Rapid Report

Confocal calcium imaging reveals an ionotropic P2 nucleotide receptor in the paranodal membrane of rat Schwann cells

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1. The paranodal Schwann cell region is of major importance for the function of a myelinated axon. In the present study we searched for a possible ionotropic effect of extracellular ATP in this Schwann cell compartment.

2. Whole-cell patch-clamp recordings from cultured rat Schwann cells revealed that ATP and 2′-3′-*O*-(4-benzoylbenzoyl)-adenosine 5′-triphosphate (BzATP) induced a non-specific cation current. The effect of ATP was much enhanced in a Ca^{2+}- and Mg^{2+}-free solution. ADP, UTP and α,β-methylene adenosine 5′-triphosphate (α,β-meATP) had no effect.

3. Confocal Ca^{2+} imaging of myelinating Schwann cells in isolated rat spinal roots showed a BzATP-induced rise in the free intracellular Ca^{2+} concentration in the paranodal Schwann cell cytoplasm whereas α,β-meATP and 2-(methylthio)-adenosine 5′-triphosphate were without effect. In contrast to the known metabotropic effect of UTP on these Schwann cell regions, the BzATP-induced Ca^{2+} signal was not transient, was unaffected by depletion of intracellular Ca^{2+} stores and dependent on the presence of extracellular Ca^{2+}.

4. These results suggest that an ionotropic ATP receptor with electrophysiological and pharmacological characteristics of the $P2X_7$ subtype of nucleotide receptors is functionally active in myelinating Schwann cells of peripheral nerves. Such a receptor might contribute to Schwann cell reactions in nerve injury or neuropathy.

The paranodal Schwann cell region is of major functional importance for the physiology and/or pathophysiology of peripheral nerves. Although receptors for neuroligands in this area of the Schwann cell membrane might contribute to hitherto unknown interactions of Schwann cells and axons or might be a key element for the responses of Schwann cells following axonal injury or in the pathogenesis of neuropathies, knowledge of such receptors is still fragmentary. At present, there is experimental evidence for the presence of metabotropic ATP receptors. Such P2Y receptors have been found in the paranodal and other cell regions of Schwann cells *in situ* (Lyons *et al.* 1995; Robitaille, 1995; Green *et al.* 1997; Mayer *et al.* 1997, 1998), as well as in Schwann cells in culture (Lyons *et al.* 1994; Berti-Mattera *et al.* 1996; Ansselin *et al.* 1997; Jeftinija & Jeftinija, 1998).

In addition to P2Y receptors, ATP and/or related nucleotides act at ionotropic (P2X) receptors (for review, see North & Barnard, 1997; Burnstock, 1998; Ralevic & Burnstock, 1998). Up to now, only one study has indicated the presence of an ionotropic ATP receptor on Schwann cells. Amédée & Despeyroux (1995) observed an ATP-induced inward current in cultured mouse Schwann cells. However, the concentration of ATP necessary to induce this effect (K_d of 8·4 mM) was about 1000-fold higher than that required to activate intracellular Ca^{2+} transients by P2Y nucleotide receptors and the authors did not classify the underlying receptor. Furthermore, the possibility of an ionotropic effect of ATP in Schwann cells in intact peripheral nerve tissue was not tested. Recently, several important advances have been made in the area of ionotropic P2X nucleotide receptors. In particular, the mRNA and the protein of the $P2X_7$ receptor have been isolated from a brain cDNA library (Collo *et al.* 1997). The $P2X_7$ nucleotide receptor, in contrast to the other P2X subtypes, is activated only by high (>0·3 mM; North & Barnard, 1997) concentrations of ATP and, therefore, might underlie the ATP-induced current observed in Schwann cells by Amédée & Despeyroux (1995). The active ligand is suggested to be the tetrabasic acid ATP^{4-}, which is present as approximately 1% of the relatively high concentration of ATP (Ralevic & Burnstock,

