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Stimulation of Ca^{2+} influx through ATP receptors on rat brain synaptosomes: identification of functional $P2X_7$ receptor subtypes

*,[1]Paul M. Lundy, [1]Murray G. Hamilton, [2]Lei Mi, [2]Wenrong Gong, [1]Cory Vair, [1]Thomas W. Sawyer & [1]Robert Frew

[1]Medical Therapy Group, Defence Research Establishment Suffield, P.O. Box 4000, Medicine Hat, Alberta, Canada T1A 8K6 and [2]Kinchyle Enterprises, Medicine Hat, Alberta, Canada T1A 8K6

1 ATP receptors of the P2X class have previously been identified on autonomic nerve endings and on a limited population of CNS neurons.

2 In the present study P2X receptors on mammalian cortical synaptosomes have been identified by a variety of functional and biochemical studies. In choline buffer ATP analogues caused concentration/time dependent Ca^{2+} influx. Relative to the effects caused by ATP, benzoylbenzoyl ATP (BzATP) was about seven times more active than ATP while 2-me-S-ATP and ATPγS were much less active. α,β-me- ATP and β,γ-me-ATP were virtually inactive. In sucrose buffer, relative to choline buffer, the activity of BzATP was more than doubled while activity in sodium buffer was reduced. Moreover, the P2X antagonists PPADS or Brilliant Blue G both significantly attenuated influx. These observations suggest the presence of P2X receptors on synaptosomes which subserve Ca^{2+} influx. This activity profile of the ATP analogues and the response to blocking agents are characteristic of responses of $P2X_7$ receptors.

3 Influx was unaffected by the VSCC inhibitors ω-CTx-MVIIC and (−) 202–791, indicating that ATP induced Ca^{2+} influx occurred primarily through P2X receptors.

4 $P2X_7$ receptor protein was identified by Western blotting and immunohistochemical staining. Purified preparations were devoid of significant concentrations of GFAP or the microglial marker OX-42 but contained greatly enriched amounts of syntaxin and SNAP 25.

5 The various pharmacological and biochemical studies were all consistent with the presence of functional $P2X_7$ receptors.

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Abbreviations: ATP, adenosine 5′triphosphate; ATPγS, adenosine 5′-O-(3-thiophosphate); BBG, Brilliant Blue G; BzATP, 2′&3′-O-(4-benzoylbenzoyl) adenosine 5′ triphosphate; ω-CTx-MVIIC, ω-conotoxin-MVIIC; GFAP, glial fibrillary acidic protein; HBSS, Hanks balanced salt solution; α,β-me-ATP, α,β-methylene-adenosine triphosphate; β,γ-me-ATP, β,γ methylene-L-adenosine triphosphate; 2-me-S-ATP, 2-methylthioadenosine triphosphate; PBS, phosphate buffered saline; PBST, phosphate buffered saline plus tween; PPADS, pyridoxal-phosphate-6-azophenyl-2′- 4′ disulphonic acid; VSCCs, voltage sensitive calcium channels.

Introduction

Ionotropic P2X purinergic receptors are located on a large number of different cell membranes and are believed to transduce the effects of extracellular ATP (adenosine triphosphate). There are currently seven identified, cloned subtypes of the P2X receptor. Some of these subtypes appear to mediate fast excitatory transmission in the peripheral autonomic nervous system between neurons and/or between neurons and smooth muscle cells (Evans *et al*., 1992; Silinsky *et al*., 1992; Galligan & Bertrand, 1994). ATP evokes the release of other autonomic neurotransmitters such as noradrenaline from peripheral neurons through activation of P2X receptor subtypes, which function as ATP gated ion channels or pores (Boehm *et al*., 1995; Gibb & Halliday, 1996; Boehm, 1999; von Külgelgen *et al*., 1993; 1999; Sperlágh *et al*., 2000). Therefore, ATP receptors operating as cation channels may serve as an important alternate mechanism to the voltage sensitive calcium channels (VSCCs), through which the organism initiates or controls the release of neurotransmitters from a variety of autonomic nerves.

Evidence describing the presence of most P2X receptor subtypes on neurons in a number of brain areas exists (Ueno *et al*., 1992; Edwards *et al*., 1992; Séguéla *et al*., 1996; Collo *et al*., 1996, 1997; Burnstock, 1999; Kanjhan *et al*., 1999). A role for undefined types of presynaptic P2X receptor subtypes in neurotransmission at a very few, well defined neuronal junctions has also been described (Motin & Bennett, 1995; Kidd *et al*., 1998; Lê *et al*., 1998; Khakh and Henderson, 1998, 2000; Gu & MacDermott, 1997; Hugel & Schlicter, 2000). Very recently, important new work has offered proof of $P2X_3$ as well as other undefined subtypes in midbrain synaptosomes (Diáz-Hernández *et al*., 2001; Gómez-Villafuertes *et al*., 2001).

In the present study, pharmacological and molecular biological evidence is presented for the existence of functional

*Author for correspondence; E-mail: Paul.Lundy@dres.dnd.ca

P2X receptors, specifically of the $P2X_7$ subtype (Surprenant *et al*., 1996) on purified synaptosomal preparations of rat cortex. We have employed a number of pharmacological manipulations of responses proposed as reliable measures of P2X receptor function (Michel *et al*., 1996; 1998; Virginio *et al*., 1997; Jiang *et al*., 2000; Hibell *et al*., 2001). We have also employed Western blotting techniques and immunohistochemistry to confirm the presence of specific P2X subtypes in order to support the identification of receptors carried out by pharmacological methods. These findings constitute a variety of evidence for the existence and functional significance of $P2X_7$ receptors on central presynaptic elements.

Methods

Preparation of synaptosomes

Rat (male, Sprague–Dawley, wt 250–300 g) crude synaptosomes were prepared by homogenization of brain cortex in ice-cold 0.32 M sucrose using six strokes of a teflon/glass homogenizer. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C. The supernatant was decanted and centrifuged at $12{,}400 \times g$ for 25 min and the resulting pellet (P_2) was resuspended in one of three different buffers of composition outlined below for the subsequent measurement of ATP induced calcium influx.

