The K-ATP channel regulates the effect of Ca^{2+} on gap junction permeability in cultured astrocytes

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Abstract **Using the scrape-loading technique we show that tolbutamide and glybenzcyclamide, two inhibitors of the K^+ channel sensitive to ATP (K-ATP channel), partially prevent the inhibition of gap junction permeability promoted by Ca^{2+} in cultured astrocytes. This effect was dose-dependent, reaching a maximum at 400 μM and 1 μM of tolbutamide and glybenzcyclamide, respectively. The presence of the Ca^{2+} ionophore A-23187 strongly reduced the concentration of Ca^{2+} required to block gap junction permeability but did not abolish the effect of tolbutamide and glybenzcyclamide. These results suggest that the effect of these two compounds are not brought about by control of the intracellular concentration of Ca^{2+} but probably by the promotion of plasma membrane depolarization.**

Key words: Brain; Sulfonylurea; Intercellular communication; Lucifer yellow; Glial cell

1. Introduction

Astrocytes are extensively coupled through the intermembrane channels known as gap junctions [1–3]. These intercellular channels allow the passage from cell to cell of ions and small molecules, such as second messengers [4] or energy metabolites [5,6] (reviewed in [7,8]). The permeability of gap junctions is subject to regulation, and plays an important role in brain metabolism and development [9]. A number of physiological factors have been described as regulators of the permeability of gap junctions in astrocytes (reviewed in [10]; see also [11,12]). Although the ultimate mechanism through which these agents inhibit gap junction permeability is not known, the activation of protein kinase C, the release of arachidonic acid and/or the increase in cytoplasmic Ca^{2+} concentrations have been proposed as possible transduction pathways (for a review see [10]). Thus, gap junction permeability in astrocytes is inhibited by Ca^{2+} although the effect of this cation is prevented by the depolarization of the plasma membrane caused by either increased extracellular K^+ concentrations or the activation of ionotropic glutamate receptors [13].

One of the mechanisms involved in preserving membrane potential is the activity of the K-ATP channel (for a review see [14]). The K-ATP channel is composed of two subunits, KIR (inwardly rectifying K^+ channel) and SUR (sulfonylurea receptor) [15], the latter conferring the channel sensitivity to ATP, sulfonylureas, and to an endogenous ligand, endosulfine. The existence of the K-ATP channel has been reported in the CNS [16], which is sensitive to sulfonylureas [17,18] and to endosulfine [19].

In a previous work we showed that the decrease in intracellular concentrations of ATP due to treatment with antimycin diminishes gap junction permeability in cultured rat astrocytes through a mechanism that is mediated by Ca^{2+} [11]. Because the intracellular concentrations of ATP regulate the open/closed state of the K-ATP channel and the subsequent change in membrane potential, the aim of this work was to investigate whether the K-ATP channel modulates gap junction permeability in astrocytes.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, poly-L-lysine, DNase, bovine serum albumin (BSA), ionophore A-23187, tolbutamide, valinomycin and lucifer yellow were purchased from Sigma (St. Louis, MO, USA). Fetal calf serum (FCS) and trypsin were obtained from Serva Boehringer Ingelheim (Heidelberg, Germany). Glybenzcyclamide was obtained from Aldrich (Milwaukee, WI, USA). Other chemicals were purchased from Sigma or Merck (Darmstadt, Germany).

2.2. Animals

Albino Wistar rats, fed ad libitum on a stock laboratory diet (49.8% carbohydrates, 23.5% protein, 3.7% fat, 5.5% minerals and added vitamins and amino acids; w/w) were used for the experiments. Rats were maintained on a 12 h light-dark cycle.

