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Chelerythrine and other benzophenanthridine alkaloids block the human $P2X_7$ receptor

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1 Extracellular ATP can activate a cation-selective channel/pore on human B-lymphocytes, known as the $P2X_7$ receptor. Activation of this receptor is linked to PLD stimulation. We have used ATP-induced $^{86}Rb^+$ (K^+) efflux to examine the effect of benzophenanthridine alkaloids on $P2X_7$ channel/pore function in human B-lymphocytes.

2 Both ATP and the nucleotide analogue 2′-3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP) induced an $^{86}Rb^+$ efflux, which was completely inhibited by the isoquinoline derivative 1-(*N*,*O*-bis[5-isoquinolinesulphonyl]-*N*-methyl-L-tyrosyl)-4-phenylpiperazine (KN-62), a potent $P2X_7$ receptor antagonist.

3 The benzophenanthridine alkaloid chelerythrine, a potent PKC inhibitor, inhibited the ATP-induced $^{86}Rb^+$ efflux by $73.4 \pm 3.5\%$ and with an IC_{50} of $5.6 \pm 2.3\,\mu M$. Similarly, other members of this family of compounds, sanguinarine and berberine, blocked the ATP-induced $^{86}Rb^+$ efflux by 58.8 ± 4.8 and $61.1 \pm 8.0\%$, respectively.

4 Concentration–effect curves to ATP estimated an EC_{50} value of $78\,\mu M$ and in the presence of 5 and $10\,\mu M$ chelerythrine this increased slightly to 110 and $150\,\mu M$, respectively, which fits a noncompetitive inhibitor profile for chelerythrine.

5 Chelerythrine at $10\,\mu M$ was effective at inhibiting the ATP-induced PLD stimulation in B-lymphocytes by $94.2 \pm 21.9\%$ and the phorbol 12-myristate 13-acetate-induced PLD stimulation by $68.2 \pm 7.4\%$.

6 This study demonstrates that chelerythrine in addition to PKC inhibition has a noncompetitive inhibitory action on the $P2X_7$ receptor itself.
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Keywords: Purinergic receptor; lymphocyte; extracellular ATP; phospholipase D; chelerythrine; cation flux

Abbreviations: BzATP, 2′-3′-*O*-(4-benzoylbenzoyl)-ATP; CLL, chronic lymphocytic leukaemia; KN-62, 1-(*N*, *O*-bis[5-isoquinolinesulphonyl]-*N*-methyl-L-tyrosyl)-4-phenylpiperazine; PBut, phosphatidylbutanol; PMA, phorbol 12-myristate 13-acetate; $^{86}Rb^+$, rubidium-86

Introduction

The $P2X_7$ receptor is an ATP-gated channel with intracellular N- and C-termini with two transmembrane domains, and is expressed in cells of haemopoietic origin (Dubyak, 2001). The large extracellular loop contains a number of glycosylation sites and putative ATP-binding domains (Worthington *et al*., 2002). The $P2X_7$ channel is selective to cations and its opening results in an influx of Ca^{2+} and efflux of K^+ from the cell (Dubyak, 2001). ATP-mediated Ca^{2+} influx through the $P2X_7$ receptor is associated with the activation of PLD in B-lymphocytes (Gargett *et al*., 1996). However, it is not known what regulates PLD downstream of ATP-induced $P2X_7$ activation. PKC is well recognized as an important regulator of PLD (Exton, 2002). It has been recently shown that the PKC antagonist chelerythrine inhibits ATP-induced PLD activity in rat submandibular acinar gland and ductal cells (Perez-Andres *et al*., 2002; Pochet *et al*., 2003), suggesting a role for PKC in this process. These studies, however, did not determine if chelerythrine inhibited the $P2X_7$ receptor directly.

