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Extracellular K⁺ and Astrocyte Signaling via Connexin and Pannexin Channels

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Abstract

Astrocytes utilize two major pathways to achieve long distance intercellular communication. One pathway involves direct gap junction mediated signal transmission and the other consists of release of ATP through pannexin channels and excitation of purinergic receptors on nearby cells. Elevated extracellular potassium to levels occurring around hyperactive neurons affects both gap junction and pannexin1 channels. The action on Cx43 gap junctions is to increase intercellular coupling for a period that long outlasts the stimulus. This long term increase in coupling, termed “LINC”, is mediated through calcium and calmodulin dependent activation of calmodulin dependent kinase (CaMK). Pannexin1 can be activated by elevations in extracellular potassium through a mechanism that is quite different. In this case, potassium shifts activation potentials to more physiological range, thereby allowing channel opening at resting or slightly depolarized potentials. Enhanced activity of both these channel types by elevations in extracellular potassium of the magnitude occurring during periods of high neuronal activity likely has profound effects on intercellular signaling among astrocytes in the nervous system.

Keywords

Gap junction; Dye-coupling; Calcium waves; ATP release

Introduction

In the central nervous system (CNS), extracellular K⁺ fluctuations occur under physiological and pathological conditions. Local, physiological variations in extracellular K⁺ concentration ([K⁺]_o) occur following neuronal activity which can lead to increases of this ion from a basal level of 3 mM to up to 10–12 mM, the ceiling level, depending on the brain region [1–10]. This ceiling, or plateau level, is exceeded only under pathological conditions [11, 12]. Pathological levels of extracellular K⁺ are attributed to hyperexcitability and or cell death such as occurs during epileptiform activity, when [K⁺]_o reaches 15 mM, and during anoxia, hypoglycemia, and spreading depression, when [K⁺]_o may increase to 30–80 mM [4, 13, 14].

Here we present a brief overview of what is known about the responses of astrocytes to changes in extracellular K⁺ levels, emphasizing the contribution of gap junction proteins (connexins and pannexins) to astrocyte signaling.

Glia Responses to Extracellular K⁺ Fluctuations

It has been known for a long time that astrocytes are involved in the maintenance of $[K^+]_o$ via the uptake of K⁺ through ion channels, transporters and its dissipation through the so called K⁺ spatial buffering process [15-17]. Moreover, elevated extracellular K⁺ alters several astrocytic processes with important implications for the progression of the disease state. For instance, membrane depolarization, accumulation of K⁺ and cell swelling are some of the effects that elevated extracellular K⁺ induces in astrocytes [18, 19]. As a result of elevated extracellular K⁺, there is increased glycolysis in astrocytes due to the stimulation of Na⁺/K⁺-ATPase by the cation [20]. In response to membrane depolarization there is intracellular pH alkalization due to influx of HCO₃⁻ via the Na⁺/2HCO₃⁻ cotransporter [21-24]. Such change in intracellular pH also leads to alterations in enzymatic activity, especially those related to glycolysis, which is increased [25]. Accumulation of K⁺ in astrocytes has also been related to enhanced glycolysis via stimulation of the enzyme pyruvate carboxylase [26]. Thus, through several convergent mechanisms, elevation of extracellular K⁺ results in altered astrocyte metabolism.

Elevated extracellular K⁺ and/or membrane depolarization of astrocytes leads to the influx of Ca²⁺ through dihydropyridine sensitive voltage activated Ca²⁺ channels [27, 28]. Calcium elevation in astrocytes has been implicated in several processes, including modulation of gap junctional communication, “gliotransmitter” release and intercellular calcium waves, the last being a mechanism by which astrocytes communicate with one another over long distances.

Gap Junction Proteins in Astrocytes: Connexins and Pannexins

Gap junctions in mammals are formed by the connexin (Cx) family of transmembrane proteins. These proteins, of which there are about twenty family members, are named according to the molecular weights in kDa that are predicted from their cDNAs; thus, Cx43 is a protein with a molecular weight of about 43,000 Da. Although gap junctions are also found in multicellular invertebrates, they are formed by a different family of proteins, the innexins that has no sequence homology with connexins. Searching vertebrate data bases for innexin homologues to innexins, Yuri Panchin et al. [29, 30] found three cDNAs encoding proteins they termed “pannexins”, a designation highlighting expression of homologous genes throughout the animal kingdom. Of these three proteins, termed “pannexins 1, 2, 3”, pannexin1 (Panx1) is known to form channels; these channels allow passage of moderately large ions from inside the cell to the extracellular space, but likely do not form gap junction channels between cells [31].

