**Effects of Bis(2-chloroethyl)sulfide on ATP Receptor-Mediated Responses of the Rat Vas Deferens: Possible Relationship to Cytotoxicity**

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**ABSTRACT**

Extracellular ATP is a broad-spectrum cytotoxic agent that produces effects via cell surface P2 purinoceptors. The ligand-gated P2X purinoceptor subtype has very high sequence homology with the R1P-2 gene, which encodes for apoptosis. The P2X RNA found in rat vas deferens is expressed preferentially by apoptotic thymocytes. P2X purinoceptor-mediated phasic (twich) motor responses of the isolated rat vas deferens to neurogenic or exogenous ATP were rapidly, specifically and irreversibly potentiated by bis(2-chloroethyl)sulfide (HD 10–100 μM). Both untreated and HD-potentiated neurogenic responses were Ca²⁺ dependent, blocked in the absence of Ca²⁺ plus 0.1 mM EGTA, by the neuronal Ca²⁺ channel blocker ω-conotoxin-MVIIC (3 μM), by the P2 purinoceptor antagonist suramin (100 μM) and by tetrodotoxin (100 nM). HD also potentiated the effects of ATP on isolated guinea pig taenia caecum, where the nucleotide acts at G protein-coupled P2Y purinoceptor subtypes to cause relaxation. HD failed to inhibit the metabolism of ATP by ecto-ATPase in vas deferens or to cause the release of endogenous ATP. Potentiation of the twitch response to electric field stimulation by HD was attenuated or eliminated in tissues excised from rats previously challenged with topically applied HD, suggesting that HD absorbed into the systemic circulation had already effected maximal potentiation of ATP responses before *in vitro* testing. The physiological consequences of HD-induced potentiation of the extracellular actions of ATP are discussed in relation to apoptosis and necrosis.

HD is a chemical warfare agent whose military use has been documented as recently as 1985 during the Iran-Iraq war (United Nations, 1986, 1987, 1988). Cutaneous exposure to this agent causes blistering of the skin, whereas systemic uptake results in cytotoxic effects occurring in a variety of different organ systems, including the respiratory and gastrointestinal tracts, reproductive organs, central nervous system and immune system (Papirmeister et al., 1991; Calvet et al., 1994; Ray et al., 1995; Sawyer et al., 1995; Dacre and Goldman, 1996). Although this compound has been extensively studied for several decades, the mechanism by which it causes cell death and vesication is not known, and antidotes against these effects do not exist. The systemic toxicity caused by this agent is even more poorly understood.

Sulfur and nitrogen mustards alkylate critical sites on DNA (Papirmeister et al., 1985; Papirmeister et al., 1991; Mol and Van der Schans, 1992). The activation by Ca²⁺ of intracellular enzymes such as phospholipases, proteases and endonucleases (Papirmeister, 1994), which are responsible for structural and functional cellular viability, has led to the suggestion that Ca²⁺ might play an integral role in the initiation of cell death brought on by a number of insults, including exposure to HD (Orrenius and Nicotera, 1987; Ray et al., 1994, 1995). It also is quite clear that the exposure of different types of cells to HD does indeed result in the elevation of [Ca²⁺], Concentration-dependent, irreversible elevations in [Ca²⁺] after HD exposure have been reported in human keratinocytes and fibroblasts (Hamilton et al., in press; Hua et al., 1993; Ray et al., 1994, 1995, but also see Mol and Smith, 1996). In addition to these findings, it is interesting to note that chelators of [Ca²⁺] have been reported to offer marked protection from HD-induced cell death (Ray et al., 1996). There is an increasing realization that Ca²⁺ may play a critical role in HD toxicity, although little effort has been expended to provide an explanation for the [Ca²⁺] increase.

During investigations of the effects of HD on responses of isolated neuroeffector preparations to nerve stimulation and to drugs, we noted that responses of the rat vas deferens to electric field stimulation and to ATP were markedly and specifically potentiated by HD (Lundy et al., 1996). These findings were of considerable interest because extracellular ATP and HD share common properties in that ATP is considered a potent broad-spectrum cytotoxic agent in its own

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**ABBREVIATIONS:** HD, bis(2-chloroethyl)sulfide; PG, prostaglandin; [Ca²⁺], intracellular calcium concentration; NANC, nonadrenergic, noncholinergic; APCPP, α,β-methylene ATP.