Chelerythrine is the only member of the benzophenanthridine alkaloids with potent and selective PKC inhibitory actions (IC_{50} $0.66\,\mu M$ in a permeabilized cell system) (Herbert *et al*., 1990). Structurally related compounds, sanguinarine and berberine, do not display such potent inhibition of PKC (Wang *et al*., 1997). However, chelerythrine also inhibits enzymes such as alanine aminotransferase and Na^+/K^+-ATPase independently of PKC (Cohen *et al*., 1978; Walterova *et al*., 1981), raising the possibility that chelerythrine may interact with other molecules such as the $P2X_7$ receptor. In this study, we demonstrate that chelerythrine blocks the ATP-induced cation fluxes mediated by the $P2X_7$ receptor, as well as the ATP-induced stimulation of PLD in human B-lymphocytes.

Methods

Source and preparation of human peripheral blood B-lymphocytes

Blood was collected from patients with informed consent and with approval from the Wentworth Area Health Service

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Human Ethics Committee (Penrith, Australia). Peripheral blood B-lymphocytes from nine different patients with chronic lymphocytic leukaemia (CLL) were isolated by density centrifugation as described (Wiley *et al*., 1992). Cells were either resuspended in NaCl medium (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 0.1% w v^{-1} BSA, 5 mM D-glucose) for rubidium-86 ($^{86}Rb^+$) loading or in supplemented RPMI-1640 medium containing 10% foetal calf serum, 2 mM L-glutamine and 5 μg ml^{-1} gentamicin for [^{3}H]oleic acid labelling.

$^{86}Rb^+$ efflux measurements

ATP-induced $^{86}Rb^+$ efflux from B-lymphocytes (5×10^6 cells ml^{-1}) was performed as described (Wiley *et al*., 2003). $^{86}Rb^+$-loaded cells were incubated for 5 min at 37°C in the presence or absence of inhibitor, before the addition of agonist for 4 min. $^{86}Rb^+$ efflux was expressed as $(1-N_t/N_{total})$, where N_t was the level of cell-associated radioactivity at time t (determined by Cerenkov counting) and N_{total} the amount of cell-associated radioactivity at time zero. The data were linearized by log transformation calculation of series time constants.

Phospholipase D assay

B-lymphocytes (1×10^7 cells ml^{-1}) were cultured at 37°C/5% CO_2 overnight in supplemented RPMI-1640 medium containing [^{3}H]oleic acid (2–5 μCi ml^{-1}). Labelled cells were resuspended in KCl medium (150 mM KCl, 10 mM HEPES, 5 mM D-glucose, 0.1% w v^{-1} BSA, pH 7.5) containing 1 mM $BaCl_2$ and pre-incubated for 5 min in the presence of the primary alcohol, 1-butanol (30 mM), which yields a stable phosphatidylalcohol end product following PLD stimulation. The cells were then incubated in the presence or absence of chelerythrine (10 μM) for 15 min at 37°C before incubation in the presence or absence of ATP (500 μM) or phorbol 12-myristate 13-acetate (PMA; 0.1 μM) for a further 15 min. Membrane lipids were extracted and the level of phosphatidylbutanol (PBut) was determined as described (Gargett *et al*., 1996).

Statistics

Differences in $^{86}Rb^+$ efflux and PLD activity were compared using the two-tailed unpaired Student's *t*-test.

Materials

ATP, BzATP, $BaCl_2$, HEPES, D-glucose, BSA, L-glutamine, gentamicin, RPMI-1640 medium, PMA, iodine crystals, organic solvents and Hyperfilm MS were from Sigma Chemical Co., U.S.A. (St Louis, MO, U.S.A.). Foetal calf serum was from Life Technologies (Grand Island, NY, U.S.A.). Chelerythrine chloride and 1-[*N*,*O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) were from BIOMOL (Plymouth Meeting, PA, U.S.A.). Ficoll-Paque ($d = 1.077$) was obtained from Pharmacia (Uppsala, Sweden). [9,10-^{3}H]oleic acid (5 mCi ml^{-1}; specific radioactivity 10 Ci mmol^{-1}) and ^{86}RbCl (1.5 mCi ml^{-1}; specific radioactivity 3 Ci mmol^{-1}) were purchased from Amersham International (Little Chalfont, Buckinghamshire, U.K.). Di-*n*-butyl phthalate and di-isooctyl phthalate from BDH Chemicals (Poole, England) were blended 80 : 20 (v v^{-1}) to give a mixture of density 1.030 g ml^{-1}. LK6D thin-layer chromatography plates were from Whatman (Maidstone, Kent, U.K.). En^{3}Hance autoradiography spray was from DUPONT (Boston, MA, U.S.A.).

