Characterization of Extracellular H^+ Fluxes from Isolated Glia and Slices of the Vertebrate Retina

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THESIS

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LIST OF ABBREVIATIONS

ADP	adenosine 5′-diphosphate
AMPA	(Aminomethyl)phosphonic acid
ATP	adenosine 5’-triphosphate
cAMP	cyclic adenosine 5’-monophosphate
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
DIDS	4,4’-Diisothiocyanatostilbene-2,2’-disulfonic acid
DL-TBOA	DL-threo-β-Benzyloxyaspartic acid
DMSO	dimethyl sulfoxide
EAAT	excitatory amino acid transporter
EIPA	5-(N-Ethyl-N-isopropyl)amiloride
GABA	γ-Aminobutyric acid
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
IP3	Inositol triphosphate
ISM	ion selective microelectrode
KA	kainic acid
MS-222	ethyl 3-aminobenzoate methanesulfonate (tricane)
OPL	outer plexiform layer
PPADS	pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid)
SCH28080	2-methyl-8-(phenylmethoxy)imidazo[1,2-a]pyridine-3-acetonitrile
SEM	standard error of the mean

UTP	uridine 5′-triphosphate
U-73122	1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione
2-APB	2-Aminoethoxydiphenylborane

SUMMARY

Glial cells are crucial for the proper execution of various processes within the nervous system; from the proper development of early synaptic connections to modulation of neuronal signaling in adulthood. The exact mechanisms underlying this bi-directional communication are still not fully understood, yet there is mounting evidence in the literature, which suggests active modulations of neuronal signaling by glia. In the retina for example, glial cells have been shown to modulate ganglion cell spiking, possibly through the release of gliotransmitters, such as ATP. Another possible mechanism proposed in this thesis, through which glia could modulate neuronal behavior, is by mediating changes in the extracellular acidity. In the vertebrate retina, extracellular pH has been extensively shown to be modulatory to photoreceptor calcium channel activity and subsequent glutamate release. Although the majority of attention has been given to retinal neurons, particularly horizontal cells, for being the potential mediators of these extracellular acidity changes, work by Jacoby et al. has consistently shown results opposite of the ones predicted by the horizontal cell-mediated feedback hypothesis.

In the work presented here shows that retinal Müller glial cells are capable of changing the extracellular pH, which could potentially have an impact on retinal physiology and neuronal signaling. Extracellular changes in proton concentrations were monitored with H^+-selective microelectrodes employed in a self-referencing manner. It was found that Müller glial cells respond to extracellular ATP and glutamate by changing the extracellular H^+ concentrations. ATP induced extracellular acidification mediated by

Müller cells isolated from the retinae of tiger salamander and various other vertebrates, including two Macaque species and human. The precise membrane player responsible for the acid extrusion was not identified despite persistent efforts. Yet, a partial mechanism was characterized. The observed extracellular acidification was shown to be due to the activation of purinergic receptors of the P2 family by extracellular ATP. The activation of these receptors then lead to intracellular calcium increases, which, as shown in this thesis, seemed to be due to the activation of the PLC-IP3 intracellular signaling pathway. The ATP-induced extracellular acidification was also observed in slices of salamander and goldfish when recordings with the ion selective microelectrodes were performed just above the outer plexiform layer. In slices, the acidification also depended on the activation of P2 receptors.

Externally applied glutamate also induced changes in the extracellular acidity, which were mediated by Müller glial cell. These changes had the opposite polarity of those induced by ATP: glutamate lead to a transient alkalinization of the extracellular milieu. The glutamate-induced alkalinization was observed from isolated Müller cells and slices, and the experimental results suggest it was mediated by the activation of glutamate transporters. Interestingly, the transient extracellular alkalinization induced by bath-applied glutamate was followed by a sustained extracellular acidification, which was particularly pronounced when recordings were done in retinal slices. Glutamate transporters did not seem to be responsible for the acidification component of the

glutamate-induced acidity changes, yet data suggested that activation of AMPA/kainate receptors was involved.

The data presented here describes the ability of retinal Müller glial cells to respond to external neurotransmitters by mediating changes in the extracellular environment though changes in extracellular acidity. In the context of the importance of extracellular pH in the outer retina, which has been amply established in the literature, and lack of unanimously accepted consensus on the retinal cell type responsible for these pH changes, the results described in this thesis provide the foundation for further investigation of a novel hypothesis implicating Müller cells as contributors to physiologically relevant changes in extracellular acidity in the retina.

I. INTRODUCTION

I. 1. Neuron-glia interactions

Historically, glial cells have been thought of as the cells providing metabolic and structural support for neurons without active contributions to signaling, neuronal behavior and ultimately output of networks (Kang J et al., 1998). Brain processes, such as plasticity, learning and memory, have been attributed solely to neurons with no direct involvement of glia, despite the fact that glial numbers have been suggested to surpass neuronal ones substantially (Temburni and Jacob, 2001). In recent decades the classical view of glial cells has been changing due to the climbing numbers of studies providing evidence for active roles of glia in modulating neuronal signaling under physiological and pathological conditions. Neuron-glia interactions are essential for the normal processing of information in the nervous system. Intimate structural and functional relationships between neurons and glia govern crucial neurobiological processes in the developing and mature nervous system.

The first electrophysiological studies on glial cells done fifty years ago showed that glial cell membranes rested at very hyperpolarized potentials (Kuffler et al., 1966; Orkand et al, 1966 cited in Olsen et al., 2015). For a long time there was a general acceptance within the scientific community that because glial membrane potential rested at very negative values without any significant changes, those cells did not actively respond to neuronally derived bioactive molecules in order to induce physiological responses involved in signaling. It is now believed that glial cells sense neurotransmitters released by neurons and reciprocate to neuronal signaling by activation of intracellular messaging pathways and release of bioactive molecules, including gliotransmitters. For example, in vitro studies have shown that hippocampal

astrocytes express neuronal nicotinic acetylcholine receptors, and their activation raises calcium levels within glial cells via mechanisms different from the ones in neurons (Temburni et al., 2001). Astrocytes have also been shown to express functional glutamate receptors which, when activated, lead to increased levels of intracellular calcium. Astrocytes express other receptor types as well, such as those sensitive to opioids, GABA, purines, etc. Experiments on retinal and hippocampal slices reveal that activation of astrocytes raises their internal calcium levels, and induces gliotransmitter release, which can ultimately modulate the electrical behavior of surrounding neurons. Astrocytes also have been shown to release glutamate, which, at least in culture, can activate neurons (see, for instance, Temburni et al., 2001 for review). The release of glutamate from glial cells could serve as an amplifier of the signal at the postsynaptic cell.

Bidirectional communication between neurons and glia has been shown to have modulatory effects on neuronal signaling. Astrocytes are strategically positioned in close proximity to neuronal synaptic points of communication to be able to not only sense neurotransmitters released by neurons but also to respond to these chemical signals by release of various receptor ligands and ultimately alter neuronal activity. Release of glutamate by astrocytes can enhance synaptic transmission whereas release of ATP can be inhibitory to neuronal activity (Allen and Barres, 2005). Experiments performed on hippocampal slices and cultures revealed that the inhibitory effects of astrocyte-derived ATP were due to extracellular break-down of ATP to adenosine, which activated presynaptic adenosine receptors, reduced presynaptic calcium influx and ultimately reduced neuronal vesicular release (Allen and Barres, 2005). The inhibitory effects of

extracellular adenosine were not restricted to a single synapse, but rather were observed over a wide area of multiple synapses. Whether this could have resulted from adenosine diffusion, numerous points of ATP release or even release from multiple glial cells was not established, but the possibility that multiple astrocytes were activated is not that far-fetched. It has been shown that glial cells, including astrocytes, can “talk” to one another via junctional channels or release of intercellular messengers. Spreading intracellular calcium waves have been observed in astrocytes after the activation of a single astrocyte in hippocampus (Sul et al., 2004 as cited in Allen and Barres, 2005) as well as retina (Newman, 2001).

Activated astrocytes have been shown to exert modulatory effects on neuronal NMDA receptor currents. When intracellular calcium was elevated in astroglia in slices from somatosensory cortex of mice by activating PAR-1 and α1-noradrenaline receptors, a burst of P2X receptor-mediated activity and smaller amplitude NMDA-mediated currents were recorded from neurons. The frequency of the purinergic currents was reduced in animal models in which the astroglial exocytotic machinery was impaired, suggesting that exocytosis from astrocytes was necessary for the observed modulatory effects on neuronal NMDAR activity (Lalo et al., 2016). The P2XR-associated currents were also reduced in a P2X4R-KO animal model. The same study revealed that the threshold for LTP induction in somatosensory cortex was reduced in a P2XR4-KO model, in which only two theta-burst stimulations were required for LTP induction compared to wild-type. Application of pharmacological inhibitors of P2X4R mimicked results with KO mice in that they also lowered the LTP induction threshold (Lalo et al., 2016). These data emphasize the ability of astroglia to modulate not only neuronal

signaling but also processes fundamental in the brain's ability to adapt and change. Moreover, the study emphasizes the importance of signaling through P2 purinergic receptors via glia-derived ATP.

The exact mechanism via which glial cells release gliotransmitters has not been fully established. Studies, primarily on cultured astrocytes, have suggested multiple mechanisms of release, including hemi-channels, volume-regulated ion channels, vesicular calcium dependent release, as well as calcium-independent mechanisms (Allen and Barres, 2005). Cultured astrocytes have been shown to release glutamate and aspartate via the opening of volume-regulated ion channels, which release is correlated with intracellular calcium rise and enhanced by extracellular ATP acting via purinergic (P2) receptors (Kimelberg, HK, 2004). Under experimental conditions, gliotransmitter release was observed from non-swollen as well as slightly swollen cells suggesting that this mechanism might be active not only during pathological conditions, such as ischemia, but under normal synaptic signaling which could cause small astrocytic volume changes (Kimelberg, 2004) and subsequent release of modulatory neuroactive molecules.

Calcium rises in astrocytes have been induced by various receptor agonists and have been correlated with the release of gliotransmitters, which have been shown to activate neuronal receptors, regulate probability of vesicle release, synaptic plasticity threshold, etc (Bazargani and Attwell, 2016). The calcium transients detected in astroglia show spatial and temporal variability. Calcium transients in the soma are much less frequent, slower and predominantly dependent on activation of IP3R2 receptors on internal store membranes, whereas calcium transients in parts distant from the soma,

e.g. tips of processes, exhibit faster kinetics, higher frequency of incidence and show only partial dependence on IP3R2 receptors but greater reliance on membrane channel-operated calcium entry, possibly through TRPA1 (transient receptor potential Ankyrin type 1) (Bazargani and Attwell, 2016). Simultaneous recordings from astrocytes and pyramidal neurons in brain slices form rats revealed that astroglia were capable of modulating neuronal behavior (Kang, 1998). Astrocytes were stimulated with a train of depolarizing pulses and miniature IPSCs in neurons were monitored. Upon glial stimulation, the frequency of mIPSPs in pyramidal neurons increased. Further experiments showed that stimulated astrocytes exhibited an increase in internal calcium levels, which propagated to surrounding glial cells in the form of a calcium wave. Glial calcium transients were also observed when GABAergic interneurons in the slices were stimulated. The calcium increases were blocked by thapsigargin, an agent that prevents reuptake of calcium into intracellular stores, and high BAPTA, a calcium chelator, in the pipette. The prevention of internal calcium increases in stimulated astroglia were correlated with failure of stimulated astrocytes to increase the frequency of mIPSCs in pyramidal neurons (Kang, 1998).

Active neuron-glia communication and calcium rises in glia have also been shown to be important for proper synapse formation. A large number of glutamatergic connections made by climbing fibers on dendrites of Purkinje cells in the cerebellum are ensheathed by the processes of Bergmann glial cells. When this wrapping was experimentally decreased, the number of synapses increased. On the other hand, when the expression of AMPA receptor subunits on Bergmann glial cells was altered to make them impermeable to calcium, more than one climbing fiber remained synaptically

coupled to the same Purkinjie cell (Terni et al., 2016), which coupling normally goes through developmental pruning down to one climbing fiber per Purkinjie cell. These experiments suggest that glia-neuron signaling is crucial for the normal developmental synapse strengthening and pruning.

In summary, a large number of studies over the last two decades have provided substantial evidence for an active communication between neurons and glia. This "talk" between glial and neurons is believed to play a role in modulating neurobiological processes, which until not long ago had been attributed solely to neuron-autonomous phenomena and neuron-neuron interactions. Yet, the precise mechanisms through which glia modulate neuronal behavior and signaling are not fully understood.

I. 2. Retinal structural and functional architecture: Focus on Müller glia.

In the vertebrate retina, much like in other brain areas as mentioned above, glial cells also perform vital functions and not only provide a chemical environment to accommodate neuron-mediated information processing and maintain neuronal health, but also play active roles in neuronal signaling.

Müller cells and astrocytes comprise the macroglia of the vertebrate retina. Müller cells are the radial glia of the retina and the principal retinal macroglia spanning almost the whole thickness of the neural retina while astrocytes are restricted to the nerve fiber layer (Newman, 2004). Müller cells provide structure and, along with neurons, make up the smallest functional unit in retinal forward signaling (Reichenbach and Bringmann, 2013). A Müller cell, depicted in the retinal schematic shown in Figure 1, is comprised of

several sections based on its morphology: an apical tuft, whose many fine processes infiltrate the outer nuclear layer of the retina and surround cell bodies of photoreceptors; an outer process; a cell body, which lies within the inner nuclear layer; an inner process; and an endfoot, which is the end thickened terminus of the inner process. There are numerous, very thin processes which emerge from the Müller cell laterally. The apical ends of all Müller cells make up the outer limiting membrane whereas the end feet make up the inner limiting membrane separating the neural retina from the vitreous body of the eye.

Müller cells span all layers of the neural retina (Figure 1) which houses specialized photosensitive cells, the photoreceptors, and is where primary processing and refinement of visual information takes place. It is located in the back of the eye, which means light has to pass through more anterior parts of the eye before reaching the retina. Light initially goes through the cornea, the lens and vitreous humor, and then has to penetrate through all retinal layers before reaching the photoreceptors. Once photoreceptors have sensed photons, the signal is converted into an electrochemical signal propagated down the next classes of retinal neurons and ultimately to the ganglion cells whose axons comprise the optic nerve. The optic nerve then takes the information out of the retina and up to higher brain areas for further processing (Kandel et al., 2000; Rodieck, 1998).

The retinal tissue is translucent and in humans is estimated to be about 0.2mm thick at the fovea. (Chan et al., 2006). The retina is a network of cells organized in laminar or layer fashion (Figure 1). The layer at the very back of the retina contains the outer and inner segments of photoreceptors, OS/IS. Working our way forward in an

anterior direction, we find the outer nuclear layer, or ONL, which contains the cell bodies of photoreceptors. Photoreceptors make synaptic connections with second order neurons – horizontal and bipolar cells – in a layer called the outer plexiform layer, or OPL. The next synaptic layer is the inner plexiform layer and is where bipolar cells and amacrine cells synapse onto ganglion cells. Between the two synaptic layers are located the cell bodies of amacrine, bipolar and horizontal cells, as well as the somatas of glial Müller cells. The cell bodies of the ganglion cells comprise their own layer, the ganglion cell layer (Kandel et al., 2000; Rodieck, 1998).

Photoreceptors do not fire action potentials; instead, they operate with graded changes in membrane potential. Photoreceptors are continuously depolarized in the dark. When light is turned on and photons are absorbed by pigments within the photoreceptor outer segments called opsins, a cascade of intracellular events lead to the closing of membrane cation channels and subsequent hyperpolarization (Kolb, 2003). When depolarized, photoreceptors release glutamate onto bipolar and horizontal cell dendrites. There are two subsets of bipolar cells based on their voltage behavior in response to light. ON-bipolar cells respond to light with membrane depolarization whereas OFF-bipolar cells hyperpolarize in the presence of light. The discrepancy in the responses is believed to stem from differential expression of glutamate receptor subtypes. Subsets of ganglion cells also exhibit opposite polarities in their responses to light: ON-ganglion cells depolarize while OFF-ganglion cells hyperpolarize (Kolb, 2003). Bipolar cells are the physical link of information flow between photoreceptors (rods and cones) and ganglion cells. Amacrine and horizontal cells have lateral projections and are believed to play roles in a mechanism known as lateral inhibition. Lateral inhibition is

important for the development of the architecture of receptive fields in the retina, both at the outer plexiform layer where horizontal cells' projections are found and in the inner plexiform layer where the laterally spanning projections are those of amacrine cells (Kandel et al., 2000; Rodieck, 1998).

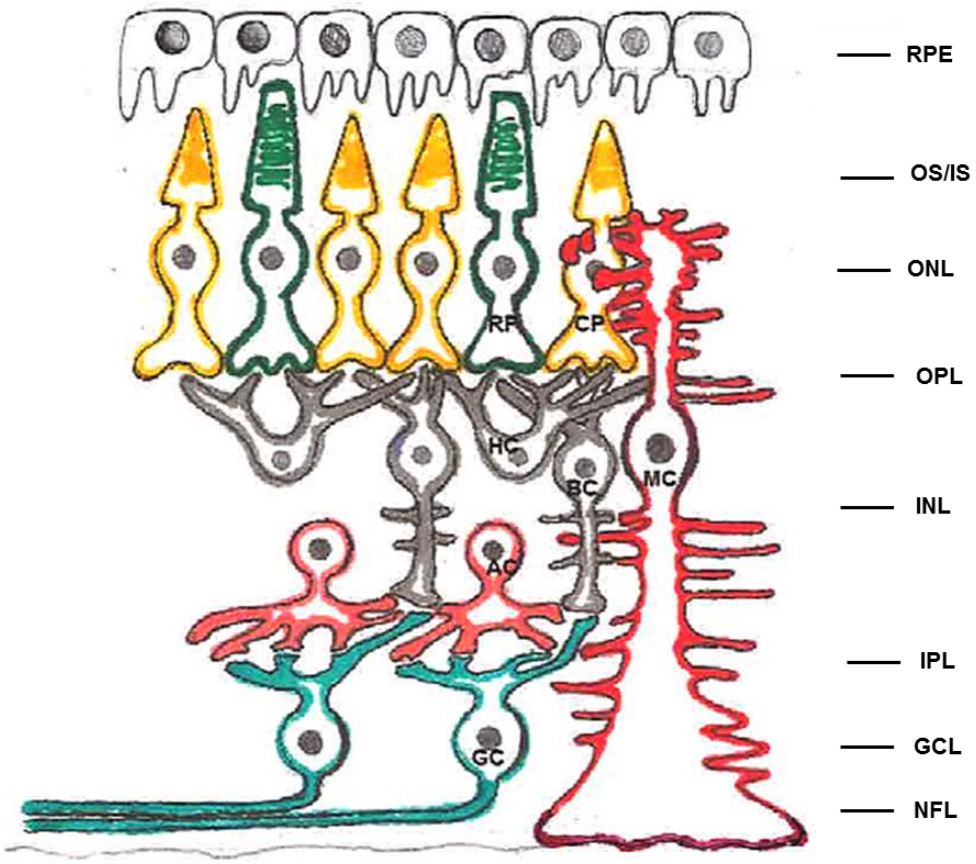


Figure 1. Schematic of the vertebrate retina, cross-section. The vertebrate retina has a laminar organization with distinct layers, the retinal pigment epithelium (RPE) is depicted on top. **OS/IS**: Outer segments/Inner segments; **ONL**: Outer Nuclear Layer; **OPL**: Outer Plexiform Layer; **INL**: Inner Nuclear Layer; **IPL**: Inner Plexiform Layer; **GCL**: Ganglion Cell Layer; **NFL**: Optic Nerve Fiber Layer. Various retinal neurons and glia are depicted in this diagram; **CP**: Cone Photoreceptor; **RP**: Rod Photoreceptor; **HC**: Horizontal Cell; **BC**: Bipolar Cell; **AC**: Amacrine Cell; **GC**: Ganglion Cell; **MC**: Müller Cell. (After Wässle, 2004 and Reichenbach & Bringmann, 2013).

All retinal neurons are vitally dependent on Müller glial cells. In normal physiology, these cells play essential roles in metabolism by providing trophic substances to neurons and removing waste products, extracellular volume regulation, ion and water homeostasis, supply of neurotransmitter pre-cursors, vascular regulation, modulation of ganglion cell spiking, etc. (Bringmann and Wiedemann, 2011). Müller cells respond to all pathogenic stimuli. In some cases, reactive Müller cells can be neuroprotective and in others they can contribute to neuronal degeneration, especially during a massive gliosis. Müller cells also act as optical fibers which guide light through the thickness of the retina and towards photoreceptors at the back, reducing photon scattering (Reichenbach and Bringmann, 2013). They are also believed to respond to changes in extracellular osmolarity (expected during neuronal activity) or glutamate by activating ATP-mediated autocrine mechanisms to prevent cellular swelling and possible subsequent retinal damage (Voigt et al., 2015).

Glutamate is the primary excitatory neurotransmitter in the retina as well as the rest of the central nervous system (Skytt et al., 2016). In the retina, Müller cells possess glutamate transporters (EAATs; EAAT-1 and -2) and are responsible for the bulk uptake of extracellular glutamate (Newman, 2004). Glial glutamate transporters, more notably the excitatory amino acid transporter-1 (EAAT-1), uptake sodium ions as well as protons along with glutamate in exchange for a potassium ion. These transporters are electrogenic as well as voltage-dependent with a smaller conductance at more depolarized membrane potentials (Levy et al., 1998). The proper operation of glial glutamate transporters is crucial for maintaining levels of extracellular glutamate below excitotoxic, thus preventing neuronal damage. The removal of glutamate efficiently by

retinal Müller glia also assures that laterally diffusing glutamate would not activate parallel vertical pathways of signaling. By uptake of excess glutamate, Müller cells partake in signaling by regulating its termination, and also play an important role in enhancing visual acuity and reducing noise by preventing activation of parallel pathways by spilled-over glutamate. The importance of efficient operation of glutamate transporters in the retina is demonstrated by studies in which pharmacological block of glutamate uptake results in larger and prolonged EPSCs in ganglion cells (Newman, 2004).

Müller glia actively communicate with each other as well as with surrounding retinal neurons. Stimulation of retinal astrocytes and Müller cells has been shown to cause propagating calcium waves across glial cells in rat retinal slices and wholemounts (Newman, 2001). Internal calcium transients in Müller cells can be elicited by application of various drugs with ATP and ATPγS having the highest potency in eliciting the responses. Studies have suggested two different mechanisms through which calcium waves are propagated amongst astrocytes and amongst Müller glia. Experiments have concluded that astrocytes communicate these calcium responses to one another likely through diffusion of intracellular messengers, possibly IP3, through gap junctions, whereas Müller cells seem to elicit calcium transients in neighboring Müller glia by release of intercellular messengers, possibly ATP (Newman, 2004). As stimulation of Müller cells not only causes propagating Ca^{2+} waves, but also leads to an ATP wave as detected by a luciferin-luciferase assay (Newman, 2001). The experimentally achieved increases in internal calcium in Müller cells have also been suggested to be present in retina in response to naturally occurring physiological stimuli. The calcium transients in

Müller glia in retinal wholemounts induced by low micromolar ATP are enhanced by application of light stimuli (Newman, 2004). These calcium transients are observed in the cells' endfeet and processes in the inner nuclear layer. Application of tetrodotoxin (TTX) abolishes the light-evoked calcium responses suggesting that they result from neuronally derived signals, probably from spiking ganglion cells. Similar results supporting the above-stated notion are achieved with electrical stimulation of ganglion cells, which is closely followed by calcium transients in Müller cells in the absence but not in the presence of TTX (Newman, 2004). In whole mounts of rat retina, application of various agonists (ATP, ATPγS, dopamine) caused an increase in glial cytosolic calcium levels and ganglion cell hyperpolarization and the generation of an outward current (Newman, 2003). The events recorded from neuronal somatas in the ganglion cell layer closely followed calcium increases in glia. Spontaneous ganglion cell spiking activity was also inhibited and its inhibition correlated with glial calcium increases. Application of cadmium, which should inhibit neuronal signaling, did not alter the hyperpolarizing current recorded from neurons suggesting that the observed altered neuronal behavior was due to glial modulatory inputs. By preferential activation of astrocytes and Müller cells, Newman concluded that Müller cells and not astrocytes are responsible for the inhibitory effects observed on neurons and proposed a mechanism through ATP release from Müller cells and subsequent extracellular ATP degradation to adenosine with subsequent activation of A1 receptors on ganglion cells (Newman, 2003). To complicate matters, glial stimulation does not always lead to neuronal inhibition. In some ganglion cells, glial stimulation enhances light evoked EPSCs while in others it depresses EPSCs (Newman, 2004).

I. 3. Protons as neuromodulators in the vertebrate retina

The exact molecular mechanisms that govern the processes of glia-induced neuronal activity modulation and neuronal activity mediated glial calcium rises in the retina remain unclear. One mechanism by which activation of glial cells could alter neuronal activity could be by altering levels of extracellular acidity. Glial cells are known to possess a voltage-dependent sodium/bicarbonate exchanger, which has been proposed to regulate extracellular levels of pH and alter neuronal activity (Newman, 1996).

In the retina, neuromodulation at the level of the outer plexiform layer where photoreceptors make synaptic connections with horizontal and bipolar cells has been proposed to be mediated by protons. Numerous studies have shown that the calcium channels at photoreceptor terminals in the OPL have a very high sensitivity to changes in extracellular pH. Experimental manipulations of extracellular pH tightly correlates with changes in the calcium channel activation curves and the amount of current inflow carried out by photoreceptor Ca2+ channels. When the pH of the extracellular medium is reduced, the activation curve of calcium channel is shifted to the right towards more depolarized levels and the amount of current inflow is reduced. (Kleinschmidt, 1991; Barnes 1993). The exact opposite is observed when the outside medium is alkalinized. In experiments where bicarbonate is replaced by HEPES, which is a faster pH buffer with a pKa around physiological pH, feedback is also reduced presumably due to the fast capture of any additional protons released. This proposed modulation of photoreceptor calcium channel activity and subsequent glutamate release is known as the proton hypothesis for lateral inhibition.

Lateral inhibition is a fundamental process in the retina. It enhances contrast and edge detection during the flow of visual information mediated by photoreceptors. Lateral inhibition is believed to define center-surround fields in the outer retina. Receptive fields are believed to have a characteristic functional architecture where neural pathways in the center are activated by sensory stimuli and the surround portions of the receptive field, where overlaps occur, are inhibited. This surround inhibition in a way enhances the center activation and reduces noise. The source of the modulatory protons in the outer retina is still heavily debated. Evidence for horizontal cell-mediated changes in photoreceptor behavior come from experiments in which direct experimental hyperpolarization of horizontal cells changes the activation curve of photoreceptor calcium channels to more hyperpolarized levels meaning an increased glutamate release from photoreceptors, or in other words reduction in the inhibition on calcium channels. Similar results were obtained with experiments in which the tissue was illuminated and voltage responses of cones lacking outer segments were monitored. The results from those experiments provided yet more evidence in support of HC(Horizontal cell)-mediated cone responses since the effects of phototransduction in these cells were eliminated by removing the cells' outer segments. Paired recordings from horizontal cells and cone photoreceptors reveal that when horizontal cells are experimentally depolarized, the Ca^{2+} currents measured in connecting cones are reduced. The reduction of the Ca^{2+} currents in cones in response to voltage changes in horizontal cells is abolished when the pH buffering of the bathing solution is increased by adding HEPES (Cadetti and Thoreson, 2006). Results with HEPES have been critiqued because this pH buffer is known to have non-specific effects outside of its primary pH buffering use

(Kramer and Davenport, 2015). HEPES has been shown to block hemichannels, which some would argue could be why feedback is reduced. But when Tris buffers are used, which are not known to have an effect on hemichannels, feedback is still reduced. It has been speculated that the effects of HEPES could be due to the buffer's ability to enter cells and acidify the cytosol; yet similar results have been obtained with buffers which do not change the intracellular pH.

A large body of evidence points to horizontal cells being the retinal cell type mediating feedback to photoreceptors by changing the pH in the synaptic cleft (Figure 2). Some of the most recent and convincing work done by Wang et al. (2014) shows that when horizontal cells in transgenic zebrafish are selectively activated (depolarized), the pH in the synaptic cleft decreases. The changes in pH are detected by CalipHluorin, a pH-sensitive green fluorescent protein fused to the outer region of a subunit of photoreceptor calcium channels. When FMRFamide receptor channels were artificially expressed in horizontal cells only and these cells were then activated by exogenously applied FMRFamide, CalipHluorin reported a drop in the pH of the solution at the very terminal. This change in pH was modulated by application of bafilomycin A1, which is a blocker of V-ATPases. The conclusion of the authors was that depolarization of horizontal cells (such as the one occurring during periods of dark and increased photoreceptor activity and glutamate release) leads to direct acidification of the cleft by horizontal cells, possibly through activation of V-ATPAses.

There is also evidence that strongly argues against the hypothesis that horizontal cells release protons in response to depolarization induced by glutamate released by photoreceptors. Molina et al (2004) demonstrated an extracellular alkalinization

produced by activated horizontal cells. Self-referencing experiments on isolated horizontal cells also show that depolarization of horizontal cells by high extracellular K^+ and glutamate receptor agonist, also lead to an extracellular alkalinization mediated by horizontal cells. Imaging work done by Jacoby et al. (2012; 2014) painted a similar picture showing an extracellular alkalinization.

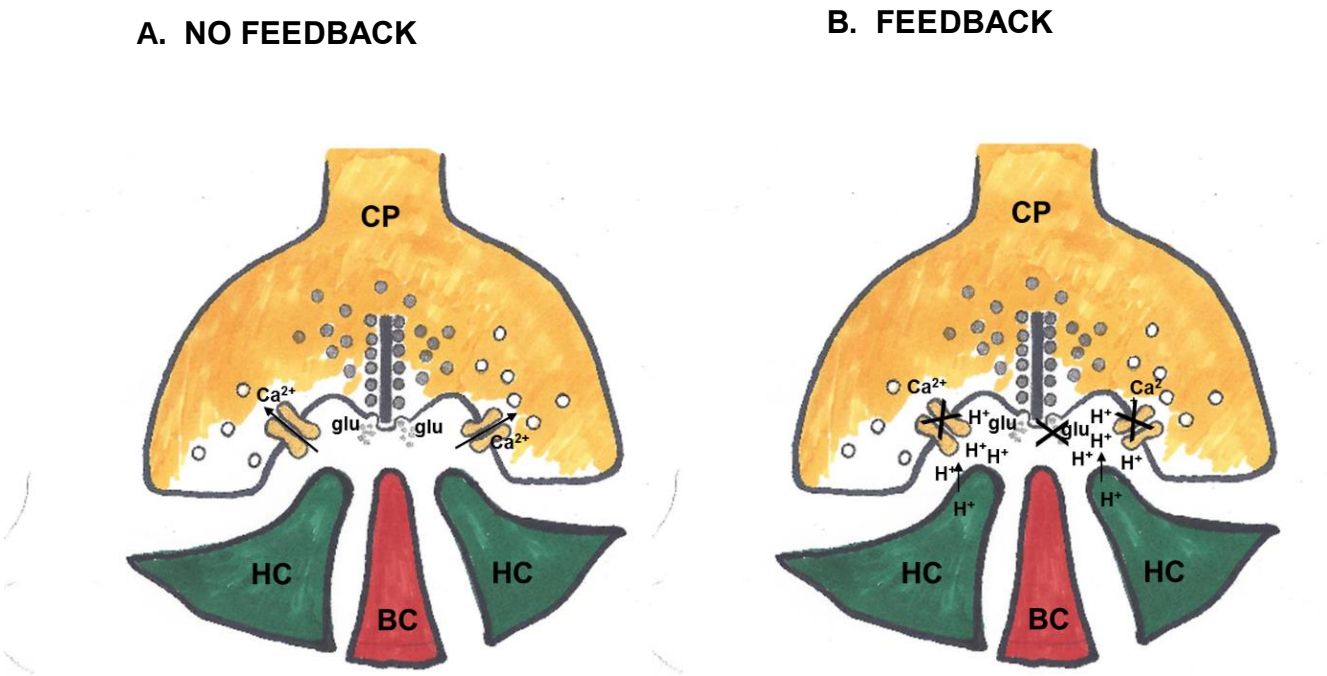


Figure 2. Schematic of horizontal cell-mediated negative feedback as predicted by the proton hypothesis. (A) A cone pedicle (CP) with invaginating dendrites of horizontal cells (HC) and a bipolar cell (BC). When the membrane of the cone terminal is depolarized, calcium enters the terminal through voltage-gated Ca2+ channels (predominantly L-type), synaptic vesicles move down the ribbon, glutamate is released. No feedback is depicted. (B) A cone pedicle with invaginating horizontal cells (HC) and a bipolar cell (BC). Feedback from horizontal cells to photoreceptors according to the proton hypothesis assumes horizontal cell-mediated acidification of the synaptic cleft. The increase in H+ is sensed by pH-sensitive Ca2+ channels on photoreceptor terminals, Ca2+ flux into the terminal is reduced, hence glutamate exocytosis is reduced. (After Kramer R and Davenport R, 2015)

Whereas there is undoubtedly evidence to support the notion that photoreceptor calcium channels are sensitive to pH changes and voltage changes in horizontal cells lead to changes in photoreceptor voltages and calcium channel activity, there is also evidence that comes from self-referencing recordings from isolated horizontal cells that shows that depolarization of horizontal cells results in an extracellular alkalinization - an effect opposite of the one predicted by the proton hypothesis (Jacoby et al, 2012; Jacoby et al, 2014). Moreover, experiments with the pH-sensitive dye HAF show a consistent intracellular acidification, in line with the extracellular alkalinization detected with H+-selective microelectrodes, in response to cell depolarization. In these experiments no domains of local extracellular pH changes were observed (Jacoby et al., 2014).