In astrocytes, Cx43 is the major gap junction protein, although Cx30 is also expressed, with a delayed developmental time course [32-34]. In addition, low level expression of a few other connexins is likely. Cx43 forms gap junctions between the tips of astrocyte processes and between astrocytes and oligodendrocytes, forming what has been termed a panglial syncytium [35]. It has been claimed that Cx43 can also form channels (“hemichannels”) that open between the cell interior and extracellular space, although in most cases the participation of Panx1 in this process has not been ruled out. Due to lack of specificity of pharmacological agents, distinguishing Panx1 from Cx43 channels is often difficult.

Both connexin and Panx1 channels are permeable to molecules up to a molecular weight of nearly 1 kDa, which includes a number of second messenger molecules such as ATP, cAMP and Ca²⁺ as well as metabolites [36, 37].

Role of Gap Junctions in K⁺ Spatial Buffering

Astrocytes have long been implicated in K⁺ clearance from the extracellular space. The presence of Na⁺/K⁺-ATPase which is highly sensitive to variations in $[K^+]_o$ together with

the Na⁺/K⁺/2Cl cotransporter [38, 39] and the inwardly rectifying K⁺ channels which are located at perisynaptic processes of astrocytes [40-42], play major roles in K⁺ clearance [43-46].

The membrane potential of astrocytes in vivo ranges from −110 to −60 mV and varies with the [K⁺]_o according to the Nernst equation [16, 47-50] due to their high K⁺ permeability. It was based on this property that Kuffler et al. [47] proposed the K⁺ spatial buffering model in which localized elevation in [K⁺]_o was hypothesized to generate a current flow that would be dissipated at sites where [K⁺]_o was low (Fig. 1).

As originally proposed by Orkand et al. [16], two conditions are necessary for efficient K⁺ spatial buffering: (1) the glial cells should form a syncytium in which K⁺ currents can traverse relatively long distances; and (2) these cells should be highly and selectively permeable to K⁺, which both enters and exits through glial cell membranes. One feature of astrocytes that may contribute to enhance their role in spatial buffering is the presence of gap junction channels, which provide electrotonic and ionic continuity among the spatially extended astrocytes necessary for the spread of current to nondepolarized regions of the syncytium [51, 52]. Both in situ and in vitro experiments have shown that astrocytes are extensively coupled to one another [53], with Cx43 and Cx30 being the main gap junction proteins at the appositional membranes [54-56].

One major problem with this model regards the length constant of the membrane, which is small and would not support current flow beyond the distance at which difference in membrane potential that drives the current is dissipated. Using dual voltage clamp recordings to measure the transfer of currents from one astrocyte to another in the hippocampus, Xu et al. [57] estimated that only 20 % of the injected current in one astrocyte cell body will reach the neighboring cell, equivalent to a distance of 50 μm.

Potassium spatial buffering is well established for the Müller cells of the retina, where the regional distribution of distinct K⁺ channels along the longitudinal axis of this cell that extends from the inner to the outer plexiform layers favors K⁺ siphoning [58, 59]. However, in CNS astrocytes the importance of gap junction intercellular channels to K⁺ buffering has been assessed recently using mice lacking Cx30 and Cx43 in astrocytes [60]. In this study it was found that gap junctional communication contributed to the radial dissipation of K⁺ in the stratum lacunosum moleculare but did not do so in hippocampal astrocytes located in the stratum radiatum [60].

Extracellular K⁺ Modulates Gap Junctional Communication

Although it is still not totally resolved whether gap junctional communication between astrocytes can effectively contribute to extracellular K⁺ homeostasis, there is strong evidence indicating that extracellular K⁺ fluctuations modulate astrocyte intercellular communication. High levels of extracellular K⁺ have been shown to increase coupling in vitro between cultured astrocytes [28, 61] and in situ between astrocytes of the olfactory glomeruli [62].

The increase in dye-coupling reported in cultured astrocytes was shown to be concentration and time dependent [28]. After 30 min exposure to 10, 25 and 50 mM K⁺ solutions, a time point in which changes in coupling reached a plateau, there was about a 20, 30 and 60 % increase in dye coupling, respectively [28]. More interestingly, we found that this increase in dye-coupling long outlasted the stimulation, such that even after 90 min of 50 mM K⁺ washout, coupling was not restored to the original, control levels [28]; see Fig. 2). We termed this phenomenon long term increase in coupling (LINC; [28]).

Several mechanisms have been proposed by which extracellular K^+ can increase coupling between astrocytes, including the effect of membrane potential changes on gap junction channels [61], changes in intracellular pH resultant from the depolarization-induced cytoplasmic alkalization [24], and changes in phosphorylation state of one of the astrocyte gap junction proteins, connexin43 (Cx43) [28, 63, 64]. Considering that gap junction channels in vertebrates are basically insensitive to membrane potential, being gated only by transjunctional voltages [65, 66], and that conductance of Cx43 gap junction channels is maximal at pH 7.3, being reduced by acidification [67], changes in phosphorylation state of Cx43 are more likely to be a mechanism by which dye-coupling is increased following high K^+ exposure. Indeed, we have shown that calcium-calmodulin kinase (CaMK) is implicated in the long term increase in coupling seen after exposure of spinal cord astrocytes to elevated extracellular K^+ [28]. Since no changes in Cx43 protein expression levels or distribution were detected in astrocytes following exposure to elevated extracellular K^+ , the increased coupling was attributed to increased number of open gap junction channels [53], likely mediated by the action of this kinase.