Purified synaptosomes were prepared using the Percoll® gradient method (Dunkley *et al*., 1988). Briefly, the S_1 sucrose/EDTA supernatant was layered over a discontinuous Percoll® (Sigma, St Louis, MO, U.S.A.) gradient consisting of 2 ml each of 3, 10, 15 and 23% (v v^{-1}) Percoll® dissolved in 0.32 M sucrose containing 100 mg l^{-1} EDTA. This was centrifuged at $20{,}000 \times g$ for 5 min at 4°C in a Beckman preparative centrifuge. The 10/15% and 15/23% interfaces were combined and diluted 4 fold with Hanks balanced salt solution (HBSS) at pH 7.3, and then centrifuged at $12{,}500 \times g$ for 25 min. The resulting pellet was resuspended in HBSS. Aliquots of the Percoll® purified synaptosomes were washed twice with 10 vol of HBSS (composition see below) to ensure the removal of all Percoll® and deposited on treated glass microscope slides (500 μl, Cytospin) and used for fluorescence histochemistry (outlined below). The composition of HBSS was as follows (in mM): KCl 5.4, KH_2PO_4 0.5, NaCl 136, $NaHPO_4$ $0{\cdot}7H_20$ 0.34, D-glucose 5.6 and $CaCl_2$ 1.

Calcium influx

The first series of experiments were carried out in choline buffer (composition outlined below) and provided the results described in the first two figures, as well as parts of subsequent figures as described in the text where appropriate. Ca^{2+} influx was carried out according to the method of Blaustein (1975), with modifications (Lundy *et al*., 1991). In those cases where drug pretreatments were required, the synaptosomes were incubated at 30°C in the presence or absence of antagonist drugs for 15 min. Experiments were carried out using synaptosomes diluted to obtain a final concentration of about 1 mg protein ml^{-1}. Following drug pre-treatment, a 100 μl aliquot of the synaptosomal suspension was quickly injected into an equal volume of resting buffer (5 mM K^+), depolarizing buffer (25 mM K^+, final concentration), or resting buffer to which ATP or its analogues had been added (various concentrations), all containing 0.5 μCi $^{45}Ca^{2+}$ (New England Nuclear, Boston, MA, U.S.A.). Triplicate estimates of basal and of K^+ stimulated Ca^{2+} influx (3 s or adenine nucleotide stimulated influx (60 s) were carried out and terminated by rapid dilution of the buffer with 4 ml ice-cold Ca^{2+}-free buffer containing 4 mM EGTA. Each suspension was rapidly filtered under vacuum through 0.45 μM membrane filters (Gelman Science, Ann Arbor, MI, U.S.A.) using a filtration apparatus (Hoeffer Scientific, San Francisco, CA, U.S.A.). The membrane filters were washed twice with 5 ml normal resting buffer to which excess Ca^{2+} had been added. Membrane filters were allowed to dry, placed in scintillation cocktail, and counted on a Wallac 1500 scintillation counter. Basal influx was subtracted from K^+ or nucleotide-stimulated influx and results were expressed as nmoles Ca^{2+} influx mg protein ml^{-1}. Experimental protocols were designed so that the effects of ATP, as well as five nucleotide analogues could be examined on each synaptosomal preparation. K^+-evoked Ca^{2+} influx was included to assess the viability of each preparation. Subsequent to the first series of experiments, either the sodium chloride or the sucrose-based systems were also used.

Buffer systems

Three buffers were used in the study of calcium influx. The first buffer was choline based and was a buffer that had been utilized in this laboratory for studies of calcium influx for several years. The other two were used as the result of reports in the literature concerning ionic effects on $P2X_7$ receptor activity:

(a) Choline based buffer was of the same composition used previously in this laboratory and is one of three different buffers employed in the measurement of calcium influx. In the results section the various buffers used are identified where appropriate. Choline buffer was made with the following composition (in mM): Choline Cl 132, KCl 5, $MgCl_2$ 1.3, $CaCl_2$ 1.5, NaH_2PO_4 12, D-glucose 10, HEPES 20, brought to pH 7.4 with TRIS base. Choline chloride was originally substituted for NaCl to negate the effects of Na^+/Ca^{2+} exchange (Blaustein, 1975) but also choline has been recently reported to interfere less with ATP induced effects on $P2X_7$ receptors than sodium rich buffer systems (Michel *et al*., 1996).

(b) Sodium based buffer was identical to the choline buffer but sodium chloride was substituted for choline chloride.

(c) Sucrose buffer was a low ionic strength medium described by Michel *et al*. (1996) and Surprenant *et al*. (1996) in order to maintain lower concentrations of certain ions which interfered with agonist activity. It consisted of the following (in mM): sucrose 280, $CaCl_2$ 0.5, KCl 5, D-Glucose 10, HEPES 10, N-methyl-D-glucamine 5, with the pH adjusted to 7.4.

Fluorescence histochemistry

Immunohistochemical staining was conducted as follows: slides were rinsed in phosphate buffered saline (PBS) for 10 min and blocking/diluent buffer was applied (PBS, 1.5% normal goat

serum (NGS) was applied for 20 min. Primary antibodies (anti-$P2X_7$, anti-syntaxin, and anti-SNAP-25, all rabbit (Alomone Labs, Jerusalem, Israel and Chemicon International Inc., Temecula, CA, U.S.A.) and anti- OX-42 (mouse, ICN Biochemicals, Aurora, OH, U.S.A.) were applied at 1/300 dilution with diluent buffer and incubated for 4 h at room temperature. Slides were rinsed three times with PBS. Secondary antibodies (Oregon Green 488-labelled goat anti-rabbit IgG and goat anti-mouse IgG, Molecular Probes, Eugene, OR, U.S.A.) were applied to sections at 1/200 dilution with diluent buffer for 2 h under the same conditions. Following incubation, the slides were washed in three changes of PBS and coverslipped using Prolong anti-fade mounting media (Molecular Probes, Eugene, OR, U.S.A.).

A negative procedural control, in which the primary antibody was omitted and replaced with diluent buffer only, was used with each series. Slides were visualized using fluorescence microscopy and representative images taken with a Spot 2 digital camera (24 bit colour images, Diagnostic Instruments Inc., Sterling Heights, MI, U.S.A.) Protein concentration was determined using the BCA Protein Assay Reagent kit (Pierce Chemical Co., Rockford, IL, U.S.A.) by the method of Bradford (1976).

