Varicosity-Schwann Cell Interactions Mediated by ATP in the Mouse Vas Deferens

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Lin, Y. Q. and M. R. Bennett. Varicosity-Schwann cell interactions mediated by ATP in the mouse vas deferens. J Neurophysiol 93: 2787–2796, 2005; doi:10.1152/jn.00772.2004. Schwann cells, from a variety of sources, are known to possess P2Y purinergic metabotropic receptors. However, it is not known if Schwann cells associated with autonomic nerve terminals possess such receptors and if so whether these receptors are activated by the endogenous release of ATP from the nerve terminals. We show that such Schwann cells in the vas deferens give evoked calcium transients on nerve stimulation. These transients are mediated, at least in part, by the endogenous release of ATP, which acts on Schwann cell P2Y receptors to release calcium from the cells. This work suggests the possibility that Schwann cells are active participants in the process of junctional transmission in the autonomic nervous system.

METHODS

Preparation and drugs

All experiments were performed on the vas deferens of mice (strain BALB/c), 5–6 wk old. Animals were anesthetized with CO₂, then killed by cervical fracture. Animal ethics approval was granted by the Animal Care Ethics Committee, University of Sydney. Both vas deferentia were dissected free along with the connective tissue and placed into a dissection bath coated with silicone elastomer (Sylgard, Dow Corning, Midland, MI) in the bottom of a Perspex chamber and immersed in Ringer solution. The Ringer solution contained (in mM) 136.9 NaCl, 2.7 KCl, 14.9 NaHCO₃, 1.5 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂, and 7.8 glucose. This solution was always oxygenated with a mixture of 95% O₂-5% CO₂ to maintain the pH between 7.1 and 7.4. After carefully removing connective tissue from the surface of the vas deferens, a longitudinal cut was made along its length so as to allow it to be pinned out as a rectangular sheet in the bath solution. From this point, the preparations were left in fresh Ringer solution for ~30–60 min at room temperature (18–20°C) before carrying out any further procedures.

All drugs used were from Sigma products (Sigma, Australia). Apyrase, ATP, caffeine, EGTA, and suramin (sodium salt) were prepared each experimental day. Nifedipine, prozasin (hydrochloride), PPADS, TTX, uridine 5-triphosphate; trisodium salt hydrate (UTP), and U-73122 were made as stock solutions. These were stored at −20°C, stored at 4°C, and diluted to final concentration before using. Nifedipine (10 μM) and 2 μM prozasin were always added in Ringer solution at 30 min before experimental recordings. The effects of other drugs (Apyrase, EGTA, suramin, PPADS, TTX, CGS-15943, U-73122, and U3343) were always examined 30 min after application.

Ca²⁺ indicator loading procedures

Loading Ca²⁺ indicators into SCs can be achieved either by microelectrode injection or by bath loading with acetoxymethyl (AM) ester. For microelectrode injection, the tissue was pinned to a 3-mL capacity Perspex recording bath that had a thin layer of Sylgard in the bottom. The tissue chamber was continuously perfused with warm (30–33°C) Ringer solution at a rate of 3 mL/min. The nuclear region of the somata (cell body) of Schwann cells on the surface of the vas deferens could be identified under transmitted light. Iontophoretic

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injection was used to fill Schwann cells with fluorescent Ca\(^{2+}\) indicator dye through a sharp microelectrode. The microelectrode was filled with loading solution of composition 1 M K-acetate with 5–10 μM Oregon Green 488 bis-(p-aminophenox)Y,N,N',N'-tetraacetic acid (BAPTA)-1 dextran Ca\(^{2+}\) indicator (O-6798, Molecular Probes, Eugene, OR). After achieving a stable impalement, a positive current (\(<0.1\) nA) was applied through the microelectrode controlled by an Axoclamp 2B voltage-clamp amplifier (Axon Instruments, Union City, CA) over a period of 30–90 s. For AM ester loading, the preparations were incubated in a loading solution, containing 2–4 μM fluo-3 AM, the membrane-permeant form of the fluorescent Ca\(^{2+}\) indicator (F-14218, Molecular Probes), for a period of 150–180 min at 24–26°C. Sometimes, 0.02% (wt/vol) of pluronic acid (F-127, Molecular Probes) was used to facilitate cell loading of AM ester (see Jahromi et al. 1992). To maintain physiological pH, the loading solution was continually bubbled with 95% O\(_2\)-5% CO\(_2\). The Ca\(^{2+}\) indicator loaded vas deferens was then placed in a beaker filled with bubbling Ringer solution or pinned down in the recording bath where it was washed for ≥45 min at room temperature before any further experimental procedures to remove any nonspecific staining. All preceding procedures were performed in the dark.

**Whole-mount immunolabeling**

Vas deferens were fixed using 2% paraformaldehyde for 25 min, then washed for ≥30 min with PBS (pH 7.4). Preparations were then permeabilized in −20°C methanol for 10 min and washed again with PBS for 45 min. The vas deferens were then rinsed with PBS containing 0.3% triton X-100 and 0.2% bovine serum albumin for another 60 min to block nonspecific staining. Vas deferens were incubated in 1:1,000 diluted primary antibodies S-100, Anti-S-100, (Monoclonal Anti-S-100, β-subunit, clone SH-B1; S2532, Sigma) overnight at room temperature. After incubation in the primary antibody, the vas deferens was rinsed in three changes of PBS for 30 min, then incubated in diluted (1:500) secondary antibody [Alexa Fluor 488 IgG (H+L), A11029, Sigma] for 90 min. The preparations were finally rinsed for 40 min with PBS before further studies. For negative control experiments, preparations were treated similarly, except that the primary antibody was omitted.

