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ORIGINAL ARTICLE

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ATP acting on P_{2Y} receptors triggers calcium mobilization in primary cultures of rat neurohypophysial astrocytes (pituicytes)

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Abstract The effect of adenosine triphosphate (ATP) on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of cultured neurohypophysial astrocytes (pituicytes) was studied by fluorescence videomicroscopy. ATP evoked a $[Ca^{2+}]_i$ increase, which was dose dependent in the 2.5–50 µM range (EC_{50}=4.3 µM). The ATP-evoked $[Ca^{2+}]_i$ rise was not modified during the first minute following the removal of external Ca^{2+}. Application of 500 nM thapsigargin inhibited the ATP-dependent $[Ca^{2+}]_i$ increase. Caffeine (10 mM) and ryanodine (1 µM) did not affect the ATP-induced $[Ca^{2+}]_i$ rise. The pituicytes responded to various P_2 purinoceptor agonists with the following order of potency: ATP=ATP[γ-*S*]=2-MeSATP≥ADP, where ATP[γ-*S*] is adenosine 5′-*O*-(3-thiotriphosphate) and 2-MeSATP is 2-methylthio-adenosine-5′-triphosphate. Adenosine, AMP, α,β-methylene adenosine-5′-triphosphate (α,β-MeATP), β,γ methylene adenosine-5′-triphosphate (β,γ-MeATP) and uridine 5′-triphosphate (UTP) were ineffective. The P_2 purinoceptor antagonists blocked the ATP-evoked $[Ca^{2+}]_i$ increase with the following selectivity: RB-2>suramin>PPADS, where RB-2 is Reactive Blue 2 and PPADS is pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid. The ATP-evoked $[Ca^{2+}]_i$ increase was substantially blocked by pertussis toxin treatment, suggesting that it might be mediated by a pertussis-toxin-sensitive G protein. The phospholipase C (PLC) inhibitor U-73122 (0.5 µM) abolished the ATP-evoked $[Ca^{2+}]_i$ rise, whereas its inactive stereoisomer U-73343 (0.5 µM) remained ineffective. Our results indicate that, in rat cultured pituicytes, ATP stimulation induces an increase in $[Ca^{2+}]_i$ due to PLC-mediated release from intracellular stores through activation of a pertussis-toxin-sensitive, G-protein-linked P_{2Y} receptor.

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Introduction

Glial cells of the nervous system are intimately associated with neurons, and thus are in a prime location for receiving any released neurotransmitters from the nerve terminals. From these observations, some fundamental questions arise: are astrocytes in close proximity to the nerve terminals simply to take up and metabolize excess neurotransmitter or do astrocytes have complex receptor-mediated responses to the neurotransmitters? Once methods for making purified astroglial cultured were developed [27], glial cells were shown to express a wide variety of functional receptors for various neurotransmitters (for review see [19]). More recently, the study of astrocytes in situ has progressed considerably and the glial cell responsiveness to neurotransmitters and related molecules has been confirmed (for review, see [33]).

Adenosine triphosphate (ATP) is recognized as an intercellular signalling molecule in the central nervous system [13]. The responses to ATP are not restricted to neurons; several other types of cell have been shown to respond to ATP including astrocytes [8, 22, 32, 36], microglia [29] and oligodendrocytes [24, 36].

We have shown in a previous study that extracellular ATP triggers an intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increase in, and vasopressin release from, neurohypophysial nerve terminals via a P_{2X} receptor [41]. We proposed that ATP, co-packaged and co-released with neurohypophysial neuropeptides, could act as a paracrine and autocrine messenger, stimulating the secretion of vasopressin from neurohypophysial terminals. In the neurohypophysis the glial compartment is composed of two different cell types. Of the non-endothelial cells, 19% are microglial cells which resemble microglia of the central nervous system [34] whereas the majority of glial cells are specialized astrocytes referred to as pituicytes [35,

38]. The presence of synaptoid contacts onto these astroglial cells [6], their proximity to neuronal elements, and their remarkable morphological plasticity during periods of high neurohormone demand [17] suggest that these cells of the neurohypophysis may be involved in modulating the neurosecretory process [5].

To test this hypothesis, and assess the effects of extracellular ATP in neurohypophysial glial cells, we took advantage of an explant culture model of the rat neurohypophysis [4]. We found that extracellular ATP triggers an $[Ca^{2+}]_i$ increase in cultured pituicytes, and we characterized the P_2 purinergic receptor involved in the $[Ca^{2+}]_i$ response.

Materials and methods

Cell culture

Adult male Wistar rats (150–200 g) maintained with ad libitum food and water were asphyxiated by CO_2 and killed by decapitation with a guillotine following the guidelines laid down by the French/European ethics committee. The pituitaries were quickly removed into sterile HEPES-buffered salt solution (HBSS) containing (in mM): NaCl 137, KCl 5.4, KH_2PO_4 0.4, $NaHCO_3$ 4.2, Na_2HPO_4 0.3, $MgCl_2$ 0.5, $MgSO_4$ 0.4, $CaCl_2$ 1.25, HEPES 10, D-glucose 0.5, bovine serum albumin 0.5 mg/ml, penicillin (100 units/ml) and streptomycin (100 µg/ml) and adjusted to pH 7.3 with NaOH. The final osmolarity of the solution was 300 mosmol/l. Under a dissecting microscope, the anterior and intermediate lobes were removed and the neural lobe was cut into eight equal pieces. Each piece of tissue explant was placed on a collagen-coated 35-mm-diameter Petri dish containing 1 ml sterile culture medium (Dulbecco's modified Eagles Medium; Gibco, UK) supplemented with 10% fetal bovine serum. As previously described [18], such explants established monolayers after 1 week. Cell cultures were allowed to grow in an incubator (37°C, 5% CO_2) for 6–8 days before being used in calcium-imaging experiments.

Immunocytochemistry

The cell cultures were characterized using an indirect immunofluorescence technique with a primary antibody directed against glial fibrillary acidic protein (GFAP). Cell cultures were rinsed three times for 5 min in phosphate-buffered saline [PBS, pH 7.2 containing (mM): Na_2HPO_4 6.5, KH_2PO_4 1.5, NaCl 140, $MgCl_2$ 0.5, KCl 3, $CaCl_2$ 1], and fixed with 4% paraformaldehyde for 20 min. After three 5-min rinses in PBS, cultures were incubated for 2 h at room temperature or overnight at 4°C with the primary antibody (mouse monoclonal antibody GFAP, 1:100; Sigma-Aldrich, France) diluted in PBS containing 0.1% bovine serum albumin and 0.1% Triton X-100 (Sigma-Aldrich).

After three 5-min washes in PBS, incubation with the second antibody [anti-mouse IgG–fluorescein isothiocyanate (FITC) conjugate 1:100, Sigma-Aldrich] was performed in PBS containing 0.1% bovine serum albumin and 0.1% Triton X-100 at room temperature for 2 h. Unspecific labelling was determined in control experiments performed by omitting the first antibody. Labelled cultures were generally observed with a Reichert Polyvar microscope. When the immunostaining was performed on Fura-2-loaded cells, the fluorescence was observed with a Zeiss ICM405 inverted microscope.