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right and as such is undergoing clinical trials as a cytotoxic cancer chemotherapeutic drug (Zanovello et al., 1990; Pizzo et al., 1991; Zheng et al., 1991; Dubyak and El-Moatassim, 1993; Rapaport, 1993; Williams, 1996). The mechanisms of toxicity of ATP appear to closely mimic those thought to be operative in the cytotoxic actions of HD. Thus, both ATP and HD appear to elevate [Ca\(^{++}\)]\(_i\), which in turn may lead to activation of proteases, phospholipases and endonucleases. Activation of these enzymes may initiate membrane structural and DNA damage sufficient to cause cell death.

The biochemical and cytotoxic properties of ATP are not random or indiscriminate effects on cell membranes but rather are mediated through discrete ATP receptors (P2 purinoceptors), which when activated by ATP or its analogs mediate the elevation of [Ca\(^{++}\)]\(_i\), levels by opening a membrane channel or stimulating [Ca\(^{++}\)], release. One important type of P2 purinoceptor that we have studied, the P2X receptor, is a ligand-gated cationic channel. This particular purinoceptor bears strong sequence homology to the apoptotic protein expressed by the RP-2 gene (Owens et al., 1991; Brake et al., 1995; Suprenant et al., 1995; Valera et al., 1995). ATP also activates the P2Z purinoceptor found on a variety of immune cells that undergo cell death after exposure to either ATP or HD. The P2Z purinoceptor has been causally linked to apoptosis in a variety of cell types (Di Virgilio, 1995; Chiozzi et al., 1996).

In the present study, we provide evidence for a specific interaction between HD and ATP-evoked responses whereby HD enhances the effects of the nucleotide. This action appears to be mediated through a common pathway involving various P2 purinoceptor subtypes, Ca\(^{++}\) influx and cell death. The mechanisms underlying HD enhancement of ATP effects may provide insight into the mechanisms involved in HD-induced cell death, vescication and other biological effects.

Materials and Methods

**Contractility studies.** The vas deferentia, paired anococcygeus muscles and strips of urinary bladder were obtained from male Sprague-Dawley rats (150–200 g). Taenia caeci strips or longitudinal smooth muscle myenteric plexus preparations were obtained from male Hartley guinea pigs (300–350 g) and prepared for contractility studies according to established procedures (Lundy and Frew, 1994). Tissues were mounted under 1 g tension in 5-ml organ baths containing Krebs-Henseleit solution of the following composition (mM): NaCl, 116, KCl 5.4, CaCl\(_2\), 1.5, MgCl\(_2\), 1.2, NaHCO\(_3\), 25 and d-glucose 11, pH 7.4; maintained at 37°C, and continuously aerated with 95% O\(_2\), 5% CO\(_2\). One end of each tissue was anchored, and the other was attached by a thread to a Harvard Apparatus (South Natick, MA) smooth muscle transducer. Auxotonic recording of responses to electric field stimulation and to drugs was displayed via a Rikadenki (Tokyo, Japan) chart recorder. After a 60-min equilibration period, responses to continuous electric field stimulation were evoked in vas deferens preparations using parallel platinum electrodes. Squarewave pulses were delivered using supramaximal voltage at 1-msec duration and 5 Hz for 1 sec every 30 sec using a Grass (Quincy, MA) S88 stimulator. The contraction resulting from stimulation of the sympathetic nerves of the vas deferens is biphasic, consisting of an initial phasic (purinergic) and a secondary tonic (adrenergic) component (Swedin, 1971), which are sensitive to P2 purinoceptor and alpha adrenoceptor blockade, respectively. The stimulation parameters that we chose elicited a response that is predominantly purinergic. Ca\(^{++}\)-free Krebs' solution was prepared by omission of Ca\(^{++}\) and addition of 0.1 mM EGTA. Responses to ATP were examined by exogenous addition of the nucleotide to quiescent tissues for 30 sec followed by an exchange of bath fluid. The time course of ATP contractions is similar to that of the rapid phasic component of neurogenic contractions. Fifteen minutes was allowed to elapse between responses. Noncumulative concentration-effect curves to the contractile effects of ATP were constructed as described by Pedan et al. (1982). One of each pair of vas deferens was treated with 100 μM HD, with the other serving as control. Contractions to ATP were normalized by expressing them as a percentage of the contraction to 20 mM K\(^+\) administered at the onset of each experiment. Care was exercised to ensure rapid and reproducible additions of the nucleotide to the isolated organ baths. Concentration-effect curves were carried out in the presence of the P1 receptor antagonist 8-phenyltheophylline.