Western blot characterization of P2X receptors

Pheochromocytoma PC12 cells (CRL-1721, American Type Culture Collection, Manassas, VA, U.S.A.), vas deferentia, cortical synaptosomes (both crude P_2 and purified homogenates), or whole brain homogenates from the rat were incubated on ice for 20 min in 300–500 μl 1× SDS gel loading buffer of the following composition: 50 mM Tris-HCl (pH 8.6), 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol. Tissues were centrifuged at $12{,}000 \times g$ for 3 min at 4°C and the supernatant was transferred to a fresh tube. Protein was measured using Coomassie protein reagent (Pierce, Rockford, IL, U.S.A.) according to the method of Bradford (1976). The protein samples were immersed in boiling water for 5 min and equal amounts of protein (20 μg of each) were electrophoresed on 8% SDS polyacrylamide gels and transblotted onto a nitro-cellulose membrane. Prestained protein standards (Bio-Rad, Mississauga, Ontario, Canada) were used to visualize successful transfer and to measure molecular weight of the subsequent signal. The membrane was blocked with PBS containing 0.1% Tween (PBST) and 5% skimmed milk overnight at 4°C, then washed four times at 10 min intervals with PBST. Washed membranes were incubated with 1 : 300 dilutions of anti-$P2X_1$, anti-$P2X_2$ (Alomone Labs, Jerusalem, Israel), or anti-$P2X_7$ antibody (Alomone Labs, or Chemicon International, Temecula, CA, U.S.A.) in PBST solutions for 90 min. They were then rewashed and incubated with a peroxidase labelled anti-rabbit antibody (1 : 3000 dilution, included in ECL-kit Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). The membranes were washed again and protein visualized with the enhanced chemiluminescence reagents according to the instructions of the manufacturer.

In order to test the purity of synaptosomes obtained by the Percoll® gradient method, we detected the expression of the synaptosome specific proteins syntaxin, SNAP-25, and glial fibrillary acidic protein (GFAP) using a Western blot. Rabbit anti-syntaxin, anti -SNAP-25 (Alomone Labs. Jerusalem, Israel) and mouse anti-GFAP (Pharmingen Labs, Mississauga, Ontario, Canada), and the corresponding cognate antibodies (Amersham Pharmacia, Quebec, Canada) were used as primary and secondary antibodies in experiments according to the above-mentioned Western blot protocol. 12% SDS-Poly-acrylamide gels were used for syntaxin, SNAP-25 and GFAP.

Drugs

The following drugs were used: Adenosine 5-triphosphate disodium (ATP), adenosine 5′-O-(3-thiotriphosphate) (ATPγS), 2-methylthio-ATP, 2′ & 3′-O-(4-benzoylbenzoyl)-ATP (BzATP), α,β-methylene ATP, β,γ-methylene-L-ATP; pyridoxal-phosphate-6-azophenyl-2-disulphonic acid tetra sodium (PPADS), ω-conotoxin-MVIIC (ω-CTx-MVIIC) and Brilliant Blue G (BBG) (all purchased from Sigma St Louis, MO, U.S.A). All drugs were dissolved in distilled H_2O and added to the appropriate incubation buffer.

Statistics

Comparisons among data were carried out using a two-way ANOVA. In certain cases a Student's *t*-test was performed where appropriate, as stated in the results. Probability values less than 0.05 were considered significant.

Results

Initial experiments were performed in choline buffer in which cortical synaptosomes were exposed to increasing concentrations of ATP or BzATP (Figure 1A) for 60 s, which was determined as the optimal incubation period (Figure 1B). The maximally effective concentration required for ATP and BzATP stimulated influx (stimulated *minus* resting $^{45}Ca^{2+}$ influx) was determined (nMoles Ca^{2+} mg protein^{-1}). Both nucleotides evoked a dose dependent influx of extracellular Ca^{2+}. Of the agonists examined, only ATP and BzATP acted as full agonists for which an EC_{50} could be calculated. The maximal responses for the two full agonists was achieved at 1 mM ATP or 100 μM BzATP. Dose-effect curves to 2-methylthioadenosine triphosphate (2-me-S-ATP) and to adenosine 5′-O-(3- thiophosphate, (ATPγS) were also carried out however these analogues acted as partial agonists and produced shallow dose response curves which did not reach maximal values at the highest concentration examined (1 mM). Therefore, each analogue was tested at the concentration of ATP which produced maximal effects (ATP EC_{100} or 1 mM). The results in Figure 2 indicate that at 1 mM the order of agonist efficacy was BzATP = ATP > 2-me-S-ATP > ATPγS. The nucleotides α, β-methylene adenosine triphosphate (α,β-me-ATP) and β, γ-methylene-L-adenosine triphosphate (β,γ-me-ATP) were inactive. EC_{50} values for ATP and BzATP were calculated to be 214 and 30 μM (about a 7 fold difference) respectively. Therefore for the two agonists for which an EC_{50} was available, BzATP was considerably more potent (about seven times) than ATP. In each experiment some synaptosomes were exposed to 25 mM K^+ for 3 s to confirm synaptosomal viability and to demonstrate the effective block of neuronal VSCCs by ω-conotoxin-MVIIC (ω-CTx-MVIIC). ATP induced influx

