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QUALITATIVE DIFFERENCES IN $[Ca^{2+}]_i$ INCREASES AND $InsP_3$ GENERATION FOLLOWING STIMULATION OF N1E-115 CELLS WITH MICROMOLAR AND MILLIMOLAR ATP

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Abstract—Addition of ATP (100 μM) to populations of the neuronal cell line N1E-115 caused a transient increase in intracellular calcium ($[Ca^{2+}]_i$) which rapidly reached a peak (maximum of 243 ± 7 nM above basal) and returned to basal after approximately 50 sec. The response was concentration-dependent (EC_{50} 21 ± 4 μM) and was unchanged when calcium was omitted from the extracellular medium. Transient increases in D-*myo*-inositol-1,4,5-trisphosphate levels ($InsP_3$) were also observed over a similar time period. Addition of millimolar ATP, however, produced characteristically different responses; $[Ca^{2+}]_i$ again increased rapidly (reaching a maximum of 639 ± 23 nM above basal) but returned to a new maintained plateau (274 ± 34 nM) which was abolished by the inorganic calcium channel blocker, nickel (1 mM), and omission of calcium from the extracellular medium. $InsP_3$ levels were also maintained but were, however, unaffected by nickel or removal of extracellular calcium. The qualitative difference in the mechanism of calcium elevation produced with millimolar ATP, compared with lower concentrations, suggests that the N1E-115 cells might also contain a low affinity P_2 receptor coupled with a calcium channel.

ATP is released from the cytoplasm of several cell types including platelets and neurones. It can act directly at the neuroeffector junction and has been suggested to act as a modulator of synaptic transmission by increasing or decreasing the release of other neurotransmitters [1]. Evidence for the existence of "purinergic" nerves was derived from the observation that a non-cholinergic/non-adrenergic component of the autonomic nervous system was associated with smooth muscle in the gastrointestinal tract and elsewhere. ATP was suggested to be the neurotransmitter involved following its detection after nerve stimulation and the presence at neuroeffector junctions of ATP-metabolizing enzymes [2]. Furthermore, ATP has been found to be co-released with noradrenaline and acetyl choline from nerve endings isolated from the CNS [3]. The concentration of ATP in the synaptic cleft has been estimated as low micromolar during routine transmission. However, during repetitive stimulation [4] it has been suggested that concentrations may increase considerably.

ATP exerts its actions via P_2 receptors [5] found on the surface of many different cells including the neuronal cell lines N1E-115 and NG108-15 [6, 7]. Four sub-divisions of P_2-purinergic receptors have been proposed, based on the pharmacological potencies of various nucleotides and other criteria: P_{2X} and P_{2Y} which mediate contraction and relaxation, respectively [8]; P_{2t} which are involved in platelet aggregation [9] and P_{2z} which mediate the permeabilizing effect of ATP^{4-} [10]. However, further sub-types of P_2 receptors which do not fit into the current classification have also recently been suggested to exist in a number of cells and cell lines [11–13, 7].

We have reported previously the existence of a nucleotide receptor in the murine neuroblastoma clone N1E-115, linked to D-*myo*-inositol-1,4,5-trisphosphate ($InsP_3$‡) generation and subsequent mobilization of intracellular calcium ($[Ca^{2+}]_i$). This receptor is activated by both ATP and UTP, with similar potencies and efficacies, and does not appear to fit in with the present classification of P_2 receptors [7]. In the present study, we have observed a concentration-dependent biphasic response to ATP in populations of N1E-115 cells, which at millimolar concentrations of the nucleotide involves a secondary entry of calcium across the plasma membrane.

MATERIALS AND METHODS

Cell culture. N1E-115 cells, passages 30–47, were cultured in 75 cm^2 flasks in Dulbecco's modified Eagles medium (with glutamine) containing 5% foetal calf serum without antibiotics.

Calcium measurements. $[Ca^{2+}]_i$ was measured as described previously [14]. Briefly, the monolayers from two near-confluent flasks were detached using Pucks D1 solution (glucose, 5.5 mM; KCl, 5.4 mM; sucrose, 58.4 mM; Na_2HPO_4, 0.17 M; NaCl, 138 mM and KH_2PO_4, 0.22 mM) and resuspended in a simple saline HEPES buffer ($CaCl_2$, 2 mM; NaCl, 145 mM; glucose, 10 mM; KCl, 5 mM; $MgSO_4$, 1 mM and HEPES, 10 mM; pH 7.45). This was followed by incubation with fura-2 acetoxy methyl ester (5 μM)

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‡ Abbreviations: $InsP_3$, D-*myo*-inositol-1,4,5-trisphosphate; $[Ca^{2+}]_i$, intracellular calcium; PDBu, β-phorbol-12,13 dibutyrate.

at 37° (in the presence of 5% foetal calf serum) for 20 min and for a further 5 min following a 3-fold dilution (to ensure maximum hydrolysis of ester to the acid form). At the end of this loading period, excess dye was removed by centrifugation, and the cells were resuspended in fresh buffer (no serum) and left at room temperature until use. Each calcium time course was preceded by a rapid spin in a microcentrifuge followed by resuspension in fresh buffer.

