British Journal of Pharmacology (1996) 119, 1045-1053 © 1996 Stockton Press All rights reserved 0007-1188/96 $12.00

Fading and rebound of currents induced by ATP in PC12 cells

*R. Giniatullin, L. Khiroug, *M. Talantova & [1]A. Nistri

Biophysics Sector and INFM Unit, International School for Advanced Studies (SISSA), 34013 Trieste, Italy [1,]*Department of Physiology, Kazan Medical University, Kazan, Tatarstan, Russia

1 Patch clamp recording (whole cell configuration) was used to study the action of ATP on rat phaeochromocytoma (PC12) cells usually held at −70 mV and rapidly superfused with buffered saline. ATP (0.5, 1 or 5 mM), applied from micropipettes by pressure application with brief (≤50 ms) pulses, induced inward currents with rapid onset and decay. ADP and α,β-methylene ATP were ineffective.

2 ATP (5 mM) applied with pulses >200 ms long elicited a complex current response characterized by a rapid peak which faded and was followed by a strong current rebound (lasting several s) as soon as the application was terminated. This type of response was readily replicated as long as ATP applications were spaced at 2-3 min intervals. The amplitude of peak and rebound currents was dependent on the length of pressure pulse and was similarly depressed by bath application of a threshold dose (25 μM) of ATP. Rapid fading and rebound of ATP-induced membrane currents were also observed when the Y-tube method was used for applying this agonist.

3 The reversal potential for peak and rebound currents was the same while the time constant values for peak fading and rebound onset were insensitive to changes in membrane potential between −70 and −40 mV. When ATP was applied to a cell clamped at depolarized potential, no current was observed but rapid return of the membrane potential to −70 mV immediately at the end of ATP application was associated with a large rebound current.

4 Brief (20 ms) application of ATP during the onset of the rebound current strongly and transiently suppressed it. The same application performed during the gradual decay of the rebound wave elicited a transient inward current which was much smaller and shorter than the one observed when the cell was in its resting state. Application of 2 s ATP pulses at 20 s intervals equally reduced the initial peak and rebound currents which recovered at the same rate.

5 The present data are interpreted according to a scheme which suggests two types of ATP receptor desensitization. The first one (D1) would be characterized by fast kinetics and low agonist affinity; rapid recovery from D1 would then be manifested as current rebound presumably due to receptor reactivation. The second desensitized state (D2) has slow kinetics and high affinity for the agonist: it is therefore typically seen with sustained application of a low dose of ATP. It is proposed that desensitization and its recovery can influence the time course of membrane responses mediated by purinoceptors.

Keywords: ATP; desensitization; purinoceptor; patch clamp

Introduction

ATP-activated receptors (termed P_2 receptors) are widely distributed in various tissues and are involved in functions such as muscle contraction, neurosecretion, etc. (Burnstock & Kennedy, 1985; 1986; Burnstock, 1990; Lewis *et al.*, 1995). In general, P_2 receptors can be subdivided into two main subclasses, termed P_{2X} and P_{2Y}, respectively (Burnstock & Kennedy, 1985). P_{2X} receptors are ligand-gated channels (see review by Humphrey *et al.*, 1995) while P_{2Y} receptors are coupled to G-proteins and operate via intracellular second messenger pathways (Burnstock, 1990). Considerable advance in the understanding of the structural properties of P_{2X} receptors has been gained by recent cloning studies which have demonstrated the existence of at least six subtypes (see Collo *et al.*, 1996). Like many other ligand-gated receptor channels, P_{2X} receptors are known to elicit responses which fade upon continuous agonist application. Such a phenomenon is presumably due to receptor desensitization which displays tissue-dependent characteristics (Lewis *et al.*, 1995). Other mechanisms might also be responsible for this phenomenon, such as, for instance, agonist-induced open channel block as first suggested for nicotinic acetylcholine (ACh) receptors (Sine & Steinbach, 1984; Ogden & Colquhoun, 1985; Colquhoun & Ogden, 1988). Curiously, on some cells, the cholinergic response fade can be followed by a rebound of activity immediately after removal of the agonist (Maconochie & Knight, 1992). It is uncertain whether purinoceptors display analogous behaviour.

Purinoceptors present on rat PC12 cells are of special interest in view of the fact that, although they are ligand-gated channels which mediate cell depolarization (Nakazawa *et al.*, 1990; Nakazawa & Inoue, 1992), they are pharmacologically dissimilar from typical P_2 receptors of peripheral tissues (Raha *et al.*, 1993; Kim & Rabin, 1994) and their structure has recently been elucidated with cloning studies (see for example Brake *et al.*, 1994). Since little is known about the properties of ATP receptor desensitization on rat phaeochromocytoma cells, the present study examined the characteristics of the response fade and whether it was followed by a rebound.

Methods

Cell preparation

Rat PC12 cells (15-20 μm body diameter) were kindly provided by the C.N.R. Institute of Neurobiology, Rome, Italy. Cells were grown in a 5% CO_2 atmosphere at 37°C in RPMI medium (Gibco; Milan, Italy) supplemented with 10% heat inactivated horse serum (Gibco; Milan, Italy) and 5% foetal calf serum (Seromed; Berlin, Germany) with addition of penicillin/streptomycin (200 units ml^{-1}). Culture medium was changed twice a week. On the day of the experiment, cells were detached from the bottom of the dish and dissociated by passing them through a 0.9 mm o.d. needle, and subsequently

[1] Author for correspondence.

transferred to 24 mm Petri dishes covered with poly-L-lysine (1.25 mg ml^{-1}). After 40–60 min the culture medium was replaced with control solution containing (in mM): NaCl 132, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, glucose 10, HEPES 10 (pH was adjusted to 7.4 with NaOH).

Patch clamp recording

Experiments were performed on single cells in continuously superfused Petri dishes (perfusion rate was set at 5–10 ml min^{-1} to aid drug removal; room temperature throughout) mounted on the stage of an inverted Nikon TDM microscope. Patch pipettes were pulled from thin glass (1.5 mm o.d.) and had resistance of 1.5–3 MΩ when filled (in mM) with CsCl 120, EGTA 5, HEPES 20, $MgCl_2$ 1 and Mg_2ATP_3 3. Cells were usually voltage-clamped at −70 mV (unless otherwise indicated) in the whole-cell configuration with GΩ seals. On a sample of cells ($n=7$) used for the present experiments uncompensated series resistance was 37±2 MΩ (this value was compensated by up to 80%) while cell capacitance was 7±0.5 pF. ATP and its analogues ADP and α,β-methylene ATP (purchased from Sigma Chemical Co., Milan, Italy) were diluted in control solution and, in the majority of experiments, delivered by pressure application (10–20 p.s.i.) from similar pipettes (located about 20 μm away from the recorded cell) using a Picospritzer II (General Valve Co., Fairfields, New Jersey, U.S.A.). In accordance with the method of Zhang *et al.* (1994), tests were run to estimate the speed of drug delivery and removal by measuring the time course of changes in the liquid junction potential of a recording pipette (filled with 150 mM KCl) placed in the external solution following pressure application (5 ms–10 s) of KCl (75 mM) from a nearby (10–30 μm away) pipette. With such a method, peak concentrations were estimated to be reached within 15–20 ms, represented approximately a 3.7–4 fold dilution of the pressure pipette dose (extrapolated from plots of potential amplitude against various concentrations of KCl), remained at plateau during application, and disappeared with a decay time constant of 46.5±1.4 ms ($n=6$) due to the fast superfusion rate. Some experiments were also carried out with a different method for rapid application and removal of ATP: this consisted of the so-called 'Y-tube' technique (Akaike *et al.*, 1991) by which a known concentration of agonist is quickly delivered by gravity and equally quickly removed by suction through the same Y-tube. Electrophysiological responses were recorded with a List L/M-PC amplifier (List, Darmstadt), filtered at 1 kHz and stored on disk using pCLAMP software (5.5 version; Axon Instruments, Foster City, California, U.S.A.). Responses were measured in terms of amplitude, or exponential onset and decay (fitted with the programme Clampfit from the same software). Graphs were constructed with Origin software (Microcal Software Inc, version 2.94). Data are presented as mean±standard error (s.e.).

