Inhibition of astrocyte gap junctional communication by ATP depletion is reversed by calcium sequestration

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Abstract **We have studied the possible role of cellular energy status in the regulation of gap junction permeability in rat astrocytes in primary culture. Incubation with the mitochondrial respiratory chain inhibitor antimycin (5 ng/ml) for 16 h caused a significant decrease in ATP concentrations. This effect was accompanied by a dose-dependent inhibition of gap junction permeability as assessed by the scrape-loading/Lucifer yellow transfer technique. No cell death was observed following this treatment. Restoration of cellular ATP levels by a further 24 h incubation in antimycin-free medium reversed the inhibition of Lucifer yellow transfer caused by antimycin. The inhibition of Lucifer yellow transfer brought about by antimycin treatment was also reversed by a short incubation of the cells with the calcium chelator EGTA plus the calcium ionophore A23187. These results suggest that ATP depletion causes a reversible inhibition of gap junction permeability through a calcium-mediated mechanism.**

Key words: Astrocyte; Gap junction; ATP; Antimycin; Calcium

1. Introduction

Gap junction channels are membrane conduits of adjacent cells that provide direct communication for the diffusion of cytoplasmic ions and small molecules between cells without access to the extracellular space [1]. Within the central nervous system, astrocytes are well coupled by gap junctions [2], whose permeability is regulated by endothelins [3,4] and cAMP/Ca^{2+} mediated transduction mechanisms [5–8]. Recently, it has been reported that gap junction permeability plays a role in the regulation of astrocyte metabolism [4].

The major gap junction protein in astrocytes, connexin43 [2], is phosphorylated by a cAMP-dependent protein kinase mechanism [9]. It has recently been reported that brain connexin43 is predominantly phosphorylated but is dephosphorylated immediately after death [10], suggesting that the energy status of the cell might regulate intercelullar communication. In addition, the turnover of phosphorylated connexin43 is rapid [11], suggesting the need for a continuous endogenous supply of ATP for cAMP-dependent protein kinase activity. In view of such evidence, the aim of the present work was to elucidate whether endogenous ATP concentration regulates intercellular communication.

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Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) and Earle's balanced salt solution (EBSS) were from Sigma Chemical Co. (St. Louis, MO., USA). Fetal calf serum was obtained from Boehringer Ingelheim (Heidelberg, Germany). Other substrates, enzymes and coenzymes were purchased from Sigma, Boehringer or Merck (Darmstadt, Germany).

2.2. Cell culture

Astrocytes in primary culture were prepared from the forebrains of neonatal (1–2-day-old) Wistar rats as previously described [12]. Cells were plated at a density of 2.5×10^5 cells/cm^2 in 8 cm^2 poly(L-lysine)-coated tissue culture plastics in DMEM supplemented with 10% (v/v) fetal calf serum. Cells were incubated at 37°C in a humidified atmosphere containing 95% air/5% CO_2 for 13–15 days with medium change twice a week. At the time of the experiment, cells were confluent and were characterized by immunostaining with glial fibrillary acidic protein [13].

2.3. Cell treatments

For exposure to antimycin, after 13–15 days in culture astrocytes were washed with PBS and incubated for 16 h in 2 ml of DMEM in the absence (control) or presence of antimycin (1–5 ng/ml). In all experiments, control cells received equal amounts of the vehicle (1 μl ethanol). Following incubation, DMEM was collected for lactate dehydrogenase (LDH) activity measurement and Lucifer yellow transfer quantitation. For the reversibility experiments, cells were incubated with antimycin (5 ng/ml) for 16 h, washed with phosphate-buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 1.7 mM NaH_2PO_4, pH 7.4) and incubated for a further 24 h in antimycin-free DMEM.

For exposure to EGTA/A23187, antimycin (5 ng/ml)-treated astrocytes were washed with ionic solution and incubated in ionic solution containing 1 mM EGTA plus 5 μM A23187 (Sigma) for 5 min at room temperature. Control cells received the same treatment. Lucifer yellow transfer was assessed as described below.

To estimate cell death [14], LDH activity was determined in cell-free DMEM as reported by Vassault [15]. The percentage of lactate dehydrogenase released into the media was calculated according to the following formula: (LDH activity in the media/total LDH activity)×100, where total LDH activity represents LDH activity in cells and media. Total LDH was determined after disruption with 0.1% (v/v; final concentration) Triton X-100.

