

Intracellular spermine prevents acid-induced uncoupling of Cx43 gap junction channels

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Polyamines (PAs), such as spermine and spermidine, modulate the activity of numerous receptors and channels in the central nervous system (CNS) and are stored in glial cells; however, little attention has been paid to their role in the regulation of connexin (Cx)-based gap junction channels. We have previously shown that PAs facilitate diffusion of Lucifer Yellow through astrocytic gap junctions in acute brain slices; therefore, we hypothesized that spermine can regulate Cx43-mediated (as the most abundant Cx in astrocytes) gap junctional communication. We used electrophysiological patch-clamp recording from paired Novikoff cells endogenously expressing Cx43 and HeLaCx43-EGFP transfectants to study pH-dependent modulation of cell–cell coupling in the presence or absence of PAs. Our results showed (i) a higher increase in gap junctional communication at higher concentrations of cytoplasmic spermine, and (ii) that spermine prevented uncoupling of gap junctions at low intracellular pH. Taken together, we conclude that spermine enhances

Cx43-mediated gap junctional communication and may preserve neuronal excitability during ischemia and trauma when pH in the brain acidifies. We, therefore, suggest a new role of spermine in the regulation of a Cx43-based network under (patho)physiological conditions. *NeuroReport* 26:528–532 Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

NeuroReport 2015, 26:528–532

Keywords: Cx43 gap junction channels, polyamines, spermine

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Received 11 March 2015 accepted 28 April 2015

Introduction

Polyamines (PAs) are involved in numerous physiological processes including gene expression, protein and nucleic acid function and synthesis, protection against oxidative stress [1,2], and increasing longevity [3–5]. The brain contains large amounts of PAs, mainly spermidine and spermine [6–8], and an age-dependent depletion of PAs has been reported [4,5,9]. Trauma also causes release and loss of PAs in the brain [6]. Intriguingly, spermidine and spermine are not synthesized in glial cells in the adult brain [10]; however, they accumulate almost exclusively in glia. Astrocytes rather than neurons contain spermidine and spermine in the cortex and hippocampus [7], as well as in Müller and Bergmann glial cells in the retina [11,12] and cerebellum [7], respectively. This suggests that PAs are transported into the glia from external sources through blood and cerebrospinal fluid [1,2,8] to be further diffused through the glial network [8,13]. PAs can regulate glial [8, 12–16] and other cell receptors/channels [17,18] and transporters [14,15] intracellularly and extracellularly.

Intracellular PAs induce a voltage-dependent block of inwardly rectifying potassium (Kir) channels, nicotinic

acetylcholine receptors, GluA-2-lacking glutamate receptor channels, GluN1/GluN2 *N*-methyl-D-aspartic acid receptor channels, olfactory cyclic nucleotide-gated cation channels, voltage-gated sodium channels, and transient receptor potential channels of the vanilloid subfamily, the melastatin subfamily, and canonical [8]. In addition, *N*-methyl-D-aspartic acid and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor/Kainate channels show a double rectification and potentiation in the presence of intracellular and extracellular PAs, because PAs can weaken hydrogen block of such channels [8,18]. To this list can be added PA-sensitive connexin (Cx) channels mediating cell-to-cell exchange of ions and macromolecules through gap junctions in glia [13, 19,20]. As most of the mentioned neuronal receptors and channels have also been found in glial cells [8], it is evident that PAs plays a similar regulatory role in astrocytes as well.

The glial network or panglial syncytium consists of Cx26, Cx30, Cx32, Cx43, Cx45, and Cx47, which have been shown to be expressed in glia and are responsible for electrical and molecular signaling. Among these Cxs, Cx43 and Cx30 play a major role because their knockout resulted in a lack of gap junctional communication in astrocytes [21]. Although PAs have been shown to increase gap junctional communication between astrocytes [13], the mechanism underlying this endogenous regulation remains to be determined.

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In this study, we used Novikoff cells endogenously expressing Cx43, as well as HeLaCx43 stable transfectants to investigate the interaction between PAs, acidification, and gap junctional communication. We found that spermine increased Cx43 junctional conductance (g_j) in a concentration-dependent manner and reversed acidification-induced uncoupling.

Materials and methods

Cell lines and cell cultures

Novikoff cells (a rat hepatoma cell line) that endogenously express Cx43 [22] were seeded in uncoated 75 cm² flasks at a density of 60 000 cells/cm², and the culture medium was exchanged every 3 days. At confluence, the cells were dissociated by trypsinization and reseeded onto glass coverslips in 35-mm Petri dishes. The cells were maintained in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, Missouri, USA) supplemented with 10 mM glucose, 2 mM L-glutamine, 10% fetal calf serum, and 200 IU/ml penicillin/200 µg/ml streptomycin at 37°C (5% CO₂, 95% air).