In vitro, high extracellular K^+ induces an increase in cytosolic calcium levels in astrocytes through voltage-activated Ca^{2+} channels sensitive to dihydropyridine compounds [28, 68-71]. Preventing the influx of calcium with the L-type calcium channel blocker nifedipine or potentiating this influx by the use of Bay-K-8644, an L-type calcium channel opener, resulted in the prevention and potentiation of K^+ -induced increase in coupling, respectively [28]. Moreover, calmidazolium, a calmodulin inhibitor, and KN93, a CaMK inhibitor, prevented the K^+ effect [28]. Thus, these studies implicated a CaMK pathway in the K^+ -induced increase in coupling in spinal cord astrocytes. The fast onset and persistence for long periods of time of this coupling increase is likely to contribute to the K^+ buffering capacity of astrocytes, by increasing the effective volume of the interconnected astrocyte network.

In contrast to astrocytes cultured from neonatal mice where Cx43 plays a major role in astrocyte coupling, at postnatal ages increased dye-coupling in astrocytes from the olfactory glomeruli in response to elevated extracellular K^+ seems to depend on Cx30 gap junction channels, given that Cx30 knockout mice did not show such plasticity [62]. Although the mechanism involved in this effect of K^+ on Cx30 is not yet known, activation of inwardly rectifying K^+ (Kir) channels seems to play an important role as evidenced by the prevention of K^+ -induced increased coupling in olfactory glomerulus astrocytes in barium-treated preparations [62].

Extracellular K^+ Activates Panx1 Channels

Differently from connexins, pannexins do not form gap junctions but, at least for Panx1, these channels form plasma membrane channels that are permeable to relatively large molecules, such as ATP. In response to membrane depolarization, cytosolic Ca^{2+} elevation, mechanical stretch or following P2X₇ receptor stimulation, Panx1 channels are open (for review see [72, 73]). Recently, we found that in cultured astrocytes and neurons, elevated extracellular K^+ activates Panx1 channels independent of membrane potential [74]. Similarly to what was found in cell culture systems, in hippocampal slices exposed to 10 mM K^+ artificial cerebro-spinal fluid (aCSF), activation of Panx1 channels was evidenced by the induction of a pathway permeable to relatively large fluorescent molecules and to ATP, both of which were abrogated in slices from transgenic mice lacking Panx1 [75]. These observations led to the hypothesis that in response to intense neuronal activity, elevated extracellular K^+ may activate Panx1 channels which in turn by releasing ATP contributes to further neuronal excitability through its action upon ionotropic P2X receptors. In favor of this hypothesis is the evidence that mice lacking Panx1 channels display shorter periods of status epilepticus following kainic acid (KA) injection than do wild-type mice

[75]. Moreover, 2 h after KA injections, Panx1-null mice were shown to release 60% less ATP than wild-type mice [75]. Thus, these studies suggest that Panx1 channels may be regarded as suitable candidates to perform the dual function, being both sensors of the extracellular ionic environment and paracrine releasing sites.

Extracellular K⁺ Modulates Intercellular Ca²⁺ Signaling

Transmission of calcium signals between astrocytes (Fig. 3) depends on gap junctional communication and on paracrine signaling activating membrane receptors, predominantly the ATP-sensitive receptors (for review see [76, 77]). Gap junction channels contribute to this process by allowing the diffusion of Ca²⁺ mobilizing second messengers generated in the stimulated cell to cross the cell boundaries and enter the cytosol of neighboring non-stimulated coupled cells. The paracrine component involved in the transmission of calcium signals between astrocytes comprises sites by which signaling molecules (ATP, glutamate, etc.) are released and activate their cognate membrane receptors. Among several molecular candidates involved in the release of ATP from astrocytes, we have shown that Panx1 channels contribute to this process, especially under conditions in which P2X₇ receptors are involved [72, 78].

