15], and DIDS diminished the stimulation by ATP of Ca^{2+}-sensitive K^+ efflux from a lymphocyte hybridoma cell line (Soltoff SP, unpublished results).

The reduction of the inhibitory effect of DIDS produced by exposure to TNP-ATP (Table 1A) suggests that DIDS binds to the ATP binding site of the P_{2Z} purinoceptor. In accord with the DIDS-promoted reduction in [^{32}P]ATP binding [4], this indicates that the mechanism of DIDS inhibition is at the step of receptor binding. Thus, DIDS functions as a purinoceptor antagonist.

Another compound that has been utilized as a purinergic antagonist to block P_2-type purinoceptors in other tissues is Reactive Blue 2 [2], which blocks the binding of nucleotides to a number of proteins [16]. Since Reactive Blue 2 [13] and DIDS (Fig. 1) both blocked the ATP-stimulated entry of $^{45}Ca^{2+}$ into the parotid cell and both reduced the binding of [^{32}P]ATP to intact parotid cells [4, 12], we postulated that they might interact at the same ATP-binding site on the parotid cell membrane. Pretreatment of the cells with Reactive Blue 2, a reversible blocker of the response to ATP, substantially blunted the covalent reaction of DIDS with the plasma membrane

Table 1. Reduction of the covalent binding of DIDS to the P_{2Z} purinoceptor by an ATP analog and Reactive Blue 2

	% Inhibition of $^{45}Ca^{2+}$ uptake rate
(A) Protection by TNP-ATP*	
TNP-ATP	2.7 ± 4.0
DIDS	55.6 ± 2.2
TNP-ATP + DIDS	17.4 ± 5.0
(B) Protection by Reactive Blue 2†	
Reactive Blue 2	4.7 ± 5.9
DIDS	43.9 ± 8.3
Reactive Blue 2 + DIDS	15.0 ± 0.6

Values are means ± SEM, N = 3.

* Parotid acinar cells were exposed to TNP-ATP (1000 μM), DIDS (100 μM) both, or neither (no additions) (see Materials and Methods). After the cells were washed, the ATP (300 μM)-stimulated $^{45}Ca^{2+}$ entry rate was measured in the absence of the added compounds. These rates were normalized to that measured in cells not pre-exposed to any of these compounds.

† Parotid acinar cells were exposed to Reactive Blue 2 (200 μM), DIDS (40 μM), both, or neither (no additions). After the cells were washed, the ATP (300 μM)-stimulated $^{45}Ca^{2+}$ entry rate was measured in the absence of inhibitors. These rates were normalized to that measured in cells not exposed to any of these compounds.

(Table 1B). These results suggest that Reactive Blue 2 binds to the DIDS/ATP binding site on the parotid P_{2Z} purinoceptor.

Our studies demonstrate that DIDS can irreversibly inhibit the effect of ATP (Table 1), although some of the inhibition was reversible if sufficient time was not allowed for the completion of covalent modification of the purinoceptor (see Results). Various studies have shown that DIDS reacts covalently with proteins. DIDS has been used to inhibit several Cl^- transport proteins, including Cl^- channels and the Cl^-/HCO_3^- exchanger (Band 3). The isothiocyanate groups of DIDS and H_2DIDS covalently bind to lysine residues on the anion exchanger in red blood cells [17]. EITC also covalently binds to and blocks the anion exchanger [18]. Other studies demonstrated that isothiocyanate stilbenes may prevent the binding of ATP to proteins, including the Na^+,K^+-ATPase [14].

In addition to establishing compounds useful for characterizing the effects mediated by purinoceptors, the identification of specific ligands will be extremely helpful in the quantification, isolation, and purification of these ATP- and DIDS-binding receptors. Purinoceptors have not been well quantified, in part due to the lack of appropriate ligands. The number of DIDS-sensitive ATP binding sites on parotid cells was 1.2 pmol/mg total cell protein, estimated at 0.46 pmol/10^6 cells or about 2.8×10^5 sites/cell [6]. This value is likely to be an upper estimate of the purinoceptor density on parotid cells. Other investigators have reported higher numbers of purinoceptors on other cells. Keppens and De Wulf [19] examined ATP binding to hepatocyte purinoceptors using [^{35}S]ATP and found 5 pmol/10^6 cells (3×10^6 sites/cell). Cooper *et al.* [20] found that [^{35}S]ADP-β-S bound with high affinity to the putative P_{2Y} receptor on turkey erythrocyte membranes at 2–4 pmol/mg purified plasma membrane protein.

The ability of DIDS to inhibit the stimulation by ATP of $^{45}Ca^{2+}$ influx into parotid cells (this paper) and to reduce [^{32}P]ATP binding to intact parotid cells [4] is further evidence that ATP activates a purinoceptor-operated cation channel in parotid acinar cells [7]. Our data do not allow us to distinguish a model in which the DIDS-sensitive ATP binding site is also part of the channel, analogous to ACh binding to the nicotinic ACh receptor/channel, from a model in which the ATP binding site is separate from the channel. Since the effects of ATP on parotid cells do not appear to be mediated by GTP-binding protein-regulated second messengers [4], the presumed binding and channel domains must be in close proximity to one another.

In summary, we have shown that isothiocyanate groups are critical to the effectiveness of stilbene disulfonate compounds in blocking purinoceptors on parotid acinar cells, and that isothiocyanate compounds other than stilbene disulfonates are effective antagonists. These observations suggest that these compounds may be effective covalent antagonists for P_2 receptors found on other cells, and may be useful in purinoceptor identification and purification.

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