Results

Characteristics of membrane currents induced by ATP

At −70 mV holding potential brief (10–50 ms) applications of ATP from pipettes containing 0.5, 1 or 5 mM ATP induced inward currents as shown in Figure 1a for a 5 mM solution.

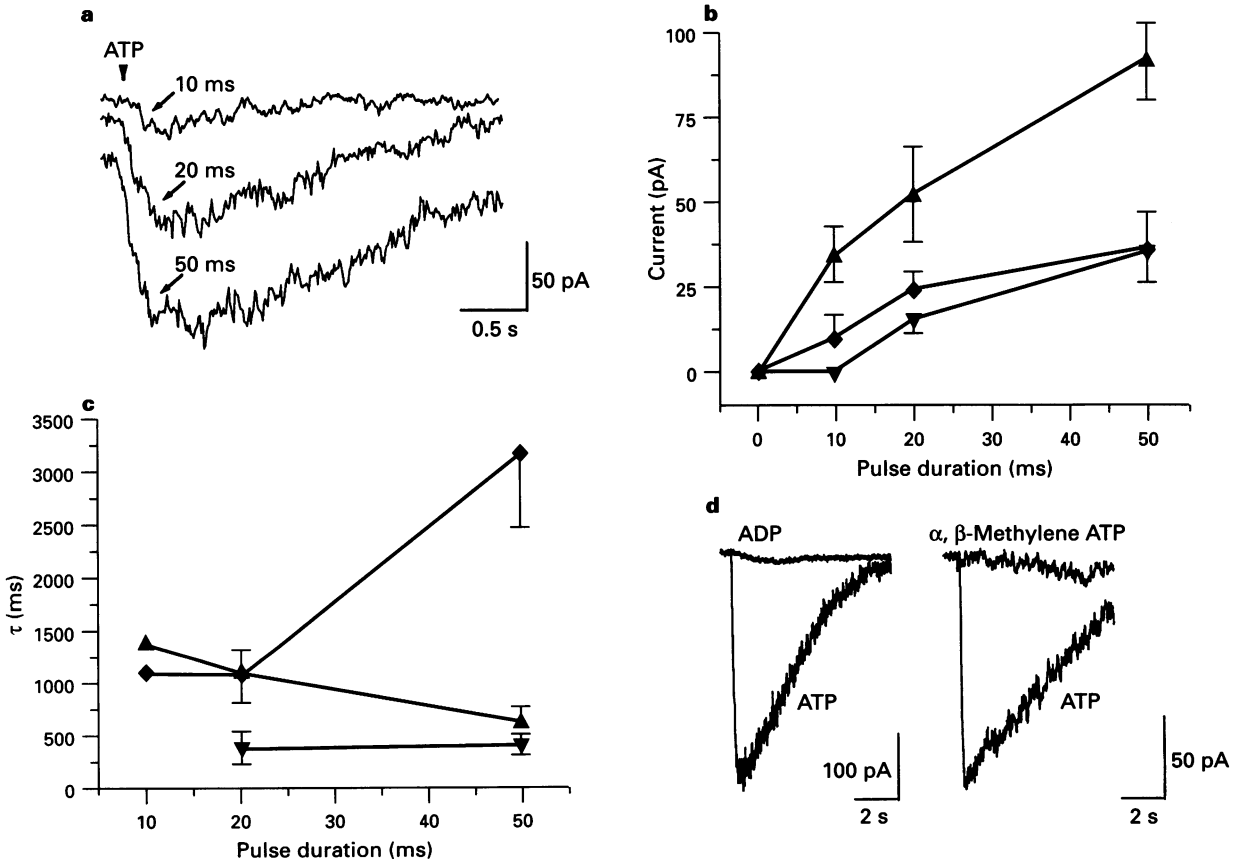

Figure 1 Membrane currents induced by pressure application of ATP. (a) Dependence of amplitude and time course of the current induced by 5 mM ATP on pulse duration (indicated in ms alongside tracings). Arrowhead indicates the time of ATP application. (b) Dependence of peak current amplitude on pulse duration for 0.5 mM (▼), 1 mM (◆), and 5 mM (▲) ATP. Datapoints are from 2–4 cells. (c) Plot of the timeconstant (τ) of the response monoexponential decay against pulse duration for different concentrations of ATP. Symbols, abbreviations and error bars as in (b). (d) comparison of effectiveness of ADP and α,β-methylene ATP versus that of ATP. Drugs were applied by 20 ms pressure pulses from 5 mM solutions (different cells in left and right panels).

Tracings in Figure 1a indicate that the amplitude and time course of ATP-induced currents were related to pulse duration for the same pipette solution. On a sample of 6 cells tested with 20 ms applications from 5 mM solution the currents displayed a 10–90% rise time of 163±14 ms and a slower decay (1130±202 ms time constant, τ, for monoexponential decline). Application time-effect curves depict the relation obtained when 0.5, 1 or 5 mM solutions were used on a sample of cells (Figure 1b). Under these conditions the largest effects were observed with 50 ms applications from 5 mM ATP solution. Pressure applications from 0.5 or 1 mM solutions evoked comparatively smaller amplitude responses (Figure 1b). The time constant values for monoexponential decay of the inward currents possessed a complex dependence on the pulse duration as shown in Figure 1c: when 0.5 mM solution was used there was no apparent variation, whereas the time constant value grew for increases in duration of pulses with the 1 mM solution and attenuated with the 5 mM one. With pulses >50 ms from a 5 mM solution the current decay became multiexponential (not shown). Pressure applications of 5 mM solutions of either ADP or α,β-methylene ATP produced only weak responses which in the example of Figure 1d were <3% of those elicited by ATP at the same concentration. The responses to short pulses of ATP were stable and highly reproducible at applications rates as high as 1 every 60 s.

