British Journal of Pharmacology (1998) 125, 235–237
http://www.stockton-press.co.uk/bjp

SPECIAL REPORT

Fading and rebound of $P2X_2$ currents at millimolar ATP concentrations caused by low pH

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When ATP is applied at high concentrations (above 1 mM) to PC12 cells, it produces a rapidly desensitizing peak current followed by a rebound of the current after termination of the ATP application. We expressed $P2X_2$ receptors, which are thought to mediate the ATP currents of PC12 cells, in HEK293 cells and studied the effects of acidification on this 'fading and rebound' phenomenon. We found that the desensitization disappeared after adjusting the low pH (<5.0) of the millimolar ATP concentrations to a more physiological value (pH 7.3). Furthermore, the fading and rebound could also be induced at much lower ATP concentrations by decreasing the pH of the ATP containing application solutions. Thus, it appears this phenomenon is not caused directly by high concentrations of ATP, but is due to a concomitant acidification that occurs when high concentrations of ATP are dissolved in only moderately buffered application solutions.

Keywords: ATP; $P2X_2$ receptor; desensitization; proton block; PC12 cells

Introduction $P2X_2$ receptors belong to the family of ATP ligand-gated ion channels. Seven subunits of this family have meanwhile been cloned ($P2X_{1-7}$, Buell *et al*., 1996). $P2X_2$ receptors have been cloned from PC12 cells (Brake *et al*., 1994) and, when expressed in HEK293 cells, exhibit characteristics typical of currents induced by ATP in PC12 cells. Thus, they have similar affinity for ATP and other ATP analogues and are, unlike $P2X_1$ and $P2X_3$ receptors, typically non desensitizing at EC_{50} values (Evans *et al*., 1995; Surprenant *et al*., 1995). Recently, however, a rapidly desensitizing current in PC12 cells has been reported when ATP was applied by U-tube or by pressure application from a pipette in concentrations above 1 mM (Giniatullin *et al*., 1996). This desensitizing current was followed, upon termination of the ATP application, by a long lasting second current. The two currents have respectively been termed the 'fading' and the 'rebound' ATP currents. Further reports have meanwhile appeared describing factors that influence this phenomenon while trying to address its underlying mechanism (Giniatullin *et al*., 1996; Khiroug *et al*., 1997a, b). We decided to determine if a similar phenomenon occurs in cloned $P2X_2$ receptors expressed in HEK293 cells and, as it has been found that changes in extracellular pH can drastically alter the ligand affinity of $P2X_2$ receptors (King *et al*., 1996; Stoop *et al*., 1997), to examine if it is sensitive to acidification of the extracellular medium.

Methods Complementary DNA encoding the $P2X_2$ receptor, originally cloned from rat phaeochromocytoma cells (Brake *et al*., 1994, gift of D. Julius, University of California at San Francisco) and subcloned in pCDNA3 (Stratagene, La Jolla, CA, U.S.A.), was used to stably transfect HEK293 cells (Kawashima *et al*., 1998). Whole-cell recording were made from HEK293 cells using an Axopatch 200 patch-clamp amplifier (Axon Instruments, Foster City, CA, U.S.A.). Patch pipettes (4–7 MΩ) contained (in mM): potassium aspartate 145, EGTA 11, HEPES 5 and NaCl 5, the external solution contained (in mM): NaCl 145, KCl 2, $MgCl_2$ 1, $CaCl_2$ 2.5, HEPES 10 and glucose 10. pH values of internal and external solutions were adjusted with NaOH or HCl to pH 7.3 unless indicated otherwise. Agonists were applied using a fast-flow U-tube delivery system which resulted in a fast onset equilibration within 10–100 ms, but a variable offset depending on bath superfusion rate, placement of U-tube relative to cell etc. (Fenwick *et al*., 1982). All currents were recorded at a holding potential of −60 mV. Reproducible responses were obtained with these receptors by applying ATP for 2 s at intervals of 2–3 min. ATP disodium salt (Na_2H_2ATP) was obtained from Sigma Chemical Co. (U.K.).

Results We applied ATP to cloned $P2X_2$ receptors, stably expressed in HEK293 cells, and obtained currents with time courses characteristic of a U-tube perfusion (Figure 1a and d). The offset of these currents is typically slow, due to a gradual replacement of ATP with perfusion medium that results from the continuous superfusion of the bath. We measured the peak currents evoked by ATP concentrations from 0.1 to 100 μM and found an expected EC_{50} value of 12 ± 2 μM ($n=5$, see also Stoop *et al*., 1997). The currents remained at this point still non desensitizing, even at saturating concentrations of up to 100 M ATP. However, when we applied ATP at concentrations of 3 mM and higher, a rapid desensitization started to occur followed by a rebound current at the end of the application very similar to the findings by Giniatullin *et al*. (1996). (Figure 1b). In fact, an identical desensitization and rebound phenomenon was also observed in PC12 cells in our lab at ATP concentrations $\geqslant 1$ mM (A. Surprenant, personal communication). Since the ATP had been dissolved in external solution without subsequent pH adjustment, we measured the pH of the ATP solutions and found a marked decrease in pH for ATP concentrations of 3 mM and higher (to as low as pH 4.2 in a 10 mM ATP solution). After adjusting the 10 mM ATP solution to pH 7.3, the rapid desensitization and rebound completely disappeared (Figure 1c). Application of this pH-adjusted ATP solution to