1998). Thus, reducing the extracellular divalent cation concentration increases agonist potency. Furthermore, it is known that BzATP is a potent agonist at $P2X_7$ nucleotide receptors (Wiley *et al.* 1994). Interestingly, some of the cellular responses described following activation of $P2X_7$ receptors are apoptosis (Chow *et al.* 1997; Ferrari *et al.* 1997*a*) and cytokine release (Ferrari *et al.* 1997*b*). These phenomena are well known in Schwann cells but there is little understanding of the underlying mechanism. Using BzATP as a pharmacological tool we have now re-investigated the ATP-induced membrane current in cultured Schwann cells. In addition, we have used confocal calcium imaging to explore the possible presence of $P2X_7$ receptors in intact peripheral nerve preparations. The data indicate that $P2X_7$ receptors are in fact functionally active in the paranodal Schwann cell region of myelinated nerve fibres.

METHODS

Preparation of Schwann cell cultures

Schwann cells were obtained from sciatic nerves of 2-day-old Wistar rats of both sexes that were killed by decapitation after anaesthesia with pentobarbital (0·1 mg g^{-1}, I.P.). All animal experiments were performed in accordance with national guidelines. The nerves were dissociated enzymatically by 0·25% collagenase type I and 0·25% trypsin and mechanically by trituration. The suspension obtained was pelleted by centrifugation (1000 r.p.m., 10 min) and the pellet resuspended in medium. The cells were plated on uncoated Falcon culture dishes (35 mm, Becton Dickinson Labware, Oxnard, CA, USA) with a medium containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS) and 500 U ml^{-1} penicillin–streptomycin (Sigma). Contaminating fibroblasts were removed by application of 10 μM cytosine arabinoside for 3 days. After that, medium was changed to proliferation medium containing DMEM, 10% FCS, 0·5% forskolin, 0·5 mM 3-isobutyl-1-methylxanthine, 2·5 μg ml^{-1} insulin, 5 ng ml^{-1} rhGGF2 (Cambridge Neuroscience, Cambridge, MA, USA), and 500 U ml^{-1} penicillin–streptomycin. Cultured cells were used for experiments 8–14 days after preparation.

Electrophysiology

Membrane currents were studied using the patch-clamp technique in the whole-cell configuration. Cells were maintained in culture dishes on the stage of an inverted microscope (Axiovert 35, Zeiss, Jena, Germany) at room temperature. Patch pipettes were pulled (DMZ puller, Zeitz, Augsburg, Germany) from borosilicate glass tubes (GC150F-10, Clark Electromedical Instruments, Pangbourne, UK). Electrode tip resistance was 6·2 ± 0·2 MΩ ($n = 40$) and the series resistance of 17·8 ± 1·7 MΩ was compensated to 74·4 ± 0·9% ($n = 27$). The mean membrane capacitance of the cultured Schwann cells was determined as 29·8 ± 3·6 pF ($n = 27$).

The standard bath solution contained (mM): 140 NaCl, 3·0 KCl, 1·2 $MgCl_2$, 2·2 $CaCl_2$, 10 Hepes, 10 glucose; pH adjusted to 7·4 with NaOH. In some of the experiments, a Ca^{2+}- and Mg^{2+}-free standard bath solution was used. Patch pipettes were filled with the following solution (mM): 140 potassium gluconate, 3 $MgCl_2$, 5 EGTA, 2 Na_2ATP, 2 Na_2GTP, 5 Hepes; pH adjusted to 7·25 with KOH. Drugs were applied by a rapid application (Y-tube) method (Nakagawa *et al.* 1991). Recordings were made with an Axopatch 200 amplifier (Axon Instruments). pCLAMP 6 software (Axon Instruments) was used for the generation of the different pulse protocols and the acquisition and analysis of data.