For ATP determination, cells were quickly washed with ice-cold PBS and 1 ml of 0.3 M $HClO_4$ was added immediately. After scraping, wells were washed with a further 1 ml of 0.3 M $HClO_4$. The pooled perchloric extracts were neutralized by adding 1 ml of 0.5 M $HKCO_3$. After 1 h in an ice-bath, extracts were centrifuged and supernatants frozen at −75°C until ATP assay. ATP was determined in the neutralized perchloric cell extracts by the luciferin/luciferase bioluminescence method [16] using a commercially available kit (Sigma) and following the manufacturer's instructions.

2.4. Determination of gap junction permeability

Gap junction permeability was determined by the scrape-loading technique described by El-Fouly et al. [17]. Cells were washed with an ionic solution (130 M NaCl, 2.8 mM KCl, 2 mM $MgCl_2$ 10 mM HEPES, pH 7.2) at room temperature (≈22°C). Scrape-loading was carried out with a blade in a solution of 1 mg/ml of Lucifer yellow CH (Sigma), a small (457 Da), highly fluorescent dye that passes through

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gap junctions, but not through the plasma membrane from loaded cells to neighbouring ones. After 2 min, the dye solution was removed and the cells were carefully washed with excess ionic solution. Optionally, a mixture of Lucifer yellow (1 mg/ml) and Rhodamine dextran (Sigma) (1 mg/ml) was used. Because gap junctions are impermeant to Rhodamine dextran (≈10 kDa), the localization of this dye is restricted to the initially loaded cells. In all experiments, 8 min after scraping microphotographs (Kodak Tmax 400 ASA) were taken using an inverted fluorescent microscope equipped with appropriate filters (Diaphot, Nikon). Estimation of junction permeability was based on the method described by Giaume et al. [18] (see also [19]). In each trial, 3–5 side-by-side microphotographs were taken in the center of the culture dish. Negatives were digitized on a CD-ROM and microphotographs were analyzed using an image analyzer software (NIH Image, Wayne Rasband, National Institutes of Health, USA). Data were quantified by measuring the fluorescent areas of the digitized images by calculating the surface occupied by the dye. Threshold detection for Lucifer yellow was determined by measurements of background intensity in dish areas in which no dye was present. For each experimental condition, the size of the fluorescent area was averaged from the data obtained in the adjacent images. The effects caused by the different treatments on junction permeability were analyzed quantitatively by measuring the fluorescence area under each experimental condition, expressing this value as a percentage of that obtained with the controls [19].

2.5. Protein determination

Protein concentration was determined in 0.5 M NaOH-digested cell extracts by the method of Lowry et al. [20], using bovine serum albumin as standard.

2.6. Statistical analysis

Results are expressed as the mean ± S.E.M. values for the number of culture preparations indicated in the legends. Statistical analysis was carried out by Student's *t*-test. In all cases, $p < 0.05$ was considered significant.

3. Results

3.1. Effect of antimycin on gap junction permeability

Astrocytes were well coupled, as shown by the high proportion of fluorescent cells after scrape-loading with Lucifer yellow (Fig. 1a) [17]. Fluorescence intensity gradually decreased from the loaded cells to a distance of about 10 cell diameters. When a mixture of Lucifer yellow (to which gap junction channels are permeable) plus Rhodamine dextran (to which gap junction channels are impermeable) was used, the fluorescence area observed after scrape-loading with the latter dye was restricted to the initially loaded cells (Fig. 1b). Incubation of astrocytes with increasing concentrations of antimycin (1 to 5 ng/ml) for 16 h resulted in a dose-dependent decrease in the fluorescent area occupied by Lucifer yellow (Figs. 1b and 2).

