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PHARMACOLOGICAL AND MOLECULAR CHARACTERIZATION OF ATP-SENSITIVE K^+ CONDUCTANCES IN CART AND NPY/AgRP EXPRESSING NEURONS OF THE HYPOTHALAMIC ARCUATE NUCLEUS

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Abstract—The role of hypothalamic ATP-sensitive potassium channels in the maintenance of energy homeostasis has been extensively explored. However, how these channels are incorporated into the neuronal networks of the arcuate nucleus remains unclear. Whole-cell patch-clamp recordings from rat arcuate nucleus neurons in hypothalamic slice preparations revealed widespread expression of functional ATP-sensitive potassium channels within the nucleus. ATP-sensitive potassium channels were expressed in orexigenic neuropeptide Y/agouti-related protein (NPY/AgRP) and ghrelin-sensitive neurons and in anorexigenic cocaine-and-amphetamine regulated transcript (CART) neurons. In 70% of the arcuate nucleus neurons recorded, exposure to glucose-free bathing medium induced inhibition of electrical excitability, the response being characterized by membrane hyperpolarization, a reduction in neuronal input resistance and a reversal potential consistent with opening of potassium channels. These effects were reversible upon re-introduction of glucose to the bathing medium or upon exposure to the ATP-sensitive potassium channel blockers tolbutamide or glibenclamide. The potassium channel opener diazoxide, but not pinacidil, also induced a tolbutamide and glibenclamide-sensitive inhibition of electrical excitability. Single-cell reverse transcription–polymerase chain reaction revealed expression of mRNA for sulfonylurea receptor 1 but not sulfonylurea receptor 2 subunits of ATP-sensitive potassium channels. Thus, rat arcuate nucleus neurons, including those involved in functionally antagonistic orexigenic and anorexigenic pathways express functional ATP-sensitive potassium channels which include sulfonylurea receptor 1 subunits. These data indicate a crucial role for these ion channels in central sensing of metabolic and energy status. However, further studies are needed to clarify the differential roles of these channels, the organization of signaling pathways that regulate them and how they operate in functionally opposing cell types.

Key words: hypothalamic slice, whole-cell patch-clamp, energy balance, glucose.

The hypothalamic arcuate nucleus (ARC) plays a key role in the maintenance of energy homeostasis by integrating metabolic signals of central and peripheral origin (Cone et al., 2001). The ARC is considered circumventricular and as such neurons in this nucleus are well suited to detect circulating factors including glucose, insulin and leptin and formulate an appropriate output including changes in electrical excitability (Spanswick et al., 1997, 2000; Cowley et al., 2001; van den Top et al., 2004). ATP-sensitive potassium channels (K_{ATP}) in ARC and ventromedial nucleus of the hypothalamus (VMH) neurons are a target through which these circulating factors convey their metabolic message to the CNS (Ashford et al., 1990a; Spanswick et al., 1997, 2000). Moreover, ARC neurons expressing K_{ATP} channels are essential components of the central mechanisms controlling the effect of insulin on hepatic glucose production (Obici et al., 2002a). Classically, K_{ATP} channels have been suggested to be a key substrate by which the brain senses changes in glucose levels. Specialized neurons in the CNS sense extracellular glucose concentrations and modulate their electrical activity appropriately (Anand et al., 1964; Oomura et al., 1964). Neurons that increase their electrical activity in response to increasing extracellular glucose, so-called glucose responsive neurons, are present in the ARC (Oomura et al., 1969; Spanswick et al., 1997). However, at present a detailed pharmacological and molecular characterization of K_{ATP} channels expressed in ARC neurons is lacking.