Preparation of isolated rat spinal roots

Wistar rats (200–400 g) were anaesthetized by I.P. injection (1·5 g kg^{-1}) and later killed by intracardiac injection of urethane (1·5 g kg^{-1}). Spinal roots were removed and split mechanically into two or three fibre bundles. After preparation, the nerves were loaded with the fluorescent dyes using the membrane-permeant AM esters. Stock solutions of 0·9 mM Calcium Green-1 AM and 1 mM Fura Red AM were prepared by dissolving 50 μg of each in 20 μl dimethylsulphoxide (DMSO) plus 20 μl Pluronic F-127 20%. The isolated fascicles were incubated for 90 min at 37 °C in 3 ml Hepes-buffered saline to which 20 μl of each stock solution had been added (final concentration 6 μM Calcium Green-1 and 7 μM Fura Red). After incubation, the nerves were washed thoroughly and stored at 4–8 °C until use.

Confocal Ca^{2+} imaging

The method used for confocal Ca^{2+} imaging has been described before (Mayer *et al.* 1997). In brief, the optics consist of an upright microscope (Olympus BX50WI) in combination with a Bio-Rad MRC-1024 confocal imaging system. The spinal root fascicles were fastened in an organ bath and confocal images were collected at 530 nm (emission of Calcium Green-1) and 660 nm (emission of Fura Red) after excitation at 488 nm at intervals of 5–10 s. An elevation in the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increases Calcium Green emission at 530 nm (F_{530}), but decreases Fura Red emission at 660 nm (F_{660}). Therefore, the mean grey values F_{530} and F_{660} of selected regions of interest were used to calculate the emission ratio: $R' = F_{530}/F_{660}$. Ratio values are always given normalized $R = R'/R_0$, i.e. divided by a reference value R_0 obtained immediately before drug administration.

Chemicals

The organ bath was continuously perfused with a bathing solution containing (mM): 118 NaCl, 3·0 KCl, 1·5 $CaCl_2$, 1·0 $MgCl_2$, 5 D-glucose, 25 $NaHCO_3$, and 1·2 NaH_2PO_4; gassed with carbogen (95% O_2–5% CO_2). ADP, ATP, 2′-3′-*O*-(4-benzoylbenzoyl)-adenosine 5′-triphosphate (BzATP), α,β-methylene adenosine 5′-triphosphate (α,β-meATP), 2-(methylthio)-adenosine 5′-triphosphate (2-MeSATP), and uridine 5′-triphosphate (UTP) were purchased from Sigma. Calcium Green-1 AM, Fura Red AM amd Pluronic F-127 were from Molecular Probes.

Statistics

All results are expressed as means ± S.E.M. with n being the sample size.

RESULTS

Electrophysiological observations on cultured Schwann cells

After the whole-cell configuration was achieved, slow voltage ramps (−140 to 80 mV in 2 s) were applied to cultured rat Schwann cells in the control bathing solution and during addition of ATP and BzATP. Both agonists produced an increase in membrane conductance. In the case of ATP (1 mM; Fig. 1*A*), this effect was more prominent at negative membrane potentials, whereas BzATP (150 μM; Fig. 1*B*) enhanced the membrane conductance over the entire voltage range tested. The reversal potential for the BzATP-induced

current in a Cl^--free bathing solution was $-1{\cdot}7 \pm 2{\cdot}9$ mV ($n = 5$) which indicates an increase in a non-specific cation conductance. The kinetics of the current produced by ATP and BzATP were measured at a constant holding potential (Fig. 1*C* and *D*) and showed a rapid onset and no desensitization (Fig. 1*D*). Quantitatively, the inward current at a membrane potential of −80 mV was analysed in 15 Schwann cells responsive to ATP and/or BzATP (total number of cells tested: $n = 52$). ATP (1 mM) induced a current of $-2{\cdot}46 \pm 0{\cdot}5$ pA pF^{-1} ($n = 6$) that was enhanced in a Ca^{2+}- and Mg^{2+}-free solution to $-7{\cdot}25 \pm 1{\cdot}7$ pA pF^{-1} ($n = 11$; ATP 0·3 mM). BzATP (150 μM) was more potent than ATP; however, the BzATP-induced current ($-12{\cdot}69 \pm 4{\cdot}2$ pA pF^{-1}; $n = 9$) did not show clear augmentation in a Ca^{2+}- and Mg^{2+}-free solution. We also tested the effects of ADP, UTP (1 mM each), and of α,β-meATP (0·4 mM); these agonists at different types of P2 receptors did not change the membrane conductance of cultured rat Schwann cells ($n = 3$–6).