Dye loading and $[Ca^{2+}]_i$ measurement

Cultures were removed from the incubator, rinsed three times in serum-free physiological saline solution (PSS) containing (in mM): NaCl 135, KCl 5, $MgCl_2$ 2, $MgSO_4$ 0.4, $CaCl_2$ 2, HEPES 10, D-glucose 5.6 and adjusted to pH 7.3 with NaOH. Cells were loaded by incubation with 1 ml of PSS containing 5 µM of the acetoxymethyl ester of Fura-2 (Fura-2/AM, Molecular Probes, Eugene, Ore., USA) in dimethyl sulphoxide carrier (0.1% final concentration) for 30 min at 37°C. They were then rinsed three times to remove the extracellular Fura-2/AM. After the loading procedure, the Petri dish was attached to the stage of a Zeiss ICM405 inverted microscope.

The optical system was composed of a Zeiss 40× objective which was used for epifluorescence measurements with a 75-W xenon lamp. The excitation beam was filtered through narrow-band filters (350 and 380 nm, Oriel, Conn., USA) mounted in a motorized wheel (Lambda 10-2, Sutter Instrument, Calif., USA) equipped with a shutter in order to control the exposure times. The incident and the emitted fluorescence radiation were separated through a Zeiss chromatic beam splitter. Fluorescence emission was selected through a 510-nm narrow-band filter (Oriel). The transmitted light images were viewed by an intensified camera (Extended ISIS, Photonic Science, Sussex, UK). The 8-bit Extended ISIS camera was equipped with an integration module in order to maximize the signal-to-noise ratio. The video signal from the camera was sent to an image processor integrated in a DT2867 image card (Data Translation, Data Translation, Mass., USA) installed in a Pentium 100 PC computer. The processor converts the video signal into 512 lines by 768 square pixels per line by 8 bits per pixel. The 8-bit information for each pixel represents one of the 256 possible grey levels, ranging from 0 (for black) to 255 (for white). Image acquisition and analysis were performed by AIW V2.0 software (Axon, USA). The acquisition rate was one image every 5 s. The final calculations were made using Excel software (Microsoft, Mass., USA).

A rapid change in the superfusion solution was accomplished with a 6-port distribution valve connected to solution reservoirs, and attached to a micromanipulator-controlled outlet at the top of the chamber.

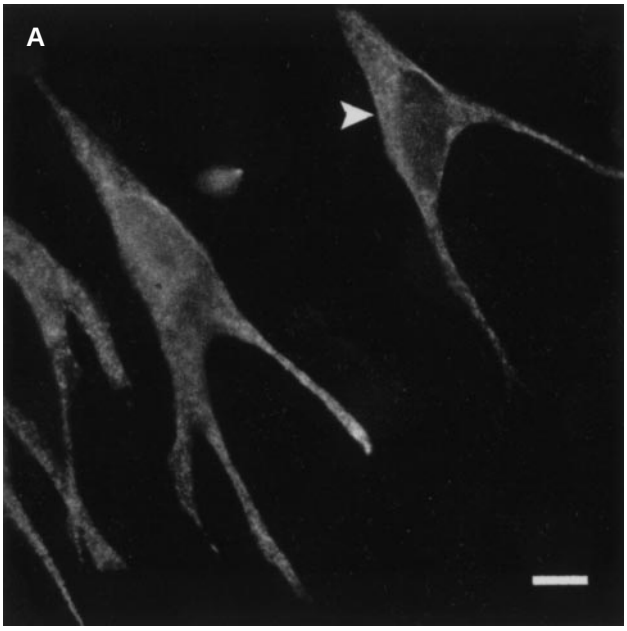
The ratio of emitted fluorescence at the two excitation wavelengths (350 nm and 380 nm) was calculated for each frame on a pixel-by-pixel basis. The calibration of the 350/380-nm ratio in terms of free Ca^{2+} concentration was based on the procedure described by Grynkiewicz et al. [16]. The calibration constants (R_{min}, R_{max} and F_o/F_s) were determined in the same experimental set-up using ionomycin permeabilization of the dye-loaded cells.

Selection of cells

Recordings of $[Ca^{2+}]_i$ were made from individual cultured pituicytes. To minimize the possibility of Ca^{2+} responses caused by signalling between cells [45], recordings were made from isolated cells in subconfluent regions located at the periphery of the cultures. To identify the cell types in primary cultures, we used the indirect immunofluorescence technique with an antibody directed against GFAP. Fluorescence microscopy revealed that the majority of cultured cells were immunostained around the nuclei. Moreover the fluorescence often extended to short cytoplasmic processes. In some experiments the immunostaining with anti-GFAP was performed after the Fura-2 imaging technique. Under these conditions the ATP stimulated-cells were also GFAP positive (Fig. 1).

Solutions and drug application

The PSS described above was the control medium. Stock solutions of drugs were diluted in this control medium. The following drugs were purchased from Sigma-Aldrich: adenosine; adenosine 5′-monophosphate (AMP); adenosine 5′-diphosphate (ADP); adenosine 5′-triphosphate (ATP); adenosine 5′-*O*-[3-thiotriphosphate] (ATP[γ-*S*]); α,β-methylene adenosine-5′-triphosphate (α,β-MeATP); β,γ-methylene adenosine-5′-triphosphate (β,γ-MeATP); uridine 5′-triphosphate (UTP); pyridoxal-phosphate-6-azophenyl-2′,4′-



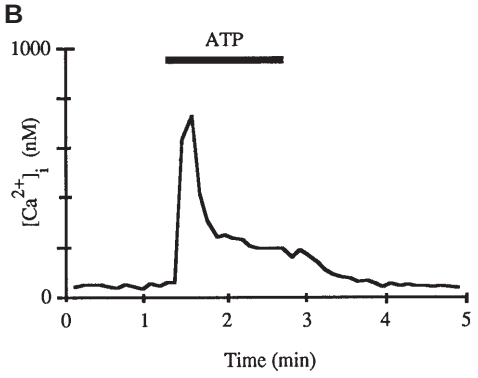
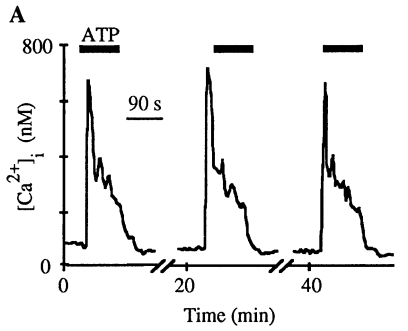
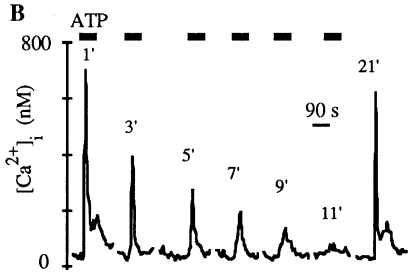