All experiments were carried out using a Perkin–Elmer LS 50 Spectrometer, with excitation ratioing between 340 and 380 nm, recording at 500 nm. The time-course for each calcium measurement was 200 sec with drugs added in 10 μL aliquots.

Manganese entry. In some experiments recordings were made at an excitation wavelength of 360 nm (the "isobestic point"). Recordings at this wavelength are independent of calcium and depend only on the concentration of the dye itself. Manganese (Mn^{2+}) quenches fura-2 fluorescence at all wavelengths and under resting conditions is largely excluded from the cytoplasm. Following agonist stimulation it is believed to enter via a similar pathway to that of calcium ions. Thus, excitation at 360 nm, in the presence of extracellular Mn^{2+}, can be used to investigate entry of calcium into the cytoplasm [15–18]. Mn^{2+} (1 mM) was added at the beginning of the time course, in the absence of extracellular Ca^{2+} ions. Recordings of individual wavelengths were made over a time course of 200 sec.

Calibration. At the end of each time course, ionomycin (20 μM) was added followed by EGTA (6.25 mM, pH greater than 8.5) in order to calculate R_{max} and R_{min} [19]. Autofluorescence was determined using a separate cuvette following the addition of manganese (5 mM) after the ionophore ionomycin (20 μM). Using these values and those obtained with fura-2 free acid, $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz *et al.* [19].

Measurement of $InsP_3$. This was carried out using minor modifications of the radioreceptor method as described previously [20]. Briefly, N1E-115 cells (approx. 5×10^5 cells/mL) were preincubated in simple saline buffer for 10 min at 37°. Initially, aliquots of the cell suspension were removed at intervals in order to measure basal $InsP_3$. The agonist was then added and further aliquots taken at specific time points (initially every 10 sec). Ice-cold perchloric acid (7.5%) was used to stop the reaction (at each time point), the samples were neutralized with a calibrated amount of $KHCO_3$ (1.2 M) and the protein was separated by centrifugation. $InsP_3$ was quantified in the supernatant layer by a radioreceptor assay using a bovine adrenal-cortical binding protein at 4°. Authentic $InsP_3$ (10^{-10}–10^{-14} mol) in neutralized perchloric acid buffer was used to construct a standard curve for displacement of bound [^{3}H]$InsP_3$. The bound [^{3}H]$InsP_3$ was separated by rapid filtration and quantified by liquid-scintillation spectrometry.

The protein pellet was digested in NaOH (0.5 M) and estimated by the method of Bradford [21].

Materials. N1E-115 cells were supplied by Porton Down (Wiltshire, U.K.). Cell culture flasks were obtained from Costar with Dulbecco's modified Eagles medium and foetal calf serum from NBL Ltd (Cramlington, U.K.). β-Phorbol-12,13 dibutyrate (PDBu) was supplied by the Sigma Chemical Co. (Poole, U.K.) with fura-2 acetoxy methylester and ionomycin from Calbiochem/Novobiochem (Nottingham, U.K.). All nucleotides were supplied by Boehringer (Lewes, U.K.) with $InsP_3$ from Amersham (U.K.) and [^{3}H]$InsP_3$ from NEN Dupont (Stevenage, U.K.).

Statistics and data analysis. EC_{50} and IC_{50} (concentrations of drug producing 50% of maximal stimulation and inhibition, respectively) values were obtained by computer-assisted curve fitting by use of the computer program Graph-Pad (ISI). Calcium data was captured using the ICBC program supplied by Perkin–Elmer and imported to the graphics program Sigma-Plot (Jandel). Free ion concentrations were estimated using the Eqcal program (Biosoft, Elsevier). Significance testing was carried out using an unpaired Student's *t*-test.

