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Role of intracellular calcium in fast and slow desensitization of P_2-receptors in PC12 cells

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1 Combined whole-cell patch clamp recording and confocal laser scanning microscopy of $[Ca^{2+}]_i$ transients were performed on single PC12 cells to study any correlation between membrane currents induced by ATP and elevation in $[Ca^{2+}]_i$. ATP was applied by pressure from micropipettes near the recorded PC12 cells continuously superfused at a fast rate.

2 Brief (20 ms) pulses of ATP elicited monophasic inward currents and $[Ca^{2+}]_i$ increases. Long applications (2 s) of ATP (5 mM) evoked peak currents which rapidly faded during the pulse and were followed by a large rebound current, interpreted as due to rapid desensitization and recovery of P_2-receptors. The associated $[Ca^{2+}]_i$ increase grew monotonically to a peak reached only after the occurrence of the current rebound, indicating that it is unlikely this cation has a role in fast desensitization.

3 Both membrane currents and $[Ca^{2+}]_i$ transients were linearly dependent on holding membrane potential, suggesting that Ca^{2+} influx is the predominant cause of $[Ca^{2+}]_i$ elevation. This view was supported by experiments carried out in Ca^{2+}-free solution.

4 Brief pulses of ATP applied after a desensitizing pulse (2 s) of the same elicited smaller inward currents and $[Ca^{2+}]_i$ rises indicating a role for $[Ca^{2+}]_i$ in controlling slow desensitization of P_2-receptors.

5 This notion was confirmed in experiments with various $[Ca^{2+}]_i$ chelators which differentially affected slow desensitization in relation to their buffering capacity, while sparing fast receptor desensitization.

6 These results suggest a role for $[Ca^{2+}]_i$ in slow rather than fast desensitization of P_2-receptors, thus proposing this divalent cation as an intracellular factor able to provide an efficient and reversible control over receptor activity induced by ATP.

Keywords: ATP; desensitization; P_2-receptors; patch clamp; confocal microscopy; intracellular calcium

Introduction

In the last decade the superfamily of ligand-gated receptors has been extended to include the P_{2X}-receptors for which adenosine 5′-triphosphate (ATP) is the natural agonist (Surprenant *et al*., 1995). This type of P_2-receptor has been found in a variety of central and peripheral neurones (Chen *et al*., 1995; Lewis *et al*., 1995). In phaeochromocytoma (PC12) cells, which are closely related to sympathetic neurones, ATP acts via P_{2X}-receptors to evoke membrane depolarization and subsequent secretion of catecholamines (Inoue *et al*., 1989).

A common feature of most ligand gated receptors is their desensitization following exposure to agonist. This phenomenon is presently the subject of intense investigation since it seems to play a physiological role in controlling the amplitude and duration of action of several neurotransmitters (Jones & Westbrook, 1996). The kinetic properties of desensitization have amply been described by Katz & Thesleff (1957) for the action of ACh on the neuromuscular junction. Subsequent work has shown that for different ligand-gated channels desensitization is a complex process which may comprise two or three components of distinct time course (Boyd, 1987; Cachelin & Colquhoun, 1989; Celentano & Wong, 1994). PC12 P_2-receptors appear to be included in such a group since they have recently been found to possess two types of desensitization, namely a fast one occurring in the region of one hundred millisecond and a slow one with typical onset/offset rates in the region of tens of seconds (Giniatullin *et al*., 1996). The mechanism responsible for P_{2X}-receptor desensitization is currently obscure. In the case of nicotinic receptors, desensitization has initially been shown to be accelerated by raising extracellular Ca^{2+} (Magazanik & Vyskočil, 1970). Subsequent work has indicated the important role of intracellular Ca^{2+} in promoting desensitization (Miledi, 1980). Furthermore, the influx of Ca^{2+} via ACh-activated nicotinic receptors has been proposed as a crucial factor for the regulation of nicotinic receptor activity (Mulle *et al*., 1992). Since P_{2X} receptors are even more permeable to Ca^{2+} than nicotinic receptors (Rogers & Dani, 1995) it seems plausible that this divalent cation could rise intracellularly to levels high enough to influence desensitization of P_{2X}-receptors. The present study thus sought to investigate changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and membrane currents by combining confocal imaging with whole-cell patch clamping of PC12 cells following application of high concentrations of ATP. This approach was complemented by experiments with different internal Ca^{2+} chelators while recording membrane currents induced by ATP. The data from imaging and internal Ca^{2+} chelators concur to suggest that slow desensitization of P_2-receptors is closely related to increases in $[Ca^{2+}]_i$.

Methods

Patch-clamp recording

PC12 cells, kindly provided by the C.N.R. Institute of Neurobiology, Rome, were grown as previously described (Giniatullin *et al*., 1996). After being plated on a Petri dish covered with poly-L-lysine (1.25 mg ml^{-1}) they were mounted on the stage of an inverted Nikon Diaphot microscope and continuously superfused (5–10 ml min^{-1}) with control solution containing (in mM): NaCl 132, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10 and HEPES 10 (pH was adjusted to 7.4 with

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NaOH). 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, HEPES 20, $MgCl_2$ 1 and Mg_2ATP_3 3. Whenever the whole cell patch clamp condition was used in combination with confocal $[Ca^{2+}]_i$ imaging, the Ca^{2+}-sensitive dye fluo-3 (25 μM) was added to the patch electrode; the pH was then adjusted to 7.2 with CsOH. In some experiments, Ca^{2+} buffers such as BAPTA or EGTA were added to the internal solution or any exogenous buffer omitted altogether (as indicated in the Results section). Cells were usually voltage-clamped at −70 mV (unless otherwise indicated) in the whole-cell configuration after GΩ seals had been obtained. Membrane currents, collected after an 8 min period of stabilization after the whole-cell condition had been attained, were recorded with a List L/M-PC amplifier (List, Darmstadt), filtered at 1 kHz and acquired on disc by pCLAMP software (5.5 version; Axon Instruments, Foster City, California). Membrane currents were measured in terms of amplitude and exponential decay.