This dissertation presents evidence that another retinal cell type is capable of generating large changes in extracellular H^+, which could potentially resolve some of the previously mentioned debate over the source of protons in the outer retina. This cell type is the retinal glial Müller cell. Extracellular ATP, which is believed to be co-released with other neurotransmitters or by itself, induces large extracellular acidifications from isolated Müller cells and retinal slices. The data points to ATP acting through P2 purinergic receptors and mobilizing intracellular calcium through PLC-IP3 signaling. The ATP-driven response is also not unique to Müller cells of tiger salamander, but also observed from retinal glia of various vertebrate species. This work also shows that glutamate can induce extracellular transient alkalinization and sustained acidification responses from isolated Müller cells and slices. The transient alkalinization seems to be mediated by glutamate transporters, whereas the data suggests that the acidification in slices is downstream of glutamate receptor activation.

II. METHODOLOGY

II. 1. Animals

All experiments were performed with protocols approved by the Institutional Animal Care and Use Committee (IACUS) and the Animal Care Committee (ACC) at the University of Illinois at Chicago, the Marine Biological Laboratories and the University of Nebraska, and comply with the federal guidelines listed in the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Larval tiger salamanders (*Ambystoma tigrinum*) 5 - 10 inches were obtained from Charles D. Sullivan Co. Inc. (Nashville, TN). Channel catfish (*Ictalurus puntatus*), 6-10 inches in length, were obtained from Osage Catfisheries, MO. Goldfish were purchased from Dave's Pets N' Stuff, Oak Park, IL. Lampreys and tissues of Sprague-Dawley rats were provided by Dr. Simon Alford's laboratory. Macaque eyes (*Macaca fascicularis* and *Macaca mulatta*) were provided by the Biologic Resource Laboratories (BRL) at the University of Illinois at Chicago. Human donor eyes were provided by Eversight Illinois.

II. 2. Retinal cell dissociation

Larval tiger salamanders were housed at 4-7^{o}C for up 2 months. Animals were anesthetized for 20 min (3g/gal MS-222 (Argent), 7.5 g/gal NaHCO3) and double-pithed. Eyes were removed and hemisected; eyecups were immersed in 5ml salamander dissociation solution (mM: 110 NaCl, 1.5 KCl, 0.5 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.40) containing 1.2 mg/ml papain (LS003119, Worthington) and 0.8 mg/ml cysteine. The retina was peeled off and let sit in the papain solution on a gently vibrating plate for 25 min, then rinsed 6-8 times in dissociation solution and mechanically triturated with a 5-ml

pipette. One drop of the cell suspension was placed in 35-mm culture dishes (Falcon 3001) preloaded with plating saline containing (mM): 110 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 10 glucose, 10 HEPES, pH 7.6 and then stored at 10^oC during the day of experiments. A similar protocol was followed for dissociations of lamprey, rat, catfish, and skate using appropriate Ringer's solutions. In dissociations of human and monkey retinal cells, enzymatic digestion with papain was done with the retina maintained at 37^0C and gently bubbling the Ringer's solution with oxygen.

II. 3. Retinal slice preparation

Animals were euthanized as described above. The eyecup was hemisected and the posterior eyecup with the retina attached cut in half with a razor blade. The eyecup was then flipped onto Millipore filter paper and the sclera removed. The retina and filter paper were placed on a Millipore filter holder and suction applied to flatten the retina while dropping on Ringer's solution to keep the tissue moist. The filter paper was then trimmed and placed on a custom-made slicing chamber filled with saline with the paper secured at either end with vacuum grease (Van Hook and Thoreson, 2013). The tissue was sliced using a Stoelting slicer yielding 200-250 µm sections. Retinal slices were transferred to a recording chamber by creating a fluid bridge between the slicing and recording chambers and turned 90 degrees.

II. 4. Preparation of H^+-selective microelectrodes

Microelectrodes were prepared in a fashion similar to the protocol described by Molina et al., 2004. Microelectrodes were produced by pulling glass capillary tubes (King Precision Glass, Inc.) with outer diameter (OD) of 1.65 mm and inner diameter (ID) of 1.15 mm using a Sutter Instruments Flaming/Brown micropipette puller model P-97. The resulting micropipettes' diameters were between 2 and 4 µm. The glass micropipettes were then placed under a beaker in an oven at 180-200^{o}C for at least 4-6 hours, often overnight, to remove any moisture from the glass surface. The micropipettes were then silanized with the vapor produced by injecting 0.1-0.2 µl of N, N-dimethyltrimethylsilyalamine (Fluka) in the upside-down beaker under a hood and the drying process was continued for another roughly 0.5hr at which point glass pipettes were pulled out of the oven to cool down. The silanized glass pipettes were filled with 100 mM KCl with a pH of 7.5 buffered with 10mM HEPES. The fluid was forced all the way to the tip of the pipettes via an air pressure injection through a tubing connected to the back of the glass pipette. The pipette tips were then front-filled with H^+ selective ionophore (Hydrogen ionophore 1 – cocktail B, Fluka). Glass capillary tubes (same OD/ID as above) were also used for glass electrodes. Four inch-long tubes were cut in two and the ends of each half were then heat-bent at a 45-60^o angle. A solution containing 3% agar and 3M NaCl was poured onto the glass tubes and brought to a boil in a microwave several consecutive times to ensure complete internal fill of the glass electrodes with the agar/NaCl solution. The electrodes were then set aside to cool down.

II. 5. Self-referencing measurements of extracellular H^+ concentration

Extracellular proton concentrations were measured in a self-referencing manner (Smith and Trimarchi, 2001) with equipment developed by the BioCurrents Research Center at the Marine Biological Laboratories in Woods Hole, MA. Self-referencing measurements from isolated cells were made by placing the tip of an H^+-selective electrode 1-2 µm from the cell membrane, recording the voltage generated, then moving the microelectrode 30 µm away parallel to the bottom of the dish, taking a second reading, and then subtracting the second reading from that obtained close to the cell. The electrode was moved alternately between these two points at a rate of 3 Hz to minimize stirring of the solution. This differential recording procedure eliminates slow electrical drift present in ion selective electrodes and increases their useful sensitivity by ~1000X (Smith and Trimarchi, 2000). The electrical drift happens at a relatively slow rate, slower than the time it takes the electrode to measure the ion concentration at the recording and measuring points and subtract the latter from the former. This is very important as a non-changing electrical drift at both points is crucial for ensuring a correct differential. For recordings with retinal slices, the microelectrode was placed ~ 2 µm above the outer plexiform layer and alternately moved to a position 30 µm vertically above the slice. Control background differential readings were made in every experiment with microelectrodes far removed from cell (200 µm) or tissue (600 µm); recordings in which differential responses were not close to zero at these control locations were discarded. Recordings were made in mechanically quiet solutions since superfusion of solution washes away the H^+ gradient between the two points of electrode movement. Drugs were applied by hand pipettor to either dish or slice; in all such

experiments, a control bolus of Ringer's solution without drug was first applied to ensure that responses observed did not result from simple mechanical application and stirring of the solution. For some experiments with cells and slice, drugs were applied or washed out by a full solution exchange: the majority of the solution in the culture dish was drawn up through tubing attached to a syringe, leaving behind just enough solution coating the dish's bottom to ensure a continuous aqueous environment for the cells; full solution exchanges were ordinarily repeated three times. Recordings were performed in plating solution in which 10 mM HEPES was substituted with 1 mM HEPES with the difference in osmolarity compensated by the addition of sucrose. All experiments were conducted at room temperature (~18–25°C).

Control experiments were conducted to ensure that drugs at the concentrations applied did not alter the sensitivity of our H^+ sensors; microelectrodes were calibrated with standard pH solutions of 6.00, 7.00 and 8.00 and the Nernstian slope of electrodes determined. None of the agents used in this study altered the slope of the electrodes at the concentrations used. We note however, that concentrations of DIDS of 500 µM or greater did reduce the sensitivity of the sensors, while concentrations of 300 µM or less had no significant effect (slopes per pH unit in µV: Ringers alone, 57 ± 0.5 (N=21); DIDS at 100 µM, 60 ± 0.8 (N=11), 250 µM, 56 ± 1.7 (N=5); 300 µM, 58.6 ± 1 (N=7); 500 µM, 42 ± 0.9 (N=9); 1 mM, 28 ± 1 (N=5).

II. 6. Calcium imaging and chemicals

The calcium indicator dye Oregon Green 488 BAPTA-1, AM (Invitrogen Molecular Probes, Eugene, OR, USA) was employed to monitor intracellular calcium changes in isolated cells. A stock solution was created by dissolving 50 µg Oregon Green in 20 µl dimethylsulfoxide (DMSO) with 20% pluronic F-127 detergent (ThermoFisher, P-3000MP). Stock solutions of Oregon Green were diluted in salamander Ringer's solution to a final concentration of 5 µM. Cells were incubated in Ringer's solution containing Oregon-Green in the dark for 10-14 min at room temperature. The dye was washed off with clean Ringer's solution and cells were then imaged. Epifluorescent images were collected from a CCD camera (Zeiss 503 mono) mounted onto a compound microscope (Zeiss Axio observer Z1). A Colibri LED system stimulated loaded cells with 470 and 505nm light. Images were filtered through a 535 nm bandpass filter (30 nm, 46 HE YFP) with binning 2 x 2. Images were acquired every 4 s with ~150ms exposure using Axiovision software. During experiments, loaded cells were continually superfused and test agents were applied with a 3-barrell Warner fast-step superfusion system with an exchange rate of less than 100 ms.

Chemicals used to prepare Ringer's solutions were obtained from Sigma Aldrich and Fisher Scientific. All chemicals used in experiments were obtained from Sigma Aldrich except the following: 4, 4'-Diisothiocyanatostilbene-2,2-disulfonic acid disodium salt, DIDS (6588, Setareh Biotech, LLC); U-73343 (B5110027), 2-APB (B5060114), and CNQX (ab120046) were purchased from ABCAM; TBOA (1223) was purchased from Tocris; forskolin (F-9929) and bafilomycin-A1 (B-1080) was purchased from LC Laboratories

II. 7. Data treatment and statistical analysis

Student's paired (unpaired, where specified) t-tests were used throughout to determine statistical significance; data are presented throughout as mean ± standard error of the mean (SEM) with individual P values provided. For most experiments, standing flux values reflect the average of the full length of the recording prior to drug application. Transient electrical artifacts resulting from opening the Faraday cage and dropping in drugs by hand pipette have been eliminated from data traces and appear as spaces in the traces. In some cases, the ATP-induced acidification took some time to reach a plateau; in these instances, the average of the plateau during the last at least 20 points was taken. All data were statistically analyzed and graphically displayed using Prism 5 (GraphPad Software). In histograms, a single asterisk indicates significance greater than 0.05, and two asterisks indicate significance at or greater than 0.01.

III. ACTIVATION OF RETINAL GLIAL (MüLLER) CELLS BY EXTRACELLULAR ATP INDUCES PRONOUNCED EXTRACELLULAR ACIDIFICATION

III. 1. SUMMARY

Small alterations in extracellular acidity are potentially important modulators of neuronal signaling within the vertebrate retina. Using self-referencing H^+-selective microelectrodes, we show that activation of retinal Müller (glial) cells of the tiger salamander by micromolar concentrations of extracellular ATP induces a pronounced extracellular acidification. ADP and UTP at micromolar concentrations were also potent stimulators of extracellular acidification, but adenosine was not. The extracellular acidification induced by ATP was significantly reduced by the P2 receptor blockers suramin and PPADS suggesting activation of P2 receptors. Bath-applied ATP induced an intracellular rise in calcium in Müller cells; both the calcium rise and the extracellular acidification were attenuated when calcium re-loading into the endoplasmic reticulum was inhibited by thapsigargin and when the PLC-IP3 signaling pathway was disrupted with 2-APB and U73122. The anion transport inhibitor DIDS also markedly reduced ATP-induced acidification. ATP-induced extracellular acidifications were also observed from Müller cells isolated from human, rat, monkey, skate and lamprey retinae, suggesting a highly evolutionarily conserved mechanism of potential general importance. Extracellular ATP also induced an extracellular acidification at the level of the outer plexiform layer in retinal slices of tiger salamander which was significantly reduced by suramin and PPADS. These data suggest that proton flux mediated by ATP-activation of Müller cells and of other glia as well may be a key mechanism modulating neuronal signaling in the vertebrate retina and throughout the brain.

III. 2. INTRODUCTION

Modulation of synaptic transmission and regulation of cellular excitability play essential roles in sensory system function. An especially potent and underappreciated mechanism of modulation of synaptic transmission and cellular excitability involves small alterations of extracellular levels of H^+. A large body of evidence suggests that changes in extracellular H^+ in the outer retina may play an important role in shaping the response properties of retinal neurons (cf. (Hirasawa et al., 2012; Kramer and Davenport, 2015) for review). Enriching the pH buffering capacity of the extracellular solution blocks the ability of horizontal cells to induce shifts in the calcium currents in photoreceptors and reduces calcium signals in photoreceptor synaptic terminals (Hirasawa and Kaneko, 2003; Vessey et al., 2005). External alkalinization increases photoreceptor calcium currents and shifts the activation of the calcium conductance to more negative voltages (Barnes et al., 1993; Hirasawa and Kaneko, 2003). Simultaneous paired recordings from horizontal and photoreceptor cells in retinal slices reveal that direct depolarization of horizontal cells induces a rightward shift of the calcium conductance activation curve that is abolished by enhancing the extracellular pH buffering capacity (Cadetti and Thoreson, 2006). Particularly strong evidence for a role for H^+ in inhibiting photoreceptor synaptic terminals has come from experiments fusing the H^+-sensitive fluorescent molecule pHluorin onto the extracellular portion of cone calcium channel subunits expressed in photoreceptors. Measurements in the intact retina of such transgenic zebrafish reveal alterations in fluorescence whose magnitude, direction and spatial dependence are

consistent with the hypothesis that changes in H^+ significantly impact retinal signals (Wang et al., 2014).

Much of the work cited above has been used to suggest that H^+ is released from horizontal cells when they are depolarized by glutamate, the photoreceptor neurotransmitter, and that this extracellular acidification is essential in creating the surround portion of the classic center-surround receptive fields of retinal neurons (Hirasawa et al., 2012; Kramer and Davenport, 2015). However, measurements of extracellular H^+ changes from isolated horizontal cells using self-referencing H^+-selective microelectrodes argues consistently against this hypothesis (Jacoby et al., 2012; Kreitzer et al., 2012; Jacoby et al., 2014). Regardless of species tested or type of horizontal cell examined, glutamate consistently induces an extracellular alkalinization that appears to result from activation of a plasmalemma calcium pump (PMCA) that expels intracellular calcium ions and takes extracellular H^+ into the cells. More recent work using the H^+-sensitive fluorescent dye HAF corroborates the alkalinization observed using self-referencing (Jacoby et al., 2014). Moreover, analysis of the spatial distribution of HAF fluorescence indicates a global alkalinization around the cell, with no signs of discrete centers of acidification. Taken together, the data with self-referencing electrodes and pH-sensitive fluorophores argues strongly against the hypothesis that depolarization of horizontal cells leads to an extracellular acidification in the retina.

In the current work, results are obtained that suggest a potential resolution of this apparent conflict between the potent effects of extracellular H^+ on retinal responses and the work arguing against proton release from horizontal cells as the proximate cause of extracellular acidification. Here, it is shown that an altogether different retinal cell type

has the potential to be a potent source of extracellular acidification. That cell is not a neuron – it is the radial glial cell of the retina, the Müller cell, which extensively enwraps and envelops all retinal neurons and their synaptic interconnections, and is well placed to be able to modulate the release of neurotransmitter by retinal neurons.

III. 3. RESULTS

Isolated tiger salamander Müller cells were identified by their distinctive morphology, characterized by an apical end with a tuft-like outer process, a cell body, an elongated and relatively thick inner process, and a wide stubby end foot (Newman, 1984). H^+-selective microelectrodes were typically placed approximately 1-2 µm from the cell membrane where the apical tip of the cell and cell body fused (Figure 3A); a differential recording was obtained by moving the electrode to a position 30 µm distant and subtracting the recordings obtained at the near and distal positions. Unstimulated cells displayed a standing differential signal averaging 28 µV ± 4 (N=8), indicating that the area adjacent to the cell membrane was slightly more acidic than the reference point located 30 µm away. The black trace in Figure 3B shows that 100 µM ATP induced a marked increase in extracellular acidification adjacent to the cell membrane. The mean differential electrode response from salamander Müller cells was 112 µV ± 22 to 1 µM ATP; 225 µV ±41 to 10 µM ATP, and 614 µV ± 91 to 100 µM ATP (N=8). The red trace in Figure 1B shows the typical response observed from a catfish horizontal cell. Prior to stimulation, the extracellular solution adjacent to the cell was slightly acidic. Stimulation of the cell with 100 µM glutamate lead to an extracellular alkalinization, a response

previously observed from horizontal cells of catfish, goldfish and skate (Molina et al., 2004; Kreitzer et al., 2007; 2012).

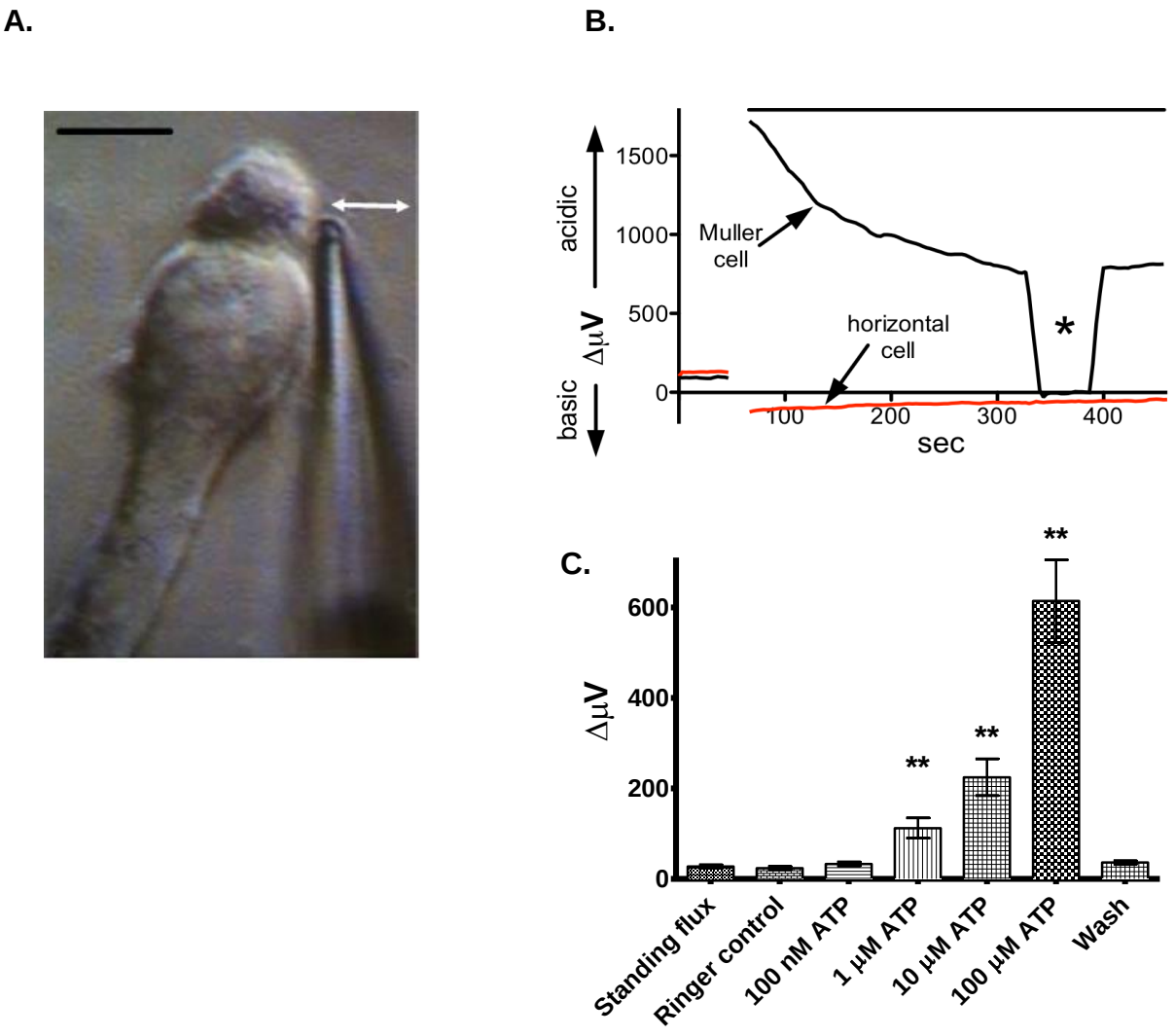


Figure 3. Extracellular ATP induces a significant extracellular acidification from isolated Müller cells. (A) An isolated Müller cell with a H^+-selective microelectrode positioned next to the apical end of the cell. Scale bar: 20 μm; double-headed arrow depicts the direction of electrode movement as it alternately records the potential established by protons adjacent to the cell and 30 μm away. (B) Response from a single isolated Müller cell to 100 μM ATP (black trace). Red trace: alkalinization response of a catfish horizontal cell to 100 μM glutamate. Top bar represents duration of drug application. Asterisk: background control readings taken 200 μm above the cell. (C) Mean data from 8 trials in response to various extracellular ATP concentrations: error bars represent SEMs; data was analyzed with a paired 2-way t-test. Bars represent mean values ± SEM. (Experiments in 3C by David Swygart, Ryan Kaufman and Matthew Kreitzer, Indiana Wesleyan University).

Because of the focus on H^+-mediated alterations in signaling in the outer plexiform layer, most recordings were made with sensors placed adjacent to the apical tuft / cell body junction of Müller cells. However, application of extracellular ATP also elicited a pronounced extracellular acidification at the end foot of Müller as well. With self-referencing electrodes placed directly adjacent to the cell endfoot, 100 µM ATP increased the H^+ signal to 450 µV ± 78 (N=5) from a pre-stimulus value of 28 ± 49 µV (p=.008).

ADP and UTP also elicited a large extracellular acidification from Müller cells. 100 µM UTP induced a differential response of 762 ± 256 µV from a mean standing flux reading of 61 ± 23 µV, p=0.003 (N=4); 10 µM UTP acidified the extracellular space from a standing flux of 148 ± 16 to 264 ± 27 µV, p=0.002 (N=6). In six other tested cells, 100 µM ADP caused an acidification, from a standing flux of 41 ± 6 µV to 235 ± 18 µV, p<0.0001 (N=6); 10 µM ADP caused an extracellular acidification from 106 ± 32 µV to 178 ± 21µV, p=0.03 (N=4). However, 100 µM adenosine did not alter extracellular acidity. In 5 cells examined, the signal obtained in the presence of 100 µM adenosine (8 ± 3 µV) was not significantly different from the standing baseline signal of 7 ± 3 µV, p=0.77; in these same cells, 10 µM ATP increased the signal to 115 ± 20 µV, p=0.006.

The purinergic receptor antagonists suramin and PPADS significantly reduced the extracellular acidification induced by extracellular ATP. Figure 4A shows responses from one Müller cell to application of 10 µM ATP first in the presence of 200 µM suramin and 200 µM PPADS and then after washout of the blockers; the cell produced little response when the blockers were present, but ATP produced a robust extracellular acidification following washout of the blockers. The mean standing signal from seven cells in the

presence of the blockers was 45 ± 7μV before and 55 ± 8 μV following the application of 10 μM ATP (p=0.13); upon washout of suramin and PPADS, the baseline signal was 48 ± 8 μV and 10 μM ATP now elicited a response of 198 ± 35 μV (p=0.002; Figure 2C). The lack of an effect by adenosine, coupled with the reduction of the ATP-induced acidification by PPADS and suramin and activation by UTP and ADP, suggest that the changes in extracellular acidification are mediated by activation of metabotropic P2Y receptors known to be present on these cells (Keirstead and Miller, 1997).

The ATP-induced extracellular acidification was also markedly inhibited by DIDS, a non-selective anion exchange antagonist. 300 μM DIDS abolished the ability of 10 μM ATP to alter extracellular H^+ levels (Figure 4B, 4D). The mean standing acidification in the presence of DIDS was 9 ± 14 μV before and 29 ± 8 μV after the application of 10 μM ATP (p=0.06, N=6). DIDS also significantly reduced the acidification normally induced by 100 μM ATP. The standing flux was also often reduced after exposure of the cells to DIDS. In some cases, the standing H^+ flux remained at a more alkaline level after DIDS application; in other cases, as illustrated in the figure, it recovered to baseline levels. Means in DIDS were calculated based on full traces in DIDS; those were compared to means of full traces of the standing fluxes after control applications of Ringer's because means of the standing flux after Ringer's slightly differed from those before. On average, the standing flux decreased from 43 ± 9 μV before to 9 ± 14 μV after exposure of the cells to DIDS (p=0.028).

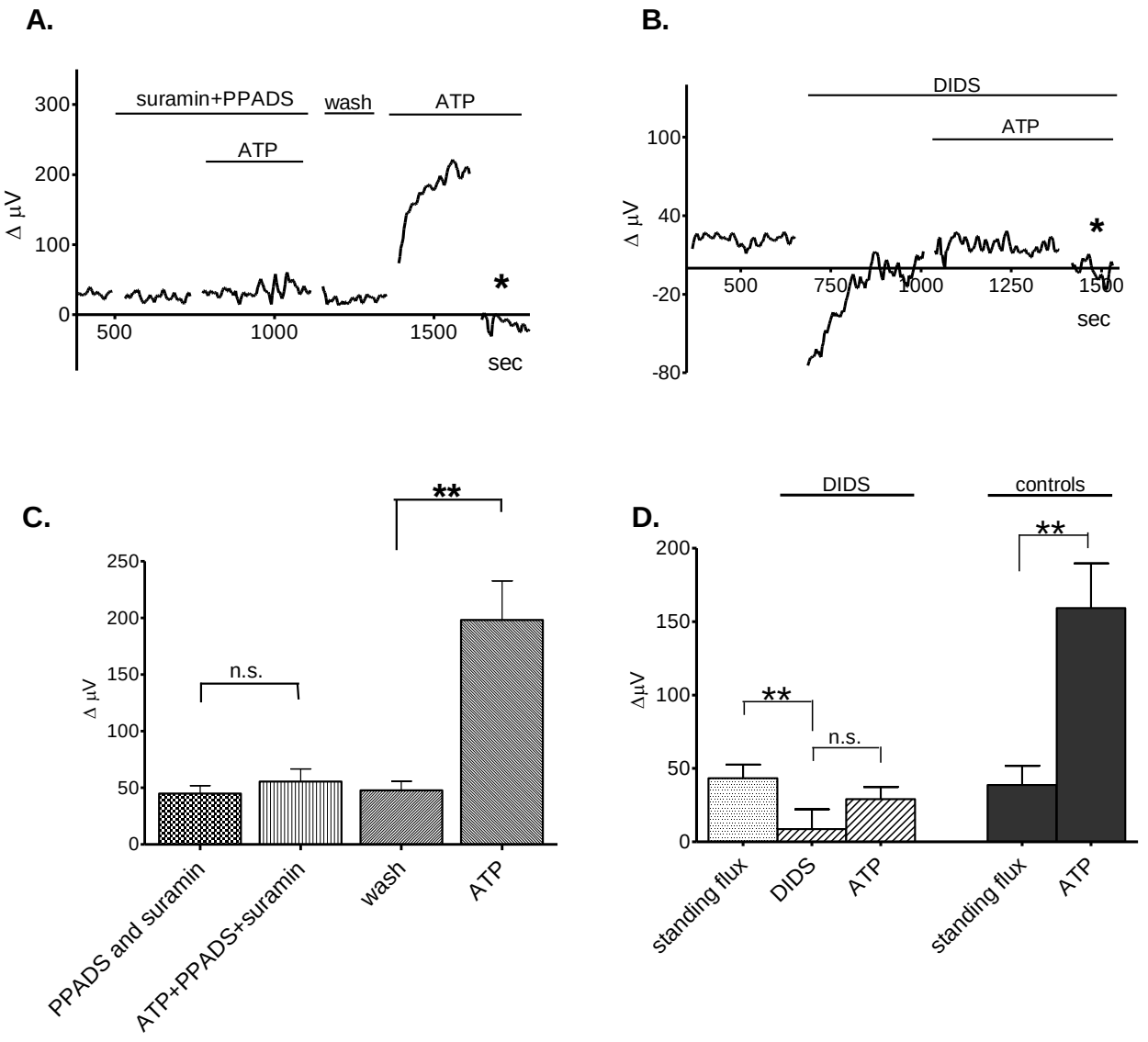


Figure 4. The purinergic receptor antagonists PPADS and suramin, and the anion transport blocker, DIDS, significantly reduce the ATP-induced extracellular acidification from isolated Müller cells. (A) A representative trace from a single Müller cell shows a significant extracellular acidification in response to 10 µM ATP that is significantly reduced by 200 µM PPADS and 200 µM suramin; asterisk indicates a background control reading. (B) 300 µM DIDS significantly reduces the extracellular acidification in response to 10 µM ATP. (C) Mean responses to 10 µM ATP with or without suramin and PPADS in the bath; N=7, error bars represent SEMs. (D) Mean responses to 10 µM ATP with 300 µM DIDS (N=6) in the presence of DIDS and 5 trials of bath-applied 10 µM ATP.

The extracellular acidification from Müller cells exposed to bath-applied ATP was dependent on rises of cytosolic calcium from intracellular stores. 1 μM thapsigargin, which inhibits calcium ATPases on the endoplasmic reticulum and prevents reloading of calcium into the endoplasmic reticulum, effectively eliminated ATP-induced increases in intracellular calcium reported by Oregon green ($p = 0.0008$, N=10; Figure 5A, C). Thapsigargin also significantly reduced the ATP-induced extracellular acidifications measured with self-referencing electrodes (Figure 5B, D). In 6 trials the mean acidification response to 1 μM ATP of 105 ± 19 μV was reduced to 24 ± 6 μV in the presence of 1 μM thapsigargin, ($p = 0.005$). In addition, the intracellular calcium rise ($p <0.0001$, N=9) and extracellular acidification ($p=0.03$, N=5) normally produced by ATP were also inhibited by 100 μM 2-APB, which blocks IP3 receptor-mediated calcium flux, (Figure 5C, D). Finally, 25 μM of U-73122, which inhibits activation of G-protein coupled PLC, also significantly reduced intracellular calcium signals ($p<0.0001$, N=9) and extracellular acidifications ($p=0.004$, N=7).

The extracellular acidification induced by bath-applied ATP was also observed in Müller cells isolated from a number of other species. Figure 6 shows responses from Müller cells isolated from human donor tissue, rat, and lamprey. Seven out of nine human Müller cells (~78%) showed clear responses to ATP with detectable increases in extracellular acidity (Figure 6A). The mean standing flux increased from 62 ± 9 μV before to 257 ± 41 μV after application of 100 μM ATP, ($p=0.001$). Five out of six (~83%) rat Müller cells responded to 100 μM ATP (Figure 6B). The mean standing flux was 5 ± 7 μV, and 100 μM ATP induced an increase in extracellular acidity to 262 ± 54 μV, a difference that was also statistically significant ($p=0.006$).