Fig. 1A, B Immunostaining with anti-glial fibrillary acidic protein (anti-GFAP) and intracellular free calcium levels as a function of time in adult rat explant cultures of the neural lobe. **A** Pituicytes after 10 days of culture are GFAP-immunoreactive and are representative of the cells that were used for calcium imaging (*scale bar*=10 µm). **B** Calcium response to 100 µM ATP recorded in the Fura-2-loaded pituicyte seen in **A** (*arrow*) before the immunostaining experiment





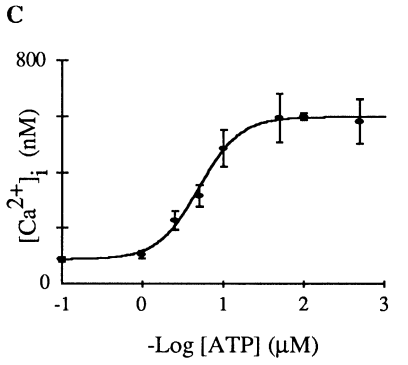


Fig. 2A–C Effects of externally applied ATP on $[Ca^{2+}]_i$ in Fura-2-loaded cultured pituicytes. **A** Reproducible calcium responses of a single cell by applications of ATP (100 µM) separated by 20-min resting periods. **B** Reduction in the calcium response of a single cell by consecutive applications of ATP (100 µM). Two minutes elapsed between the different ATP applications, during which primary cultures of rat cultured pituicytes were superfused with physiological saline solution (*PSS*). **C** Dose/response curve for ATP in cultured pituicytes. Each *point* is the mean of the peak Ca^{2+} response to the ATP at each concentration; *vertical lines* show SEM. EC_{50} was 4.3 µM

disulphonic acid (PPADS); Cibacron Blue 3GA formerly listed as Reactive Blue 2 (RB-2); thapsigargin; pertussis toxin (PTX).

Suramin and 2-methylthio-adenosine-5′-triphosphate (2-MeS-ATP) were purchased from ICN Pharmaceuticals (France). 1-[6-[[17-beta-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1-*H*-pyrrole-2,5-dione (U-73122) and 1-{6-{[17 beta-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl}-2,5-pyrrolidine-dione (U-73343) were purchased from Biomol Research (France). Fura-2/AM was from Molecular Probes.

Statistical analysis

Each experiment was performed between 4 and 34 times, and involved between 2 and 19 rats. The experimental results were analysed by the Mann-Whitney non-parametric test using Instat 2.01 for Macintosh (GraphPad Software, USA). Results are expressed as means ±SEM. Half-maximal concentration (EC_{50}) values were calculated by fitting a sigmoid function to the data using Cigale F2.2 for Macintosh (M. Bordes, IPMC, France).

Results

Effects of ATP stimulation

The resting $[Ca^{2+}]_i$ was 70±2 nM (*n*=284). Exposure to extracellular ATP (100 µM) induced an immediate increase in $[Ca^{2+}]_i$ in 89.2±4.6% of the cells analysed (544±15 nM above the resting level, *n*=284). The response usually started with a initial peak response reaching 614±15 nM, followed by a more or less “sustained plateau phase”, and then $[Ca^{2+}]_i$ returned to near the basal levels once the ATP was washed out (Figs. 1, 2). In order to evoke reproducible responses it was necessary to allow an inter-

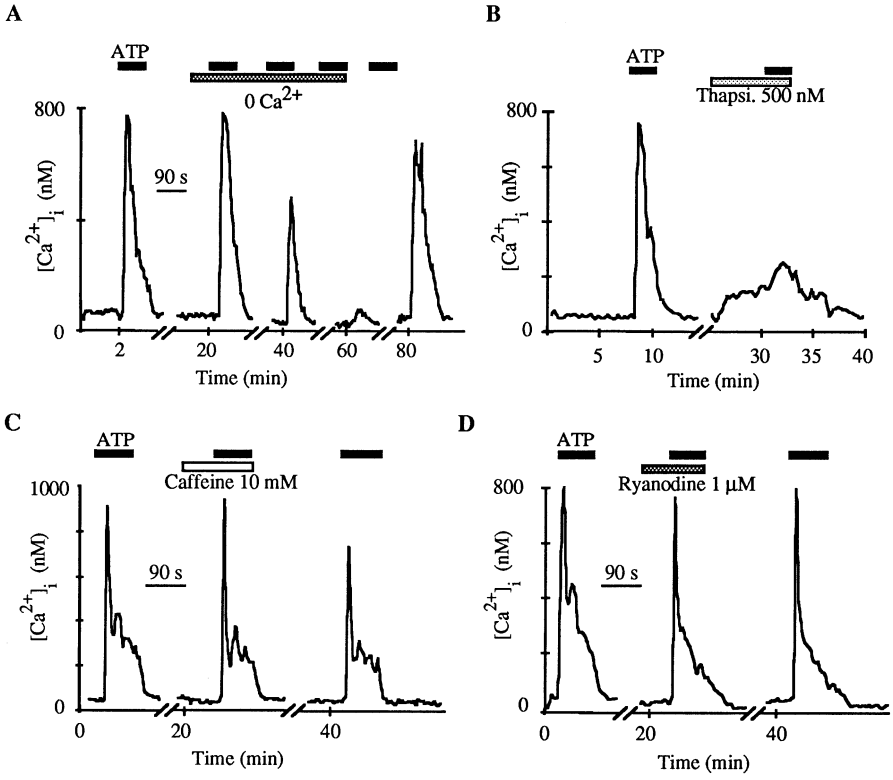


Fig. 3A–D Effects of the absence of extracellular calcium, of thapsigargin, caffeine and ryanodine on the ATP-dependent $[Ca^{2+}]_i$ increase. **A** Dependence of ATP responses on extracellular calcium. ATP (100 µM) was applied to a single cell in the presence and absence (*hatched bar*) of calcium. Each stimulation was separated by 20-min resting periods. The *black bar* indicates the application of ATP. **B** Application of ATP (100 µM), as indicated by the *black bars*, elicited responses in cultured pituicytes before and during the application of 500 nM thapsigargin. Thapsigargin application is indicated by the *long hatched bar*. Depletion of intracellular Ca^{2+} stores with the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin prevents ATP-induced responses. **C, D** ATP (100 µM) responses were elicited in cultured pituicytes, before, during and after the application of 10 mM caffeine (**C**) or 1 µM ryanodine (**D**) as indicated by the *black bars*. Caffeine (*open bar*) and ryanodine (*hatched bar*) do not affect the resting $[Ca^{2+}]_i$ and the ATP-dependent $[Ca^{2+}]_i$ elevations

val of about 20 min between repeated applications of ATP. With this interval the peak amplitude of the responses was similar (1st, 634±29 nM; 2nd, 627±37 nM; 3rd, 630±26 nM, *n*=26, Fig. 2A). However, successive ATP stimulations separated by only 2-min intervals resulted in a considerable run-down (1st, 695±48 nM; 2nd, 329±41 nM; 3rd, 241±28 nM; 4th, 203±24 nM; 5th, 169±22 nM; 6th, 104±11 nM, *n*=20, Fig. 2B). This effect was reversible after a 20-min rinse with PSS (445±35 nM, *n*=20, Fig. 2B).