**Local ATP, UTP application**

Agonists were applied locally on Schwann cells using 2-μm tip diameter micropipettes, containing either ATP or UTP solutions. A micropipette was positioned at a SC soma (20–30 μm away with ~30° angle) under visual control at high magnification (>40 water-immersion objective). Local ejection of either ATP or UTP was achieved by giving a short pulse (150–250 ms) of positive air pressure (<10 psi) to the micropipette with a Picospirtzer II (CV General Valve, Fairfield, NJ). The actual time for ejection was controlled by a Master-8 programmable pulse generator (AMPI, Jerusalem, Israel), which was triggered by computer. ATP or UTP were ejected from the micropipette in the opposite direction to that of the incoming flow of bath solution. Schwann cells were first examined for their response to the application of drugs at the edge of the vas deferens furthest removed from the incoming solution; subsequently SCs were examined for responses at successive sites, ≥60–100 μm apart, further removed from this edge. This ensured that SCs were not repeatedly exposed to drug application. Concentrations of 10 μM of ATP and UTP were generally used as higher concentrations (100 μM) could initiate muscle contraction. Pressure application of Ringer solution alone did not induce any Ca\(^{2+}\) changes in SCs.

**Stimulation of sympathetic nerve**

The stimulation electrode consisted of two parallel fine epoxy-insulated tungsten wires separated by 40–80 μm. After we chose a loaded Schwann cell, the Y-shaped tip of the stimulation electrode was placed on the surface of the preparation, 30–40 μm away from the cell and straddling strings of varicosities. Electrical current was generated from an isolated stimulator (Digitimer, model DS2) using a pulse duration of 0.08 ms. The voltage applied (always <20 V) was adjusted so as to avoid causing muscle contraction. Stimulation trains of 5 Hz for 10 s were most often used and these were controlled by a Master-8 pulse generator.

**Ca\(^{2+}\) imaging of Schwann cells**

Fluo-3 AM fluorescent dye was excited using a mercury arc lamp reflected light fluorescence attachment on an upright Olympus Optical microscope (model BX50WI, Tokyo, Japan). An Olympus Optical fluorescein wide band filter WIB, which combined dichroic mirror DMS05, excitation filter BP460-490 (model U-MIB), and barrier filter BA515IF (model MBA530W25) was used to view fluo-3 AM. To minimize photo damage, a 6% neutral density filter was always inserted in the excitation path, and sometimes another 25% filter was added to attenuate to 1.5% of maximum intensity. Fluorescence was observed through an Olympus Optical ×40 water-immersion objective (model LUM PlanFIIR 0.80 numerical aperture; Olympus) using a CCD camera (model WV-BP-310; Panasonic, Secaucus, NJ). Images were acquired using a Scion (Frederick, MD) LG3 frame grabber. Images were taken and processed by using National Institutes of Health Image 1.63 program (available from the user remote location http://rsb.info.nih.gov/nih-image/). Images that demonstrated the distribution of SCs (for example, Figs. 1 and 2) were taken by integrating 32 frames. Ca\(^{2+}\) transients of SCs were captured using single frames at rates of 5 frames/s, and the exact time of frame acquisition stamped on each image. All recording were performed at 31–33°C.

Images were analyzed using the National Institutes of Health Image 1.63 program. On the first control image, a region of interest was chosen that encompassed a single SC. The mean intensity from a region of interest of exactly the same size and geometry was measured on all images of a given cell. Any movement of the cell over the course of the experiment was corrected for by an automated procedure, written in the macro programming language of National Institutes of Health Image, which tracked the cell between every frame based on the strongest local fluorescent signal compared with the background. The recording data were rejected if the SC had a vertical movement during image capturing as indicated by a spot adjacent to the SC of interest. The relative changes in fluorescence intensity in a SC were expressed as ΔF/Fo = 100 × (F – Fm)/Fm, where F is the fluorescence intensity after ATP or UTP application or nerve stimulation, and Fm is the averaged fluorescence intensity of a cell over a 10-s period before any nerve stimulation or drug application. Failure to obtain a change in Ca\(^{2+}\) (Δ[Ca\(^{2+}\)]\(_{i}\)) was taken to occur if the peak pixel intensity was <4 times the SD of the background. These experimental data were rejected if there was dye saturation. The intensity of dye loading in SCs, in general, was not uniform, even between nearby SCs located on the same string.

**Statistics**

Results are presents as means ± SE. A Student’s unpaired t-test was used when data were compared between different groups by using the Microsoft Excel software. Statistical significance was determined at a confidence level of 95%. N\(_i\) indicates the number of preparations used, whereas N\(_c\) refers the number of cells studied.
RESULTS

Distribution of Schwann cells on the surface of the vas deferens

Exposure of the vas deferens to the calcium indicator fluo-3 AM for 180 min reveals a network of stained Schwann cells (SCs) with a heavily stained cell body after exposure of the tissue to the calcium indicator fluo-3 AM; the lower axon bundle possesses several SCs and their processes at different transverse sections along the length of the bundle. Single SCs are also seen passing from one nerve trunk to another. B: SCs, with prominent cell bodies, are found in strings that occasionally branch. C: a string of terminal varicosities that have been labeled together with an associated string of SCs. D: a single SC body together with strings of terminal varicosities. E: a single SC labeled with fluo-3 AM (a) and later labeled with anti S-100 (b) after washing out the calcium indicator over 4 h. Calibration, 5 µm. All these figures were integrated from 32 frames under a 25% neutral density filter.