Confocal microscopy imaging of $[Ca^{2+}]_i$

For confocal microscopy imaging in the visible light excitation range we used the Ca^{2+}-sensitive dye fluo-3. Although with this molecule it is not possible to perform ratiometric measurements to express quantitatively changes in $[Ca^{2+}]_i$, it offers advantages in terms of fast kinetics, selectivity and linearity of response in relation to free Ca^{2+} (Minta *et al*., 1989) and, as such, it is commonly used to image $[Ca^{2+}]_i$ in living cells (Kao *et al*., 1989; Rathouz & Berg, 1994; Markram *et al*., 1995; Andjus *et al*., 1996). Ca^{2+} imaging was performed in intact cells or in combination with patch clamp recording. In the first case cells were incubated for 45–60 min at 37°C with the dye (4 μM; cell permeant methylester form (AM) from Molecular Probes, Eugene, Oregon) added to control solution, and then washed three times before the experiment. Pilot tests showed that 4 μM fluo-3/AM provided an optimal loading of PC12 cells in terms of signal to noise ratio and reproducibility of Ca^{2+} responses. When confocal Ca^{2+} studies were performed in combination with patch clamping, 25 μM fluo-3 (cell impermeant form, pentapotassium salt; Molecular Probes) was applied via the patch pipette. This concentration was selected to provide optimal signal to noise ratio without substantial buffering of free Ca^{2+}. On a few occasions cells pre-loaded with fluo-3/AM were subsequently patched with a pipette filled with fluo-3 for simultaneous imaging of Ca^{2+} in intact and patched pairs of cell. Regardless of the method of delivery of fluo-3 to the cell cytoplasm the baseline fluorescence level was consistently the same in absolute terms. Fluo-3 emission was induced by the Ar-Kr laser of the MultiProbe 2001 confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, California) with the 488 nm band, and detected by a photomultiplier tube with a combination of 510 nm high-pass and 530±30 nm band-pass filters. Under these conditions no dye bleaching was observed. Images were digitized as 64×64 pixel arrays with pixel size 0.6 μm, and a confocal aperture of 200 μm. Fluorescent signals were analysed over the area of the whole pericaryon central optical section in the 32-line rapid scan mode (temporal resolution of 320 ms per scan) by use of ImageSpace 3.10 software (Molecular Dynamics). The signal transients were expressed as fractional amplitude ($\Delta F/F_0$; where F_0 is the baseline fluorescence level, and ΔF is the rise over the baseline), with the rise time from 10 to 90% of the peak amplitude and decay time constant. All data are presented as mean±s.e.mean with statistical significance assessed by means of paired *t* test (for normally distributed data) or ANOVA test (for non-parametric data).

Drug application

ATP (Na salt; purchased from Sigma Chemical Co., Milan) was diluted in control solution and delivered by pressure application (10–20 p.s.i.) from glass micropipettes (located about 15 μm away from the recorded cell) by a Picospritzer II (General Valve Co., Fairfields, New Jersey). Great care was taken to ensure that pressure application of ATP did not produce any visible distortion of cell shape which could compromise Ca^{2+} imaging. Rate of drug delivery and removal were estimated as previously described (Giniatullin *et al*., 1996; see also Sugasawa *et al*., 1996) and yielded peak concentrations reached within 15–20 ms. Tests with combined patch clamp recording (with the pipette containing 5 mM EGTA or 10 mM BAPTA) and confocal $[Ca^{2+}]_i$ imaging failed to reveal any increase in $[Ca^{2+}]_i$ following pulse application of ATP (5 mM), suggesting that adequate Ca^{2+} buffering was provided by these chelators.

Results

Membrane currents and $[Ca^{2+}]_i$ transients induced by ATP

Membrane currents and associated changes in $[Ca^{2+}]_i$ were studied in combined experiments by whole-cell patch clamp recording and confocal Ca^{2+} imaging. An example of this approach is shown in Figure 1, at −70 mV holding potential, application of ATP from pipettes containing 0.5 or 5 mM concentrations induced inward currents (Figure 1Ab and Bb) and rises in $[Ca^{2+}]_i$ (Figure 1Aa and Ba) of a PC12 cell. In this case, application of a 0.5 mM solution of ATP (either 20 ms or 2 s pulse; arrows show applications for $[Ca^{2+}]_i$ responses while arrowhead or bar shows application for current responses) induced monophasic responses with rapid onset and gradual decline, although there was an apparent lag between the peak of the inward currents and that of the $[Ca^{2+}]_i$ transients (the latter peaking at 6.7 s for 20 ms or 8.3 s for 2 s pulse after current peak; note that the timescale for $[Ca^{2+}]_i$ is much slower than that for membrane currents). When the ATP concentration was 5 mM application of a 20 ms pulse generated a monophasic response comprising a 364 pA inward current and an associated rise (0.32 $\Delta F/F_0$) in $[Ca^{2+}]_i$ (see Figure 1Ba, b; different cell from Figure 1A). When the 5 mM ATP solution was applied for 2 s, the inward current peak (960 pA) quickly faded almost back to baseline level and was followed by a rebound current (1034 pA) immediately after the end of drug application: the latter phenomenon is considered to be due to fast receptor desensitization and recovery (Giniatullin *et al*., 1996). Unlike this biphasic nature of the inward current response, the corresponding $[Ca^{2+}]_i$ rise (although much larger in fractional amplitude and decay time constant, namely 0.64 $\Delta F/F_0$ and 40 s, respectively) had smooth onset and decline (Figure 1Ba; note slower timebase than corresponding currents in Figure 1Bb). It is worth noting that the peak $[Ca^{2+}]_i$ increase occurred with a considerable delay (21 s) with respect to peak and rebound inward currents. Since it has been shown that the development of $[Ca^{2+}]_i$ rise is related more to the current integral rather than its peak amplitude (Rathouz & Berg, 1994), it is not unexpected that the rise in $[Ca^{2+}]_i$ after 2 s ATP pulse was slower than after 20 ms pulse since the long application induced a 314 pC charge transfer during the initial peak compared to the 490 pC charge transfer induced by the brief pulse.

Since long pulses applied from 5 mM ATP solutions were reliably capable of inducing rapid receptor desensitization consisting of current fade and rebound (Giniatullin *et al*., 1996), further experiments aimed at investigating the relationship between ATP induced currents and $[Ca^{2+}]_i$ were carried out with this concentration of ATP. Nevertheless, it should be noted that, unlike in our previous patch clamp study of PC12 cells (Giniatullin *et al*., 1996), in the present investigation it was necessary to omit Ca^{2+} chelators, such as EGTA, from the pipette solution as they would have compromised $[Ca^{2+}]_i$ imaging (see Methods). Hence, since long-term changes in $[Ca^{2+}]_i$ could not be prevented, it was decided to measure responses to ATP (in the presence of fluo-3 in the