RESULTS

ATP (100 μM) caused a rapid, transient increase in $[Ca^{2+}]_i$ (Fig. 1A) which was concentration-dependent (EC_{50} of 21 ± 4 μM; N = 3) and unaffected by the omission of extracellular calcium (86 ± 9% of control response; N = 3). $InsP_3$ mass measurements (Fig. 1B) revealed a similarly transient increase to a maximum of 35 ± 3 pmol/mg (N = 3) above basal which was unchanged in the absence of extracellular calcium [7].

Addition of a higher concentration of ATP (1 mM; Fig. 2, open circles) again produced a rapid increase in $[Ca^{2+}]_i$ rising to a peak of 639 ± 23 nM ($P < 0.01$; N = 3) above basal which returned to a new maintained plateau, above pre-stimulus levels (after approximately 60 sec) of 274 ± 34 nM ($P < 0.05$; N = 3).

Source of the calcium

When ATP was removed by centrifugation following a 2 min stimulation period, the $[Ca^{2+}]_i$ returned to resting values (Fig. 2, open triangles). However, addition of bradykinin (an agonist which in this cell line is thought to release calcium only from intracellular stores; [14]) failed to have any effect. When ATP (1 mM) was added for a second time the maintained plateau was re-established.

Addition of the inorganic calcium channel blocker nickel (1 mM), before or after ATP (1 mM), removed the secondary plateau and converted the calcium change to a transient response (Fig. 3). Further, when the cells were challenged with UTP (100 μM) to discharge the intracellular stores, a second response to ATP (1 mM) was only obtained when calcium was included in the extracellular medium (Fig. 4).

It should be noted that there was some variability in the absolute $[Ca^{2+}]_i$ increases from experiment to experiment (e.g. Figs 2 and 3A) although the qualitative responses to ATP were very similar. In some experiments (e.g. Fig. 3A) there was also an upward drift, most evident after 90 sec. This is probably not a direct of ATP since similar drifts can be observed in untreated cells (data not shown) and

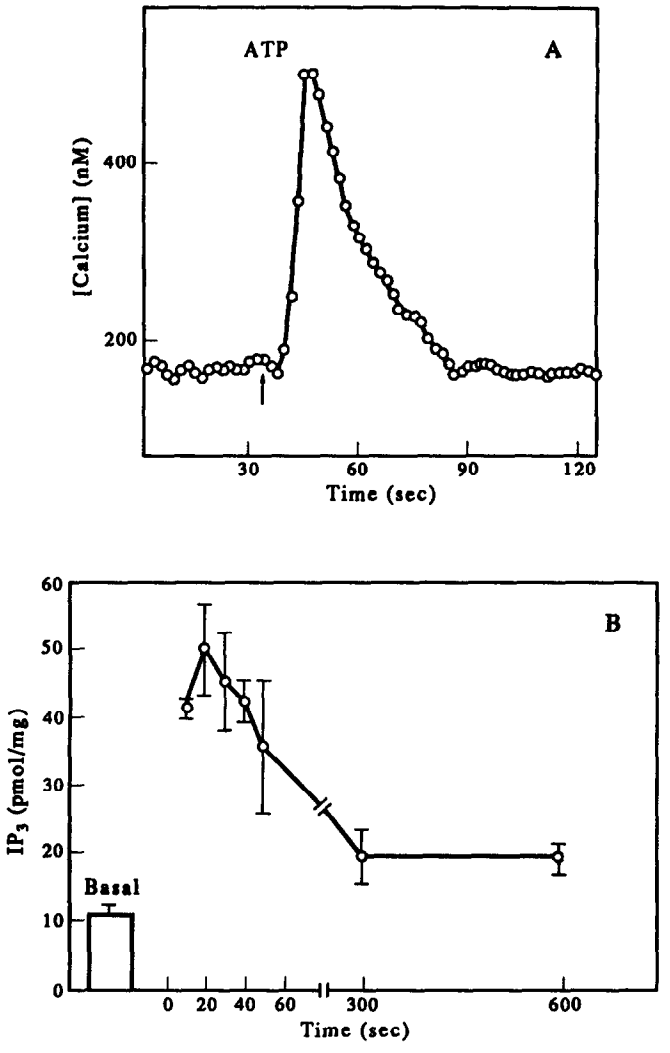

Fig. 1. The upper panel shows the effect of ATP (100 μM) on $[Ca^{2+}]_i$ in populations of fura-2-loaded N1E-115 cells. Fluorescence values were measured following excitation at 340 and 380 nm and the ratio was used to determine $[Ca^{2+}]_i$. The graph is typical of two others. The drug was added approximately at the time indicated (this is the same for all subsequent calcium traces). The lower panel represents the time course for $InsP_3$ generation (pmol/mg) following the addition of ATP (100 μM). Basal levels are represented by the histogram. The data are means of three separate determinations; vertical error bars represent SEM.

this probably reflects either an outward flux of fura-2 or an inward leakage of calcium.