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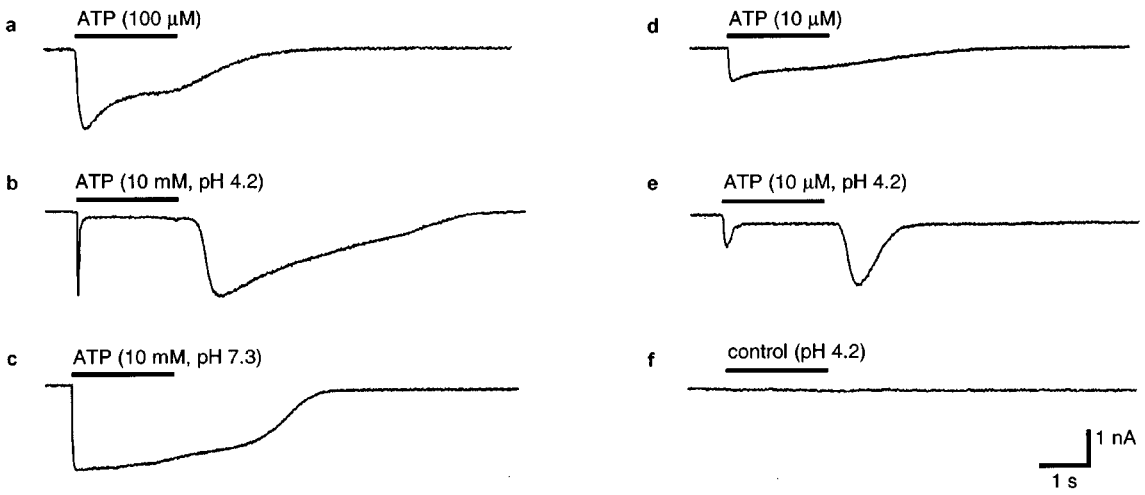

Figure 1 Examples of membrane currents induced by U-tube application of different concentrations of ATP (as indicated) at pH 4.2 or 7.3 (total number of experiments $n=4-6$ per trace). Duration of ATP application indicated by bars above each trace.

the same cell now resulted in an ATP current similar to the one induced by 100 μM ATP, with differences typically expected from a U-tube perfusion with a high concentration of ligand: The current induced by 10 mM ATP exhibited a faster rise time than the one obtained with 100 μM ATP and lasted longer at the end of the perfusion due to the slower wash-out of ATP.

We then asked whether we could induce a similar desensitization at lower ATP concentrations by decreasing the pH of the ATP solution. We first measured currents induced by a 10 μM concentration of ATP which had not undergone any measurable acidification by ATP (Figure 1d). Subsequently, we decreased the pH of this 10 μM ATP solution to pH 4.2 and measured the currents induced by ATP at this lower pH. As can be seen in Figure 1e, a desensitizing current could now also be observed that showed a rebound upon removal of ATP administration, resembling the trace with the unadjusted 10 mM ATP application (Figure 1b). In order to verify that these effects were not due to proton-gated ion channels (Waldmann *et al*., 1997), we applied a solution of pH 4.2 that did not contain any ATP to the same cell. Perfusion with this low pH solution alone did not evoke any currents (Figure 1f), thus excluding any effects from proton-gated ion channels.

Discussion Our results show that the desensitization of the $P2X_2$-activated current during application and the rebound of the current after application of concentrated ATP can be eliminated by adjusting pH values of the ATP solutions to physiological levels. Inversely, they can be induced at low ATP concentrations by decreasing the pH of the ATP solution. Thus, it appears the fading and rebound is not caused by the high concentration of ATP, but is due to the low pH of the application solution.

How can we explain this pH effect? In a recent study (Stoop *et al*., 1997) we have shown that extracellular acidification ot levels as low as pH 5.3 increases the ATP affinity of $P2X_2$ receptors (see also King *et al*., 1996). During these experiments, we had also found that a further decrease of the pH in the external medium and in the U-tube to values below pH 5.0 lead to a drastic block of the ATP induced current, which could not be overcome by increasing concentrations of ATP (unpublished observations). We here postulate that the fast desensitization is in fact a proton block that occurs very quickly after U-tube application of ATP concentrations of 3 mM (or higher), which are causing an acidification of the external solution to a pH of 5.0 (and lower). The rebound of the current following the end of the ATP application consequently results from the gradual washout of ATP after U-tube exposure which leads to a simultaneously less acidic environment. When the pH value increases above pH 5.0, the proton block is relieved and remaining ATP can at this point still open the $P2X_2$ channels. In fact, the still relatively low pH may at the same time increase the affinity for ATP, thus giving rise to a long lasting and large rebound current.

We would like to thank D. Estoppey and D. Fahmi for technical assistance with the cell cultures and Drs R.A. North and A. Surprenant for support and helpful advice.

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(Received April 6, 1998
Accepted June 15, 1998)