FIG. 1. Distribution of varicosities and Schwann cells on the surface of the vas deferens. A: small axon bundles possess Schwann cells (SCs) with heavily stained cell body after exposure of the tissue to the calcium indicator fluo-3 AM; the lower axon bundle possesses several SCs and their processes at different transverse sections along the length of the bundle. Single SCs are also seen passing from one nerve trunk to another. B: SCs, with prominent cell bodies, are found in strings that occasionally branch. C: a string of terminal varicosities that have been labeled together with an associated string of SCs. D: a single SC body together with strings of terminal varicosities. E: a single SC labeled with fluo-3 AM (a) and later labeled with anti S-100 (b) after washing out the calcium indicator over 4 h. Calibration, 5 µm. All these figures were integrated from 32 frames under a 25% neutral density filter.

FIG. 2. Single Schwann cells associated with axons, injected with Oregon Green 488 bis-(o-aminophenoxy)-X,N,N',N'-tetraacetic acid (BAPTA)-1. A: a Green 488 BAPTA-1-filled microelectrode impaling a SC in its cell body. In general, long thin SC processes from opposite poles of the ellipsoidal nucleus can be observed. B: on occasions, the microelectrode inadvertently impales a single varicose axon, which is then labeled with Oregon Green 488 BAPTA-1 dextran. C: the cell body of a SC and its processes filled with the calcium indicator (↓). D: the same SC as in C but under transmitted light (↓). Calibration of 5 µm in B is also for A and calibration of 5 µm in D is also for C. All figures were taken by integrating 32 frames under a 25% neutral density filter.
intensely stained cytoplasm of the cell body ~5 μm diameter (Fig. 1A). On occasions more than one SC body can be found in a cross-section through the bundle (Fig. 1A). Single SCs, sequentially arranged into a string, are observed to leave the small axon bundles at intervals along their length (Fig. 1B). These are presumably associated with single strings of varicosities that can be observed to leave small axon bundles. This was confirmed by observing, on occasions, both SCs and the strings of varicosities that accompanied them as a consequence of both being labeled with fluo-3 AM (Fig. 1, C and D). Cells filled with calcium indicator were confirmed as SCs by labeling them with anti-S100 (Fig. 1E). In general, there was nonuniformity in intensity of fluo-3 AM dye loading in SCs. This could even differ between nearby SCs located on the same string (data not be shown).

Individual SCs in a string could be injected with calcium dyes, such as Oregon Green 488 BAPTA-1 dextran, at the level of their cell bodies. Such strings of SCs were first identified with transmitted light (Fig. 2D) and a microelectrode filled with dye, positioned over a cell body, which was then impaled, filling the SC with dye (Fig. 2, A and C). In general such SCs were then observed to possess one or more thin long processes, proceeding from opposite poles of the ellipsoidal cell body (Fig. 2, A and C). On some occasions, the nearby varicose axon was also impaled (Fig. 2B), revealing the close proximity of nerve terminal and SC.

Identification of Schwann cells

To confirm our criteria for the identification of SCs, images were captured of calcium-labeled SCs on which pharmacological studies had been carried out (Fig. 1Ea). Whole preparations from which these images had been captured were then rinsed with Ringer solution for ~4 h until no calcium fluorescence could be observed. Preparations were then processed for immunohistochemistry, and the presence of anti-S-100 on the SCs previously labeled with calcium indicator revealed (Fig. 1Eb). The anti-S-100 labeling of SCs was the same as that which we have reported previously (Barden et al. 1999). These observations confirm our criteria for the identification of SCs at nerve terminals.

Spontaneous calcium transients in individual terminal Schwann cells

Occasionally, spontaneous calcium transient could be observed in SCs. Figure 3A shows nine images of a SC taken during a spontaneous calcium transient (Δ[Ca\(^{2+}\)]\(_s\)) with zero time indicating the moment of peak Δ[Ca\(^{2+}\)]\(_s\). It will be noted that before the transient the two main processes emanating from the soma of the SC are barely visible (~0.5 s), whereas during the peak of the transient (0 s) they are clearly delineated. The Δ[Ca\(^{2+}\)]\(_s\) declined following the peak over a few seconds back to baseline calcium levels. The peak Δ[Ca\(^{2+}\)]\(_s\) could be quite different for different SCs (see Fig. 3, B and C).