Results

ATP-induced $^{86}Rb^+$ efflux from B-lymphocytes is mediated by the P2X$_7$ receptor

Chelerythrine and other benzophenanthridine alkaloids are fluorescent compounds, making them unsuitable for use in fluorescent-based assays of P2X$_7$ function, such as flow cytometry and fluorimetry. Therefore, P2X$_7$ channel/pore activity was assessed by measuring ATP-induced $^{86}Rb^+$ efflux from B-lymphocytes. This method has been previously used to measure P2X$_7$ function in lymphocytes, monocytes and macrophages (Steinberg & Silverstein, 1987; Wiley *et al*., 1992; 2003; Sluyter *et al*., 2004). Over a period of 4 min, $^{86}Rb^+$ efflux followed first-order kinetics with a rate constant of 0.02 ± 0.01 min^{-1} ($n = 9$) in the absence of ATP and 0.20 ± 0.03 min^{-1} ($n = 9$) in the presence of 100 μM ATP (Figure 1). Moreover, the potent P2X$_7$ agonist BzATP (20 μM), induced a similar rate of $^{86}Rb^+$ efflux from human B-lymphocytes at a rate of 0.23 min^{-1} ($n = 2$, Figure 1). To confirm that the ATP-induced $^{86}Rb^+$ efflux was mediated by P2X$_7$, B-lymphocytes were pre-incubated with the specific P2X$_7$ antagonist, KN-62 (Gargett & Wiley, 1997). Pre-incubation of B-lymphocytes for 5 min with 1 μM KN-62 had no effect on basal rates of $^{86}Rb^+$ efflux, but inhibited ATP-induced fluxes by 96.0% ($n = 2$, Figure 1).

Chelerythrine and its structural analogues inhibit P2X$_7$ receptor-mediated $^{86}Rb^+$ efflux in B-lymphocytes

To examine the effect of chelerythrine, a PKC inhibitor, on P2X$_7$ receptor function, we measured ATP-induced $^{86}Rb^+$ efflux. Pre-incubation of B-lymphocytes with 10 μM cheler-

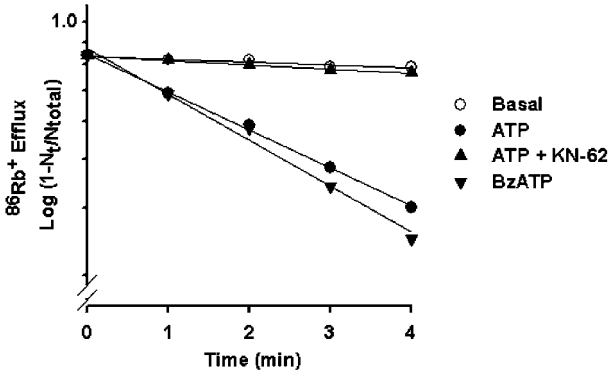

Figure 1 P2X$_7$ activation mediates $^{86}Rb^+$ efflux from human B-lymphocytes. $^{86}Rb^+$-loaded CLL B-lymphocytes in KCl (for ATP) or NaCl (for BzATP) medium supplemented with 20 μM $CaCl_2$ were pre-incubated at 37°C for 5 min in the absence or presence of 1 μM KN-62, before the addition of 100 μM ATP or 20 μM BzATP for a further 4 min. The data are one representative of nine experiments for ATP and one representative of two experiments for BzATP and KN-62.

ythrine inhibited the $^{86}Rb^+$ efflux induced by 100 μM ATP by 73.4±3.5% ($n=13$, $P<0.001$, Figure 2). At 100 μM ATP, chelerythrine inhibited $^{86}Rb^+$ efflux with an IC_{50} of 5.6±2.3 μM (data not shown). The Hill coefficient for ATP was 2.1 in the presence of chelerythrine and 2.0 in its absence. Pre-incubation of B-lymphocytes with 10 μM chelerythrine also inhibited the $^{86}Rb^+$ efflux induced by 20 μM BzATP by 51% (data not shown).