Manganese entry

Manganese (Mn^{2+}; 1 mM) was added to the incubation medium in order to investigate the possibility of agonist-stimulated entry. When recordings were made at excitation wavelengths of 340 and 360 nm, there was a sharp drop in both signals immediately after addition of Mn^{2+} (due to quenching of extracellular fura-2) followed by a slower decline probably due to a small leakage of Mn^{2+} into the cells. Addition of ATP (1 mM), in the absence of extracellular calcium, resulted in a rapid transient increase in the 340 nm signal with only a slight change in the 360 nm signal (Fig. 5A). A similar fall in the 360 nm signal was observed in the absence of Mn^{2+} (control; Fig. 5B) suggesting slight calcium sensitivity at this wavelength. A significant entry of Mn^{2+} was, however, observed following addition of the calcium ionophore ionomycin (20 μM; Fig. 5A).

Phorbol pre-treatment

Pre-incubation (20 min during fura-2 loading period) with the phorbol ester PDBu (1.5 μM) caused a significant reduction in the response to UTP (42 ± 4% of control; 100 μM). However, the calcium response to ATP (1 mM), added approximately 1 min later, was unaffected (105 ± 12% of control response). A similar result

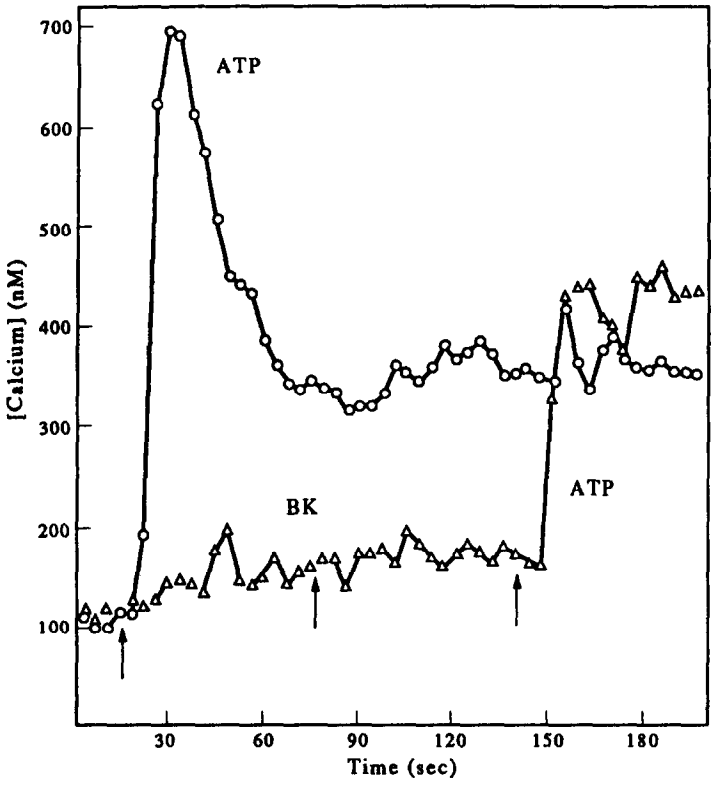

Fig. 2. The effect of ATP (1 mM) on $[Ca^{2+}]_i$ in populations of fura-2-loaded N1E-115 cells (open circles). Fluorescence values were measured followed excitation at 340 and 380 nm and the ratio used to determine $[Ca^{2+}]_i$. The graph is typical of two others. The superimposed trace (open triangles) represents cells pre-treated with ATP (1 mM) for 120 sec, followed by resuspension in fresh buffer (no ATP). The cells were then immediately challenged with bradykinin (BK; 100 nM) and ATP (1 mM).

was obtained when the cells were challenged with ATP (100 μM) followed by millimolar ATP (data not shown).

$InsP_3$ mass measurements

Mass measurements of $InsP_3$ showed a rapid increase in production peaking at 10–20 sec after addition of the agonist (ATP; 1 mM) and maintained significantly above basal levels for the time course of the experiment (Fig. 6A). Similarly maintained responses were observed when calcium was omitted from the extracellular medium (Fig. 6B) and the cells were pre-incubated with the inorganic calcium channel blocker, nickel (1 mM; Fig. 6C).

