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ATP Causes Release of Intracellular Ca^{2+} via the Phospholipase Cβ/IP_3 Pathway in Astrocytes from the Dorsal Spinal Cord

Michael W. Salter and J. L. Hicks

Division of Neuroscience, Hospital for Sick Children, Department of Physiology, University of Toronto, Toronto, Ontario, Canada

Calcium signaling within astrocytes in the CNS may play a role comparable to that of electrical signaling within neurons. ATP is a molecule known to produce Ca^{2+} responses in astrocytes, and has been implicated as a mediator of intercellular Ca^{2+} signaling in other types of nonexcitable cells. We characterized the signal transduction pathway for ATP-evoked Ca^{2+} responses in cultured astrocytes from the dorsal spinal cord. Nearly 100% of these astrocytes respond to extracellularly applied ATP, which causes release of Ca^{2+} from an intracellular pool that is sensitive to thapsigargin and insensitive to caffeine. We found that intracellular administration of IP_3 also caused release of Ca^{2+} from a thapsigargin-sensitive intracellular pool, and that IP_3 abolished the response to ATP. The ATP-evoked Ca^{2+} response was blocked by the IP_3 receptor antagonist heparin, applied intracellularly, but not by N-desulfated heparin, which is not an antagonist at these receptors. The Ca^{2+} response caused by ATP was also blocked by a phospholipase C inhibitor, U-73122, but not by its inactive analog, U-73343. Increases in $[Ca^{2+}]_i$ were elicited by intracellular application of activators of heterotrimeric G-proteins, GTPγS and AlF_4^-. On the other hand, $[Ca^{2+}]_i$ was unaffected by a G-protein inhibitor, GDPβS, but it did abolish the Ca^{2+} response to ATP. Pretreating the cultures with pertussis toxin did not affect responses to ATP. Our results indicate that in astrocytes ATP-evoked release of intracellular Ca^{2+} is mediated by IP_3 produced as a result of activating phospholipase C coupled to ATP receptors via a G-protein that is insensitive to pertussis toxin. ATP is known to be released under physiological and pathological circumstances, and therefore signaling via the PLC-IP_3 pathway in astrocytes is a potentially important mechanism by which ATP may play a role in CNS function.

[Key words: fura-2, spinal dorsal horn, P_2 purinergic receptors, heparin, U-73122, GTPγS, GDPβS, aluminum fluoride, thapsigargin, G-proteins, pertussis toxin]

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Correspondence should be addressed to Dr. Michael W. Salter, Division of Neuroscience, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8.

Adenosine 5′-triphosphate (ATP) released into the extracellular space in the mammalian CNS has been suggested to act as an intercellular signaling molecule (Phillis and Wu, 1981; Stone, 1981; Burnstock, 1990; Salter et al., 1993). One functional role for ATP may be as a chemical mediator of fast excitatory transmission at central synapses. Evidence supporting this role comes from biochemical studies showing the presence of ATP in synaptic vesicles (Poisner and Trifaró, 1982), and its release in a Ca^{2+}-dependent manner from both synaptosomes (White et al., 1985) and CNS slice preparations (Wieraszko et al., 1989). Electrophysiological studies *in vivo* (Phillis and Wu, 1981; Fyffe and Perl, 1984; Salter and Henry, 1985) and *in vitro* (Jahr and Jessell, 1983; Illes and Norenberg, 1993) have demonstrated that neurons from numerous CNS regions are excited by ATP. Moreover, evidence for excitatory postsynaptic currents mediated by ATP has been obtained in the CNS, specifically in the medial habenula (Edwards et al., 1992). However, the actions of ATP are not limited to neurons. Astrocytes (Neary et al., 1988; Pearce et al., 1989; Bruner and Murphy, 1993; Salter and Hicks, 1994), microglia (Missiaen et al., 1994; Nörenberg et al., 1994), and oligodendrocytes (Kastritsis and McCarthy, 1993; Salter and Hicks, 1994) have each been shown to respond to ATP. Because non-neuronal cells as well as neurons are responsive to ATP, this suggests that a role as an excitatory neurotransmitter is but one of many potential signaling roles for ATP released in the CNS.

In cells that are not electrically excitable, such as non-neuronal cells in the CNS, a change in the concentration of intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) is particularly important for transducing and transmitting signals (Berridge, 1993; Putney and Bird, 1993). Indeed, in astrocytes intracellular Ca^{2+} responses and subsequent intercellular transmission of Ca^{2+} signals, often by means of Ca^{2+} waves, have been suggested as an information processing system that may complement the electrical signaling system in neurons (Cornell-Bell et al., 1990; Dani et al., 1992). One molecule that has been implicated in mediating transmission of Ca^{2+} signals between nonexcitable mast cells in culture system (Osipchuk and Cahalan, 1992) is ATP.

ATP-evoked increases in $[Ca^{2+}]_i$ in astrocytes have been shown in cells from cerebral cortex (Neary et al., 1988; Pearce et al., 1989; Bruner and Murphy, 1993) and dorsal spinal cord (Salter and Hicks, 1994). Five subtypes of P_2 purinergic (ATP) receptors—P_{2X}, P_{2Y}, P_{2Z}, P_{2T}, and P_{2U}—have been described (Burnstock, 1990; O'Connor et al., 1991), and studies on the potency of ATP analogs have suggested that the increase in $[Ca^{2+}]_i$ in astrocytes is due to activation of the P_{2Y} subtype (Kastritsis et al., 1992; Salter and Hicks, 1994). ATP-evoked increases in $[Ca^{2+}]_i$ are highly prevalent in astrocytes from the dorsal

spinal cord with greater than 99% of cells responding to ATP or other P_{2Y} receptor agonists (Salter and Hicks, 1994). Such $[Ca^{2+}]$ responses could play important roles in physiological or pathological processes within the spinal cord.

Increases in $[Ca^{2+}]_i$ produced by ATP have been correlated with increases in inositol phospholipid turnover and with inositol 1,4,5 trisphosphate (IP_3) itself in studies of astrocytes (Pearce et al., 1989; Kastritsis et al., 1992). This correlation has led to speculation that in astrocytes IP_3 may be a mediator of $[Ca^{2+}]$ release produced by ATP, although a causal relationship has not been established. Evidence indicates that P_{2Y} receptors may be coupled to additional signal transduction pathways that might affect $[Ca^{2+}]_i$ secondarily (Boyer et al., 1993; Bruner and Murphy, 1993).

In order to establish the signal transduction pathway for the ATP-evoked increase in $[Ca^{2+}]_i$ in astrocytes we have used whole-cell patch-clamp recording with simultaneous optical measurement of $[Ca^{2+}]_i$. We have taken advantage of the exchange dialysis that occurs during whole-cell recording in order to administer agents into the cells. We report here the first direct evidence that activation of P_2 purinergic receptors raises $[Ca^{2+}]_i$ via the phospholipase C/IP_3 pathway.

Preliminary results of this study have been reported in part (Salter and Hicks, 1992).

Materials and Methods

The methods used for cell culture, measuring $[Ca^{2+}]_i$, and simultaneous whole-cell patch-clamp recording have been described in detail previously (Salter and Hicks, 1994).

Cell cultures. Cultures of dorsal spinal cord were prepared from fetal Wistar rats (embryonic day 17–19) taken from time-pregnant females (Charles River) killed by cervical dislocation. Upon removal, the fetuses were transferred to chilled, sterile Hank's buffered salt solution, and then killed by decapitation. The entire spinal cord was removed by an anterior approach and the dorsal half of the cord was dissected by the "open book" method (Guthrie et al., 1987). The tissue was treated with trypsin and then mechanically dissociated in culture medium consisting of minimum essential medium supplemented with 10% fetal bovine serum, 10% heat-inactivated horse serum, and 1 U/ml insulin. The cells were plated onto collagen-coated Aclar 33C plastic, which transmits visible and near-visible ultraviolet light. Four days after plating the cultures were treated for 24 hr with 5′-fluoro-2-deoxyuridine (25 μg/ml) and uridine (50 μg/ml). Cultures were used for recording 6–10 d after plating. At this time, the astrocyte layer was still subconfluent and individual cells were readily visible. Media and sera were from Gibco Canada Inc. (Burlington, Ontario).