Current fade and rebound

Figure 2a shows that 20 ms ATP (5 mM) application (filled arrowheads above tracings) induced a large inward current with a rise time of 205 ms. Increasing the duration of the ATP pulse to 200 ms (filled horizontal bar) generated an initial current with smaller peak (see thick tracing; rise time = 130 ms) than that observed with the shorter application (medium thickness tracing; arrowhead) and was followed by fast current fade (see filled arrow). Immediately after the end of 200 ms drug delivery the response rapidly increased (termed rebound) up to the level observed with the 20 ms application (Figure 2a, filled arrow) despite fast superfusion of the cell which rapidly lowered extracellular ATP. When the pressure pulse was 2 s long (open bar above tracings), the initial current peak was the same as with the 200 ms application (rise time was also very similar, i.e. 140 ms) and then faded to a plateau level (open arrow). In such a case the current decline from its peak value could be fitted by two exponentials ($\tau_1 = 95 \pm 15$ ms; $\tau_2 = 4.3 \pm 0.2$ s; $n = 5$). Immediately after the end of the 2 s drug application current rebound also developed. This pattern of current fade and rebound was readily reproduced as long as ATP pulses were applied at 2–3 min intervals. It is also interesting to note that in the example of Figure 2a the τ_1 value for current fade shortened from 1097 to 264 ms when the pressure application time was varied from 0.2 to 2 s, while the corresponding τ value for rebound onset increased from 1148 to 7994 ms. Figure 2b shows the relation between the latency of the rebound (from the start of the pulse) and the pulse duration (0.2–10 s): this was found to be linear and in no instance did the rebound develop before the end of the application.

The unexpected observation of a large current rebound raised the possibility that this response was somehow caused by the method of pressure application of the agonist and perhaps its incomplete removal. In order to rule out this potential artefact, ATP (5 mM) was also applied by the Y-tube method (Akaike *et al.*, 1991). Furthermore, it was equally important to check the time course of the signals produced in blank experiments by KCl applied either by pulse pressure or by the Y-tube technique, since these observations can suggest the time profile of agonist presence in the external medium (Zhang *et al.*, 1994). Figure 3 shows a representative example of records obtained following pressure application of KCl (a(i)) or 5 mM ATP (a(ii)): the ATP current peak was generated slightly slower than the maximum

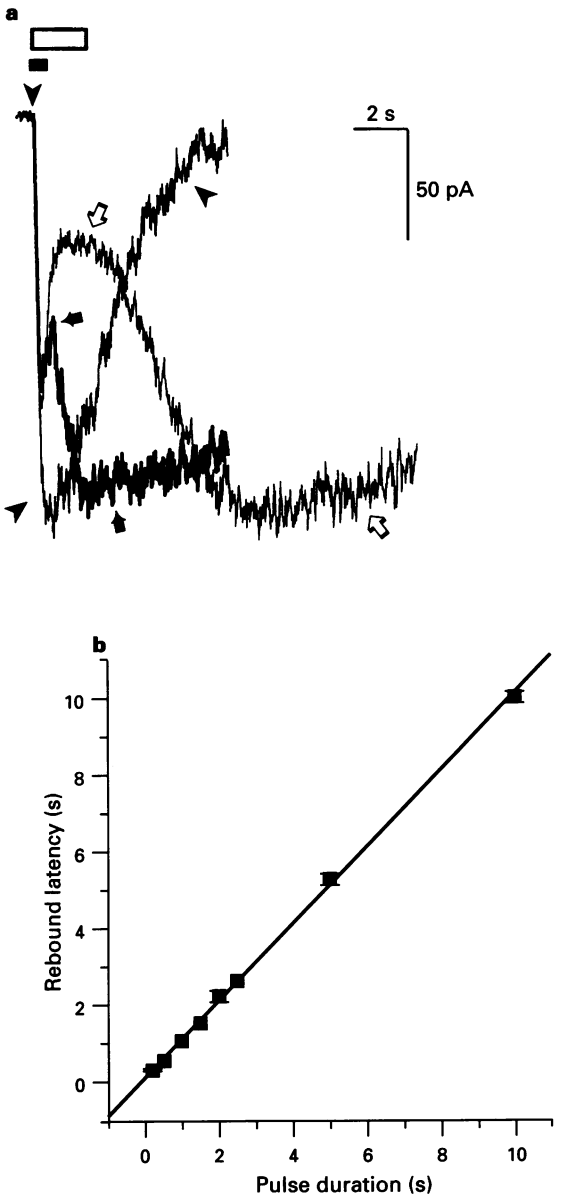

Figure 2 Current fade and rebound with 5 mM ATP pulse longer than 100 ms. (a) Superimposed tracings of responses evoked by 20 ms (filled arrowheads and medium thickness trace), 200 mM (thick trace and filled arrows), and 2000 ms (thin trace and open arrows) pulses of 5 mM ATP. Above baseline arrowhead indicates time of 20 ms ATP application, while filled and open bars show the duration of 200 ms and 2000 ms pulses, respectively. (b) Correlation between pulse duration and latency for onset of rebound; latency was measured as the time interval from the beginning of the pulse to the crossover of the rebound current slope with the current level at the end of the pulse. Data points (from 2–4 cells) are fitted with a linear equation where Y = 0.0887 + 0.9996 *X (0.9996 is the correlation coefficient).

of the KCl-induced signal while the ATP rebound current appeared when the KCl signal had already dissipated. Figure 3b shows comparative data obtained with the Y-tube application of KCl (b(i)) or ATP (5 mM (b(ii)). Also in this case the ATP-induced current peak had an onset similar to that of the KCl signal and showed a rebound at the time when the KCl-induced liquid junction change had disappeared. Similar observations were obtained in three cells. These results confirm that the ATP current rebound was observed with two different methods of application and was not dependent on slow agonist washout.

The relation between ATP-induced peak current and rebound was explored in a more systematic fashion as indicated

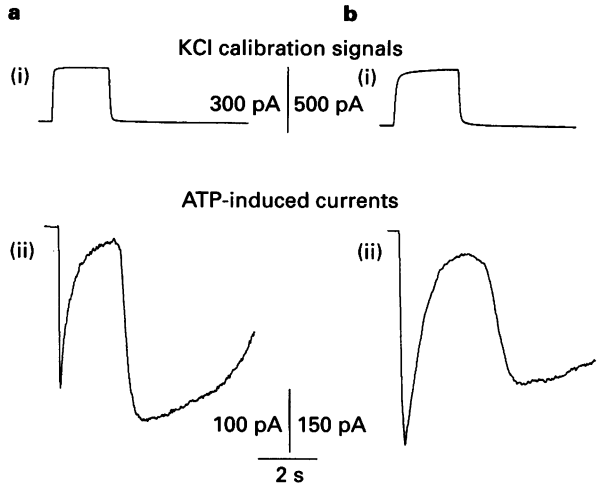

Figure 3 ATP (5 mM)-induced current fade and rebound following pressure pulse (a) or Y-tube (b) application. (a) Comparison of the time course of the liquid junction change induced by pressure application of KCl (KCl calibration signal, (i)) with that of 5 mM ATP-induced membrane current elicited with the same method of application (ii). (b) (Different cell from a) shows comparable results obtained with Y-tube application. Note that both pressure pulse and Y-tube application of 5 mM ATP caused current fade and rebound.