DISCUSSION

We have shown previously that ATP at concentrations of up to 100 μM in the neuronal cell line N1E-115 causes a transient rise in both $[Ca^{2+}]_i$ and $InsP_3$, which is unaffected by omission of calcium from the extracellular medium, and which is sensitive to pre-treatment with the phorbol ester, β-phorbol-12,13 dibutyrate [7]. In this report, we present evidence that stimulation with a higher concentration of ATP (1 mM) involves sustained calcium entry accompanied by a maintained increase in $InsP_3$ levels.

Calcium entry?

The calcium rise following stimulation with 100 μM ATP in N1E-115 cells is mediated via activation of an atypical P_2-purinergic receptor (neither P_{2X} nor P_{2Y}; [7]), due to mobilization of calcium from intracellular stores. ATP produced a transient increase in $[Ca^{2+}]_i$ which returned to pre-stimulus levels 50 sec after agonist addition. A similarly transient increase in $InsP_3$ generation was also observed (Fig. 1). However, when the cells were challenged with a higher concentration of ATP (1 mM) the initial calcium spike was followed by a secondary plateau phase which remained significantly above pre-stimulus values for the duration of the experiment (Fig. 2). The response was reversible since removal of the agonist by centrifugation resulted in $[Ca^{2+}]_i$ returning to the pre-stimulus level (Fig. 2). However, addition of bradykinin (previously shown to release calcium only from intracellular stores [14]) failed to have any significant effect, suggesting the stores of $[Ca^{2+}]_i$ had been discharged. The plateau was reestablished following a second addition of ATP (1 mM), indicating that this sustained phase did not require the presence of stored intracellular calcium. Addition of the inorganic calcium channel blocker, nickel (before or after ATP), converted the change in $[Ca^{2+}]_i$ back to a transient response, indicating a secondary entry of

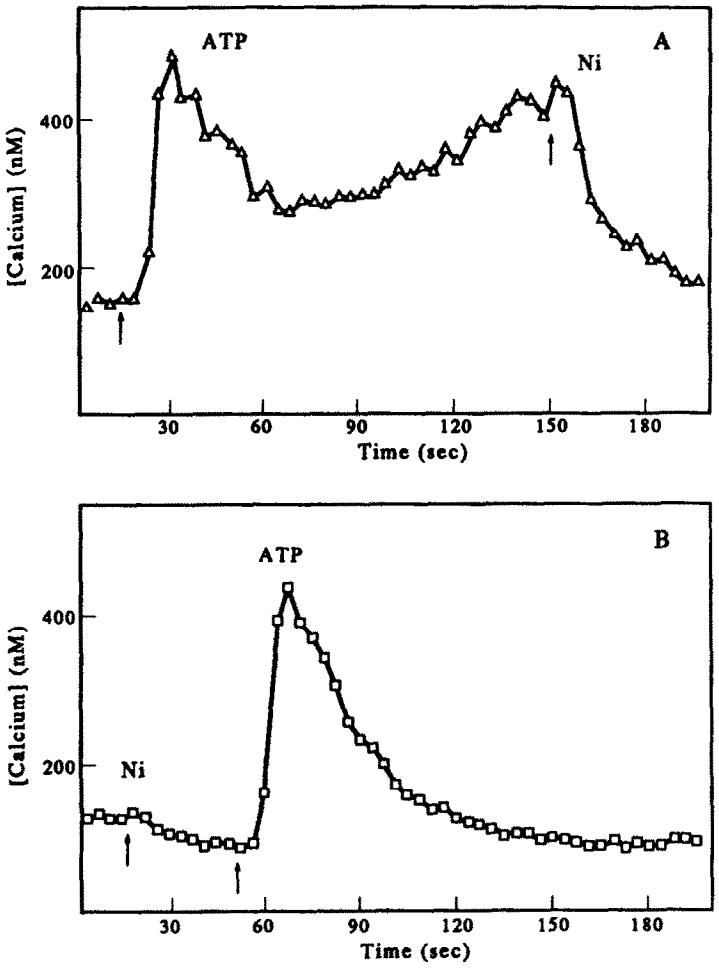

Fig. 3. The effect of the inorganic channel blocker nickel (Ni; 1 mM) on the calcium response to ATP (1 mM). Addition of nickel before ATP (panel B) prevents the secondary plateau phase. Similarly, its addition after ATP (panel A) decreases $[Ca^{2+}]_i$ back to pre-stimulus levels. Each graph is typical of two others.

calcium across the plasma membrane, which was not evident when the cells were stimulated with lower concentrations of ATP. Furthermore, the secondary response was dependent on extracellular calcium; addition of ATP (1 mM), after discharge of the intracellular stores by UTP (100 μM), failed to have any significant effect in nominally calcium-free medium (Fig. 3).