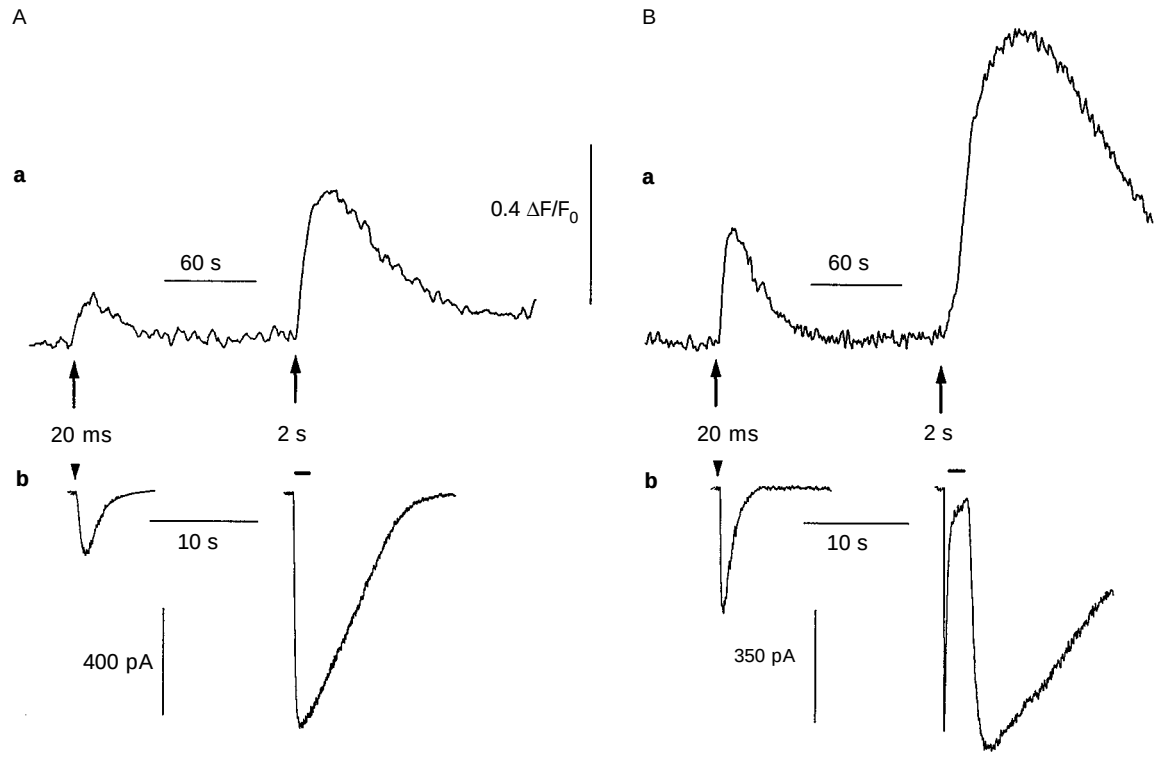

Figure 1 Effects of ATP on $[Ca^{2+}]_i$ transients and membrane currents of PC12 cells. (A) Combined recording of $[Ca^{2+}]_i$ response (a) and inward current (b) induced by either 20 ms (arrowhead) or 2 s (horizontal bar) application of ATP (0.5 mM). Note different timescale for (a) and (b). ATP applications in (a) are indicated by arrows. (B) Responses produced by 5 mM ATP on a different PC12 cell. Application times and symbols are the same as in (A). Timescale in (a) is much slower than in (b). Note that a 2 s application of ATP induced a large peak current which faded during the application and was followed by a large rebound immediately after termination of ATP pressure pulse.

internal solution) for not more than 20 min after the stabilization period (see Methods); during this time these responses were as reproducible as those of intact (non-patched) cells loaded with fluo-3/AM (see below).

Figure 2 shows, for a sample of 9 cells, that the use of either brief (10–50 ms) or long (2 s) pulses of 5 mM ATP led to significant differences in the increase in $[Ca^{2+}]_i$ and its rise and decay times. Long pulses elicited responses of a larger amplitude (Figure 2a; $P<0.05$) and longer rise time (Figure 2b; $P<0.005$). Interestingly, the parameter which differed most between the responses to brief and long pulses was the rise time (see Figure 2b) for $[Ca^{2+}]_i$ transients (5.5 fold increment); this behaviour was the opposite of that seen when ATP-induced membrane currents were investigated (see for example Figure 1B). Time constant values of the $[Ca^{2+}]_i$ transient decay were also significantly different (Figure 2c; $P<0.05$) between brief and long pulses of ATP delivered to the same cells.

Source of $[Ca^{2+}]_i$ rise induced by ATP

The relatively slow characteristics of $[Ca^{2+}]_i$ changes produced by ATP raised the possibility that they were caused by the voltage-clamp condition which prevented activation of voltage-dependent Ca^{2+} channels by membrane depolarization. It therefore seemed of interest to compare the $[Ca^{2+}]_i$ changes induced in patched and intact cells. This was studied with ATP pulses of 20 ms or 2 s duration. Figure 3a, c shows the effect of brief (20 ms) pulses of ATP (see arrowheads); they induced monophasic $[Ca^{2+}]_i$ responses in both patched and intact cells. The peak amplitude of the $[Ca^{2+}]_i$ rise was larger in the intact than in the voltage-clamped cell (0.81 ± 0.23 and 0.27 ± 0.04 $\Delta F/F_0$, respectively, for a sample of 9 cells), and its rate of rise was faster (0.61 ± 0.1 s vs 2.82 ± 0.28 s, respectively, $n=9$).

However, a major difference appeared when responses produced by the 2 s ATP application were compared (see horizontal bar in Figure 3b, d). In the example shown in Figure 3b, d we imaged a pair of neighbouring cells (at the same distance from the puffer pipette), both loaded with the Ca^{2+}-sensitive dye though only one (shown in b) was patched. In the intact cell there was an initial, rapid rise (labelled as A_1 in Figure 3d) in $[Ca^{2+}]_i$ followed by a decline and a much larger and slower increase (labelled as A_2 in Figure 3d). On the other hand, the response of the adjacent, patched cell had a monophasically slow onset and offset (Figure 3b). The intact cell displayed a much larger peak rise in $[Ca^{2+}]_i$ (1.9 vs 0.76 $\Delta F/F_0$) although in either cell the time from the end of the ATP pulse to the maximum increase in $[Ca^{2+}]_i$ was approximately the same (4.3 s vs 4.7 s). The inset of Figure 3d shows that the initial peak (A_1) was consistently smaller than the second one (A_2) for a sample of 9 intact cells. It is worth noting that the amplitude of the maximal peak (A_2) evoked by 2 s ATP pulse was significantly ($P<0.05$) larger (by 73%) than the corresponding peak of patch-clamped cells (see Figure 2a), although the decay time constant of the $[Ca^{2+}]_i$ transient was the same (27.1 ± 9.1 vs 30.6 ± 8.1; $n=9$). The difference between the amplitudes of the $[Ca^{2+}]_i$ rises in intact compared to patched cells suggested that the response measured from intact cells partly comprised a component due to voltage-dependent Ca^{2+} channels, possibly activated by ATP-induced depolarization. On the other hand, the similar decay time course indicated that it was not determined by voltage-activated channels, and that the mechanisms responsible for removal of $[Ca^{2+}]_i$ following a long pulse of ATP were not substantially disturbed in patch-clamped cells.

