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Properties and Physiological Function of Ca^{2+} Dependent K^{+} Currents in Uniglomerular Olfactory Projection Neurons

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ABSTRACT

Ca^{2+}-activated potassium currents ($I_{K(Ca)}$) are an important link between the intracellular signaling system and the membrane potential, which shapes intrinsic electrophysiological properties. To better understand the ionic mechanisms that mediate intrinsic firing properties of olfactory uniglomerular projection neurons (uPNs), we used whole-cell patch clamp recordings in an intact, adult brain preparation of the male cockroach *Periplaneta americana* to analyze $I_{K(Ca)}$. In the insect brain uPNs form the principal pathway from the antennal lobe to the protocerebrum, where centers for multimodal sensory processing and learning are located. In uPNs the activation of $I_{K(Ca)}$ was clearly voltage and Ca^{2+} dependent. Thus, under physiological conditions $I_{K(Ca)}$ is strongly dependent on the Ca^{2+} influx kinetics and on the membrane potential. The biophysical characterization suggests that $I_{K(Ca)}$ is generated by BK channels. An SK channel generated current could not be detected. $I_{K(Ca)}$ was sensitive to charybdotoxin and iberiotoxin but not to apamin. The functional role of $I_{K(Ca)}$ was analyzed in occlusion experiments under current clamp, in which portions of $I_{K(Ca)}$ were blocked by charybdotoxin or iberiotoxin. Blockade of $I_{K(Ca)}$ showed that $I_{K(Ca)}$ contributes significantly to the intrinsic electrophysiological properties such as the action potential waveform and membrane excitability.

KEYWORDS: Antennal lobe, chemosensory, glomerulus, olfaction, *Periplaneta americana*

INTRODUCTION

In insects odors are detected by antennal sensory neurons and are initially processed in the first synaptic relay, the antennal lobe (AL), which is the functional equivalent of the mammalian olfactory bulb (reviewed in Bargmann 2006; Davis 2004; Galizia and Rössler 2010; Hildebrand and Shepherd 1997; Strausfeld and Hildebrand 1999; Vosshall and Stocker 2007; Wilson and Mainen 2006). The integrated olfactory information is then conveyed to higher order processing centers in the protocerebrum by antennal lobe projection neurons (PNs), the analog of the mammalian mitral/tufted cells (Su et al. 2009). The most prominent and most studied group of PNs are the uniglomerular projection neurons (uPNs), whose morphology and response properties are well described in various insect species. uPNs are considered the only cholinergic input to the mushroom body calyx (Fusca et al. 2013; Yasuyama et al. 1995, 2002). Typically, each uPN receives synaptic input from olfactory sensory neurons and local interneurons in a single AL glomerulus. Each uPN sends an axonal projection into the mushroom body calyx and/ or the lateral horn where it terminates with synaptic boutons. The tuning profiles and odor responses of uPNs are determined by synaptic inputs from the sensory and local interneurons together with their intrinsic electrophysiological properties. A given uPN can respond to odorants of many different chemical classes, typically with odor specific elaborate patterns of excitation and spiking that could also include periods of inhibition (Broome et al. 2006; Christensen et al. 1998; Kanzaki et al. 1989; Lemon and Getz 1998; Mazor and Laurent 2005; Stopfer et al. 2003; Watanabe et al. 2012; Wilson et al. 2004).

While the odor response profiles of uPNs are generally well documented we have only limited information about the intrinsic firing properties and the cellular mechanisms that determine them. However, in depth knowledge of the cell-type specific intrinsic electrophysiological properties and the membrane conductances that mediate them is an important prerequisite towards a detailed understanding of the cellular basis of olfactory information processing. Here we analyzed the Ca^{2+} dependent K^+ outward currents ($I_{K(Ca)}$) of uPNs, which play a crucial role in controlling action potential waveform and neuronal

84 excitability (Adelman et al. 2012; Berkefeld et al. 2010; Faber and Sah 2003; Ghatta et al.

85 2006; Greffrath et al. 2004; Vergara et al. 1998).