The cDNA constructs encoding Cx43-EGFP were made using HeLa cells as described by Bukauskas *et al.* [22]. HeLa cells transfected with cDNAs encoding rat Cx43 or rat Cx43 with EGFP attached to the C terminus (Cx43-EGFP) were maintained in culture as described by Bukauskas *et al.* [22]. Cx43-EGFP cells could be recognized by their fluorescence at 530 nm.

For electrophysiological analysis the cells were seeded onto coverslips placed in culture dishes at 1000 cells/cm² 16–24 h before the experiment.

Patch clamp from cell pairs in cell cultures

Coverslips with cultured cells were transferred to a recording chamber (RC-27L; Warner Instr. Corp., Hamden, Connecticut, USA) adapted on the stage of an Olympus upright microscope (Olympus, Shinjuku-ku, Tokyo, Japan) with infrared and fluorescence attachments. Cells were visualized using the Nomarski optical infrared attachment equipped with DIC (BX51WI; Olympus) and a DP30BW digital camera with DP Controller software (Olympus).

Two piezoelectric micromanipulators (MX7500 with MC-1000 drive; Siskiyou Inc., Grants Pass, Oregon, USA) were used for precise positioning of the micropipettes. Whole-cell recordings were performed in pairs of Novikoff and HeLaCx43-EGFP cells using a dual whole-cell voltage clamp. The extracellular perfusion solution (extracellular solution; Sigma-Aldridge, St. Louis, Missouri, USA) contained (in mM): NaCl, 140; CaCl₂, 2.5; MgCl₂, 2; HEPES, 10; and KCl, 3 (osmolarity was kept stable at 308 mosmol/l). HEKA amplifiers (EPC-10, three channels, Germany) were used to acquire, store, and analyze the data obtained from pairs of cells.

Junctional current (I_j), measured in cell 2 was divided by voltage (V_j), applied to cell 1 to calculate junctional

conductance (g_j). Data records were digitized at 5 kHz and filtered at 1 kHz.

Electrodes and intracellular solutions

Patch pipettes were fabricated from Clark capillaries (outer diameter, 1.5 mm/inner diameter, 0.86 mm; #300058; Harvard Apparatus, Holliston, Massachusetts, USA) using a P-97 puller (Sutter Instr. Co., Novato, California, USA). For dual whole-cell voltage clamp recording, the patch pipettes were filled with a pipette solution containing: KCl, 140 mM; HEPES-KOH, 10 mM; EGTA, 2 mM; CaCl₂, 0.2 mM; MgCl₂, 1 mM; and pH 7.2 spermine, 100 µM–10 mM. The resistance of the pipette was in the range of 5–8 MΩ. Intracellular pH was adjusted by lowering the pH in the pipettes to pH 6 using MES buffer. To measure intracellular pH, cells were transfected with EGFP, and intensity of the EGFP fluorescence was used as criteria to estimate pH_i.

Data analysis

Data were analyzed using Origin 9.1 software (OriginLab, Northampton, Massachusetts, USA) and are reported as mean ± SEM. Significant differences between groups of data were evaluated using *t*-tests ($P < 0.05$).