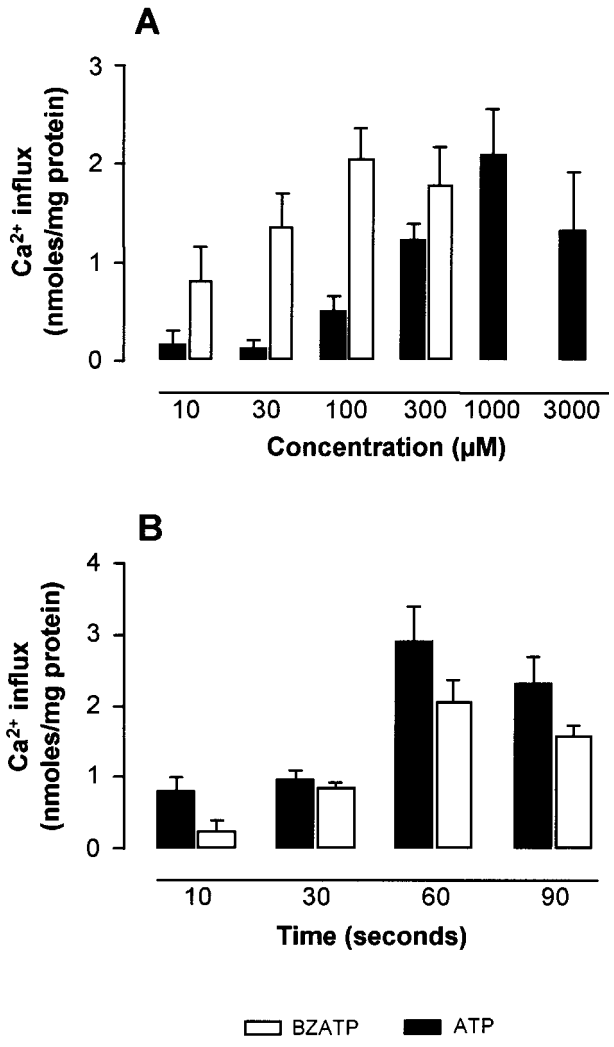

Figure 1 Concentration and time dependence of ATP and BzATP-evoked Ca^{2+} influx in rat brain cortical synaptosomes carried out in choline buffer as outlined in Methods. (A) ATP (solid bars) or BzATP (open bars) were incubated with the synaptosomes for 60 s at the concentrations indicated and calcium influx was measured. (B) Time dependence of ATP and BzATP-evoked Ca^{2+} influx. Synaptosomes were incubated with maximally effective concentrations of ATP (1 mM) or BzATP (100 μM) for 10–90 s as indicated. Values shown are mean ± s.e.mean, $n=4$.

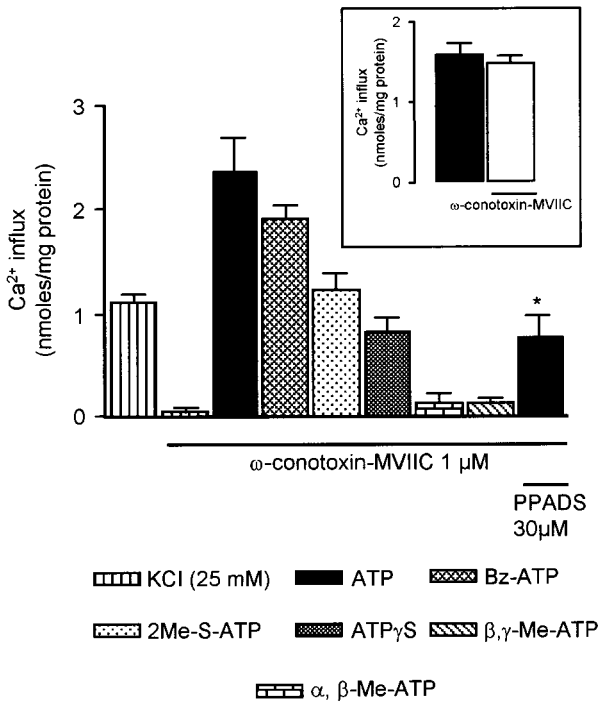

Figure 2 K^+ and adenine nucleotide-evoked Ca^{2+} influx in rat brain cortical synaptosomes. K^+-evoked and adenine nucleotide induced influx was measured in the presence and absence of (ω-CTx-MVIIC, 1 μM) as indicated. ATP evoked influx (solid bar, third bar from left) is shown in the absence and presence (solid bar, far right side) of PPADS (30 μM) $n=4$. Synaptosomes were exposed to 25 mM K^+ for 3 s and 1 mM of the nucleotides for 60 s. The influx induced by maximally effective concentrations of 1 mM ATP or BzATP were not significantly different from one another. Inset: shows lack of effect of ω-CTx-MVIIC on ATP-evoked Ca^{2+} influx ($^*P<0.05$, $n=4$).

(Figure 2 inset) was unaffected by a concentration of the VSCC blocker ω-CTx MVIIC (1 μM) sufficient to completely inhibit influx through all of the recognized neuronal VSCCs (Hillyard *et al*., 1992; Lundy *et al*., 1994, see also Figure 2). ATP stimulated influx was also unaffected in synaptosomes treated with the L-type calcium channel blocker (−)202–791 (Lundy *et al*., 1991, present results not shown).

The P2X receptor antagonist pyridoxal-phosphate-6-azophenyl-2′-4′ disulphonic acid (PPADS) (Lambrecht, 1996) at a concentration of 30 μM markedly reduced ATP-evoked Ca^{2+} influx (Figure 2).

Since $P2X_7$ agonist activity has been reported to be greater in physiological buffers containing low concentrations of cations, experiments were repeated in two additional cation modified buffers (see Methods). The dose-effect curve for BzATP-stimulated influx was shifted to the left in sucrose, as compared to both choline and Na^+ based buffers; (difference $P<0.001$, 2-way ANOVA), confirming previous studies concerning $P2X_7$ receptor sensitivity in ionic media (Michel *et al*., 1998; Virginio *et al*., 1997). Results are shown in Figure 3A.

Brilliant Blue G (BBG), an antagonist selective for $P2X_7$ receptors (Jiang *et al*., 2000; Hibell *et al*., 2001) showed concentration related inhibitory activity towards BzATP-stimulated influx ($P<0.01$, at 1 μM, Figure 3B).

In order to provide more definitive data concerning the receptor subtype involved in the observed influx, lysates and homogenates were prepared from both whole brain and purified synaptosomes and were probed with antibodies for $P2X_2$ and $P2X_7$ receptors (Figure 4). $P2X_2$ receptors were detected in whole brain homogenates (Br) but not in purified synaptosomes (Syn) (Figure 4A). Lysates of whole PC-12 cells served as a positive control for the presence of $P2X_2$ receptors (Brake *et al*., 1994). $P2X_7$ receptor protein was strongly expressed in both whole brain (Br) homogenates and in synaptosomal preparations (Syn) (Figure 4B). Homogenates of vas deferens (Vas) were used as negative controls for the presence of $P2X_7$ receptors.