Pituicytes were challenged with increasing concentrations of ATP, from 100 nM to 500 µM. ATP concentrations of 100 nM and 1 µM did not modify the $[Ca^{2+}]_i$ (100 nM: 75.8±6.5 nM, *P*=0.49, *n*=19; and 1 µM: 79.5±6.5 nM, *P*=0.42, *n*=21), while an ATP concentration of 2.5 µM was effective (225±33 nM, *n*=15, Fig. 2C). The response was then proportional to the ATP concentration (5 µM ATP, 314±40 nM; 10 µM ATP, 487±67 nM; *n*=18 and 23, Fig. 2C) up to a maximal effect at 50 µM (595±88 nM, *n*=10, Fig. 2C). No further effect was observed with higher ATP concentrations (*P*=0.94 and 0.93; 100 µM: 601±13 nM, *n*=276 and 500 µM: 582±80 nM; *n*=4). The experimentally determined EC_{50} for ATP was 4.3 µM.

Source of Ca^{2+} in the ATP-evoked response

In order to determine whether extracellular Ca^{2+} is required to produce the responses to ATP, we compared responses evoked in extracellular solution containing 2 mM Ca^{2+} versus those obtained in Ca^{2+}-free solution containing EGTA (500 µM). After 1 min in Ca^{2+}-free PSS the initial peak response observed in the presence of 100 µM ATP was completely unaffected, both in its time course and its magnitude (ATP with Ca^{2+}, 634.4±40.4 nM; first ATP challenge without Ca^{2+}, 595.4±51.2 nM, *P*=0.57, *n*=15, Fig. 3A). These results suggest that extracellular Ca^{2+} is not required and that the Ca^{2+} response to ATP is caused by release of Ca^{2+} from an intracellular pool. Nevertheless, although the initial ATP-induced response was of comparable magnitude to that found in the presence of extracellular Ca^{2+}, subsequent responses showed a considerable degree of run-down (276±40 nM for the 2nd ATP stimulation in the absence of external Ca^{2+}, and 167±52 nM for the 3rd, *n*=15, Fig. 3A).

Further evidence for the involvement of intracellular Ca^{2+} stores in the generation of the ATP-induced response was obtained using thapsigargin, an irreversible inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase,

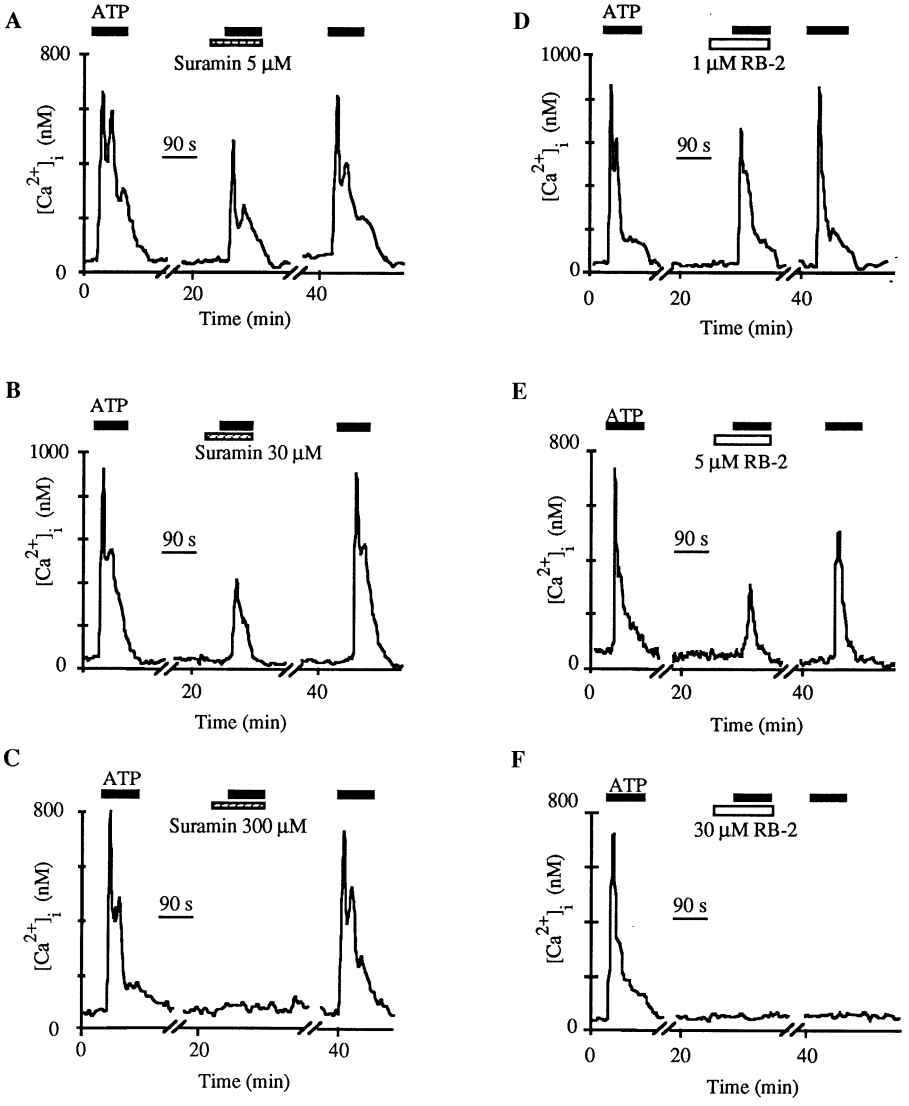


Fig. 4 Effects of suramin (**A**–**C**) and Reactive Blue-2 (**D**–**F**) on ATP-dependent $[Ca^{2+}]_i$ increase. Cells were incubated with suramin at different concentrations (**A**, 5 µM; **B**, 30 µM; **C**, 300 µM). In all three cases, $[Ca^{2+}]_i$ profiles were observed after exposure to 100 µM ATP. The same cell, after washing, was incubated with suramin for 1 min and then subjected to ATP. The effect of ATP was completely abolished when incubated with 300 µM suramin. Note that the inhibitions observed in the presence of suramin were reversible. **D**–**F** Block of ATP responses by the specific P_{2Y} purinoceptor antagonist, Reactive Blue-2 (*RB-2*). Cells were incubated with RB-2 at different concentrations (**D**, 1 µM; **E**, 5 µM; **F**, 30 µM). In all three cases, $[Ca^{2+}]_i$ profiles were observed after 100 µM ATP. The same cell, after washing, was incubated with RB-2 for 1 min and then subjected to ATP. The effect of ATP was completely abrogated when incubated with 30 µM RB-2. Note that the inhibitions observed in presence of 30 µM RB-2 were completely irreversible, and only partially reversible in presence of 5 µM RB-2

which accumulates Ca^{2+} in the lumen of internal membrane-bound stores. Thapsigargin (500 nM) alone caused a small and slow increase in cultured pituicytes' $[Ca^{2+}]_i$ (228±24 nM, *n*=12, Fig. 3B). This rise of Ca^{2+} is consistent with the known action of this compound and has been seen in various cell types [39]. After 5 min of pretreatment with thapsigargin, the response to 100 µM ATP was markedly reduced (control ATP, 641±78 nM; ATP plus thapsigargin, 323±32 nM, *n*=12, Fig. 3B) and further applications of ATP failed to elicit any response.