Simultaneous measurement of $[Ca^{2+}]_i$ and whole-cell patch recording. Intracellular $[Ca^{2+}]$ was measured photometrically from single astrocytes using the fluorescent calcium indicator dye fura-2 (Molecular Probes, Inc., Eugene, OR). In most experiments $[Ca^{2+}]_i$ was measured before as well as during whole-cell recording; therefore, it was necessary to preload the cells with fura-2. This was done by incubating the cultures for 75–90 min at room temperature in recording medium supplemented with fura-2 acetoxymethyl ester (fura-2 AM; 2 μM) and bovine serum albumin (0.5%, Sigma). The cells were then rinsed thoroughly in the extracellular recording solution containing (mM) NaCl, 140; KCl, 5.4; *N*-2-hydroxyethylpiperazine sulfonic acid (HEPES), 25; glucose, 33; $CaCl_2$, 1.3; $MgCl_2$, 1; tetrodotoxin (TTX), 0.001; pH 7.35, osmolarity 310–320 mOsm. The cultures were placed in the recording chamber on a Nikon Diaphot microscope. All experiments were done at room temperature (20–22°C).

Excitation light was from a 75 W xenon arc lamp. The light was passed alternatingly through 340 or 380 nm bandpass filters (Omega Optical, Brattleboro, VT) via a spinning mirrored chopper. The excitation light was transmitted via a quartz fiber-optic cable coupled to the epifluorescence adapter of the microscope. The light was directed toward the cell by means of a DM400 dichroic mirror and was focused through a 40× fluor oil-immersion objective (Nikon). The emitted light was reflected off a DM580 dichroic mirror and passed through a 510 nm bandpass filter. Light was collected by a photomultiplier tube detector, operating in a single photon counting mode, which sent a TTL signal to an IBM PC compatible computer. Fluorescence signals were sampled at a rate of 20 Hz. The signals were acquired, stored, and analyzed using hardware and OSCAR software (Photon Technologies Inc., London, Ontario). An adjustable rectangular diaphragm was used to sample light emitted from an individual cell.

Intracellular $[Ca^{2+}]$ was determined from the ratio (R) of the light emitted at 340/380 nm excitation wavelengths. R was calculated after subtracting background readings measured in a region of the dish which did not contain any cells (Thayer et al., 1988). Free $[Ca^{2+}]$ was calculated using the formula $[Ca^{2+}] = K\,(R - R_{min})/(R_{max} - R)$, where R_{min} is the minimum ratio corresponding to fully unbound dye, R_{max} is the maximum ratio for the fully bound dye, and K is the product of the dissociation constant for fura-2 and the ratio of bound to free dye at 380 nm excitation. R_{min}, R_{max}, and K were determined by *in vitro* titration using the method described by Grynkiewicz et al. (1985). In the calibration, ratio measurements were made in a series of solutions with a fixed concentration of EGTA and varying amounts of added Ca^{2+}. The free concentration of Ca^{2+} in these solutions was calculated from the EGTA stability constants.

During the period when fluorescence and electrical signals were measured, the cells were visualized continuously under Hoffman modulation contrast (Modulation Optics, East Hills, NY), This was done by transilluminating the cells with light which had been passed through an A600 barrier filter. The transmitted light passed through the DM580 mirror and was sent to a CCD camera. The images were observed on a standard television monitor. The cross talk introduced by transilluminating the cells was less than 0.1% of the typical fluorescence signal and therefore did not interfere with the measurements of $[Ca^{2+}]_i$. To minimize photobleaching of fura-2 and possible cell damage due to UV irradiation, unnecessary exposure to the excitation light was eliminated during periods of recovery or changing solutions by closing a shutter in front of the xenon lamp. During these periods $[Ca^{2+}]_i$ could not be measured and this is shown in the figures by small discontinuities in the recordings.

Patch-clamp recordings in the whole-cell configuration were made while simultaneously measuring $[Ca^{2+}]_i$. Patch pipettes were constructed from thin-walled borosilicate glass and the electrode resistances were 3–5 MΩ. The recording pipettes were filled with an intracellular recording solution containing (mM) fura-2 pentapotassium salt, 0.05; KCl, 140; HEPES, 10; EGTA, 0.1; $MgCl_2$, 1; pH 7.26. The low concentration of EGTA was included to prevent overloading the cells with Ca^{2+} (Thayer et al., 1988).

Recordings were made using an EPC-7 amplifier (Adams and List, Westbury, NY) and cells were studied under voltage-clamp conditions with the membrane potential held at −60 mV unless otherwise indicated. Membrane current and voltage were monitored on a chart recorder (Gould Inc.) and were stored along with trigger pulses on videotape (VR 100A; Instrutech Inc., Great Neck, NY). Also, membrane current was sampled on an analog channel of the hardware used for acquiring the fluorescence data. Trigger pulses for the drug applications were acquired with this hardware. The trigger pulses were used for display purposes to align records of $[Ca^{2+}]_i$ with those of membrane current which were resampled from tape at 0.1–1 kHz on an IBM PC compatible computer using pCLAMP hardware and software (Axon Instruments, Foster City, CA).

Selection of cells and electrophysiological characteristics. Astrocytes were selected for patch-clamp recording on the basis of characteristic morphological features described in Salter and Hicks (1994). We have shown that cells with such morphological features express the astrocyte marker glial fibrillary acidic protein (GFAP). Also, such cells do not generate action potentials, even in the absence of TTX, nor do they show postsynaptic currents. The patch pipette was placed onto the cell membrane near the nucleus. After rupturing the membrane patch, the series resistance was 8–20 MΩ and was compensated approximately 50%. The input resistance of the cells studied was in the range of 100–300 MΩ. We tried to minimize possible problems of voltage clamping cells with extensive processes by avoiding cells larger than approximately 75 μm in diameter. Also, ATP was directed onto the region of the cell near the recording pipette where control of membrane potential would be expected to be the best. In preliminary experiments Ca^{2+} responses to ATP were found to be consistent when the membrane potential was varied in the range of −80–−20 mV. Thus, even though it is possible that the membrane potential might not have been con-

trolled optimally, this could not account for effects on Ca^{2+} responses obtained during whole-cell recording.

Application of ATP and other drugs. ATP was made up as a 1 M stock solution with the pH adjusted to 7.4. The stock solution was divided into single-use aliquots and stored at −70°C. Aliquots were thawed immediately prior to use and working solutions were made by diluting the stock solution in extracellular recording media. ATP (1–10 μM) was applied by pressure (10–50 kPa; Picospritzer II, General Valve, Fairfield, NJ) from a glass micropipette, the tip of which was located approximately 20–30 μm from the cell under study.

The following compounds were tested by adding them to the intracellular recording solution and administering them by exchange diffusion during whole-cell recording: D-myo-inositol 1,4,5 trisphosphate (IP_3), heparin, N-desulfated heparin, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS), or NaF and $AlCl_3$. IP_3, fura-2, and fura-2 AM were obtained from Molecular Probes, NaF and $AlCl_3$ were from Fluka, and the other compounds were from Sigma.

Other drugs tested were thapsigargin, 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U-73122), and 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione (U-73343), which are membrane-permeant compounds. They were made up as stock solutions in DMSO and stored at −70°C. Just before use the stock solution was diluted in the extracellular recording media. The compounds were applied by exchanging the entire bath volume 5–6 times. The final concentration of DMSO never exceeded 0.1%; no effects of DMSO were observed even at a concentration of 1%. Thapsigargin was from Research Biochemicals Inc. (Natick, MA); U-73122 and U-73343 were from Biomol Research Labs, Inc. (Plymouth Meeting, PA).

Pertussis toxin experiments. In order to examine the effect of pertussis toxin on responses to ATP, fresh medium containing 1 μg/ml pertussis toxin (Sigma) or its vehicle (glycerol 0.5%, Na_2HPO_4 0.5 mM, NaCl 1 mM; final concentration) was added to the cultures. After incubating the cultures for 20–24 hr at 37°C, the response of control and treated cells was determined to a test application of ATP (3 μM, 5 sec). The effectiveness of pertussis toxin in ADP-ribosylating substrate proteins was verified by means of an *in vitro* ADP-ribosylation assay (Ribeiro-Neto et al., 1987; Wong et al., 1988) using cell homogenates from control and treated cells. Cultures were rinsed with phosphate-buffered saline (in mM: 137 NaCl, 3 KCl, 8.1 Na_2HPO_4, 1.5 KH_2PO_4, 0.5 $MgCl_2$, 1 $CaCl_2$; pH 7.4) and cells were then scraped from the dishes and homogenized in Tris-HCl 100 mM, pH 8. The homogenate was incubated with ^{32}P-nicotinamide-adenine dinucleotide (^{32}P-NAD; 0.5 μCi) with or without activated pertussis toxin (2.5 μg) at 30°C for 30 min. Proteins were solubilized in SDS sample buffer and separated by electrophoresis on a 12.5% SDS-polyacrylamide gel with 10 μg protein per lane. The gel was stained with Coomassie blue, destained, dried, and subjected to autoradiography for 24 hr. The ADP-ribosylation assay was performed in duplicate.