A more controlled way to assess the role of voltage-dependent Ca^{2+} channels in the persistent rise in the $[Ca^{2+}]_i$ was to apply depolarizing voltage steps to patched cells. For this purpose we used 5 s long voltage commands from -70 mV holding potential to -20 mV test potential (which should be sufficient to activate high threshold Ca^{2+} currents; Di Virgilio

et al., 1987; Fasolato *et al*., 1990). Under these conditions ($n=3$ cells) the $[Ca^{2+}]_i$ rise displayed a monotonic, rapid (0.88 ± 0.21 s) increase to a peak level ($\Delta F/F_0$ 0.46 ± 0.13) from which it recovered with a decay time constant of 9.6 ± 2.1 s. These responses from patched cells had onset/offset kinetics similar to those of the $[Ca^{2+}]_i$ rise observed in unclamped cells either with a brief ATP pulse or early during a 2 s ATP pulse (A_1 peak). These data imply that, while membrane depolarization by ATP could increase $[Ca^{2+}]_i$ via activation of voltage-dependent Ca^{2+} channels in intact cells, this phenomenon could not be detected when cells were clamped at a holding potential of -70 mV.

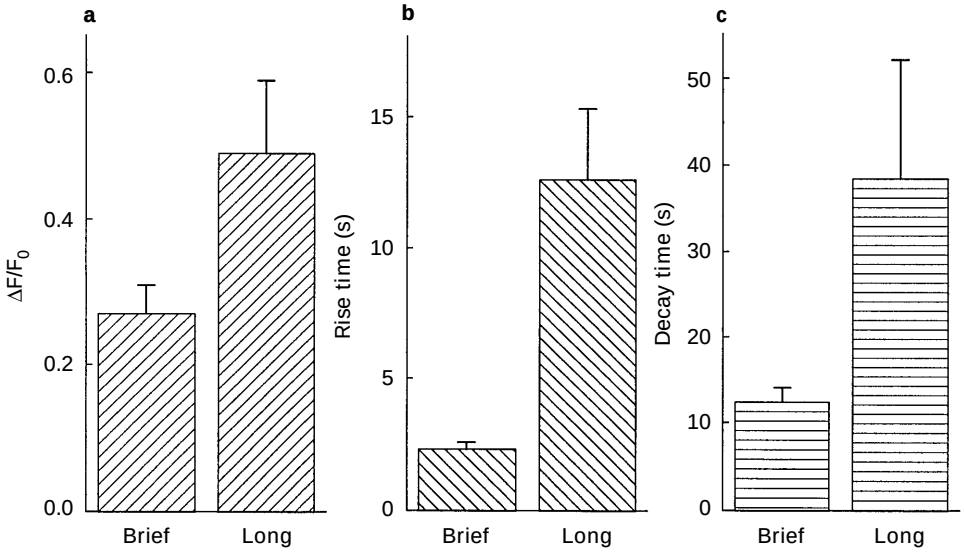

Figure 2 $[Ca^{2+}]_i$ changes following brief (20–50 ms) or long (2 s) pulses of 5 mM ATP to patch-clamped PC12 cells. (a) Fractional changes in peak amplitude of $[Ca^{2+}]_i$ responses. (b) Time constant of $[Ca^{2+}]_i$ rises. (c) Decay time constant of $[Ca^{2+}]_i$ transients. Data are from 9 cells. In (a), (b) and (c) the difference between the pair of histograms was significant ($P<0.05$, 0.005, and 0.05, respectively) when analysed by ANOVA (a) or paired t test (b and c).

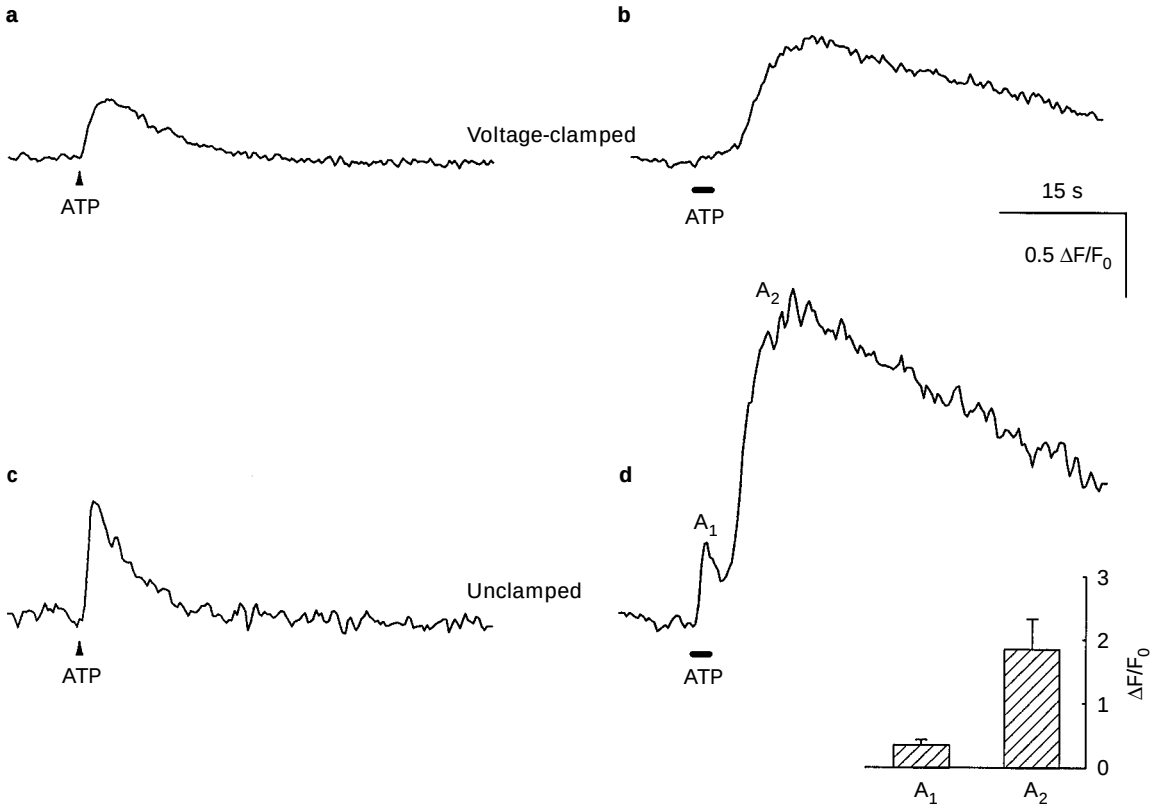

Figure 3 Comparison of $[Ca^{2+}]_i$ transients in patched or intact PC12 cells. (a) and (c) Responses induced by 20 ms ATP (5 mM) pulse on two different cells; (b) and (d) responses recorded simultaneously from a pair of cells in which only one (b) was patched (voltage clamped at -70 mV). Note faster and biphasic nature of $[Ca^{2+}]_i$ response of unclamped cell (d). ATP (5 mM) was applied for 2 s from a pressure pipette equidistant from both cells. Inset to (d) shows average values for the first (A_1) or the second (A_2) peak of the $[Ca^{2+}]_i$ response of intact cells ($n=9$).