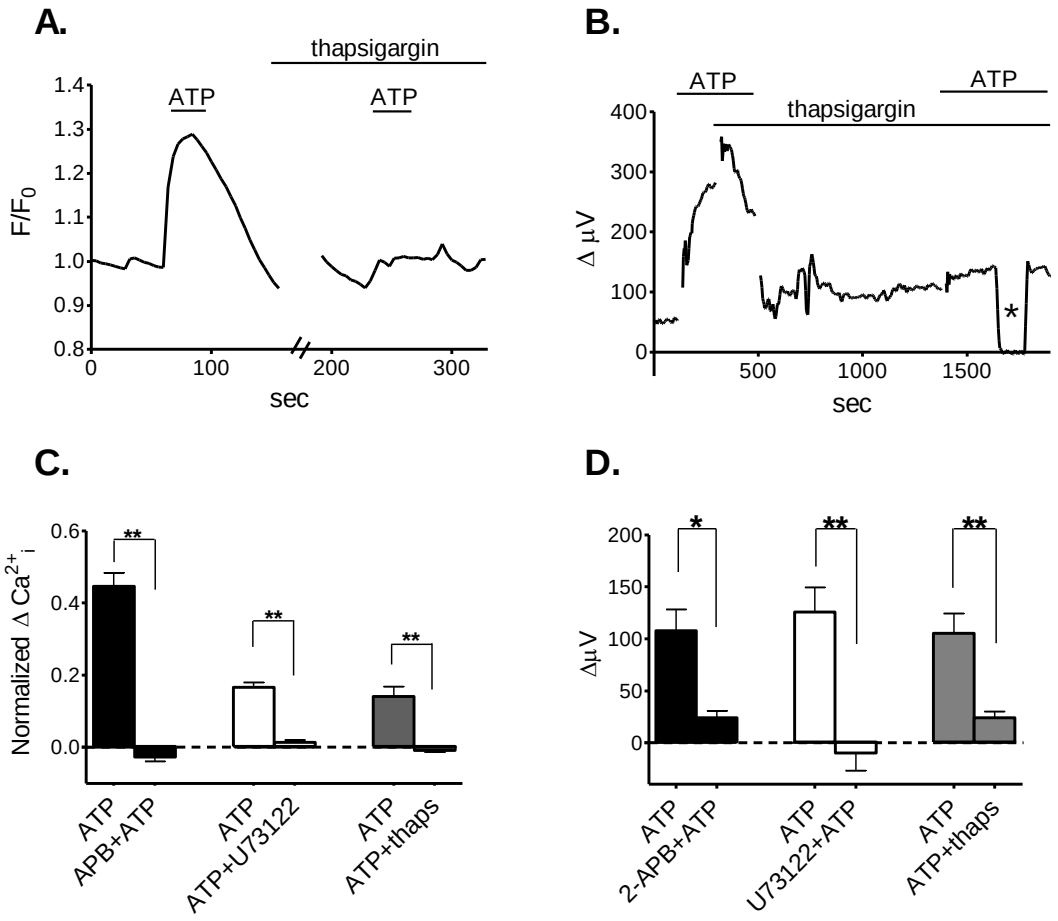


Figure 5. 1 μM ATP causes an increase in the intracellular calcium concentration in acutely isolated Müller cells, as well as increases in extracellular acidity measured via self-referencing. (A) 1 μM ATP causes an increase in intracellular calcium as reported by Oregon green. Subsequent ATP-induced intracellular calcium increase is blocked by thapsigargin, second ATP application 15 minutes after thapsigargin treatment; 15-minute incubation in thapsigargin is indicated by the double-crossed x-axis. (B) A representative trace from a single self-referencing recording from an isolated Müller cell shows that thapsigargin also reduces the extracellular acidification induced by 1μM ATP from Müller cells as reported from self-referencing measurements; asterisk: background control. (C) Mean responses from imaging data for ATP induced responses in 2-APB, U73122 and thapsigargin. Data was normalized to the last fluorescence data point before treatment. (D) Mean responses with self-referencing. ATP readings represent absolute recorded values minus values of the standing flux. (Experiments by Chad Heer, Marin Young and Matthew Kreitzer, Indiana Wesleyan University).

Six out of seven lamprey Müller cells (~86%) also responded to 100 μM ATP (Figure 5C). The signal increased from -0.971 ± 13 μV in plain Ringer's solution to 88 ± 21 μV in response to ATP (N=7, p=0.0217). Similar results were obtained from isolated Müller cells of skate, channel catfish, and two Macaque species, 5 cells from Macaca mullata (baseline signal 14 ± 8 μV; in ATP, 306 ± 83 μV; p = 0.0241) and six cells from Macaca fascicularis (35 ± 6 μV; response of 3 cells to 85 μM ATP was 222 ± 48 μV and response of 3 cells to 100 μM ATP was 318 ± 83 μV, p = 0.004)

Next, changes in extracellular H^+ concentration from retinal slices in response to exogenously applied ATP were examined (Figure 7). The H^+-selective microelectrode was positioned several microns above the outer plexiform layer and a reference point was taken 30 μm away in a vertical direction. Responses to application of 100 μM ATP were examined, with the expectation that ecto-ATPases present in the tissue would likely reduce the actual concentration reaching the tissue. In eight slices, pipette-applied 100 μM ATP caused a robust extracellular acidification. The mean acidification response increased from a standing, already acidic flux of 1000 ± 67 μV to 2227 ± 313 μV, p = 0.0021. The response to ATP was significantly reduced in the presence of the ATP receptor blockers suramin and PPADS. Application of PPADS and suramin alone caused a slight decrease in the standing flux from 1032 ± 111 μV to 832 ± 78 μV. Although this decrease was not significantly different from the standing flux before stimulation, the difference between the standing flux in the ATP receptor blockers and the signal in subsequent addition of ATP (1112 ± 164 μV) to statistically significant levels, p = 0.0482. However, the response induced by ATP in the absence of PPADS and suramin was much larger than that induced when the blockers were present.

Similar results were obtained from goldfish slices, where H+-sensitive microelectrodes positioned above the outer plexiform layer detected a robust extracellular acidification induced by 100 μM ATP. The average H+ flux at the level of the outer plexiform layer prior to stimulation was 1205 ± 90 μV from six slices, and increased to 1856 ± 87 μV upon application of 100 μM ATP ($p=.0001$). This ATP-induced acidification was significantly reduced in the presence of 200 μM suramin and PPADS (1064 ± 99 μV).

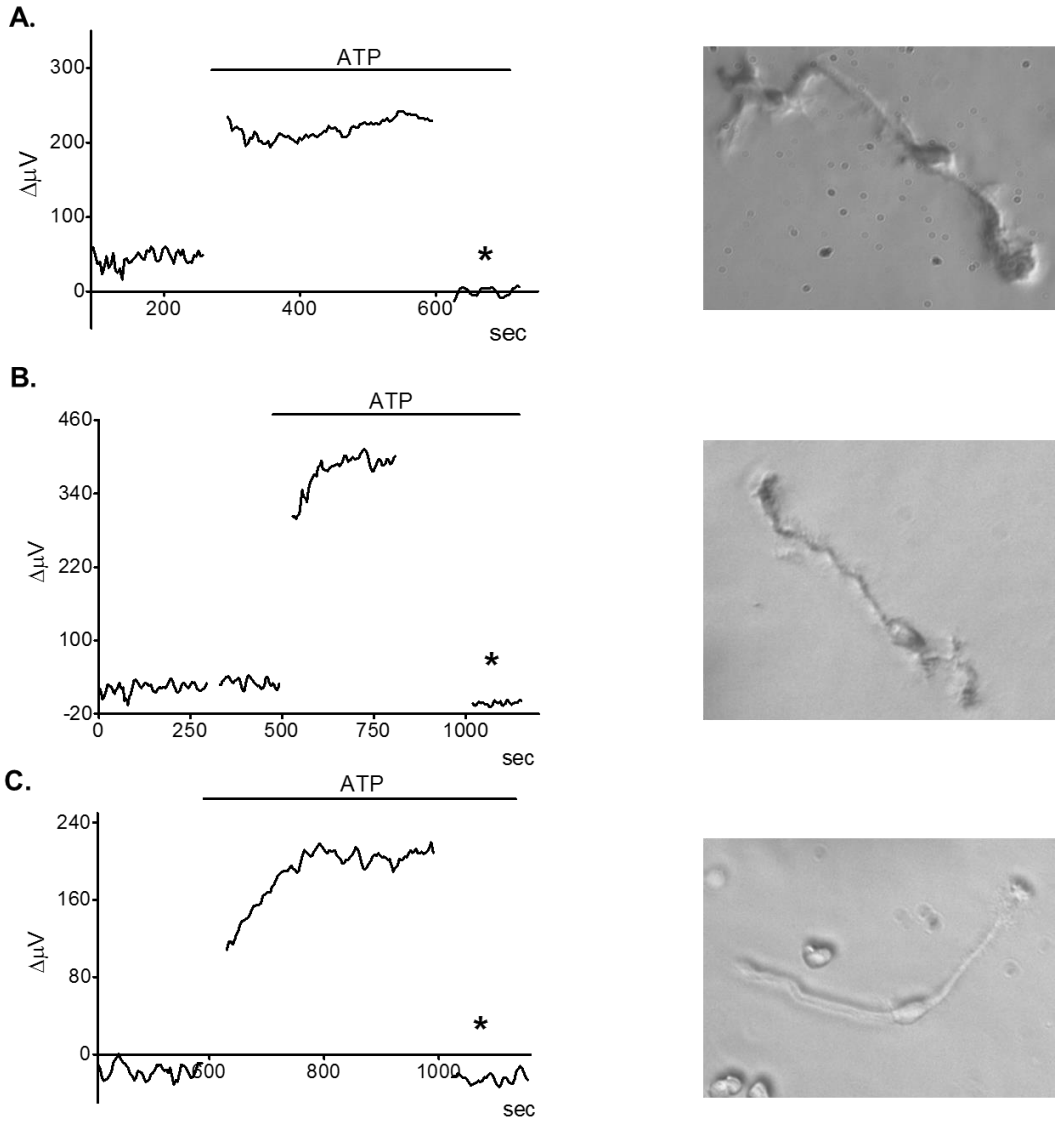


Figure 6. 100μM ATP causes an extracellular acidification from isolated Müller cells from human retina (A), Sprague-Dawley rat retina (B) and lamprey retina (C). An image of an isolated Müller cell from each species is to the right of each respective trace; asterisks indicate background controls.

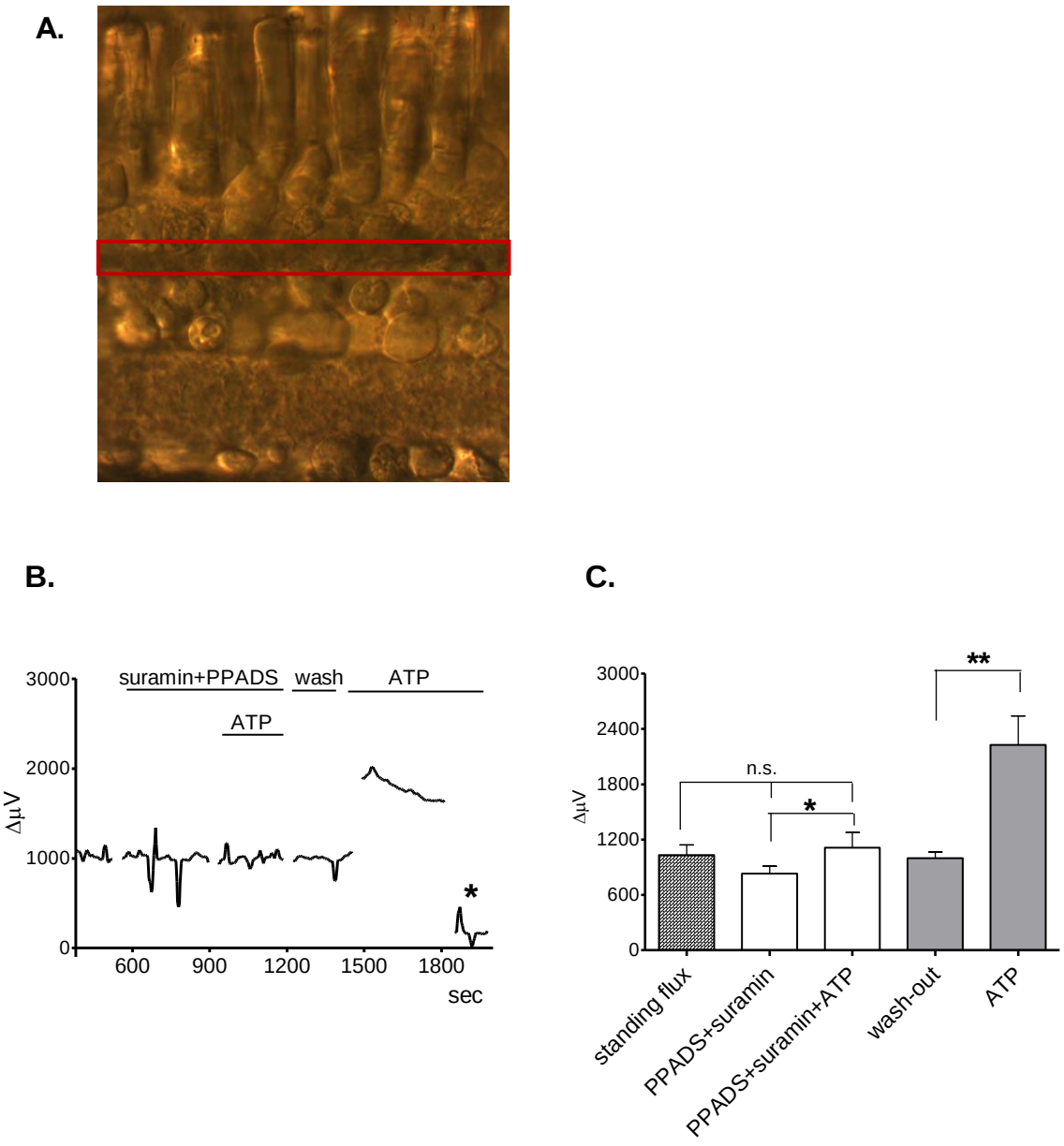


Figure 7. Bath application of 100 µM ATP causes an extracellular acidification from salamander retinal slice, which is abolished in the presence of 300 µM PPADS and 300 µM suramin. (A) Retinal cross-section of a tiger salamander retina; red box highlights the outer plexiform layer (OPL). (B) A representative trace from an individual self-referencing recording from a retinal slice with the microelectrode positioned just above the OPL; asterisk indicates a background control reading taken 600 µm above the retinal slice. (C) Mean data from eight slices. ATP induced a significantly smaller acidification in the background of suramin and PPADS than it did in plain Ringer's solution.

III. 4. DISCUSSION

Small alterations in the extracellular concentration of H^+ have profound modulatory effects on neuronal transmission within the retina (Kleinschmidt, 1991; Barnes et al., 1993; DeVries, 2001; Hirasawa and Kaneko, 2003). The ATP-induced extracellular acidification from Müller cells has the potential to act as a key molecular mechanism mediating neuronal inhibition in the retina in general and in the outer plexiform layer specifically. The magnitude of the ATP-induced acidification is similar to that detected from cells and tissues known to be potent proton pumpers, such as the vas deferens (Breton et al., 1998), where changes in extracellular H^+ concentration play an essential role in activation of sperm. The ATP-induced extracellular acidification detected in retinal slices is likely mediated by Müller cells; self-referencing recordings with H^+-selective microelectrodes from photoreceptors isolated from tiger salamander have not shown significant alteration in extracellular H^+ upon application of ATP, nor have we observed ATP-induced alterations in extracellular H^+ from horizontal cells of catfish, or horizontal cells, bipolar cells or photoreceptors of goldfish.

A recent study suggests that extracellular ATP exerts its effects on synaptic transmission in the retina by increasing pH buffering in the synaptic cleft (Vroman et al., 2014). The increase in extracellular acidity observed from isolated Müller cells and in retinal slices cannot be explained by such a mechanism. The reduction of the extracellular acidification by the P2 antagonists suramin and PPADS implicate activation of ATP receptors; the reduction by agents that block rises in intracellular calcium also do not fit the buffering hypothesis. Extracellular acidifications are also observed at

concentrations of extracellular ATP as low as 1 µM, a concentration not likely to alter significantly the pH buffering capacity. The block of ATP-induced extracellular acidification by the anion transport inhibitor DIDS also cannot be explained by simple increases in extracellular pH buffering. Müller cells possess a voltage-sensitive, sodium-coupled bicarbonate transporter that is capable of acidifying the extracellular fluid (Newman, 1991; 1996). However, the bicarbonate transporter is unlikely to account for the acidification since in all of the experiments reported here, 1 mM HEPES was the only pH buffer. While the underlying molecular mechanism(s) or transporter(s) is not yet clearly established, the block by DIDS suggests a link to an anion-coupled transport system, although caution is needed with this assessment, since DIDS has been suggested to inhibit ATP-activated receptors directly (Mamedova et al., 2004).

The high sensitivity of photoreceptor calcium channels to small changes in extracellular H^+ has led to the hypothesis that the surround portion of the classic center-surround receptive fields of retinal neurons are due to alterations in extracellular acidity mediated by retinal horizontal cells (Thoreson and Mangel, 2012; Kramer and Davenport, 2015). According to this hypothesis, glutamate released by photoreceptors depolarizes horizontal cells; the depolarization leads to increased release of protons from horizontal cells and inhibition of calcium influx through the photoreceptor calcium channels, with consequent reduction in glutamate release. As shown in Figure 1, self-referencing recordings from isolated horizontal cells show that application of glutamate leads to an extracellular alkalinization – precisely the opposite result expected - which is likely due to the activation of the plasmalemma PMCA $Ca2+/H^+$ antiporter; calcium extrusion is coupled to the influx of H+. Two previous studies (Jouhou et al., 2007;

Trenholm and Baldridge, 2010) using the pH-sensitive dye HAF purporting to claim that isolated horizontal cells acidify the extracellular fluid when depolarized, likely were measuring changes in intracellular H^+ instead, since the HAF loading protocol used in those studies led to large amounts of the dye in the cell's interior (Jacoby et al., 2012). Under loading conditions in which HAF is restricted to plasma membrane, glutamate induces a change in HAF fluorescence indicative of a uniform alkalinization around the cell, in agreement with self-referencing H^+ electrode studies (Jacoby et al., 2014). Two additional sets of experimental results have been taken as strong evidence in support of the hypothesis that depolarization of horizontal cells induces an acidification in the synaptic cleft. First, simultaneous paired recordings from horizontal and photoreceptor cells in retinal slices reveal that direct depolarization of horizontal cells induces a rightward shift of the calcium conductance activation curve of photoreceptors which is abolished by enhancing extracellular pH buffering capacity (Cadetti and Thoreson, 2006; Thoreson et al., 2008). More recently, Kramer and colleagues fused a pH-sensitive form of GFP (pHluorin) onto the extracellular side of a cone calcium channel subunit (dubbing the compound "CalipHluorin") and measured light-elicited changes in fluorescent intensity indicative of an acidification when horizontal cells are expected to be depolarized (Wang et al., 2014). An extracellular acidification reported by calipHlourin in the photoreceptors was also observed in retinal slices when genetically modified zebrafish horizontal cells containing FMRF-amide receptors were depolarized by FMRFamide. These results might be accounted for by indirect activation of the neighboring Müller cells. Synaptic vesicles possess high concentrations of ATP which can be released to the extracellular space by fusion of vesicles with the plasma

membrane (cf Burnstock and Verkhratsky, 2012) for review). Although horizontal cells do not make significant numbers of conventional chemical synapses onto the terminals of photoreceptors in most species examined, they do make numerous conventional chemical synapses with bipolar cells (Werblin and Dowling, 1969; Sakai and Naka, 1986). Experiments in mammalian retinal horizontal cells also suggest that disruption of vesicular release of GABA by horizontal cells by deleting vesicular GABA transporters impairs feedback modulation of photoreceptor calcium channels (Hirano et al., 2016). Depolarization of horizontal cells may thus lead to vesicular fusion and the release of ATP, and indirect activation of neighboring Müller cell processes, with a consequent increase in Müller cell-mediated extracellular acidification.

If the above line of reasoning is correct, what then is the likely physiological impact of protons released by Müller cells? There are at least two important possibilities. First, Müller cells possess a glutamate transporter which removes glutamate from the synaptic cleft and keeps extracellular glutamate levels low. This transporter induces a rapid and marked extracellular alkalinization likely due to co-transport of protons with glutamate into the cell (cf (Grewer and Rauen, 2005) for review). Removal of protons within the synaptic cleft has the potential to limit glutamate uptake. It may be that Müller cells provide extracellular protons in part to ensure continued removal of excess glutamate. Second, it may be that H^+ release from Müller cells acts as an essential feedback mechanism to limit over-excitability in the retina. According to this idea, ATP co-released with glutamate from photoreceptor synaptic vesicles activates neighboring Müller cells, which acidify the extracellular solution; protons bind to photoreceptor calcium channels, reduce calcium influx, decrease vesicular fusion and depress release

of the excitatory neurotransmitter glutamate. This mechanism can potentially explain an aspect of retinal function that has long puzzled retinal physiologists. Photoreceptors in the dark are believed to be tonically depolarized and constantly liberating glutamate; why should this release be highest in the dark, and why does this expected continuous release not induce glutamate-mediated neuronal excitotoxicity, as observed in other areas of the brain when high levels of glutamate are present? It may be that glutamate release from tonically depolarized photoreceptors is accompanied by co-release of ATP, leading to extracellular acidification mediated by Müller cells, and that this release of acid from Müller cells acts as a form of automatic gain control, decreasing calcium influx into synaptic terminals and limiting neurotransmitter release from retinal neurons without altering the voltages of the cells themselves. Such control of synaptic gain by glial-mediated alterations in extracellular acidity may occur in the inner plexiform layer as well.

There is considerable previous evidence that activation of Müller cells leads to modulation of retinal neurons (Newman and Zahs, 1998; Newman, 2003; 2004a; 2004b). The pattern of modulation is complex, with some classes of retinal neurons showing inhibition upon glial stimulation and others showing enhances in light-stimulated action potentials. In addition to uptake of neurotransmitter in the synaptic cleft by Müller cell transporters, multiple molecular mechanisms have been suggested to be involved in this modulation of neuronal activity by activation of Müller cells, with ATP playing a leading role but with evidence also for the involvement of glutamate, adenosine, GABA, glycine and serine. The data presented here implicate H+ release from Müller cells as a potentially important neuromodulator and gliotransmitter regulating synaptic transmission in the retina.

It may further be that H^+-mediated gain control is not limited to Müller cells in the retina, but could be a general mechanism to limit neuronal excitability used by glial cells throughout the brain. In support of this hypothesis, preliminary self-referencing experiments reveal that extracellular ATP also induces an increase in extracellular H^+ from glial cells cultured from cortex and hippocampus. These findings may impact on an additional question that has bedeviled the field of neuroscience for many years: what is/are the molecular mechanisms by which glial cells modulate excitability within the nervous system? These results point to H^+ as a key "gliotransmitter" capable of modulating neuronal activity that may limit and prevent neuroexcitotoxicity within the nervous system.

IV. EXTENSIONS AND ELABORATIONS

IV. 1. SUMMARY

The extracellular acidification observed from isolated Müller cells in response to bath-applied ATP could play a role in various processes, such as regulatory cell volume decrease during high neuronal activity, establishing an acidic environment as means of tuning down neuronal activity and preventing the extracellular build-up of excitatory glutamate wither by affecting calcium channel activity or by ensuring efficient glutamate uptake, etc. To examine the role of the extracellular acidification, it is important to establish the identity of the molecular mediators of H+ efflux as well as the nature of the mechanisms associated with the activation of the process(es). Similarly to ATP, UTP and ADP, known agonists or purinergic receptors, induce an extracellular acidification, whereas adenosine, a ligand to P1 adenosine receptors, fails to do so. The extracellular acidification and acidic standing flux are reduced in 0 extracellular sodium, DIDS and probenecid, but not in low extracellular chloride, bafilomycin-A1 or SCH28080. Forskolin is able to induce a small extracellular acidifying response, which response is absent in dibutyryl-cAMP. A robust acidification is also induced by the application of a hyperosmotic Ringer's solution. The ATP-induced acidification is also observed from Müller cells isolated from various other species: lamprey, channel catfish, rat, two macaque species, and human, as well as from slices of salamander and goldfish retinae. These results suggest that the ATP-induced acidification is mediated by the activation of P2 purinergic receptors with no contribution of adenosine receptors. It exhibits dependence on extracellular sodium, but not chloride, yet DIDS data does suggest activation of anion transport, which could include chloride efflux of transport of organic osmolytes. Activation of membrane H+ pumps does not seem to be responsible for the

H+ efflux from these cells. Under the current experimental conditions, intracellular levels of cAMP do not seem to be part of the signaling mechanism, unlike the activation of the PLC-IP3 pathway as suggested in chapter III. The detection of the acidification in other species suggests that is a general and conserved response of retinal Müller cells to ATP across various vertebral classes of animals.

IV. 2. INTRODUCTION

ATP can act as a ligand to ATP receptors of the P2Y, metabortropic, and P2X, ionotropic subtypes. P2X receptors are ligand-gated ion channels which are permeant to Na+, K+ and Ca2+, and at least 7 members have been identified so far. Some P2X receptors, such as the P2X7, have been shown to form large-conductance pores when physically associated with pannexins. During the formation of such a large pore, larger molecules have been shown to be able to pass through. P2Y receptors, on the other hand, are metabotropic, G-protein coupled receptors, execute functions via activating G proteins and intracellular messaging pathways. At least 8 P2Y receptors have been identified so far. ATP is not the only ligand to P2 receptors; various subtypes are also gated by ADP, as well as pyrimidines such as UTP and UDP. Depending on the ligand and on the particular P2Y receptor subtype activated, intracellular events downstream of receptor activation can lead to PLC activation with or without IP3 productions, intracellular calcium rises, and activation or inhibition of adenylyl cyclase. The expression pattern or P2Y receptors vary based on tissue and the pharmacology of these receptors is far from ideal. Broad-spectrum inhibitors block some P2X and some P2Y receptors,

but not others. Also, some inhibitors, such as suramin, have been shown to also directly affect membrane ion channels (Lechner and Boehm, 2004).

Müller cells of various species throughout the vertebrate classes have been shown to express P2 receptors. Studies which have employed a battery of agonists and antagonists have concluded that P2Y1, 2, 6 and 11, and possibly P2Y 4 and 13 are expressed on the membranes of tiger salamander Müller cells (see Reifel et al, 2003).

Activation of P2 receptors has been shown to be involved in cellular regulatory mechanisms, such as regulatory volume decrease after osmotic swelling, which is believed to be mediated by increased potassium release. P2Y receptor signaling increases intracellular calcium and other intracellular messenger molecules, whose various targets can involve membrane channels and transporters.

Nucleotides are believed to be released by cells from various tissues. The standard notion is that once in the extracellular space these nucleotides are quickly broken down by ectoenzymes. As mentioned earlier, ATP and ADP act on P2 receptors, but there is another class of receptors which are activated by the final breakdown product of ATP, adenosine. These receptors comprise the P1 class, are coupled to G proteins, and also act through intracellular messengers.

In this work, I have attempted to provide evidence that the observed extracellular acidification is due to ATP acting on receptors of the P2 family. In this work, the functions of various ion transporters and pumps were manipulated by either applying known inhibitors or by altering extracellular ion concentrations. This work also shines light onto possible intracellular molecules/pathways immobilized downstream of ATP receptor

activation. The ability of Müller cells of species other than the tiger salamander to acidify the extracellular space in response to ATP were investigated.

IV. 3. RESULTS

IV. 3. 1. In search for the molecular mediators of extracellular acidification: ion transporters and pumps.

Potential dependence of the observed extracellular acidification from ATP-stimulated Müller cells on extracellular sodium was examined by performing self-referencing measurements in bathing Ringer's solution lacking added sodium. 110mM NaCl were replaced with 110mM choline chloride. In zero extracellular Na+ conditions, ATP still caused an extracellular acidification, but was greatly reduced in amplitude (Figure 8). In six trials from isolated Müller cells from the tiger salamander the mean standing flux in 0 Na+ was 11 ± 3 μV compared to 69 ± 7 μV in the presence of 100mM extracellular sodium, $p<0.001$. In zero extracellular sodium, the mean acidification response to 10 μM ATP was 59 ± 12 μV compared to the acidification response in sodium-containing Ringer's, 207 ± 20 μV, $p<0.01$. The introduction of sodium into the dish alone caused a transient and significant acidification which eventually stabilized at what is reported here as the standing flux in sodium-containing Ringer's. The mean peak of the acidification caused by introduction of Na+ was 241 ± 30 μV, $p<0.001$. Under zero extracellular sodium conditions, the standing flux was virtually absent as there was no significant difference between it and the background control. Even though the overall extracellular acidity was greatly reduced when sodium was omitted from the extracellular

bathing solution, the percent increase induced by ATP from standing acidity levels was actually higher in zero extracellular sodium, 460% versus 200% increase in 110mM NaCl. The ATP-induced extracellular acidification was not significantly affected by 20 μM EIPA, a hydrogen/sodium exchanger blocker. In three cells 20 μM ATP still produced a pronounced extracellular acidification in the background of EIPA (Figure 9A, B). The mean standing flux was 22 ± 9 μV after EIPA application and ATP produced a mean extracellular acidification of 238 ± 5 μV, p=0.001. When the standing flux levels before and after EIPA application were compared in eight trials (Figure 9C), there was a significant difference between the extracellular acidity in clean Ringer's and after the application of 20 μM EIPA. EIPA reduced the extracellular acidity from 33 ± 7 μV to 19 ± 3 μV, p=0.03 (N=8).

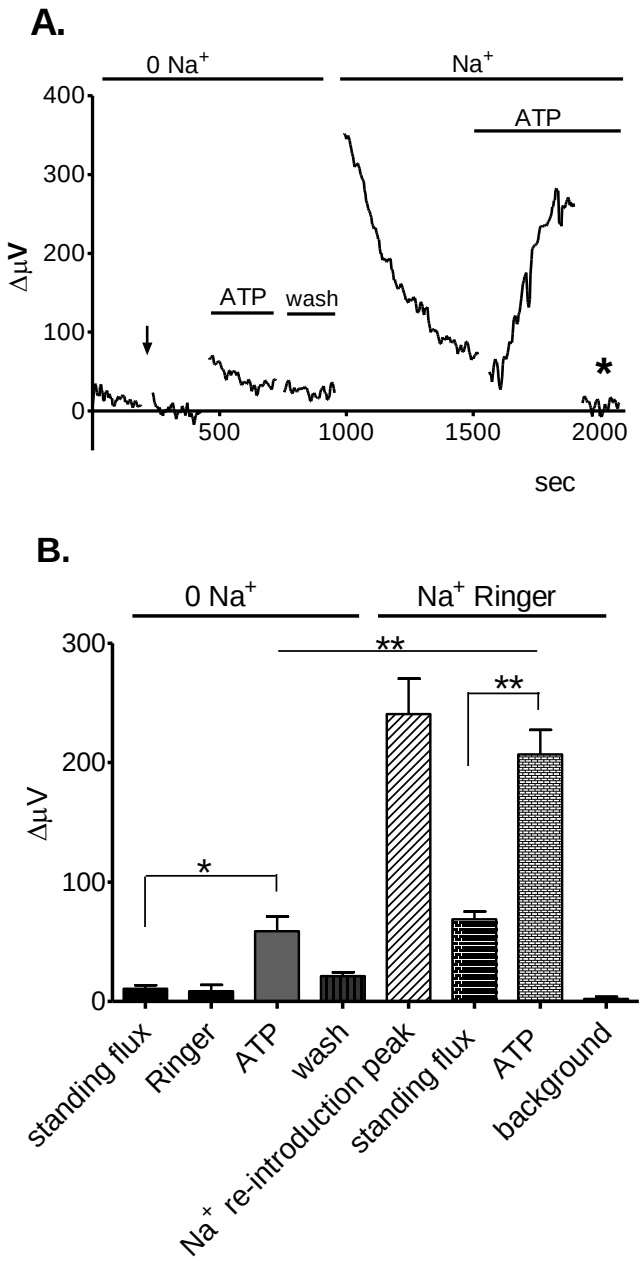


Figure 8. Omission of sodium from the extracellular bathing solution significantly reduces the standing acidic flux and the extracellular acidification induced by 10 μM ATP. (A) A representative trace from a single recording from an isolated Müller cell in 0 extracellular sodium and after sodium reintroduction; note the robust transient acidification caused by the reintroduction of sodium. (B) Mean data from six trials. The standing flux and the extracellular acidification induced by 10 μM ATP are significantly reduced when sodium is omitted from the bathing solution.