The occurrence of $P2X_7$ receptors in brain has been attributed to their presence on microglia (Collo *et al*., 1997). However, contamination by non-neuronal elements was not detected in purified synaptosomes prepared by similar methods as those described here (Daniels & Vickroy, 1998;

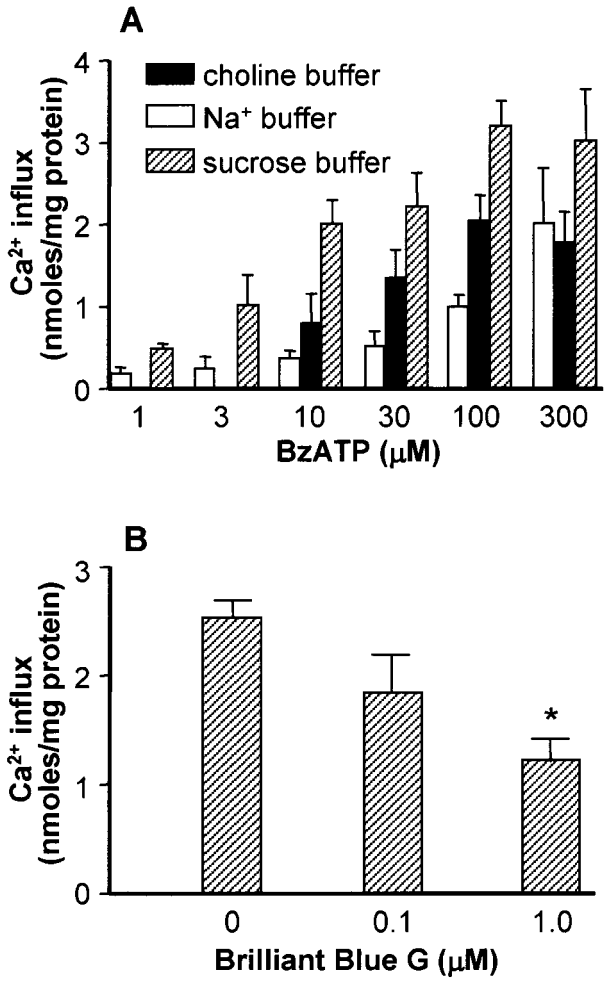

Figure 3 (A) Calcium influx was measured in synaptosomal preparations exposed to increasing concentrations of BzATP in three incubating solutions of different ionic composition. Each bar represents the mean ± s.e.mean, obtained in three experiments carried out in triplicate. BzATP induced influx was significantly greater in sucrose buffer than in the high ionic strength sodium or choline based buffers (2-way ANOVA, $P<0.001$). (B) Calcium influx initiated by BzATP in synaptosomal preparations incubated in the presence of the selective $P2X_7$ antagonist Brilliant Blue G. The influx represented by the bars is the mean ± s.e.mean of three experiments carried out in triplicate. The asterisk denotes a significant reduction in influx as compared to that caused by BzATP alone ($P<0.01$, Student's *t*-test).

Hoogland *et al*., 1999). However, to preclude this possibility, we examined the presence of contaminants using specific antibodies to $P2X_7$ receptors and OX-42 (specific for microglia). Background immunofluorescence was examined in whole brain (Figure 5A) and synaptosomes (Figure 5B).

Immunofluorescence for $P2X_7$ receptors was located in brain and synaptosomes (Figure 5C, D) while OX-42 fluorescence was evident in whole brain (Figure 5E) but was absent in synaptosomes. The fluorescence in individual synaptosomes appears not to be uniform, probably as the result of the fact that they were concentrated (cytospun) and therefore could be at slightly different planes in the field or in some cases overlap one another. In similar experiments we also detected GFAP immunofluorescence in cortical tissue but not in purified synaptosomes (results not shown).

Although the lack of activity of the $P2X_1$ agonist α,β-me-ATP appears to preclude a role for $P2X_1$ receptors in ATP-stimulated Ca^{2+} influx, $P2X_1$ receptors were weakly detected by Western blot techniques in whole brain and in purified synaptosomal preparations. Rat vas deferens was used as a positive control for $P2X_1$ receptors (results not shown).

Western blots obtained using purified synaptosomal homogenates probed with anti-SNAP 25, anti-syntaxin and anti-GFAP antibodies, provided no evidence for GFAP in the purified synaptosomal preparation, but both synaptic markers were clearly evident (Figure 6). The purity of synaptic elements was further confirmed using immunohistochemistry to visualize the neuronal markers using fluorescent antibodies against SNAP-25 and syntaxin. These findings, which are in accord with the Western blot data, are shown in Figure 7.

Discussion

The present study offers several lines of evidence, which suggests the existence of P2X receptors on presynaptic elements of mammalian brain. In these studies we have examined the effects of ATP analogues on $^{45}Ca^{2+}$ influx, which provides a reliable functional assay for characterizing P2X receptor activity (Michel *et al*., 1996; 1998). In previous studies, the potency order of ATP and related nucleotides has been used to define P2X receptor subtypes pharmacologically in a variety of tissues (Abbracchio & Burnstock, 1994; Fredholm *et al*., 1994; 1997; Burnstock, 1999). In the present study, the synaptosomal calcium influx initiated by ATP or ATP analogues suggested both the presence of P2X receptors and a role for a specific subtype.

Dose response curves for calcium influx following ATP and BzATP were constructed. With the exception of BzATP, the remaining analogues were all compared at the same concentration at which ATP produced its maximal effect (the EC_{100}.). This is a similar method of comparison to that employed by other investigators as the result of similar problems obtaining EC_{50} values for these agonists (Surprenant *et al*., 1996; King *et al*., 1996; von Külgelgen *et al*., 1997; Troadec *et al*., 1998; Wiley *et al*., 1998; Chung *et al*., 2000). BzATP was about seven times more effective in evoking calcium influx than ATP while 2-me-S-ATP and ATPαS were less active than ATP probably as the result of their partial agonist properties at the $P2X_7$ receptor (Wiley *et al*., 1998). These results together with the inactivity of α,β,methylene-adenosine 5′triphosphate, (α,β-me-ATP), and β,γ-methylene-L-adenosine triphosphate (β,γ-me-ATP) were consistent with the activation of a $P2X_7$ receptor subtype (Abbracchio & Burnstock, 1994; Wiley *et al*., 1996; 1998; Surprenant *et al*., 1996; Watling, 1998).