It therefore seemed feasible that the increase in $[Ca^{2+}]_i$ observed under patch clamp conditions following ATP application resulted from Ca^{2+} permeation through influx via activated P_2-receptors, intracellular Ca^{2+} release, or a combination of the two. If the rise in $[Ca^{2+}]_i$ depended exclusively on Ca^{2+} release from the intracellular compartment, one might expect it to be relatively insensitive to changes in membrane potential (Sugasawa *et al*., 1996) which, on the other hand, should influence transmembrane Ca^{2+} influx by changing its driving force (Reber *et al*., 1992). Figure 4 shows an experiment in which a 2 s pulse of ATP was applied to a cell clamped at holding potentials of −40, −70 or −100 mV. As the holding potential was made more negative, there was a progressive increase in $[Ca^{2+}]_i$ (see Figure 4Aa) and in the inward current (Figure 4Ab). Plotting the integral of the membrane current (which is related to the changes in $[Ca^{2+}]_i$; cf Rathouz & Berg, 1994) or the peak amplitude of $[Ca^{2+}]_i$ against holding potential (Figure 4B) yielded an approximately linear relationship for both parameters, suggesting that the magnitude of the $[Ca^{2+}]_i$ transient was dependent on membrane voltage and thus perhaps related to Ca^{2+} influx. This notion was confirmed in another set of experiments in which changes in $[Ca^{2+}]_i$ in control or Ca^{2+}-free external solution were measured in either intact or patched cells. After 4 min removal of external Ca^{2+} (plus addition of 10 mM EGTA to the superfusion solution) the peak $[Ca^{2+}]_i$ rise induced by a 2 s pulse of ATP was reduced from 1.84 ± 0.49 to 0.083 ± 0.032 ($n = 9$) $\Delta F/F_0$ in intact cells, and from 0.49 ± 0.1 to 0.04 ± 0.02 ($n = 3$) $\Delta F/F_0$ in patch-clamped cells. These results indicate that in intact or patched PC12 cells the increase in $[Ca^{2+}]_i$ is predominantly dependent on extracellular Ca^{2+}.

Role of $[Ca^{2+}]_i$ in ATP receptor desensitization

It was previously observed (Giniatullin *et al*., 1996) that ATP receptors apparently possess two distinct types of desensitization: a fast one (developing in the ms range) and responsible for the rapid current fade, and a slower one (developing in the range of tens of s) which determines a sustained depression of responsiveness to subsequent ATP applications. In order to study how changes in $[Ca^{2+}]_i$ developed during desensitization, brief pulses of ATP were repetitively applied at time intervals (15–60 s) which allowed reproducibility of responses and were then followed by a 2 s long, desensitizing application of ATP after which the brief pulse sequence recommenced. With this protocol it was advantageous to study the characteristics of slow desensitization. Conversely, because the apparent $[Ca^{2+}]_i$ increase during the 2 s application of ATP was too small in voltage-clamped cells (see Figure 1Ba and Figure 3b) to study the role of $[Ca^{2+}]_i$ in fast desensitization, the latter process was investigated with an alternative approach (based on intracellular Ca^{2+} buffering) as described later. In the example of Figure 5Aa, $[Ca^{2+}]_i$ transients followed the inward currents evoked by repetitive ATP pulses (see Figure 5Ab showing absolute amplitude values of membrane current on the same time scale) up to the application of a long pulse of ATP. The latter was associated with a large rise in $[Ca^{2+}]_i$ which in this cell declined with a 109 s time constant and was accompanied by a persistent depression of inward currents and $[Ca^{2+}]_i$ changes. Figure 5B shows the relationship between the time constant for the $[Ca^{2+}]_i$ increase and the half-time for recovery of the inward currents due to repetitive brief applications of ATP after a long pulse of the same ($n = 6$). Data could be fitted by a linear relationship with $r = 0.963$. These observations thus indicated that there was an association between recovery from slow desensitization of P_2-receptors and $[Ca^{2+}]_i$.

In order to study the influence of $[Ca^{2+}]_i$ on fast desensitization, various Ca^{2+} chelators (applied intracellularly via the patch electrode) were used during the measurement of membrane currents induced by ATP. As the index of fast desensitization the time constant of the fast fading of the membrane current to the 2 s pulse of 5 mM ATP was used. Sample tracings are shown in Figure 6Aa in which responses observed in

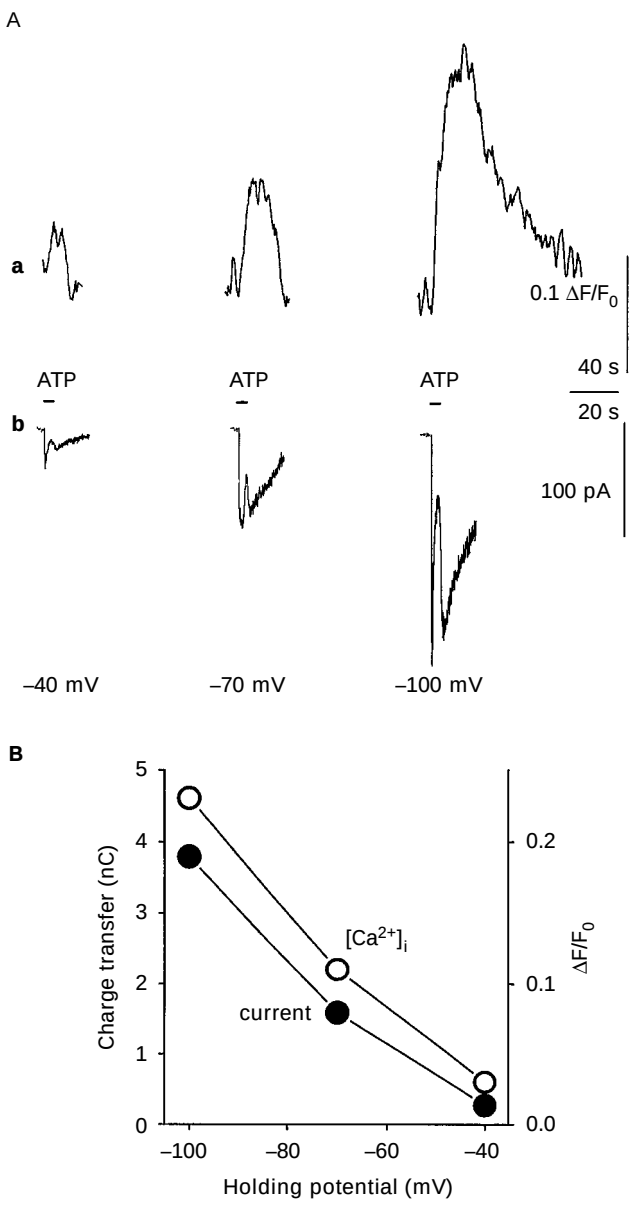

Figure 4 Effect of changes in holding potential on $[Ca^{2+}]_i$ transients and membrane currents induced by 2 s application of 5 mM ATP. (A) Simultaneous recording of $[Ca^{2+}]_i$ transients (a) and inward currents (b) at the holding potentials indicated at the bottom of the tracings. Note different timescale in (a) and (b). (B) Plot of holding potential against the charge transfer ((●) left ordinate scale) of the ATP current or the peak increase in $[Ca^{2+}]_i$ ((○) right ordinate scale). Note approximately linear relationship between either response to membrane potential.

control solution (with no internal buffers added) were essentially similar to those observed after at least 8 min whole-cell patch recording with an electrode containing 10 mM BAPTA. Figure 6Ab shows that the fading time constant of the initial peak current was approximately the same in the absence and presence of the internal chelators, 25 μM fluo-3, 5 mM EGTA and 10 mM BAPTA.