Nature of the channel

Manganese has been used by Hallam and coworkers [15–17] and Jacob [18] as a tool to investigate entry of calcium across the plasma membrane. Its use is dependent on the fact that it quenches fura-2 fluorescence at all wavelengths and that it enters cells via a similar pathway to that of calcium. Recording at 360 nm (the "isobestic" or "calcium-insensitive" wavelength) in the presence of extracellular Mn^{2+} (1 mM) and the absence of calcium allows its passage into cells to be monitored. Following addition of ATP (1 mM) there was a transient rise in the 340 nm signal (Fig. 5A), indicative of intracellular calcium release, with only a slight change in the 360 nm signal. This would suggest, when taken together with the blockade of calcium entry in the presence of nickel, that ATP promotes calcium entry via a Mn^{2+}-insensitive pathway. There are other examples of calcium channels which do not allow the passage of Mn^{2+} [22, 23]. More importantly, however, this is evidence against ATP simply forming "pores" in the plasma membrane. The calcium ionophore, ionomycin, is known to act in this way and following its addition there was significant entry of Mn^{2+} resulting in quenching of the intracellular signal. There was a small change in the 360 nm signal following addition of ATP (1 mM) but this was probably due to slight calcium sensitivity of the dye at an excitation wavelength of 360 nm, as repeating the experiment in the absence of Mn^{2+}, but recording using the same wavelengths, produced a similar result (Fig. 5B).

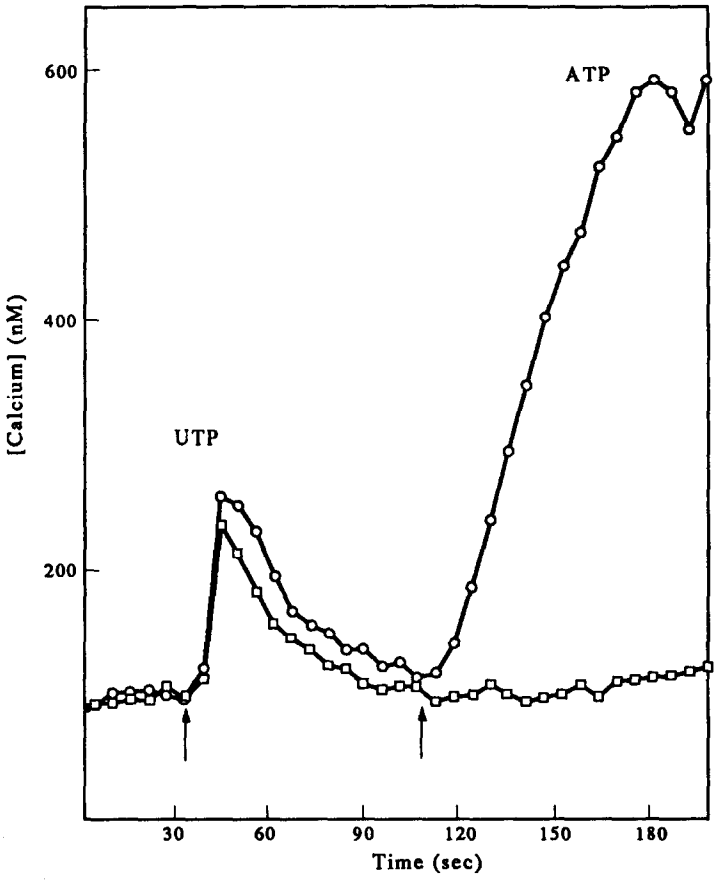

Fig. 4. The importance of extracellular calcium in the calcium response to ATP (1 mM). The cells were challenged with a maximally effective concentration of UTP (100 μM) to discharge intracellular stores, resulting in a transient increase in $[Ca^{2+}]_i$. This was followed by addition of ATP (1 mM) which stimulated a secondary influx of calcium (open circles). The superimposed trace (open squares) represents the same experiment with calcium omitted from the extracellular medium. The secondary rise in $[Ca^{2+}]_i$ did not occur under these conditions. Each graph is representative of two others.