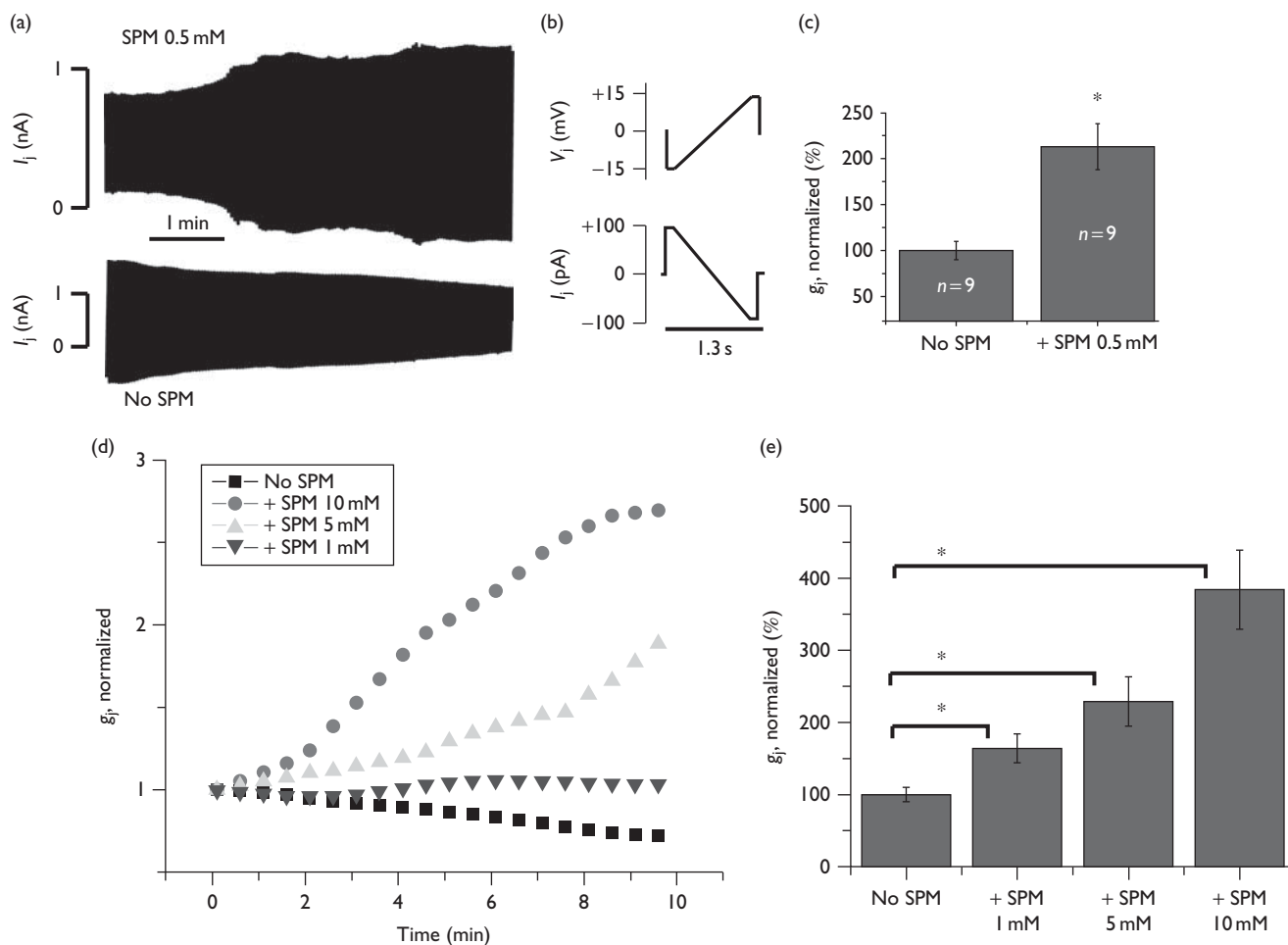
Results

Spermine enhances Cx43-mediated gap junctional communication

Recently, in acute brain slices it was shown that spermine added intracellularly facilitated the spread of Lucifer Yellow in the astrocytic syncytium [13]. The number of fluorescent cells increased about 10-fold [13] when spermine (1 mM) was added in the astrocyte. The most abundant Cx in astrocytes is Cx43 [21]; therefore, we focused on Cx43-expressing cells and examined the effect of spermine on junctional current (I_j) using whole-cell patch-clamp recordings from Novikoff and HeLaCx43-EGFP cell pairs.

Typically it takes about 2 min to dialyze and fully replenish glial cells with PAs through a patch pipette [16]. We found enhanced gap junctional communication in Novikoff (Fig. 1d and e) and HeLaCx43-EGFP cells (Fig. 1a–c; by 214 ± 25%, $P < 0.05$, $n = 9$) with the presence of spermine in both pipettes. In contrast, if patch pipettes did not contain spermine, we observed a run-down of I_j (Fig. 1a and d). The latter suggests that washout of endogenous PAs from cells (any proliferating cells contain endogenous PAs) triggers moderate uncoupling of the cells. While keeping the internal pH_i stable at 7.2, we observed that the gap junctional communication increased with a rise in spermine concentration in the pipette solution from 1 to 5 and 10 mM by 164 ± 20, 229 ± 34, and 384 ± 55%, respectively ($P < 0.05$, $n = 5$ in each group; Fig. 1d and e).