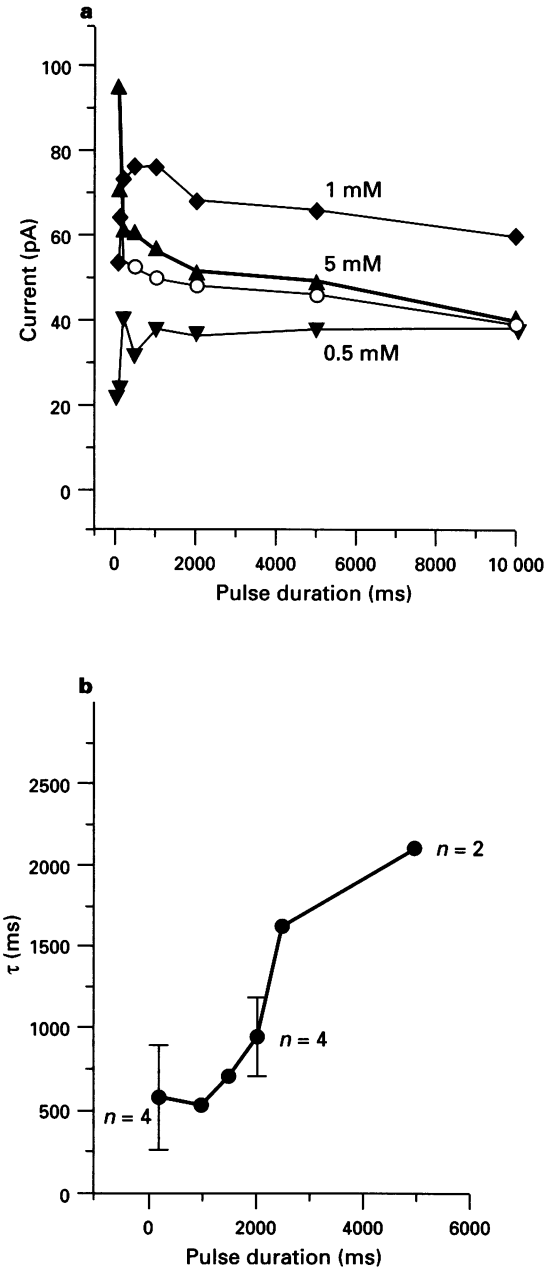

Figure 4 Current peak, rebound amplitude and onset of rebound are dependent on ATP pulse duration. (a) Dependence of current initial peak (filled symbols) and rising phase of the rebound current (whenever present; ○) amplitudes on pulse duration for three pipette concentrations of ATP (0.5 mM, ▼; 1 mM ◆; 5 mM ▲). All data are from the same representative cell on which responses were usually tested twice: (b) Dependence of the time constant (τ) of rebound response evoked by 5 mM ATP on pressure pulse duration. n = number of cells.

in Figure 4a which presents the plot of the amplitude of the first peak (filled symbols) and of the rebound (whenever present; open symbols) currents versus pulse duration for solutions containing 0.5, 1 or 5 mM ATP (all data from the same cell). With a 5 mM solution, the largest response was found with a 100 ms pulse since longer pulses elicited smaller peak responses and were associated with a rebound response of approximately similar amplitude. On average, on a sample of 7 cells a 2 s pulse (5 mM ATP) induced a current peak of 136 ± 43 pA and a rebound of 99 ± 28 pA, that is the rebound was $75 \pm 12\%$ of the peak current. When pulses were several s long, neither the current peak nor the rebound consistently changed in amplitude, indicating that an apparently steady-state condition had been reached. Responses elicited by 0.5 or 1 mM solutions were not followed by a rebound even if some peak current responses (e.g. those to 0.2–1 s pulse from 1 mM solution), though submaximal in absolute terms, were larger than the ones elicited by the 5 mM solution for equivalent pulse duration. Only in 1/11 cells did 0.5 or 1 mM solutions evoke rebound effects.

As indicated by the sample tracings of Figure 2a the rate of rise of the rebound wave was faster when the application was 200 ms instead of 2 s. The graph of Figure 4b quantifies this phenomenon by plotting τ values for the rising phase of the rebound current against ATP pulse duration, though the relation was not linear.

Should ATP current fade and rebound have a common origin, they might share similar sensitivity not only to pressure-applied ATP but also to bath application of the same substance. In Figure 5 this issue was examined by bath-application of a small concentration of ATP (25 μM) which *per se* produced only a minimal inward current (5 pA) that remained stable for about 10 min: subsequent application of a 2 s pulse from a 5 mM ATP solution produced a smaller peak response (37% of control) and rebound (48%) although the ratio between rebound and first peak remained essentially similar before (94%) and during (121%) bath-application of ATP. It is interesting also to note that the current faded to the same absolute level regardless of the presence of bath-applied ATP (13 pA in control solution and 12 pA in ATP solution). In summary then, the rebound wave required a pulse application of longer duration than that needed for maximal responses, was similar in size to the first peak with which it shared comparable sensitivity to ambient ATP, and developed more slowly as the pulse length was increased.

Voltage-dependence of ATP-induced inward currents

The inward rectification of the ATP current at holding potentials less negative than −40 mV (see Khakh *et al.*, 1995) prevented a systematic analysis of the voltage-sensitivity of the current fade and rebound. Nevertheless, within the apparently linear range of the current-voltage relation the slope conductance (calculated between −80 and −40 mV) was relatively similar, being 2.8 ± 0.4 nS for the first peak and

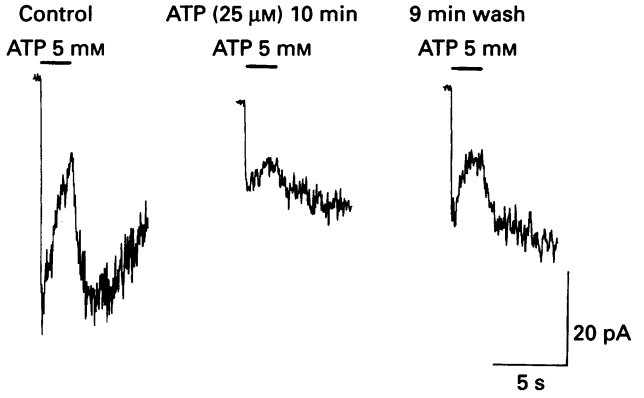

Figure 5 Bath application of 25 μM ATP affects response to 2 s pressure application of 5 mM ATP. Pressure application duration is indicated by the horizontal bars; the time of wash is calculated from the beginning of bath exchange with control solution. In the presence of 25 μM ATP both initial peak and rebound are reduced while there is a modest downward shift of the baseline indicating a slight inward current due to bath-applied ATP.

2.4 ± 0.8 nS for the rebound ($n=3$). Furthermore, the apparently null potential for the rebound wave was -34.6 ± 4.7 mV, a value close to that found for the first peak (-28.4 ± 6.4; $n=4$) following a 2 s pulse of ATP. Figure 6a shows a plot of τ_1 for peak current fade (solid squares) and of τ for rebound onset (solid circles) versus holding membrane potential. These two parameters showed differential dependence on changes in membrane potential only in the range -120 to -90 mV as the τ_1 values for current fade grew with depolarization while τ values for rebound decreased. At less negative membrane potentials (from -70 to -40 mV) neither parameter changed. Figure 6b shows the result obtained with a different voltage clamp protocol: ATP was initially applied for 2 s to the cell clamped at -70 mV to produce a fading response followed by rebound (thick current trace); after a recovery of about 3 min the cell was clamped at $+40$ mV and did not display a response during the application of ATP (owing to strong current rectification). Immediately after the end of ATP delivery the membrane potential was returned to -70 mV (indicated by arrow in Figure 6b) and a rebound similar to the one previously observed became apparent. These data imply that the rebound phenomenon was independent from actual current flow through activated purinoceptors.