Phorbol sensitivity

We have previously shown that calcium mobilization from intracellular stores via activation of P_2 receptors by ATP (100 μM) is subject to inhibition by phorbol esters [24] which is presumed to be due to protein kinase C activation. This effect is common for many receptor-mediated phosphoinositidase C signal transduction pathways [25]. However, from the data it would appear that the calcium entry is unaffected by pre-treatment with PDBu. Although the target of PDBu as an inhibitor of calcium mobilization is not known, this provides additional evidence that the rise in $[Ca^{2+}]_i$, in the presence of higher concentrations of ATP, is qualitatively different from that observed in the presence of ATP concentrations of up to 100 μM, and that the plateau phase does not result from sustained mobilization of $[Ca^{2+}]_i$.

ATP is known to chelate metal cations [26] [for example, using the computer program, Eqcal, estimations for the free concentrations of calcium (2 mM added) and magnesium (1 mM added) in the presence of 1 mM ATP were 1.3×10^{-3} and 7.3×10^{-4}, respectively] and this might compromise, to some extent, interpretation of some of the data presented. These changes in free ion concentrations would not appear to be functionally important, however, because even at the highest concentration of ATP (1 mM) there was still sufficient free calcium (Fig. 2) and manganese (after the addition of ionomycin; Fig. 5) to enter the cells and sufficient free nickel (Fig. 3) to block the entry of calcium.

$InsP_3$ production

Increasing the concentration of ATP similarly produced a maintained accumulation of $InsP_3$ (Fig. 6A). However, unlike the calcium entry component, the maintained $InsP_3$ response was unaffected by the omission of extracellular calcium, or by the inclusion of the inorganic calcium channel blocker, nickel, into the extracellular medium (Fig. 6B and C). Thus, secondary, calcium-stimulated phosphatidyl inositol 4,5-bisphosphate hydrolysis would appear to be unlikely as a continuing source of $InsP_3$.

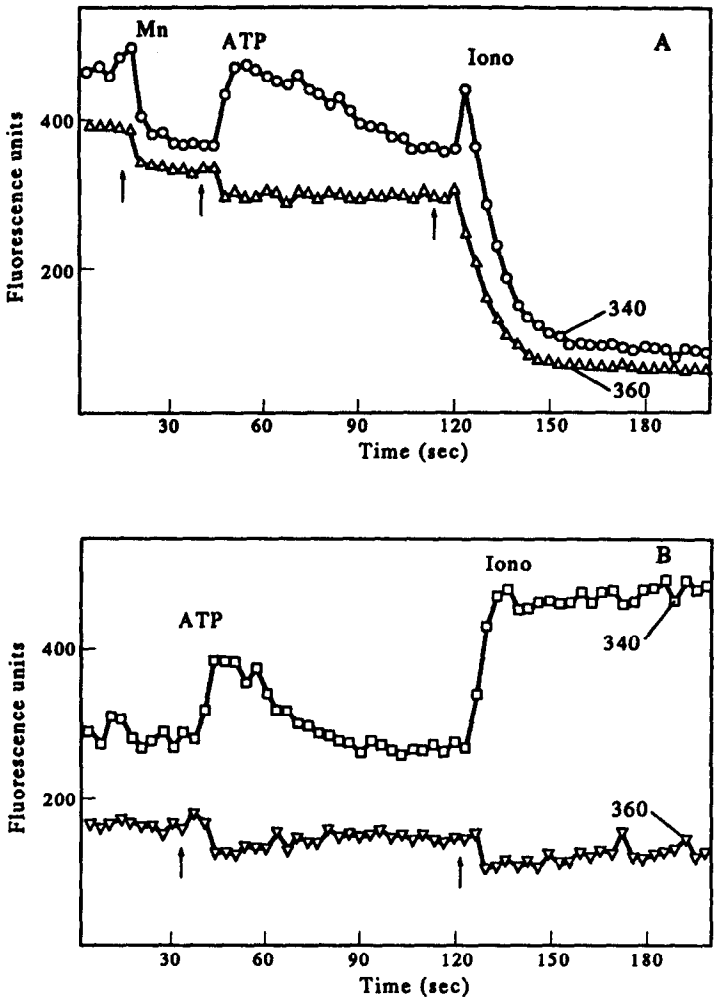

Fig. 5. Single wavelength recordings in fura-2-loaded N1E-115 cells suspended in nominally calcium-free medium (see text). The emission wavelength was set at 500 nm with excitation at 340 (open circles) and 360 (open triangles) nm. In the upper panel Mn^{2+} (1 mM) was added at the time indicated followed by ATP (1 mM) and the ionophore ionomycin (20 μM). The agonist caused only a slight change in the 360 nm signal unlike ionomycin which stimulated significant entry of Mn^{2+} (represented by a drop in the 360 nm signal). This graph is typical of two other experiments. The lower panel represents a control situation in the absence of extracellular Mn^{2+} (340 nm open squares, 360 nm open triangles). The small drop in the 360 nm signal was still present following addition of ATP, indicating some calcium sensitivity at this wavelength.