2.3. Cell cultures

Astrocytes in primary culture were prepared from the brains of 1–2-day-old Wistar rats as previously described [20]. In brief, animals were decapitated and their brains immediately excised. After removing the meninges and blood vessels, the forebrains were placed in Earle's balanced solution containing 20 μg/ml DNase and 0.3% (w/v) BSA. The tissue was minced, washed, centrifuged and incubated in 0.025% (w/v) trypsin and 60 μg/ml DNase for 15 min at 37°C in a shaking water bath. Trypsinization was completed by adding DMEM containing 10% (v/v) FCS. The tissue was then dissociated by trituration, passing it eight times through a siliconized Pasteur pipette and the supernatant cell suspension was recovered. This procedure was repeated and the resulting cell suspension was centrifuged. Culture medium was DMEM supplemented with 10% (v/v) FCS, penicillin (50 U/ml) and streptomycin (50 μg/ml). The cell pellet was resuspended in a known volume of culture medium and plated on poly-L-lysine-coated culture flasks (150 cm^2) at a density of 10^5 cells/cm^2. Cells were incubated at 37°C in an atmosphere of 95% air/5% CO_2 with 90–95% humidity. After 5 days cells were replated onto petri dishes (diameter 3.5 cm) at a density of 1.5×10^6 cells per petri dish. Cytosine arabinoside (10 μM) was added to the culture medium for 2 days to avoid the growth of microglia and cells from the O-2A (oligodendrocyte and type-2 astrocyte) lineage. The culture medium was renewed with a fresh one twice a week. Scrape-loading experiments were performed 2 weeks after seeding, once the cells were highly coupled. Under our experimental conditions, when confluence was reached, 90–95% of the

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BSA, bovine serum albumin; K-ATP, K^+ channel sensitive to ATP; KIR, inwardly rectifying K^+ channel; SUR, sulfonylurea receptor

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cells were astrocytes, as determined by immunostaining against glial fibrillary acidic protein [20,21].

2.4. Assessment of junctional communication

Gap junction permeability was determined by the scrape-loading/dye transfer technique described by El-Fouly et al. [22]. In brief, the culture medium was removed and cells were washed with an ionic solution (130 mM NaCl, 2.8 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 mM HEPES, pH 7.2) and preincubated at room temperature (~22°C) in ionic solution. After 5 min, this solution was removed and cells were further incubated in the same ionic solution without Ca^{2+} [23] for 2 min. Scrape-loading was performed by scraping the cell layer with a broken razor blade in the Ca^{2+}-free ionic solution containing 1 mg/ml of lucifer yellow CH (Sigma), a highly fluorescent dye that passes through gap junctions from loaded cells to neighboring ones, but not through the plasma membrane. After 1 min, the dye solution was removed and the cells were carefully washed with excess ionic solution. Eight minutes after scraping, photomicrographs (Kodak T_{max} 400 ASA) were taken using an inverted fluorescent microscope equipped with appropriate filters (Diaphot, Nikon).

To estimate gap junction permeability, three side-by-side photomicrographs of the center of the dish were taken. Negatives were digitized on a scanner and microphotographs were analyzed using image-analyzer software (NIH Image, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Data were quantified by measuring the fluorescent areas of the digitized images by computation of the surface occupied by the dye. All pictures were large enough to cover the limit of dye diffusion. In each experiment, the fluorescent area occupied by the first line of loaded cells was subtracted from the rest of the experimental conditions. The fluorescent area found in the untreated cells was considered 100% (control). The effects caused by the different treatments on junction permeability were expressed as percentages of the control. For each experimental condition, the size of the fluorescent area was averaged from the data obtained in the adjacent images.

2.5. Cell treatments

The compounds tested were $CaCl_2$ (0.01–5 mM), 130 mM KCl, tolbutamide (100–600 μM), glybenzcyclamide (0.1–1 μM), 2 μM valinomycin and 5 μM of the Ca^{2+} ionophore A-23187. Stock solutions of these compounds were dissolved in water except the Ca^{2+} ionophore A-23187 and valinomycin, which were dissolved in dimethyl sulfoxide and absolute ethanol, respectively. Tolbutamide and glybenzcyclamide were preincubated for 30 min in the culture medium at 37°C. The effects of valinomycin and A-23187 were tested by preincubation for 10 min in the ionic solution at room temperature. All these compounds were added to the solutions used throughout the scrape-loading procedure. The effects of $CaCl_2$ and KCl on gap junction permeability were tested by adding them, at the desired concentrations, to the ionic solution containing lucifer yellow. In the solutions containing 130 mM KCl, NaCl was reduced to 5 mM. For each experimental condition, an equal amount of the vehicle was used as a control. The effects caused by the different treatments on junction permeability were analyzed quantitatively by measuring the fluorescent area under each experimental condition, expressing this value as a percentage of that obtained with the controls. Statistical analyses were performed using Student's *t*-test.