We also investigated the process of slow desensitization when minimal (with 25 μM fluo-3) or strong (with 5 mM EGTA or 10 mM BAPTA) Ca^{2+} buffering was used. Slow desensitization was taken as % depression of the test inward current (elicited by 20 ms ATP pulses) at 30 s from the start of a 2 s desensitizing pulse when the rebound current had fully dissipated. Figure 6Ba, b shows an example of the current responses induced by a 20 ms pulse before (left), 30 s (middle), or 2 min (right) after a 2 s pulse of ATP. When the pipette

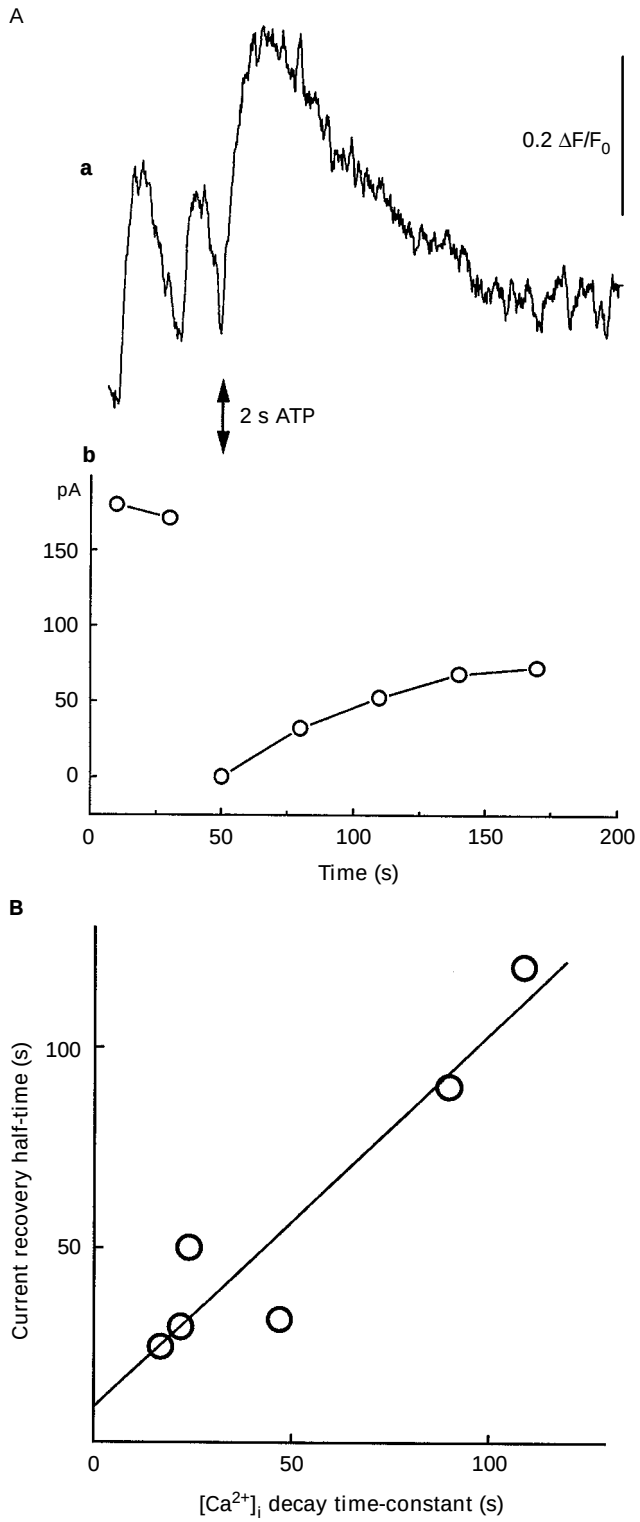

Figure 5 Changes in $[Ca^{2+}]_i$ level and inward currents produced by ATP (5 mM). (A,a) $[Ca^{2+}]_i$ transients induced by 20 ms pulses of ATP before and after a 2 s ATP pulse (doubleheaded arrow); (b) corresponding current amplitudes recorded from the same cell following the same protocol as above. Current amplitudes are expressed in absolute values disregarding their polarity. Timescale in (b) applies also to (a). (B) Time constant of $[Ca^{2+}]_i$ decay plotted against half-time of current recovery (obtained from graphs such as the one in (Ab)). Datapoints refer to different cells.

contained fluo-3 the test current was almost suppressed (on average it was reduced to $30.0 \pm 7.6\%$ of the first pulse current; $n = 7$; see Figure 6Bc), while with BAPTA-containing pipettes the test current was not reduced (on average $92.2 \pm 5.1\%$; $n = 5$; Figure 6Bc). With an EGTA-containing pipette the test current was slightly reduced to $73 \pm 4.6\%$ of the first response (Figure 6Bc; $n = 3$). Since recording stability with pipettes containing no Ca^{2+} buffers was often brief, it was difficult to obtain comparable observations when $[Ca^{2+}]_i$ was allowed to rise in the absence of intracellular chelators.

Discussion

The principal finding of the present study was the novel demonstration of a close association between $[Ca^{2+}]_i$ and slow desensitization of P_2-receptors activated by high doses of ATP in PC12 cells. Conversely, the process of fast desensitization of the same receptors appeared to be independent of $[Ca^{2+}]_i$.

Characteristics of membrane currents elicited by high doses of ATP

P_2 receptors on PC12 cells are heterogeneous and comprise two broad classes, one mediating fast cationic fluxes (Nakazawa *et al*., 1990) and the other one coupled to intracellular second messengers (Grohovaz *et al*., 1991): these receptor subtypes correspond to those generally termed P_{2X} and P_{2Y}, respectively (Burnstock & Kennedy, 1985; Kennedy, 1990). Molecular biology and pharmacological investigations have indicated that PC12 cells express a P_{2X_2}-receptor subtype (Collo *et al*., 1996) which produces fast responses due to opening of non-specific cationic channels (Nakazawa *et al*., 1990). With slow rates of ATP application (by bath superfusion) to PC12 cells only a slowly developing desensitization of P_{2X}-receptors has been found after sustained agonist applications (see for example Nakazawa *et al*., 1990; Nakazawa & Inoue, 1992; Reber *et al*., 1992). In the case of P_{2X_3}-receptors of ganglion cells, when the ATP concentrations are in the micromolar range and applied via a rapid delivery system, fast desensitization can develop (Robertson *et al*., 1996). We have recently observed that fast application of millimolar concentrations of ATP to continuously superfused PC12 cells elicits peak inward currents rapidly fading and immediately followed by current rebound at the end of the ATP application (Giniatullin *et al*., 1996). This phenomenon was interpreted as due to rapid desensitization of ATP receptors by high doses of ATP which quickly returned to their active state as soon as the agonist was removed: this process is thus distinct from slow desensitization which is gradually manifested following either closely spaced applications or bath administered low doses of the agonist (Giniatullin *et al*., 1996).