Evoked calcium transients in individual Schwann cells

Stimulating the intramural sympathetic nerve supply within the vas deferens with single impulses did not give rise to Δ[Ca\(^{2+}\)]\(_s\) in any of the SCs studied (N\(_s\) = 22 from N\(_p\) = 6), whereas short trains of impulses at ≥5 Hz frequencies did give rise to Δ[Ca\(^{2+}\)]\(_s\) in 25.6% of the SCs (N\(_s\) = 156 from N\(_p\) = 40) examined. No Δ[Ca\(^{2+}\)]\(_s\) were observed in any SCs (N\(_s\) = 77 from N\(_p\) = 6) during trains of impulses in the presence of TTX (300 nM). Figure 4A shows the changes in Δ[Ca\(^{2+}\)]\(_s\) in the nuclear regions of two SCs after nerve stimulation commencing at 0 s for 10 s at 5 Hz. There is a gradual increase in Δ[Ca\(^{2+}\)]\(_s\) throughout the 10-s period of stimulation with both SCs showing a decline in Δ[Ca\(^{2+}\)]\(_s\) after the end of stimulation. However, the rate of Δ[Ca\(^{2+}\)]\(_s\) decline is different between different SCs (Fig. 4B). While the Δ[Ca\(^{2+}\)]\(_s\) in one SC has declined by 70% of its peak value 40 s after the end of stimulation the Δ[Ca\(^{2+}\)]\(_s\) of the other SC had only declined by ~25% (Fig. 4B). Furthermore, the peak Δ[Ca\(^{2+}\)]\(_s\) due to nerve stimulation also varies greatly at different SCs. Figure 4C shows the changes in Δ[Ca\(^{2+}\)]\(_s\) of two other SCs that responded to short trains of impulses (10 s at 5 Hz) with small changes in Δ[Ca\(^{2+}\)]\(_s\). Notice that even 25 s after the end of stimulation, the Δ[Ca\(^{2+}\)]\(_s\) of one of the SCs has only declined slightly, whereas that of the remaining SC is approaching the base line level of Δ[Ca\(^{2+}\)]\(_s\), observed at the beginning of stimulation. The mean time constant of decline of Δ[Ca\(^{2+}\)]\(_s\) due to nerve stimulation was 25.7 ± 4.9 s (N\(_s\) = 32 from N\(_p\) = 31). The Δ[Ca\(^{2+}\)]\(_s\) in response to nerve stimulation possesses on average a time constant significantly longer than that of the spontaneous Δ[Ca\(^{2+}\)]\(_s\) (P < 0.001). The frequency distribution of the peak Δ[Ca\(^{2+}\)]\(_s\) observed in all SCs studied (N\(_s\) = 156 from N\(_p\) = 40), that gave Δ[Ca\(^{2+}\)]\(_s\) to nerve stimulation, is skewed (Fig. 4D).

The Δ[Ca\(^{2+}\)]\(_s\) responses to successive trains of nerve impulses greatly declined in amplitude. Figure 5A shows that at intervals of 30 s between trains of impulses of 10 s at 5 Hz the Δ[Ca\(^{2+}\)]\(_s\) to the second train was only 51.6 ± 8.2% of that for the first train (N\(_s\) = 7 from N\(_p\) = 6; Fig. 5B). Further trains of nerve impulse at the same intervals gave no responses.

Effect of blocking purinergic receptors on the nerve evoked Δ[Ca\(^{2+}\)]\(_s\) in Schwann cells

We next determined if ATP, a principal transmitter released from sympathetic nerve terminals in the vas deferens, acts on SCs during nerve stimulation to generate Δ[Ca\(^{2+}\)]\(_s\). Suramin nonspecifically blocks most of ionotropic P2X receptors as well as metabotropic P2Y receptors (Ralevic and Burnstock 1998). Incubating the vas deferens in suramin (100 μM) for >30 min before stimulation of the sympathetic nerves at 5 or 10 Hz for 10 s greatly decreased the amplitude of Δ[Ca\(^{2+}\)]\(_s\) (N\(_s\) = 146 from N\(_p\) = 22; compare the frequency histogram in Fig. 4E of peak Δ[Ca\(^{2+}\)]\(_s\) in the presence of suramin with that in D in its absence). The successful responses to nerve stimulation decreased from 25.64 to 10.96% (P < 0.001), and most Δ[Ca\(^{2+}\)]\(_s\) were very small or negligible compared with controls (mean peak of 1.59 ± 0.29 compared with control peak of 3.58 ± 0.83; P < 0.05). Apyrase (5 U/ml), which removes ATP, blocked Δ[Ca\(^{2+}\)]\(_s\) in response to nerve stimulation (N\(_s\) = 79 from N\(_p\) = 6).

Purinergic receptors on Schwann cells that evoke Δ[Ca\(^{2+}\)]\(_s\)

The Δ[Ca\(^{2+}\)]\(_s\) response of SCs to purinergic receptor agonists was next determined by pressure ejection of ATP (10 μM) or UTP (10 μM; an agonist at P2Y2, P2Y4, and P2Y6...
receptors) onto them. Local application of ATP by such ejection yielded a \(\Delta [Ca^{2+}]_i\) in 77.2% of SCs \((N_s = 57 \text{ from } N_p = 15, \text{ see Fig. 6A, a and d). In the case of local application of UTP, 76.9% of SCs responded \((N_s = 26 \text{ from } N_p = 7, \text{ see Fig. 6A, b and d).}