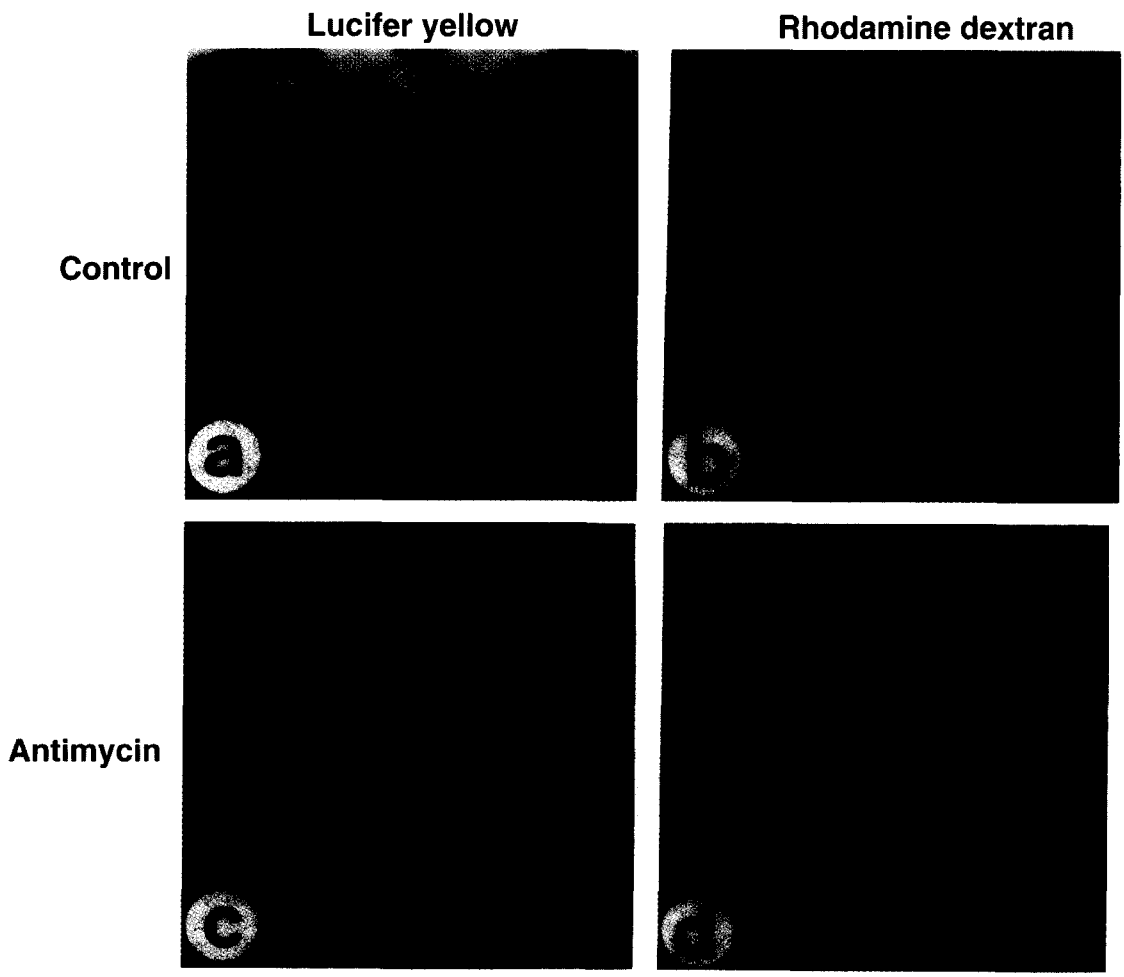

Fig. 1. Fluorescence microphotographs of cultured rat astrocytes after scrape-loading with a mixture of Lucifer yellow and Rhodamine dextran. Astrocytes were incubated in the absence (a,b) or presence (c,d) of 5 ng/ml of antimycin. Microphotographs were taken from the same area using appropriate filters for Lucifer yellow (a,c) or Rhodamine dextran (b,d) to visualize each fluorescent probe. Bar = 25 μm.

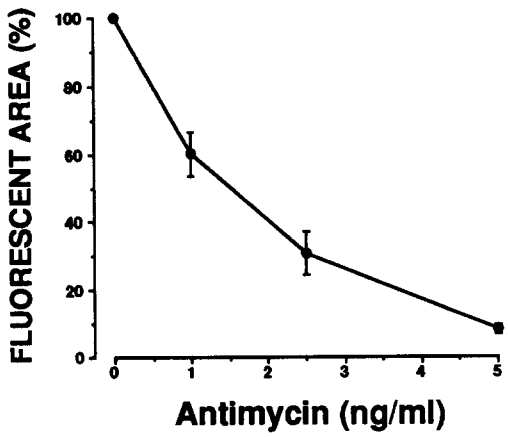

Fig. 2. Antimycin-mediated inhibition of gap junction permeability in astrocytes in primary culture. Confluent cells were incubated with antimycin at the indicated concentrations for 16 h at 37°C and gap junction permeability (expressed as the percentage of fluorescent area) was determined as described in Section 2. Results are the mean ± S.E.M. values for five experiments.