86   Since Ca^{2+} is an important messenger of the intracellular signaling system, Ca^{2+}

87 dependent ion channels constitute an important link between the intracellular status of

88 neurons and their membrane potential. Vertebrates and invertebrates express several types

89 of Ca^{2+} dependent potassium (K_{Ca}) channels in most neuron types, where they mediate Ca^{2+}

90 dependent hyperpolarization and contribute to the resting membrane potential, spike wave

91 form, and spike frequency. Historically, three main types of K_{Ca} channels have been

92 categorized by their biophysical and pharmacological properties: 1) small conductance (SK)-,

93 2) intermediate conductance (IK)-, and 3) big conductance (BK or maxi K) channels

94 (Adelman et al. 2012; Berkefeld et al. 2010; Faber and Sah 2003). Molecular cloning helped

95 to reveal sequence relationships and made structure/function studies possible (Sah and

96 Faber 2002; Stocker 2004). The SK family has three members ($K_{Ca}2.1$-2.3) and is closely

97 related with the IK channel ($K_{Ca}3.1$). These channels are activated by cytosolic Ca^{2+} and are

98 not voltage-sensitive. Their activation by intracellular Ca^{2+} is mediated by calmodulin that is

99 constitutively bound to the C-terminus. In contrast, the BK channel ($K_{Ca}1.1$) is activated by

100 both voltage and cytosolic Ca^{2+}. The activation by Ca^{2+} is not calmodulin dependent but

101 mediated directly by cytoplasmic Ca^{2+} binding sites (Berkefeld et al. 2010; Fakler and

102 Adelman 2008; Grunnet and Kaufmann 2004; Lee and Cui 2010; Salkoff et al. 2006).

103   Here we aimed to characterize Ca^{2+} dependent K^+ currents $I_{K(Ca)}$ of uPNs in the AL of

104 the cockroach *Periplaneta americana*. First, we characterized the general functional

105 properties of $I_{K(Ca)}$ in uPNs with whole-cell voltage clamp recordings in an intact brain

106 preparation. Second, we used occlusion experiments, in which portions of $I_{K(Ca)}$ were

107 pharmacologically blocked, to better understand the functional role of $I_{K(Ca)}$ in shaping the

108 electrophysiological properties of uPNs.

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METHODS

Preparation

P. americana were reared in crowded colonies at 27 °C under a 12 : 12 h light/ dark photoperiod regimen, on a diet of dry rodent food, oatmeal, and water. All experiments were performed with adult males. Chemicals, unless stated otherwise, were obtained from Applichem (Darmstadt, Germany) or Sigma-Aldrich (Taufkirchen, Germany) and were of 'pro analysis' grade. The intact brain preparation was based on an approach described previously (Husch et al. 2009; Kloppenburg et al. 1999a), in which the entire olfactory network was left intact. Animals were anaesthetized by CO_2, placed in a custom built holder, and the head was immobilized with tape (Tesa ExtraPower Gewebeband, Tesa, Hamburg, Germany). The head capsule was opened by cutting a window between the two compound eyes at the bases of the antennae. The brain was dissected in extracellular saline (see below) and pinned in a Sylgard-coated (Dow Corning Corp., Midland, Michigan, USA) recording chamber. To minimize synaptic input to AL neurons, the antennae were removed. To gain access to the recording site and to facilitate the penetration of pharmacological agents into the tissue, we desheathed parts of the AL using fine forceps. Some preparations were also enzymatically treated with a mixture of papain (0.3 mg·ml^{-1}, P4762, Sigma) and L-cysteine (1 mg·ml^{-1}, 30090, Fluka) dissolved in 'normal' saline (~ 2 min, 37°C). For electrophysiological recordings, the somata of the AL neurons were visualized with a fixed stage upright microscope (BX51WI, Olympus, Hamburg, Germany) using a water-immersion objective (UMPLFL, 40x, 0.8 numerical aperture [NA], 3.3 mm working distance, Olympus) and infrared differential interference contrast optics (Dodt and Zieglgänsberger, 1994).