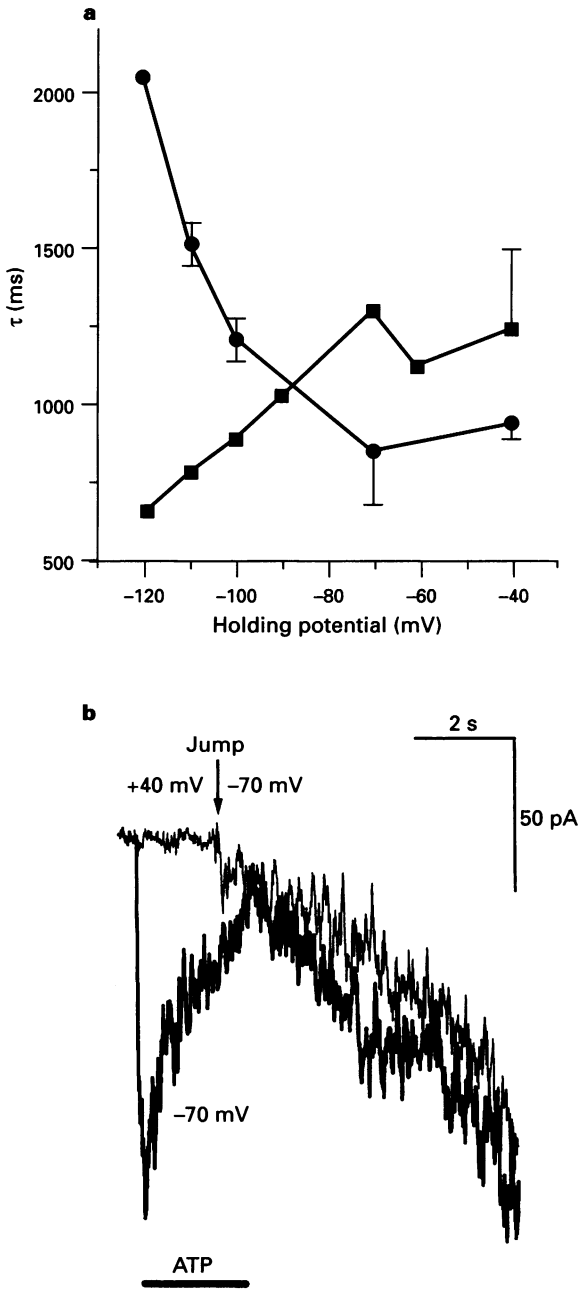

Figure 6 Voltage-dependence of current fade and rebound. (a) Plot of first time constant of current fade (■) and time constant of rebound (●) against holding membrane potential. Data from 2–4 cells. (b) Thick tracing shows current fade and rebound after 2 s application of 5 mM ATP to a cell clamped at -70 mV, while the thin tracing shows the response from the same cell clamped at -40 mV during the 2 s application of ATP, after which the holding potential was stepwise changed to -70 mV (the time of potential jump is indicated by the arrow). With either holding potential the rebound was similar. The horizontal bar corresponds to the time of pressure application of ATP.

Sensitivity of ATP currents to paired-pulse or repetitive applications

Figure 7 shows the effects of a short (20 ms) application of 5 mM ATP either alone (a) or at different intervals (c, d) after a long (2 s) pulse of ATP which produced fade and rebound (b). When the short application (arrowhead) coincided with the developing phase of the rebound (Figure 7c) there was a transient current suppression which lasted for 900–1500 ms and was then followed by the rebound wave. When the short pulse of ATP was given during the decay phase of the rebound (arrow in Figure 7d) but at the same absolute current level as before, the response consisted of a small inward current. Hence, the timing of the short application of ATP meant either suppression or non-linear summation of the response: this result is shown in Figure 7e where the absolute current response produced by 20 ms ATP with respect to the preceding current level is plotted as a function of its interval from the long 2 s application. Similar findings were obtained from three cells. In control conditions the standard frequency of 2 s pluse 5 mM ATP applications was once every 2–3 min to prevent gradual decline of the response. In fact, when 10 mM ATP was pressure-applied for 2 s, responses run down even if spaced at 3 min intervals (data not shown). Figure 8 presents an example of the progressive change in response to 5 mM ATP (2 s pulse application) when the frequency of application was increased first to once every 60 s (see Figure 8b) and then to once every 20 s (Figure 8c). In the latter case the initial current peak was 28% of control while the rebound wave was 42% (recovery was obtained 30 min later; Figure 8d). Figure 8e shows a graph of the time course of peak and rebound currents induced by applying ATP (5 mM) at various intervals: it is apparent that there was a parallel decline (and recovery) for both components of the response.

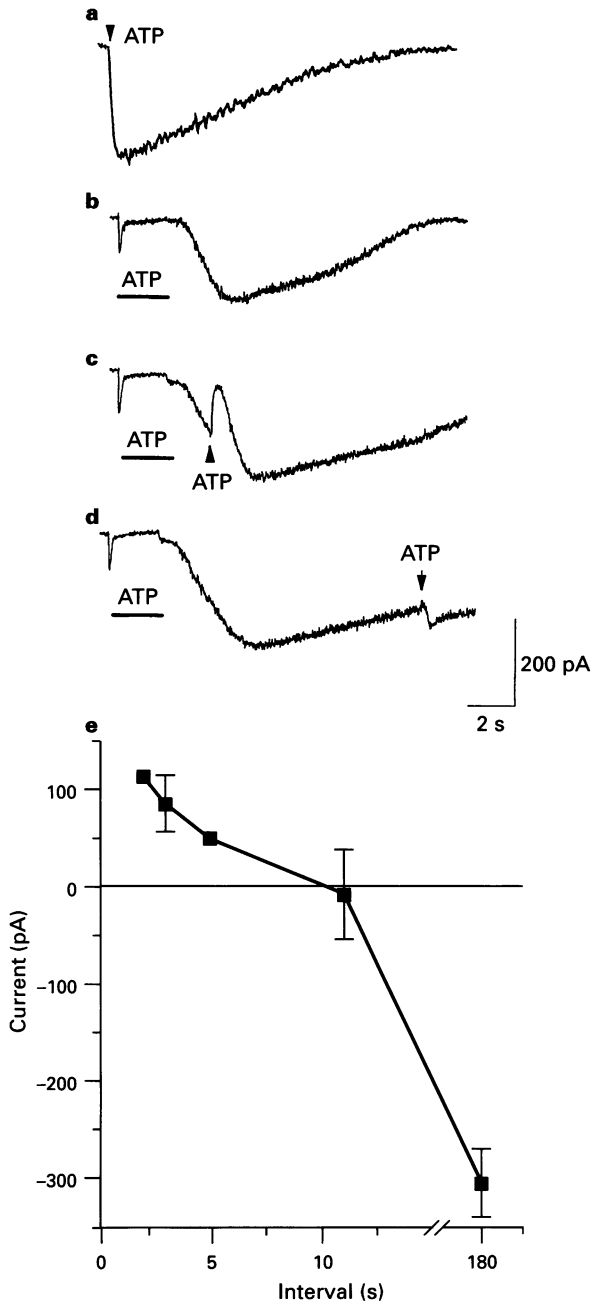

Figure 7 Brief application of ATP can influence rebound response. (a) Large, nondecremental response evoked by 20 ms pulse of ATP (5 mM). The arrowhead indicates the time of application; (b) biphasic response evoked by 2 s application of ATP (5 mM) indicated by the horizontal bar; (c) 20 ms (arrowhead) pulse of ATP applied 2 s after the end of 2 s application (horizontal bar) transiently suppressed rebound current (observed as a outwardly-directed current after which rebound development continued); (d) 20 ms (arrowhead) pulse of ATP applied 11 s after the end of 2 s pulse (horizontal bar) evoked a small inward current; (e) responses (expressed in terms of absolute current amplitude with respect to preceding current level) to 20 ms application of ATP are plotted versus the interval between the end of 2 s application and the delivery of 20 ms pulse. Data are repeated measurements (2–4) from the same cell.