Confocal calcium imaging of Schwann cells *in situ*

Bath application of BzATP produced a rise in $[Ca^{2+}]_i$ in the paranodal Schwann cell region of myelinated nerve fibres in isolated rat spinal roots (Fig. 2). Quantitatively, 150 μM BzATP produced a mean change in the emission ratio of Calcium Green and Fura Red of $46{\cdot}5 \pm 6{\cdot}6$% in 27 paranodes responsive to BzATP. Eleven paranodes (28%) were considered non-responsive, i.e. the changes in the ratio were less than 5%. In contrast, α,β-meATP (0·3 mM; $n = 11$) and 2-MeSATP (0·1 mM; $n = 7$), both agonists tested at various P2X receptors, did not induce a rise in $[Ca^{2+}]_i$ in the paranodal cytoplasm of BzATP-sensitive Schwann cells.

The BzATP-induced Ca^{2+} signal differed in three aspects from the Ca^{2+} transient produced by bath application of UTP. Previously, UTP has been found to activate metabotropic $P2Y_2$ receptors in myelinated Schwann cells (Mayer *et al.* 1997, 1998). First, the effect of BzATP on $[Ca^{2+}]_i$ was not transient. This is illustrated in Fig. 2 which shows one of three experiments in which BzATP and UTP were both applied for 3 min. The effect of UTP on $[Ca^{2+}]_i$ was transient, i.e. the Ca^{2+} signal returned to the baseline level despite the continuing presence of the receptor agonist. In contrast, the rise in $[Ca^{2+}]_i$ produced by BzATP was maintained: it remained elevated until the end of the application time.

The second difference between BzATP and UTP was the source of Ca^{2+} for the rise in $[Ca^{2+}]_i$. As illustrated in Fig. 3, the Ca^{2+} signal produced by BzATP was completely absent in a Ca^{2+}-free bath solution ($n = 7$), whereas the UTP-induced Ca^{2+} transient was only slightly reduced under these circumstances (to 80 ± 12% of control; $n = 3$). Finally, the effects of BzATP and UTP differed in their dependence on intracellular Ca^{2+} stores. Cyclopiazonic acid (CPA, 5 μM), an inhibitor of the Ca^{2+}-ATPase of the