3. Results

Astrocytes were extensively coupled, as shown by the high number of fluorescent cells observed after scrape-loading with lucifer yellow (Fig. 1a). Fig. 1b shows that in the presence of 1.5 mM $CaCl_2$, lucifer yellow was restricted to the first row of loaded cells, indicating that junctional communication in as-

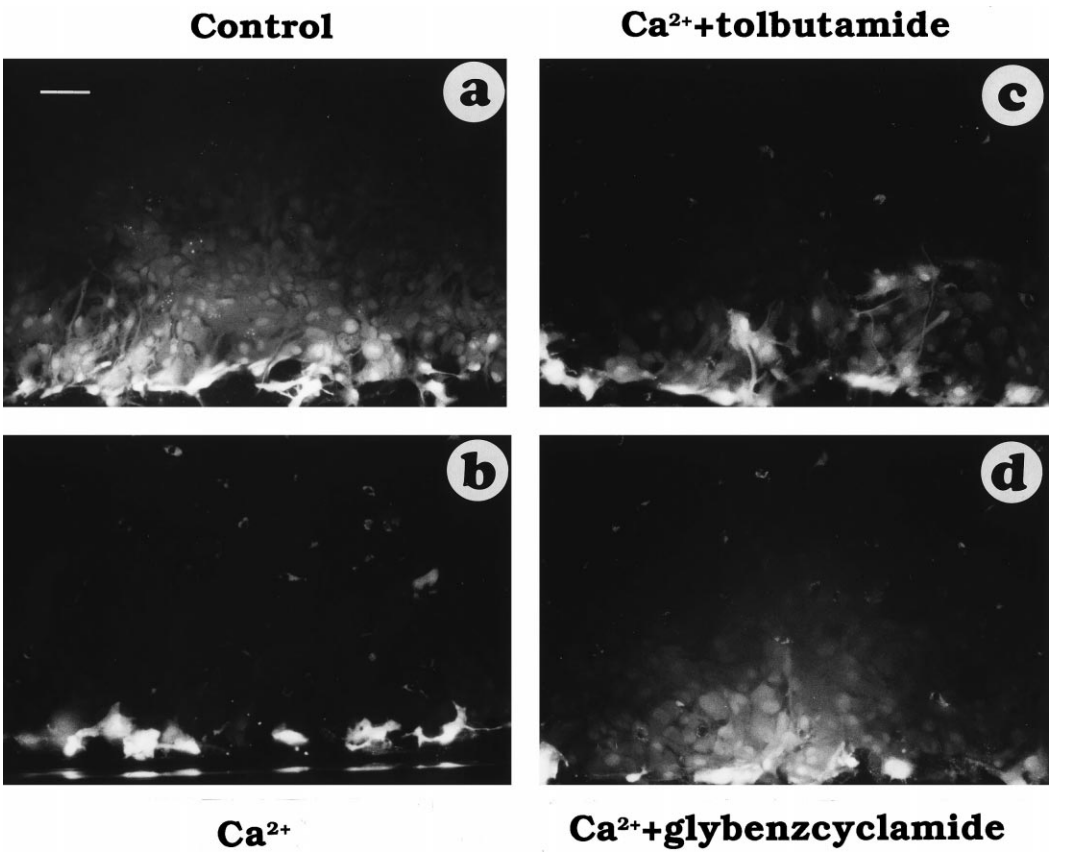

Fig. 1. Fluorescence photomicrographs of cultured rat astrocytes after scrape-loading. Astrocytes were incubated in the absence (a, b) or in the presence of 400 μM tolbutamide (c) or 1 μM glybenzcyclamide (d). Gap junction permeability was determined by the scrape-loading technique as described in Section 2. Scrape-loading was performed in the ionic solution containing lucifer yellow, in the absence (a) or in the presence of 1.5 mM $CaCl_2$ (b, c, d). Microphotographs were taken using appropriate filters for lucifer yellow. Bar = 70 μm.