However, it is possible that the calcium entry component is under the control of an $InsP_3$ or possibly $InsP_4$-mediated [27] calcium channel. Indeed, it is not known whether or not the maintained level of $InsP_3$ is due to its continued synthesis or to inhibition of its metabolism. Such possibilities require further investigation.

In summary, addition of ATP (1 mM) to populations of the neuronal cell line N1E-115 resulted in a primary mobilization of calcium from intracellular stores followed by secondary entry of calcium via a nickel-sensitive but manganese-insensitive pathway. The calcium response was accompanied by, but was apparently not the result of, maintained $InsP_3$ levels. The physiological relevance of these results is unclear, but high extracellular ATP concentrations may be achieved during trauma and repetitive nerve stimulation [4, 10]. Furthermore, if a similar situation exists in purinergic cells *in vivo*, the resultant effects may be important contributory factors in subsequent cell lysis and death, as has been proposed for increased calcium entry due to excitatory amino acids such as *N*-methyl D-aspartate [28]. The qualitative change in the nature of the calcium response from a transient to a sustained increase occurs over a very restricted ATP concentration range, and it is tempting to speculate that this might be involved in the triggering of a cytotoxic as opposed to a rapid signalling response.

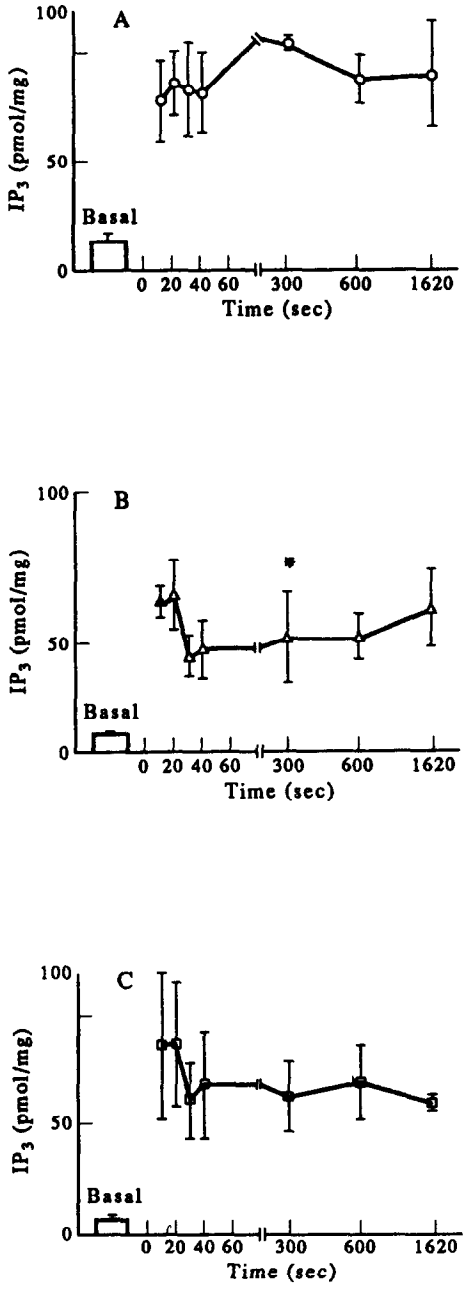

Fig. 6. The effects of extracellular calcium and nickel on the $InsP_3$ response to ATP (1 mM). The upper panel represents the time course for $InsP_3$ generation following addition of ATP (1 mM) in the presence of extracellular calcium (2 mM). The graph shows $InsP_3$ formation (pmol/mg) as a function of time. $InsP_3$ levels remained elevated above basal for the time course of the experiment. Data are means of three separate determinations using cells from the same passage; vertical error bars represent SEM. Similar results were obtained when the experiments were repeated using cells from a different passage. Panels B and C show the effects of nominally calcium-free medium and the inorganic channel blocker nickel (1 mM), respectively, on the same response. In both cases, $InsP_3$ levels failed to return to basal even after 27 min. All $InsP_3$ values measured after the addition of ATP were statistically different from basal levels (Student's unpaired *t*-test; $P < 0.05$) with the exception of the point marked with an asterisk.

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