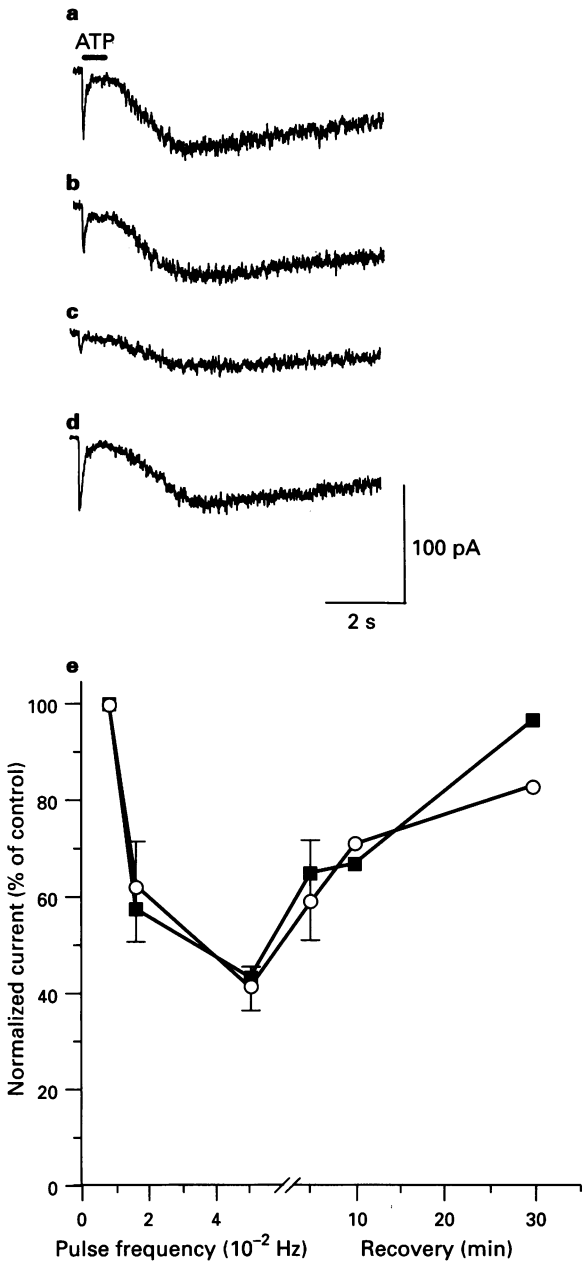

Figure 8 Responses to repetitive 2 s applications (indicated by horizontal line) of 5 mM ATP. (a–d) Show progressive decline in inward currents when application interval was changed from 2 min (a) to 1 min (b) and to 20 s (c) with recovery after 30 min wash (d); (e) plot of current amplitude (as % of control) against pulse frequency (Hz) between successive applications of 5 mM ATP. Data points after break in graph indicate responses at various intervals after the high frequency tests (recovery; note that abscissa scale is now in min). (■) Initial peak currents, (○) rebound currents. Data are from 3–7 cells.

Discussion

The main observation of the present study is the complex current response comprising a rapid peak followed by fading and rebound when high concentrations of ATP were used on PC12 cells. Such complex ATP-induced currents might have been due to either heterogeneity of purinoceptors or distinct kinetic states of a single receptor type. On single smooth muscle cells, ATP activates complex macroscopic current responses via ligand-gated (P_{2X} subtype) and G-protein coupled (P_{2Y} subtype) receptors (Xiong *et al.*, 1991). Previous studies of PC12 cells have shown that these cells, although endowed with G-protein coupled purinoceptors (Grohovaz *et al.*, 1991), typically display ATP-induced inward currents via activation of ligand-gated receptors (Nakazawa *et al.*, 1990; Nakazawa & Inoue, 1992; Brake *et al.*, 1994). In the present experiments on PC12 cells the current peak and rebound induced by ATP were probably caused by activation of a single type of ligand-gated receptor (presumably of the P_{2X} class) for the following reasons: (i) both current components exhibited similar voltage-

sensitivity; (ii) the biphasic current response was not reproduced by ADP or α,β-methylene ATP which have a different degree of effectiveness on P_{2X} receptors of various tissues (Kim & Rabin, 1994; Surprenant *et al.*, 1995); (iii) in control solution the rebound always appeared after the end of the pulse application even if the delivery time varied by one order of magnitude, thus making it unlikely that the rebound was due to delayed, concentration-dependent activation of a separate receptor system; (iv) the initial peak and the rebound wave decreased approximately to the same extent in the presence of bath-applied ATP.

Characteristics of ATP current rebound

ATP current rebound has not apparently been described before. Previous studies of native or cloned PC12 forms of purinoceptors (Nakazawa *et al.*, 1990; Nakazawa & Inoue, 1992; Brake *et al.*, 1994; King *et al.*, 1996) did not detect current rebound, probably because lower ATP concentrations and slow rates of ATP application (e.g. by bath superfusion) were used. In the present experiments current rebound was observed with ATP pipette concentrations >1 mM and pulse duration $\geqslant 200$ ms (15–20 ms solution exchange time). One might suggest that with an even faster drug delivery system the rebound phenomenon might develop with lower concentrations of ATP. Indeed, a similar rebound effect has been observed in ACh-activated channels from bovine adrenal chromaffin cells with >1 ms agonist application (Maconochie & Knight, 1992). The present data suggest that fading and rebound of ATP currents originated from some common mechanism. In fact, the latency and the onset time constant of the rebound were proportional to the ATP pulse duration which also determined the extent of current fading. Furthermore, the rate of rebound development was inversely related to the fading rate of the first peak response.