**Ecto-ATPase activity in vas deferens.** The rat vas deferens has high levels of ecto-ATPase activity (Harris, 1972). The abilities of the vas deferens ecto-ATPase activity to hydrolyze ATP, and of HD to inhibit hydrolysis were examined by monitoring the production of inorganic phosphate (P\(_i\)). Briefly, whole desheathed vas deferentia prepared as for organ bath experiments were incubated in 24-well culture dishes containing 1 mM ATP (in 250 μl of Krebs' buffer) and shaken continuously at 37°C for 30 min. Incubation was stopped by removing the Krebs' buffer and adding it to 0.9 ml of a 2.5% (w/v) solution of sodium dodecyl sulfate for P\(_i\) assay. For the assay, 1 ml of ammonium molybdate in 2 N HCl and 0.1 ml of 16% (w/v) Fiske and SubbaRow reducing agent were added to the samples (Zaganshin et al., 1995). The P\(_i\) produced was measured spectrophotometrically at 700 nM. Protocols routinely included controls to monitor spontaneous ATP breakdown in Krebs' buffer, as well as spontaneous ATP release from tissues.

**Effect of HD on neurotransmitter release.** Basal and HD-evoked neurotransmitter release from rat vas deferentia was measured. Neuroneurotransmitter stores were labeled using 3H-(-)-norepinephrine (13.8 Ci/mmol, Dupont New England Nuclear, Mississauga, Ontario, Canada) for 20 min in the presence of pargyline (10 μM) to inhibit monoamine oxidase. Three pairs of labeled vas deferentia were loaded into each of six tissue chambers of a SP-600 superfusion system equipped with built-in platinum electrodes connected to a Grass S88 stimulator (Brandel, Gaithersburg, MD) and superfused at a flow rate of 0.5 ml/min with oxygenated Krebs' solution maintained at 37°C containing pargyline, as above. Imipramine (1 μM) and cortisol (10 μM) were included to inhibit neuronal and extraneuronal reuptake of norepinephrine, respectively. After 20 min of superfusion to obtain stable basal 3H-efflux levels, the first of a total of 14 fractions was collected, each at 2-min intervals. At 10 min (fraction 5), tissues were stimulated with a submaximal stimulus: 10 Hz, 1 msec, at supramaximal voltage for 60 sec (S\(_1\)). One of each pair of tissues was then exposed to HD (100 μM) for 15 min, at which time all tissues were restimulated (S\(_2\)). At the end of each experiment, tissues were solubilized in 0.2 ml of Protosol (Dupont New England Nuclear) at 50°C overnight. Superfusates and the 3H remaining in tissues were prepared for counting by scintillation spectrometry. 3H-Release was calculated and expressed as a fraction of the total 3H present in the tissues at the onset of each collection period and is referred to as fractional release.

**In vivo studies.** Neat HD was applied (250 mg/kg) to the backs of rats that had been previously shaved and then depilated using Neet depilatory cream, Boyle-Midway, Toronto, Ontario, Canada. At 240 min after HD exposure, the vas deferentia were excised and mounted for contractility studies as described above. Animals were used in accordance with the guidelines of The Canadian Council on Animal Care (Guide to the Care and Use of Experimental Animals, Vol. 1, 1993). All experimental protocols were approved by the institutional animal care committee.