The suggestion of two distinct processes of desensitization (fast and slow) promoted us to consider the mechanism(s) responsible for these phenomena. In the case of nicotinic receptors, desensitization is strongly influenced by intracellular Ca^{2+} (Miledi, 1980). Since ATP is known to increase $[Ca^{2+}]_i$ of PC12 cells (Grohovaz *et al*., 1991; Reber *et al*., 1992; Raha *et al*., 1993), this divalent cation seemed to be a likely candidate to control the process of P_2-receptor desensitization. For this purpose simultaneous measurements of ATP-activated membrane currents and $[Ca^{2+}]_i$ transients were performed with combined whole-cell patch recording and confocal microscopy on the same cell. In order to ensure focal and rapid exposure of the cells to ATP, this substance was delivered by pressure applications via a micropipette positioned closely to the recorded cell.

Time course of $[Ca^{2+}]_i$ changes evoked by ATP

While brief applications of ATP elicited monophasic $[Ca^{2+}]_i$ rises in intact as well as patch clamped cells (in which this response was consistently slower), the biphasic time course of the changes in $[Ca^{2+}]_i$ induced by 2 s application of ATP to intact cells was reminiscent of the two-component current observed with the same application protocol in patch clamped cells. This apparent similarity might have suggested a possible role for $[Ca^{2+}]_i$ in current fading and rebound. However, this

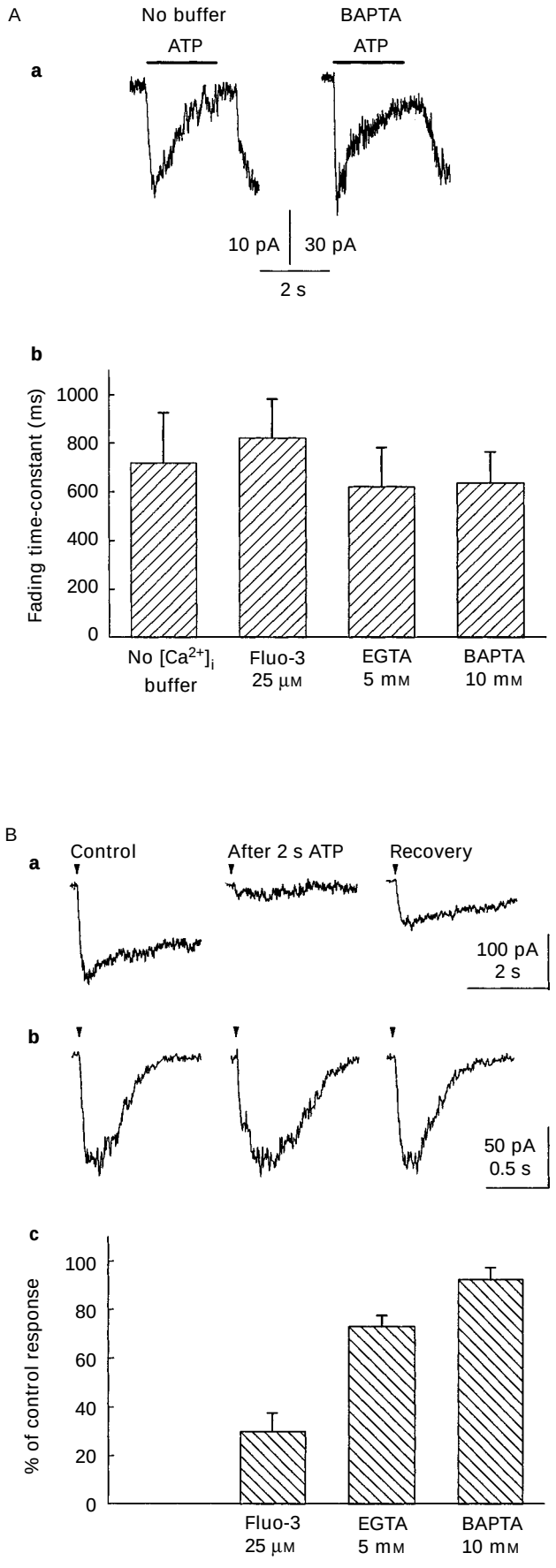

Figure 6 Effect of $[Ca^{2+}]_i$ buffers on membrane currents induced by 5 mM ATP. (A,a) Comparison of current fading evoked by 2 s ATP pulse (horizontal bar) recorded in a cell without addition of $[Ca^{2+}]_i$ buffers (left) with a cell recorded with a BAPTA containing pipette (right). Note similar fading process during ATP application; (b) time constant of peak current fading from cells recorded without addition of intracellular buffers ($n=7$), or with indicated buffers (fluo-3, $n=13$; EGTA, $n=16$; BAPTA, $n=18$). Note similar results despite different pipette solutions. (B,a) Responses to 20 ms ATP pulse before (control), 30 s after a 2 s ATP pulse (middle), and 2 min recovery (right) recorded from a cell patch-clamped with a fluo-3 (25 μM)-containing pipette; (b) comparable data obtained from another cell patch clamped with a BAPTA (10 mM)-containing pipette; (c) depicts degree of slow desensitization (expressed as % of response to 20 ms ATP 30 s after 2 s ATP pulse) of cells recorded with fluo-3 ($n=7$), EGTA ($n=3$) or BAPTA ($n=7$)-containing pipettes. Note that slow desensitization was minimal in the presence of BAPTA and very large in the presence of fluo-3. The average value for fluo-3 was significantly different from the one for EGTA ($P<0.05$) and BAPTA ($P<0.05$), while the difference between BAPTA and EGTA data was not significant ($P>0.05$).

suggestion is unlikely since, under voltage clamp conditions when this initial $[Ca^{2+}]_i$ rise was absent, the current peak fading and rebound were reliably present, implying that they could occur in the absence of comparable changes in $[Ca^{2+}]_i$. It seems more likely that the fast increase in $[Ca^{2+}]_i$ was due to influx of this ion via voltage gated Ca^{2+} channels, which are known to be activated by depolarization during ATP application (Di Virgilio *et al*., 1987). This conclusion is supported by the fast temporal characteristics of the initial peak of $[Ca^{2+}]_i$ transient in intact cells which resembled rapid rise and decay of $[Ca^{2+}]_i$ responses to voltage steps applied to patch clamped cells.

There are several factors determining the decay of $[Ca^{2+}]_i$ transients, such as Na^+/Ca^{2+} exchange, Ca^{2+} pumps and intracellular Ca^{2+} sequestration (Neher & Augustine, 1992; Markram *et al*., 1995; Park *et al*., 1996). However, it is worth noting that the time course of $[Ca^{2+}]_i$ transient decay after a long pulse of ATP was similar for patch clamped and intact cells, indicating that Ca^{2+} removal mechanisms in the cells under patch clamp conditions (with a 25 μM fluo-3 containing pipette) were not disrupted.