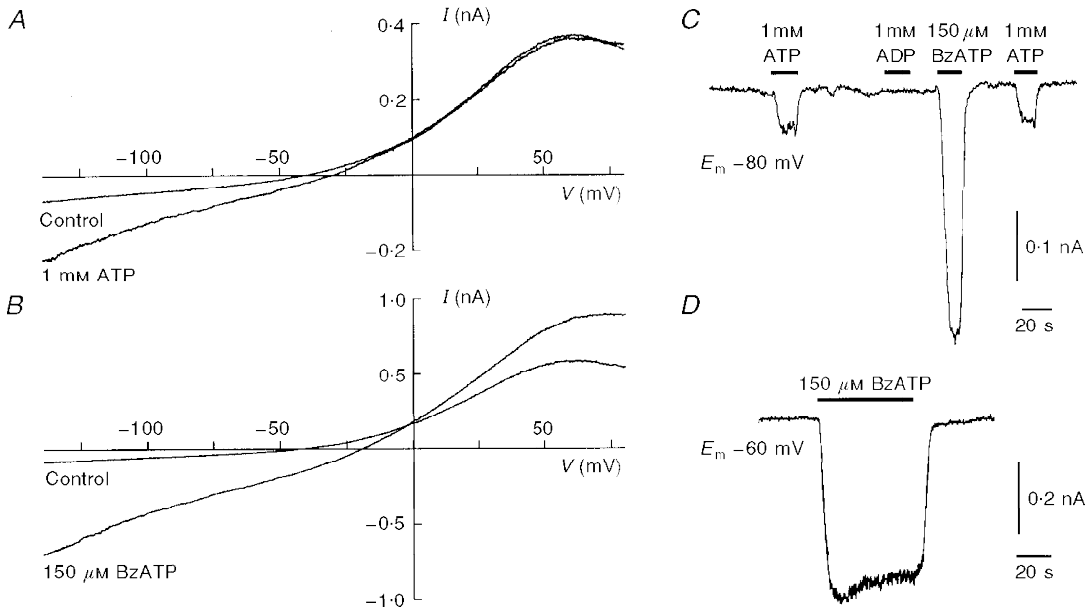

Figure 1. ATP and BzATP induce membrane currents in cultured rat Schwann cells

The illustrated examples from four different Schwann cells are representative for the effects of ATP and BzATP. *A* and *B*, membrane currents induced by slow voltage ramps from −140 to 80 mV in 2 s were recorded from two different cultured rat Schwann cells before and during bath application of ATP (1 mM) and BzATP (150 μM). Note the different scaling of the current axes. *C* and *D*, the membranes of these Schwann cells were voltage clamped at a constant holding potential (E_m) and changes in the holding current were recorded during bath application of different P2 nucleotide receptor agonists.

sarco/endoplasmic reticulum, was added to the bathing solution 5 min before and during the application of the two P2 nucleotide receptor agonists (Fig. 4). CPA blocked the effect of UTP ($n = 4$) whereas the BzATP-induced Ca^{2+} signal was not much changed (to $95 \pm 18\%$ of control; $n = 12$).

DISCUSSION

The action of ATP at $P2X_7$ receptors differs in three important respects from those at other P2X receptors (Evans *et al.* 1998). First, the effective concentrations of ATP are 30- to 100-fold higher. Second, removal of extracellular Mg^{2+} and Ca^{2+} potentiates the inward current. Third, BzATP is more potent than ATP. The ATP-induced current observed in the present study showed all these characteristics and, therefore, indicates the presence of $P2X_7$ receptors on cultured rat Schwann cells. This view is supported by the lack of effect of α,β-meATP, an agonist on $P2X_1$ and $P2X_3$ receptors (Evans *et al.* 1998). An ATP-induced, $P2X_7$-like non-specific cation current has also been found in other glial cells such as mouse microglia (Haas *et al.* 1996; Chessell *et al.* 1997) and rat astrocytes (Ballerini *et al.* 1996). Moreover, the previously described ATP-induced current in cultured mouse Schwann cells (Amédée & Despeyroux, 1995) seems to be due to activation of a $P2X_7$-like nucleotide receptor although the effects of BzATP and low divalent cations were not tested in their study. So far, the classification as $P2X_7$ is based on electrophysiological and pharmacological criteria. To our knowledge, the presence of mRNA and/or of the $P2X_7$ protein itself has not been reported for Schwann cells.

It is well known that observations on cultured Schwann cells do not necessarily resemble the situation of these cells in an intact peripheral nerve tissue. For example, the cultures used in the present study were prepared from postnatal (P2) rats and it is, therefore, unclear whether $P2X_7$ receptors are active in an intact adult peripheral nerve. Schwann cells *in situ* can be analysed with a combination of ion-sensitive fluorescent dyes and confocal microscopy. An important advantage of this method is that Ca^{2+}-sensitive dyes are taken up preferentially into Schwann cells but not into axons of myelinated nerve fibres (Lev-Ram & Ellisman, 1995; Lyons *et al.* 1995). In the present study, we observed a BzATP-induced intracellular Ca^{2+} signal in paranodal Schwann cell regions. However, in contrast to the previously described metabotropic effects of ATP analogues (Mayer *et al.* 1997), BzATP induced a rise in $[Ca^{2+}]_i$ which was entirely dependent on the presence of extracellular Ca^{2+} and was not