**Drugs.** Tetrodotoxin, ATP, α, β-methylene ATP, guanethidine sulfate, atropine sulfate, EGTA, cortisol, pargyline HCl and imipramine HCl were all purchased from Sigma Chemical (St Louis, MO). α-Conotoxin-MVIIC was obtained from Bachem (Torrance, CA).
8-Phenyltheophylline and suramin were obtained from Research Biochemicals (Natick, MA). PGF$_{2\alpha}$ was a gift from Upjohn, (Kalama-zoo, MI). Sulphur mustard (HD, NATO STANAG designation) was prepared at Defence Research Establishment Suffield at $>$98% purity. All drugs were dissolved in glass-distilled H$_2$O. 8-Phenyltheo-phylline was dissolved at 10 mM in 80% methanol containing 0.2 M NaOH, and aqueous dilutions were made from this solution. HD was dissolved in absolute ethanol, and small aliquots (0.1% v/v) were added to the medium used to bath the tissues.

Results

Continuous stimulation of rat vas deferens preparations at 5 Hz, 1-msec pulse width, for 1 sec every 30 sec at supramaximal voltage, evoked reproducible twitch responses, which were due to release of the cotransmitter ATP from intrinsic sympathetic nerves, as judged by the ability of the P2 purinergic receptor antagonist suramin to inhibit the response and the insensitivity of the response to alpha-adrenoceptor blockers.

Addition of HD to the Krebs’ solution used to bath the continuously stimulated vas deferens markedly, selectively and irreversibly potentiated the ATP-mediated phasic response evoked by electric field stimulation (fig. 1). Threshold and maximal potentiating effects of HD were observed at 10 and 100 µM, respectively. Onset of potentiation was rapid and usually fully expressed by 15-min. HD undergoes chemical degradation in aqueous solution, so it is no longer active and usually fully expressed by 15-min. HD undergoes chemical degradation in aqueous solution, so it is no longer active after several minutes. We noted in our experiments that compared with a 15-min exposure of the vas deferens to HD, a 60-sec exposure produced potentiation of comparable magnitude when examined 60 min after the initial addition and washout of HD (data not shown). No effects of HD on the quiescent tone of the unstimulated vas deferens were observed during this 15-min treatment period. In contrast to the vas deferens, HD inhibited neurogenic sympathetic noradrenergic responses of rat anococcygeus muscles evoked by similar parameters of field stimulation and failed to potentiate cholinergic responses of the guinea pig ileum longitudinal smooth muscle myenteric plexus preparation continuously stimulated at 0.2 Hz (table 1). HD enhanced the magnitude of motor responses to exogenously added ATP (100 µM) in rat vas deferens (P2X purinergic receptor mediated) and relaxant responses to ATP in guanethidine- and atropine-treated, PGF$_{2\alpha}$-contracted strips of guinea pig taenia caeci (P2Y purinergic receptor mediated) (fig. 2). The vehicle (ethanol, 0.01% v/v) was without effect on the neurogenic responses of these tissues or on their responses to exogenous ATP.

Concentration-effect curves in response to the contractile effects of ATP carried out in the presence of 8-phenyltheophylline indicated an increase in the maximum contractile response after HD treatment without changing the apparent ED$_{50}$ value of the ATP curve (fig. 3). In accordance with the study of Fedan et al. (1982), contractions to ATP did not achieve a maximum level at millimolar concentrations.

Both the neurogenic twitch response and the HD-enhanced twitch response of the vas deferens were Ca$^{++}$ dependent and totally inhibited by the neuronal Ca$^{++}$ channel antagonist ω-conotoxin-MVIIC (3 µM) or by removal of Ca$^{++}$ from the medium. Similarly, responses were inhibited in the presence of suramin (100 µM, 30 min), a selective inhibitor of purinergic transmission in the rat vas deferens (Mallard et al., 1992). Typical traces are shown in figure 4. Responses of all tissues to electric field stimulation were tetrodotoxin (100 nM) sensitive.

A modest potentiating of responses to electric field stimulation by HD (100 µM) was observed in rat urinary bladder, in which the response to electric field stimulation has both cholinergic and purinergic components (table 1). There was some indication that HD had additional effects on the quiescent tone of both the rat bladder and the guinea pig ileum. These effects were not investigated further.