Results

Intracellular $[Ca^{2+}]$ was measured in more than 100 individual astrocytes. ATP (1–10 μM) produced an increase in $[Ca^{2+}]_i$, and in cells which were not subject to patch-clamp recording the Ca^{2+} responses were consistent with repeated applications of ATP (Salter and Hicks, 1994). As shown in Figure 1*A*, the response to ATP was unaffected by removing Ca^{2+} and including EGTA (0.1 mM) in the extracellular solution, indicating that ATP caused release of Ca^{2+} from intracellular stores. Figure 1*B* illustrates that ATP also produced increases in $[Ca^{2+}]_i$ during whole-cell recording when the intracellular solution consisted of a minimal balanced salt solution containing a low concentration of EGTA (0.1 mM). Under these recording conditions the amplitude of the responses during whole-cell recording was within 10–15% of that of responses evoked before patch recording, and the Ca^{2+} responses were stable for up to 30 min after attaining whole-cell recording. Also, the baseline level of $[Ca^{2+}]_i$ was maintained near the level prior to rupturing the membrane patch, typically 50–100 nM, throughout the period of whole-cell recording.

The diffusional exchange between the intracellular and pipette

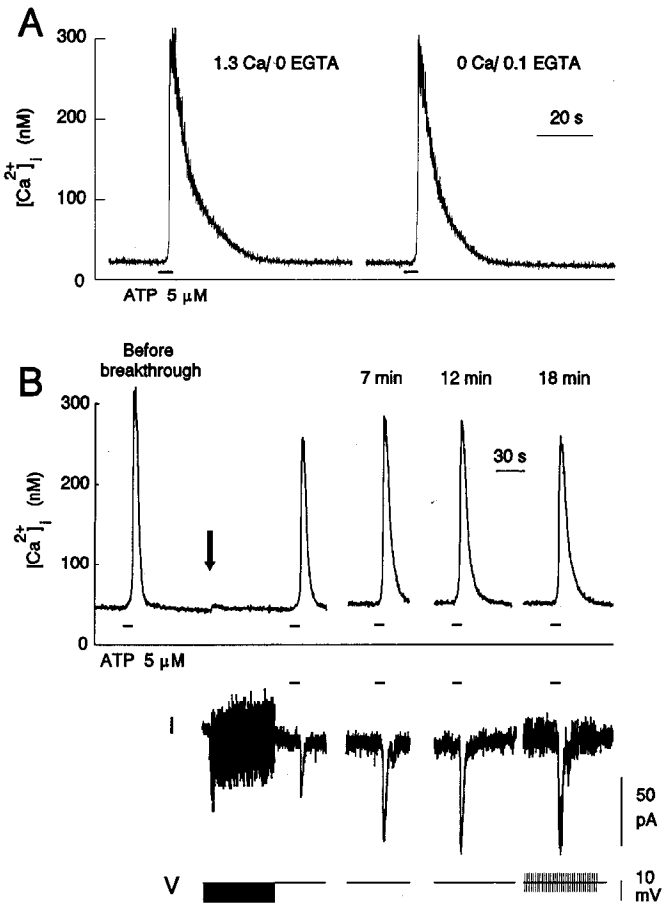

Figure 1. ATP causes an increase in $[Ca^{2+}]_i$ in individual spinal dorsal astrocytes that is not dependent upon the presence of extracellular Ca^{2+} and that is stable during whole-cell patch-clamp recording. *A* shows traces of $[Ca^{2+}]_i$ during recording from an astrocyte that was not subject to whole-cell patch clamp. On the *left* is an example of the Ca^{2+} response to application of ATP (5 μM, 5 sec) when the extracellular recording solution contained 1.3 mM Ca^{2+}. The bath was exchanged with extracellular solution containing no added Ca^{2+} and 0.1 mM EGTA. Two minutes after the bath had been exchanged, ATP was applied from a second pipette in which it was dissolved in the 0 Ca^{2+}/0.1 mM EGTA extracellular solution. *B,* Simultaneous optical and electrophysiological recording from another astrocyte. The record of $[Ca^{2+}]_i$ is shown in the *upper trace.* The *lower traces* show corresponding records of membrane current (*I*) and voltage (*V*). ATP (5 μM, 5 sec) was applied after the recording pipette had been sealed to the membrane but before rupturing the patch. The time of patch rupture and "breakthrough" to whole-cell recording is illustrated by the *arrow* above the $[Ca^{2+}]_i$ trace. The times indicate the time after breakthrough when ATP was applied. Hyperpolarizing voltage steps (10 mV, 10 msec) were made repeatedly around the time of breakthrough in order to do series resistance and whole-cell capacitance. The *horizontal bars* below the $[Ca^{2+}]_i$ traces and above the current records show the period of ATP application.

solutions that occurs during whole-cell recording was utilized to confirm that Ca^{2+} responses were due to increases in $[Ca^{2+}]_i$ in the cytoplasm rather than in compartmentalized intracellular regions. In some experiments cells were loaded via fura-2 AM, but fura-2-free acid was not included in the intracellular recording solution. More than 90% of the fluorescent signal was lost from the cells by dialysis during such experiments, indicating that the vast majority of the dye was localized in the freely diffusible cytoplasmic compartment of the cells. In other experiments, to minimize possible intracellular sequestration of fura-2, cells were loaded only by including fura-2-free acid in the

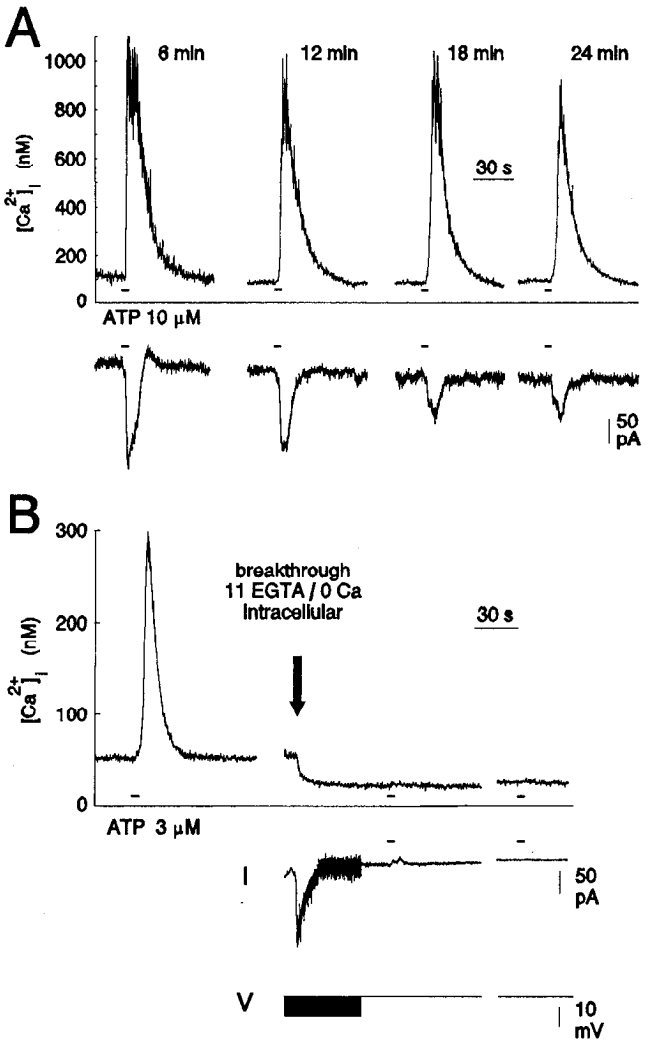

Figure 2. ATP-evoked increases in $[Ca^{2+}]_i$ are consistent in astrocytes not loaded with fura-2 AM (*A*), and the effect of increasing the Ca^{2+} buffering capacity of the intracellular solution (*B*). *A* shows records of $[Ca^{2+}]_i$ and current from an astrocyte when only the intracellular recording solution contained fura-2. ATP (10 μM, 5 sec; *horizontal bars*) was first applied only after sufficient fura-2 had diffused into the cell to yield a satisfactory signal:noise ratio. This occurred about 6 min after the breakthrough. Three subsequent applications of ATP were made at the times indicated. In *B*, the records are from an astrocyte when the concentration of EGTA in the intracellular solution was 11 mM. ATP (3 μM, 10 sec) was applied before, 1 min, and 6 min after breakthrough. Note that upon breakthrough a transient inward current was evoked that was never observed when the EGTA concentration was 0.1 mM.