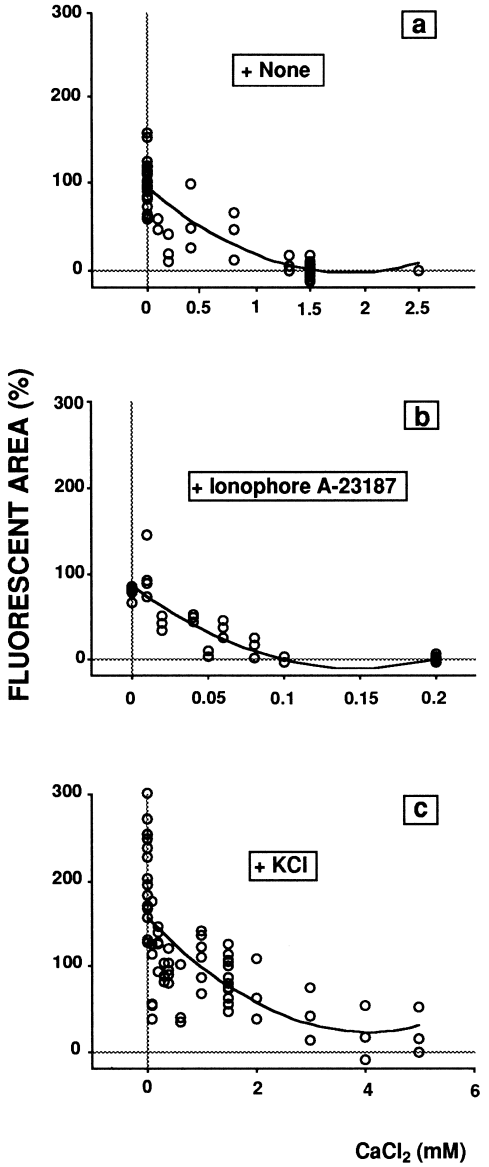

Fig. 2. Effect of increasing Ca^{2+} concentrations on gap junction permeability in cultured astrocytes. Gap junction permeability was determined by the scrape-loading technique as described in Section 2. Increasing concentrations of $CaCl_2$ were added to the ionic solution containing lucifer yellow (a, b, c). Some experiments were carried out in the presence of 5 μM of the Ca^{2+} ionophore A-23187 (b). Some scrapes were performed with the ionic solution containing lucifer yellow, 130 mM KCl and 5 mM NaCl (c). The lucifer yellow fluorescent area was quantified and the results from at least three different cultures were expressed as percentages of the controls. The values were adjusted to the following functions:
(a) $y = 95.1 - 0.11x + 2.8 \times 10^{-5}x^2$, $P < 0.001$;
(b) $y = 88.6 - 1.4x + 5.0 \times 10^{-3}x^2$, $P < 0.001$;
(c) $y = 158 - 0.07x + 8.2 \times 10^{-6}x^2$, $P < 0.001$.

trocytes was inhibited. Our results show that $CaCl_2$ inhibited gap junction communication in a dose-dependent manner (Fig. 2a), the maximum effect being reached when the concentration of $CaCl_2$ in the scrape solution was about 1.5 mM. In order to facilitate the access of Ca^{2+} into the cell, the astrocytes were treated with the Ca^{2+} ionophore A-23187 and the scrape was then performed in a solution with increasing concentrations of $CaCl_2$ (Fig. 2b). Under these circumstances, the presence of $CaCl_2$ decreased the lucifer yellow fluorescent area in a dose-dependent manner, the maximum effect being reached at concentrations of $CaCl_2$ of about 0.1 mM. Thus, the presence of the Ca^{2+} ionophore decreased the concentration of $CaCl_2$ required for the inhibition of gap junction permeability by approximately 15-fold.