HD had no effect on basal or stimulated [$^3$H]-(-)-norepinephrine efflux. Effects of HD on basal or electrically evoked release of [$^3$H] from superfused rat vas deferens labeled with [$^3$H]-(-)-norepinephrine are shown in figure 5.

HD at (0.1 mM) and above (1 mM) concentrations that enhanced the twitch response to electric field stimulation or to exogenous ATP failed to modify ecto-ATPase activity in the vas deferens, in that the ability of the tissue to hydrolyze ATP was not attenuated by HD. Results of inorganic phosphorus assays are shown in figure 6. Contractions to the ecto-ATPase resistant ATP analog APCPP were potentiated by HD 100 µM (fig. 7).

Potentiation of neurogenic responses of rat vas deferens by exogenously added HD (100 µM) was markedly attenuated in vas deferens removed from HD-pretreated rats (250 mg/kg cutaneous for 240 min). Results are presented in table 1, and the tracings of one such experiment are shown in figure 8.

Discussion

Cell surface ATP receptors (P2 purinoceptors) mediate the effects of extracellular ATP and include two major families, designated P2X and P2Y (Abbrachio and Burnstock, 1994; Fredholm et al., 1994). Additional purinoceptor subtypes have been characterized, including the P2Z, an ATP-gated ion pore (Di Virgilio, 1995), which was recently designated a P2X$_7$ purinoceptor (Suprenant et al., 1996), and P2U, a purinoceptor sensitive to ATP and UTP (Harden et al., 1995). P2X purinoceptors are membrane-bound ligand-gated cationic channels that mediate ATP-induced influx of extracellular...
Ca$^{2+}$ into cells and occur on a wide variety of cell types, including the smooth muscle cells of the rat vas deferens. In the rat vas deferens, neurogenic or exogenous ATP evokes a Ca$^{2+}$-dependent contraction by activating postjunctional P2X purinoceptors located on the smooth muscle cells. The P2X purinoceptor antagonist suramin (Mallard et al., 1992), the neuronal Ca$^{2+}$ channel blocker $\nu$-conotoxin-MVIIC (Hill-yard et al., 1992) and the Na$^+$ channel blocker tetrodotoxin completely abolish the contraction to electric field stimulation, confirming that the neurogenic response of the vas deferens examined was sympathetic purinergic, Ca$^{2+}$-dependent and action potential evoked.

Under physiological conditions, ATP is rapidly degraded by ATP-metabolizing ecto enzymes located on the postjunctional membrane (Harris, 1972; Gordon, 1986; Ziganshin et al., 1994). The hydrolysis of ATP by ecto-ATPase quickly terminates the tissue response, and in the case of the rat vas deferens, it can be considered a neurotransmitter-metabolizing enzyme. The potentiation of the twitch response to electric field stimulation by HD observed in our experiments (fig. 1) could result from (1) HD-evoked release of ATP from prejunctional or postjunctional sites, (2) inhibition of ATP metabolism by inhibition of ecto-ATPases, (3) direct stimulation of P2 purinoceptors by HD or (4) induction of a conformational change in the P2X purinoceptor to increase ATP binding. Each of these possibilities was considered. The observation that HD per se does not contract the smooth muscle of the vas deferens suggests that HD is not releasing ATP from either prejunctional or postjunctional sites. Furthermore, in superfusion studies, HD failed to modify basal or electrically evoked release of $[^3]$H from superfused $[^3]$H-(−)-norepinephrine-labeled vas deferens. Because norepinephrine and ATP are cotransmitters released from sympathetic

### TABLE 1

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Pretreatment</th>
<th>HD potentiation</th>
<th>Motor response</th>
</tr>
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<tbody>
<tr>
<td>Rat vas deferens</td>
<td>None</td>
<td>199.3 ± 19.0 (27)</td>
<td>Purinergic</td>
</tr>
<tr>
<td>Rat ves deferens</td>
<td>250 mg/kg HD cutaneous</td>
<td>19.0 ± 2.9 (5)</td>
<td>Cholinergic/NANC</td>
</tr>
<tr>
<td>Rat bladder</td>
<td>None</td>
<td>38.4 ± 16.4 (5)</td>
<td>Adrenergic</td>
</tr>
<tr>
<td>Rat anococcygeus muscle</td>
<td>None</td>
<td>0 (5)</td>
<td>Cholinergic</td>
</tr>
<tr>
<td>Guinea pig ileum myenteric plexus</td>
<td>None</td>
<td>0 (5)</td>
<td>Cholinergic</td>
</tr>
</tbody>
</table>

Results are reported as mean ± S.E.M.