In one set of experiments, untreated astrocytes were exposed to antimycin (5 ng/ml) for 5 min and Lucifer yellow transfer was determined immediately thereafter. Under these conditions, the fluorescent area occupied by the dye was unchanged by the presence of antimycin 94.9 ± 8.2% of the control, $n = 3$). LDH release, which is an index of cellular death, was unchanged by antimycin treatment (0.92 ± 0.4% release of total LDH, $n = 5$). Inhibition of mitochondrial respiration is known to increase lactate release [21], which might acidify the cell culture medium and inhibit gap junction permeability [22]. However, incubation of untreated cells with lactate at similar concentrations to those found in the culture medium after 16 h of antimycin (5 ng/ml) treatment (≈6 mM), did not affect Lucifer yellow transfer (results not shown).

3.2. Effect of antimycin on cellular ATP concentration

Astrocytes were incubated with 5 ng/ml antimycin at 37°C for 16 h and ATP concentrations were determined. Cellular ATP concentrations were modestly but significantly decreased by this treatment (13%; Fig. 3B). Parallel incubations were carried out in which the medium was replaced by fresh DMEM after 16 h and cells were incubated for a further 24 h. Under these conditions, ATP concentrations in cells previously exposed to antimycin were not significantly different from the control ones (Fig. 3B). Restoration of ATP concentrations was accompanied by the reversal of antimycin-induced Lucifer yellow transfer inhibition (Fig. 3A).

3.3. Effect of calcium sequestration on antimycin-induced gap junction inhibition

Antimycin-treated cells were incubated with 1 mM of the calcium chelator EGTA plus 5 μM of the calcium ionophore A23187 for 5 min and scrape/Lucifer yellow loading was carried out as described above. As shown in Fig. 4, the presence of EGTA plus A23187 completely restored the inhibition of gap junction permeability caused by antimycin.

4. Discussion

In the present work we describe the antimycin-induced, dose-dependent inhibition of intercellular communication through gap junctions in astrocytes in primary culture. This effect may be attributed to the inhibition of ATP synthesis, since gap junction permeability was unaffected by a short incubation with antimycin. Moreover, antimycin-dependent inhibition of gap junction permeability was accompanied by a decrease in endogenous ATP concentrations. These results suggest that energy status may play an important role in regulating intercellular communication. Previous work has shown that cell death, and hence depletion of cellular energy stores, is accompanied by irreversible gap junction inhibition, possibly as a consequence of connexin43 dephosphorylation [10]. Under our experimental conditions, however, cell death did not occur. Moreover, replacement of antimycin-containing medium with fresh medium restored both intracellular ATP concentrations and Lucifer yellow transfer, suggesting that the effect of antimycin on gap junction permeability is reversible.