The $P2X_2$ receptor subtype has previously been identified on mammalian CNS neurons (see Kanjhan *et al*., 1999 and references therein; Collo *et al*., 1996). This receptor subtype has also been proposed to exist both on peripheral nerve endings and a percentage of neurohypophysial neurons (Troadec *et al*., 1998; Boehm, 1999). These previous studies raise the possibility that $P2X_2$ receptors might be responsible for the calcium influx observed in this synaptosomal preparation. However several observations argue that $P2X_2$ receptors do not exist on presynaptic areas in the cortex and

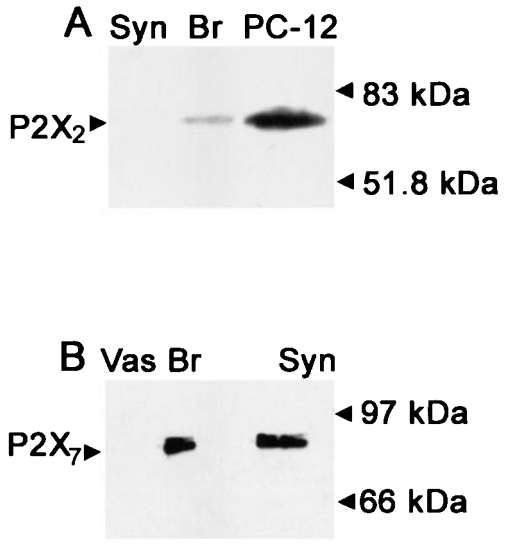

Figure 4 Western blots carried out to detect the presence of $P2X_2$ and $P2X_7$ receptor protein in rat cortical homogenates and synaptosomal preparations. Cortical homogenates and purified synaptosomes were prepared and treated with antibodies to $P2X_2$ and $P2X_7$ receptors. (A) Shows intense staining for $P2X_2$ receptors in PC-12 cells which were used as a positive control, weak detection in brain homogenates (Br), but no evidence of $P2X_2$ subtypes were found in purified synaptosomes (Syn). (B) Shows $P2X_7$ protein in brain homogenates and purified synaptosomes, but not in rat *vas deferens* (Vas) where $P2X_1$ receptors have been located.

are not responsible for the calcium influx observed in the present study. For example, there is ample evidence that BzATP is a much weaker agonist at $P2X_2$ receptors than ATP, 2-me-S-ATP and ATPγS (King *et al*., 1996; Bianchi *et al*., 1999; Michel *et al*., 1996). In the present work, BzATP was found to be approximately seven times more effective as an agonist than ATP, the next most active nucleotide, which was in turn much more active than were any of the other analogues. Moreover western blots from whole brain showed the presence of $P2X_2$ receptors while the synaptosomal preparation did not. The lack of evidence of $P2X_2$ receptors in the synaptosomes corresponds with other important recent studies in which these receptors also could not be identified on midbrain synaptosomes (Gómez-Villafuertes *et al*., 2001; Diáz-Hernández *et al*., 2001). Differences between the existence of the same functional structures like $P2X_2$ receptors on peripheral and central nerve endings may be analogous to the situation with calcium channels. In the periphery, the N type VSCC is found almost exclusively in nerve endings whereas the P/Q type appears to be much more prevalent centrally (Lundy & Frew, 1996).

Several other lines of functional evidence all point to the existence of $P2X_7$ receptors on synaptosomes. Ionic composition of the buffer in which P2X receptor/agonist interactions are measured, has a major impact on the effectiveness of these interactions, particularly on the activity of the $P2X_7$ receptor subtype (Michel *et al*., 1996; 1998; 1999; Surprenant *et al*., 1996; Virginio *et al*., 1997). Sodium has been suggested to cause an increase in the interference in $P2X_7$ agonist/receptor interactions (see ref. above and Wiley *et al*., 1992; Hibell *et al*., 2001). In our studies, BzATP was much more active in the sucrose based buffer than in either sodium or choline based buffers. The response profile is in good agreement with the activity of BzATP at $P2X_7$ receptors described in previous studies (Michel *et al*., 1996; 1999). BBG and PPADS also inhibited agonist induced calcium influx. BBG, a selective $P2X_7$ inhibitor (Jiang *et al*., 2000) was found in this study, to be an effective inhibitor of calcium influx at concentrations similar to those reported previously to be effective (Hibell *et al*., 2001). The inhibitory effects of BBG at these relatively low concentrations, further mitigates against the presence of most of the other receptor subtypes with the possible exception of $P2X_2$.

Voltage and ligand gated ion channels are the principal Ca^{2+} entry pathways in excitable cells in general and in synaptosomes in particular (Bean, 1992; Benham, 1992). Previous studies have indicated different interactions between P2X receptors and VSCCs. For example, ATP has been reported to activate Ca^{2+} influx by activating both P2X receptors and also VSCCs in some (Rogers *et al*., 1997; von Külgelgen *et al*., 1999; Boehm, 1999) but not all peripheral preparations (Sperlágh *et al*., 2000). In the CNS, the relative role played by calcium channels and P2X receptors in response to nucleotides depends on the neurons examined (Gu & MacDermott, 1997; Khakh and Henderson 1998; 2000; Hugel & Schlicter, 2000). Influx through VSCCs and subsequent neurotransmitter release in rat cortical synaptosomes occurs via activation of three major types of VSCCs (N, P and Q) which are inhibited by ω-CTx-MVIIC (Hillyard *et al*., 1992; Wheeler *et al*., 1994; Lundy *et al*., 1994). In the present study, in order to determine whether nucleotide induced influx was the result of the activation of VSCCs, experiments were carried out in the presence of ω-CTx-MVIIC and the L-type channel blocker (−)202-791. The lack of effect of ω-CTx-MVIIC and (−)202-791 on nucleotide-evoked Ca^{2+} influx would appear to preclude ATP induced activation of VSCCs and suggests that the Ca^{2+} entry observed in the current study occurred directly *via* P2X receptors. These results and those in the literature are consistent with the proposal that various central neuronal populations may utilize different mechanisms to control Ca^{2+} influx following stimulation by ATP and related nucleotides. Therefore, nucleotide induced influx may be the result of activation of VSCCs, activation of P2X receptors or a combination of these two mechanisms, depending on the neurons and the species examined (Khakh and Henderson, 1998; 2000; Gu & MacDermott, 1997; Pintor *et al*., 1999; Hugel & Schlicter, 2000; Nörenberg & Illes, 2000).