In order to confirm previous works reporting that junctional inhibition brought about by Ca^{2+} is dependent on membrane polarity [13], some experiments were carried out after depolarization of the plasma membrane. To do so, the K^+ concentration was increased (130 mM KCl) while the Na^+ concentration was lowered (5 mM NaCl) in the ionic solution containing lucifer yellow. Again, the scrape was performed in this solution with increasing concentrations of $CaCl_2$ (Fig. 2c). Under these circumstances, $CaCl_2$ was able to reduce the lucifer yellow fluorescent area in a dose-dependent manner, although the concentration of $CaCl_2$ needed to reach a maximum effect was approximately 4 mM, i.e. nearly threefold higher than the concentration of $CaCl_2$ required in the presence of low K^+ concentrations. Moreover, in the presence

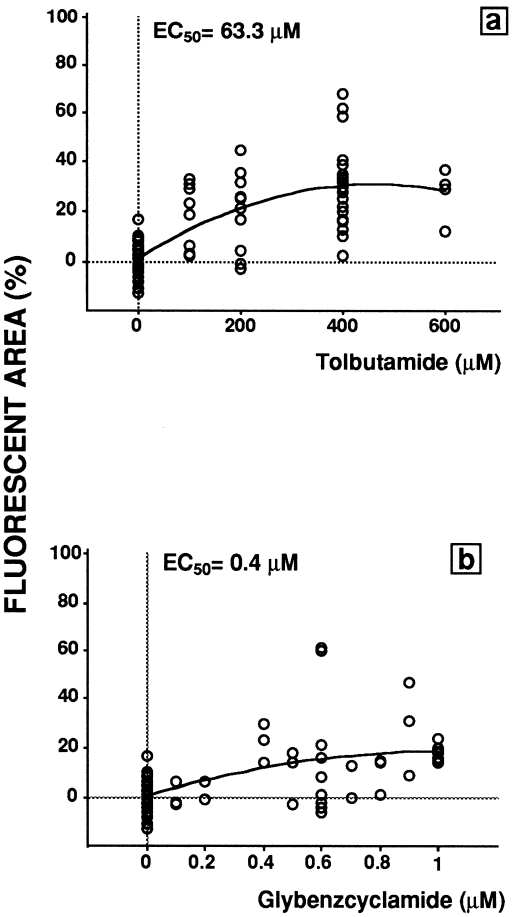

Fig. 3. Effect of tolbutamide and glybenzcyclamide in the Ca^{2+}-elicited inhibition of gap junction permeability. Astrocytes were incubated with increasing concentrations of tolbutamide (a) or glybenzcyclamide (b). 1.5 mM $CaCl_2$ was added to the ionic solution containing lucifer yellow. Scrape-loading was performed as described in Section 2. The lucifer yellow fluorescent area was quantified and the results from at least three different cultures were expressed as percentages of the controls. The values were adjusted to the following functions: (a) $y = 1.25 + 0.13x - 1.4 \times 10^{-4}x^2$, $P < 0.001$; (b) $y = 0.5 + 0.04x - 17 \times 10^{-5}x^2$, $P < 0.001$. EC_{50}, concentration that produces 50% of the maximal effect.