recording pipette. As illustrated in Figure 2*A*, ATP evoked reproducible and stable increases in $[Ca^{2+}]_i$ in cells loaded with fura-2-free acid. Also, the baseline level of $[Ca^{2+}]_i$ was found to be in the range of that of cells loaded with fura-2 AM. In experiments when the buffering capacity of the intracellular solution was elevated by increasing the concentration of EGTA from 0.1 to 11 mM, there was a rapid decrease in the baseline level of $[Ca^{2+}]_i$ within seconds after rupturing the patch (Fig. 2*B*). Moreover, the ATP-evoked increase in $[Ca^{2+}]_i$ was abolished within 1 min following the breakthrough, presumably because released Ca^{2+} was buffered by EGTA. These observations indicate that it is Ca^{2+} in the cytoplasm that is being measured. Moreover, agents introduced into the astrocytes via the patch pipette can diffuse into regions of the cell to affect the ATP-evoked increase in $[Ca^{2+}]_i$.

In contrast to the Ca^{2+} responses, the membrane currents produced by ATP showed considerable cell-to-cell variability. Also, as illustrated in Figures 1 and 2, the ATP-evoked currents typically were not stable during the recordings. Rather, there was a progressive change in the amplitude of the currents, which may indicate that the currents are regulated by diffusible intracellular factors lost from the cell during dialysis. The currents produced by ATP and their possible intracellular regulation are examined elsewhere (M. W. Salter, in preparation) and the remainder of the present study focuses on the signal transduction pathway of the ATP-induced increases in $[Ca^{2+}]_i$.

IP_3 evokes release of intracellular Ca^{2+}

If IP_3 is the ultimate effector of the ATP-evoked Ca^{2+} response, then it should be possible to demonstrate an IP_3-releasable pool of Ca^{2+} in the astrocytes. The presence of such an IP_3-sensitive Ca^{2+} pool was examined by including IP_3 (1, 10, or 30 μM) in the intracellular solution as illustrated in Figure 3. At the lowest concentration of IP_3 (1 μM), rupture of the patch was followed by a small, transient increase in $[Ca^{2+}]_i$ of less than 50 nM. Larger transient increases in $[Ca^{2+}]_i$, up to 1000 nM, were evoked when the higher concentrations of IP_3 were used (e.g., Fig. 3*A*). Intracellular $[Ca^{2+}]$ typically reached a peak 10–20 sec after attaining whole-cell recording, and then declined over the next 30–60 sec to reach a level above baseline $[Ca^{2+}]_i$. During the sustained phase $[Ca^{2+}]_i$ was 130 ± 15 (mean ± SEM) and 350 ± 150 nM above the baseline levels with 10 μM (n = 6 cells) and 30 μM (n = 3 cells) IP_3, respectively. As shown in Figure 3*B*, a large transient rise in $[Ca^{2+}]_i$ was also elicited by including IP_3 (10 μM) in the intracellular solution when the extracellular medium contained no Ca^{2+} and 0.1 mM EGTA (n = 5 cells).

We have previously shown that ATP-evoked release of $[Ca^{2+}]_i$ is blocked by thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase (Salter and Hicks, 1994). To determine whether thapsigargin could also block IP_3-evoked Ca^{2+} release, we treated the cells with thapsigargin (1 μM) prior to recording. As illustrated in Figure 3*C*, thapsigargin prevented the increases in $[Ca^{2+}]_i$ produced by 10 μM IP_3; similar results were obtained in all cells tested (n = 4). Thus, the astrocytes from the dorsal spinal cord appear to contain thapsigargin-sensitive stores of intracellular Ca^{2+} that can be released by IP_3. Furthermore, we found that when ATP was applied 10–15 min after breakthrough when IP_3 (10 μM) was present, the ATP-evoked increase in $[Ca^{2+}]_i$ was eliminated (n = 2 cells).

Heparin blocks Ca^{2+} response to ATP

The IP_3 receptor antagonist heparin (Cullen et al., 1988; Nilsson et al., 1988) was used in order to investigate whether activation of these receptors is a necessary step in the intracellular pathway for increase in $[Ca^{2+}]_i$ caused by ATP. As shown in the example in Figure 4*A*, when heparin (1 mg/ml) was included in the intracellular solution the Ca^{2+} response to ATP was decreased by 94% within 2 min after patch rupture, and the response was abolished by 7 min into the recording. In all cells tested (n = 8), heparin reduced the response to ATP at 7 min by more than 90%. In contrast, intracellular administration of N-desulfated heparin (1 mg/ml), which is not an IP_3 receptor antagonist (Missiaen et al., 1991), had no effect on the ATP-evoked increase in $[Ca^{2+}]_i$ (Fig. 4*B,C*; n = 3).

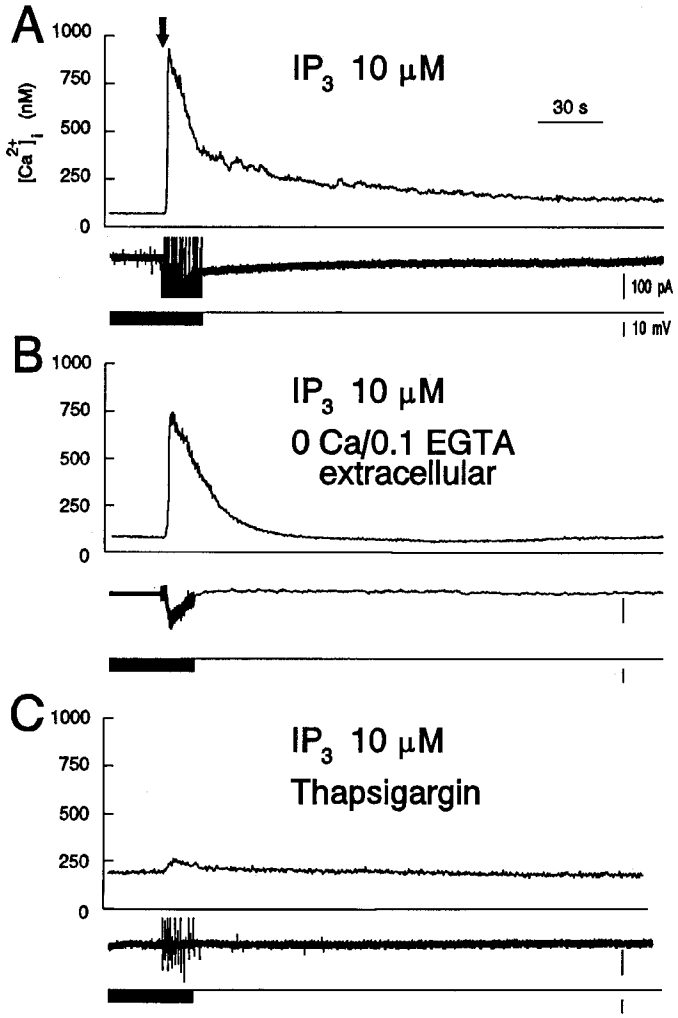

Figure 3. IP_3 causes release of Ca^{2+} from an intracellular pool that is sensitive to thapsigargin. Continuous recordings of $[Ca^{2+}]_i$, current (*I*), and membrane potential (*V*) are shown around the time of breakthrough for three individual astrocytes. In all cases IP_3 (10 μM) was added to the intracellular recording solution. In *A*, the extracellular solution contained 1.3 mM Ca^{2+} throughout the recording period. On the other hand, in *B*, just prior to the start of the record but after the formation of the seal, the bath volume was exchanged with extracellular solution with no added Ca^{2+} and 0.1 mM EGTA. *C* shows records from a cell after a 5 min bath application of thapsigargin (1 μM). All of the recordings are aligned so that the time of breakthrough for the three cells is indicated by the *arrow* above the uppermost $[Ca^{2+}]_i$ trace.