In contrast, neither ryanodine or caffeine had an effect on the resting $[Ca^{2+}]_i$ in these cells (basal 65±4 nM versus 10 mM caffeine, 49±3 nM, *n*=16; *P*=0.44; basal 40±5 nM versus 1 µM ryanodine, 30±5 nM, *n*=7; *P*=0.38; Fig. 3C, D). This suggests that the cells do not express the ryanodine receptor-mediated calcium-induced calcium-release mechanism. Accordingly, after a 5-min exposure to ryanodine or caffeine, the subsequent addition of ATP yielded a response indistinguishable from the control one (control ATP, 702±116 nM; ATP plus 10 mM caffeine, 711±78 nM, *n*=16; control ATP 931±67 nM; ATP plus 1 µM ryanodine, 850±83 nM, *n*=7, Fig. 3C, D).

Effects of purinoceptor agonists on $[Ca^{2+}]_i$

To determine the receptor type involved in the ATP-induced increase in $[Ca^{2+}]_i$, we tested the effects of various purinoceptor agonists on $[Ca^{2+}]_i$. Adenosine (100 µM) and AMP (100 µM) were ineffective in increasing $[Ca^{2+}]_i$ (Table 1). These two compounds are known to be more effective than ATP in stimulating P_1-type purinergic receptors, suggesting that P_1 receptors are not involved in mediating the ATP-evoked calcium response of astrocytes.

α,β-MeATP, β,γ-MeATP and UTP had no effect on $[Ca^{2+}]_i$ (Table 1). 2-MeSATP (100 µM) and ATP were equally effective in increasing the $[Ca^{2+}]_i$ of pituicytes (*P*=0.8, Table 1). ATP[γ-*S*] (100 µM) elicited a response as large as that observed with ATP (100 µM) (*P*=0.6, Table 1). Application of ADP produced a $[Ca^{2+}]_i$ increase in 19 of the 27 tested cells (Table 1). In the sensitive

Table 1 Pharmacology of the agonists of the pituicyte Ca^{2+} response

Compound	Peak $[Ca^{2+}]_i$ (nM)
ATP	614±15 (284)
ATP[γ-*S*]	735±56 (11)**
2-Methylthio-ATP	404±47 (26)**
ADP sensitive	511±46 (19)**
ADP insensitive	63±9 (8)*
Adenosine	71±12 (11)*
5′-AMP	72±5 (31)*
UTP	88±5 (34)*
α,β-Methylene ATP	45±4 (17)*
β,γ-Methylene ATP	104±8 (30)*

The values are presented as mean ±SEM (number of analysed cells)
** Not significantly different of the ATP-induced $[Ca^{2+}]_i$ increase (Mann-Whitney test)
* Not significantly different of the resting $[Ca^{2+}]_i$ (Mann-Whitney test)

cells, ADP evoked a characteristic $[Ca^{2+}]_i$ response which was not significantly different from ATP-induced $[Ca^{2+}]_i$ response (*P*=0.3, Table 1).

Effects of purinoceptor antagonists on ATP-induced Ca^{2+} mobilization

We next investigated the effects of several P_2 purinoceptor antagonists on the $[Ca^{2+}]_i$ increase induced by 100 µM ATP. Suramin, a trypanocidal drug, is known to be a broad spectrum competitive inhibitor of P_2-purinoceptors [12]. We found that applying suramin (5 µM) weakly inhibited (15%) the test ATP response (ATP control, 750±94 nM; ATP plus suramin, 592±61 nM, *P*=0.04, *n*=29, Fig. 4A). At 30 µM, suramin induced a greater inhibition of the test ATP response (78%, ATP control, 741±30 nM; ATP plus suramin, 161±42 nM, *n*=21, Fig. 4B). A strong inhibition of the $[Ca^{2+}]_i$ response (92±1%) was recorded in the presence of 300 µM suramin (ATP control, 581±40 nM; plus suramin, 80±7 nM, *n*=30). The effect of suramin was reversible (Fig. 4A–C).

The effects of RB-2, a selective P_{2Y} antagonist [7], were tested on the $[Ca^{2+}]_i$ increase induced by 100 µM ATP. At 1 µM, RB-2 inhibited the test ATP response by 37% (ATP control, 836±103 nM; plus RB-2, 444±63 nM; *n*=15; Fig. 4D) and a further block of 62% was obtained when RB-2 was given at 5 µM (ATP control, 628±79 nM; plus RB-2, 305±47 nM; *n*=20, Fig. 4E). Figure 4F shows that when applied at 30 µM, RB-2 reduced the ATP response by 88% (RB-2, 82±9 nM versus 489±56 nM for control, *n*=17). At this concentration the effect of RB-2 was irreversible (Fig. 4F).

PPADS has been reported to antagonize P_{2X}- and P_{2Y}-purinoceptor-mediated responses in many cases (for review [30]). As shown in Fig. 5, 100 µM PPADS decreased the Ca^{2+} response to ATP by 76% compared to the control (ATP control, 808±51 nM; plus PPADS, 235±48 nM; *n*=12). The above results indicates that the rank order of inhibition of the ATP-evoked Ca^{2+} response was RB-2>PPADS>suramin.