To determine if the inhibitory effect of chelerythrine on the $P2X_7$ receptor was PKC dependent, we studied two structural analogues of chelerythrine, sanguinarine, which has poor PKC inhibitory activity with an IC_{50} of 217 μM, and berberine, which has no known PKC inhibitory actions (Wang *et al*., 1997). Both sanguinarine and berberine at 10 μM inhibited ATP-induced $^{86}Rb^+$ efflux by 58.8±4.8% ($n=3$, $P<0.02$) and 61.1±8.0% ($n=3$, $P<0.02$), respectively (Figure 2). These analogues had no significant effect on the rate of $^{86}Rb^+$ efflux in the absence of ATP (data not shown).

Inhibition of the $P2X_7$ receptor by chelerythrine is noncompetitive

To establish whether the inhibition of chelerythrine was competitive or noncompetitive, the $^{86}Rb^+$ efflux was measured over a range of ATP concentrations in the absence or presence of 5 or 10 μM chelerythrine (Figure 3). The addition of ATP (25–1000 μM) increased $^{86}Rb^+$ efflux in a concentration-dependent manner (Figure 3). Maximum rates of $^{86}Rb^+$ efflux were observed at ATP concentrations above 200 μM. Chelerythrine was an effective inhibitor at all ATP concentrations tested. The EC_{50} for ATP was 78 μM in the absence of inhibitor. In the presence of 5 and 10 μM chelerythrine, the EC_{50} for ATP rose modestly to 110 and 150 μM, respectively. However, the maximum response to ATP was reduced by 35.1% in the presence of 5 μM chelerythrine and further reduced by 64.9% in the presence of 10 μM chelerythrine. The data are consistent with the idea that the inhibitory action of chelerythrine is predominantly noncompetitive. Similar results were also obtained with berberine, which has no known PKC inhibitory action (data not shown).

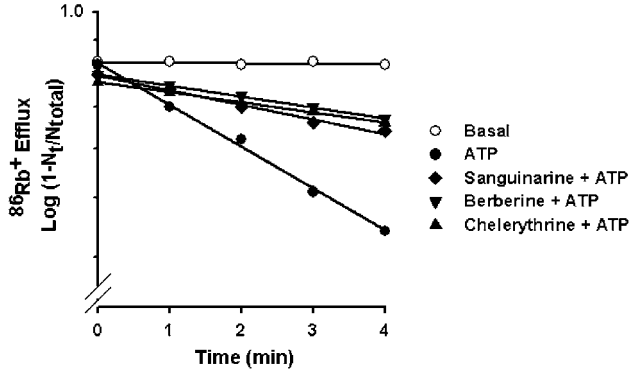

Figure 2 Chelerythrine and its structural analogues, sanguinarine and berberine, block ATP-induced $^{86}Rb^+$ efflux. $^{86}Rb^+$-loaded CLL B-lymphocytes in KCl medium containing 20 μM $CaCl_2$ were pre-incubated for 5 min at 37°C in the presence of 10 μM sanguinarine, 10 μM berberine or 10 μM chelerythrine, or in the absence of inhibitors, before the addition of 100 μM ATP for a further 4 min. The data are one representative of 13 experiments for chelerythrine and three experiments for sanguinarine and berberine.