ATP-evoked increase in $[Ca^{2+}]_i$ is prevented by U-73122

To investigate whether the Ca^{2+} response to ATP was produced by activation of phospholipase C (PLC), we examined the effects of the PLC inhibitor U-73122 and its inactive analog U-73343 (Smith et al., 1990). Because both of the analogs are membrane permeant they were applied in the bathing solution and their effects were examined in astrocytes that were not subject to whole-cell recording. As shown in Figure 5*A*, application of U-73343 (5 μM) did not affect the Ca^{2+} response to ATP. On the other hand, the response was reduced by 92% by applying U-73122 at the same concentration. After washing out U-73122, the Ca^{2+} response returned to preapplication level, indicating that the depression is reversible. As illustrated in Figure 5*B*, the average amplitude response to a test application of ATP (5 μM, 10 sec) was significantly depressed in cells treated with U-73122 (n = 8 cells) as compared with cells treated with U-73343 (n = 10 cells). In six of the eight cells treated with U-73122 the ATP-evoked Ca^{2+} responses were abolished.

Effects of GTPγS, AlF_4^- and GDPβS

The possible involvement of coupling via heterotrimeric G-proteins in the Ca^{2+} response to ATP was investigated by determining the effects of activating or inhibiting G-proteins pharmacologically. GTPγS was used as it is a stable analog of GTP known to cause sustained activation of G-proteins (Gilman, 1987). In experiments (n = 5) when GTPγS (500 μM) was included in the intracellular solution, there was a rapid increase in $[Ca^{2+}]_i$ after rupturing of the patch and then oscillations in the level of $[Ca^{2+}]_i$ (Fig. 6*A*). On the other hand, GTP itself did not affect $[Ca^{2+}]_i$ (n = 3 cells). Moreover, as illustrated in Figure 6*B*, intracellular application of another activator of heterotrimeric G-proteins, AlF_4^- (Sternweis and Gilman, 1982), also produced an oscillating rise in $[Ca^{2+}]_i$. AlF_4^- caused increases in $[Ca^{2+}]_i$ in all cells tested (n = 8). Thus, it appears that activation of heterotrimeric G-proteins is sufficient to increase $[Ca^{2+}]_i$.

Heterotrimeric G-protein function is known to be inhibited by the stable GDP analog GDPβS (Hall, 1990), and therefore the effect of this analog on the Ca^{2+} response evoked by ATP was examined. GDPβS (100 or 500 μM) had no effect on the baseline level of $[Ca^{2+}]_i$ in the astrocytes. However, as shown in Figure 7*A*, 2 min after GDPβS (500 μM) began entering the cells the Ca^{2+} response to ATP was decreased by 84%, and 5 min later the response was depressed by more than 98%. ATP-evoked increases in $[Ca^{2+}]_i$ were also attenuated by a lower concentration (100 μM) of GDPβS (Fig. 7*B*). Taken together, these results indicate that the Ca^{2+} response to ATP is mediated via a G-protein coupling.

Lack of effect of pertussis toxin on responses to ATP

The subtype of G-protein involved in the response to ATP was examined by the use of pertussis toxin, which catalyzes ADP ribosylation, and thereby produces inactivation of the α subunit of several classes of G-protein (Yamane and Fung, 1993). Cultures were treated for 20–24 hr with pertussis toxin (1 μg/ml) or vehicle control, and a standardized application of ATP (3 μM, 5 sec) was used to determine the responses of astrocytes. All astrocytes tested in control and pertussis toxin–treated cultures, n = 26 and 14 cells, respectively, responded to ATP; examples of responses are shown in Figure 8, *A* and *B*. There was no significant difference in the peak level of $[Ca^{2+}]_i$ evoked by ATP in the control versus treated cells (Fig. 8*C*), indicating that treatment with pertussis toxin had not affected the Ca^{2+} response to ATP. The effectiveness of the pertussis toxin treatment in causing ADP ribosylation was confirmed by an *in vitro* ADP-ribosylation assay. With proteins from control cultures, pertussis toxin caused *in vitro* ADP ribosylation of a band with apparent molecular mass of 40 kDa (left lane in Fig. 8*D*), typical of that of G-protein α subunits (Gilman, 1987; Yamane and Fung, 1993). In contrast, *in vitro* ADP ribosylation of this protein was absent with proteins from cells that had been pretreated with pertussis toxin (second lane from the left, Fig. 8*D*). This result indicates that treating the cultures with pertussis toxin had caused complete ADP ribosylation of this protein. Taken together, these results indicate that the G-protein mediating the Ca^{2+} response to ATP is insensitive to pertussis toxin.

Discussion

Our present results indicate that astrocytes from the dorsal spinal cord possess an intracellular pool of Ca^{2+} that can be released

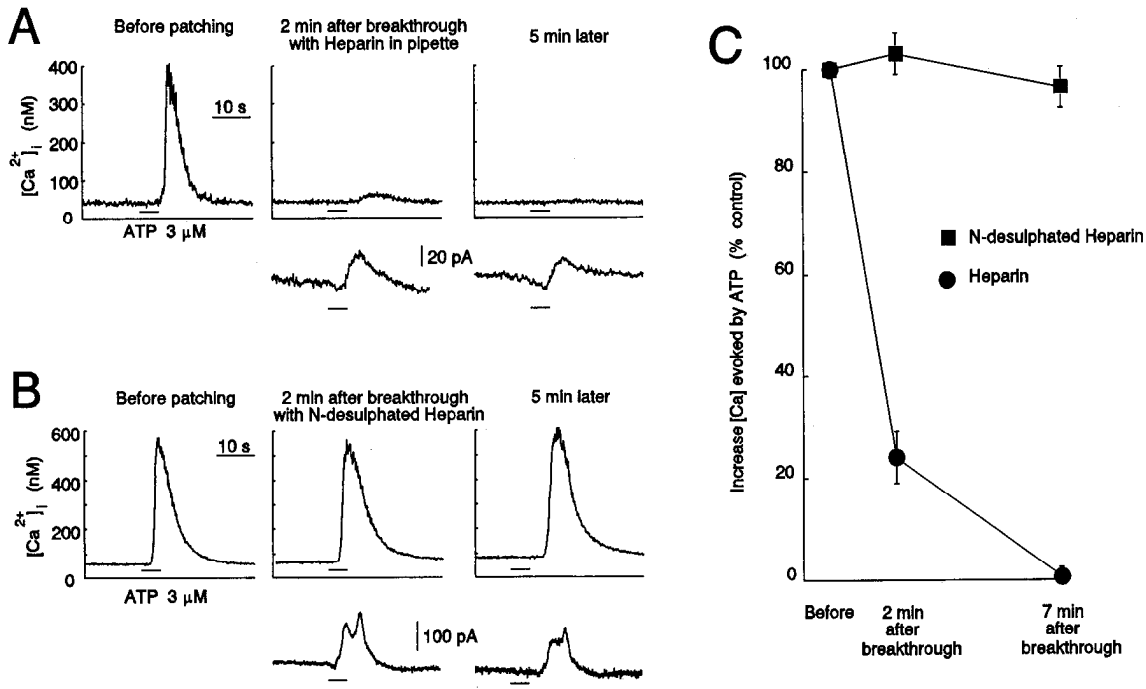

Figure 4. $[Ca^{2+}]$ responses to ATP are blocked by intracellular heparin but not by N-desulfated heparin. *A* and *B* show recordings of $[Ca^{2+}]_i$ (*upper traces*) and current (*lower traces*) from two individual astrocytes. The intracellular solution was supplemented with heparin (1 mg/ml; *A*) or N-desulfated heparin (1 mg/ml; *B*). ATP (3 μM) was applied before, 2 min, and 7 min after breakthrough, during the periods indicated by the *horizontal bars*. In the graph in *C*, the average percentage increase in $[Ca^{2+}]_i$ evoked by ATP is plotted for cells when the intracellular solution contained heparin (n = 8) or N-desulfated heparin (n = 3). Each value is the peak increase in $[Ca^{2+}]_i$ expressed as a percentage of a control response to an application of ATP made before breakthrough. Error bars are ± SEM.

by IP_3. We show that this pool, like that mediating the ATP-evoked release of intracellular Ca^{2+}, is depleted by the Ca^{2+}-ATPase inhibitor thapsigargin. The Ca^{2+} response to ATP was found to be abolished by administering IP_3 intracellularly, possibly because of depletion of Ca^{2+} from the IP_3-sensitive pool. In addition, the response to ATP was blocked by the IP_3 receptor antagonist heparin, whereas the response was unaffected by N-desulfated heparin. Moreover, the response to ATP is pre-