Potential dependence of the observed ATP-induced extracellular acidification on extracellular chloride was examined by performing self-referencing measurements from isolated Müller cells when the majority of extracellular chloride was removed (Figure 10). 110 mM NaCl in the Ringer's were replaced with 110 mM Na isethionate. There was still extracellular chloride present in the Ringer's solution, in low milimolar concentratiton, which was derived from the magnesium, potassium and calcium salts in the Ringer's. It was assumed that if the observed effect was driven by a membrane transporter which was dependent on chloride influx, removing most of the extracellular chloride would significantly impede the ability of the transporter. Data from six trials showed that under very low extracellular chloride conditions 100 µM ATP still induced a very potent extracellular acidification (Figure 10B), which was not significantly different from the acidification produced by ATP in normal extracellular chloride concentrations (unpaired t-test analysis of two populations from the same retinal dissociation). Under low $[Cl^-]_e$, 100 µM ATP increased the extracellular acidity from a mean standing flux of 61 ± 16 µV to 514 ± 111 µV, p=0.0087. Under normal extracellular chloride conditions, the standing flux increased from 33 ± 5 µV before to 642 ± 135 µV after application of 100 ATP µM, p=0.0098, N=5 (Figure 10C,D)

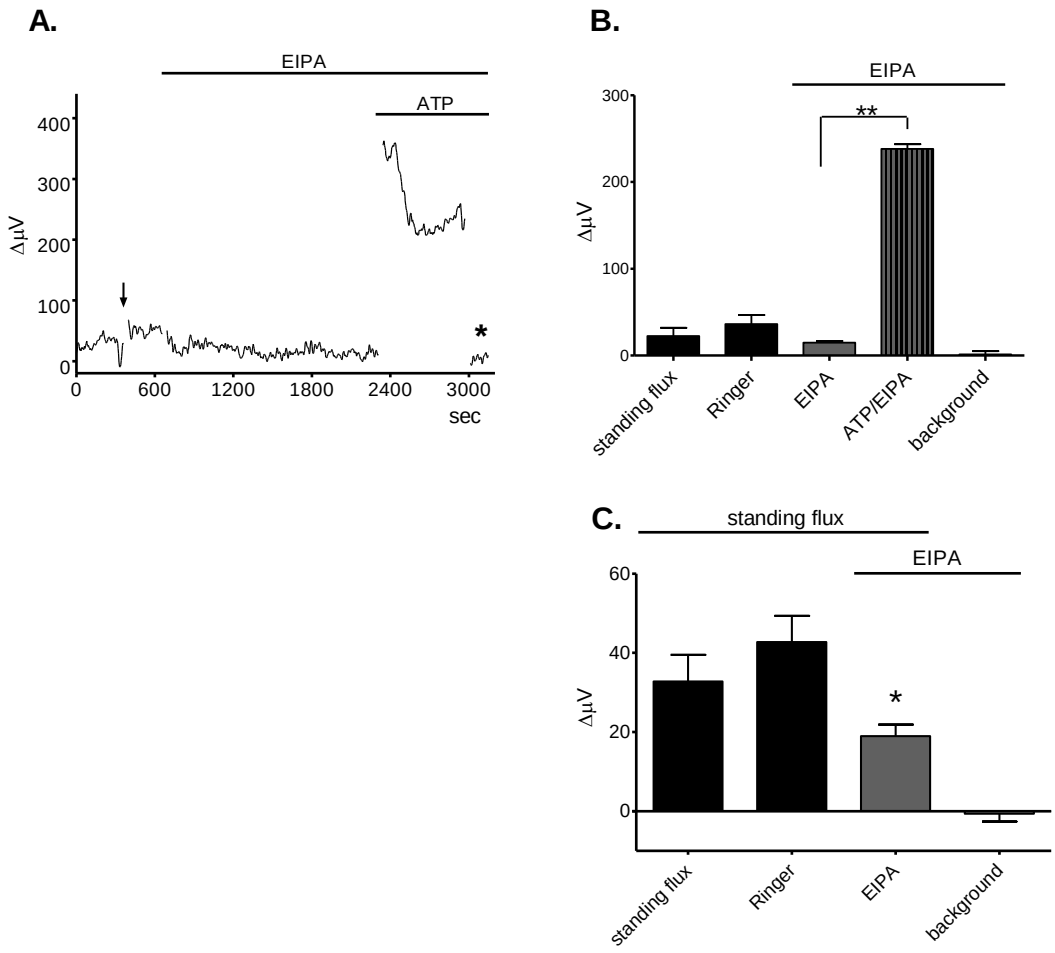


Figure 9. 20 μM ATP still produces a pronounced extracellular alkalinization from Müller cells isolated from tiger salamander retina in the presence of the Na^+/H^+ exchanger blocker EIPA. (A) A representative trace from a single recording with a self-referencing H^+-selective microelectrode. (B) Mean data from three trials still shows a pronounced ATP-induced extracellular acidification in the presence of EIPA. (C) The standing acidic flux still persists in the background of EIPA; it is however reduced when compared to the Ringer's control but not when compared to the standing flux before the control Ringer's application, N=8.

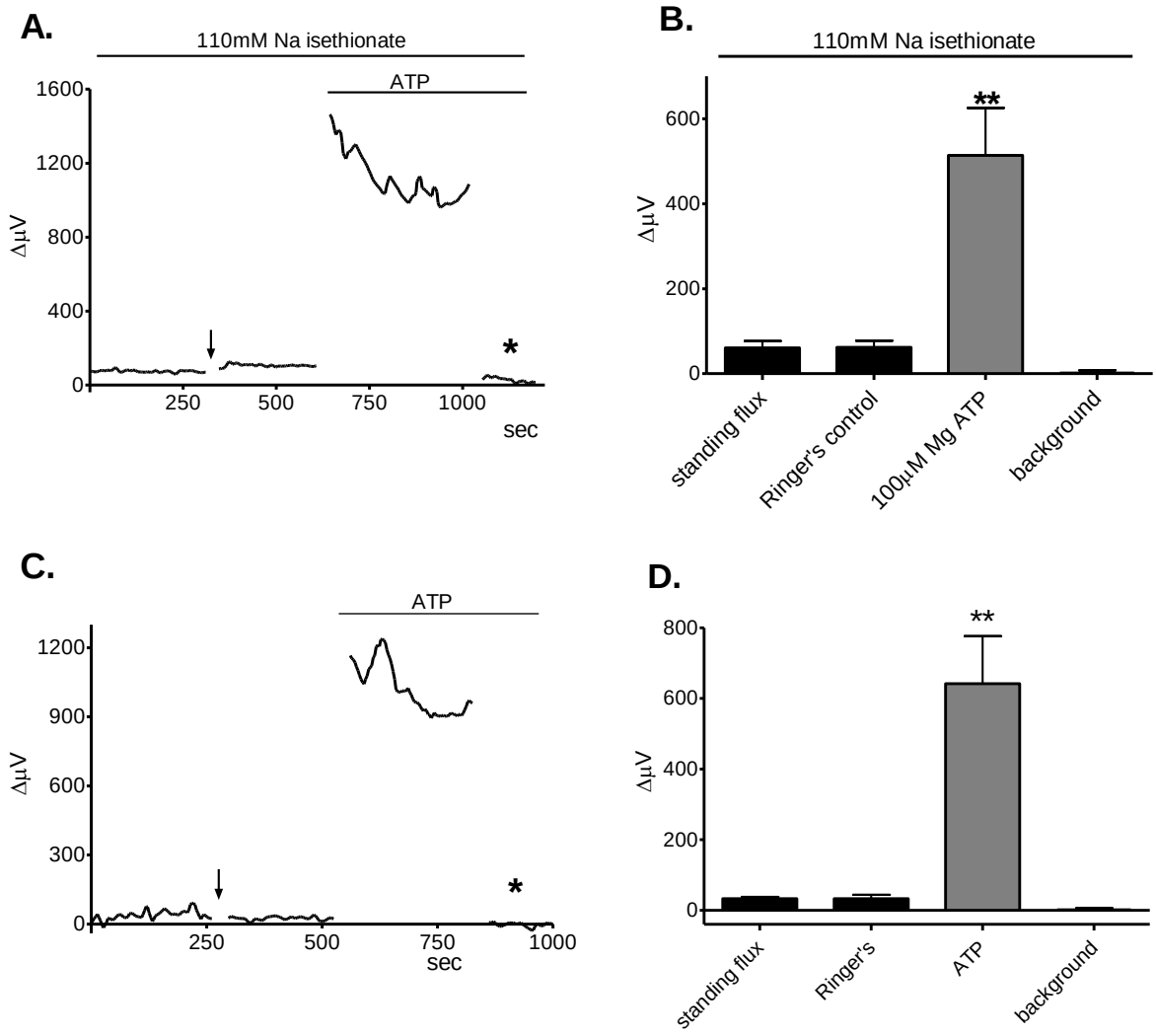


Figure 10. 100 μM ATP still causes an extracellular acidification from isolated salamander Müller cells when 100 mM NaCl is replaced by 100 mM Na isethionate in the Ringer's solution. (A) A single recording from an isolated Müller cell shows an increase in the extracellular acidity after ATP application in low $[Cl^-]_e$. (B) Mean data from six trials show a significant increase in extracellular acidity in response to 100 μM ATP in low extracellular chloride conditions. (C) A single recording from the same cell dissociation in normal Ringer's shows an increase in extracellular acidity in response to 100 μM ATP. (D) Mean data from control experiments in normal Ringer's from the same cell dissociation show a significant increase in extracellular acidity induced by bath application of ATP, N=5.

Possible involvement of pannexin channels was also explored, since pannexins had been shown to associate with certain P2 receptor subtypes to form a large-pore complex capable of mediating the transport of larger molecules. The question of whether pannexins were involved was addressed with the use of a pharmacological agent, probenecid, which had been known to be inhibitory at pannexin-1 channels. These experiments were performed following two different experimental paradigms; in one set of experiments the cells were pre-incubated in probenecid for a period of 20-40 minutes before the onset of recordings, and in another set of experiments the probenecid was added to the dishes during the recordings. 100 μM ATP (Figuer 11A) and 10 μM ATP (Figure 11B, C) still caused a significant extracellular acidification from Müller cells which had been pre-incubated in 1 mM probenecid. The mean standing flux from three cells pre-incubated in probenecid was 12 ± 10 μV, which was increased to 91 ± 11 μV after the addition of 10 μM ATP, $p=0.0433$. In experiments where 1 mM probenecid was introduced in the dish after bath application of 10 μM ATP, the extracellular acidification induced by ATP was larger before the introduction of probenecid into the dish (Figure 12). The mean standing flux from four cells was 6 ± 15 μV in clean Ringer's. ATP alone increased the voltage differential signal to 161 ± 6 μV, $p=0.0018$. The voltage signal subsequently decreased to 100 ± 17 μV after probenecid was added to the ATP bathing the cells, which was still significantly higher than the standing flux ($p=0.0022$), but slightly lower than the acidification produced by ATP alone, $p=0.0365$. When the responses to 10 μM ATP in both experimental paradigms were then compared (Figure 12C) with an unpaired t-test (since the comparison was performed between two different experimental populations), the ATP-induced acidification produced by Müller cells pre-incubated in 1

mM probenecid was significantly smaller than the one produced by cells bathed in clean Ringer ($p=0.002$); yet there was no statistical difference between both populations when the ATP-induced acidification from cells pre-incubated in probenecid was compared to the extracellular H^+ change recorded from cells exposed to probenecid after ATP exposure.

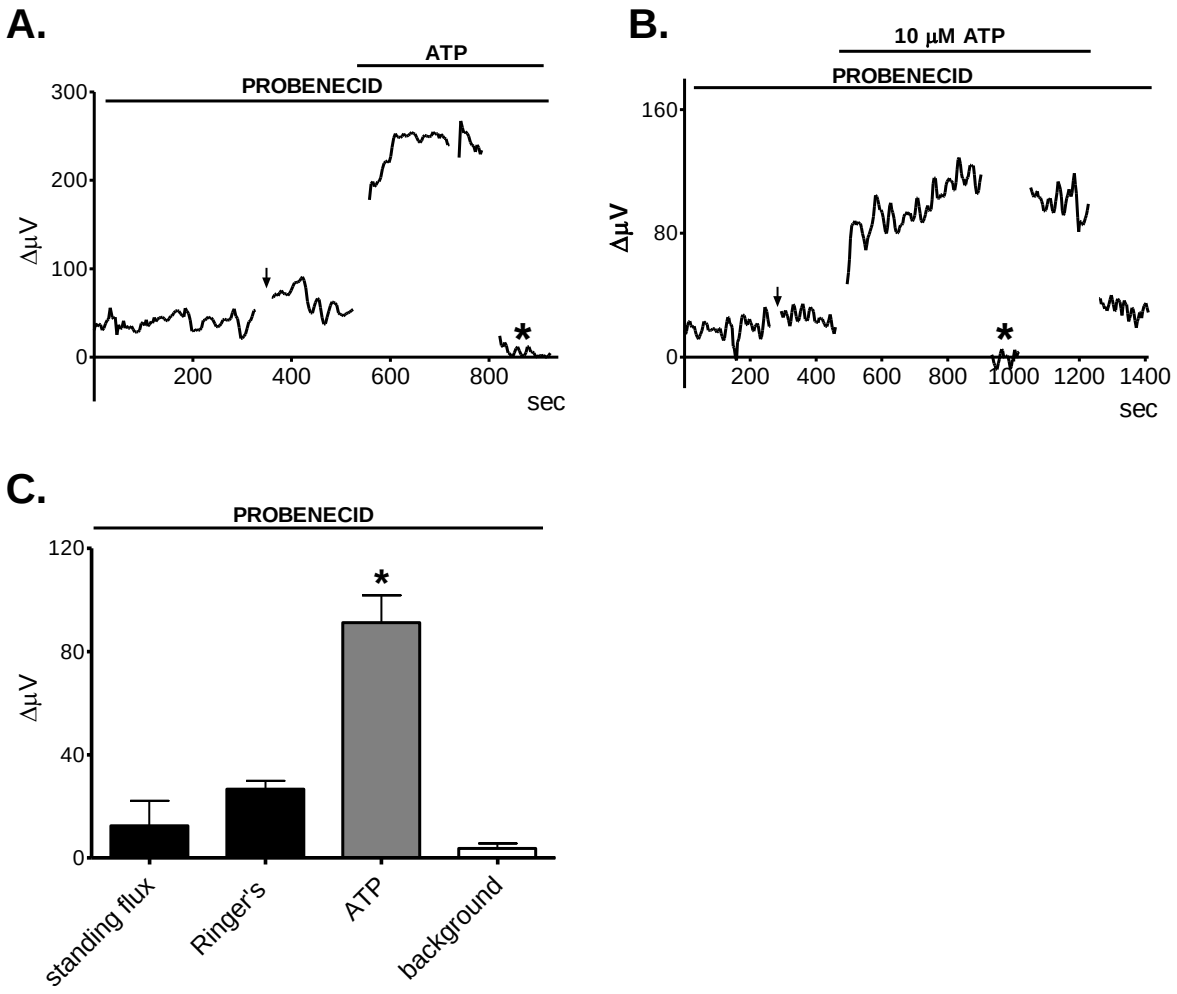


Figure 11. ATP still induces an extracellular acidification from isolated salamander Müller cells in the presence of probenecid. (A) A representative trace from a single self-referencing recording shows an increased extracellular acidification in response to 100 μM ATP in the background of 1mM probenecid. Asterisks indicate background controls 200 μm above the cell. (B) A representative trace from a single self-referencing recording shows an increased extracellular acidification in response to 10 μM ATP in the background of 1mM probenecid. (C) Mean data from three trials show that the acidification caused by 10 μM ATP is still significant in cells pre-incubated in 1 mM probenecid.

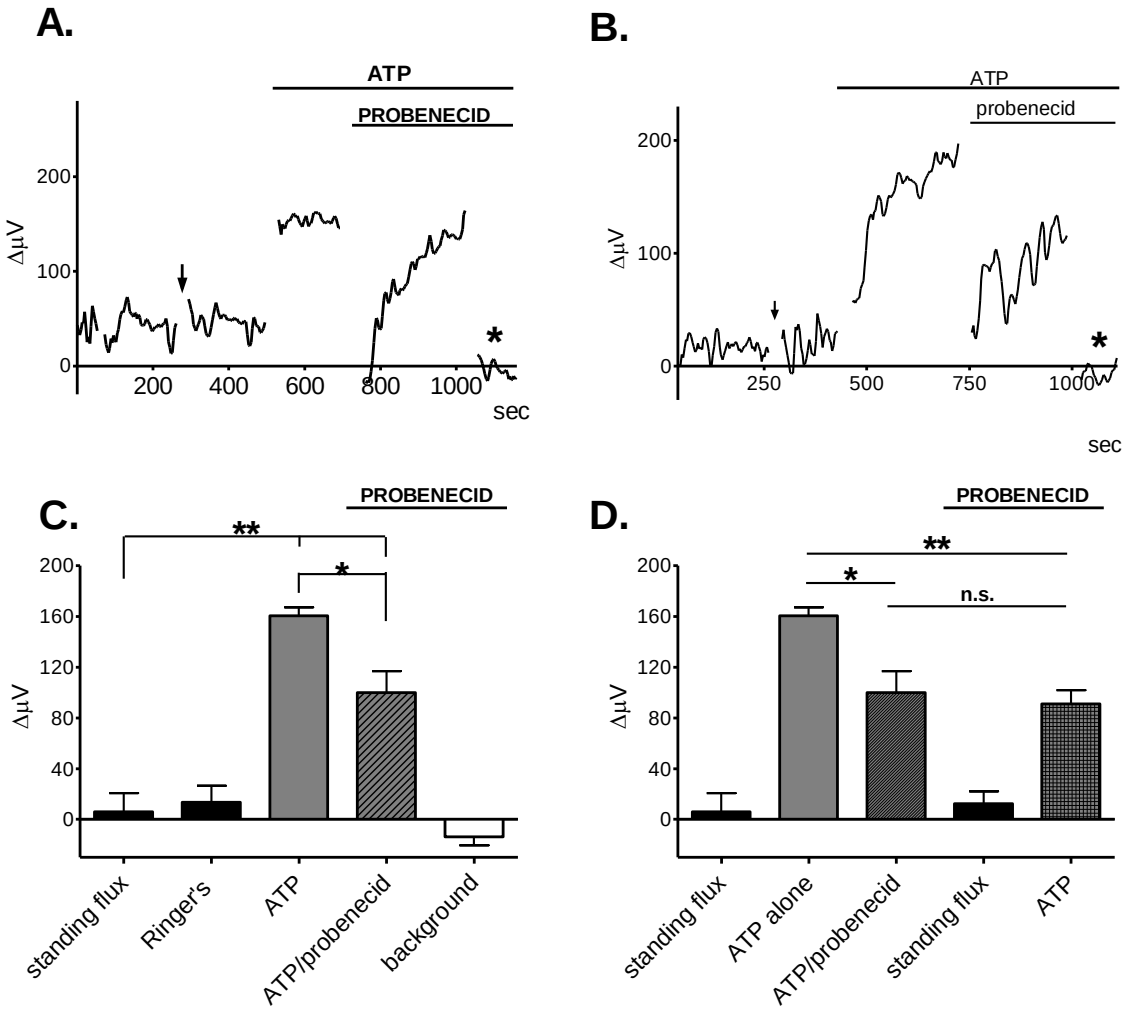


Figure 12. Probenecid reduces the ATP-induced an extracellular acidification from isolated salamander Müller cells. (A, B) Representative traces from single self-referencing recordings show an increased extracellular acidification in response to 10 μM ATP, which is transiently reduced by 1mM probenecid. Asterisks indicate background controls 200 μm above the cell; downward arrows: Ringer's controls. (C) Mean data from four trials show that the acidification caused by 10 μM ATP is reduced yet still significant in 1 mM probenecid. (D) Mean data from the two populations in Figures 11 and 12. First three columns are from Figure 12 and the last two columns are from Figure 11. The comparison between the two populations was done with an unpaired t-test, while all comparison within the same population were done with a paired t-test.

Studies on epithelial cell transmembrane transport have demonstrated that the H^+ ATPases are pumps capable of causing a significant extracellular acidification. Moreover, it has been suggested, based on experimental data, that H+ ATPases on horizontal cells could be responsible for acidifying the extracellular space during the horizontal cell-to-cone photoreceptor negative feedback according to the proton hypothesis. Whether the observed ATP-induced extracellular acidification from isolated Müller cells was due to the activity of vacuolar H+ ATPases was tested. In the presence of 400 nM bafilomycin-A1, an inhibitor of vacuolar H^+ ATPases, 100 μM ATP still managed to induce a very significant extracellular acidification from isolated salamander Müller cells (Figure 13). In six trials, the signal increased from a mean standing acidifying flux of 88 ± 31 μV before to 765 ± 83 μV after ATP application, p=0.0009. There was no difference in the standing acidity between pre- and post- bafilomycin application.

A possible role for the H+/K+ ATPase (gastric proton pump) in the observed extracellular acidification from isolated Müller cells was also examined in the presence of an inhibitor of gastric proton pumps, SCH28080. 10 μM ATP still induced a significant acidification in the presence of SCH28080 (Figure 14). Isolated Müller cells were pre-incubated in 130 μM SCH28080 for a period of 25-40 minutes before the onset of experiments. In the presence of the drug, 10 μM ATP induced a significant extracellular acidification and increased the H+ electrode's voltage signal from a standing acidic flux of 128 ± 25 μV to 349 ± 32 μV, p=0.0193 (N=4).

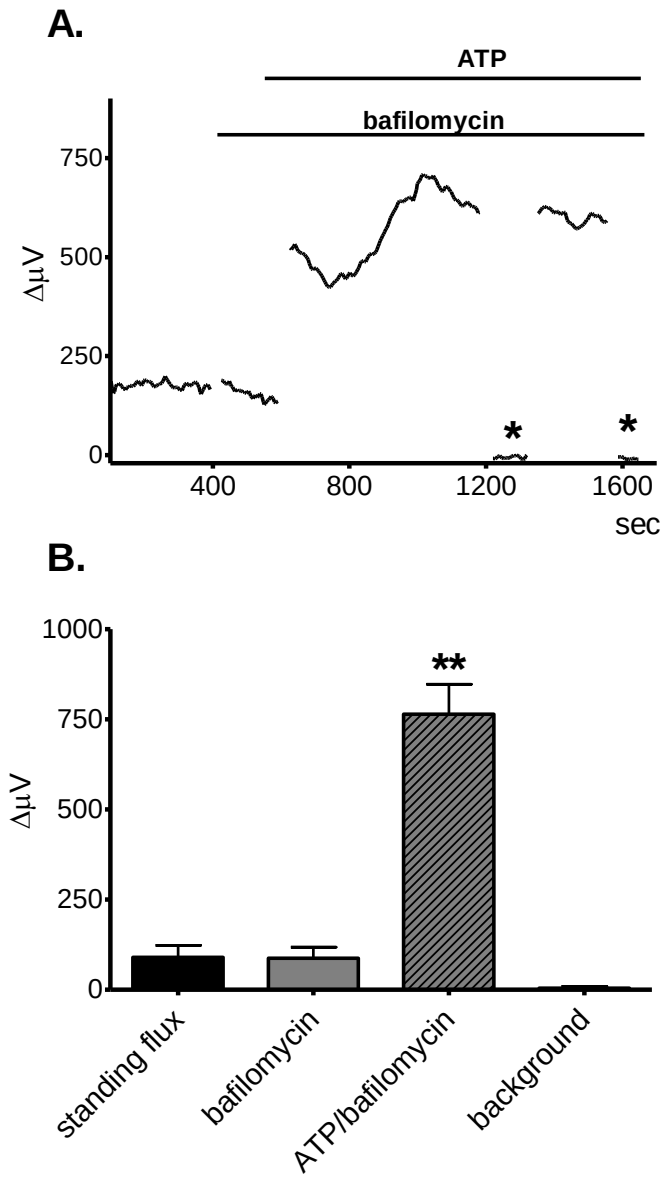


Figure 13. Bafilomycin-A1 fails to inhibit the ATP-induced extracellular acidification from Müller cells isolated from T. salamander. (A) A representative trace from a single self-referencing recording shows a robust extracellular acidification in response to 100 μM ATP in the background of 400 nM bafilomycin-A1. Asterisks indicate background controls 200 μm above the cell. (B) Mean data from six trials show a significant increase in extracellular acidity in response to ATP in the presence of bafilomycin-A1.

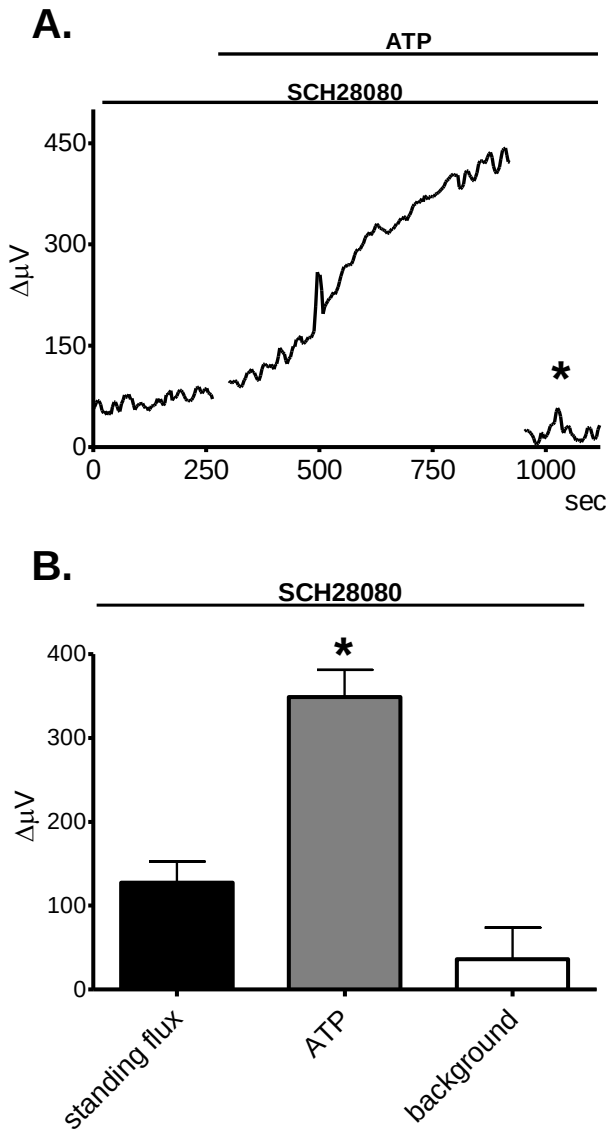


Figure 14. SCH28080, a H+/K+ ATPase inhibitor, fails to inhibit the ATP-induced extracellular acidification from Müller cells isolated from T. salamander. (A) A representative trace from a single self-referencing recording shows a robust extracellular acidification in response to 10 μM ATP in the background of 130 μM SCH28080. Asterisks indicate background controls 200 μm above the cell. (B) Mean data from four trials show a significant increase in extracellular acidity in response to ATP in the presence of SCH28080.

The effect of extracellular osmolarity on the observed H+ fluxes from these cells was also examined. Müller cells have been shown to possess a mechanism responsible for regulatory volume changes when the osmolarity of the extracellular medium fluctuates, especially with increased neuronal activity. Autocrine ATP signaling has been speculated to be the mechanism through which these cells maintain their volume and prevent swelling. Preliminary experiments were performed in which extracellular H+ changes were recorded from isolated Müller cells in isosmotic and hyperosmotic solutions. Extracellular osmolarity was increased by either increasing extracellular NaCl from 200 mOsm to 400 mosm (Figure 15B), which increased the osmolarity of the normal Ringer's solution by 200mOsm, or by adding 100 mM of sucrose to the Ringer's which increased the osmolarity by 100 mOsm (Figure 15A). The introduction of hyperosmotic Ringer's to isolated Müller cells caused a pronounced extracellular acidification (Figure 15C). The mean standing flux in isosmotic Ringer's solution was 37 ± 14 μV (N=3). Increasing the extracellular osmolarity by 100-200 milliosmoles significantly increased the standing acidic flux to 132 ± 18 μV, $p=0.0023$.

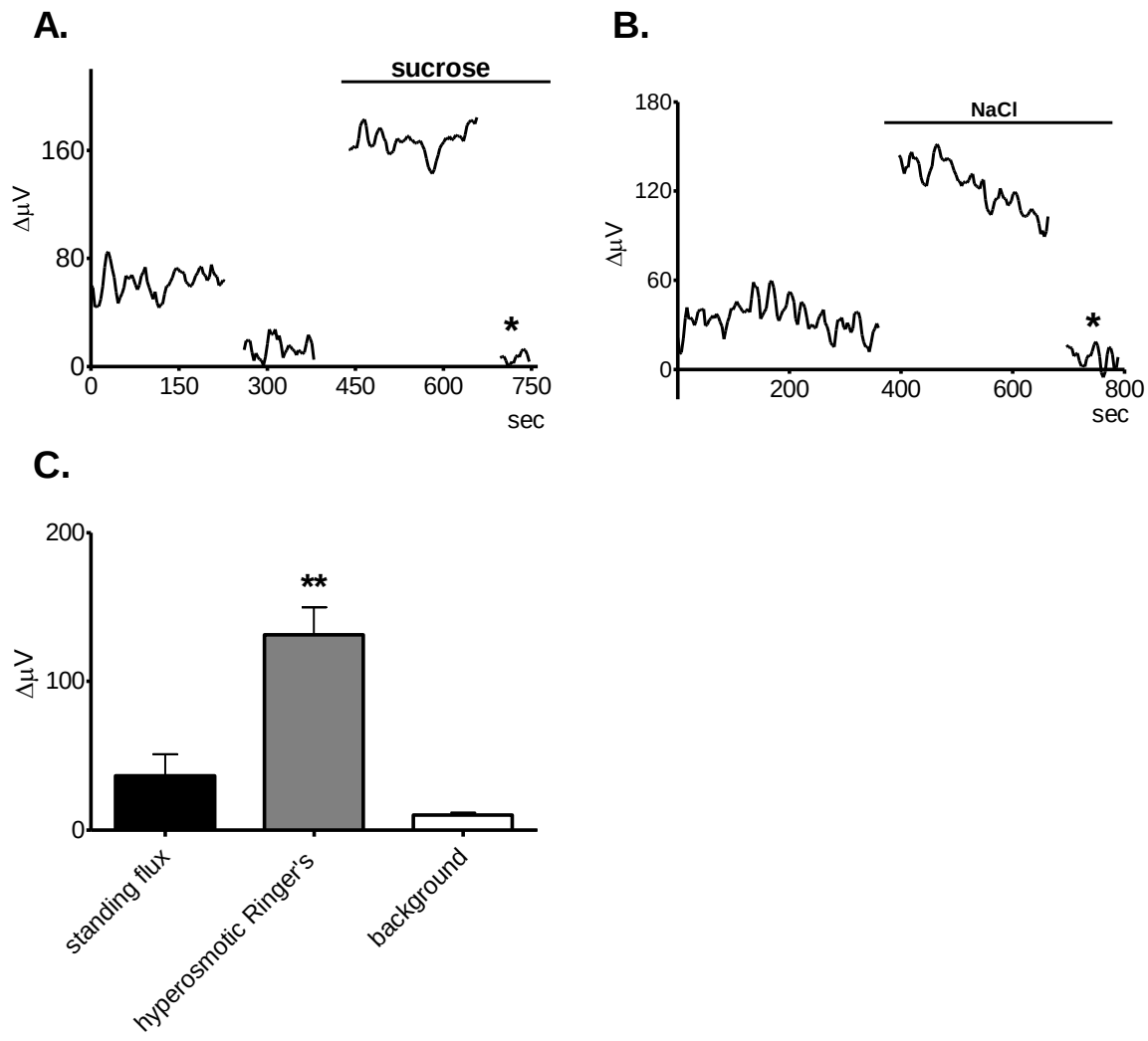


Figure 15. Isolated Müller cells acidify the extracellular bath after exposure to a hyperosmotic extracellular environment. (A) A representative trace from a single self-referencing recording shows a robust extracellular acidification in response to the addition of 100 mOsm of sucrose to the bathing Ringer's. (B) A representative trace from a single self-referencing recording shows a robust extracellular acidification in response to the addition of 200 mOsm of NaCl to the bath solution. Asterisks indicate background controls 200 μm above the cells. (C) Mean data from three trials show a significant increase in extracellular acidity in response to extracellular increase in osmolarity.

