C36

Direct measurement of adenosine release during epileptiform activity in the *in vitro* rat hippocampus using an enzyme-based adenosine sensor

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The purine nucleoside adenosine is released during seizure activity and has been described as an endogenous anticonvulsant that raises the threshold for seizure generation or aids in the termination of seizures. The proposed neuroprotective properties of adenosine are primarily achieved through inhibition of glutamate release via presynaptic A_1 adenosine receptors. In an *in vitro* model of epilepsy, using 600 μm transverse hippocampal slices prepared from 16-22 day old rats killed humanely by cervical dislocation, seizures were induced by means of a 2 s, 60 Hz stimulus train delivered to stratum radiatum of the CA1 region in nominally Mg^{2+}-free aCSF at 30–31 °C. In control conditions the stimulus initiated epileptiform activity, which persisted for 21.3 ± 3.4 s (mean ± SEM; n=10) and caused a profound (79.7 ± 3.8%; n=10), transient (5–6 min) depression of synaptic transmission. Upon application of CPT, a selective adenosine A_1 receptor antagonist, the duration of evoked seizures was dramatically prolonged (41.0 ± 7.3s; paired t-test; $p = 0.002$) whilst the depression of synaptic transmission was significantly reduced (28.0 ± 7.3%; paired t-test; $p < 0.001$). This confirms that adenosine is released and inhibits seizures via activation of A_1 receptors. To measure directly the release of adenosine during epileptiform activity we used a miniature adenosine sensor, which relies on the enzymatic conversion of adenosine to H_2O_2 (Dale, 1998; Dale *et al.*, 2000). The net signal obtained from the sensor, placed on the surface of area CA1, revealed that extracellular adenosine rises rapidly following the stimulus train. Within one minute of the onset of the seizure the peak level of adenosine measured 2.4 ± 0.6 μM (n = 4) and the time course of recovery from the post-ictal depression of synaptic transmission corresponded closely with the clearance of endogenous adenosine. Adenosine release was also observed during spontaneous epileptiform activity, either in control conditions or in the presence of CPT, and argues against stimulus-induced electroporation of axons as the source of extracellular adenosine. These results reveal the time course of adenosine release during brief periods of epileptiform activity and strengthen the importance of endogenous adenosine in the termination of seizures.

Dale N (1998) *JPhysiol* **511**, 265-272

Dale N *et al.* (2000) *JPhysiol* ***526****, 143-155*

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C37

Role of adenosine in the depression and recovery of excitatory synaptic transmission in area CA1 of rat hippocampus after *in vitro* ischemia.

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Adenosine is released in response to cerebral ischemia when it is believed to exert a neuroprotective influence by inhibiting glutamate release via activation of adenosine A_1 receptors. To correlate adenosine production during *in vitro* ischemia with excitatory synaptic transmission we have measured directly extracellular adenosine whilst recording glutamatergic field excitatory postsynaptic potentials (fEPSPs) from area CA1 of the rat hippocampal slice. Slices were prepared from 16–23 day old rats killed humanely by cervical dislocation and maintained at 32 °C in aCSF gassed with 95 % O_2, 5 % CO_2 (pH 7.4). *In vitro* ischemia was induced via oxygen/glucose deprivation (OGD; replaced by 95 % N_2, 5 % CO_2 and 10 mM sucrose, respectively). In control conditions the fEPSPs were fully depressed by 99 ± 2 % (mean ± SEM, n = 4) after 6.25 min of OGD, whereas in the presence of 8-CPT (1 μM, n = 4), a competitive A_1 receptor antagonist, the fEPSPs were only depressed by 32 ± 3 % (unpaired t-test, $p = 0.0012$) demonstrating that the depression of glutamatergic transmission was largely dependent on A_1 receptor activation. To measure directly adenosine release in response to OGD an enzyme-based adenosine sensor was placed on the surface of area CA1 (Dale *et al.*, 2000). In control experiments after exposure to OGD of 6.25 minutes (n = 3) the adenosine released 5 minutes after OGD was 13.88 ± 2.2 μM, with an estimated IC_{50} = 0.64 ± 0.4 μM, after which transmission slowly recovered to 77 ± 0.3 % after 1 hr of re-oxygenation. To examine the basis of the protracted recovery of the fEPSP, 1 μM 8-CPT (n = 3) was applied to the slice 22 minutes after reoxygenation after which the fEPSP rapidly increased to close to baseline values (ie 100 %, of which 20 % of the increase can be attributed to basal (ie non OGD-induced) adenosine), compared to 23 ± 1 % of control immediately prior to 8-CPT. 8-CPT had no effect on extracellular adenosine. When two periods of OGD (8.5 – 9.5 min; n= 4) were given, recovery of the fEPSP after the first measured 63 ± 4 % at 40 min, whereas after only 26 min of recovery the second OGD resulted in 85 ± 1 % recovery of the fEPSP. This was associated with reduced adenosine release during the second OGD (12.1 ± 1.8 μM) compared to the first (20 ± 4 μM). These data suggest that elevated extracellular adenosine can account for the protracted recovery of transmission after OGD and that the accelerated recovery of synaptic transmission after repeated OGD can be due to adenosine depletion.