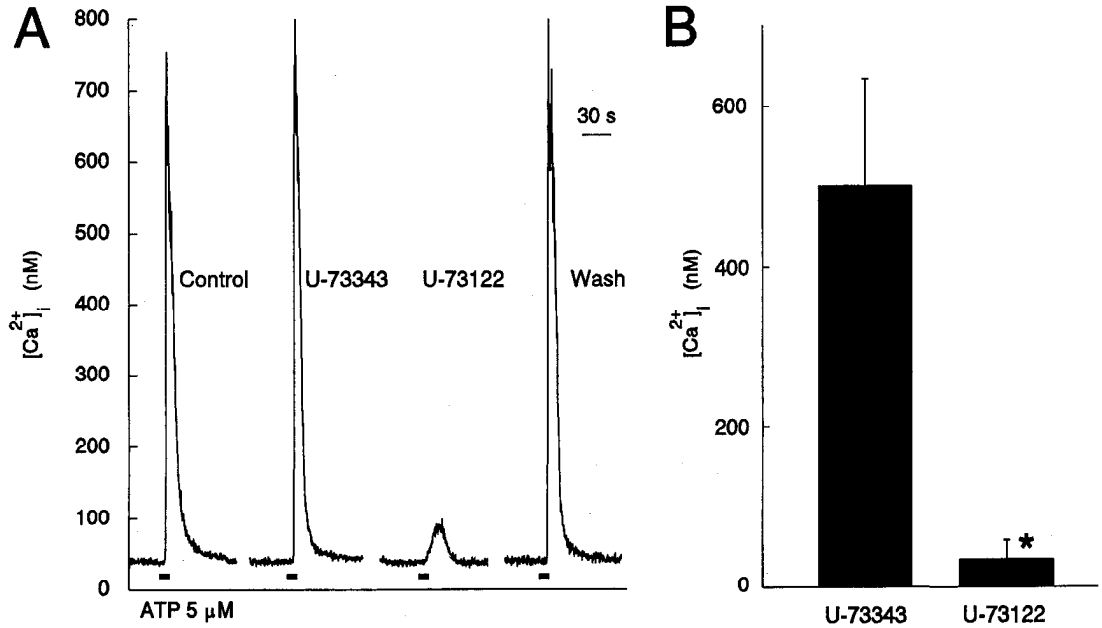

Figure 5. Effects of U-73343 and U-73122 on ATP-evoked increase in $[Ca^{2+}]_i$. *A*, The traces show $[Ca^{2+}]_i$ recorded from one astrocyte. ATP (5 μM) was applied as indicated by the *bars* below the traces. The applications of ATP were made before drug administration (*Control*), after a 30 min incubation with U-73343 (5 μM), after removing U-73343 and incubating for 30 min with U-73122 (5 μM), and 30 min after washing the dish with control extracellular solution (*Wash*). The mean of the peak increase in $[Ca^{2+}]_i$ evoked by ATP in cells treated with U-73343 (n = 8) or U-73122 (n = 10) is plotted in the graph in *B*. The error bars show ± 1 SEM. * $p < 0.01$, Student's t test.

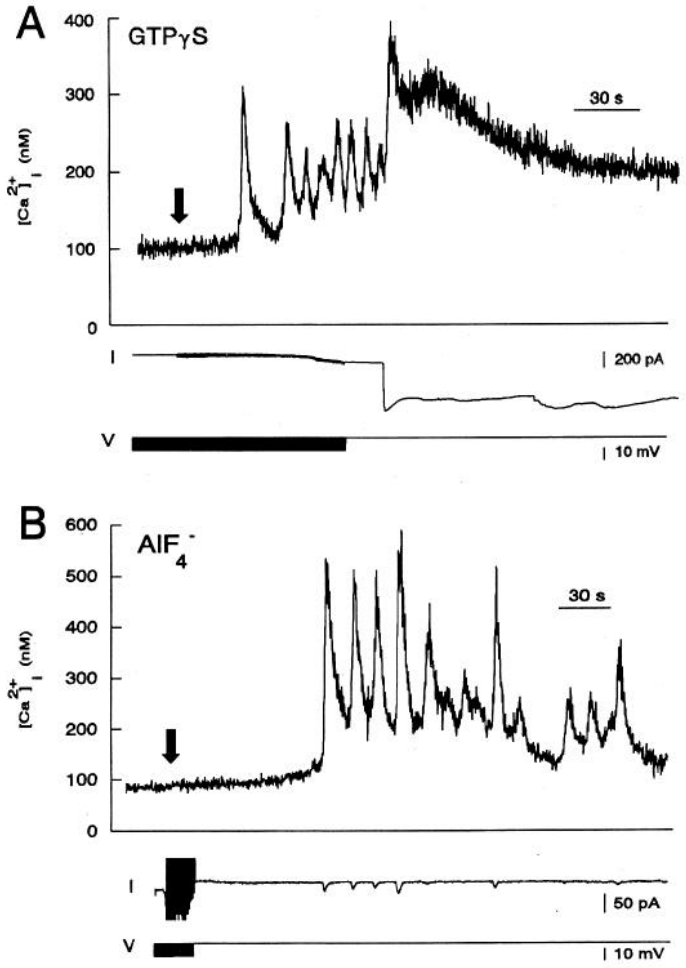

Figure 6. Effects of intracellular administration of G-protein activators GTPγS or AlF_4^-. Recordings of $[Ca^{2+}]_i$, current (*I*), and membrane potential (*V*) are shown for two single astrocytes. GTPγS (500 μM), or NaF (5 mM) and $AlCl_3$ (50 μM), were added to the intracellular solution in *A* and *B*, respectively. The time of breakthrough is indicated by the *arrow* above each of the traces of $[Ca^{2+}]_i$.

vented by the PLC inhibitor U-73122, but not by its inactive analog, U-73343. Taken together, these results indicate that activation of the PLC/IP_3 pathway is both necessary and sufficient to account for the ATP-evoked release of intracellular Ca^{2+}.

We have reported that the apparent rank order of potency for increasing $[Ca^{2+}]_i$ in dorsal spinal astrocytes is 2-methylthioATP > ATP ≈ ADP ≫ β,γmethyleneATP = AMP = adenosine (Salter and Hicks, 1994). This order suggests that the Ca^{2+} response is due to activation of the P_{2Y} subtype of purinergic receptor. Other studies on effects of ATP on astrocytes from other regions of the CNS (Pearce et al., 1989; Kastritsis et al., 1992), on a glioma cell line (Lin and Chuang, 1993), and on peripheral tissues (Boyer et al., 1989; O'Connor et al., 1991; Gerwins and Fredholm, 1992) have indicated only that there is a correlation between P_{2Y} receptor-evoked increases in $[Ca^{2+}]_i$ and in IP_3. Thus, the present study is the first to demonstrate that the PLC/IP_3 pathway mediates the release of intracellular Ca^{2+} produced by activating P_{2Y} receptors.

We found that IP_3 caused a large transient increase in $[Ca^{2+}]_i$ that persisted upon removing extracellular Ca^{2+}, which indicates that astrocytes have an IP_3-sensitive pool of intracellular Ca^{2+}. It has been speculated that it is release of Ca^{2+} from an IP_3-sensitive pool that is responsible for the Ca^{2+} responses of astrocytes from a number of CNS regions to a variety of putative signaling molecules such as bradykinin, acetylcholine, noradrenaline, histamine, and glutamate (McCarthy and Salm, 1991; Hösli and Hösli, 1993; Shao and McCarthy, 1993). Therefore, the present demonstration of IP_3-sensitive Ca^{2+} stores establishes a fundamental link for a diversity of Ca^{2+} signaling pathways in astrocytes.