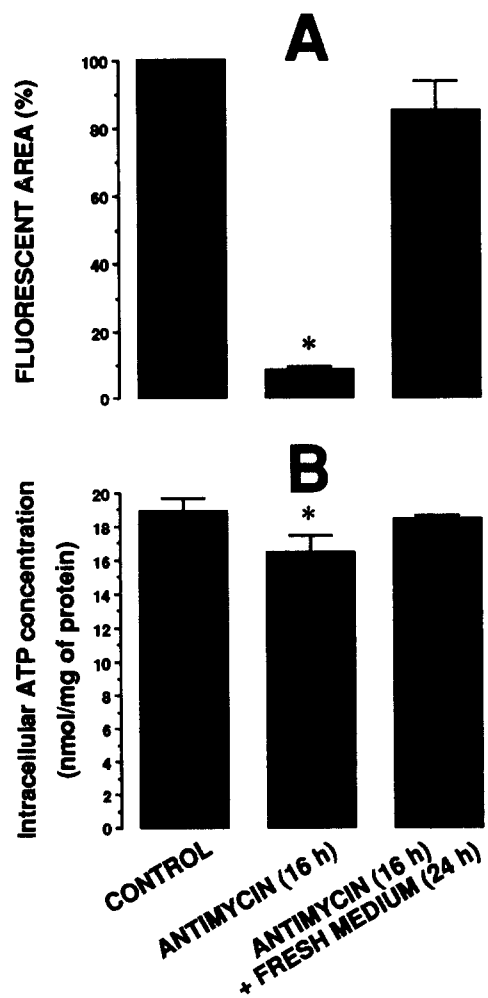

Fig. 3. Reversibility of the inhibition of gap junction permeability by antimycin and intracellular ATP concentration. Confluent cells were incubated with antimycin (5 ng/ml) for 16 h at 37°C and the permeability of gap junctions (expressed as the percentage of fluorescent area) (A) and intracellular ATP concentration (B) were measured as described in Section 2. In one set of experiments, antimycin-treated cells were washed, incubated in fresh medium for a further 24 h and the permeability of gap junctions and intracellular ATP concentration were determined. Results are the mean ± S.E.M. values for five experiments. *Significantly different as compared to antimycin (16 h)+fresh medium (24 h).

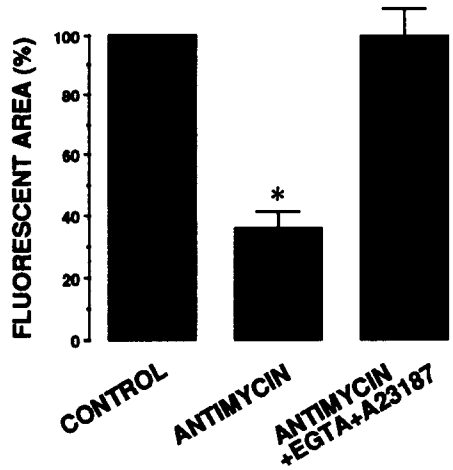

Fig. 4. Effect of calcium sequestration on antimycin-mediated inhibition of gap junction permeability. Confluent cells were incubated with antimycin (5 ng/ml) for 16 h at 37°C. Gap junction permeability (expressed as the percentage of fluorescent area) was assessed in either untreated or antimycin-treated cells after a 5 min incubation period in the absence (control and antimycin) or presence of EGTA (1 mM) plus A23187 (5 μM) (antimycin+EGTA+A23187) as described in Section 2. Results are the mean ± S.E.M. values for five experiments. *Significantly different as compared to antimycin+EGTA+A23187.

The mechanism underlying the inhibition of gap junction permeability by ATP depletion is elusive. Extracellular ATP administration causes an inhibition of gap junction permeability [23], probably through a transient rise in cytosolic free calcium through the activation of the P_2 receptor [24]. Since free calcium is a well-known inhibitor of gap junctional communication [25], the effect of ATP could be mediated by this cation. In agreement with this, the plasma membrane depolarization, an index of gap junction inhibition, observed after extracellular ATP administration has been attributed to the activation of an ion channel-mediated increase in cytosolic free calcium [26]. In contrast to this, it has also been reported that extracellularly administered ATP opens the gap junctional channel [27], an effect which has been suggested to be mediated by the direct reaction of ATP with gap junction proteins [28].

In addition to the effects caused by exogenous ATP, it might be speculated that the decrease in ATP availability would limit substrate supply for cAMP-dependent protein kinase activity, which maintains the coupling through gap junctions [11]. However, under our conditions, the decrease in ATP due to antimycin treatment was relatively small ($\approx$13%) as compared to the strong inhibition of gap junction permeability ($\approx$75–80%). Therefore, an alternative mechanism for ATP depletion-mediated inhibition of gap junction permeability should be suggested. In this context, Harold and Walz [29] have reported that the depletion of endogenous concentrations of ATP depolarizes the astrocyte plasma membrane. This has been interpreted as the occurrence of cell death-induced membrane disintegration [29]. However, our results show that the gap junction permeability inhibition caused by intracellular ATP deficiency is reversible, an observation that cannot be explained in terms of cell death. Moreover, the effects observed in our experiments may be calcium-mediated since the antimycin-mediated inhibition of gap junction permeability was completely prevented by calcium depletion. Accordingly, it may be suggested that ATP deficiency facilitates cytosolic free calcium accumulation, presumably through decreases in the levels of calcium in the endoplasmic reticulum or other reservoirs due to the inhibition of Ca^{2+}-ATPase [30]. In conclusion, our results suggest that a mild depletion of endogenous ATP concentration reversibly inhibits intercellular communication through gap junctions by a calcium-mediated mechanism.

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