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C39

Characterisation of the pathways involved in elevating $[Ca^{2+}]_i$ following PAR2 activation in cultured rat hippocampal neurones.

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Protease-activated receptor 2 (PAR2) is widely distributed throughout the central nervous system (CNS) and has been shown to play a role in neurogenic inflammation and nociception (MacFarlane et al, 2001). In hippocampal cultures, chronic activation of PAR2 leads to cell death and is associated with elevated $[Ca^{2+}]_i$ (Smith-Swintosky et al, 1997) but the signalling mechanisms underlying this remain unclear. In the present study, we have examined the pathways underlying PAR2 $[Ca^{2+}]_i$ responses in cultured hippocampal neurones, using fluorescence-imaging techniques.Cultures of rat hippocampal neurones were prepared from humanely killed rats and $[Ca^{2+}]_i$ measured as described previously (Shanley et al, 2002). Experiments were performed on cells >13 days in culture, continually perfused with a standard HEPES-buffered saline containing TTX (0.5μmM). All drugs were applied via the perfusate. Data are expressed as mean ± S.E.M. of fluorescent ratio arbitrary units (a.u.) and statistical significance determined using Student's paired t-tests, n = number of neurones obtained from at least two separate cultures.In the majority of neurones, application of trypsin (80nM) elevated $[Ca^{2+}]_i$ from control levels by 0.35 ± 0.05 a.u. (n=17/20, P<0.01). In addition, the selective PAR2 peptide agonists, SLIGRL (80μM) and ASKH95 (80μM), both elevated $[Ca^{2+}]_i$ from control levels by 0.15 ± 0.02 a.u. (n=26, P<0.01) and 0.23 ± 0.03 a.u. (n=15, P<0.01) respectively. In Ca^{2+}-free solution, SLIGRL (80μM) increased $[Ca^{2+}]_i$, however responses were moderately reduced (20 ± 5 % reduction, n=10, P<0.01). Application of SLIGRL following the depletion of intracellular Ca^{2+} stores with cyclopiazonic acid (30μM) had little effect on $[Ca^{2+}]_i$ (97 ± 4% reduction, n=10, P<0.01) indicating intracellular stores as the primary source of the elevated $[Ca^{2+}]_i$. Inhibition of PLC and IP3 receptors by U73122 (1μM) and 2-aminoethoxydiphenyl borate (20μM) also reduced SLIGRL responses (87 ± 4% reduction, n=10, P<0.01 and 91 ± 5% reduction, n=10, P<0.01, respectively).These data reveal that activation of PAR2 in neurones elevates $[Ca^{2+}]_i$, via the PLC / IP3 pathway. Thus PAR2 may play an important role in modulating Ca^{2+}-dependent processes within the hippocampus.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C40

The gap junction blocker carbenoxolone affects sensory processing and motor pattern generation in *Xenopus* embryos.