ATP current fading and rebound can be accounted for by receptor desensitization

In the light of the above considerations it seems interesting to discuss first why responses to ATP faded since this approach might provide a unitary hypothesis to account for the fade and rebound phenomena. In general, there are two possible mechanisms for current fading under the action of high concentrations of agonist, namely desensitization (Katz & Thesleff, 1957) and open channel block (Sine & Steinbach, 1984; Ogden & Colquhoun, 1985). Fading and rebound of ACh receptor activity in the presence of agonist high concentrations was attributed by Maconochie & Knight (1992) to blocking and unblocking of open channels by ACh itself. Analogous suggestions were advanced for comparable phenomena induced by excitatory amino acids on cultured neurones (Vlachova *et al.*, 1987) and for the agonist action of (+)-tubocurarine on mutant ACh receptors (Bertrand *et al.*, 1992). In the present study some arguments stand against open channel block/unblock as the most plausible explanation for current fading and rebound induced by ATP. First, it is known that the action of open channel blockers is highly voltage-dependent (Adams, 1976; Neher & Steinbach, 1978; Sine & Steinbach, 1984; Ogden & Colquhoun, 1985). On PC12 cells the τ_1 value for peak current fading (and τ for rebound onset) did not vary for membrane potentials less negative than -70 mV. This accords with the observations of Dudel *et al.* (1992) who have found significant open channel block by ACh of nicotinic receptors only at potentials more hyperpolarized than -80 mV. Moreover, the rebound phenomenon was the same when the cell was clamped at $+40$ mV or at -70 mV during the ATP application. It means that the rebound phenomenon was independent of current flow through ATP-activated channels. Second, ATP molecules are negatively charged and thus unlikely to be attracted to cationic channels (see for example, Neher, 1983). These findings thus favor fast desensitization as the main mechanism for current fading in the presence of high concentrations of ATP. For a more direct identification of the underlying mechanisms it will, however, be necessary to perform single channel recording. Assuming that desensitization was the predominant process, the onset of the rebound phenomenon at the end of ATP application might have reflected the recovery from fast desensitization of purinoceptors. Fast desensitization was observed not only as quick current fading during continuous application of a high concentration of ATP but also when a short (20 ms) pulse of ATP (applied during the onset of the rebound) rapidly suppressed the slowly-developing inward current. Short application during the decline of the rebound current did not produce any profound, additional desensitization but only a small inward current. It seems likely that the different responses induced by short pulses of ATP reflected the dissimilar concentration of ATP at receptor level immediately before these brief applications. Fast desensitization can also explain the reduced peak amplitude of inward currents induced by long vs brief pulses of ATP: in fact, since the lengthening of the ATP pulse is expected to augment the agonist dose delivered to the cell, desensitization which is known to be time- and concentration-dependent (Katz & Thesleff, 1957; Dudel *et al.*, 1992) would develop rapidly to curtail the inward current peak.

In addition to such a process of fast desensitization which developed quickly within hundreds of ms, slow desensitization (over a span of some min in terms of onset and recovery) was also present: this phenomenon would be analogous to the one reported for nicotinic receptors (Sakmann *et al.*, 1980) and, in the present study, was manifested as a reversible decrease in responses induced by 5 mM ATP after bath application of low doses of the same agonist (cf. Katz & Thesleff, 1957) or when ATP was applied repetitively (0.05 Hz). These data are also in agreement with observations by Nakazawa *et al.* (1990) who, using a slow drug application system, reported slow rate of desensitization and recovery in these cells.

A model for receptor activation, desensitization and reactivation

The present data can be described by a model which takes into account the experimental observations of rapid fade and rebound of the ATP current plus the classical description of desensitization according to Katz & Thesleff (1957). It should be emphasized that this is only an operational scheme to provide a framework for further studies. Accordingly:

$$\begin{array}{ccc} A + R & \longleftrightarrow & AR^* \longleftrightarrow AD1 \\ \uparrow\downarrow & & \uparrow\downarrow \\ A + D2 & \longleftrightarrow & AD2 \end{array}$$

where A is the agonist (ATP), R and R^* are the resting and activated receptors, and D1 and D2 are receptors in fast and slow desensitized states, respectively. For sake of simplicity this scheme implies that one ATP molecule is sufficient for receptor activation and that ATP-bound receptors are either in an active conformation or desensitized.

According to this model, in the presence of large concentrations of ATP, the ATP-activated receptor (AR^*) might quickly turn to the AD1 state (experimentally observed as rapid current fading) from which recovery to the AR^* state (experimentally observed as current rebound developing with an order of magnitude slower than the initial fading) would ensue only after ATP delivery in view of fast superfusion which aided removal of free agonist molecules. The rebound phenomenon might therefore be based on reopening of channels as they dwell in an active state before entering a resting state. Since high concentrations of ATP were required to observe rapid desensitization and rebound, D1 presumably represents a low-affinity receptor conformation. This property would

explain why with pipette concentration of ATP $\leqslant 1$ mM current fading and rebound were very rarely seen. Fast desensitization (D1) thus appeared to be the predominant phenomenon during the action of high concentrations of ATP as the model implies fast transition to and from the D1 state. On the other hand, the D2 state might be induced by the continuous presence of low concentrations of ATP activating only a small fraction of channels. This phenomenon would be typically observed following sustained bath application of low doses of ATP which depressed peak and rebound currents evoked by a large concentration of ATP to a similar degree by reducing the number of available receptors for ATP binding. Hence, the D2 state would represent a high-affinity receptor conformation. Might the D2 state have a role in shaping responses to low concentrations of pressure-applied ATP? This seems unlikely because with the lowest pipette concentration of ATP tested (0.5 mM) the inward current declined monoexponentially with the same τ value even if the pulse duration (and thus the agonist concentration) was more than doubled. Curiously, when the pipette solution was 1 mM, the τ of current decline increased with a longer pulse application (50 ms): perhaps this phenomenon reflected recruitment of distant receptors by an increasing dose of agonist. Further dose increments reduced the τ value as desensitization presumably limited the response duration.

Functional implications of current desensitization and rebound

The rebound process observed in our experiments can be regarded as a novel manifestation of ATP buffering by desensitzed receptors. Such a buffering might be observed with either exogenously-applied agonists or endogenous transmitter. Thus, agonist buffering by desensitized $GABA_A$ receptors has been shown with exogenously-applied GABA (Jones & Westbrook, 1995) and has led to the proposal that reopening of GABA channels after desensitization may be the physiological mechanism which produces a long-lasting component of the transmitter-induced inhibitory current. However, the functional consequence of agonist buffering might be not only current prolongation or biphasic current responses as found in the present study but also shortening of agonist-evoked currents (Magazanik *et al.*, 1990; Giniatullin *et al.*, 1993). The outcome would depend on factors such as receptor affinity, and efficiency of any agonist breakdown mechanism. In the case of purinoceptors the ATP current rebound might be expected to prolong purinergic synaptic currents when the ATP concentration reaches the mM range perhaps as a result of impaired ecto-ATPase activity. Such a current rebound might then be an amplifying mechanisms to support sustained Ca^{2+} influx through the ATP-gated channels which are permeable to this cation (Humphrey *et al.*, 1995).

We thank Prof. A. Cattaneo and Ms Anna Di Luzio for supplying PC12 cells, and Mr Massimo Righi and Ms Gabriella Rossi for cell culturing facilities and technical support. We are also grateful to Dr Laura Ballerini for her help with the Y-tube experiments. This work was supported by a grant from the Istituto Nazionale di Fiscia della Materia (INFM) and from Consiglio Nazionale delle Ricerche (CNR). R.G. and M.T. are grateful to the Russian Foundation for Fundamental Research (RFFR) for financial support. L.K. gratefully acknowledges a PhD studentship from the International Center for Genetic Engineering and Biotechnology (ICGEB).

References

ADAMS, P.R. (1976). Drug blockade of open end-plate channels. *J. Physiol.*, **260**, 531–552.