IV. 3. 2. Evidence that activation of P2 and not P1 receptors is responsible for the extracellular acidification.

To test whether the observed increase in extracellular acidification could be due to break-down of the bath-applied ATP to adenosine (and phosphate groups), which could subsequently activate P1 receptors, 100 μM adenosine was bath-applied to isolated Müller cells (Figure 16A,B) and retinal slices(Figure 16C, D). In five trials with isolated Müller glia adenosine alone did not cause a significant change in the extracellular acidity compared to the standing flux before application, $p=0.77$. Subsequent application of 10 μM ATP on these same cells induced the typical increase in the extracellular acidity seen after ATP application. The standing flux increased from 8 ± 3 μV in adenosine alone to 115 ± 20 μV after the introduction of ATP, $p=0.004$. Adenosine also did not induce an extracellular H+ change from slices (N=5). The mean standing flux was 737 ± 73 μV after control applications of Ringer's and 721 ± 75 μV after the application of 100 μM adenosine, $p=0.1007$.

Two other agonists of P2 receptors, UTP and ADP, also produced an increase in extracellular acidity when applied to the solution bathing Müller cells isolated from the tiger salamander retina. 10 μM ADP caused a significant increase in extracellular acidity from isolated Müller cells detected via self-referencing (Figure 17A). In four trials the self-referencing signal increased from a mean value of 106 ± 32 μV before 10 μM ADP application to 178 ± 21 μV after, $p=0.03$ (Figure 17B). 100 μM ADP caused an even more robust increase in extracellular acidity (Figure 17C, D). In six trials, the signal increased from a mean standing flux of 41 ± 6 μV before to a mean of 235 ± 18 μV after ADP application, $p<0.0001$. In the case of 10 μM ADP, there was no difference between

the means of the whole-length signals in the presence of the drug and only those segments which constituted the maximal responses. In the case of 100 μM ADP, the mean of the maximal responses was significantly higher than the mean of the full-length signals in the background of the drug, p=0.007.

Bath-application of 10 μM UTP induced a significant increase in the extracellular acidity measured from isolated Müller cells (Figure 18A,B). In six cells, the standing flux increased from a mean value of 149 ± 16 μV before to 267 ± 30 μV after the application of 10 μM UTP, p=0.003, with a significant difference between the mean of the full-length signals and the mean of the maximal UTP-induced response, p=0.015. The magnitudes of the signals induced by 100 μM UTP were even larger (Figure 18C,D). In four cells, 100 μM UTP increased the standing acidic flux from 60 ± 23 μV before to 867 ± 182 μV after UTP application, p=0.021.

More evidence that ATP acts through P2 receptors comes from experiments described in chapter III, Figure 4, in which suramin and PPADS, broad-spectrum inhibitors of P2 receptors, significantly reduce the ATP-induced extracellular acidification. Preliminary results from whole-cell voltage clamp experiments on isolated Müller cells did not indicate any obvious P2X-driven currents at resting potentials (-80mV) activated by ATP application.

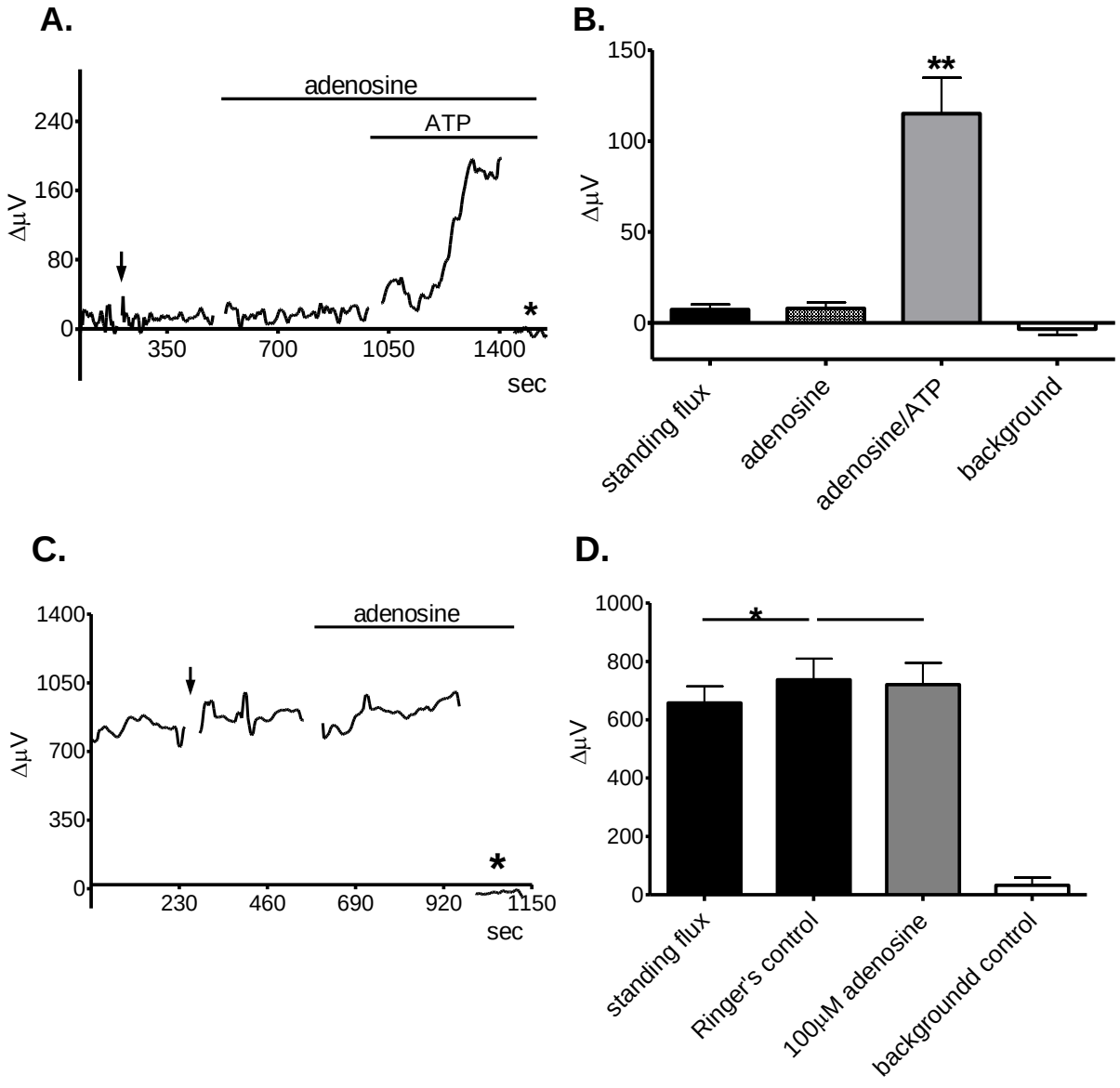


Figure 16. Adenosine does not induce an extracellular change in H+ from isolated Müller cells or retinal slices. (A) A representative trace from a single recording from an isolated Müller cell exposed to 100 μM adenosine first and then 10 μM ATP. (B) Mean data from five trials show lack of response to adenosine alone, but a large acidification in response to ATP. (C) A representative trace from a recording from a slice with bath-applied adenosine. Arrow indicates a control application of Ringer's solution; asterisk: background control. (D) Mean data shows that 100 μM adenosine does not induce an extracellular change in H+ from retinal slices, N=5.

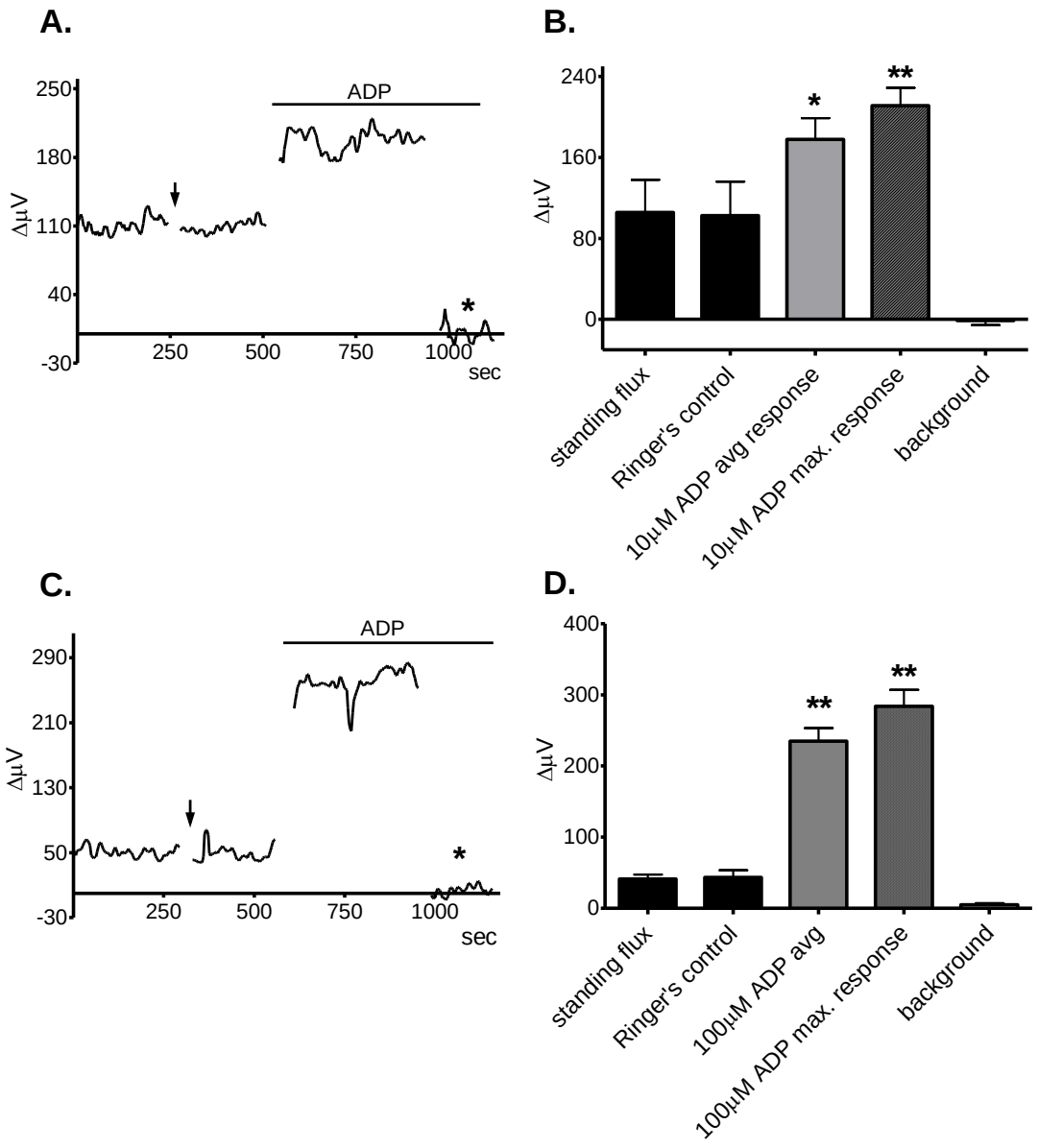


Figure 17. Bath application of 10 μM and 100 μM adenosine diphosphate (ADP) induces an extracellular acidification when recording from isolated Müller cells with H+-selective microelectrodes. (A) A representative trace from a recording from an isolated Müller cell exposed to 10 μM ADP. (B) Mean data from four cells shows an increase in extracellular acidity when cells are exposed to 10 μM ADP. (C) A representative trace from a single recording from a Müller cell exposed to 100 μM ADP. (D) Mean data from six trials shows an even larger extracellular acidification in response to 100 μM ADP.

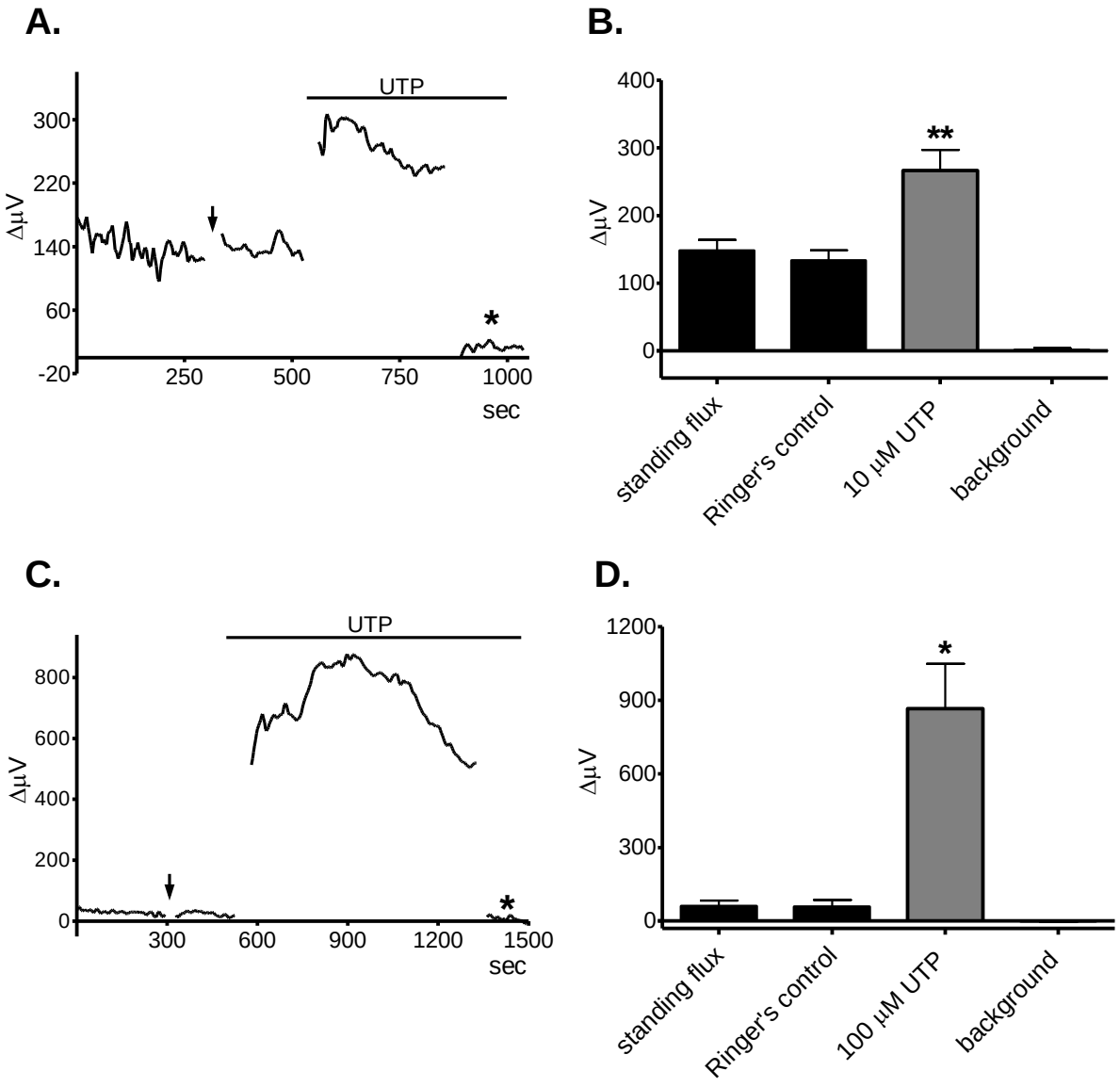


Figure 18. Bath applications of 10 μM and 100 μM uridine-5'-triphosphate (UTP) also induce robust extracellular acidification response from isolated Müller cells. (A) A representative trace from a single Müller cell exposed to 10 μM UTP. (B) Mean data from six trials show a significant extracellular acidification detected from Müller cells after application of 10 μM UTP. (C) A representative trace from a single Müller cell's response to 100 μM UTP. (D) Mean data from four trials show increased extracellular acidity in response to 100 μM UTP.

IV. 3. 3. Intracellular mechanisms underlying the extracellular acidification induced by ATP.

As shown in the previous chapter, our data strongly suggests that the extracellularly applied ATP activates (likely through activation of P2YRs) a PLC-dependent intracellular pathway which mobilizes calcium from intracellular stores. The extracellular acidification is reduced when calcium efflux through IP3-sensitive calcium channels is inhibited.

Caffeine has the ability to permeate cells and has been shown to mobilize intracellular calcium through ryanodine receptor (RyR)-mediated increases in cytosolic calcium. To test whether caffeine alone could mimic the effect seen with ATP by raising intracellular calcium levels, I applied 2 mM caffeine onto culture dishes containing dissociated retinal cells from tiger salamander. I performed self-referencing measurements from dissociated Müller cells exposed to caffeine. Application of caffeine alone did not alter extracellular self-referencing signals. Subsequent application of 50 μM ATP caused a significant increase in the extracellular acidity. The mean signal increased from 17 ± 5 μV in caffeine alone to 137 ± 24 μV in 50 μM ATP in caffeine background, $p=0.002$, $N=6$.

Activation of metabotropic P2 receptors leads to the activation of a variety of intracellular signaling molecules and pathways depending on the particular subtypes of P2 receptors being stimulated. Some P2 receptor subtypes activate adenylyl cyclase (AC) which increases cAMP concentrations, others inhibit the activity of the enzyme, thus preventing the rise of cAMP. To test whether the activation of AC could be part of the intracellular pathway in Müller cells necessary for the observed extracellular

acidification to occur, forskolin, a cell-permeable activator of adenylyl cyclase, was bath-applied to isolated Müller cells (Figure 20). Application of 88 μM forskolin to five isolated Müller cells increased the standing acidic flux after the Ringer's control from a mean of 6 ± 17 μV to 41 ± 11 μV, p=0.0347. The signal in forskolin additionally increased to 621 ± 127 μV after application of 50 μM ATP in the background of, p=0.0094.

To further investigate the possibility that activation of AC and a rise in cAMP concentrations was the pathway involved in Müller cells acidifying the extracellular space following ATP application and likely P2 receptor activation, dibutyryl-cAMP was applied (Figure 21). Dibutyryl is a cell-permeable analog of cAMP which has the ability to activate kinases which are otherwise activated by the endogenous cAMP. In five trials, dibutyryl alone (250 μM) did not significantly change the extracellular acidic flux, mean value of 49 ± 20 μV, p=0.394. In the background of dibutyryl, ATP still induced a pronounced extracellular acidification. Forty and fifty micromolar ATP increased the acidic flux in dibutyryl from 52 ± 15 μV to 384 ± 47 μV, p=0.0034. In three of those trials, in which only 40 μM ATP was applied, the increase in acidity was still detectable regardless the low N, p=0.048

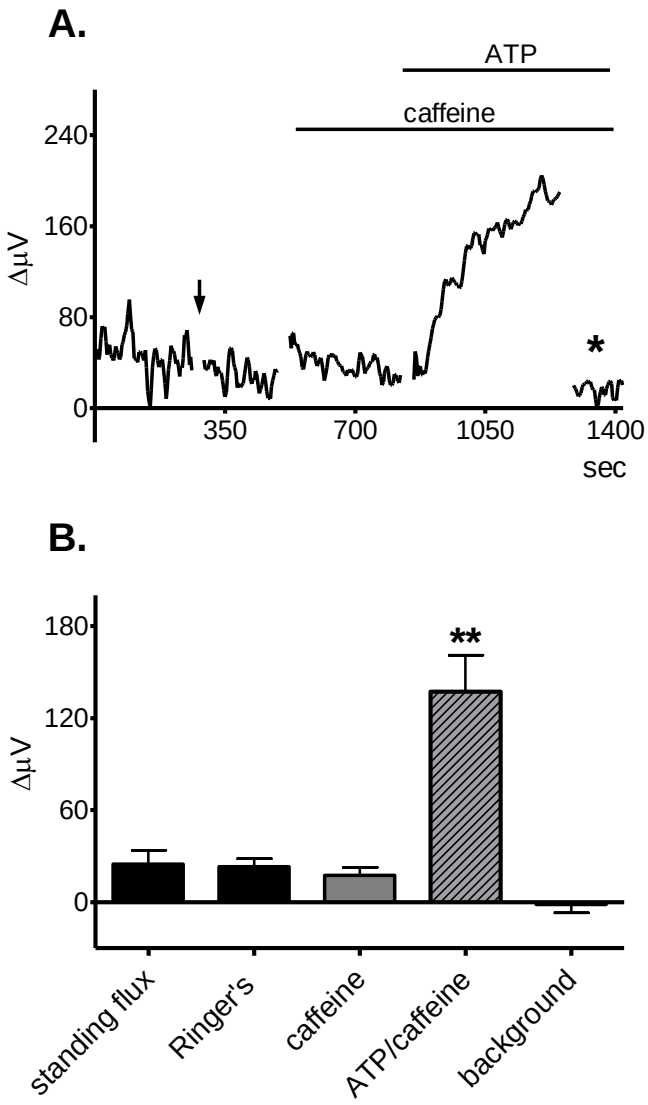


Figure 19. 50 μM ATP induces a significant extracellular acidification in the background of 2mM caffeine. Caffeine (2 mM) alone does not change the extracellular acidity measured from isolated Müller cells.. (A) A representative trace from a single self-referencing recording depicts an increase in extracellular acidity in response to ATP in the background of caffeine, but not to caffeine alone. (B) Mean data show a significant increase in extracellular acidity triggered by bath-application of 50 μM ATP, N=6.

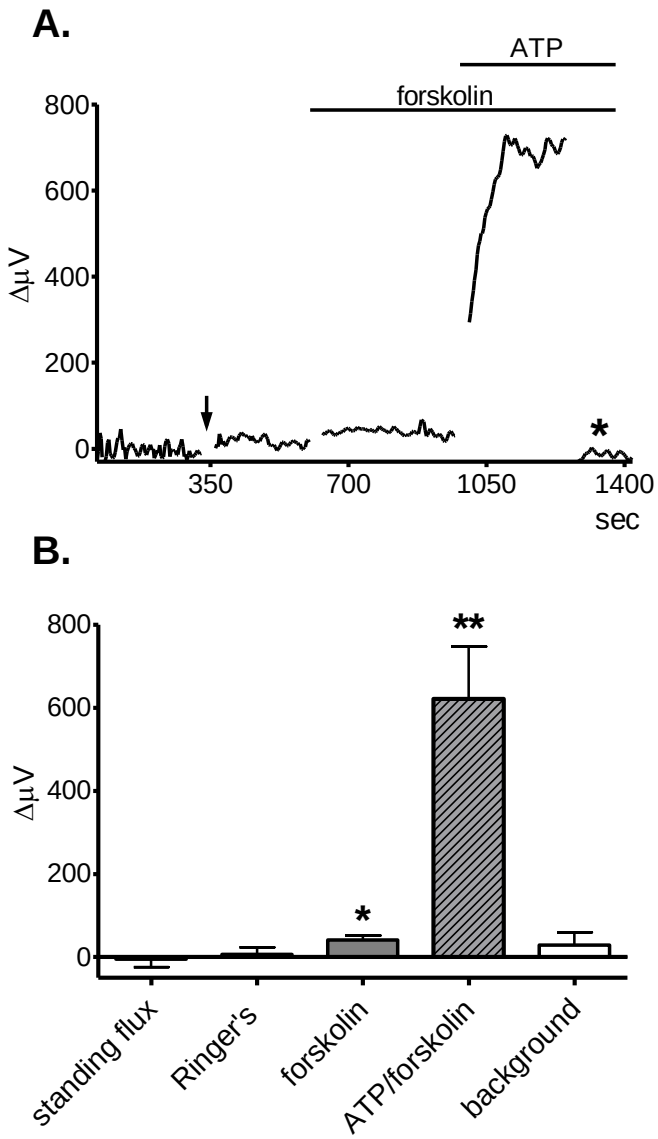


Figure 20. Forskolin alone induced a much smaller extracellular acidification compared to the one induced by ATP form isolated Müller cells. (A) A representative trace from a single self-referencing recording depicts a slight increase in extracellular acidity in response to 88 μM forskolin and even more robust increase after a subsequent application of 50 μM ATP. (B) Mean data show a significant increase in extracellular acidity triggered by bath-application of ATP in the background of forskolin; forskolin alone (88 μM) caused an increase in extracellular acidity, but this increase was much smaller than the one caused by ATP, N=5.

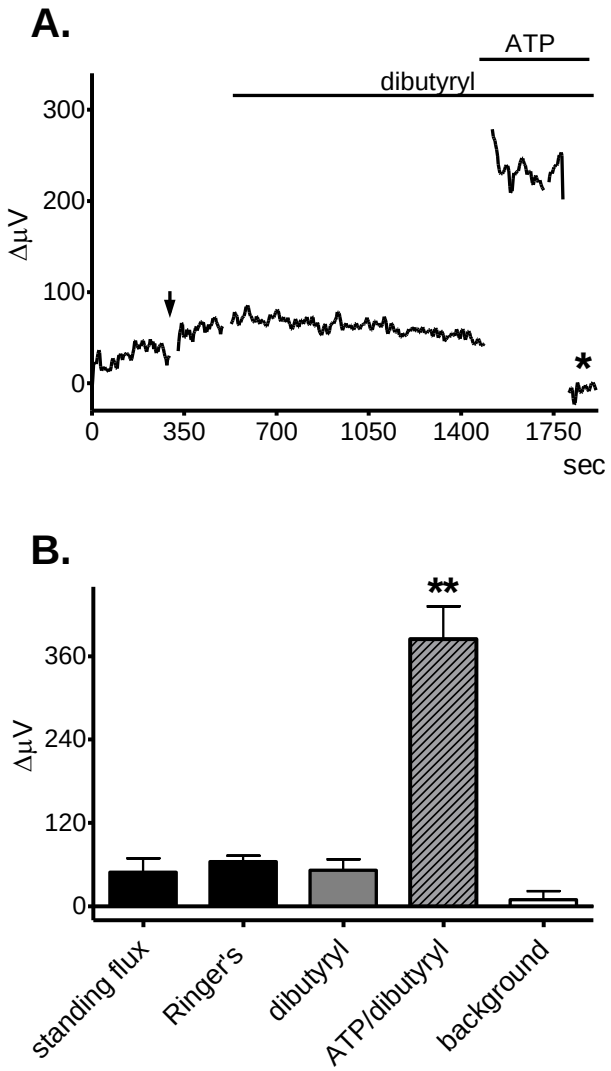


Figure 21. Dibutyryl-cAMP alone did not induce any change in extracellular H+ measured from isolated Müller cells in which 40-50 μM ATP still caused a significant increase in extracellular acidity. (A) A representative trace from a single self-referencing recording depicts an increase in extracellular acidity in response to ATP in the background of dibutyryl. (B) Mean data show a significant increase in extracellular acidity triggered by bath-application of 40-50 μM ATP in the background of 250 μM dibutyryl; dibutyryl alone does not cause a change in extracellular acidity, N=5.

IV. 3. 4. Effects of glutamate, GABA and dopamine on changes of extracellular acidity mediated by isolated Müller cells

The ability of Müller cells isolated from tiger salamander to change the acidity of the extracellular medium in response to other neuro-active molecules was investigated. Glutamate is the predominant neurotransmitter in the retina, but GABA and dopamine are also present in retinal neurons.

Glutamate, bath-applied on isolated Müller cells, caused the opposite effect of bath-applied ATP: it lead to a very prominent, transient extracellular alkalinization, which was also followed by an extracellular acidification but the magnitude of the acidification signals generated was significantly smaller than the ones induced by extracellular ATP. Glutamate data is presented in chapter V.

Preliminary experiments with GABA (473 - 617 μM) (Figure 22A,C) and dopamine (167 – 250 μM) (Figure 22B, D) indicate a lack of the ability of these two neurotransmitters to induce a change in extracellular pH mediated by Müller cells, such as the changes observed with ATP and glutamate applications. Even though the results are very preliminary (GABA, N=2; dopamine N=3), trends can be observed. ATP was applied to the cells as a positive control and typical ATP-induced increases in acidity were observed.

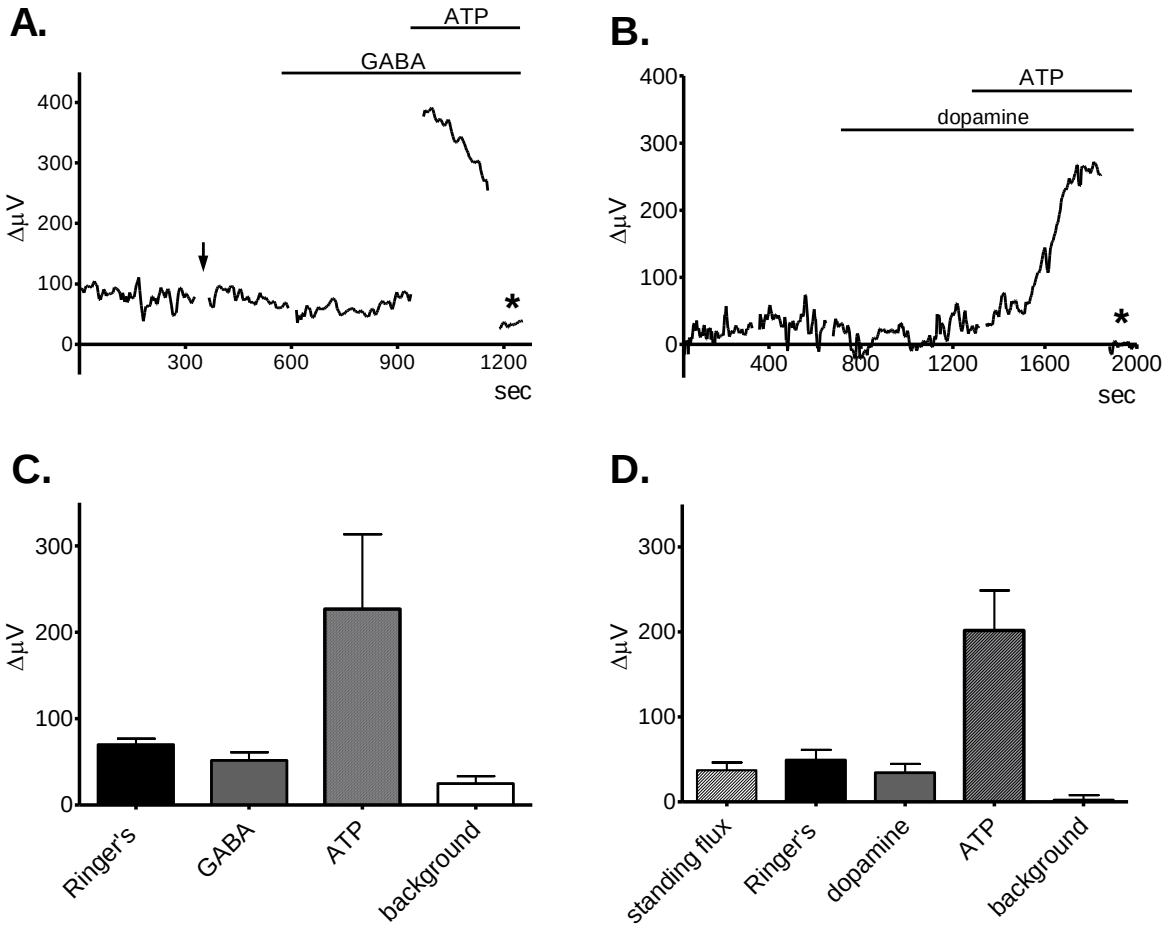


Figure 22. Extracellular GABA and dopamine fail to induce H+ fluxes from isolated salamander Müller cells. (A) A representative trace from a single self-referencing recording from a cell exposed to 617 μM GABA and subsequent 25 μM ATP for a positive control. (B) A representative trace from a single recording from a cell exposed to 167 μM dopamine and subsequent 40 μM ATP for a positive control. (C) Mean data from only 2 trials with GABA. (D) Mean data from 3 trials with dopamine, 167-250 μM. Samples too small for statistical analyses, yet bar graphs show obvious trends.