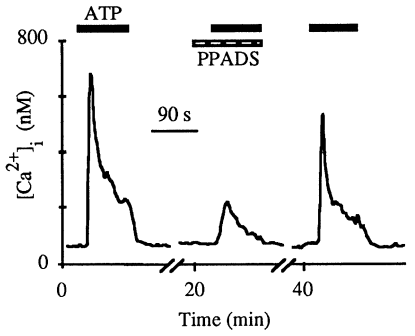


Fig. 5 Sensitivity of ATP-dependent $[Ca^{2+}]_i$ increase to pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (*PPADS*) in cultured pituicytes. Cultured pituicytes were incubated with 100 µM PPADS after a control 100 µM ATP-induced $[Ca^{2+}]_i$ increase. After washing, the same cell was incubated with PPADS for 1 min and then subjected to ATP. The effect of ATP was partially inhibited and the inhibition observed in presence of PPADS was reversible

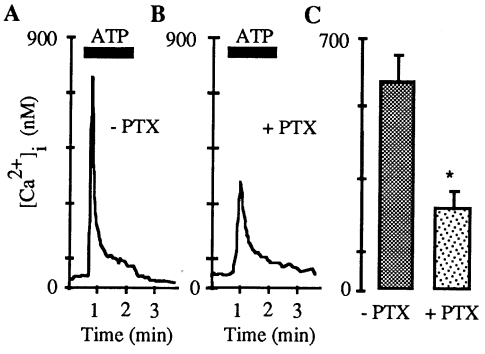


Fig. 6A–C Effects of pertussis toxin (*PTX*) on the ATP-dependent $[Ca^{2+}]_i$ increase in cultured pituicytes. **A** Representative trace showing the $[Ca^{2+}]_i$ rise evoked by 100 µM ATP. **B** Representative trace of the ATP response after pretreatment for 20 h with pertussis toxin (1 µg/ml). **C** Diagram representing data pooled from two independent experiments similar to those shown in **A** and **B** (*P<0.04)

The nature of the coupling between the ATP receptor and $[Ca^{2+}]_i$ elevation

For metabotropic receptors that elicit a release of Ca^{2+} from thapsigargin-sensitive intracellular stores, the receptors are typically coupled to phospholipase C (PLC) via G proteins [3]. In order to determine whether a G protein sensitive to PTX (1 µg/ml) mediates the response to ATP, we compared responses to a standard application of ATP (100 µM) in cells treated with PTX versus with vehicle alone (Fig. 6). PTX significantly reduced the ATP-evoked $[Ca^{2+}]_i$ increase by 40% (PTX, 364±47 nM, *n*=26, versus 568±70 nM for the ATP control, *n*=16, Fig. 6).

At 500 nM, U-73122, an inhibitor of PLC, inhibited the effect of 100 µM ATP on $[Ca^{2+}]_i$ by 86% when preincubated for 1 min (U-73122, 111±18 nM versus 628±66 nM for the control, *n*=43, Fig. 7), and by 99% when preincubated for 15 min (U-73122, 5±4 nM versus 657±65 nM for the control, *n*=6). Therefore, Ca^{2+} release is probably mediated by products of phosphatidyl inositol turnover. U-73343 (500 nM), the inactive stereoisomer of U-

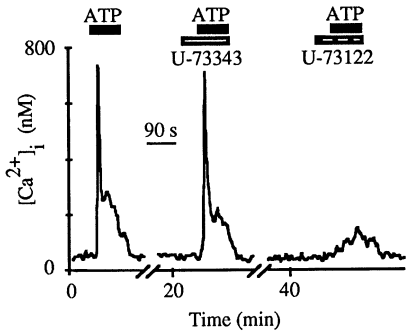


Fig. 7 Effects of U-73122 and U-73343 on the ATP-dependent $[Ca^{2+}]_i$ increase in cultured pituicytes. The *traces* show $[Ca^{2+}]_i$ recorded from one pituicyte. ATP (100 μM) was applied as indicated by the *bars* above the *traces*. ATP was applied before drug administration (*control*), after a 1-min incubation with U-73343 (0.5 μM), after removing U-73343 and incubating for 1 min with U-73122 (0.5 μM)

73122, had no effect on the ATP-evoked $[Ca^{2+}]_i$ increase (ATP, 519±129 nM; ATP+U-73343, 596±147 nM, *n*=12, Fig. 7).

Discussion

In the present study we have examined the effects of ATP, as well as agonists and antagonists, on $[Ca^{2+}]_i$ and on the different Ca^{2+} signalling pathways in cultured pituicytes from adult rat neurohypophysial explants.

We observed cells growing out from the explant, as previously described in primary culture from adult rat neurohypophysis [4, 18]. The cells showed positive immunoreactivity with antibodies against GFAP (Fig. 1), as already described [4]. This is good evidence for identifying these cells as pituicytes, since the presence of GFAP in pituicytes in situ has been abundantly described [35, 38]. To further confirm the presence of pituicytes, we tested the effectiveness of vasopressin (AVP) in elevating $[Ca^{2+}]_i$ on Fura-2-loaded cultured explants. We reproduced the results (data not shown) previously described by Hatton et al. [18], showing the activation of a V_1-receptor on these cells.

In this work, we have demonstrated that ATP triggers a rapid $[Ca^{2+}]_i$ increase in cultured pituicytes, which returns to basal levels when ATP is removed. The $[Ca^{2+}]_i$ increase evoked by extracellular ATP is dose-dependent in the range of 2.5–50 μM. The observed EC_{50} was 4.8 μM. The ATP-dependent $[Ca^{2+}]_i$ increase was due to discharge from an intracellular pool, as shown by experiments performed in the absence of external Ca^{2+}. We showed that this pool is depleted by the Ca^{2+}-ATPase inhibitor thapsigargin and is caffeine and ryanodine insensitive.

The use of inhibitors indicates that suramin, a non-selective P_2 antagonist, inhibits the ATP-dependent $[Ca^{2+}]_i$ increases measured in cultured pituicytes. PPADS only partially inhibited the ATP-induced $[Ca^{2+}]_i$ responses. This drug has been shown to act as a potent antagonist of P_{2X} receptors, and also, with a lower potency, as an antagonist of P_{2Y} receptors (for review see [30]). This compound has been reported to block the P_{2Y} receptor-mediated, ATP-induced intracellular Ca^{2+} mobilization by a non-specific mechanism that probably involves the inhibition of inositol-1,4,5-trisphosphate ($InsP_3$) channels [46]. RB-2, a selectively P_{2Y} subtype antagonist [7], was most effective at inhibiting ATP-dependent $[Ca^{2+}]_i$ increases in pituicytes.

Several lines of evidence indicate that the ATP-evoked $[Ca^{2+}]_i$ increases are mediated by receptors belonging to the P_{2Y} receptor family. First, the presence of external Ca^{2+} is not necessary to induce ATP-dependent $[Ca^{2+}]_i$ increases, which rules out the intervention of ligand-gated P_{2X} channels. Second, Ca^{2+} rises were reduced by PTX, (a G protein blocker of the Gi/o family), consistent with the proposal that all P_{2Y} receptors are G-protein-coupled receptors [1]. In particular, the P_{2Y} receptors identified in this study are coupled to stimulation of PLC, as suggested by the abolition of $[Ca^{2+}]_i$ responses by the PLC inhibitor, U-73122, but not by its inactive analogue, U-73343 [15, 37]. Finally ATP-evoked Ca^{2+} increases are inhibited by RB-2, strongly indicating that the pituicyte purinoceptor belongs to the P_{2Y} receptor family [7].