ATP may be metabolized to adenosine by ecto-enzymes existing in the junctional cleft (Salter et al. 1993; Ziganshin et al. 1994). Thus ATP may elicit \(\Delta [Ca^{2+}]_i\) through the action of adenosine. However, it is unlikely that adenosine receptors play a major role in contributing to \(\Delta [Ca^{2+}]_i\) in the vas deferens SCs. Local ejection of 10 \(\mu M\) ATP onto SCs in the presence of 10 \(\mu M\) CGS-15943, a nonselective adenosine receptors antagonist, gave \(\Delta [Ca^{2+}]_i\) in 71.5% of SCs studied, and these had an average peak \(\Delta F/F\) size of 7.35 \pm 0.16 \((N_s = 52 \text{ from } N_p = 3)\) which is not significantly different to the effects of ATP in the absence of the antagonist \((P = 0.68)\). Furthermore, local application of adenosine \((20 \mu M\) gave \(\Delta [Ca^{2+}]_i\) responses in only 16.7% of SCs \((N_s = 48 \text{ from } N_p = 6)\). No \(\Delta [Ca^{2+}]_i\) was observed in response to local application of 20 \(\mu M\) of adenosine to SCs after exposure of the vas deferens for 40 min to CGS-15943 \((N_s = 21 \text{ from } N_p = 4)\).

The preceding observations prompted us to investigate whether purinergic receptors provide a major contribution to the eliciting of \(\Delta [Ca^{2+}]_i\) due to action of ATP. To test the involvement, two nonspecific purinergic receptors antagonists were used, suramin and PPADS. These two antagonists have been reported to be effective at blocking most P2X and P2Y receptor subtypes (Ralevic and Burnstock 1998). The percentage of SCs responding to local application of ATP in the presence of suramin \((100 \mu M)\), that is had a \(\Delta F/F > 0.8\), was only slightly reduced compared with controls \((61.8\% \text{ compared with } 77.2\%; P < 0.02; N_s = 34 \text{ from } N_p = 5; \text{ see Fig. 6Ad})\), although the amplitude of \(\Delta F/F\) was much smaller \((2.98 \pm 1.16 \text{ compared with } 7.97 \pm 1.06, P < 0.05; \text{ see Fig. 6Ac})\). Thus the frequency distribution of SCs responding to ATP with a particular peak \(\Delta [Ca^{2+}]_i\) in the presence of suramin (Fig. 6Bb) was very skewed toward low \(\Delta [Ca^{2+}]_i\) in the presence of suramin (Fig. 6Bb).

The effect of PPADS, an antagonist of P2X1 to P2X3, P2X5, P2X7, P2Y1, P2Y4, and P2Y6 receptors, was also tested. To avoid unnecessary side effects of PPADS, such as inhibiting IP3-induced \([Ca^{2+}]_i\) mobilization we started with 5 \(\mu M\) concentration. The percentage of SCs responding to local application of ATP decreased from 77.2 to 65.2% \((N_s = 23 \text{ from } N_p = 4)\), but this was not significant \((P > 0.2)\). However, the mean peak \(\Delta F/F\) was smaller than controls (compare Fig. 6B, c with a). It dropped from control value of 7.97 \pm 1.06 to 4.38 \pm 1.56 \((P < 0.05)\). The percentage of success in responding to ATP in the presence of 10 \(\mu M\) PPADS was significantly decreased to 21.4% \((P < 0.0001)\). The averaged \(\Delta F/F\) peak in SCs that did respond also declined in this concentration of PPADS to 1.15 \pm 0.16 \((N_s = 28 \text{ from } N_p = 4; P < 0.0001)\). The distribution of \(\Delta F/F\) is indicated in Fig. 6Bc \((5 \mu M\) PPADS). Application of ATP in the presence of the adenosine receptor blocker CGS-15943 \((10 \mu M)\) gave similar \(\Delta [Ca^{2+}]_i\) as in the absence of the blocker (percentage of cells responding, 71.2%; \(\Delta F/F\) of 7.35 \pm 0.16, \(P > 0.5; N_s = 52 \text{ from } N_p = 3; \text{ compare Fig. 6B, d with a})\). The results suggest that purinergic receptors play a major role in eliciting \(\Delta [Ca^{2+}]_i\) in vas deferens and this involves primarily P2 receptors.

FIG. 3. Spontaneous calcium transients in Schwann cells. A: an individual SC was impaled with sharp microelectrodes filled with Oregon Green 488 BAPTA-1 dextran. Images show spontaneous changes in \(\Delta [Ca^{2+}]_i\) in this SC at 9 different times: prior to \((-0.5 \text{ s})\), at the peak of \(\Delta [Ca^{2+}]_i\) \((0 \text{ s})\), and during the subsequent decline in calcium intensity \((0.5–3.5 \text{ s})\). In the scale at the right, the top part corresponds to the greatest measured fluorescence intensity and thus to the highest \(\Delta [Ca^{2+}]_i\); the scale is linear with respect to the fluorescence intensity. Scale bar, 5 \(\mu M\). B–C: the quantitative changes in spontaneous \(\Delta [Ca^{2+}]_i\) of SCs. B and C show the \(\Delta [Ca^{2+}]_i\) for 2 different SCs.
Test/conditioning applications of ATP to SCs at an interval of 30 s gave test/Ca\textsuperscript{2+}\textsubscript{SCs} responses that were only about a half of the conditioning C\textsuperscript{2+}\textsubscript{SCs} response (56.7 ± 3.6%, N\textsubscript{s} = 7 from N\textsubscript{p} = 3; see Fig. 5, C and D), much the same as did repeated nerve stimulation (51.6 ± 8.2%, N\textsubscript{s} = 7 from N\textsubscript{p} = 5; see Fig. 5, A and B).