Origin of $[Ca^{2+}]_i$ rises induced by ATP

Since the main part of $[Ca^{2+}]_i$ rise in intact cells appeared to be due to influx through voltage-gated Ca^{2+} channels activated by ATP-induced depolarization, the remaining component constituting $[Ca^{2+}]_i$ responses in patch clamped cells might have been caused either by influx of this cation via ionotropic P_2-receptors (Nakazawa & Inoue, 1992; Reber *et al*., 1992; Raha *et al*., 1993), by its release from intracellular Ca^{2+} stores (Grohovaz *et al*., 1991; Zacchetti *et al*., 1991), or by a combination of the two. In our experiments the $[Ca^{2+}]_i$ elevation in both intact and patch clamped cells was dependent on extracellular Ca^{2+}, as suggested by the near absence of $[Ca^{2+}]_i$ increase in the cells superfused with Ca^{2+}-free external solution (see also Nakazawa & Inoue, 1992; Reber *et al*., 1992). This observation suggests the predominant role of transmembrane Ca^{2+} influx and while not excluding intracellular Ca^{2+} release (previously shown for these cells; Grohovaz *et al*., 1991; Zacchetti *et al*., 1991), it implies that if the latter took place, it must have been dependent on Ca^{2+} influx. However, the steep, linear-dependence of $[Ca^{2+}]_i$ transients on the holding potential of patch clamped cells (see present study and Reber *et al*., 1992) indicates a minimal contribution by intracellular Ca^{2+} release which is expected to be voltage-independent (Sugasawa *et al*., 1996).

Changes in $[Ca^{2+}]_i$ during fast and slow desensitization of ATP receptors

Several lines of evidence suggest that fast desensitization of ATP-gated channels of PC12 cells took place independently of $[Ca^{2+}]_i$ variations. Firstly, by use of combined Ca^{2+} imaging and patch clamp recording, there was only a negligible $[Ca^{2+}]_i$ rise during fading of the membrane current corresponding to

fast desensitization. Secondly, even stronger evidence was provided by experiments on $[Ca^{2+}]_i$ buffering with different (or no) Ca^{2+} chelators. In this case, the onset of fast desensitization (measured as fading rate of the current induced by 2 s pulse of ATP) was the same in a large sample of cells dialysed in the absence and presence of various Ca^{2+} buffers, such as the fast acting BAPTA (Tsien, 1981), the relatively slow acting EGTA and a low concentration of the BAPTA-derived fluorescence dye fluo-3.

In contrast to fast desensitization, the process of slow desensitization was found to be highly Ca^{2+}-dependent. This conclusion is supported by the same arguments used to discard a major role of $[Ca^{2+}]_i$ in fast desensitization. In fact, as indicated by simultaneous recording of $[Ca^{2+}]_i$ and membrane currents induced by ATP, the time-course of $[Ca^{2+}]_i$ after a desensitizing application of this agent developed over the same time scale which might have allowed Ca^{2+} to interact with desensitized receptors. An important finding was the high correlation between the decay of ATP-induced $[Ca^{2+}]_i$ transients and the recovery from relatively long-lasting desensitization in a sample of cells in which these two phenomena always varied in a similar fashion. The role of Ca^{2+} in slow desensitization was further supported by experiments in which slow desensitization was evaluated in the presence of different intracellular Ca^{2+} chelators. Thus, the degree of sensitivity loss after a 2 s pulse of ATP was the largest with a low concentration of fluo-3 (which presumably allowed more Ca^{2+} to interact with P_2-receptors from the cytoplasmic side) than with EGTA or BAPTA. The Ca^{2+}-independence of fast desensitization, on the one hand, and the high Ca^{2+}-dependence of slow desensitization, on the other, accord with a similar role of $[Ca^{2+}]_i$ in the fast and slow phases of desensitization of ACh receptors on skeletal muscle (Cachelin & Colquhoun, 1989).

Two possible hypotheses have been suggested for the precise mechanism of Ca^{2+} action on slow desensitization. Firstly, Ca^{2+} might switch on various protein kinases which can influence receptor activity by phosphorylation of certain subunits (Kozawa *et al*., 1995). Alternatively, if the temporal overlap between the persistence of $[Ca^{2+}]_i$ rise (after 2 s pulse of ATP) and the changes in receptor sensitivity is taken into consideration, a direct action of intracellular Ca^{2+} on the sensitivity of P_2-receptors without intervention of other second messengers would be possible. Further experiments will be necessary to clarify this issue.

Physiological implication of desensitization of ATP receptors

The action of ATP on PC12 cells cannot be taken, in general, as representative of the many different effects of this substance on central and peripheral neurones. Nevertheless, the accessibility and stability of P_2-receptors in PC12 cells make them a convenient model to provide data useful to interpret phenomena such as desensitization known to occur in other neurones. This is so because the P_{2X_2}-receptor expressed by PC12 cells is also found in tissues such as brain, spinal cord, certain ganglia and adrenal medulla (Collo *et al*., 1996). The phenomenon of desensitization seems to play a physiological role in shaping synaptic responses and in limiting activation of postsynaptic membrane receptors for several neurotransmitters (Jones & Westbrook, 1996). A process such as fast desensitization of ATP-activated receptors of PC12 cells (Giniatullin *et al*., 1996) would then be expected to prolong the macroscopic response of P_2-receptors activated by synaptically released ATP in a manner analogous to the action of γ-aminobutyric acid (GABA) which, by inducing rapid and reversible state transitions of its channels, extends synaptic inhibition of hippocampal neurones (Jones & Westbrook, 1995).

In contrast to fast desensitization, slow desensitization might serve as an efficient process to limit excitability of neurones during repetitive stimulation of ATP releasing nerve fibres. The functional role of Ca^{2+} in slow desensitization of P_2-receptors of PC12 cells was probably underestimated by the present study because voltage clamping prevented a more substantial $[Ca^{2+}]_i$ rise via voltage activated Ca^{2+}-channels. In our previous patch clamp study (Giniatullin *et al*., 1996), like in many other similar investigations concerning P_{2X} receptors (Bean, 1990; Nakazawa *et al*., 1990; Nakazawa & Inoue, 1992; Valera *et al*., 1994; Khakh *et al*., 1995), whole-cell patch clamping with pipettes containing relatively high levels of Ca^{2+} buffers was used. It is likely that this condition also led to an underestimation of the slow desensitization. These considerations therefore reinforce the important role of Ca^{2+} in regulating slow desensitization in physiological conditions. Since $[Ca^{2+}]_i$ levels can be affected by a variety of intracellular second messengers, it seems plausible that the process of $[Ca^{2+}]_i$-dependent slow desensitization is modulated according to the different functional states of the cell.

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