Fig. 1



Effect of spermine (SPM) on Cx43 gap junction channels. (a) Examples of junctional current (I_j) records from HeLaCx43-EGFP cell pairs in response to a repeated step-ramp-step protocol shown in (b). The presence of SPM in the pipette solution caused an increase, whereas the absence of SPM produced a decrease in I_j over time. (b) Voltage (V_j) protocol used to study I_j changes over time. (c) Summarized data after 10 min of recording in the presence or absence of 0.5 mM SPM in the pipette solution. Gap junctional conductance was calculated and normalized. Error bars represent SEM. *Significant difference ($P < 0.05$, $n = 9$ in each group). (d) Averaged junctional conductance (g_j) measurements in Novikoff cell pairs using different intracellular concentrations of SPM: no SPM, 1 mM SPM, 5 mM SPM, and 10 mM SPM. (e) Summarized data of experiments shown in (d) at 10 min of the recordings. Error bars represent SEM. *Significant difference between groups ($P < 0.05$, $n = 5$ in each group).

Spermine prevents acidification-induced uncoupling of Cx43 gap junctions

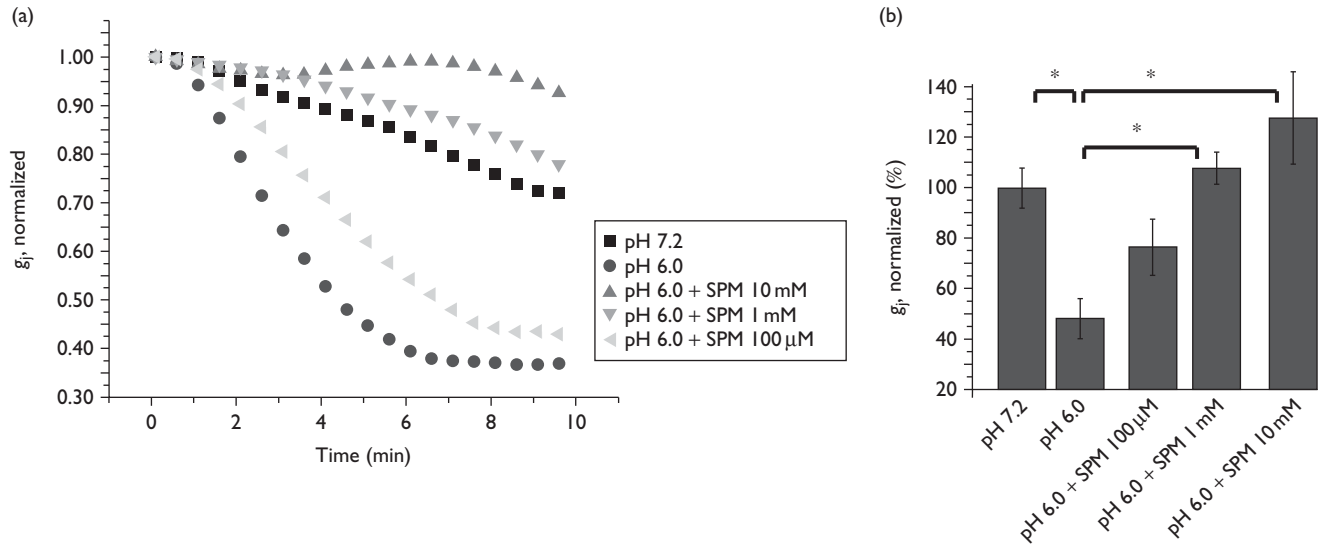
Hydrogen cations (H^+) are known blockers of Cxs, including Cx43 [23,24]. We found that PAs not only increase Cx43-mediated gap junctional communication (Fig. 1), but also protect Cx43 gap junctions from acidification-induced uncoupling (Fig. 2). For this, we recorded I_j from Novikoff cells, in which internal acidification was induced by reducing the pH_i of the pipette solution to 6 (Fig. 2). Within a few minutes after patch opening, I_j declined on average to $35 \pm 10\%$ ($P < 0.05$, $n = 4$; Fig. 2a and b). This g_j reduction was rescued by PAs added to the pipette (Fig. 2a and b). At a concentration of spermine greater than 1 mM, the blocking effect at $pH_i = 6$ was fully removed (Fig. 2a and b).

Discussion

Our data (Figs 1 and 2) show that spermine (i) produces robust enhancement of communication through Cx43 gap junctions under normal conditions and (ii) can unblock closure of these gap junctions during cytoplasmic acidification of Cx43-expressing cells. A similar enhancement of astrocyte-to-astrocyte communication under normal conditions was observed in brain slices [13]. Astrocytes also predominantly express Cx43 in addition to Cx30 [21]. Taken together, these data show the unique capability of spermine to enhance Cx43-mediated gap junctions; the specific channels expressed in brain astrocytes, not in neurons.