The present study shows that the apparent rank order of potency for increasing $[Ca^{2+}]_i$ in rat cultured pituicytes is ATP=ATP-[γ-*S*]=2-MeSATP≥ADP. This pharmacology exhibits a selectivity quite different to that classically described for cloned P2Y receptors. The inactivity of the pyrimidines and the weak efficacy of the ADP found in pituicytes are not described for the P_{2Y} purinoceptors already cloned [30], and are consistent with the presence of a new subtype of P_{2Y} receptors. Nevertheless, the rank order of agonist potencies for P_{2Y} receptors was recently redefined [30]. The presence of contaminating nucleotides in commercial preparations, the hydrolysis of nucleotides by endogenous ecto-enzymes and the presence of endogenous purinoceptors in some expression systems have recently led to a re-evaluation of the nucleotide selectivities of P_{2Y} purinoceptors [30]. In fact, the purinoceptor of the cultured pituicytes may be a variant of a known P_{2Y} receptor, or a new subtype. However, a molecular biological approach is necessary to elucidate this point.

The next question is the physiological source of the ligand for this (or these) receptor(s). A number of lines of circumstantial evidence suggest that the neurohypophysial secretion of ATP may activate this purinoceptor. If indeed ATP exerts effects on pituicyte $[Ca^{2+}]_i$ in vivo, as observed here in vitro, the most immediate source of ATP appears to be the neurohypophysial terminals, since ATP is present in many secretory vesicles [48] and has been specifically localized within the neurosecretory granules in the neurohypophysis ([14] and unpublished results). The stimulation of neuropeptide release is associated with a significant increase of neurosecretory granule exocytosis, and a subsequent rise in ATP release (unpublished results). In the neurohypophysis, the intragranular ATP

concentration was found to be 2 mM [14]. After stimulation, ATP would be expected to achieve an extracellular concentration of 4–40 µM in the vicinity of the terminals (see [10]). Morphological evidence from intact neurohypophysis suggests that the pituicytes are intimately associated with the nerve terminals [42]. Therefore, the pituicytes would be in an ideal position to detect changes in the ATP concentrations resulting from neurosecretion activity. In the present study, the ATP dose dependency was in the same range of magnitude as that of the P_{2X2} purinoceptor previously described in neurohypophysial nerve terminals (EC_{50}=4.8 µM) [41]. These observations support the hypothesis that ATP can act as a neuronal-glial signalling molecule within the neurohypophysis.

Since we have demonstrated the existence of an ecto-ATPase on the neurohypophysial pituicytes [40], the breakdown of ATP by this ecto-ATPase could well terminate the action of ATP on the purinoceptor.

The physiological role(s) of the ATP-evoked $[Ca^{2+}]_i$ increase in pituicytes is (are) largely unknown. One acute effect could be the activation of Ca^{2+}-activated K^+ channels in the pituicyte membrane. Such an activation has already been reported with glutamate [9] and serotonin [21] in cultured rat astrocytes, and with histamine in C6 glial cells [47]. Moreover, in cultured non-proliferating microglial cells, ATP has been shown to open K^+ channels, via the P_{2Y} receptor [20]. Finally, we postulate that the P_{2Y} purinoceptors located in pituicytes can activate Ca^{2+}-activated K^+ channels. This activation may then participate in the increase of the local external $[K^+]$. Such an increase has already been reported by Leng and Shibuki [25], who electrically stimulated the neurohypophysis in vitro. This increase has been found to enhance the excitability of the tissue [26]. The pituicytes could therefore be implicated in stimulus–secretion coupling, contributing to facilitate hormone release via an ATP-stimulated Ca^{2+}-activated K^+ conductance.

Concurrently, ATP could also be implicated in several trophic actions in pituicytes. It has been shown in primary cultures of rat astrocytes that ATP stimulates mitogen-activated protein kinase [23]. Moreover, extracellular ATP mediates stellation [28] and enhances the number of GFAP-positive astroglial cells [2, 11]. These morphological changes could then be involved in the reactive gliosis that occurs during central nervous system injury.

Under resting conditions, the pituicytes surround and engulf many of the axons and terminals [43]. Under diverse physiological stimuli, such as dehydration, parturition or lactation, there are considerable morphological changes in glia resulting in a reduction in the number of axons engulfed by pituicytes [31, 43, 44]. Consequently, the pituicyte processes retract from their usual positions along the basement membrane. This phenomenon permits the hormone-secreting terminals to have functional access to the perivascular spaces surrounding the fenestrated capillaries [30, 43, 44]. Little is known about the molecular mechanisms that regulate these morphological changes, but ATP via the activation of the P_{2Y} purinoceptor could be one of the effectors of these trophic effects.

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References

1. Abbracchio MP, Burnstock G (1994) Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Ther 64: 445–475
2. Abbracchio MP, Saffrey MJ, Hopker V, Burnstock G (1994) Modulation of astroglial cell proliferation by analogues of adenosine and ATP in primary cultures of rat striatum. Neuroscience 59:67–76
3. Berridge MJ (1993) Inositol triphosphate and calcium signalling. Nature 361:315–325
4. Bicknell RJ, Luckman SM, Inenaga K, Mason WT, Hatton GI (1989) β-Adrenergic and opioid receptors on pituicytes cultured from adult rat neurohypophysis: regulation of cell morphology. Brain Res Bull 22:379–388
5. Bicknell RJ, Boersma CJC, Van Leeuwen FW, Mason WT (1993) Calcium signalling in neurosecretory terminals and pituicytes. Ann NY Acad Sci 689:177–182
6. Boersma CJC (1993) Immunoelectron microscopic demonstration of oxytocin and vasopressin in pituicytes and in nerve terminals forming synaptoid contacts with pituicytes in the rat neural lobe. Brain Res 611:117–129
7. Burnstock G, Warland JJI (1987) P_2-purinoceptors of two types in the rabbit mesenteric artery: reactive blue 2 selectively inhibits responses mediated via the P_{2Y}- but not the P_{2X}-purinoceptor. Br J Pharmacol 90:383–391
8. Chen CC, Chen WC (1996) ATP-evoked inositol phosphates formation through activation of P2U purinergic receptors in cultured astrocytes: regulation by PKC subtypes α, δ, and θ. Glia 17:63–71
9. Chen J, Backus KH, Deitmer JW (1997) Intracellular calcium transients and potassium current oscillations evoked by glutamate in cultured rat astrocytes. J Neurosci 17:7278–7287
10. Chow RH, Von Rüden L (1995) Electrochemical detection of secretion from single cells. Single channel recording. In: Sakmann B, Neher E (eds) Single channel recording. Plenum, New York, pp 245–277
11. Ciccarelli R, Di Iorio P, Ballerini P, Ambrosini G, Giuliani P, Tiboni GM, Caciagli F (1994) Effects of exogenous ATP and related analogues on the proliferation rate of dissociated primary cultures of rat astrocytes. J Neurosci Res 39:556–566
12. Dunn PM, Blakeley AGH (1988) Suramin: a reversible P2-purinoceptor antagonist in the mouse vas deferens. Br J Pharmacol 93:243–245
13. Evans RJ, Derkach V, Surprenant A (1992) ATP mediated fast synaptic transmission in mammalian neurons. Nature 357: 503–505
14. Gratzl M, Torp-Pedersen C, Daertt DA, Treiman M, Thorn NA (1980) Isolation and characterization of secretory granules from bovine neurohypophyses. Hoppe-Seylers Z Physiol Chem 361:1615–1628
15. Green AC, Dowdall MJ, Richardson CM (1997) ATP acting on P2Y receptors triggers calcium mobilization in Schwann cells at the neuroelectrocyte junction in skate. Neuroscience 80:635–651
16. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450
17. Hatton GI, Perlmutter LS, Salm AK, Tweedle CD (1984) Dynamic neuronal-glial interactions in hypothalamus and pituitary: implications for control of hormone synthesis and release. Peptides 5:121–138
18. Hatton GI, Bicknell RJ, Hoyland J, Bunting R, Mason WT (1992) Arginine vasopressin mobilises intracellular calcium