IV. 3. 5. ATP causes extracellular pH fluxes from isolated Müller cells and slices of other vertebrate species

The effects of extracellular ATP on H+ fluxes mediated by isolated Müller cells were tester in two macaque species. Figure 23 shows data collected from Müller cells isolated from the retinae of Macaca fascicularis, or the crab-eating macaque. Self-referencing recordings revealed ATP induced an extracellular acidification from Müller cells of this macaque species. In six trials, 82.5 - 100 μM ATP significantly increased the standing acidic flux from 35 ± 6 μV to 270 ± 48 μV, p=0.004.

Similar responses were obtained from Müller cells isolated from the retinae of Macaca mullata, Rhesus macaque. Recordings of extracellulr H+ concentrations were again performed in a self-referencing format. Müller glial cells isolated from the Rhesus macaque retina also responded to external ATP with extracellular acidification (Fig. 24A). In five trials, 100 μM ATP lead to an increase in the signal from an already slightly acidic standing flux of 14 ± 8 to a significantly more mean acidic level of 306 ± 83 μV, p=0.024 (Figure 24B).

ATP also induced an extracellular acidification mediated by Müller cells isolated from the retinae of channel catfish, Ictalurus punctatus. H+ microelectrodes detected significant increases in the H+ of the extracellular milieu when 100 μM ATP was added to the bath as shown in Figure 25A. The self-referencing signal increased from 9 ± 5 μV, before to 144 ± 24 μV after ATP was introduced into the recording dish, p = 0.0006, N=7 (Figure 25B). There was no standing acidic flux detected from catfish Müller cells.

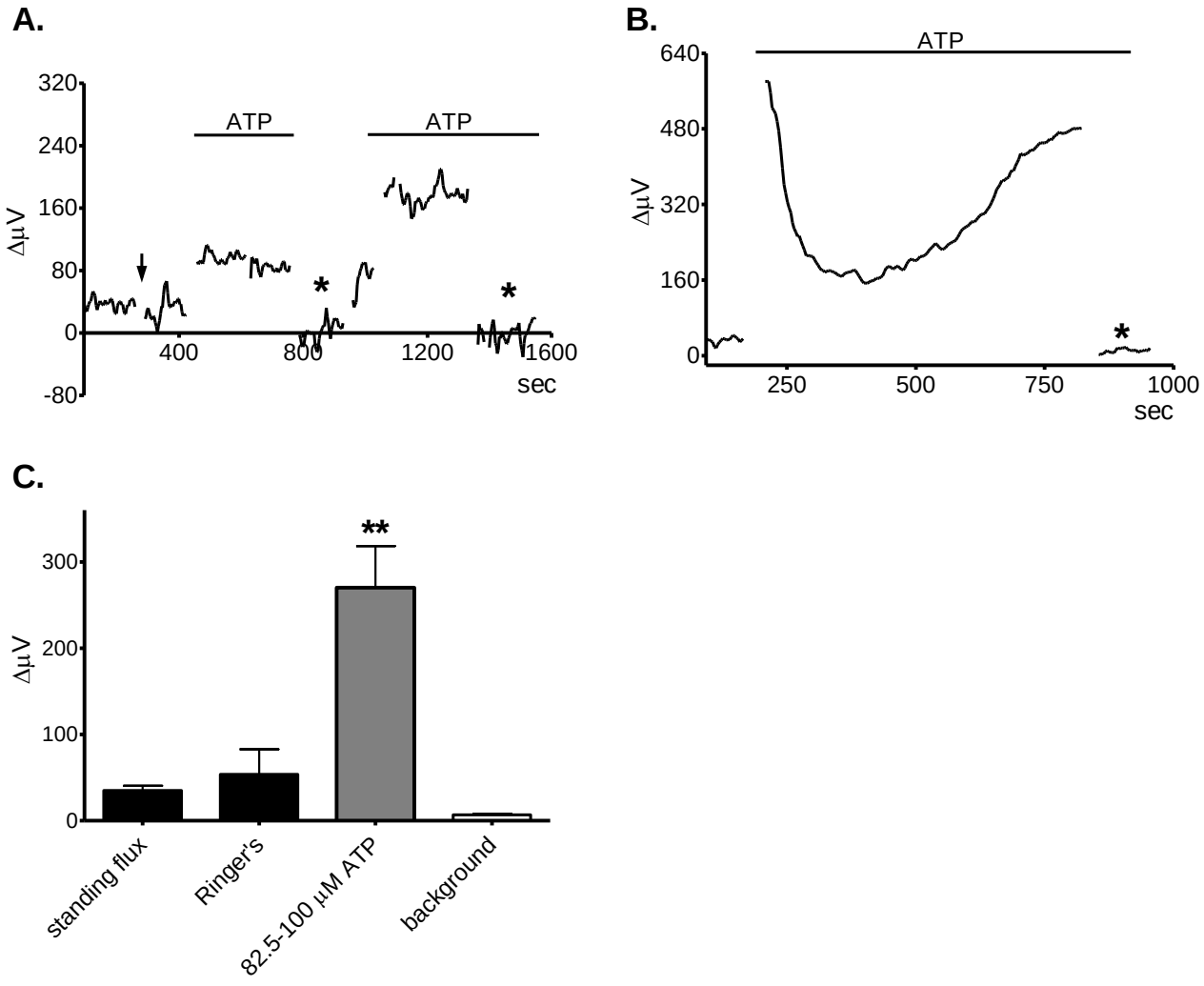


Figure 23. Bath-applied adenosine 5'-triphosphate (82.5-100 μM) induces an extracellular acidification from Müller cells isolated from Macaca fascicularis (Crab-eating macaque). (A) A representative trace from a single Müller cells exposed to 10 μM (first horizontal bar) and 82.5 μM ATP (second horizontal bar). Asterisks indicate background controls; arrow indicates the onset of the Ringer's control. (B) A representative trace from a single recording from a Müller cells exposed to 100 μM ATP. (C) Mean data show an increase in extracellular acidity measured from isolated Müller cells in response to ATP, 82.5-100 μM; N=6.

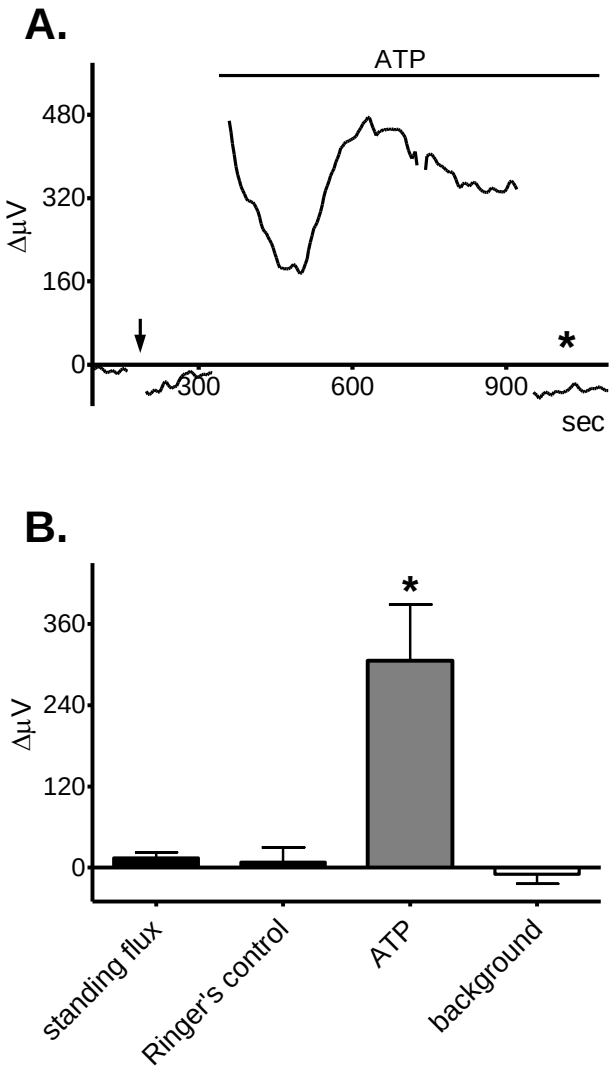


Figure 24. Bath-applied adenosine 5'-triphosphate (100 μM) induces an extracellular acidification from Müller cells isolated from Macaca mulatta (Rhesus macaque). (A) A representative trace from a single Müller cells exposed to 100 μM ATP. Asterisks indicate background controls; arrow indicates the onset of the Ringer's control. (B) Mean data show an increase in extracellular acidity measured from isolated Müller cells in response to 100 μM ATP; N=5.

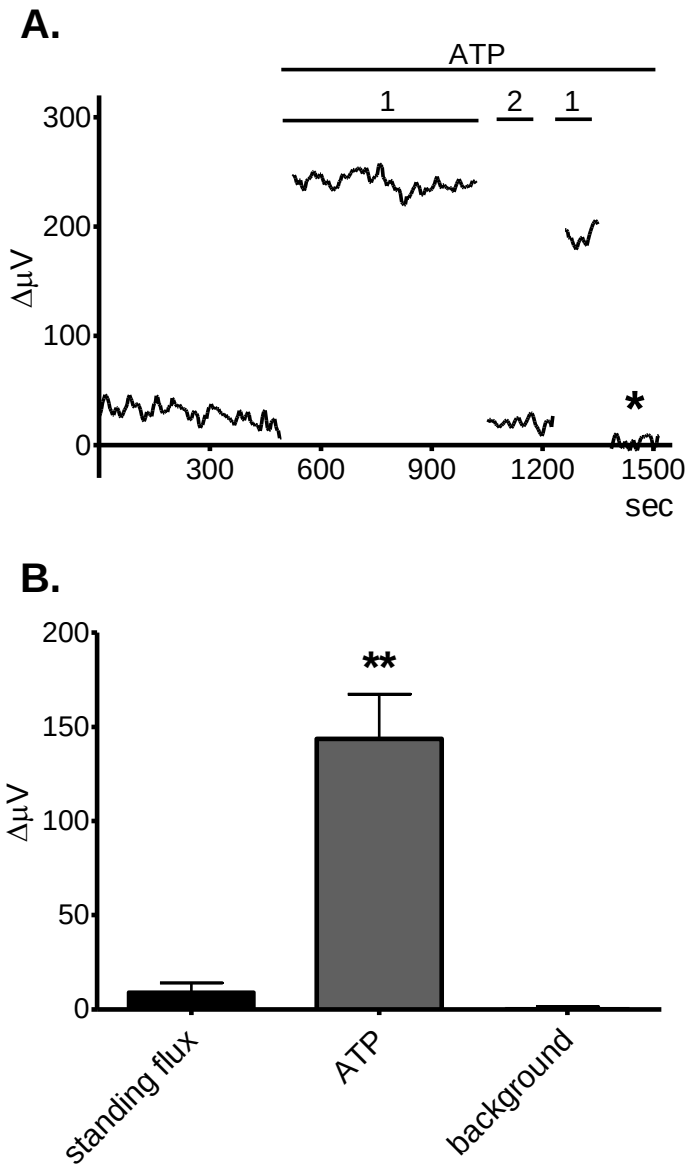


Figure 25. Bath-applied adenosine 5'-triphosphate (100 μM) induces an extracellular acidification from Müller cells isolated from Ictalurus punctatus (Channel catfish). (A) A representative trace from a single Müller cell exposed to 100 μM ATP: horizontal bar labeled "1" signifies the H+ electrode was positioned by the apical end; horizontal bar labeled "2" means the H+ electrode was recording from the end foot. Asterisk indicates background control. (B) Mean data show an increase in extracellular acidity measured in response to 100 μM ATP; N=7.

Extracellular ATP also induced significant extracellular acidifications from Müller cells isolated from human, rat and lamprey. Individual self-referencing traces are shown in Figure 6 in chapter III. Mean data are presented in Figure 22 below. Seven out of nine human Müller cells (~78%) showed clear responses to ATP with detectable increases in extracellular acidity (Figure 26A). The mean standing flux increased from 62 ± 9 μV before to 257 ± 41 μV after application of 100 μM ATP, (p=0.001). Five out of six (~83%) rat Müller cells responded to 100 μM ATP (Figure 26B). The mean standing flux was 5 ± 7 μV, and 100 μM ATP induced an increase in extracellular acidity to 262 ± 54 μV, a difference that was also statistically significant (p=0.006). Six out of seven lamprey Müller cells (~86%) also responded to 100 μM ATP (fig 26C). The signal increased from -0.971 ± 13 μV in plain Ringer's solution to 88 ± 21 μV in response to ATP (N=7, p=0.0217).

As shown in chapter III, bath-applied ATP induces a robust extracellular acidification from Müller cells isolated from tiger salamander retinae as well as from salamander slices when the H+ electrodes were positioned just above the OPL. To rule out contributions from photoreceptors in slices, whose terminals are located in the OPL, responses of isolated cone photoreceptors to 10 μM extracellular ATP via changes in extracellular H^+ were examined (Figure 27). Two locations along the cells were tested: the terminals and the cell bodies. ATP did not cause a significant change in extracellular ATP mediated by photoreceptors at either tested location, N=5 (Figure 27B). At terminals, the self-referencing signal increased from 7 ± 6 μV to 13 ± 6 μV in response to ATP, p=0.173. At cell bodies, the signal changed from 28 ± 10 μV before to 37 ± 13 μV after ATP application, p=0.284.

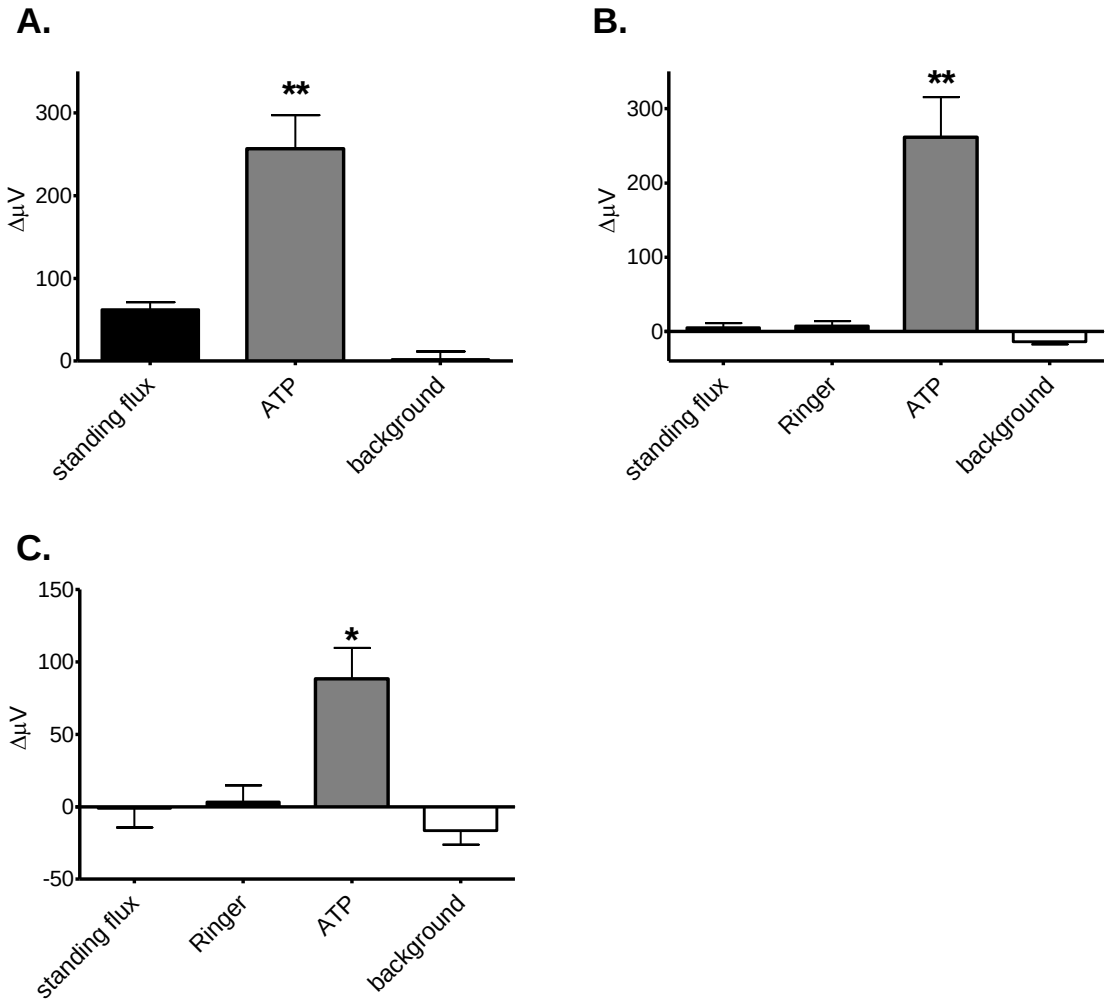


Figure 26. Bath application of 100 µM adenosine-5'- triphosphate induces a pronounced extracellular acidification from Müller cells isolated from the retinae of human, rat and lamprey. Recordings were done with H^+-selective microelectrodes. (A) Mean data from seven human Müller cells show a pronounced acidification in response to 100 µM ATP. (B) Mean data from six rat Müller cells shows an increase in extracellular acidity in response to 100 µM ATP. (C) Mean data from seven lamprey Müller cells shows an increased extracellular acidity induced by the application of 100 µM ATP.

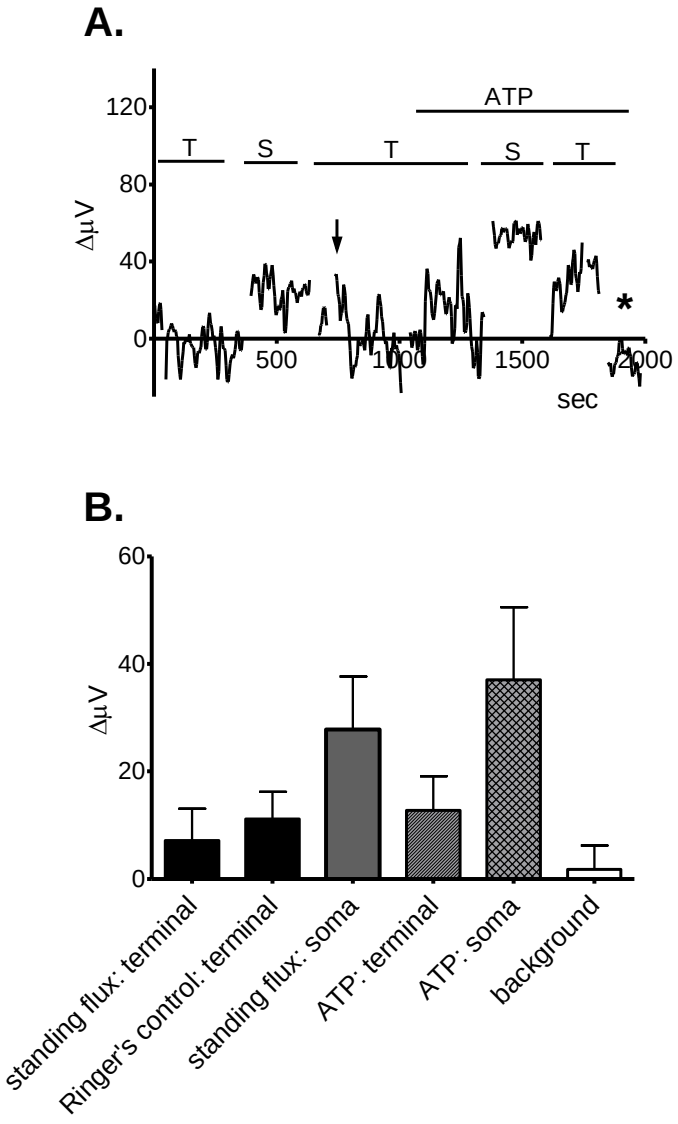


Figure 27. Bath application of 10 µM ATP does not cause a change in extracellular H^+ concentration measured from two locations on cone photoreceptors isolated from the tiger salamander retina: soma and axon terminal. (A) A representative trace from a single self-referencing recording of extracellular H+ at a photoreceptor terminal and soma. (B) Mean data shows that 10 µM ATP did not cause a significant change in the extracellular acidity at the terminals or cell bodies of photoreceptors, N=5.

The effects of extracellular ATP on H^+ fluxes measured at the level of the outer plexiform layer in slices of goldfish retinae were also examined in a similar fashion as the experiments from slices of salamander retinae. In goldfish slices, ATP also caused a robust extracellular acidification (Figure 28B). Goldfish slices exhibited a significant acidic standing flux similar to salamander slices and in both species the ATP-induced acidification was decreased in the background of the P2 receptor blockers suramin and PPADS. Moreover, in goldfish slices (Figure 28A) the sensitivity of the standing flux to suramin and PPADS seemed to be more pronounced than that of the standing flux from salamander slices. Introduction of the blockers (200 μM) to the recording chamber resulted in a drop of the signal from a mean standing level of 1205 ± 90 μV in clean Ringer's to 861 ± 34 μV in the presence of suramin and PPADS, p=0.0027, N=6 (Figure 28C), corresponding to a reduced extracellular standing acidity. ATP still caused an increase in acidity in the presence of suramin and PPADS, but this increase was smaller than the increased caused by ATP in clean Ringer's. In the background of suramin and PPADS, the voltage signal increased to 1064 ± 99 μV after the addition of 100 μM ATP to the bath solution, p= 0.0359, which actually was not statistically different from the standing acidic flux in clean Ringer's. After a wash-out, which likely resulted in an incomplete wash of all the drugs, ATP induced a much larger extracellular acidification. The signal increased from 968 ± 51 μV after the Ringer's wash to 1856 ± 87 μV after the application of 100 μM ATP, p=0.0001.

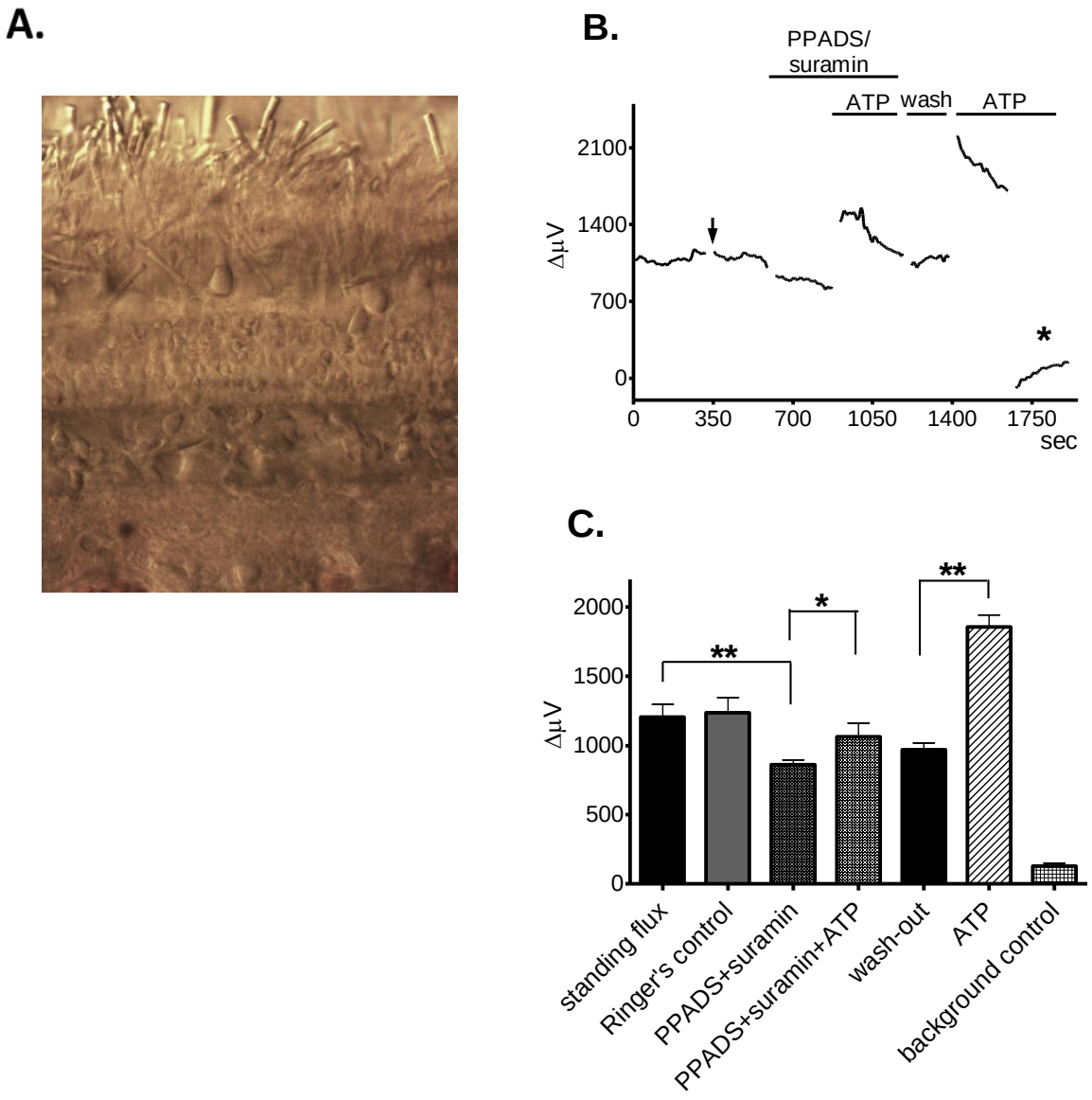


Figure 28. Bath application of 100 μM ATP causes an extracellular acidification from goldfish retinal slices; the acidification is reduced in the presence of P2 receptor blockers suramin and PPADS. Recordings were performed in a self-referencing manner with the microelectrode positioned above the outer plexiform layer/ outer nuclear layer. (A) A representative image of a cross-section (slice) through a goldfish retina. (B) A representative trace from a recording from a single slice. Arrow represents the onset of the Ringer's control; asterisk represents background control reading 600 μm above the slice. (B) Mean data from six slices shows the acidification caused by ATP is attenuated in the background of 200 μM suramin and PPADS.

IV. 4. DISCUSSION

The data presented in this chapter demonstrates that the observed extracellular acidification from isolated Müller cells in response to ATP is most likely due to activation of P2 rather than P1 receptors as application of adenosine alone resulted in no detectable changes in extracellular acidity. The variability of the voltage response to the P2 receptor ligands, ATP, UTP and ADP is likely due to differential affinities of the receptors present on Müller cells. The inhibition of the ATP-induced change in extracellular acidification by DIDS demonstrated in chapter III points to dependence on or coupling to an anion transport mechanism; yet experiments in which the extracellular chloride was reduced drastically did not inhibit the acidification response. One explanation for this could be that the transport blocked by DIDS is not that of chloride. Alternatively, DIDS could still be blocking chloride transport, but in the outward direction, rather than inward, which would explain the lack of effect seen in low chloride conditions. Replacing extracellular sodium with choline reduced the acidification induced by exogenous ATP, but did not completely remove it, yet suggesting a partial dependence on extracellular sodium. It should be noted that in those conditions, the standing flux was abolished and the percent increase in acidity with ATP was similar in sodium and sodium-free conditions even though the magnitude of responses was reduced. EIPA, a sodium/hydrogen exchanger, failed to inhibit the ATP effect and it only partly removed the standing flux, leaving a standing acidity which did not seem to be EIPA-sensitive. Even though the acidity induced by ATP was still significant in the background of the Na+/H+ exchanges blocker, it could have been still reduced to a degree. Unfortunately trial numbers were low and wash-outs and control ATP applications were not performed

on these cells. Dr. Kreitzer's lab on the other hand has data showing about a 40%-percent block of the ATP-induced responses in the presence of the Na+/H+ exchanger inhibitor amiloride.

Bafilomycin and SCH28080, which block the vacuolar H+ ATPase and the H+/K+ ATPase (aka gastric proton pump), failed to abolish the ATP-induced acidification. Probenecid, which has been shown to be inhibitory to pannexin-1 channels, seemed to reduce the extracellular acidification induced by ATP. It should be noted that probenecid also shows inhibitory actions at organic anion transporters and multidrug resistance transporters (Tsuruya et al, 2016), so the modulation of the ATP-induced acidification by probenecid reported in this dissertation could be attributed to the inhibition of the activity of the above-mentioned transporters rather than to pannexin channels. Moreover, preliminary data showed that Müller cells respond to extracellular hyperosmolarity with increases in extracellular acidity. Transmembrane transport of organic osmolytes has been implicated in regulatory cell volume changes and some organic transporters co-transport ions.

The extracellular acidification could not be induced by dibutyryl-cAMP, a cell-permeable analogue of cAMP, which could mean that elevated levels of cAMP are not involved in the intracellular mechanism responsible for the ATP-induced extracellular acidification or that increased [cAMP] is just not sufficient by itself to trigger the acidification. Another possibility is that the amount of dibutyryl-cAMP was too low or the pre-incubation time was not long enough to cause the level of intracellular dibutyryl-cAMP to rise to a needed threshold. Forskolin, a cell-permeable adenylyl cyclase (AC) activator, did induce a slight, but significant increase in the extracellular acidity measured

from isolated Müller cells. It is a bit surprising that forskolin had an effect and dibutyryl-cAMP did not, since AC catalyzes the production of cAMP from ATP. It could be that longer pre-incubation in or/and higher concentrations of dibutyryl-cAMP were needed to activate an intracellular pathway. The forskolin-induced acidification though was small compared to the one induced by external ATP, which again could be because of an inadequate forskolin concentrations/pre-incubation times or it could point to a more complex intracellular cascade of events involving multiple signaling messengers/pathway. The extracellular acidification evoked by exogenous ATP also persisted in the background of caffeine, which mobilizes calcium from intracellular stores, even though the magnitude of the mean responses seems to be smaller given the stimulating concentration of ATP. Caffeine has been used as a stimulating agent of ryanodine receptors which are IP3-insensitive. It has been shown though that in cells of certain tissues caffeine can also cause an inhibition of IP3-sensitive calcium channels on the endoplasmic reticulum. The concentration of caffeine used in these experiments is lower than the one needed for a maximal calcium release from ryanodine receptors (Sei et al, 2001), which could mean that any potential effects of caffeine could be partial.

Glutamate is the primary neurotransmitter in the outer retina, but not the only bioactive molecule released by retinal neurons. Since the acidification induced by ATP is not confined to the apical end of Müller cells but is also observed at their end feet, the effects of a variety of neurotransmitters on Müller cell-mediated changes in extracellular H+ were also considered. My experiments show that neither GABA nor dopamine induce a change in the extracellular H+ measured from isolated Müller cells. Glutamate did induce a change in the extracellular acidity measured from isolated Müller cell, but

the change was particularly pronounced in the polarity opposite of the ATP-induced changes. The effect of glutamate on isolated Müller cells and retinal slices is discussed in detail on chapter V.

ATP produces an extracellular acidification from salamander retinal slices when the H+-selective microelectrodes are positioned right above the outer plexiform layer (see chapter III). Photoreceptors have been shown to possess ATP receptors. To eliminate photoreceptors as the cells mediating the ATP-induced extracellular acidification in slices, ATP was bath-applied onto photoreceptors isolated from tiger salamander retinae. No significant changes in extracellular acidity were observed from isolated photoreceptors exposed to exogenous ATP.

Bath application of ATP also caused an extracellular acidification from goldfish retinal slices with the microelectrodes positioned above the outer plexiform layer. Suramin and PPADS did reduce the ATP effect as well as the standing acidic flux observed prior to ATP application. The interpretation of these results as to the particular cells mediating these responses in goldfish slices is a little more challenging as the responses from isolated goldfish Müller cells to ATP are variable and small.

V. DIFFERENT PATHWAYS MEDIATE THE EXTRACELLULAR ALKALINIZATION AND ACIDIFICATION INDUCED BY GLUTAMATE FROM MÜLLER GLIA AND RETINAL SLICES.

V. 1. SUMMARY

Glutamate is the primary excitatory neurotransmitter in the central nervous system, as well as in the vertebrate retina. Elevated levels of extracellular glutamate can lead to neuronal excitotoxicity and, in the retina, high levels of glutamate have been linked to various retinal pathologies and visual loss, such as diabetic retinopathy, as well as other neurological pathologies in the nervous system, such as amyotrophic lateral sclerosis (ALS), Huntington's disease, Alzheimer's disease, various brain insults, etc. (Grewer, C and Rauen T, 2005). In retinal pathologies, the excess extracellular glutamate has been correlated with impaired (or reversed) glutamate uptake into retinal glia (Müller cells) and neurons (Russo et al., 2013; Bringmann et al., 2013). Here, I show that the uptake of glutamate in salamander retinal Müller cells is associated with a transient extracellular alkalinization detected with H+ microelectrodes from isolated Müller glia and retinal slices. The alkalinization from isolated Müller glia is reduced in zero extracellular sodium and in the presence of glutamate transporter blocker TBOA in both isolated cell and slice set-ups. Glutamate application on retinal slices also caused an extracellular acidification which seemed to be mediated by a mechanism different from the glutamate transporter-associated import of H+. The extracellular acidification, but not transient alkalinization, is also induced by kainic acid application and inhibited by the kainate/AMPA receptor blocker CNQX. Purinergic receptor blockers show only a slight impact on the glutamate-induced extracellular acidification from slices. The results support the notion that glutamate transporters of the sodium-dependent family of EAATs, co-transport H+ along with Na+ and glutamate and also reveal a different mechanism,

probably mediated by the activation of AMPA/kainate receptors, responsible for extracellular acidification responses to glutamate.