The large amount of PAs in the brain [7] and retina [12] is accumulated almost exclusively in the glia, not in

Fig. 2



Spermine (SPM) rescues uncoupling of Cx43 gap junctions induced by intracellular acidification (pH_i6) of Novikoff cells. (a) Averaged and normalized junctional conductance (g_j) measured in Novikoff cells. Intracellular acidification (pH_i = 6) significantly enhanced g_j decay compared with g_j changes at pH_i = 7.2. SPM added in the patch pipette solution transformed g_j decay into g_j increase even at pH_i = 6. (b) Summarized data from (a) at 10 min of the recordings. Error bars represent SEM. *Significant difference ($P < 0.05$, $n = 4$ in each group).

neurons. Spermine accumulating in the glia can regulate neurons in two ways: (i) opening glial cell Cx43 gap junctions will contribute to activation of the glial syncytium and to better potassium, water, and glutamate buffering [21] during neuronal activity, and (ii) when spermine opens large pores made from Cx43 hemichannels in astrocytes, it will allow permeation of different neuroactive substances such as ATP, glutamate, and others through these pores. In addition, we propose that, spermine may exit the glial cytoplasm through the Cx43 hemichannels and modulate neuronal channels and receptors, because many neuronal channels are very sensitive to spermine [18]. The conditions under which spermine is liberated from cytoplasmic buffers, as well as the functional role of spermine in regulation of glial Cx43 hemichannels, require additional study.

Indeed, multiple biological effects of PAs have been reported, including increasing longevity [3], memory [4,5], cell proliferation, and differentiation; regulation of receptors and channels [16,18]; modulation of transporters [14,15], behavior, and learning; and antinociceptive, neuroprotective, antidepressant, and antioxidant effects. Glial cells and their PA-dependent proteins such as PA transporters, Kir4.1 channels, and Cx43 are involved in these processes [8]. As highlighted in numerous studies, the mechanistic aspects of PA functions in the brain remain poorly identified; however, there are many links and correlations between diseases, syndromes, and disorders of the central nervous system and PAs: glioblastoma multiforme, neuropathic pain, migraines, global amnesia, autism, depression, suicidal tendencies, stress, anxiety, sleeplessness, memory loss, and drug addiction are

among a host of devastating neurological diseases and disorders linked to the glia and PAs for which a prevention or cure must be found [8]. Epidemiological studies have demonstrated a correlation between development of Alzheimer's, Parkinson's, and Huntington's diseases, along with amyotrophic lateral sclerosis and aging, and depletion of PAs in the brain, and these data suggest the involvement of the glia and PAs in brain (dys)function [2,8,13].

Since their original discovery by Leeuwenhoek, the PAs spermine and spermidine have attracted the attention of scientists and clinicians [1,2]. Partly because of neurological complications in anticancer treatment using a block of polyamine biochemistry, this approach failed, and later studies have shown that this could have been predicted because of the existence of multiple effects of PAs on receptors and channels in the brain [8,13,17,18].

However, as free endogenous PAs in millimolar concentrations were found almost exclusively in the glia, not in neurons [7,11,12,15,16], and as PAs are colocalized with Cx43, the following questions arise: What are the mechanisms of regulating Cx43 by PAs? and What is the role of PA content (loss/restoration) in CNS function? Interestingly, whereas Cx40 is blocked by PAs [25], the Cx43 gap junctions are opened by PAs (Figs 1 and 2). Glutamate residues at positions 9 and 13 of Cx40 are responsible for spermine block. If these glutamate residues are replaced with lysine, the block by spermine is eliminated [25].

Our data spotlight wider areas in which PAs and Cx43 are both involved. Outside the central nervous system, Cx43

is most abundantly expressed in an assortment of cell types, including cardiomyocytes, epithelial cells, and hepatocytes, and it plays an important role in many (patho)physiological processes. This makes PAs attractive for study of Cx43 regulation in relevance to diseases.

Conclusion

In this study, we focused specifically on Cx43 and the polyamine spermine, and their interaction. We found that spermine augments the communication through Cx43 gap junctions under normal conditions and rescues Cx43 gap junctions from acidification-induced uncoupling. Thus, spermine not only regulates Cx43 gap junction permeability, but may prevent the Cx43-based network from uncoupling during ischemia, neuronal hyperexcitability, or other pathological conditions that lead to acidification.

Acknowledgements

This work was supported by NIH grants: SC2 GM095410 from NIGMS (to Y.V.K.); R01 NS065201 from NINDS (to S.N.S.); R01 NS072238 (to F.F.B.); V PPOHA P031S130068, G12 RR03035-Project-A from NIMHD (to J.B. and M.I.) and G12 MD007583 from NIMHD (for core facilities at UCC).

Conflicts of interest

There are no conflicts of interest.

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