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Gap junctions are intercellular channels that allow direct, low resistance, electrical and chemical communication between neighbouring cells. Such coupling, which is particularly prevalent early in development, is rapid compared to chemical synaptic transmission and allows the diffusion of relatively large molecules between cells. An important function of electrical coupling is to synchronize neuronal activity (Kiehn & Tresch, 2002). This may be less appropriate to the regulation of more mature networks, indicating that functional de-coupling of gap junctions could contribute to neural network development. In hatchling *Xenopus* frog embryos, motorneurones that are rhythmically active during swimming, are coupled to their near neighbours via electrical synapses. This coupling is thought to ensure that homonymous motorneurones all fire their single impulse per cycle synchronously (Perrins & Roberts, 1995). However, early in larval development, neurones can fire repetitively in each cycle and ventral root bursts become desynchronized, leading to the hypothesis that a reduction in gap junction coupling might be responsible. We studied the effects of a gap junction blocker, carbenoxolone (CBX; 100-200 μM) on fictive swimming and on the initiation of swimming via skin stimulation in (stage 37/38) *Xenopus* embryos.We monitored the effects of CBX on the transmission of the skin impulse through the embryos epithelium. Skin cells can generate a cardiac-like impulse of ca. 100mV amplitude and 100ms duration, which propagates to neighbouring cells via gap junctions at rates of ca. $5cm/s^{-1}$ (Roberts & Stirling, 1971). CBX reduced conduction rates through the skin; impulses generated by a current pulse applied to the caudal tail skin were delayed in reaching more rostral regions (n=9). Thus CBX can impair transmission through the electrically coupled network of skin cells. In the presence of CBX a single stimulus could lead to multiple skin impulses and impulses could sometimes occur spontaneously. Next, the effects of CBX on fictive swimming were examined. Normally, each cycle comprises a biphasic compound ventral root burst of ca. 5-7 ms. CBX reduced swim episode durations, with an associated increase in motor burst durations (n=6). The increase in burst durations is consistent with a de-synchronization of motor activity leading to the conclusion that electrical coupling is necessary to synchronize motorneurone activity. We propose that a decrease in coupling during development contributes to the maturation of larval swimming.

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C42

Astrocyte glycogen metabolism is required for neural activity during aglycaemia or intense stimulation in mouse white matter

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CNS glycogen is located in astrocytes, but its function is unclear. Glycogen metabolism supports mouse optic nerve (MON) function during aglycaemia (Brown et al., 2003), and supports function during high intensity stimulus, when ambient glucose is insufficient to meet the elevated energy demands (Brown et al., 2003). Isofagomine inhibits mouse brain glycogen phosphorylase activity (Waagepetersen et al., 2000), thus we used isofagomine to evaluate functional importance of glycogen metabolism in supporting MON axon conduction. Adult male Swiss Webster mice were killed by decapitation under deep CO_2 anaesthesia. MONs were placed in a superfusion chamber at 37°C. Axon function was assessed as CAP area, with glycogen content measured biochemically. Data presented as means S.E.M., and one-way ANOVA employed to determine significance. MONs perfused in 10 or 30 mM glucose, respectively, had glycogen content of 6.01 ± 0.65 pmoles per μg protein (n = 3), or 7.91 ± 0.64 pmoles per μg protein (n = 3; $p < 0.05$), respectively. Isofagomine (400 μM) increased glycogen content of MONs perfused in 10 or 30 mM glucose, respectively, to 9.73 ± 0.75 pmoles per μg protein (n = 4; $p < 0.05$), or 11.31 ± 1.28 pmoles per μg protein, respectively (n = 4; n.s.). The robust CAPs seen in MONs bathed in 10 mM glucose disappeared after 15.9 ± 0.4 mins (n = 6) after aglycaemia onset. Introduction of glucose free aCSF in the presence of isofagomine shortened CAP failure to 11.2 ± 0.7 mins (n = 4, $p < 0.05$). MONs bathed in 30 mM glucose aCSF, in the absence or presence of 400 μM isofagomine, respectively, displayed onset to CAP failure after 23.2 ± 2.2 mins (n = 4) or 13.5 ± 1.2 mins, respectively (n = 4, $p < 0.001$) after onset of aglycaemia.In MONs in 10 mM glucose, CAP increased then decreased during a 4 min period of 100 Hz stimulation, and fell below baseline after 3 mins (n = 4). MONs pre-treated (4 hrs) with 400 μM isofagomine showed an initial increase and then a rapid, marked decline in the CAP area below baseline after 1 min (n = 4). The results show isofagomine significantly increased MON glycogen content, but during subsequent aglycaemia latency to CAP failure declined, showing the importance of glycogen metabolism under such conditions. Additionally, inhibiting glycogen metabolism accelerated CAP failure during high intensity stimulus, implying glycogen metabolism supports axon function during intense activity in normoglycaemic ambient glucose.