**Contribution of calcium stores to C\textsuperscript{2+}\textsubscript{SCs} of Schwann cells**

The fact that local application of UTP gave a similar percentage of successful C\textsuperscript{2+}\textsubscript{SCs} responses as did ATP suggests that ATP is primarily activating metabotropic purinergic receptors. This was checked by depleting the intracellular calcium stores with 5 mM caffeine in 0 C\textsubscript{2+}o and in the presence of 1 mM EGTA in the bath solution. In this case there was no C\textsuperscript{2+}\textsubscript{SCs} response to ATP (N\textsubscript{s} = 24 from N\textsubscript{p} = 5, see Fig. 7B), although normal responses are obtained in this bath solution in the absence of caffeine (N\textsubscript{s} = 38 from N\textsubscript{p} = 5; Fig. 7A). To further examine if a G protein-PLC-IP3 cascade is involved in the C\textsuperscript{2+}\textsubscript{SCs} responses to local application of ATP, we incubated preparations with a PLC metabolism inhibitor U73122 in 0 C\textsubscript{2+}o and 1 mM EGTA solution. The C\textsuperscript{2+}\textsubscript{SCs} signals to applied ATP were abolished completely (N\textsubscript{s} = 31 from N\textsubscript{p} = 4; Fig. 7C). The inactive analog of U73122, U73343, had no effect on the C\textsuperscript{2+}\textsubscript{SCs} in response to applied ATP (percentage of cells responding, 77%; ΔF/F of 8.55 ± 1.25, P > 0.7; N\textsubscript{s} = 35 from N\textsubscript{p} = 3).
Furthermore, the SCs are an order of magnitude larger than sympathetic varicosities (see Fig. 1, C and D), so that even trains of impulses at high frequencies of stimulation which give rise to large $\Delta[Ca^{2+}]_v$ are unlikely to involve a significant contribution of $\Delta[Ca^{2+}]_v$ to $\Delta[Ca^{2+}]_s$ (O’Connor et al. 1999). In addition, we have loaded SCs by injection of the calcium indicator rather than using the acetylmethyl form and shown

**FIG. 5.** Desensitization of the response of terminal Schwann cells to nerve stimulation and applied ATP. A: the $\Delta[Ca^{2+}]_s$ in response to stimulation of the sympathetic nerves with 2 trains of impulses of 5 Hz for 10 s at 30 s apart. B: the change in peak $\Delta[Ca^{2+}]_s$ for successive trains of impulses of the kind shown in A; a 3rd train of impulses at this interval did not give any response. C: the $\Delta[Ca^{2+}]_s$ in response to the local application of ATP (10 $\mu$M, $\rightarrow$) to a SC on 2 occasions at 30 s apart. D: the change in peak $\Delta[Ca^{2+}]_s$ for successive responses to iontophoresis of ATP; a 3rd application at the same interval did not give any response.

**DISCUSSION**

**Schwann cells on the surface of the vas deferens**

Bundles of up to five axons, together with single axons, occur frequently on the surface of the mouse vas deferens as has been ascertained by examining serial sections of the muscle with the electron microscope (Cottee et al. 1996; Lavidis and Bennett 1993) or staining the mitochondria within the axon with the fluorescent dye, 3,3-diethyloxadecarboxyanine iodide (Lavidis and Bennett 1992, 1993). The study of $\Delta[Ca^{2+}]_s$ in the present work was confined to single strings of SCs, identified both on loading with calcium indicators as well as immunohistochemically with antibodies to the SC marker S100 (see Barden et al. 1999). These SCs were probably associated with single strings of varicosities, whereas strings of multiple SCs in close juxtaposition were probably associated with axon bundles. On occasion, a single string of varicosities was labeled with the calcium indicator and shown to be in close relation with a string of SCs.

**Calcium transients in the Schwann cell versus those in the varicosities**

Are the calcium transients that have been attributed to the SC (i.e., $\Delta[Ca^{2+}]_s$) partly due to calcium transients in the varicosities (i.e., $\Delta[Ca^{2+}]_v$). This is unlikely, as single impulses give rise to large $\Delta[Ca^{2+}]_v$ (Brain and Bennett 1997), whereas such impulses give no detectable $\Delta[Ca^{2+}]_s$ at all. Furthermore, the SCs are an order of magnitude larger than sympathetic varicosities (see Fig. 1, C and D), so that even trains of impulses at high frequencies of stimulation which give rise to large $\Delta[Ca^{2+}]_v$ are unlikely to involve a significant contribution of $\Delta[Ca^{2+}]_v$ to $\Delta[Ca^{2+}]_s$ (O’Connor et al. 1999). In addition, we have loaded SCs by injection of the calcium indicator rather than using the acetylmethyl form and shown