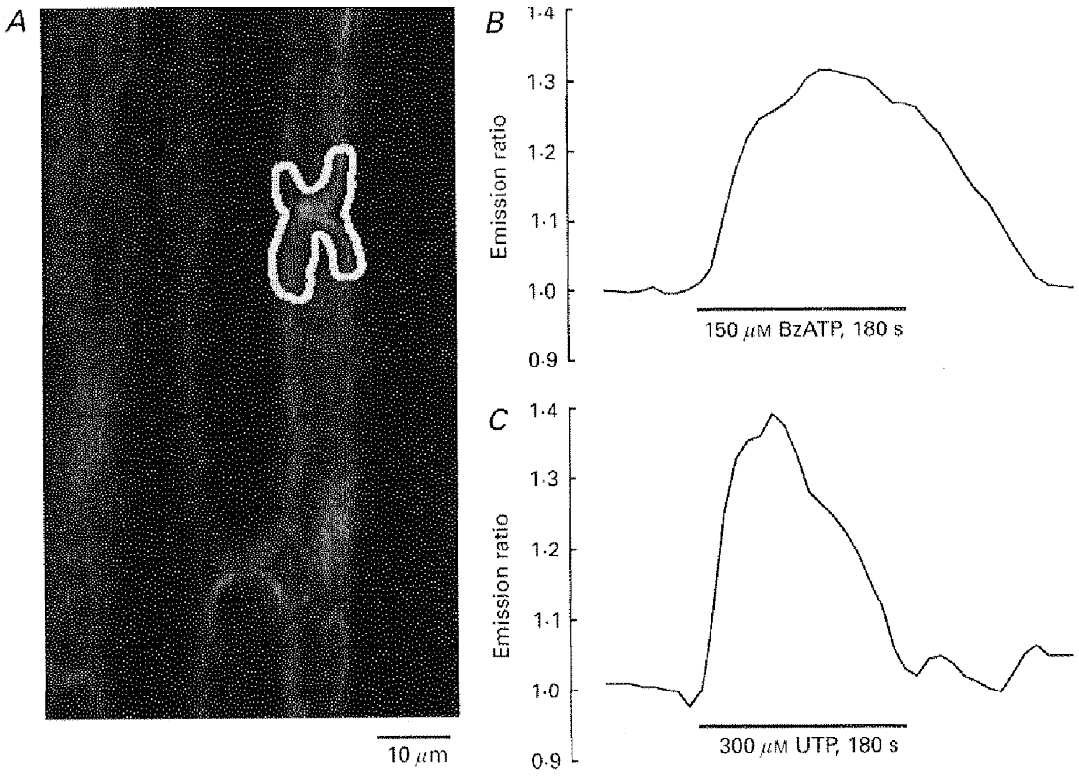

Figure 2. BzATP induces a rise in $[Ca^{2+}]_i$ in the paranodal Schwann cell cytoplasm

A, confocal image of myelinated nerve fibres within an isolated rat spinal root stained with the Ca^{2+}-sensitive dyes Calcium Green-1 and Fura Red (excitation wavelength 488 nm; emission wavelengths 530 and 660 nm, respectively). *B* and *C*, the mean grey value of the paranodal Schwann cell area (selected region of interest indicated in *A*) and its normalized ratio was measured during bath application of BzATP (150 μM; *B*) and UTP (300 μM; *C*). Note the maintained rise in $[Ca^{2+}]_i$ during application of BzATP whereas UTP induced a transient rise in $[Ca^{2+}]_i$ only.