Brown AM et al. (2003). J Physiol 549.2, 501-512.
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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C43

A membrane-targetted palmitoylated peptide replicating a phosphatidylinositol-4,5-bisphosphate binding site inhibits IK(M) and modulates muscarinic receptor-mediated inhibition

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It has been suggested that IK(M) (KCNQ/K_V7) channels are regulated by phosphatidylinositol-4,5-bisphosphate (PIP_2: Suh & Hille, 2002; Zhang et al., 2003). We have used a novel approach employing N-terminal palmitoylated decapeptides (palpeptide) to study this transduction pathway (Robbins & Brown, 2003).Rat superior cervical ganglion cells (isolated from rats killed by approved Schedule 1 methods) in primary culture were superfused at 23^0C with (mM) NaCl 144, KCl 2.5, $MgCl_2$ 0.5, $CaCl_2$ 2, Hepes 5, glucose 10, pH 7.4. The amphotericin perforated variant of the patch clamp technique was used with electrodes filled with (mM) K acetate 80, KCl 30, Hepes 40, $MgCl_2$ EGTA 3, $CaCl_2$ 1, pH 7.2. Cells were clamped at -20mV and stepped to -50mV for 1 second to generate IK(M) deactivating tail currents. All peptides were synthesised by ABC, Imperial College, London, purified to >90% with RPHPLC and dissolved in DMSO at 10mM. The original peptide (H-palmitoyl-HRQKHFEKRR-$CONH_2$) is based on the putative PIP_2 binding site on KCNQ2-5 (Zhang et al, 2003) and a range of variants produced: K4M, H5C, K8M, K4MK8M, non palmitoylated and a fluorescein labelled version.The PIP_2 palpeptide inhibited IK(M) with an IC_{50} of 1.5 ± 0.2μM (n=7); the non- palmitoylated version was ineffective up to 10μM (n=6). The muscarinic receptor antagonists, atropine (1μM) and pirenzepine (0.3μM), did not reduce the effectiveness of the PIP_2 palpeptide. PIP_2 palpeptide had no effect on other potassium currents such as IK(A) and IK(DR). The fluorescein labelled version confirmed a plasma membrane localisation of the PIP_2 palpeptide. To investigate the residues likely to be involved in PIP_2 binding we used a number of mutants and assessed their potency in inhibiting IK(M). The K4M and H5C mutants showed no significant change in potency, IC_{50}s 0.7 ± 0.1μM (n=5) and 0.9 ± 0.3μM (n=5) respectively. Conversely the K8M and the double mutant K4MK8M were significantly ($p<0.05$ ANOVA) less potent on the current (IC_{50}s 3.2 ± 0.9μM (n=5) and 3.4 ± 0.5μM (n=5) respectively). All slope values were not significantly different from each other or unity. Further, low concentrations of PIP_2 palpeptide increased the sensitivity (IC_{50}) of IK(M) to oxotremorine-M evoked inhibition from 0.8 ± 0.1μM (n=6) to 0.5 ± 0.1μM (n=6) 0.2 ± 0.04μM (n=6) at 0.1 and 0.3μM PIP_2 palpeptide respectively.We suggest that the PIP_2 palpeptide is effectively buffering PIP_2 levels in the membrane so reducing IK(M) amplitude and increasing its sensitivity to muscarinic receptor mediated inhibition. Furthermore the lysine residue near the C-terminal end of the palpeptide seems to be important for PIP_2 binding.

Robbins J & Brown DA (2003) Soc. Neuroscience Abstr. 545.1.
Suh B-C & Hille B (2002) Neuron 35: 507.
Zhang H *et al.* (2003) Neuron 37: 963.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C44

Denervation-like properties of innervated muscle fibres in the R6/2 transgenic mouse model of Huntington's Disease