In contrast to the initial transient rise in $[Ca^{2+}]_i$, the sustained increase caused by IP_3 occurred only when Ca^{2+} was present in the extracellular medium. This observation suggests that applying IP_3 may lead to Ca^{2+} influx as well as to release of intracellular Ca^{2+}. Influx of Ca^{2+} following activation of the PLC/IP_3 pathway has been reported to be due to activation of membrane Ca^{2+} channels by metabolites of IP_3 such as IP_4 in cells including endothelial cells (Lückhoff and Clapham, 1992). Alternatively, influx channels may be activated by a diffusible molecule produced by depleting IP_3-sensitive intracellular stores (Putney, 1990; Hoth and Penner, 1992); the molecule mediating the effect of depleting the Ca^{2+} stores has been partially characterized (Randriamampita and Tsien, 1993). We have found no evidence that activation of P_2 purinergic receptors causes influx of Ca^{2+} in the dorsal spinal astrocytes (Salter and Hicks, 1994), although this has been reported in purified astrocytes from the cerebral cortex (Neary et al., 1988). Our results might seem to contradict the idea that IP_3 mediates the response to ATP. However, we used short applications of ATP at low concentrations. Thus, there might have been only limited stimulation of the PLC/IP_3 pathway which was insufficient to activate the influx channels. This could have occurred either if not enough IP_3 metabolites were produced or if not enough of the stored Ca^{2+} was released. Alternatively, we expect that injecting IP_3 itself would not necessarily reproduce all of the sequelae of activating receptors. P_2 receptor activation would stimulate PLC, and thereby cause the generation of diacylglycerol in addition to IP_3. Also, P_2 receptors have been reported to be coupled to other second messenger pathways such as the phospholipase A_2 (Bruner and Murphy, 1993) or the adenylate cyclase (Boyer et al., 1993) pathways. Thus, it is conceivable that in dorsal spinal astrocytes an additional effect of stimulating P_2 receptors might be activation of a signaling pathway that suppresses the influx channels.

Blockade of the Ca^{2+} response to ATP by heparin but not by N-desulfated heparin suggests that the response is mediated via IP_3 receptors. Heparin as well as other polysulfated anions have been reported recently to activate ryanodine receptor/channels from skeletal muscle cells (Bezprozvanny et al., 1993). By activating ryanodine receptors, heparin could conceivably cause depletion of caffeine/ryanodine-sensitive intracellular Ca^{2+} stores (Ehrlich et al., 1994). In spinal dorsal astrocytes caffeine does not increase $[Ca^{2+}]_i$ in unstimulated cells, nor in cells that have been stimulated in an attempt to reveal a caffeine-sensitive pool that is empty (Salter and Hicks, 1994). Thus, these astrocytes appear not to express caffeine/ryanodine-sensitive stores. Hence, the blockade of ATP-evoked Ca^{2+} responses by heparin cannot be accounted for by an effect on ryanodine receptors.

We found that the ATP-evoked Ca^{2+} response is blocked by U-73122, an inhibitor of PLC, but not by the analog U-73343 which does not inhibit PLC. It has been reported that U-73122 does not inhibit a number of other enzymes including phospholipase A_2 (PLA_2), adenylate cyclase, and protein kinase A (Bleasdale et al., 1990; Smith et al., 1990), which suggests that these enzymes are not involved in the Ca^{2+} response to ATP. In

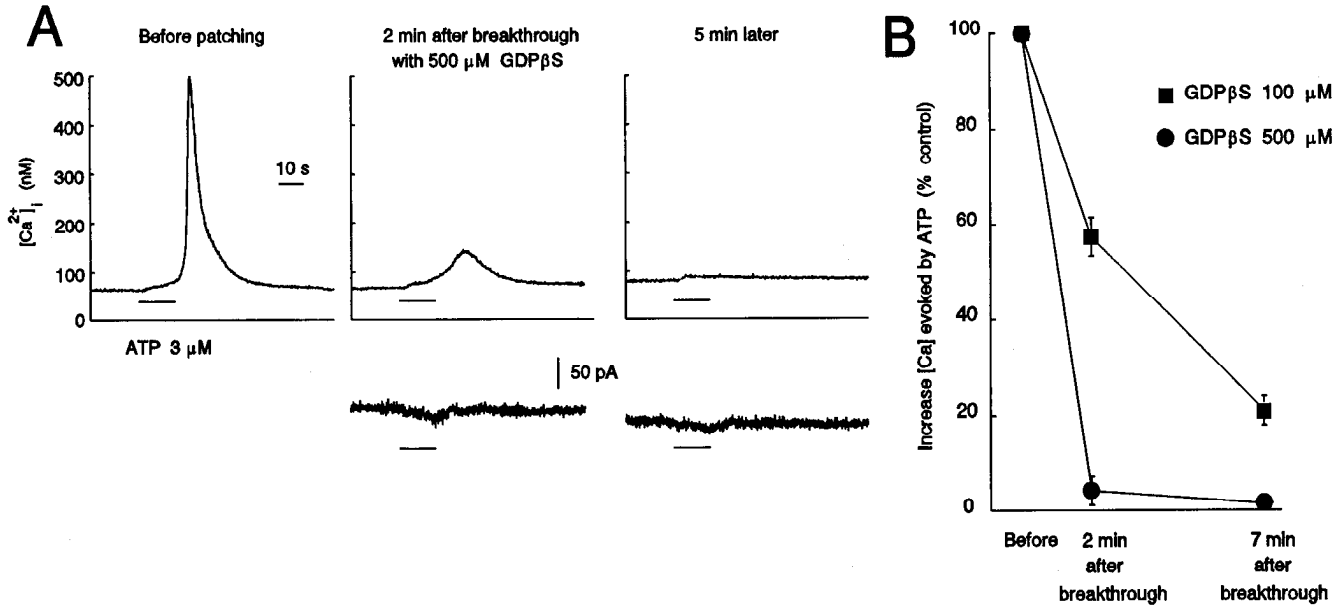

Figure 7. Intracellular GDPβS blocks Ca^{2+} response evoked by ATP. *A* shows $[Ca^{2+}]_i$ and current recordings from an astrocyte when the intracellular solution was supplemented with GDPβS (500 μM). ATP (3 μM, *horizontal bar* below the records) was applied before, 2 min, and 7 min after breakthrough. The average peak increase in $[Ca^{2+}]_i$, as a percentage of the control response, is plotted in *B*. The intracellular solution contained GDPβS at a concentration of 100 μM (n = 4 cells) or 500 μM (n = 5 cells). Error bars are ± SEM.