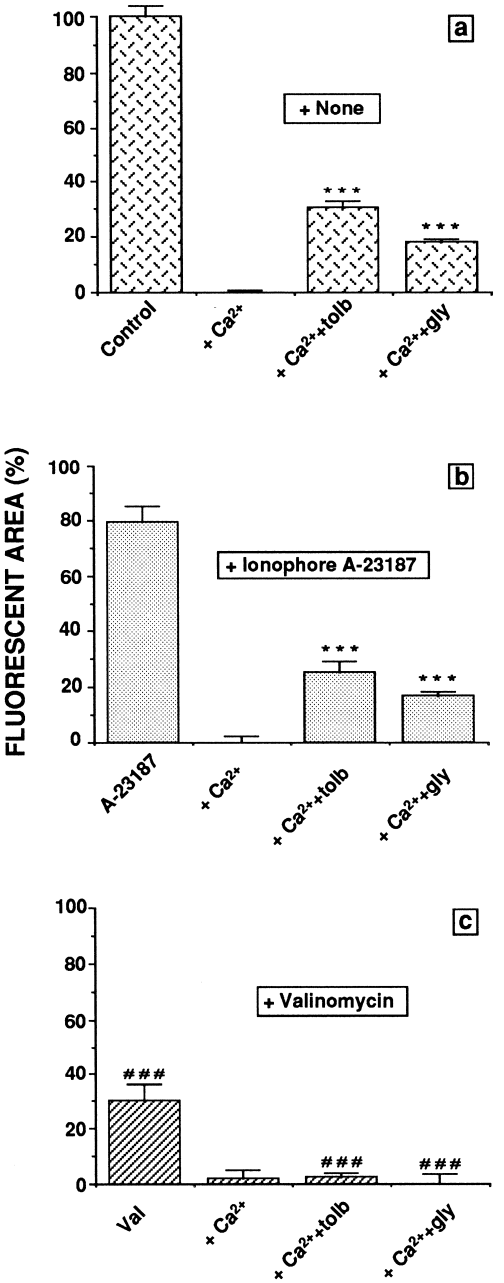

Fig. 4. Effect of sulfonylureas on the inhibition of gap junction permeability promoted by Ca^{2+} in the absence (a), or in the presence of the Ca^{2+} ionophore A-23187 (b) or valinomycin (c). Astrocytes were exposed to 1.5 mM $CaCl_2$, 1.5 mM $CaCl_2$ plus 400 μM tolbutamide or 1.5 mM $CaCl_2$ plus 1.5 μM glybenzcyclamide in the absence (a) or in the presence of 5 μM A-23187 (b) or 2 μM valinomycin (c). Scrape-loading was performed as described in Section 2. The lucifer yellow fluorescent area was quantified and the results from at least three different cultures were expressed as percentages of the controls and are means ± S.E.M. Statistical differences between the values obtained with different treatments as compared with those obtained in the presence of 1.5 mM $CaCl_2$ were expressed as $***P < 0.001$. Statistical differences between the values obtained in the absence (a) and in the presence of the Ca^{2+}-ionophore A-23187 (b; not significant) or valinomycin (c) were expressed as $P < 0.001$. Tolb, tolbutamide; gly, glybenzyclamide; Val, valinomycin.

of 130 mM KCl, lucifer yellow was never restricted to the first row of loaded cells because lucifer yellow still diffused through approximately 20% of the fluorescent area in the presence of 5 mM $CaCl_2$, (Fig. 2c). It should be mentioned that the presence of high extracellular K^+ elicited an approximately 100% increase in the fluorescent area as compared with untreated cells (Fig. 2c).

Our results show that treatment with tolbutamide (Fig. 1c) or glybenzcyclamide (Fig. 1d) partially prevents the inhibitory effect of Ca^{2+} on gap junctional permeability. Thus, when 1.5 mM $CaCl_2$ was present in the scrape solution, the lucifer yellow fluorescent area was increased from 0% to approximately 30% by the presence of increasing concentrations of tolbutamide (Fig. 3a). The effect of tolbutamide was dose-dependent and reached a maximum at tolbutamide concentrations of about 400 μM. These values were adjusted to a second order polynomial function; using this function the concentration of tolbutamide that produces 50% of its maximal effect (EC_{50}) was calculated to be 63.3 μM. It should be mentioned that treatment with 400 μM tolbutamide promoted an increase in the lucifer yellow fluorescent area of 19.8 ± 2.8% ($n = 10$; $P < 0.01$) as compared to the control. Similar results were obtained with another sulfonylurea, glybenzcyclamide, which increased the spreading of lucifer yellow, measured as fluorescent area, by 15.8 ± 2.8% ($n = 7$; $P < 0.05$). In addition, in the presence of 1.5 mM $CaCl_2$, the fluorescent area was increased from 0% to approximately 20% by increasing glybenzcyclamide concentrations (Fig. 3b). This effect was also dose-dependent, reaching a maximum at concentrations of glybenzcyclamide of about 1 μM. The data obtained from the glybenzcyclamide-treated cells were also fitted to a second order polynomial function, the EC_{50} being 0.4 μM. Therefore, the EC_{50} for glybenzcyclamide was about 1000-fold lower than that of tolbutamide, in agreement with their reported affinities for the sulfonylurea receptor [18,24].

In the presence of the Ca^{2+} ionophore A-23187, tolbutamide and glybenzcyclamide still were able to partially prevent the inhibitory effect of Ca^{2+} on astrocytic gap junction permeability (Fig. 4b). However, in the presence of valinomycin, a K^+-specific ionophore that equilibrates K^+ concentrations across the plasma membrane, sulfonylureas were not able to prevent the inhibitory effect of Ca^{2+} on gap junctional permeability (Fig. 4c).