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The R6/2 mouse line is transgenic for the first exon of the human huntingtin gene with an expanded (>150) CAG repeat. R6/2 mice develop a complex progressive neurological phenotype from around 3-4 weeks of age, that ultimately involves severe motor and cognitive dysfunction. The mice lose weight from 8 weeks of age and die of unknown causes between 16 and 18 weeks of age. From about 10 weeks of age, skeletal muscles begin to show conspicuous atrophy and ubiquitinated inclusions in myonuclei. In the present study we asked whether the progressive muscle atrophy is a consequence of progressive disuse or denervation. Behavioural observations, including measurements of forelimb grip strength, indicated that most symptomatic R6/2 mice were motivated and capable of sustained use of voluntary muscle. This was confirmed by sustained isometric tetanic tension measured in isolated hindlimb muscles after killing of the animals by cervical dislocation (in accordance with Home Office Schedule 1). Intracellular recordings from isolated flexor digitorum brevis (FDB) muscles showed robust evoked synaptic transmission at virtually all neuromuscular junctions: fewer than 10% of fibres failed to respond to nerve stimulation in R6/2 mice aged over 15 weeks. As expected from the degree of muscle atrophy, FDB muscle fibre input resistance and MEPP amplitude were significantly increased, by about two fold ($P<0.01$ in both cases; Mann-Whitney test). Resting membrane potentials became significantly less negative than normal as the muscle atrophy progressed with age, and membrane time constants were also prolonged ($P<0.01$; Mann-Whitney test). Immunocytochemistry and confocal fluorescence microscopy showed that virtually all neuromuscular junctions were normally innervated, and there were few discernible abnormalities in organisation of either axons, myelin, nerve terminals, terminal Schwann cells, ACh receptors or ACh esterase. However, although innervated, most atrophic R6/2 muscles were resistant to block by the muscle NaV1.4 channel antagonist μ-conotoxin GIIIB. They also produced contractures in response to bath-applied ACh, or to ACh applied iontophoretically to extrajunctional muscle fibre membranes. Junctional fibroblasts were also spread over the muscle fibre surfaces. We suggest that in skeletal muscle, transgenic expression of protein with an expanded polyglutamine repeat induces an innervation-independent and activity-independent switch from the normal physiological state to one resembling that induced by prolonged paralysis or muscle denervation.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C45

TRPM2 modulates oxidative stress-induced cell death in endogenously expressing rat striatal neurons and insulinoma cells

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TRPM2 is nonselective cation channel that acts as an oxidative stress sensor in cells. A splice variant of TRPM2 (TRPM2-S) modulates TRPM2 activity when coexpressed in HEK293 cells (Zhang et al, 2003). The aim of the present study was to assess the role of TRPM2 in oxidative-stress induced calcium influx and cell death in endogenously expressing cells following exposure to hydrogen peroxide (H_2O_2). Rat insulinoma CRI-G1 cells were seeded into 24-well plates at 50.000 cells per well and incubated at 37 ºC / 5% CO_2 overnight. Cells were transfected with TRPM2-S or vector, and after 48h cell death was assesssed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reduction assay. A statistically significant reduction ($F(1,46)= 9.277$, $p=0.0038$) in oxidative stress-mediated cell death was observed after 18h post-exposure to 100 μM H_2O_2 in CRI-G1 cells transfected with TRPM2-S (n=12-14 replicates pooled from three different experiments).Primary cultures of striatal neurons were prepared from humanely sacrified E18 Sprague-Dawley rat embryos. Dissociated neurons were transfected and seeded into 96-well plates at 40-60.000 cells per well and incubated at 37 ºC / 5% CO_2 for 48 h. Fluorimetric imaging of Fluo-3 AM loaded cells was used to determine intracellular free Ca^{2+} ion concentration changes. Fluorimetric imaging of Sytox Green was used to determine loss of membrane integrity as indicative of cell death. In the presence of TRPM2-S, both Ca^{2+} influx and cell death following exposure to 1 μM-3 mM H_2O_2 were inhibited in a statistically significant manner ($F(1,44)=97.40$, $p<0.0001$; and $F(1,38)=158.2$, $p<0.0001$: for Ca^{2+} influx and cell death, respectively). In summary, we have demonstrated that TRPM2 function contributes to oxidative stress-induced cell death in striatal neurons. Furthermore, our data conclusively demonstrate TRPM2 role on oxidative stress-induced cell death in pancreatic beta cells, as previously suggested by Herson et al (1999) and Hara et al (2002). Thus, TRPM2 plays a key role on calcium homeostasis and regulation of cell death following oxidative stress insult in endogenous expressing systems.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.