Hypoglycemia and increased circulating leptin/insulin concentrations are, *in vivo*, indicative of opposing physiological states while both conditions inhibit ARC neurons through the opening of K_{ATP} channels (Spanswick et al., 1997, 2000). Therefore, in order to facilitate these polar effects through a common pathway, it seems likely that K_{ATP} channels are expressed on anabolic as well as catabolic neurons. Accordingly, we hypothesized that K_{ATP} channels are expressed by multiple ARC neurons in-

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Abbreviations: ACSF, artificial cerebrospinal fluid; AgRP, agouti-related protein; ARC, hypothalamic arcuate nucleus; CART, cocaine-and-amphetamine-regulated transcript; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; Hepes, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid); K_{ATP} channels, ATP-sensitive potassium channels; KCO, potassium channel opener; KIR, inward rectifying potassium channel; Na-ATP, ATP disodium salt; NGS, normal goat serum; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; RT-PCR, reverse transcription–polymerase chain reaction; SUR1, sulfonylurea receptor 1; SUR2, sulfonylurea receptor 2; TBS-T, Tris-buffered saline containing 1% Triton X-100; TTX, tetrodotoxin; VMH, ventromedial nucleus of the hypothalamus.

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cluding the parallel but functionally antagonistic neuronal populations expressing the orexigenic neuropeptide Y/agouti-related protein (NPY/AgRP) or the anorexigenic pro-opiomelanocortin/cocaine-and-amphetamine-regulated transcript (POMC/CART) (Ollmann et al., 1997; Broberger et al., 1998; Bagnol et al., 1999). Here we describe for the first time the pharmacological and molecular properties of functional K_{ATP} channels in the ARC. Moreover, we show that K_{ATP} channels are expressed by the majority of ARC neurons (70%) including NPY/AgRP and CART neurons. A preliminary account of these findings has appeared in abstract form (Lyons et al., 2004).

EXPERIMENTAL PROCEDURES

Slice preparation

Male Wistar rats from our institutional breeding colonies, aged 4–12 weeks, were humanely killed by cervical dislocation and subsequently decapitated in agreement with UK national guidelines and in accordance with the principles and guidelines of the Canadian Council for Animal Care. Every effort was made to minimize the number of animals used and their suffering. The brain was rapidly removed and coronal 300–400 μm slices containing the ARC cut using a Vibratome (Intracel, Series 1000, Royston, UK). Slices were maintained at room temperature in oxygenated artificial cerebrospinal fluid (ACSF) for at least an hour prior to recording.

Recording and analysis

For recording, slices were transferred to a custom-made recording chamber and continuously perfused at room temperature with ACSF of the following composition (in mM): 127.0 NaCl, 1.9 KCl, 1.2 KH_2PO_4, 26.0 $NaHCO_3$, 10.0 D-glucose, 1.3 $MgCl_2$, 2.4 $CaCl_2$, equilibrated with 95% O_2, 5% CO_2, pH 7.3–7.4. Glucose-free ASCF was prepared by substituting equimolar concentrations of D-glucose with D-mannitol. Recordings were obtained from neurons located in the ARC using axopatch-1D amplifiers (Axon Instruments, Foster City, CA, USA). Patch pipettes were pulled using a horizontal puller (Sutter Instrument Co., Novato, CA, USA) from thin-walled borosilicate glass (Harvard Apparatus LTD, Edenbridge, Kent, UK) with resistances between 4 and 7 MΩ when filled with electrode solution. The pipette solution comprised (mM); 140 Kgluconate, 10 Hepes, 10 KCl, 1.0 EGTA, 2 ATP disodium salt (Na-ATP) and had a pH of 7.3. For experiments performed in the absence of intracellular ATP, Na-ATP was omitted. Whole cell recording in conjunction with single cell reverse transcription–polymerase chain reaction (RT-PCR) analyses of the neuronal cytoplasm was performed under visual control using infrared video microscopy (Lee et al., 1998). The intracellular solution used for these experiments comprised (mM); 120 Kgluconate, 10 NaCl, 2 $MgCl_2$, 0.5 K_2EGTA, 10 Hepes, 4 Na_2ATP, 0.3 Na_2GTP, pH 7.2. Current and voltage data were displayed on a digital oscilloscope (Gould DSO1602) and stored on DAT-tape (Biological DTR-1204, Intracel) with currents filtered at 1 kHz using a four-pole low-pass Bessel filter. For data analysis signals were digitized at 2–10 kHz, stored and analyzed on a personal computer running pClamp8 software (Axon Instruments). In voltage-clamp experiments neurons were held at either −40 or −60 mV and the series resistance compensated by 60–80%.