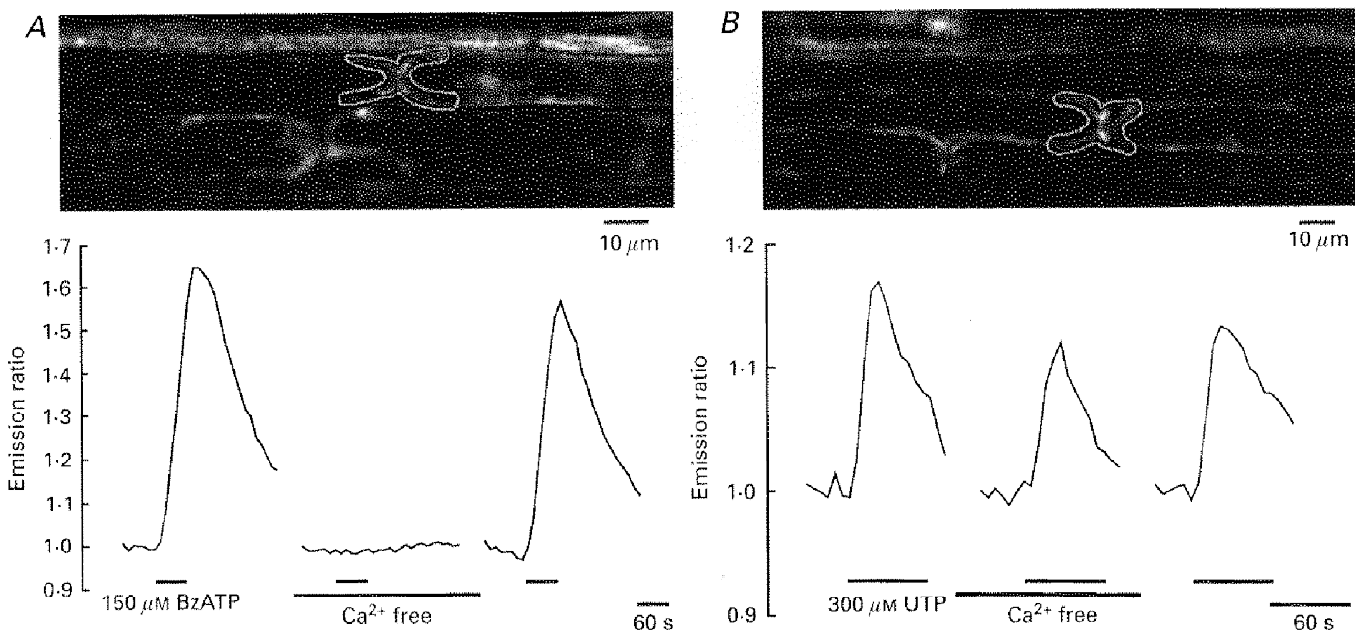

Figure 3. BzATP induces a transmembrane Ca^{2+} influx

A, the white outline in the upper panel shows a paranodal Schwann cell region within an isolated rat spinal root stained with the Ca^{2+}-sensitive dyes Calcium Green-1 and Fura Red. Changes in $[Ca^{2+}]_i$ were measured during application of BzATP (150 μM) into the standard and into a Ca^{2+}-free bathing solution. *B*, a similar experimental protocol was applied to a different rat spinal root. In this case, changes in $[Ca^{2+}]_i$ were measured during application of UTP (300 μM) into the standard and into a Ca^{2+}-free bathing solution. Note that only the BzATP-induced intracellular Ca^{2+} transient was completely and reversibly blocked during removal of extracellular Ca^{2+}.