Chelerythrine inhibits the ATP- and PMA-induced PLD activation in B-lymphocytes

To determine if chelerythrine also impairs ATP-induced PLD stimulation, in B-lymphocytes, cells were labelled with [^{3}H]oleic acid by overnight incubation and PLD activity was measured by the transphosphatidylation reaction in the presence of 1-butanol. As previously observed (Gargett *et al*., 1996; Gargett & Wiley, 1997), but with a different cohort of B-CLL patients, we found that both ATP and PMA stimulated PLD activity in B-lymphocytes (Figure 4). Pre-incubation of B-lymphocytes with chelerythrine at 10 μM abolished the ATP-induced PLD activity by 94.2±21.9% ($n=5$, $P<0.03$) and reduced the PMA-induced PLD activity by 68.2±7.4% ($n=5$, $P<0.05$; Figure 4). Chelerythrine did not inhibit the basal activity of PLD.

Discussion

In this study, we used ATP-induced $^{86}Rb^+$ efflux from B-lymphocytes to assess the effect of chelerythrine and other

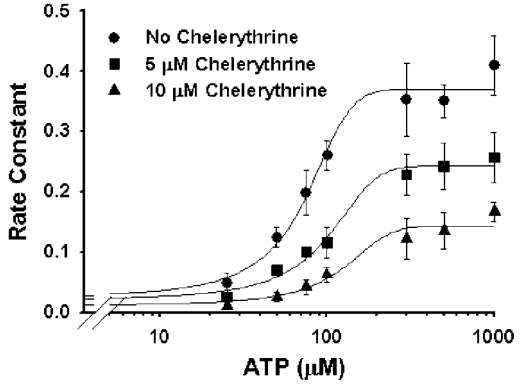

Figure 3 Allosteric noncompetitive inhibition of ATP-induced $^{86}Rb^+$ efflux by chelerythrine. $^{86}Rb^+$-loaded CLL B-lymphocytes were incubated for 5 min at 37°C in the presence of either 5 or 10 μM chelerythrine or in the absence of chelerythrine before addition of ATP, as indicated for a further 4 min. The data are presented as means ±s.e.m. from three separate experiments.

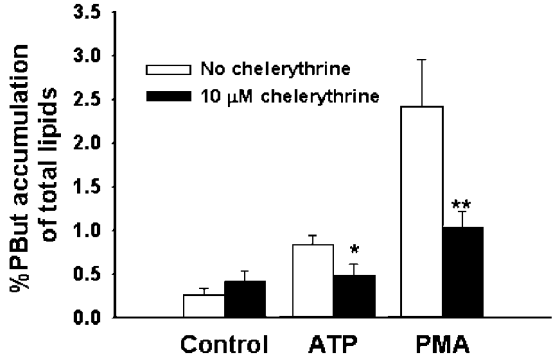

Figure 4 Chelerythrine inhibits both ATP- and PMA-induced PLD activity. [^{3}H]oleic acid-labelled CLL B-lymphocytes were incubated in KCl medium containing 1 μM $BaCl_2$ in the absence or presence of 10 μM chelerythrine for 15 min prior to the addition of 500 μM ATP or 0.1 μM PMA for a further 15 min at 37°C. The data are presented as means ±s.e.m. from five separate experiments (*$P<0.03$, **$P<0.05$, chelerythrine *versus* control).

benzophenanthridine alkaloids on $P2X_7$ channel/pore activity. In a series of preliminary experiments, other commonly used techniques such as flow cytometry and fluorimetry (Wiley *et al*., 2001) were found to be unsuitable due to the high fluorescence of chelerythrine (data not shown). The ATP-induced $^{86}Rb^+$ efflux from B-lymphocytes was mediated *via* the $P2X_7$ receptor, as this process was also stimulated by the potent $P2X_7$ agonist BzATP. In addition, the $P2X_7$ antagonist KN-62 inhibited ATP-induced $^{86}Rb^+$ efflux. Moreover, we have recently shown that ATP-induced $^{86}Rb^+$ efflux is impaired in B-lymphocytes from subjects heterozygous for a loss-of-function mutation at amino-acid 568 of the $P2X_7$ receptor (Wiley *et al*., 2003).