Drugs and solutions

Drugs used were diazoxide, glibenclamide pinacidil and tolbutamide (Sigma, Gillingham, Dorset, UK), ghrelin and orexin-A (Bachem, St. Helens, UK) and tetrodotoxin (TTX) from Alomone Laboratories (Jerusalem, Israel). Drugs dissolved in dimethyl sulfoxide (DMSO) were diluted in ACSF with a maximal concentration of 0.5% DMSO. Pinacidil was made up in ethanol resulting in a final concentration of 0.1% ethanol. Solvents did not induce effects on the electrical properties of ARC neurons (n=3). Stock solutions of other drugs were made in distilled water prior to dilution in ACSF. All drugs were bath-applied from reservoirs connected to the ACSF flow line by manually operable three-way valves.

Immunocytochemistry

Recordings were obtained utilizing an intracellular solution containing the fluorescent dye Alexa 594 (50–100 μM). Post-recording slices were fixed overnight in a 0.1 M phosphate buffer containing 4% paraformaldehyde (pH 7.4). Subsequently, slices were incubated in Tris-buffered saline containing 1% Triton X-100 (TBS-T) and 4% normal goat serum (NGS) for 1 h. Slices were incubated overnight at 4 °C in rabbit anti-CART (Phoenix Pharmaceuticals, Belmont, CA, USA, 1:1000 in TBS-T) in the presence of 2% NGS and rinsed prior to incubation in goat anti-rabbit conjugated to CY2 (Jackson ImmunoResearch Laboratories, Bar Harbor, ME, USA; 1:200). Finally, slices were rinsed and mounted for confocal microscopy using Prolong anti-fade (Molecular Probes, Eugene, OR, USA).

Single cell RT-PCR

Analysis of gene expression in single neurons has been described previously (Dixon et al., 1998; Lee et al., 1998). Briefly, somatic cytoplasm from single neurons was aspirated into the recording electrode and subsequently forced into a microtube and the RNA reverse transcribed using an anchored oligo dT primer and 200 U of MMLV reverse transcriptase (BRL) according to the manufacturer's recommendations. After 60 min at 37 °C the cDNA was stored frozen at −20 °C prior to processing. After amplification of the cDNA using *Taq* polymerase, the expression of specific genes was measured using primers designed to amplify products of between 120 and 250 base pairs in length, close to the 3′ ends of the mRNA transcripts.

The primers used were; sulfonylurea receptor 1 (SUR1; accession number L40624) forward primer (bases 4824-4842): TGAAGCAACTGCCTCCATC; reverse primer (bases 5005-4987): GAAGCTTTTCCGGCTTGTC; sulfonylurea receptor 2 (SUR2; accession number D83598), forward primer (bases 4853-4872): ACCTGCTCCAGCACAAGAAT: reverse primer (bases 4997-4976): TCTCTTCATCACAATGACCAGG; alpha tubulin (accession number V01226): forward primer (bases 300-318): CACTGGTACGTGGGTGAGG; reverse primer (bases 471-450): TTTGACATGATACAGGGACTGC; cytochrome oxidase (accession number L48209): forward primer (bases 96-116): ATCACCATTGGGCTCACTTC; reverse primer (bases 281-264): ATCCCAGGGTAAGCCAGC; synaptotagmin 1 (accession number X52772): forward primer (bases 4022-4042): AGGGGCTTTCCTATCTAAGGG; reverse primer (bases 4223-4204): GTTGGCAGTGTTGCAAGAGA. These PCR reactions were run for 45 cycles of 92 °C (denaturing, 2.5 min), 55 °C (annealing, 1.5 min) and 72 °C (extension, 1 min), followed by a final extension of 10 min at 72 °C. The PCR products were separated on 2.5% agarose gels and the product sizes were as predicted from sequences. Confirmation of the sensitivity and specificity of PCR reactions was achieved as described previously (Dixon et al., 1998). The nature of the SUR1 product was confirmed by sequencing the amplified PCR products.

Statistical analyses

All values are expressed as mean±S.E.M. The two-tailed Student's *t*-test was utilized for all statistical analysis in the paired and