V. 2. INTRODUCTION

In the outer retina glutamate is released from photoreceptors and relays visual information to second-order retinal neurons via activation of glutamate receptors located on membranes of horizontal and bipolar cells. A proposed hypothesis extrapolated from a large pool of data gathered over decades postulates that glutamate-activated horizontal cells feed information back onto photoreceptors via modulating the activity of calcium channels at photoreceptor terminals, thus regulating subsequent calcium entry-dependent glutamate exocytosis. There is a large body of evidence to suggest that the modulatory mechanism by which horizontal cells are able to do this is by changing the concentration of protons in the synaptic cleft. Recordings from photoreceptor calcium channels show that when the pH of the extracellular medium is decreased, the amount of current decreases and the calcium channel activation curve shifts to the right. The opposite has been shown when the pH of the bathing solution is increased towards a more alkaline value. When photoreceptors are exposed to brief, repetitive light stimuli in a bathing solution with a pH of 7.8, recordings from horizontal cells show hyperpolarizations during the light stimuli and depolarizations during the dark periods when photoreceptors are depolarized and releasing glutamate onto second order neurons. Shifting the pH of the bathing solution from 7.8 to 7.5 reduces the magnitude of the voltage changes in horizontal cells. Reducing the pH even further to 7.2 almost completely abolishes the voltage changes in horizontal cells. The lack of voltage

response from horizontal cells is believed to be due to a decrease in the amount of calcium entering the photoreceptors' terminals induced by the increase in acidity, leading to a decrease in glutamate release from photoreceptor and less activation of glutamate receptors on horizontal cells. More evidence in support of the hypothesis that fluctuations in the H^+ concentration at the level of the photoreceptors-second order neurons synapses modulates synaptic transmission via pH-dependent inhibition of photoreceptor calcium channels comes from experiments in which a pH sensitive fluorescent protein was fused with the extracellular portion of a subunit of a cone photoreceptor calcium channel in a transgenic zebrafish model. The authors speculate that the observed light-dependent pH changes reported by the CaliPhluorin construct are in line with horizontal cell-mediated light responses. The authors go farther into emphasizing that horizontal cells are responsible for altering extracellular pH during neuronal signaling by observing that selective depolarization of horizontal cells results in a decrease in pH at the level of the photoreceptor terminals.

In chapters III and IV I showed that the Müller glia of the retina are also capable of mediating extracellular pH changes. Self-referencing recordings with H^+-selective microelectrodes showed that Müller cells isolated from the retinae of tiger salamanders and other organisms, including two Macaque species as well as human, produced large extracellular acidifications in response to bath-applied ATP. ATP, which has been shown to be co-released with other neurotransmitters, also induced an extracellular acidification from a salamander retinal slice and in both slices and isolated Müller cells from salamander the signals were greatly reduced by the broad-spectrum P2 receptor blockers suramin and PPADS.

Müller cells possess many functions which are crucial for normal retinal physiology. Amongst them is the removal extracellular glutamate; by doing this very efficiently Müller cells reduce synaptic transmission noise and prevent the build-up of glutamate to excitotoxic levels. This uptake is mediated by sodium-dependent and independent transporters. The glutamate transporter in Müller cells responsible for a large bulk of the glutamate uptake is the sodium-dependent, electrogenic, high-affinity excitatory amino acid transporter-1 (EAAT-1): EAATs 2-5 have also been detected in retinal cells of various species with differential expression in neurons and glia. Along with glutamate, EAATs transport in three sodium ions and one proton, and transport out one potassium ion (Grewer C and Rauen T, 2005). Glutamate uptake through some EAATs has been associate with an anion conductance, but the operation of the transporter is not dependent on the anion flux, whereas the anion conductance only opens when there is glutamate uptake (Grewer C and Rauen T, 2005). Some studies claim that the glutamate transporter in retinal Müller cells is now associated with a significant chloride conductance, unlike the EAAT4 present on cone terminals (Billups and Attwell, 1996). Whole cell experiments have demonstrated that the glial glutamate transporter produces inward currents, which are steady and non-desensitizing in nature for the duration of the glutamate application. Aside from pH dependence, the transporter also exhibits a clear voltage dependence with highest conductance at very hyperpolarized voltages and a gradual reduction of the transporter-associated current with depolarizing steps (Levy et al., 1998).

In this chapter, recordings done with H^+-selective microelectrodes in a self-referencing manner show that glutamate uptake by Müller cells of the tiger salamander

retina can generate large and transient decreases in extracellular H^+ concentrations corresponding to an increase of the pH of the extracellular milieu. Glutamate uptake via EAATs caused an even larger extracellular alkalinization when recording from salamander retinal slices. Results also revealed that bath-applied glutamate lead not only to an extracellular alkalinization, but also a pronounced following acidification which seems to be independent of the activation of glutamate uptake systems, dependent on AMPA/kainate receptor activation with partial reliance on the activation of purinergic signaling systems.

V. 3. RESULTS

Isolated tiger salamander Müller cells were identified by their distinctive morphology, characterized by an apical end with a tuft-like outer process, a cell body, an elongated and relatively thick inner process, and a wide stubby endfoot (Newman, 1994). H^+-selective microelectrodes were typically placed approximately 1-2 µm from the cell membrane where the apical tip of the cell and cell body fused (Figure 29A); a differential recording was obtained by moving the electrode to a position 30 µm distant and subtracting the recordings obtained at the near and distal positions. Figure 29B shows a robust transient extracellular alkalinization induced by application of 550 µM glutamate on an isolated Müller cell (Figure 29A). The mean peak of the glutamate-induced transient alkalinization recorded from five cells was -171 ±12 µV, a significant decrease from 24 ± 5 µV, the standing flux after a Ringer's addition, $p<0.0001$ (Figure 29C). The signal recovered to and exceeded the initial readings resulting in slightly more acidic levels compared to the standing flux after Ringer's addition, 55 ± 8 µV, $p=0.0023$. In this

case, the standing flux after Ringer's control was used for comparison because the Ringer's control showed a slight difference from the standing flux before, p=0.0419. Similar results were obtained when bath-applied glutamate was reduced to 300 μM. When sodium in the extracellular bathing solution was replaced with choline the standing flux was markedly reduced and the transient alkalinization induced by 300 μM glutamate was virtually abolished (Figure 30A, B). In seven trials, the mean standing flux was 4.2 ± 3 μV, which was not statistically significant from the background control. In those conditions, glutamate did not cause a significant alkalinization or acidification.

The glutamate transporter blocker, TBOA (200μM), also significantly reduced the alkalinization produced by 300μM glutamate, p=0.0291 (Figure 30C, D).

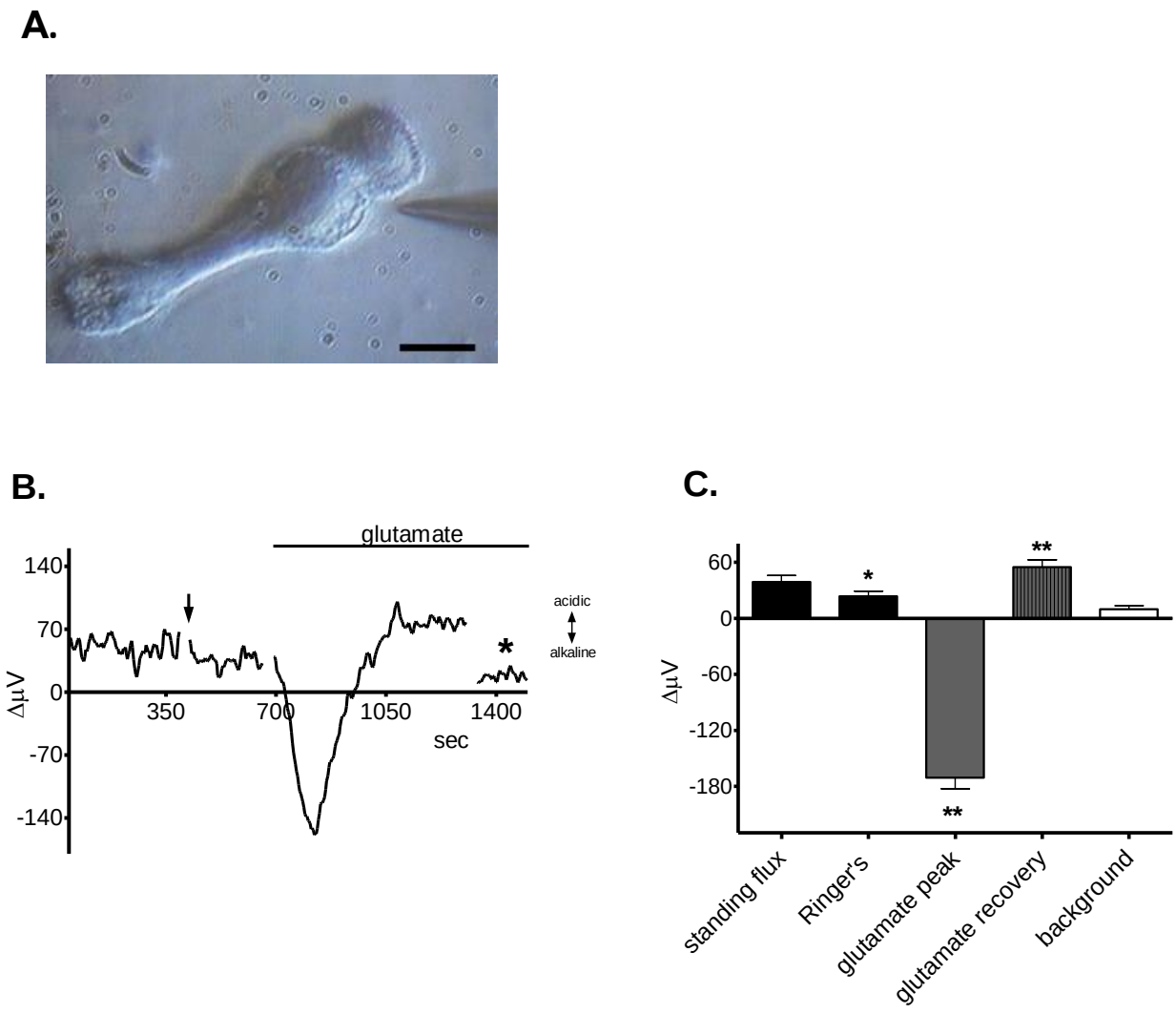


Figure 29. Bath application of 550 µM glutamate causes a transient alkalinization from isolated Müller cells of the tiger salamander retina. (A) Isolated Müller cell with a pH microelectrode positioned next to the apical end of the cell, scale bar: 20µm. (B) A representative self-referencing trace from a Müller cell shows a robust transient alkalinization in response to glutamate. (C) Mean responses of five cells exposed to 550 µM glutamate.

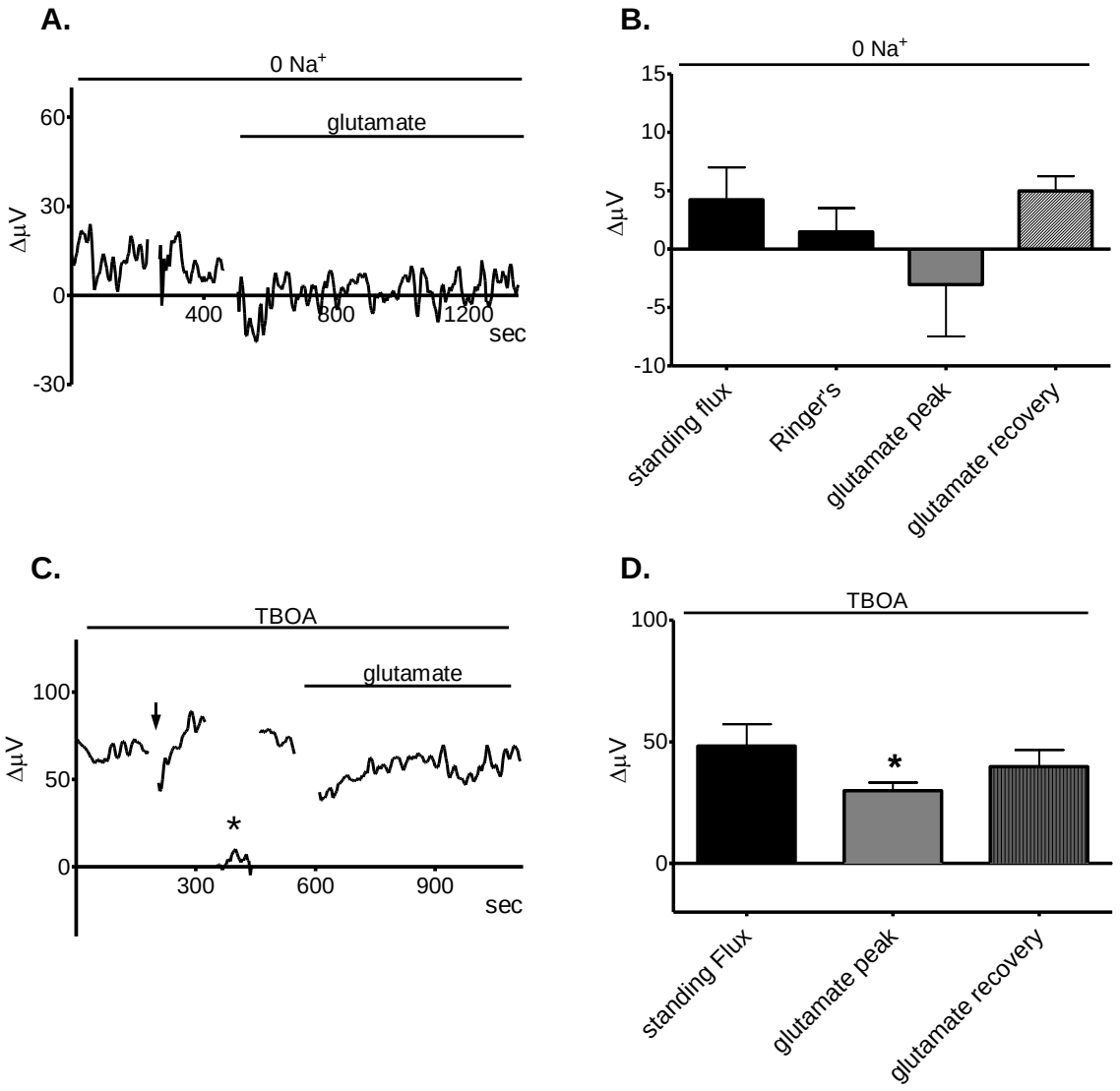
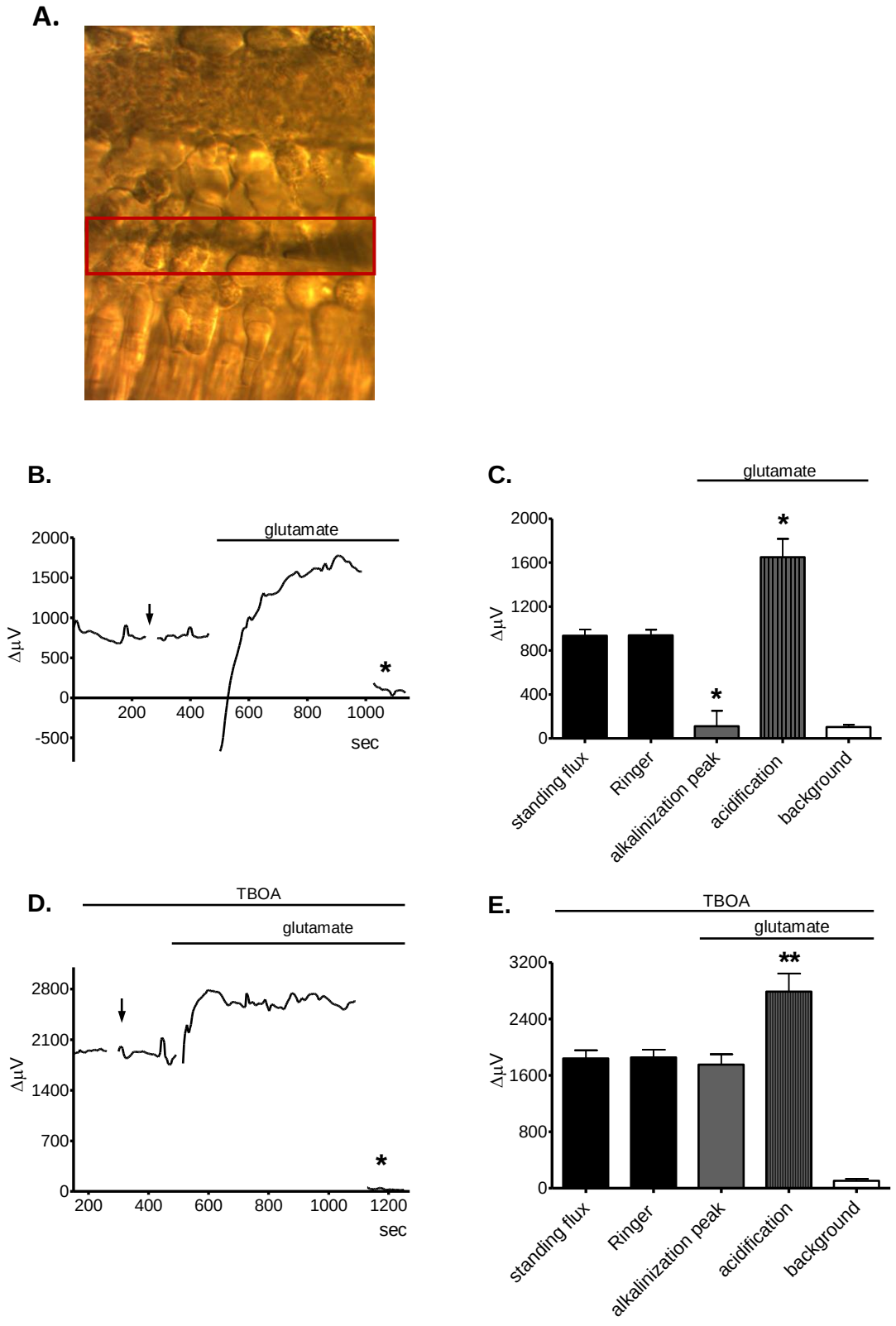


Figure 30. The transient alkalinization from isolated Müller cells induced by 300μM glutamate is dependent on extracellular sodium and is reduced in the presence of the glutamate transporter blocker TBOA. (A) A Representative trace from an individual self-referencing recording from an isolated Müller cell shows that replacing Na^+ in the bathing solution with choline reduces the glutamate-induced transient alkalinization. (B) Mean responses of seven cells in 0 Na^+. (C) A representative trace from an individual self-referencing recording shows a reduced glutamate-induced alkalinization in the background of the glutamate transporter blocker TBOA. (D) Mean data from seven cells. (Experiments in 30A, B by Ethan Naylor, Matthew Kreitzer; 30C,D Ethan Naylor, David Swygart, Ryan Kaufman, and Matthew Kreitzer, Indiana Wesleyan University).

Similar results were obtained from salamander retinal slices where glutamate also caused an extracellular transient alkalinization (Figure 31A). The H^+-sensitive electrode was positioned immediately above the outer plexiform layer (OPL) and a reference reading was obtained by moving the microelectrode 30µm above tissue. 300µM glutamate induced a large and transient alkalinization in slices, which was followed by a pronounced acidification when glutamate was still present in the bath (Figure 31B). In slices, the standing acidic flux recorded was orders of magnitude larger than the one previously recorded from isolated Müller cells. The mean standing flux recorded from 8 slices was 935 ± 56 µV and, in these slices, 300 µM glutamate induced a mean alkalinization response (peak) of 112 ± 140 µV, p=0.0001, and a mean acidification response of 1649 ± 167 µV, p=0.0011 (Fig. 31C). Similar to the data from isolated Müller cells, the glutamate-induced alkalinization in slices was drastically reduced in the presence of TBOA (Figure 31D). During these conditions, the acidification component of the signal persisted. In the presence of TBOA the standing acidification signal as well as the increased acidification in the presence of glutamate were much larger compared to experiments in which TBOA was not present in the bath. The mean standing flux in TBOA was 1842 ± 115 µV and the mean acidification component of the response to 300 µM glutamate was 2790 ± 256 µV, p=0.0012, N=7 (Figure 31E). TBOA reduced the effect of glutamate on the alkalinization component of the traces to a mean value of 1755 ± 147 µV, p=0.375 (Figure 31E). TBOA was dissolved in DMSO, so control experiments were performed to examine any changes of DMSO alone on the glutamate-induced extracellular pH effects. No such DMSO-associated changes were found.

Figure 31. Bath application of 300μM glutamate causes a transient extracellular alkalinization and a following extracellular acidification from retinal slices. Recordings were done with the pH microelectrodes positioned just above the outer plexiform layer in retinal slices (A). (B) A representative trace from a single recording showing a transient extracellular alkalinization and a subsequent sustained acidification in response to 300μM bath-applied glutamate. (C) Mean data from 8 slices. (D) The transient alkalinization is removed when slices are incubated in TBOA; a single trace. (E) Mean data of response from 7 slices pre-incubated in 200 μM TBOA and stimulated with 300 μM glutamate.


A.
B.
glutamate
2000
1500
1000
500
0
-500
ΔμV
200
400
600
800
1000
sec
*
C.
glutamate
2000
1600
1200
800
400
0
ΔμV
*
*
standing flux
Ringer
alkalinization peak
acidification
background
D.
TBOA
glutamate
2800
2100
1400
700
0
ΔμV
200
400
600
800
1000
1200
sec
*
E.
TBOA
glutamate
3200
2400
1600
800
0
ΔμV
**
standing flux
Ringer
alkalinization peak
acidification
background

It was previously shown in chapter III that bath-applied ATP caused a robust acidification from retinal slices of tiger salamanders, which acidification was reduced by the P2 receptor blockers suramin and PPADS. Here, in the presence of 200μM suramin and PPADS glutamate (300μM) still induced a robust alkalinization in a slice, but the acidification response was reduced in comparison to experiments in which the P2 receptor blockers were not present (Figure 32A, B). The mean standing flux from five slices was 1075 ± 86 μV, which was reduced to 900 ± 103 μV when suramin and PPADS were added to the bath, but the change was not statistically significant. In the presence of suramin and PPADS glutamate still caused a transient extracellular alkalinization, -55 ± 63, p=0.0003. The mean acidification from slices in the presence of glutamate, PPADS and suramin (200μM) was 1281 ± 71 μV, which was not significantly changed from the initial standing flux, but was slightly elevated compared to the already slightly decreased standing flux in the presence of suramin and PPADS alone, p=0.033. Next, we examined the effects of the P2 receptor blockers on the glutamate-induced changes in the extracellular acidity from retinal slices in the presence of the glutamate transported blocker TBOA (Figure 32C, D). In the presence of TBOA, the addition of suramin and PPADS significantly reduced the standing flux in 10 slices from 1399 ± 89 μV to 1099 ± 77 μV, p=0.0016. The subsequent addition of glutamate did not significantly alter the signal any further, 966 ± 92 μV, p=0.719. In the presence of all pharmacological agents, the acidification component of the glutamate-induced change in the trace was still present at statistically significant levels when compared to the standing flux in TBOA or the standing flux in TBOA, suramin and PPADS with a mean of 1548 ± 136 μV, p=0.306 and p=0.0016 respectively.

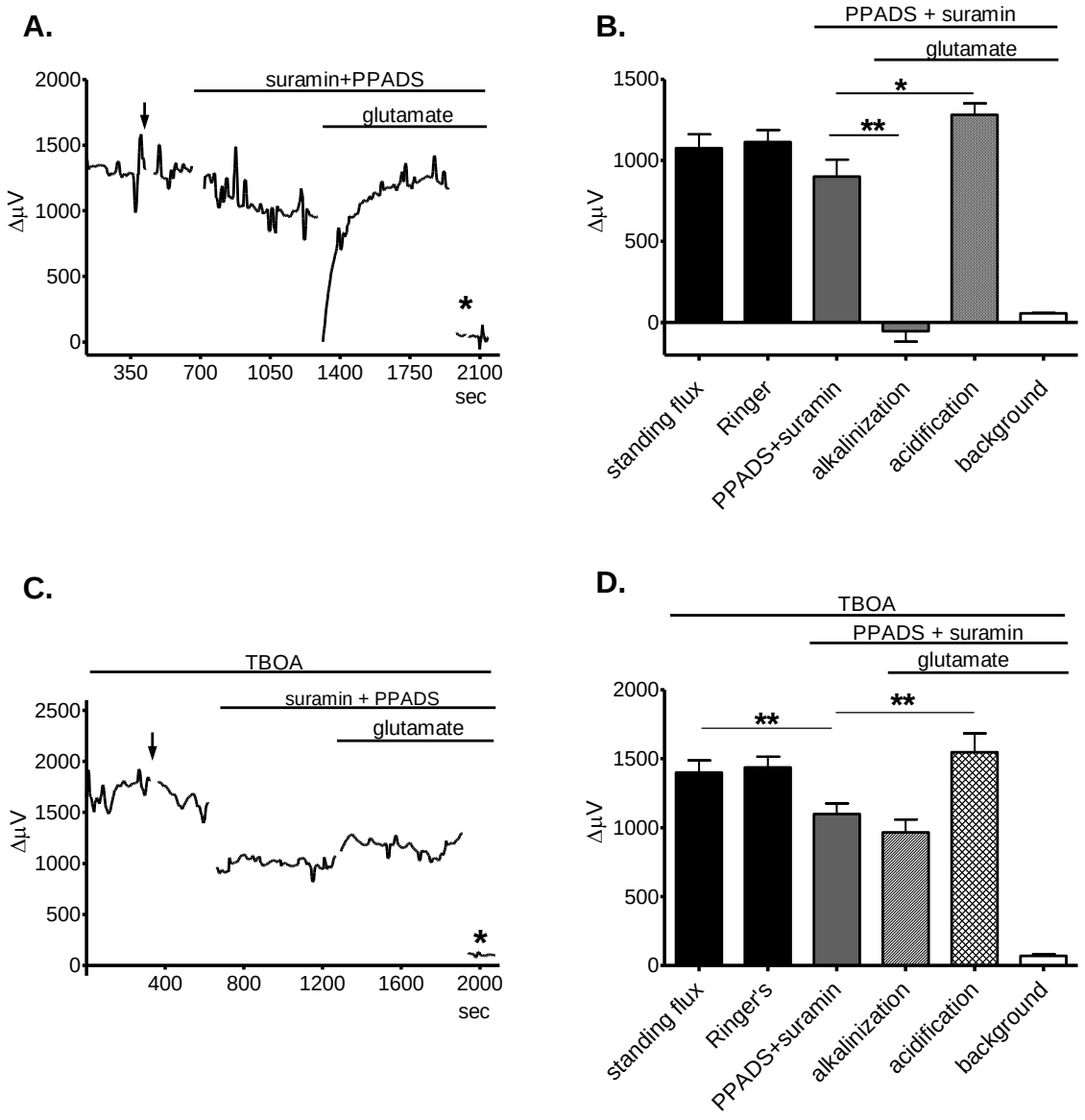


Figure 32. The extracellular acidification caused by bath application of glutamate is reduced in the presence of ATP receptors blockers suramin and PPADS (200μM) in the presence or absence of glutamate transporter blocker TBOA (200μM). (A) A representative trace from a single slice recording. (B) Mean data from 5 trials; Slices were bathed in 200 μM PPADS and suramin prior to glutamate application, 300 μM; (C) A trace depicting a single recording form a slice pre-incubated in TBOA and subsequently exposed to suramin and PPADS, and then to glutamate in the background of all the blockers. (D) Mean data from 10 trials.

To examine any potential involvement of the ionotropic AMPA/kainate receptors in the observed extracellular acidity changes in slices exposed to glutamate, eight slices were pre-incubated in 500 μM of the AMPA/kainate receptor antagonist CNQX (Figure 33). The transient alkalinization component of the trace persisted in CNQX as the standing flux alkalinized from 872 ± 99 μV in CNQX alone to -80 ± 159 μV in CNQX plus glutamate, $p<0.0001$. The acidification component slightly increased to 1039 ± 142 μV, but this change in the background of CNQX was no longer of statistical significance, $p= 0.0922$. CNQX was dissolved in pure water and its addition to the Ringer's solution resulted in slight dilution of the contents of the Ringer's. Control experiments with glutamate in the background of diluted Ringer's were performed and no significant changes were found.

To further test the notion that activation of AMPA/kainate receptors could play a role in the observed glutamate-induced extracellular acidification, we stimulated retinal slices with 200 μM kainic acid (Figure 34). In six slices, I observed a robust increase in the extracellular acidity from a standing acidic flux of 982 ± 44 μV before to 2026 ± 202 μV after the application of KA, $p=0.0018$. It should be noted that in 5 other slices 100 μM kainic acid did not alter the extracellular acidity: the standing flux increased from 1039 ± 98 μV to 1060 ± 109 μV, $p=0.7114$ (Figure 34A, B).

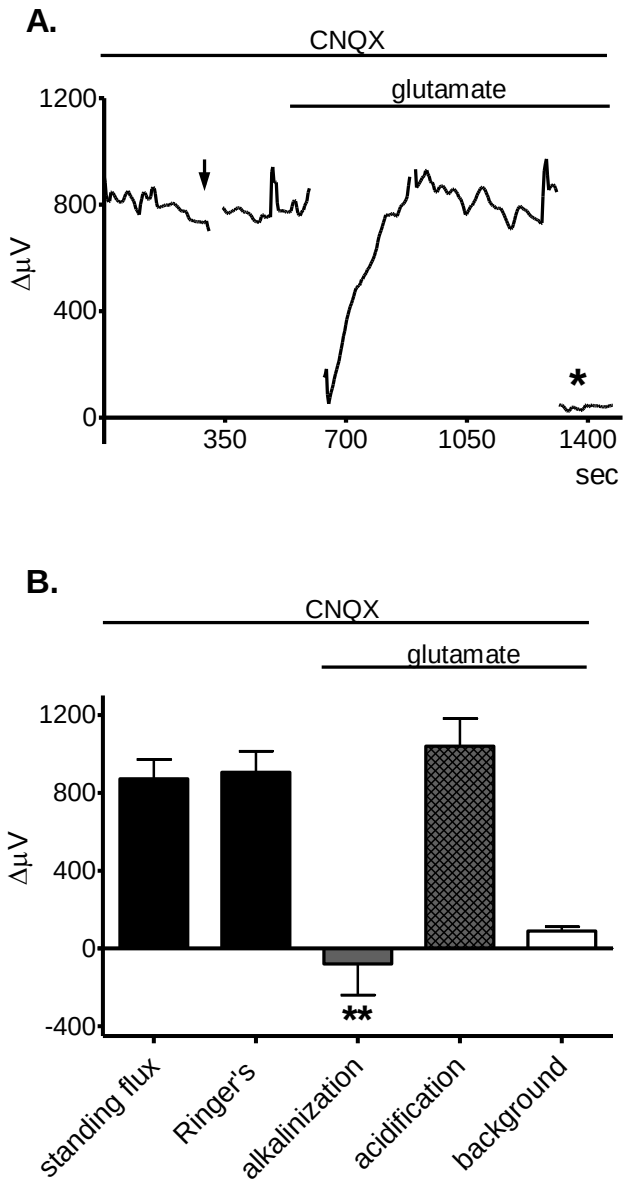


Figure 33. The AMPA receptor blocker CNQX reduces the glutamate-induced acidification recorded from retinal slices without affecting the transient alkalinization observed after glutamate application. (A) A trace from a single slice recording; glutamate still induces a robust alkalinization in the presence of CNQX, 500 μM. (B) Mean data of 8 trials: all slices were pre-incubated in 500 μM CNQX; 300 μM glutamate causes a significant and transient alkalinization as observed in previous figures. The acidification component of the glutamate-evoked changes is strongly diminished.