via V1-receptor activation in astrocytes (pituicytes) cultured from adult rat neural lobes. Brain Res 588:75–83
19. Hosli E, Hosli L (1993) Receptors for neurotransmitters on astrocytes in the mammalian central nervous system. Prog Neurobiol 40:477–506
20. Illes P, Norenberg W, Gebicke-Haerter PJ (1996) Molecular mechanisms of microglial activation. B. Voltage- and purinoceptor-operated channels in microglia. Neurochem Int 29:13–24
21. Jalonen TO, Margraf RR, Wielt DB, Charniga CJ, Linne ML, Kimelberg HK (1997) Serotonin induces inward potassium and calcium currents in rat cortical astrocytes. Brain Res 758: 69–82
22. Kastritsis CH, Salm AK, McCarty K (1992) Stimulation of the P2Y purinergic receptor on type 1 astroglia results in inositol phosphate formation and calcium mobilization. J Neurochem 58:1277–1284
23. King BF, Neary JT, Zhu Q, Wang S, Norenberg MD, Burnstock G (1996) P_2 purinoceptors in rat cortical astrocytes: expression, calcium imaging and signalling studies. Neuroscience 74:1187–1196
24. Kirischuk S, Scherer J, Kettenmann H, Verkhratsky A (1995) Activation of P_2-purinoceptors triggering Ca^{2+} release from $InsP_3$-sensitive internal stores in mammalian oligodendrocytes. J Physiol (Lond) 483:41–57
25. Leng G, Shibuki K (1987) Extracellular potassium changes in the rat neurohypophysial during activation of the magnocellular neurosecretory system. J Physiol (Lond) 392:97–111
26. Leng G, Shibuki K, Way SA (1988) Effects of raised extracellular potassium on the excitability of, and hormone release from, the isolated rat neurohypophysis. J Physiol (Lond) 399: 591–605
27. McCarthy KD, De Vellis J (1980) Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J Cell Biol 85:890–902
28. Neary JT, Norenberg MD (1992) Signaling by extracellular ATP: physiological and pathological considerations in neuronal-astrocytic interactions. Prog Brain Res 94:145–151
29. Norenberg W, Langosch JM, Gebicke-Haerter PJ, Illes P (1994) Characterization and possible function of adenosine 5′-triphosphate receptors in activated rat microglia. Br J Pharmacol 111:942–950
30. North RN, Barnard EA (1997) Nucleotide receptors. Curr Opin Neurobiol 7:346–357
31. Perlmutter LS, Hatton GI, Tweedle CD (1984) Plasticity in the “in vitro” neurohypophysis: effects of osmotic changes on pituicytes. Neuroscience 12:503–511
32. Peuchen S, Clarck JB, Duchen MR (1996) Mechanisms of intracellular calcium regulation in adult astrocytes. Neuroscience 71:871–883
33. Porter JT, McCarthy KD (1997) Astrocytic neurotransmitter receptors in situ and in vivo. Prog Neurobiol 51:439–455
34. Pow DV, Perry VH, Morris JF, Gordon S (1989) Microglia in the neurohypophysis associate with and endocytose terminal portions of neurosecretory neurons. Neuroscience 33:567–578
35. Salm AK, Hatton GI, Nilaver G (1982) Immunoreactive glial fibrillary acidic protein in pituicytes of the rat neurohypophysis. Brain Res 236:471–476
36. Salter MW, Hicks JL (1994) ATP-evoked increases in intracellular calcium in neurons and glia from the dorsal spinal cord. J Neurosci 14:1563–1575
37. Salter MW, Hicks JL (1995) ATP causes release of intracellular Ca^{2+} via the phospholipase Cb/IP_3 pathway in astrocytes from the dorsal spinal cord. J Neurosci 15:2961–2971
38. Suess U, Pliska V (1981) Identification of the pituicytes as astroglial cells by indirect immunofluorescence-staining for the glial fibrillary acidic protein. Brain Res 221:27–33
39. Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP (1990) Thapsigargin, a tumour promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc Natl Acad Sci USA 87:2466–2470
40. Thirion S, Troadec JD, Nicaise G (1996) Cytochemical localization of ecto-ATPases in rat neurohypophysis. J Histochem Cytochem 44:103–111
41. Troadec JD, Thirion S, Nicaise G, Lemos J, Dayanithi G (1998) ATP evoked increases in $[Ca^{2+}]_i$ and peptide release from rat isolated neurohypophysial terminals via a P_{2X2} purinoceptor. J Physiol (Lond) 511:89–103
42. Tweedle CD, Hatton GI (1980) Evidence for dynamic interactions between pituicytes and neurosecretory axons in the rat. Neuroscience 5:661–667
43. Tweedle CD, Hatton GI (1980) Glial cell enclosure of neurosecretory endings in the neurohypophysis of the rat. Brain Res 192:555–559
44. Tweedle CD, Hatton GI (1987) Morphological adaptability at neurosecretory axonal endings on the neurovascular contact zone of the rat neurohypophysis. Neuroscience 20:241–246
45. Venance L, Stella N, Glowinski J, Giaume C (1997) Mechanism involved in initiation and propagation of receptor-induced intercellular calcium signalling in cultured rat astrocytes. J Neurosci 17:1981–1992
46. Vigne P, Pacaud P, Urbach V, Feolde E, Breitmayer JP, Frelin C (1996) The effect of PPADS as an antagonist of inositol (1,4,5)triphosphate induced intracellular calcium mobilization. Br J Pharmacol 119:360–364
47. Weiger T, Stevens DR, Wunder L, Haas HL (1997) Histamine H1 receptors in C6 glial cells are coupled to calcium-dependent potassium channels via release of calcium from internal stores. Naunyn Schmiedebergs Arch Pharmacol 355:559–565
48. Zimmermann H (1994) Signalling via ATP in the nervous system. Trends Neurosci 17:420–426