AKAIKE, N., SHIRASAKI, T. & YAKUSHIJI, T. (1991). Quinolones and fenbuten interact with $GABA_A$ receptor in dissociated hippocampal cells of rat. *J. Neurophysiol.*, **66**, 497–504.

BERTRAND, D., DEVILLERS-THIERY, A. REVAH, F., GALZI, J.-L., HUSSY, N., MULLE, C., BERTRAND, S., BALLIVET, M. & CHANGEUX, J.-P. (1992). Unconventional pharmacology of a neuronal nicotinic receptor mutated in the channel domain. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 1261–1265.

BRAKE, A.J., WAGENBACH, M.J. & JULIUS, D. (1994). New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature*, **371**, 519–523.

BURNSTOCK, G. (1990). Purinergic mechanisms. *Ann. N.Y. Acad. Sci.*, **603**, 1–17.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P2-purinoceptors? *Gen. Pharmacol.*, **16**, 433–440.

BURNSTOCK, G. & KENNEDY, C. (1986). A dual function for adenosine-5'-triphosphate in the regulation of vascular tone. *Circ. Res.*, **58**, 319–330.

COLLO, G., NORTH, A.N., KAWASHIMA, E., MERLO-PICH, E., NEIDHART, C., SURPRENANT, A. & BUELL, G. (1996). Cloning of $P2X_5$ and $P2X_6$ receptors and the distribution and the properties of an extended family of ATP-gated ion channels. *J. Neurosci.*, **16**, 2495–2507.

COLQUHOUN, D. & OGDEN, D.C. (1988). Activation of ion channels in the frog end-plate by high concentrations of acetylcholine. *J. Physiol.*, **395**, 131–159.

DUDEL, J., FRANKE, C. & HATT, H. (1992). Rapid activation and desensitization of transmitter-liganded receptor channels by pulses of agonists. In *Ion Channels*, ed. Narahashi T., Vol. 3, pp. 207–260. New York: Plenum Press.

GINIATULLIN, R.A., KHAZIPOV, R.N., ORANSKA, T.I., VORONIN, V.A. & VYSKOCIL, F. (1993). The effect of nonquantal acetylcholine release on quantal miniature currents at mouse diaphragm. *J. Physiol.*, **466**, 105–114.

GROHOVAZ, F., ZACCHETTI, D., CLEMENTI, E., LORENZON, P., MELDOLESI, J. & FUMAGALLI, G. (1991). $[Ca^{2+}]_i$ imaging in PC12 cells: multiple response patterns to receptor activation reveal new aspects of transmembrane signalling. *J. Cell Biol.*, **113**, 6, 1341–1350.

HUMPHREY, P.P.A., BUELL, G., KENNEDY, I., KHAKH, B.S, MICHEL, A.D., SUPRENANT, A. & TREZISE, D.J. (1995). New insights on P_{2X} purinoceptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **352**, 585–596.

JONES, M.V. & WESTBROOK, G.L. (1995). Desensitized states prolong $GABA_A$ channel responses for brief agonist pulses. *Neuron*, **15**, 181–191.

KATZ, B. & THESLEFF, S. (1957). A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *J. Physiol.*, **138**, 63–80.

KHAKH, B.S., HUMPHREY, P.P.A. & SUPRENANT, A. (1995). Electrophysiological properties of P_{2X}-purinoceptors in rat superior cervical, nodose and guinea-pig coeliac neurones. *J. Physiol.*, **484**, 385–395.

KIM, W.K. & RABIN, R.A. (1994). Characterization of the purinergic P2 receptors in PC12 cells. Evidence for a novel subtype. *J. Biol. Chem.*, **269**, 6471–6477.

KING, B.F., ZIGANSHINA, L.F., PINTOR & BURNSTOCK, G. (1996). Full sensitivity of $P2X_2$ purinoceptor to ATP revealed by changing extracellular pH. *Br. J. Pharmacol.*, **117**, 1371–1373.

LEWIS, C., NEDRA, S., HOLY, C., NORTH, R.A., BULL, G. & SUPRENANT, A. (1995). Coexpression of P_{2X2} and P_{2X3} receptor subunits can account for ATP-gated currents in sensory neurons. *Nature*, **377**, 432–435.

MACONOCHIE, D.J. & KNIGHT, D.E. (1992). A study of the bovine adrenal chromaffin nicotine receptor using patch clamp and concentration-jump techniques. *J. Physiol.*, **454**, 129–153.

MAGAZANIK, L.G., SNETKOV, V.A., GINIATULLIN, R.A. & KHAZIPOV, R.N. (1990). Changes in the time course of miniature end plate currents induced by bath applied acetylcholine. *Neurosci. Lett.*, **113**, 281–285.

NAKAZAWA, K. & INOUE, K. (1992). Roles of Ca^{2+} influx through ATP-activated channels in catecholamine release from pheochromocytoma PC12 cells. *J. Neurophysiol.*, **68,** 2026–2032.

NAKAZAWA, K., FUJIMORI, K., TAKANAKA, A. & INOUE, K. (1990). An ATP-activated conductance in pheochromocytoma cells and its suppression by extracellular calcium. *J. Physiol.*, **428,** 257–272.

NEHER, E. (1983). The charge carried by single-channel currents of rat cultured muscle cells in the presence of local anaesthetics. *J. Physiol.*, **339,** 663–678.

NEHER, E., & STEINBACH, J.H. (1978). Local anesthetics transiently block currents through single acetylcholine-receptor channels. *J. Physiol.*, **277,** 153–176.

OGDEN, D.C. & COLQUHOUN, D. (1985). Ion channel block by acetylcholine, carbachol and suberyldicholine at the frog neuromuscular junction. *Proc. R. Soc. B.*, **225,** 329–355.

RAHA, S., DE SOUZA, L.R. & REED, J.K. (1993). Intracellular signalling by nucleotide receptors in PC12 pheochromocytoma cells. *J. Cell. Physiol.*, **154,** 623–630.

SAKMANN, B., PATLAK, J. & NEHER, E. (1980). Single acetylcholine activated channels show burst-kinetics in the presence of desensitizing concentrations of agonists. *Nature*, **286,** 71–73.

SINE, S.M. & STEINBACH, J.H. (1984). Agonists block currents through nicotine acetylcholine receptor channels. *Biophys. J.*, **46,** 277–283.

SUPRENANT, A., BUELL, G. & NORTH, R.A. (1995). P_{2X} receptors bring new structure to ligand-gated ion channels. *Trends Neurosci.*, **18,** 224–229.

VLACHOVA, V., VYKLICKY, L., VYKLICKY, L. JR., & VYSKOCIL, F. (1987). The action of excitatory amino acids on chick spinal cord neurones in culture. *J. Physiol.*, **386,** 425–438.

XIONG, Z., KITAMURA, K. & KURIYAMA, H. (1991). ATP activates cationic currents and modulates the calcium current through GTP-binding protein in rabbit portal vein. *J. Physiol.*, **440,** 143–165.

ZHANG, Z., VIJAYARAGHAVAN, S. & BERG, D.K. (1994). Neuronal acetylcholine receptors that bind α-bungarotoxin with high affinity function as ligand-gated ion channels. *Neuron*, **12,** 167–177.

(Received May 9, 1996
Revised July 19, 1996
Accepted July 31, 1996)