Chelerythrine inhibited the efflux of $^{86}Rb^+$ in a dose-dependent manner and acted noncompetitively with respect to ATP at the $P2X_7$ receptor. Moreover, the structural analogues of chelerythrine, sanguinarine and berberine, which have limited or no known PKC-inhibitory activity (Wang *et al*., 1997), also inhibited ATP-induced $^{86}Rb^+$ efflux. These results suggest that the inhibitory action of these compounds on the $P2X_7$ receptor is structurally related and may be a result of chelerythrine acting directly with the receptor. Chelerythrine, like KN-62, may be binding to a portion of the extracellular loop (Chen *et al*., 2002). However, it seems unlikely that chelerythrine binds to the ATP-binding site of the $P2X_7$ receptor as its locus of action on PKC is independent of the ATP active site (Herbert *et al*., 1990), consistent with chelerythrine acting as a noncompetitive inhibitor of $P2X_7$ receptor. However, given that a number of potential PKC phosphorylation sites are present in the $P2X_7$ receptor (Watters *et al*., 2001), and that phosphorylation of a conserved threonine residue in the N-terminus of $P2X_2$ and $P2X_3$ receptors is necessary for their normal function (Boue-Grabot *et al*., 2000; Paukert *et al*., 2001), we cannot exclude the possibility that a component of the inhibitory action of chelerythrine on $P2X_7$ function may involve PKC. Chelerythrine has been reported to inhibit other channels such as acetylcholine-induced K^+ channel in mouse atrial myocytes (Shi & Wang, 1999) and acetylcholine-induced currents in PC12 cells (Cho *et al*., 2001). Nevertheless, this study confirms that the $P2X_7$ receptor can be added to the list of ion channels directly blocked by chelerythrine.

In addition to ATP-induced $^{86}Rb^+$ efflux, chelerythrine also inhibited ATP-induced PLD stimulation. Chelerythrine blocked 500 μM ATP-induced PLD activity with greater efficacy than 100 μM ATP-induced $^{86}Rb^+$ efflux in human B-lymphocytes (94.2 ± 21.9 *versus* $73.4 \pm 3.5\%$, respectively) suggesting that chelerythrine not only blocks $P2X_7$ but may also act on signalling events, such as PKC, downstream of this receptor. Consistent with this idea, chelerythrine inhibited PLD activity induced by PMA, a known activator of PKC. Moreover, recent studies have indicated that ATP *via* $P2X_7$ receptor activation stimulates other kinases such as stress-activated protein kinase and Rho-effector kinase (Humphreys *et al*., 2000; Verhoef *et al*., 2003). The ATP- and PMA-induced PLD activity could not be investigated in the presence of sanguinarine or berberine, as these compounds are only soluble in alcohol and this alcohol interferes with the transphosphatidylation assay used to determine PLD activity (data not shown). Collectively, these results suggest that further studies are needed to confirm if PKC is activated by $P2X_7$ and if PKC is involved in the regulation of ATP-induced PLD stimulation *via* the $P2X_7$ receptor.

Studies using $P2X_7$-deficient mice have shown that these animals have reduced inflammatory responses (Labasi *et al*., 2002) possibly due to reduced $P2X_7$-mediated secretion of interleukin-1β from cells of the monocyte–macrophage lineage (Solle *et al*., 2001), which is due, in part, to K^+ efflux (of which Rb^+ is a surrogate) (Perregaux & Gabel, 1994). Interestingly, chelerythrine and sanguinarine have potent anti-inflammatory effects *in vivo* (Lenfeld *et al*., 1981). Thus, it is tempting to speculate that the previously described anti-inflammatory effects of these benzophenanthridine alkaloids may arise from inhibition of the $P2X_7$ receptor.

In conclusion, our data highlight the importance of ensuring that kinase inhibitors employed in studying signalling events downstream of $P2X_7$ do not affect the receptor directly.

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