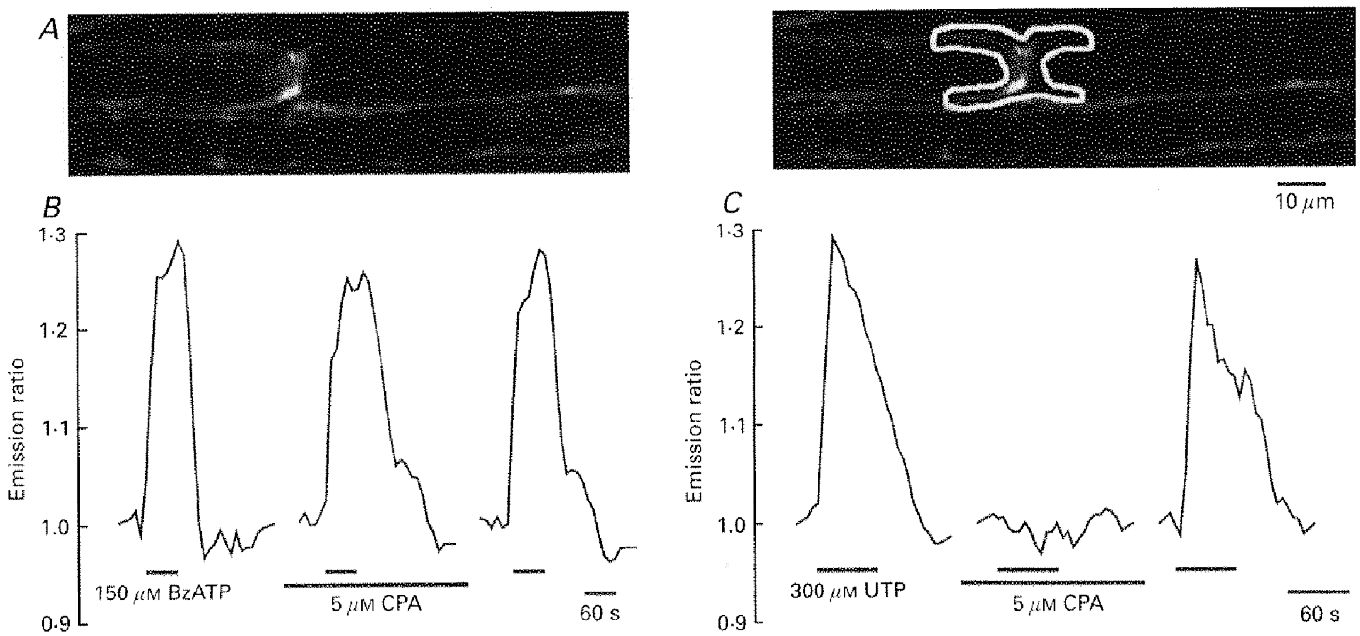

Figure 4. BzATP does not release Ca^{2+} from intracellular stores

A, confocal image of two myelinated nerve fibres within an isolated rat spinal root stained with the Ca^{2+}-sensitive dyes Calcium Green-1 and Fura Red. The mean grey value of the paranodal Schwann cell area (selected region of interest indicated in the right panel) and its ratio was used for the analysis. *B* and *C*, changes in $[Ca^{2+}]_i$ were measured during application of BzATP (150 μM) and UTP (300 μM) into the standard bathing solution and after depletion of intracellular Ca^{2+} stores with cyclopiazonic acid (CPA, 5 μM for 5 min). Note that only the UTP-induced intracellular Ca^{2+} transient was completely and reversibly blocked by CPA.

mediated by Ca^{2+} release from intracellular stores. This finding is similar to the BzATP-induced influx of extracellular Ca^{2+} observed in cultures of astrocytes (Ballerini *et al.* 1996) and microglia (Inoue *et al.* 1998). It is highly plausible, therefore, that the non-specific cation current seen during the exposure of cultured Schwann cells to BzATP is also the mechanism underlying the rise in $[Ca^{2+}]_i$ produced by BzATP in Schwann cells *in situ*. To our knowledge, this is the first description of an ionotropic ATP receptor in Schwann cells of an intact peripheral nerve. At present, we have concentrated our analysis on the paranodal Schwann cell membrane because this structure is of major importance for the function of a myelinated axon. However, P2X receptors might be present on the other Schwann cell membrane regions as well.

Although the possible function of an ionotropic ATP receptor on the paranodal Schwann cell membrane was not addressed in the present study, the following considerations may be relevant. It is not very likely that action potentials in axons release sufficient ATP to activate the $P2X_7$ receptor. In fact, in a previous study on the isolated rat vagus nerve, we did not find intracellular Ca^{2+} transients in Schwann cells during trains of axonal action potentials (Wächtler *et al.* 1998). Nevertheless, it is possible that high concentrations of extracellular ATP in combination with low concentrations of extracellular divalent cations are reached following axonal damage. In this situation, the reactions of the Schwann cells might be due to stimulation of $P2X_7$ receptors. Cellular responses attributed to $P2X_7$ receptors are apoptosis (Chow *et al.* 1997; Ferrari *et al.* 1997*a*), cytokine release (Ferrari *et al.* 1997*b*), and the formation of a permeabilizing, cytotoxic membrane pore (Surprenant *et al.* 1996). Therefore, the data obtained in the present study also suggest a possible new mechanism in the pathophysiology of nerve injury or neuropathy.

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