* Significantly different from no pretreatment (P < 0.001) Mann-Whitney test.

' Inhibition.

' Thought to be purinergic (Burnstock et al., 1972).

Number of animals is given in parentheses.

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**Fig. 2.** Effects of HD on responses to exogenous ATP in rat vas deferens and guinea pig taenia caecum. A, P2X purinoceptor-mediated contractile responses of rat vas deferens to ATP in the absence and presence of HD (100 μM) and 60 min after washout of HD from organ baths. B, P2Y purinoceptor-mediated relaxant responses of PGF$\_2$α- contracted guinea pig taenia caecum. Response to ATP (10 μM) in the absence (top left) and presence (top right) of HD and untreated time control (below). Atropine (0.5 μM) and guanethidine (5 μM) were present in the Krebs’ buffer used to bath the taenia caecum.

**Fig. 3.** Effect of HD on the ATP concentration-response relationship in the rat vas deferens (n = 4). Controls (○) and HD (100 μM) for 15 min (●). Responses are expressed as a percentage of contractions to 20 mM K$^+$. The Krebs’ solution contained 3 μM 8-phenyltheophylline. Inset, ATP controls plotted with a different scale on the y axis.
adrenergic nerve varicosities in the molar stoichiometry found within the vesicles of this tissue (Burnstock, 1990; Sperlág and Vizi, 1996), the measurement of norepinephrine release can be used as an indirect assessment of ATP release. It thus appears that the potentiating effects of HD are unlikely to be due to release of neuronal ATP (or norepinephrine).

It has been suggested that ecto-ATPase modulates purinergic transmission in the guinea pig vas deferens, and it has been shown that ecto-ATPase inhibition greatly potentiates the neurogenic responses (Kennedy et al., 1996). HD has been shown to inhibit enzymes that transfer PO₄ groups (Papirmeister et al., 1991). However, hydrolysis of ATP by rat vas deferens ecto-ATPase was not modified by HD pretreatment, suggesting that inhibition of ATP-metabolizing enzymes does not account for HD-induced potentiation of neurogenic and exogenous ATP responses. Potentiation by HD of responses to APCPP, the methylene isostere analog of ATP (in which replacement of the α,β,-anhydride oxygen by methylene confers resistance to degradation by ecto-ATPase), adds support to this view. This could be further confirmed with the use of the selective ecto-ATPase inhibitor ARL 67156 (Kennedy et al., 1996; Westfall et al., 1996), which, however, we have been unable to obtain. It is also interesting to note that HD has been reported to inhibit Ca²⁺-ATPase (Kim et al., 1995; Mol and Smith, 1996), an intracellular enzyme that helps to maintain [Ca²⁺], levels within normal concentrations. Although this mechanism likely explains the increase in Ca²⁺ levels in certain tissues, in the vas deferens studies reported here, the Ca²⁺ apparently originates from external sources. Receptor binding studies are required to assess whether HD potentiates neurogenic and exogenous ATP responses by inducing a conformational change at the P2X purinoceptor.

Contractions of the vas deferens to ATP are complex and may be mediated by multiple mechanisms (Fedan et al., 1982). Concentration-effect curves to ATP indicate an increase in the maximal contractile response after exposure to HD, with no change in the apparent ED₅₀ values (fig. 3). The presence of the P1 purinoceptor antagonist 8-phenyltheophylline in the Krebs’ solution appears to rule out a role for adenosine, a breakdown product of ATP, or adenosine receptors, in the potentiating effects of HD.