**FIG. 6.** The $\Delta[Ca^{2+}]_s$ of terminal Schwann cells in response to local application of ATP or uridine 5-triphosphate; trisodium salt hydrate (UTP). A: examples of $\Delta[Ca^{2+}]_s$ from 2 SCs after local application of ATP (10 $\mu$M) at the ↑; b: the $\Delta[Ca^{2+}]_s$ from 2 SCs in response to local application of UTP (10 $\mu$M) at the ↑; c: examples of $\Delta[Ca^{2+}]_s$ from 2 SCs in response to ATP (10 $\mu$M) at the ↑ in the presence of suramin (100 $\mu$M); d: a histogram showing the percentage of SCs that give a $\Delta[Ca^{2+}]_s$ in response to local application of ATP (77.2%, $N_s = 57, N_v = 15$), UTP (76.9%, $N_s = 26, N_v = 7$). ATP in the presence of 10 $\mu$M CGS-15943 (71.15%, $N_s = 52, N_v = 3$) and ATP in the presence of 100 $\mu$M suramin (61.6%, $N_s = 34, N_v = 5$). The vertical bars on each histogram indicate $\pm$ SE with ATP $\pm 1.33$, UTP $\pm 0.91$, ATP with CGS-15943 $\pm 0.16$ and ATP with suramin $\pm 1.38$, respectively. B: frequency histograms of the number of SCs that gave a particular size $\Delta[Ca^{2+}]_s$ in response to local application of ATP in the presence of purinergic receptor antagonists: a: ATP (10 $\mu$M) alone; b: ATP + suramin (100 $\mu$M); c: ATP + pyridoxal-phosphate-6-azophenyl-2',5'-disulfonic acid (PPADS; 5 $\mu$M); d: ATP + CGS-15943 (10 $\mu$M).
that the SCs still give large ∆[Ca²⁺]ᵢ in response to nerve stimulation even though there is no ∆[Ca²⁺]ᵢ in these circumstances.

Source of the ATP that gives rise to ∆[Ca²⁺]ᵢ

What is the source of ATP that contributes to ∆[Ca²⁺]ᵢ on nerve stimulation, given that ∆[Ca²⁺]ᵢ is reduced by suramin and assuming that the action of this drug is to block P2 receptors. There are two identified sources of ATP in the mammalian vas deferens on nerve stimulation: one of these is the nerve terminal (Sneddon et al. 1982) and the other the muscle on α-adrenoceptor or P2X receptor activation, the latter occurring even in the absence of muscle contraction (Katsuura et al. 1991; Vizi and Burnstock 1988; Vizi et al. 1992). We have not distinguished between these two possible sources of ATP in generating ∆[Ca²⁺]ᵢ. Furthermore, we cannot exclude the possibility that ATP, locally applied to the SC, releases a substance from the ensheathed nerve or muscle that then produces the ∆[Ca²⁺]ᵢ. Finally, it is known that mammalian SCs themselves can release ATP under a variety of conditions (see, for example, Liu and Bennett 2003), so that we cannot exclude the possibility that an autocrine release of ATP occurs from the SCs.

The possibility that other transmitters than ATP are contributing to the nerve evoked SC response must be entertained. Immunohistochemistry indicates that there are five different classes of pelvic neurons that innervate the rat vas deferens, of which the great preponderance are noradrenaline (NAd) and neuropeptide (NPY) containing (≈70%). The remaining neurons contain vasoactive intestinal peptide (VIP; ≈15%), NPY (5%), NPY plus VIP (5%) or galanin (Gal; ≈2%) (Keast 1992). The VIP neurons are cholinergic as are the NPY-, NPY + VIP-, and Gal-containing neurons so that ∼30% of the nerves are cholinergic (Keast 1995; Wanigasekara et al. 2003). Sympathetic neurons (receiving an innervation from the hypogastric nerve) are either noradrenergic and contain NPY or cholinergic and contain VIP. Parasympathetic neurons (receiving an innervation from the pelvic nerve) are almost all cholinergic and contain either VIP or NPY or both (Keast 1995; Wanigasekara et al. 2003). The NAd + NPY-containing neurons innervate the longitudinal and circular muscle coats as do the NPY, NPY + VIP and Gal neurons, whereas the VIP neurons, which are probably cholinergic, mainly innervate the lamina propria (Kaledycz 1998; Keast 1992). Sensory axons, containing calcitonin gene-related peptide (CGRP) and substance P, are also found in the muscle coats (Kaledycz 1998), although the proportion of axons in the rodent vas deferens that are sensory is not known. These considerations show that there are a variety of transmitters, besides ATP, that have the potential to act on SCs on stimulation of the mammalian vas deferens nerve supply.

The question arises as to why so few SCs respond to nerve stimulation (~25%) compared with those that respond to the application of ATP (~80%). The most likely reason is that we have failed to stimulate the nerves associated with the SC recorded from. Preparing the vas deferens for dye loading of SCs, and their subsequent exposure to local ATP application, involves removing some of the epimysium and perimysium. This certainly damages part of the nerve supply and so has the potential of interrupting impulse traffic between the site of intramural nerve stimulation and the area containing the SCs of interest.

Physiological frequencies of activation of Schwann cells

Is the frequency of stimulation necessary to elicit ∆[Ca²⁺]ᵢ, namely >5 Hz, is outside the physiological range? Preganglionic nerves to the mammalian superior cervical ganglion fire with a normal upper frequency of ~6 Hz (Janig 1988), reaching as high as 40 Hz during inspiration (Boczek-Funcke et al. 1992). This is mirrored by postganglionic sympathetic nerves that may fire with a rhythm reaching 6 Hz (Geber et al. 1990), with a mean within-burst firing rate of ~19 Hz (Macefield et al. 1994). In the present work, ∆[Ca²⁺]ᵢ was observed for frequencies of nerve stimulation as low as 5 Hz. This is in the range of physiological frequencies so that ∆[Ca²⁺]ᵢ might function during normal mammalian nerve activity.