Western blots revealed weak staining for the $P2X_1$ receptor (results not shown) indicating its presence in synaptosomes. $P2X_1$ receptors predominate in smooth muscle, and their existence has also been reported in limited areas of the CNS, such as the cerebellum (Kidd *et al*., 1995; Burnstock, 1999). However $P2X_1$ (or $P2X_3$) receptor mediated Ca^{2+} influx in this preparation was also precluded as the result of the lack of activity of α, β-me-ATP, a potent agonist at these receptor subtypes (Valera *et al*., 1994; rev. see Abbracchio & Burnstock, 1994). Furthermore, α,β-me-ATP, which desensitises $P2X_1$ receptors (Kasakov & Burnstock, 1983) also failed to affect subsequent BzATP-evoked influx (P.M. Lundy and R. Frew, unpublished results). In addition, calcium influx was significantly reduced by BBG at concentrations that would not be expected to be active at $P2X_1$ receptors. Effective

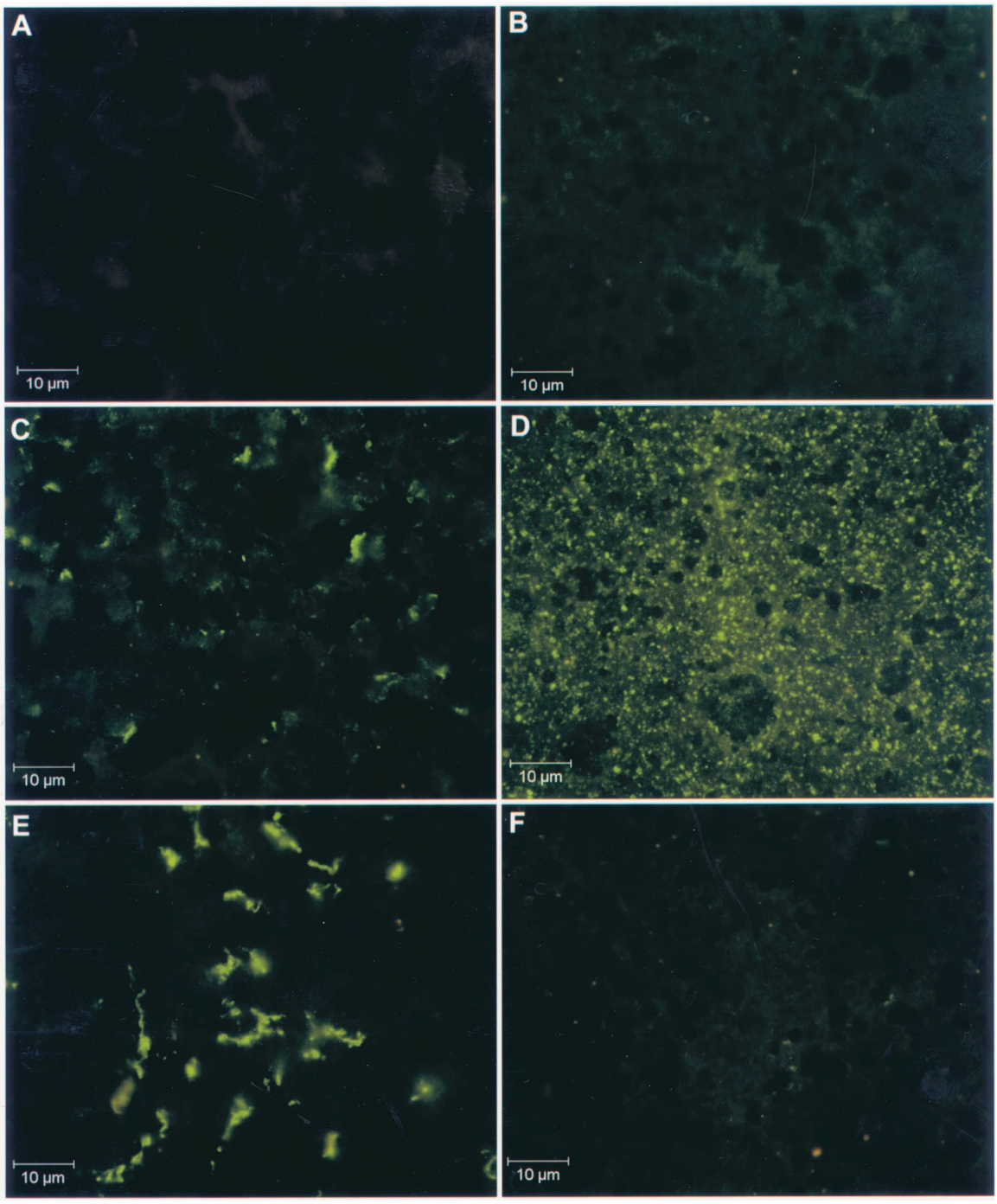

Figure 5 Immunoreactivity in cortical slices or in purified rat cortical synaptosomes treated with fluorescent antibodies to $P2X_7$ receptors or to the microglial marker OX-42. (A and B) show the background fluorescence in the absence of the primary antibodies to $P2X_7$ receptors or to OX-42 in cortical sections or synaptosomes respectively. Immunofluorescence demonstrated following treatment of cortical sections with fluorescent antibodies to $P2X_7$ receptors (C) or cytospun cortical synaptosomes (D). (E) reveals specific immunofluorescence in cortex treated with antibodies to the microglial marker OX-42. This fluorescence is absent from synaptosomal preparations (F).

antagonism of ATP-evoked Ca^{2+} influx by PPADS and the lack of selective activity of certain agonists, appears to preclude a role for $P2X_4$ and $P2X_6$ receptor subtypes (Evans *et al*., 1998). However, convincing evidence for the existence of $P2X_3$ receptors in synaptosomes from rat midbrain has been recently reported (Gómez-Villafuertes *et al*., 2001; Díaz-Hernández *et al*., 2001). Although we did not determine the proportion of $P2X_7$ receptors in our preparation, the presence of a significant proportion of $P2X_3$ receptors on cortical synaptosomes appears unlikely since a high concentration of α-β-me-ATP (1 mM) a potent $P2X_3$ agonist was inactive in the mediation of calcium influx in our studies.

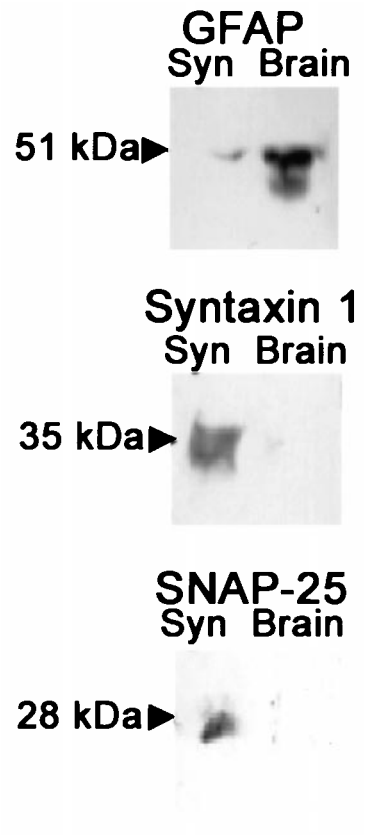

Figure 6 Western blots showing the presence of the synaptic markers syntaxin and SNAP-25 and the virtual absence of glial fibrillary acid protein (GFAP) in purified synaptosomes (Syn). Top panel: GFAP staining in whole cortical homogenates and virtual absence of staining in purified synaptosomes is shown. The figure shows the enhanced concentration of synaptic proteins syntaxin (middle panel) and SNAP-25 (bottom panel) in purified synaptosomes (Syn).