Several mechanisms may contribute to the extent to which these calcium signals can be transmitted between astrocytes, including the degree of gap junctional coupling, and the expression levels and types of purinergic receptors [56, 79]. Unexpectedly, when cultured cortical astrocytes were exposed for 30 min to 50 mM K⁺ solution, a condition that was shown to increase dye-coupling [28, 61] and to activate Panx1 channels [74], a significant decrease in the amplitude of mechanically induced calcium waves was recorded compared to that measured in control (5.4 mM) K⁺ solution (Fig. 3a, b). The amplitude of calcium waves, as measured by the fold change in Indo-1 fluorescence intensity, decreased from 2.16 ± 0.06 fold ($N=44$) to 1.47 ± 0.04 fold ($N=45$) when bathed in 50 mM K⁺ solution. Although further studies are necessary to disclose the mechanisms involved in this depressed signal transmission between astrocytes, our unpublished observation indicates that depletion of intracellular Ca²⁺ stores underlies this event. Application of thapsigargin to cultured astrocytes treated for 30 min with elevated extracellular K⁺ induced significantly lower calcium transients than in control treated cells. Further experiments using Panx1-null astrocytes should indicate the extent to which these channels contribute to store depletion by continuously releasing ATP.

Conclusions

Intracellular signaling among astrocytes utilizes a variety of mechanisms including gap junction channels, other channels of the non-junctional membrane, receptor activated channels, as well as the vesicular release of “gliotransmitters”. The role of such long range intercellular signaling is incompletely understood but certainly has the potential to provide modulation of neuronal tone in widely separated compartments throughout the brain. Moreover, because astrocytes interact with all neural cell types, heterocellular signaling will likely be affected over long distances. The studies summarized here indicate that two components of intercellular signaling among astrocytes are dramatically modified by elevated potassium.

The first pathway that is described is a long lasting increase in coupling strength that appears to be due to calcium entry and activation of CaMK activity. This change, termed LINC, appears to be due to activation of Ca²⁺ and calmodulin dependent kinase (CaMK). This phenomenon appears very similar to that reported in Mauthner cells in goldfish in which tetanic stimulation produces long lasting potentiation of both electrotonic and chemical synapses (reviewed in [80]). Studies on the mammalian homologue of the fish neuronal gap

junction protein, Cx36, indicate that Cx36 binds to and is phosphorylated by CaMKII in a calcium and calmodulin dependent process [81]. This process is strikingly analogous to that proposed to underlie long-term potentiation at chemical synapses.

The second distinct potentiating process involves action of K^+ ions on Panx1 channels, increasing their probability of opening at resting potentials. This effect is substantially larger than accounted for by changes in resting potential and is seen under voltage clamp conditions, so that it is likely to represent a direct effect of extracellular K^+ on the Panx1 channel, rather than just depolarizing the cell.

Connexin channels provide direct pathways for communication between cells, while Panx1 provides a pathway for release of moderately large signaling molecules, such as ATP, that act via autocrine/paracrine mechanisms. Potentiation of each of these independent intercellular signaling under conditions where extracellular K^+ is elevated likely extends the extent of spatial buffering, minimizing the impact of locally high neuronal activity. Moreover, such modulation of the astrocytic syncytium likely amplifies the range of glia-neuronal signaling in the brain, normalizing such processes as metabolite delivery and removal, changes in Ca^{2+} in astrocyte microdomains and may contribute to excitotoxicity and neurodegeneration.

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Fig. 1. Potassium spatial buffering model where astrocytes remove excess K^+ by current loops through the coupled syncytium [16, 47, 82, 83]

Fig. 2.

Long term increase in coupling among astrocytes exposed to elevated extracellular K^+ . **a** Time course of fluorescence recovery after photobleach (FRAP) of cytoplasmic calcein measured from six independent cultures of wild-type and Cx43-null cortical astrocytes bathed in control (5.4 mM) and high (50 mM) K^+ solutions. The time constant of recovery in wild-type astrocytes decreased from 139.7 to 35.4 s, 30 min after control (5.4 mM) extracellular K^+ solution was changed to a 50 mM K^+ solution. Note that in Cx43-null astrocytes derived from neonatal mice, there is no dye coupling in either 5.4 or 50 mM K^+ solutions. **b** Time course of FRAP measured from astrocytes after (0 and 90 min) washout of 50 mM K^+ solution. After 90 min washout, the time constant of recovery was still lower (78.6 s) than in control conditions (139.7 s), indicating elevated extracellular K^+ causes a long term change in dye coupling. For these experiments, confluent cultures of cortical astrocytes were loaded with calcein-AM and FRAP experiments performed using a Nikon real time confocal microscope, as previously described [84]

Fig. 3.

Long term exposure of astrocytes to elevated extracellular K^+ reduces the transmission of calcium waves. Confocal images of cultured cortical astrocytes exposed for 30 min to **(a)** control (5.4 mM) and **b** high (50 mM) extracellular K^+ solutions showing the increase in intracellular calcium levels following a mechanical stimulation of a single astrocyte (*arrows*). Note that the amplitude of calcium wave in astrocytes treated with 50 mM K^+ solution was greatly reduced compared to that of untreated cultures. For these experiments, astrocytes were loaded with the calcium indicator Indo1-AM and images acquired in a Nikon real time confocal microscope, as previously described [56]