Purinergic receptors on Schwann cells give rise to ∆[Ca²⁺]ᵢ

In the present work, a proportion of the ∆[Ca²⁺]ᵢ due to nerve stimulation was blocked by suramin, indicating the likelihood that it is due to the action of released ATP. Local application of ATP produced ∆[Ca²⁺]ᵢ that were antagonized by suramin and PPADs, supporting a role for P2 receptors on these SCs. It is unlikely that P1 receptors produce ∆[Ca²⁺]ᵢ as local application of adenosine produced few responses. The ∆[Ca²⁺]ᵢ in response to applied ATP was completely eliminated by blocking the IP₃ pathway or emptying the calcium stores with caffeine. Furthermore, the action of UTP in producing ∆[Ca²⁺]ᵢ, suggests that P2X2 or P2Y4 receptors are present on mammalian SCs (von Kugelgen and Wetter 2000). However, whether the P2X receptors observed immunohistochemically on these SCs by Barden et al. (1999) contribute to the ∆[Ca²⁺]ᵢ when activated by either exogenous or endogenous ATP was not ascertained.

Cultured mammalian premyelinated axons release ATP in response to impulses to increase ∆[Ca²⁺]ᵢ, in SCs (Fields and Fields 2000; for a review, see Fields and Stevens 2000) Such
observations raise the question as to whether the mechanisms involved in elevating $\Delta [Ca^{2+}]_s$ observed on nerve stimulation in the present study can be differentiated from that which occurs along the length of the axon. Terminal SCs at the amphibian neuromuscular junction can elevate $\Delta [Ca^{2+}]_s$ through the nerve terminal release of ATP that acts on P2Y2 receptors (Robitaille 1995). However, terminal SCs at the somatic mammalian neuromuscular junction elevate $\Delta [Ca^{2+}]_s$ through the nerve terminal release of adenosine, which acts on A1 adenosine receptors, whereas P2 receptors for ATP do not appear to be present (Rochon et al. 2001). This is in contrast to the present study, which shows that both nerve released and applied ATP acts directly on P2 receptors, whereas adenosine does not much affect the SCs. There is then a heterogeneity of mechanisms whereby $\Delta [Ca^{2+}]_s$ is elevated by ATP for the SCs found at mammalian autonomic compared with mammalian somatic neuromuscular junctions. Whether such heterogeneity exists for SCs at preterminal axons compared with those at the terminals with their high density of varicosities remains for investigation.

Sources of calcium for $\Delta [Ca^{2+}]_s$ in SCs

Although we have provided evidence for the $\Delta [Ca^{2+}]_s$ due to ATP arising from calcium release from internal stores, there has been no investigation of the process of SC calcium sequestration. The $\Delta [Ca^{2+}]_s$ at the amphibian neuromuscular junction due to nerve stimulation is at least in part sequestered by SC endogenous buffers (Castonguy and Robitaille 2001; see also Castonguy and Robitaille 2002). We have shown that depleting calcium from internal stores in the SC with, for example, caffeine, completely abolishes $\Delta [Ca^{2+}]_s$. Furthermore, reducing the extracellular calcium concentration to zero (with 0 calcium and EGTA) has no effect. This result would be understandable if only P2Y metabotropic receptors existed on SCs in this organ, but immunohistochemical studies show that P2X1 and P2X3 ionotropic receptors are present on these SCs (Barden et al. 1999). There is evidence for such ionotropic P2X receptors on Schwann cells at the frog neuromuscular junction (Robitaille 1995) and in mammalian nerve trunks (Coloma and Amédee 2001; Irińchi et al. 2001). It would be anticipated then that reducing the extracellular calcium to zero would at least produce a quantitative decrease in $\Delta [Ca^{2+}]_s$; however, this was not observed. Our only explanation is that the P2X receptors are quantitatively far fewer on the SCs than the P2Y receptors that generate $\Delta [Ca^{2+}]_s$.

Why do spontaneous $\Delta [Ca^{2+}]_s$ transients occur, whereas single impulses do not give rise to an observable $\Delta [Ca^{2+}]_s$? It might be that the spontaneous $\Delta [Ca^{2+}]_s$ are due to spontaneous release of $[Ca^{2+}]_s$ from internal stores of a magnitude that can be measured but that release due to single or a short train of impulses is not. The similar times courses of decay of the spontaneous $[\Delta Ca^{2+}]_s$ compared with the very different time courses of decay of the evoked $[\Delta Ca^{2+}]_s$ may be due to the former involving spontaneous release of all the calcium from a single store, whereas the latter is due to release from a variable number of stores with different rates of sequestration. The mechanisms responsible for the very different rates of decline of $\Delta [Ca^{2+}]_s$ in different SCs after trains of impulses are yet to be elucidated. Finally, we have not yet identified a physiological role for $\Delta [Ca^{2+}]_s$ at the autonomic neuromuscular junction. SCs at the somatic neuromuscular junction of amphibia participate in a nerve-impulse-evoked process that leads to modulation of transmitter release from terminals (Robitaille 1998). Whether the $\Delta [Ca^{2+}]_s$ identified in the present work participates in such a process at the autonomic neuromuscular transmitters is now open to investigation.

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References