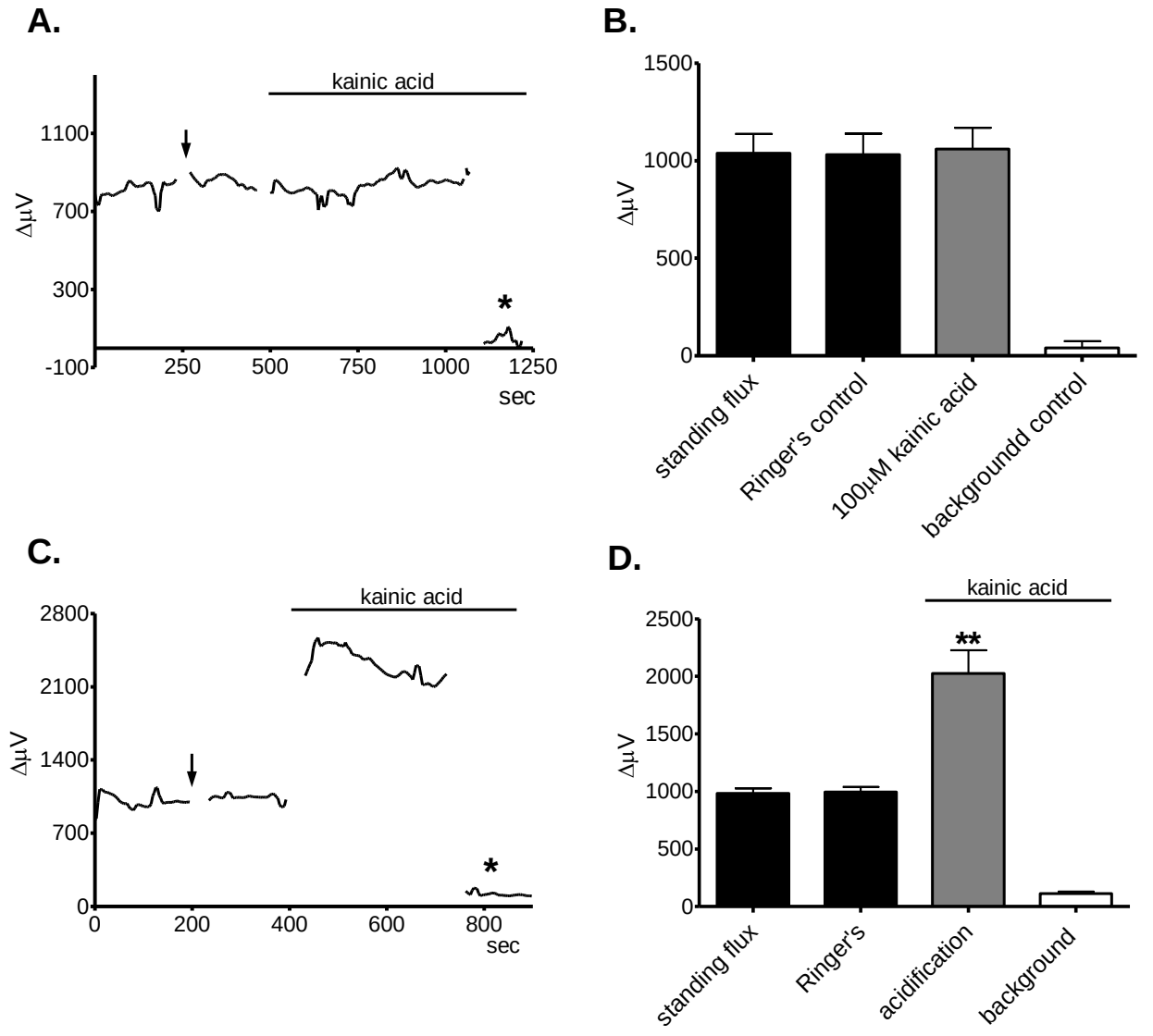


Figure 34. Kainic acid evokes an extracellular acidification from retinal slices. (A) A trace from a single slice recording depicts a high level of extracellular standing acidity and a lack of pH response to bath-applied 100 μM kainic acid detected with an H^+-selective microelectrode. (B) Mean data from 5 trials analyzed with a paired t-test. 100 μM Kainic acid failed to significantly increase the extracellular acidification recorded from slices of salamander retinae. (C)) A trace from a single slice recording depicts a high level of extracellular standing acidity and a significant increase in acidity in response to 200 μM kainic acid. (D) Mean data from 6 trials show 200 μM Kainic acid significantly increases the extracellular acidification recorded from slices of salamander retinae.

V. 4. DISCUSSION

Bath-applied glutamate caused a transient extracellular alkalinization from isolated retinal Müller cells and retinal slices of the tiger salamander. The transient alkalinization was followed by a robust extracellular acidification, which was particularly pronounced in slices. In isolated cells, the alkalinization was removed independently by both replacement of bath sodium with choline and by the addition of the glutamate transporter blocker TBOA. TBOA also reduced the transient alkalinization recorded from slices without affecting the acidification component. The P2 receptor blockers PPADS and suramin did not affect the glutamate-induced alkalinization, but the acidification component seemed to be slightly reduced than the one in the absence of the blockers. PPADS and suramin, when introduced in the slice chamber in the background of TBOA, also slightly reduced the acidification compared to the acidification in the background of glutamate alone. The P2 receptor blockers also reduced the standing flux in the background of TBOA even though the magnitude of standing acidification was not much different from the standing acidification in PPADS and suramin alone, in which case there was no significant reduction, but slices pre-incubated in TBOA seemed to exhibit more acidic standing fluxes. The TBOA data suggests that the observed transient alkalinization is due to the activity of glutamate transporters (EAATs). Studies have previously shown that Na+-dependent glutamate transporters co-transport H^+ along with glutamate and Na+, thus the H^+-selective microelectrodes used in these experiments probably reflect a transient reduction in H^+ as these ions are being pulled in. These results are different from previously reported as previous studies have not shown a transient nature of the activity of the glial glutamate transporter EAAT1. It should be

noted though that the activity of the transporter was examined with H^+-sensitive microelectrodes in this work compared to whole-cell voltage clamps in previous studies cited here. In the experiments presented here, the voltage of the cell is not controlled and any voltage changes that might be occurring post glutamate application were not monitored. Also, in whole-cell experiments examining the voltage dependence of the transporter-associated current, the internal environment of the cells was manipulated by the contents of the patch pipettes, as well as the internal pH was buffered by the pH buffer (HEPES) in the pipette (Levy et al., 1998). I previously showed that bath-applied ATP induced an extracellular acidification from salamander retinal slices as well as from isolated Müller glial cells (chapters III and IV). In those experiments, PPADS and suramin also attenuated the ATP-induced response.

Experiments on slices with the AMPA/Kainate receptor antagonist reveal no change in the extracellular alkalinization, but a robust decrease in the acidification component. The lack of inhibition on the alkalinization was not surprising since CNQX is not expected to alter the activity of glutamate transporters. The reduced extracellular acidification does suggest that activation of AMPA receptors is required in order for an acidic output to be detected. The data with P2 receptor blockers and CNQX suggests that some of the observed glutamate-induced acidification could be due to the activation of a purinergic signaling pathway downstream from glutamate receptor stimulation. Activation of kainate receptors with 200 μM kainic acid also resulted in a profound extracellular acidification, similar to the one produced after glutamate application. The alkalization component was not present in slices stimulated by kainic acid, which should be expected as EAATs would not transport it. The robust extracellular acidification

produced after introducing kainate into the slice chamber supports the interpretation of the CNQX inhibition and asserts the notion that the acidification requires activation of AMPA/kainate receptors. It should be noted that experiments with 100 μM kainic acid did not produce a significant extracellular acidification. One explanation could be that when the slices are stimulated by an agonist, such as kainic acid, to only a subpopulation of glutamate receptors, the stimulation has to be strong enough to make up for the missing component of the response otherwise produced by glutamate (e.g. other glutamate receptors not activated by kainic acid).

An interesting observation is that both the standing flux and the glutamate-induced acidification recorded in the background of TBOA seem to be higher than those recorded in Ringer's only background. The notion here is that TBOA would prevent the activation of at least some of the glutamate transporters, resulting in an increased extracellular glutamate concentration even in un-stimulated slices, presuming there is still some level of glutamate signaling taking place. Increased standing acidity in TBOA background could be mimicking the observed effects of bath-applied glutamate in clean Ringer's background with the same hypothesis at hand: glutamate activates glutamate receptors, which turns in motion the mechanism responsible for acidifying the extracellular space, possibly in part by means of purinergic signaling.

The current leading hypothesis in the retinal field as to how signal outflow from photoreceptors is modulated postulates that horizontal cells depolarized by photoreceptor-derived glutamate feed back onto photoreceptor calcium channels by changing the proton concentration in the synaptic cleft. Some of the more direct evidence comes from Wang and colleagues who express a particular ligand-gated Na^+

channel (FMRFamide channel) solely in horizontal cells in zebrafish. When these cells are then preferentially depolarized by the application of this sodium channel's ligand, FMRFamide, the authors observe an extracellular acidification as reported by CaliPhluorin, a pH-sensitive modification of GFP, which the authors attached to the extracellular side of a photoreceptor calcium channel. Wang et al. conclude that horizontal cells must be the ones mediating the pH changes in the extracellular space responsible for the regulation of the activity of photoreceptor calcium channels. Even though the results presented here support the notion that activation of glutamate receptors (e.g. on horizontal cells) at the outer retina leads to an extracellular acidification, I argue that these data do not exclude the possibility that other retinal cells are also contributing to the observed extracellular pH changes. One feasible hypothesis is that horizontal cells depolarized by glutamate from photoreceptors go on to relay the message to retinal Müller (glial) cells, for example via ATP release, which cells in turn release protons and (additionally) acidify the extracellular space.

In the previous chapters I reported that ATP application on isolated Müller cells of the salamander retina caused a very significant acidification of the extracellular space mediated by these glial cells. An extracellular acidification was also observed in ATP-stimulated slices and in both cases PPADS and suramin, broad-spectrum P2 receptor blockers, significantly reduced the ATP effect, suggesting purinergic receptor mediated responses were required for the detected changes in extracellular acidity to occur. In the work presented in this chapter, PPADS and suramin also reduced the glutamate-induced acidification in slices from tiger salamander retinae, implicating the activation of purinergic receptors, probably on Müller cells as suggested by my previous work, but not

excluding other cell types' contributions. It should be noted that PPADS and suramin did not completely abolish the increase in acidity triggered by glutamate, but the increase was smaller than the one produced by glutamate application in the absence of the P2 receptor blocker (~76% increase in Ringer's compared to ~42% in PPADS and suramin).

These data and current knowledge about EAATs suggest that the transient alkalinization produced by glutamate application reflects the activity of glutamate transporters as they pull in protons along with glutamate. The interpretation of the acidification portion of the observed glutamate-induced effects remains less clear and more speculative. Retinal horizontal cells possess glutamate receptors and respond to glutamate exocytosed by photoreceptors by changing their membrane potential to more depolarized levels. I speculate that this depolarization could trigger exocytosis of vesicles containing ATP. There is a large body of evidence in the literature to suggest that ATP can and does act as an intercellular signaling molecule. The ATP released by horizontal cells (or other cell types in the retina) could then activate ATP receptors (P2) on neighboring cells, especially Müller glia, which in turn would release protons and acidify the extracellular space based on their ATP-mediated responses described in this work. The results from the experiments presented here show that Müller cells from the tiger salamander retina capable of generating sizeable acidification responses to bath-applied ATP. The data also show that the increase in acidity measured in slices is lower when the purinergic signaling system is disrupted by PPADS and suramin. It should be noted that suramin and PPADS are broad-spectrum P2 receptor blockers, acting on members of both P2Y and P2X receptor types with varied abilities to prevent activation

of these receptors in the presence of their ligand(s). The lack of a stronger inhibition of the glutamate-induced acidification could result from an incomplete P2 receptor inhibition (for example, because other P2Rs are not greatly inhibited by suramin and PPADS).

Another possibility is that the purinergic system contributes to the generated responses only partially and the remainder of the signal could be due to non-purinergic mechanisms. Another notable observation is that slices pre-incubated in the glutamate transporter blocker TBOA exhibited larger standing fluxes than those pre-incubated in clean Ringer's. In clean Ringer's, the increase in acidification after glutamate application was 76% from standing flux levels, whereas in slices pre-incubated in TBOA this increase was 51%. One way to explain the larger standing fluxes observed in TBOA background is that extracellular glutamate in slices exposed to TBOA is higher than the extracellular glutamate levels in slices in which the up-take of the neurotransmitter into cells had not been disrupted. Higher extracellular glutamate could be activating the same mechanism(s) activated by bath-application of glutamate, hence raising the acidity levels which we observe also by bath-applying glutamate.

These findings build up on the already existing evidence that some glutamate transporters co-transport H^+ along with the neurotransmitter and show that these transporters are present in the retina, particularly on Müller cells' membranes, and have the ability to impact acidity levels significantly. The results presented here also confirm the notion that in the retina glutamate released from photoreceptors does trigger a response from one or multiple cell types which results in an extracellular acidification, hypothesized to be responsible for photoreceptor calcium channel modulation. This work, along with work described in previous chapters, suggests the possibility that this

crucial glutamate-induced acidification may at least partly involve responses from retinal Müller glia through ATP signaling.

VI. DISCUSSION

Localized changes in the extracellular pH in the vertebrate retina have been proposed to be the mechanism for negative feedback through which the regulation and modulation of synaptic activity at the level of the photoreceptor terminals occurs. A large body of evidence has implicated horizontal cells as the cell type feeding back onto photoreceptor terminals through acidification of the synaptic cleft, which reduces the conductance and shifts the activation curve of photoreceptor calcium channels. The hypothesis for proton negative feedback states that upon horizontal cell depolarization by glutamate released from photoreceptors, these neurons acidify the synaptic cleft. Yet data from the Malchow lab has consistently showed using various techniques that depolarization of horizontal cells leads to the exact opposite results: an alkalinization of the extracellular space (Jacoby et al, 2012: Jacoby et al, 2014).

The work presented here shows convincing evidence that the radial glial cells of the vertebrate retina, the Müller cells, are also capable of mediating significant changes in the extracellular pH. Presented here are large extracellular acidification responses of Müller cells of the tiger salamander retina when exogenous ATP is present in a concentration as low 1µM. Self-referencing recordings with H^+-selective microelectrodes have shown that the amplitude of the extracellular acidification correlates with increases in the concentration of ATP used. Self-referencing experiments in conjunction with the use of pharmacological agents and intracellular calcium imaging have all shed some light onto a pathway through which extracellular ATP generates the responses observed from isolated Müller cells. The persistence of an acidification response from these cells when ATP is replaced by wither UTP or ADP strongly suggests that these extracellular molecules exert their actions through purinergic receptors of the P2 family, as all three

substances are ligands to P2 receptors. Further support for this notion comes from experiments which show inhibition of the extracellular acidification with the broad-spectrum inhibitor of purinergic receptors, suramin. Lack of changes in extracellular acidity when adenosine is introduced into the bath solution rules out the involvement of P1 adenosine receptors. Imaging data from isolated Müller cells loaded with the calcium indicator Oregon Green have revealed a tight correlation between the observed extracellular acidification and intracellular calcium rises. The source of calcium seems to be intracellular stores rather than calcium entry from the extracellular space, as the intracellular calcium rises persist even with omission of Ca^{2+} from the bathing solution. Pharmacological disruption of the PLC-IP3 signaling cascade reduced both the intracellular calcium rise and the extracellular acidification, which suggests that activation of PLC and calcium release from IP3-sensitive receptors on the endoplasmic reticulum are necessary for the ATP-induced acidification to occur. The imaging data also strongly implicates a particular subclass of purinergic receptors, the metabotropic P2Y receptors which are coupled to G-proteins and can modulate the activity of PLC and lead to calcium rises independent of extracellular calcium. This work has provided substantial evidence for the hypothesis that, in the case of tiger salamander Müller cells, bath-applied ATP binds to metabotropic receptors of the P2Y class, which in turn activate PLC. This then leads to the production of IP3 and subsequent opening of IP3-sensitive calcium channels on internal stores, ultimately raising the intracellular calcium concentration and inducing extracellular acidification. What is still unknown is the exact identities of the molecular/membrane entities which directly mediate the extracellular acidification induced by ATP. What is mediating this extracellular acidification? Is

calcium directly affecting the activity of this unknown mediator (membrane proteins involved in transport?) or are there more intermediate steps between the calcium rise and the change in extracellular pH? Is it only one or could it be that multiple entities that are being activated? One hypothesis is that ion channels and/or ion transporters are being activated on the membrane or inserted into the membrane by fusing vesicles. Data with the anion transport blocker DIDS does point to a chloride/anion dependency of this mechanism since in the presence of DIDS the extracellular acidification is drastically reduced, but the data so far does not point to a particular transporter or channel. The lack of inhibition of the ATP-induced effect on extracellular pH when extracellular chloride is drastically reduced does suggest a couple of things. First, chloride fluxes might not be involved at all and the transport blocked by DIDS could be of other anions. It should be noted that all the recordings were performed in HEPES as the extracellular pH buffer, so contributions from bicarbonate transport are very unlikely. Second, the mechanism could be dependent on chloride outflow rather than inflow. Third, the remaining extracellular chloride concentration could be enough to still provide enough ions to flow into the cell. And fourth, DIDS could be having an effect on other anion transport, such as the transport of small organics (e.g. taurine). It has been established that chloride channels are not highly selective for chloride over other inorganic anions, and that they can conduct small organic anions as well. For that reason it has been suggested that they are referred to as anion channels (Duran et al., 2010). There are five families of chloride channels, some of which are chloride/H+ exchangers, such as some of the CICs, others regulate other ion channels and transporters, such as the cystic fibrosis transmembrane conductance regulator (CFTR), and some are calcium

activated chloride channels, such as the ones belonging to the TMEM16A/anoctamin family (Duran et al, 2010). So, given the diversity of chloride/anion channels, some of whom act as transporters, with their numerous permeabilities and variable modes of activation, such as by ligands or intracellular messengers, the inhibition by DIDS presented here is hard to interpret without further exploration of all above-mentioned possibilities.

To investigate the possibility that the acidification was simply due to the activation of a Na^+/H^+ exchanger, experiments in which extracellular Na^+ was replaced by choline were performed. The results from those experiments were, unfortunately, also not definitive. Even though the overall magnitudes of the signals, both the standing acidification from cells as well as the ATP-induced acidification, were reduced, the percent increase in extracellular acidity in zero sodium was similar to the one in normal sodium. The sodium dependence of the standing flux was more apparent as the proton signal was reduced by removing $[Na^+]_e$. Whether the standing flux is entirely due to the activity of the Na^+/H^+ exchanger is also debatable as experiments with EIPA, which blocks the exchanger, revealed only partial inhibition of the standing flux by EIPA. One possibility is that the standing flux was driven by multiple sodium-dependent mechanisms. Another is that if multiple isoforms of the Na^+/H^+ exchangers were present, only some were EIPA-insensitive or the inhibition by the drug had particularly slow kinetics (work from Dr. Kreitzer's lab has shown about 40% inhibition of the ATP-induced acidification by amiloride).

Müller glial cells isolated from the retinae of other species from different vertebrate classes also respond to extracellular ATP with a robust extracellular acidification. Müller

cells of lamprey, zebrafish, goldfish, catfish, skate, rat, 2 Macaque species – Macaca fascicularis and Macaca mullata – and human were isolated and exposed to ATP. Lamprey, catfish, skate, both macaque species and human Müller glia mediated an ATP-induced acidification of the extracellular space, which points to a very general response across vertebrates, which may or may not utilize the same molecular/signaling pathways that lead up to the observed response.

The interpretation of the results and the lack of contributions from other cell types made a set-up using isolated cells appropriate in order to characterize the membrane and intracellular components which played roles in the ATP-induced extracellular acidification. At the same time it was important to try and relate the responses from individual glial cells to the behavior of the retinal network they were part of, which was why retinal slices were also used. Using the self-referencing technique again, it was demonstrated that ATP also induced extracellular acidification from slices when the H+ microelectrodes were positioned right above the OPL where photoreceptors made synaptic connections with horizontal and bipolar cells. In slices, the standing acidic flux was significantly larger than the one detected from isolated Müller cells, which could be because, in a slice set-up, the H^+ electrode could be receiving H^+ outputs from multiple Müller cells in the vicinity of the electrode's tip or possibly other cell types could have contributed to the generated signals. Self-referencing recordings from isolated photoreceptors show neither a standing flux nor an ATP-mediated pH response, so their contribution to the acidic responses generated in slices is unlikely. ATP application also does not lead to H^+ responses from horizontal cells isolated from catfish. Preliminary experiments on slices show that the observed extracellular acidification may be present

not only at the level of the OPL, but also at the level of the ONL, IPL and possibly the INL. Such findings would be consistent with data from isolated Müller cells and the basic retinal architecture. Acidic fluxes have been observed along the length of Müller cells from the apical tuft, down to the endfoot and given that these cells span the whole thickness of the retina, it can be expected that acidic signals along the whole cross-section would be detected. Yet, in a slice the network of various cell types makes it challenging to pinpoint the source of the pH changes. Another parallel between the results from slice experiments and those from isolated Müller cells is the fact that in both cases the ATP-induced acidification can be attenuated with the P2 receptor blockers suramin and PPADS, suggesting that the response is driven by means of purinergic signaling in slices as well. Similar results were obtained from goldfish slices in which suramin and PPADS attenuated not only the acidification induced by bath-applied ATP, but also the standing acidic flux. Very preliminary data suggest that ATP might induce similar acidification responses from slices of rat retinae.

Despite all efforts and all data collected, the precise mediator(s) of the ATP-induced acidification from Müller cells isolated from the tiger salamander remains unresolved. Multiple ion channels and/or transporters could be involved as suggested by the zero extracellular sodium and DIDS data. Other possibilities, which were not tested, include the activation of monocarboxylate transporters (MCTs), such as the lactic acid transporter. These transporters are located on both glia and neurons and they have been shown to have a preferential direction of transport, working mainly in the inward direction when on neuronal membranes and in the outward direction when located on glia. MCTs have also been shown to conduct H^+; furthermore, it has been hypothesized

that their mode of operation is based on a transmembrane H+ gradient. (Cheeti and Lee, 2011). Inhibition of MCTs by the anion transport blocker DIDS has also been observed. Another membrane transporter, which could be involved in the mediation of the observed extracellular acidification is the taurine transporter. Taurine, β-aminoulfonic acid, is widely-distributed in the retina and although its full functional significance has not been well established, it has been shown to be crucial for proper retinal function and architecture (El-Sherbeny et al., 2004). One of the functions attributed to the taurine transporter is involvement in osmoregulation (Mongin et al., 1999). Small organic osmolytes, taurine included, have been shown to be transported through anion channels, such as the volume-regulated anion channel, VRAC, in cultured astrocytes (Mongin and Kimelberg, 2004). Moreover, ATP has been shown to enhance the activity of VRACs and the release of organic osmolytes during periods of osmotic stress. The modulation of VRACs by ATP has been suggested to be due to the activation of P2Y receptors and has been shown to be dependent on intracellular calcium with multiple calcium-dependent pathways activated (Mongin and Kimelberg, 2004). ATP application on isolated Müller cells also resulted in shape changes and occasional membrane blebbing, which changes were enhanced at higher ATP concentrations. Cellular shape changes and blebbing have been correlated with the development of chloride currents in the human chloride-secreting colonic cell line T-84 (Worrell et al., 1989). Moreover, shape changes in inner supporting cells of the rat cochlea have been correlated with the activation of ATP receptors on these cells and subsequent IP3-mediated rises in intracellular calcium levels. Membrane shrinkage due to water loss following the activation of Ca^{2+}-activated Cl^- channels has been proposed (Tritsch et al., 2010).

It has been speculated that under osmotic stress conditions cells would use transmembrane transport of small organic osmolytes to evoke obligatory water movement to prevent bulk movements of ions and avoid membrane voltage changes, etc. The taurine transporter, TauT, has been shown to be highly expressed on various retinal cells including cultured rat Müller glia. Moreover, in experiments aiming to establish any dependence of the transporter on Na+ and Cl- in cultured retinal ganglion and Müller cells, taurine uptake was strongly inhibited in the presence of [Na+]e but absence of $[Cl^-]_e$ and vice versa. The data from isolated Müller cells show smaller acidification responses in the presence of $[Cl^-]_e$ but absence of [Na+]e and even more pronounced inhibition of the acidification by DIDS, which blocks chloride and other anion transport. Moreover, some taurine transport, such as the one through the PAT1 transporter, has been shown to be coupled to protons (Anderson et al., 2009). Müller cells have been shown to possess an autocrine mechanism for osmotic-induced volume regulation via release of ATP, which subsequently binds to ATP receptors on Müller cells. This mechanism could be also valid if Müller cells sense ATP released from neurons during high neuronal activity during which transmembrane fluxes of ions could lead to changes in osmolarity. During periods of high neuronal activity, the osmolarity of the extracellular space can significantly drop because the influx of extracellular ions such as Na^+ and Ca^{2+} can exceed released K^+ significantly (Dimitriev et al., 1999 as cited in Vogler et al., 2013). Müller cells are believed to possess a glutamate-purinergic autocrine mechanism to ensure prevention of their osmotically induced swelling, as swelling would lead to a decrease of the extracellular volume, which could be excitotoxic to neurons (Dudek et al., 1990 and Chebabo et al., 1995 as cited in Vogler, S., 2013).

Calcium channels located on retinal photoreceptors' terminals have been shown to be sensitive to extracellular H^+. The proton hypothesis for negative feedback postulates that photoreceptor-derived glutamate depolarizes horizontal cells, which initiates an increase in extracellular acidity, which in turn reduces photoreceptor calcium entry through a direct H^+-calcium channel modulation, ultimately reducing glutamate release from photoreceptors. It is possible that the role of the observed Müller cell-mediated extracellular acidification is to decrease the level of neuronal activity at the OPL and maybe at other retinal layers. ATP is believed to be co-released from synaptic vesicles containing neurotransmitter. If neuronally derived ATP is able to reach processes of surrounding Müller cells, for example during high photoreceptor activity in the dark when extracellular concentrations of glutamate and ATP might reach higher levels, Müller glia could be initiating this ATP-driven response in order to acidify the extracellular space and tune down photoreceptor activity to prevent excitotoxic extracellular levels of the neurotransmitter. Some preliminary recordings of calcium currents in photoreceptors in slices of tiger salamander were gathered in the lab of Dr. Wallace Thoreson at the University of Nebraska Medical Center. The data was collected working alongside Dr. Matthew Van Hook, a postdoc in the Thoreson lab at the time. The preliminary data showed that calcium currents in photoreceptors actually got larger and their activation curves shifted to the left in response to superfused ATP (100μM), which was a result exactly opposite of the above-stated hypothesis that Müller cells might acidify the extracellular space in response to ATP in order to reduce synaptic transmission by reducing neuronal (particularly photoreceptor) calcium channels' activity. The data was preliminary and deemed insufficient to completely rule out the hypothesis.

Even if Müller cells are not the ones mediating modulation of photoreceptor calcium channels, the data does show robust increases in acidity in response to ATP in slices which cannot be ignored, especially in the context of the ongoing debate as to what cell type mediates negative feedback through H^+ changes in the outer retina. One possible explanation for the lack of modulation of calcium channel conductance in photoreceptors could be that when recording H^+ fluxes from slices using H^+-selective microelectrodes, the surface acidification detected is due to bath-applied ATP, which is not penetrating a deeper layer where maybe photoreceptor terminals are be buried. Additional physiological experiments in the context of the retinal network need to be performed.

Work presented here has also revealed that ATP is not the only extracellular molecule capable of activating Müller cells in a way in which they modulate the pH of the extracellular space. Since glutamate is the neurotransmitter believed to be released by photoreceptors, changes in extracellular H^+ in response to bath-applied glutamate were explored. Isolated Müller cells responded to glutamate with a very robust extracellular alkalinization, which is transient in nature and recovers to slightly above baseline levels even when glutamate is still in the bath. Glutamate fails to elicit a response in zero extracellular sodium. The response is also significantly attenuated in the background of TBOA, a glutamate transporter blocker. Müller cells are known to possess membrane glutamate transporters and their function is believed to be removal of excess extracellular glutamate, especially under scenarios of glutamate spill-over beyond its intended localized activity at the synapse. Glutamate transporters have been shown to uptake glutamate, along with sodium ions and protons. Self-referencing data supports

the notion that the observed transient alkalinization is due to the activity of glutamate transporters (EAATs). Preliminary experiments I conducted with high extracellular K^+ show a reduced uptake of glutamate by Müller cells reflected in a lower-magnitude alkalinization, in correlation with the notion that glutamate uptake through EAATs is electrogenic and less efficient at more depolarized potentials. Moreover, this transient alkalinization in response to glutamate persists when H^+ recordings were performed from retinal slices in which the H^+ microelectrodes were positioned at the level of the outer plexiform layer (OPL), similar to the experimental design for investigating H^+ fluxes from slices bathed in ATP (chapter III and IV). Similar to the data from isolated cells, the transient alkalinization from slices was reduced in the presence of TBOA, suggesting that in slices the reduction of extracellular H^+ is probably also due to the activity of glutamate transporters on Müller cells and possibly other cell types. An interesting observation was that the H^+ signal did not just return to the standing acidic flux, which in slices was much larger than the one detected from isolated cells, but it surpassed it and eventually reached a plateau at much more acidic levels. An acidification that followed the transient glutamate-induced alkalinization was also observed when recording from isolated Müller cells, but with a much smaller magnitude. TBOA removed the alkalinization response from slices but did not affect the acidification component suggesting the activation of a different mechanism which was not triggered by the activity of the glutamate transporters. Since involvement of glutamate transporters in the acidification response could be ruled out, it was speculated that this H^+ change must be then due to the activation of glutamate receptors. The acidification was blocked in the background of CNQX, a known inhibitor of AMPA/kainate receptors. Glutamate

receptors are present on various retinal cell types, including horizontal cells, which are the cells believed to mediate negative feedback in response to glutamate exocytosed from photoreceptors. Müller cells have been suggested to possess metabotropic glutamate receptors (MGluRs) whose activation leads to the generation and spreading of intracellular calcium waves (Keirstead and Miller, 1997), as well as AMPA/kainate receptors (Wakakura and Yamamoto, 1994). Self-referencing experiments from slices also reveal that 200 μM kainate can induce a robust extracellular acidification without the alkalinization component since kainate would not be taken up by EAATs. Interestingly, 100 μM kainic acid did not affect the extracellular acidity. It seems reasonable to conclude that the glutamate-induced extracellular acidification effect is due to the activation of glutamate receptors on retinal cells. Unfortunately, at this time it is not clear what cells are being activated by glutamate and what cells mediate the glutamate-induced acidification in slices and whether the former and the latter are the same cells. Having previously observed large extracellular acidifications from Müller cells and from slices exposed to ATP (as described in chapters III and IV), I investigated the possibility that the acidification I detected from slices bathed in glutamate could be due to glutamate-activated cells releasing ATP, which in turn was activating Müller cells. The glutamate-induced acidification detected from slices persisted in the background of the P2 receptor blockers suramin and PPADS, but it seemed as a small fraction of it was sensitive to the blockers as indicated by the slightly reduced magnitude.

If the presumptions that ATP is co-released with glutamate and that Müller cells take up excessive glutamate to prevent excitotoxicity through glutamate transporters which import H^+ along with glutamate are correct, it could be speculated that the release

of H^+ in response to ATP is to ensure that there are sufficient extracellular protons for efficient glutamate uptake.

This dissertation work has introduced strong evidence that in retina, where extracellular pH has been hypothesized to be critical for proper signal modulation, Müller glial cells are capable of generating large changes in extracellular pH in response to neuromodulators and neurotransmitters (ATP and glutamate) normally used by nerve, including retinal, cells. In the retina particularly, changes in extracellular pH have been shown to be important for the modulation of photoreceptor signaling. A lot of data over the years has shown the role of extracellular protons as neuromodulators in the outer retina, but controversial results have left the debate over which retinal cell type mediates these pH changes open. The work presented here could potentially implicate yet a new cell type in this process of proton modulation of photoreceptor activity, and that is the glial Müller cell. Many experiments have led to partial characterization of the mechanisms involved in the ATP- and glutamate-induced H^+ fluxes. Several hypotheses have been described to explain these phenomena, but further work investigating molecular identities and physiological meaning is need.

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