4. Discussion

The potassium channel sensitive to ATP (K-ATP) comprises a K^+ pore (KIR) and a regulating subunit (SUR) that confers the channel sensitivity to ATP, sulfonylureas and endosulfine (for a recent review see [15]). Our results show that the presence of tolbutamide or glybenzcyclamide, two well-known sulfonylureas, significantly decreases the inhibitory effect of Ca^{2+} on gap junction permeability in astrocytes (Figs. 1 and 3). These observations suggest that the K-ATP channel controls permeability through gap junctions in astrocytes. Indeed, the increase in extracellular K^+ concentrations also decreased the inhibitory effect of Ca^{2+} (Fig. 2c and [13]), suggesting that the effect of Ca^{2+} is associated with the occurrence of an outward flux of K^+. In addition, the presence of valinomycin, a potassium ionophore that equilibrates K^+ concentrations across the plasma membrane, abolished the preventive effect of tolbutamide or glybenzcyclamide on the inhibition of gap junction permeability caused by Ca^{2+} (Fig. 4c). This is consistent with the idea that the K-ATP

channel would control the regulation of gap junction permeability brought about by Ca^{2+}.

It could be speculated that the K-ATP channel controls gap junction permeability by modulating Ca^{2+} concentrations in the cytosol. If so, the inhibition of the K-ATP channel would prevent the entry of Ca^{2+}, resulting in an enhancement of gap junction permeability. Contrariwise, the activity of the K-ATP channel would allow Ca^{2+} entry which inhibits gap junction permeability. However, this does not seem the mechanism operating under these circumstances, because tolbutamide or glybenzcyclamide still decreased the effect of Ca^{2+} in the presence of the Ca^{2+} ionophore A-23187 (Fig. 4b), suggesting that the effect of K-ATP channel on gap junction permeability is not associated with changes in cytosolic free Ca^{2+} concentrations. Instead, membrane depolarization caused by the inhibition K-ATP channel would prevent the inhibition of gap junction permeability caused by Ca^{2+}, a fact consistent with the idea that the inhibitory effect of Ca^{2+} requires a polarized membrane [13]. In agreement with this, membrane hyperpolarization caused by valinomycin alone sharply decreased gap junction permeability (Fig. 4c; [13]) while the depolarization caused by increasing extracellular concentrations of K^+ sharply increased gap junction permeability (Fig. 2c; [13]). It may therefore be be concluded that the K-ATP channel regulates gap junction permeability by controlling membrane potential through a mechanism that is independent of but mandatory in the Ca^{2+} effect.

Because tolbutamide and glybenzcyclamide mimic the effects of ATP on the K-ATP channel, it may be suggested that intracellular ATP concentrations regulate gap junction permeability [11] through the control of the K-ATP channel. The physiological meaning of this effect is not evident but it is tempting to speculate that the energy status of the astrocyte would be able to regulate intercellular communication through gap junctions. When the energy status of the astrocyte was sufficient to sustain its own metabolic machinery, gap junction permeability would increase to allow the passage of metabolites [5,6] and signals [25] to adjacent cells. However, if the energy reserves of the astrocyte were compromised, ATP concentrations would decrease, resulting in a drop in gap junction permeability [11].

We have previously suggested that astrocytic gap junctions play an important role in 'pipelining' metabolic substrates through the CNS by acting like waterway locks that allow the establishment of a cell-to-cell metabolite gradient that fuelled the transport of substrates to targeted neurons [5,6]. If so, ATP concentrations may regulate the open/closed state of the 'locks' by controlling the activity of the K-ATP channel. Once the incoming substrates have fulfilled the energy requirements of the cells, gap junction permeability would increase to allow spared metabolic substrates to pass to adjacent cells, thus distributing energy and carbon skeletons through the CNS.

In conclusion, the K-ATP channel may play an important role in controlling the transport of ions, metabolites and signals through the CNS, thus regulating the crucial collaboration between astrocytes and neurons. This also allocates an important role to endosulfine, an endogenous putative physiological inhibitor of the K-ATP channel [19].

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