To further elucidate the nature of HD-evoked enhancement of the neurogenic responses of the vas deferens, the effects of HD on the neurogenic responses of other rat tissues were examined. HD-induced potentiation of the neurogenic motor response of the rat bladder is consistent with the NANC innervation of this tissue, in which the NANC component has been suggested to be purinergic (Burnstock et al., 1972; Hoyle and Burnstock, 1993). The rat anococcygeus muscle has a dense adrenergic innervation, and motor responses of this preparation to electric field stimulation are completely blocked by α-adrenoceptor antagonists (Gillespie, 1980), whereas responses of the guinea pig ileum longitudinal muscle are cholinergic and are completely blocked by the muscarinic antagonist atropine (Paton and Zar, 1968). The absence of a potentiating effect of HD on responses to electric field stimulation in these preparations is consistent with the hypothesis that HD induces a conformational change at the P2X purinoceptor.
consistent with the contention that HD potentiation is specific for P2 purinoceptor-mediated effects.

The G protein-coupled P2Y purinoceptor (Abbrachio and Burnstock, 1994) mediates relaxation to ATP in the precontracted guinea pig taenia caecum. HD-evoked potentiation of responses to exogenously added ATP in this tissue suggests that HD-induced potentiation is not restricted to P2X purinoceptor-mediated effects and may extend to other P2 purinoceptor subtypes.

The HD-induced potentiation of ATP-mediated responses observed in this study, and the reported ability of ATP to initiate cell death, may involve activation of P2X purinoceptors because of the close structural relationship between this purinoceptor subtype (Brake et al., 1995; Valera et al., 1995) and the RP-2 gene reported to initiate apoptosis (Owens et al., 1991).

However, other P2 purinoceptor subtypes (P2Y, P2Z, P2U) also occur on cells affected by HD, including those that mediate immunity, inflammation, growth and development and viability of skin and respiratory cells (for reviews, see Dubyak and El-Moatassim, 1993; Harden et al., 1995). Activation of these other purinoceptor subtypes, as in the case of P2X purinoceptors, also evokes an elevation of [Ca$^{2+}$], either by forming [Ca$^{2+}$]-permeable pores or by initiating phosphoinositol breakdown (Dubyak and El-Moatassim, 1993; Boehm et al., 1995; Harden et al., 1995; Rogers and Dani, 1995; Suprenant et al., 1996). Elevation of Ca$^{2+}$ levels resulting from the activation of these ATP receptors may also initiate Ca$^{2+}$-induced cellular toxicity, and it has been proposed that elaborate protective mechanisms exist to control such events (Edwards, 1996). HD may override such mechanisms. With respect to human skin keratinocytes, which are a particular target of HD, purinoceptors on these cells (and on those from other species) play an important role in the control of growth and development (Pillai and Bikle, 1992), and it is well
known that Ca\(^{2+}\) plays a critical role in the control of these processes (Sharpe et al., 1989; Pillai et al., 1990; Pillai and Bikle, 1991). Significantly, both ATP (Suter et al., 1991) and HD (Ray et al., 1994, 1995; Sawyer et al., 1996a) induce the elevation of [Ca\(^{2+}\)]\(_i\), in keratinocytes as they do in a wide variety of tissue types in which they produce their cytotoxic effects. It appears that perturbed ionic balance, followed by the activation of destructive intracellular enzymes, and the fragmentation of DNA may be the underlying causes in the extensive, general cytotoxic activity of ATP (Di Virgilio et al., 1989; Zanovello et al., 1990; Pizzo et al., 1991; Zheng et al., 1991; Zoeteweij et al., 1992; Spanzi et al., 1993). There is growing evidence to suggest that HD may also induce changes in intracellular Ca\(^{2+}\) and induce a similar cascade of events (Hamilton et al., in press; Hua et al., 1993; Papirmeister et al., 1991; Ray et al., 1995, 1996). In support of this view, we have, in preliminary studies, observed apoptotic cells in HD exposed vas deferentia (P. M. Lundy, T. W. Sawyer, B. T. Hand, R. Frew and A. J. Zubaidy, unpublished observations).

In summary, our results strongly suggest a direct link between the enhanced efficacy of ATP at P2 purinoceptors and HD exposure and the cytotoxic effects of HD. The observations that HD-induced potentiation of neurogenic responses to ATP were attenuated or absent in vas deferentia after HD exposure and the cytotoxic effects of HD. The ob-...