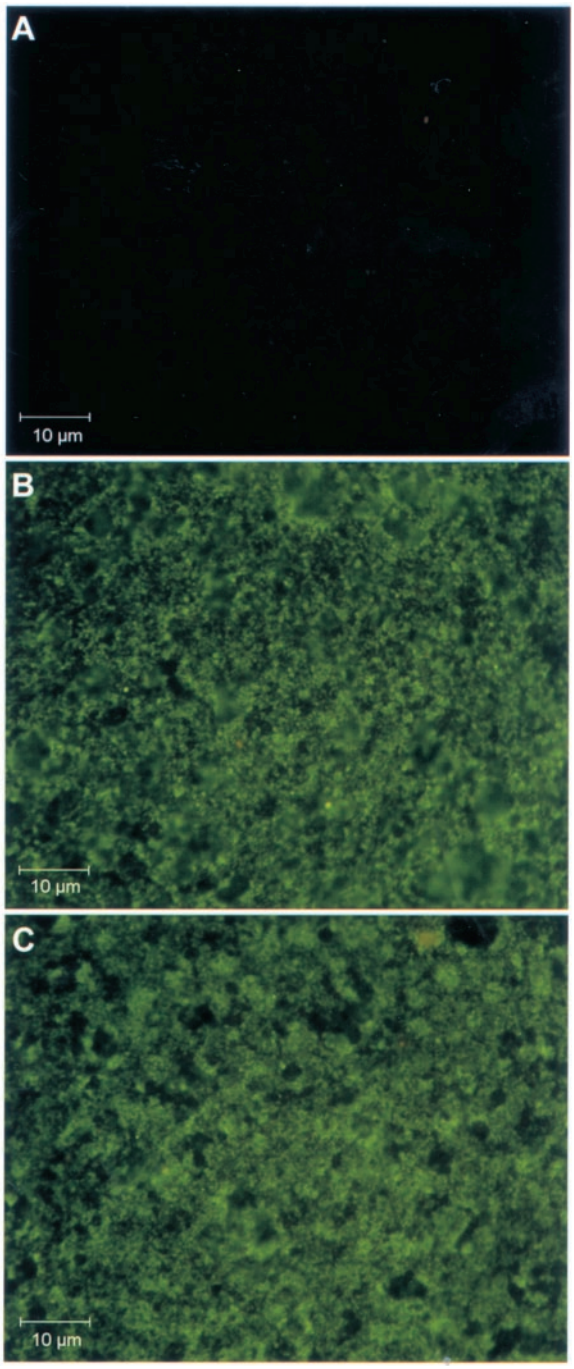

Figure 7 Immunoreactivity of purified synaptosomes treated with antibodies to the synaptic markers syntaxin and SNAP-25. (A) non-specific staining; (B) SNAP 25 staining; and (C) syntaxin staining.

However it appears clear that more than a single P2X receptor subtype likely co-exists at least in certain brain areas (Gómez-Villafuertes *et al*., 2001; Diáz-Hernández *et al*., 2001). Determination of the presence of $P2X_3$ receptors in cortical synaptosomes, or conversely of $P2X_7$ receptors in midbrain synaptosomes would be very interesting studies to carry out.

The results which demonstrated that the selective $P2X_7$ receptor agonist BzATP was of the order of seven times more potent with respect to evoking Ca^{2+} influx than ATP, the next most effective agonist, is in good agreement with a number of previous studies in other preparations containing $P2X_7$ receptors (for example, Nuttle & Dubyak, 1994; Wiley *et al*., 1996; 1998; Evans *et al*., 1998; Surprenant *et al*., 1996; Chessell *et al*., 1998; Chung *et al*., 2000). However, some doubt has been raised recently concerning the actual $P2X_7$ selectivity of BzATP. For example in certain assays, BzATP has been reported to be more potent at $P2X_1$ than at $P2X_7$ receptors (Bianchi *et al*., 1999). Reasons for differences reported among a variety of studies concerning the selectivity of BzATP are unclear, but the apparent controversy may complicate use of BzATP as a tool to distinguish between $P2X_1$ and $P2X_7$ subtypes. Oxidized ATP (2,3′-dialdehyde ATP), a $P2X_7$ receptor antagonist, could not be used to further confirm the presence of the $P2X_7$ receptor subtype since its inhibitory activity has been reported to develop only after a 2 h pretreatment period (Murgia *et al*., 1993) which is inconsistent with synaptosomal viability. Studies using KN-62, which has been reported to be a potent antagonist of the $P2X_7$ receptor of human lymphocytes (Gargett & Wiley, 1997) failed to inhibit ATP-evoked Ca^{2+} influx in synaptosomes (P.M. Lundy and R. Frew, unpublished results) likely due to differences in its inhibitory activity between rat and human receptors (Humphreys *et al*., 1998).

$P2X_7$ receptor subtypes detected in whole brain homogenates have also been attributed to their presence on microglia (Ballerini *et al*., 1996; Collo *et al*., 1997; Ferrari *et al*., 1997). It was important therefore to ensure that $P2X_7$ protein was indeed located on synaptosomal terminals and was not due to non-neuronal contaminating elements. Percoll® gradient isolation of synaptosomes has been reported to be sufficient to eliminate non-synaptosomal contaminants (Daniels & Vickroy, 1998; Dugar *et al*., 1998; Hoogland *et al*., 1999). The presence in the purified synaptosomes of the markedly enhanced concentrations of the synaptic markers syntaxin and SNAP-25, and the almost complete absence of GFAP or the microglial marker OX-42 (Brook *et al*., 2001) confirmed the lack of glial or microglial contamination in these synaptosomes.

In summary, our study clearly demonstrates that P2X receptors exist on central presynaptic nerve terminals and that the evidence presented here suggests that these receptors are of the $P2X_7$ subtype. This novel finding of $P2X_7$ receptors on cortical synaptosomes suggests that these receptor subtypes play a role in neurotransmission, and probably in other Ca^{2+} dependant events distinct from those mediated by VSCCs in presynaptic terminals in the CNS. We are currently examining the role of this receptor on BzATP induced glutamate release.

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