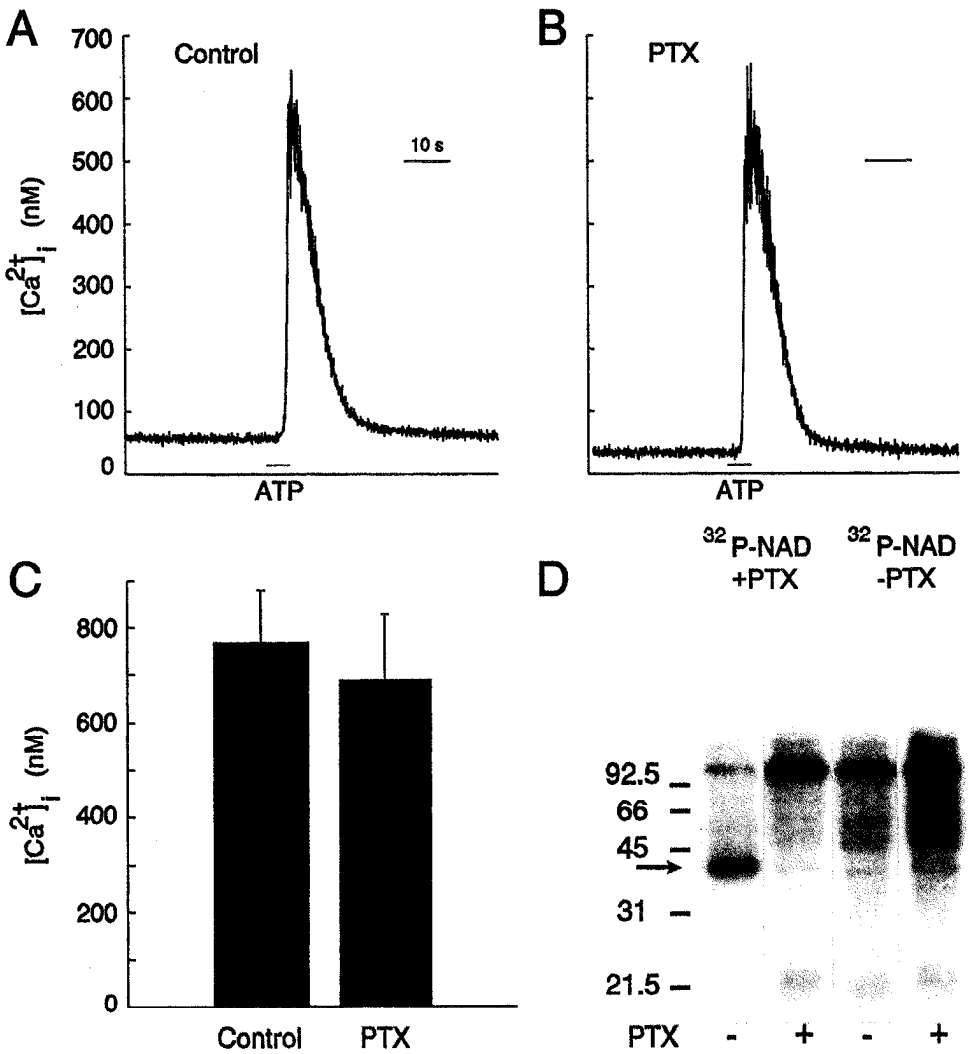

Figure 8. Pertussis toxin does not affect ATP-evoked increases in $[Ca^{2+}]_i$. Recordings of $[Ca^{2+}]_i$ from individual astrocytes from cultures treated with vehicle alone (*Control*) or with pertussis toxin (*PTX*) are shown in *A* and *B*, respectively. ATP (3 μM) was applied as indicated by the *bars* below the records. Time bars indicate 10 sec in *A* and *B*. In *C* the mean of the peak increase in $[Ca^{2+}]_i$ evoked by a standardized application of ATP (3 μM, 5 sec) for astrocytes from control (n = 26 cells) and PTX-treated (n = 14 cells) cultures. The error bars are ± SEM. *D* shows an autoradiograph of proteins separated by SDS-PAGE after incubating with ^{32}P-NAD. For the two lanes on the *left*, PTX was included (*+PTX*) in the incubation mixture, whereas for the two *right lanes* PTX was omitted (*<PTX*). Control or PTX-treated cultures were used as indicated by the − or +, respectively, below each lane. The position of migration of molecular size standards (in *K*) are shown by the *bars* to the *left* of the autoradiograph. The *arrow* indicates the position of the band from control cultures specifically labeled *in vitro* by PTX.

membrane preparations it has been observed that PLC stimulated via activating G-protein-coupled receptors, such as muscarinic receptors in neuroblastoma cells or FMLP or PAF receptors in neutrophils (Smith et al., 1990), is inhibited by U-73122 at concentrations lower than those required to inhibit PLC that has been stimulated by a direct means such as by increasing Ca^{2+} (Bleasdale et al., 1990; Thompson et al., 1991). The concentration of U-73122 we used was in the range reported to inhibit G-protein-coupled stimulation of PLC. Thus, the blockade of the Ca^{2+} response to ATP by U-73122, together with the effects of GTPγS, AlF_4^-, and GDPβS, as discussed next, indicate that the PLC isoenzyme mediating this response is a member of the PLCβ family, which has been shown to be coupled to G-proteins (Berridge, 1993). Three subtypes of PLCβ—β1, β3, and β4—have been identified in the CNS (Lee et al., 1993), indicating that there are several potential candidate PLCβ enzymes that might mediate the Ca^{2+} response to ATP.

We found that intracellular administration of GTPγS produces a large and prolonged increase in $[Ca^{2+}]_i$. We used GTPγS because biochemical studies have shown that it causes sustained activation of heterotrimeric G-proteins (Gilman, 1987). Another potential intracellular target for GTPγS is the small molecular weight G-protein class (Hall, 1993). In contrast to heterotrimeric G-proteins, small G-proteins require GTP hydrolysis for functioning and are inhibited by GTPγS. However, small G-proteins are reportedly unaffected by AlF_4^- (Kahn, 1991), whereas it promotes dissociation of α and βγ subunits, thereby activating heterotrimeric G-proteins (Higashijima et al., 1987). We found that both GTPγS and AlF_4^- produce a rise in $[Ca^{2+}]_i$, which indicates this was due to activation of heterotrimeric G-proteins. Moreover, the Ca^{2+} response to ATP was blocked by GDPβS, which has been shown to inhibit dissociation of heterotrimeric G-proteins (Gilman, 1987). Thus, our results indicate that heterotrimeric G-proteins are necessary for the P_{2Y} receptor-evoked increase in $[Ca^{2+}]_i$.

The Ca^{2+} response appears to be mediated by a G-protein that is pertussis toxin insensitive. G-proteins in the G_q/G_{11} subclass have been shown to lack the site for ADP ribosylation present in other G-protein subclasses (Strathmann and Simon, 1990). There is evidence that G_q can activate PLCβ enzymes (Taylor et al., 1991; Hepler et al., 1993) and that G_q/G_{11} is widely expressed in the CNS (Milligan, 1993). Thus, we suggest that the P_{2Y} receptor-evoked release of $[Ca^{2+}]_i$ in dorsal spinal astrocytes is mediated via a G-protein of the G_q/G_{11} subclass. It has been reported, as well, that Ca^{2+} responses to ATP in cortical astrocytes (Cejka et al., 1993) and in C6 glioma cells (Lin and Chuang, 1993) are pertussis toxin insensitive. On the other hand, in other cell types responses mediated via P_{2Y} receptors have been reported to be reduced or eliminated by pertussis toxin treatment, including activation of PLC (Dubyak et al., 1988; Gerwins and Fredholm, 1992) or PLA_2 (Boyer et al., 1993), or inhibition of adenylate cyclase (Boyer et al., 1993). Thus, P_{2Y} receptors might be coupled to G-proteins in addition to G_q/G_{11}, such as G_i or G_o. However, our results indicate that functional G_i and G_o are not necessary to produce the Ca^{2+} responses in astrocytes.

A full-length cDNA encoding for a functional P_{2Y} receptor, called P_{2Y1}, has recently been cloned from chick brain (Webb et al., 1993). From the predicted primary amino acid sequence it has been suggested that P_{2Y1} has seven transmembrane-spanning domains typical of G-protein-coupled receptors. In the chick, Northern blotting analysis has indicated that the CNS is a tissue where P_{2Y1} mRNA is expressed at high levels (Webb et al., 1993), and *in situ* hybridization has shown that this mRNA is expressed by astrocytes (Webb et al., 1994). Thus, the molecular identity of the receptor mediating the presently reported Ca^{2+} responses may be the rat homolog of chick P_{2Y1}, or possibly a related protein.

A potential role for the P_{2Y} receptor-PLC-IP_3 pathway may be in signaling from neurons to astrocytes. Neuron–astrocyte signaling has been described as a consequence of release of transmitter at synapses in the hippocampus (Dani et al., 1992), at the neuromuscular junction (Jahromi et al., 1992; Reist and Smith, 1992), and in cell culture (Murphy et al., 1993). There is strong evidence that ATP may be released from nerve terminals at synapses within the spinal dorsal horn (Sweeney et al., 1989; Sawynok et al., 1993). If astrocytes in the dorsal horn express P_{2Y} receptors that are accessible to synaptically released ATP, then it is possible that the PLC/IP_3 pathway stimulated by activating the receptors might play a physiological role in neuronal–glial signaling. The function of this pathway might be to signal astrocytes to increase metabolism (Arbones et al., 1990), to alter neurotransmitter uptake (Vernadakis, 1988), to modify astrocyte gene expression (Georgiou et al., 1994), or to secrete neuroactive molecules to provide feedback regulation of neuronal excitability and neurotransmission (Martin, 1992; Nedergaard, 1994).

Calcium signaling between astrocytes is known to be a mechanism for propagating Ca^{2+} waves within the CNS (Cornell-Bell et al., 1990). ATP has been implicated as a mediator of intercellular propagation of Ca^{2+} signals between mast cells (Osipchuk and Cahalan, 1992), another type of electrically nonexcitable cell. It is possible that ATP, and activation of the PLC/IP_3 pathway, might mediate signaling between astrocytes. Hence, a common functional role for ATP might be as a mediator of intercellular Ca^{2+} signaling for nonexcitable cells.

ATP, as well as other adenine nucleotides, is released by tissue damage (Gordon, 1986). Astrocytes are reported to respond to tissue damage through a number of processes regulated by $[Ca^{2+}]_i$ (Neary et al., 1987; Arenander et al., 1989; Condorelli et al., 1989). As we have now established that P_{2Y} receptor activation leads to increased $[Ca^{2+}]_i$ via the PLC/IP_3 pathway, its role in responses of astrocytes to pathological conditions could be investigated and potentially novel targets for therapeutic intervention could be identified.

In summary, our results indicate that ATP acting at P_{2Y} purinergic receptors on spinal dorsal astrocytes may stimulate PLCβ via coupling through a pertussis toxin–insensitive G-protein, possibly G_q/G_{11}. This causes generation of IP_3 which in turn releases Ca^{2+} from an intracellular pool that is sensitive to thapsigargin. The signal transduction pathway demonstrated in the present study may have a role in neuron–astrocyte communication in the dorsal horn of the spinal cord.

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