Whole-cell recordings

Whole-cell recordings were performed at room temperature (~24°C; RT) following the methods described by Hamill *et al.* (1981). Electrodes with tip resistances between 2.5-3.5 MΩ were fashioned from borosilicate glass (inner diameter 0.86 mm, outer diameter 1.5 mm, GB150-8P, Science Products, Hofheim, Germany) with a temperature-controlled pipette

139   puller (PIP5, HEKA-Elektronik, Lambrecht, Germany). If not stated otherwise, the recording

140   pipettes were filled with intracellular saline solution containing (in mM): 190 K-aspartate, 10

141   NaCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EDTA or EGTA, and adjusted to pH 7.2 with KOH,

142   resulting in an osmolarity of ~415 mOsm and the cells were superfused constantly with

143   extracellular saline solution containing (in mM): 185 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10

144   HEPES, 35 D-glucose. The solution was adjusted to pH 7.2 with NaOH and to 430 mOsm

145   with glucose.

146   In the experiments in which it was crucial to quantitatively clamp the free intracellular

147   Ca^{2+} to different levels, the solutions were made up with calculated Ca^{2+}/ Ca^{2+} chelator ratios

148   to reach the desired concentration range, while Ca^{2+} influx via voltage gated Ca^{2+} channels

149   was blocked by $CdCl_2$ (5 x 10^{-4} M). In these experiments the stated free intracellular Ca^{2+}

150   concentrations are measured using a Ca^{2+} electrode. The calibration of the Ca^{2+} electrode

151   was performed as described in Kay et al. 2008.

152   Whole-cell voltage- and current clamp recordings were made with an EPC9 patch

153   clamp amplifier (HEKA-Elektronik) that was controlled by the program Patch Master (version

154   2.52, HEKA-Elektronik) running under Windows. The electrophysiological data were sampled

155   at 20 kHz and low pass filtered at 2 kHz with a 4-pole Bessel-Filter. Compensation of the

156   offset potential and capacitive currents was performed using the 'automatic mode' of the

157   EPC9 amplifier. Whole-cell capacitance was determined by using the capacitance

158   compensation (C-slow) of the amplifier. The liquid junction potential between intracellular and

159   extracellular solution of 15.6 mV was calculated with Patcher's-Power-Tools plug-in

160   (http://www3.mpibpc.mpg.de/groups/neher/index.php?page=aboutppt)   for   Igor   Pro

161   (Wavemetrics, Portland, Oregon) and was also compensated. To remove uncompensated

162   leakage and capacitive currents, a p/6 protocol was used (Armstrong and Bezanilla 1974).

163   Voltage errors due to series resistance (R_S) were minimized using the Rs-compensation of

164   the EPC9. R_S was compensated between 50% and 90% with a time constant (τ) of 100 µs.

165   To reduce synaptic input during the current clamp recordings, 10 µM CNQX (6-cyano-7-

166   nitroquinoxaline-2,3-dione,   C127,   Sigma-Aldrich),   50   µM   DL-AP5   (DL-2-amino-5-

167   phosphonopentanoic acid, BN0086, Biotrend), 100 µM PTX (picrotoxin, P1675, Sigma

168   Aldrich), 5 µM CGP-54626 hydrochloride (BN0597, Biotrend), and 100 µM mecamylamine

169   hydrochloride (M9020, Sigma-Aldrich) or 50 µM tubocurarine (93750, Sigma Aldrich)) was

170   added to the extracellular saline. These chemicals block glutamate-, $GABA_A$-, $GABA_B$-, and

171   nicotinic acetylcholine receptors, respectively. Initially we used tubocurarine to block nicotinic

172   synaptic transmission. To avoid potential interactions with SK channels it was replaced by

173   mecamylamine, which has been shown to block nicotinic transmission in the antennal lobe of

174   *P. americana* (Warren and Kloppenburg 2014).

175   The protocols for each set of the voltage clamp experiments are given in RESULTS.

176   Current density was calculated as the ratio between current and whole-cell capacitance. To

177   analyze changes in electrophysiological properties during the occlusion experiments, series

178   of hyperpolarizing and depolarizing current pulses (500 ms) were applied under current

179   clamp from a membrane potential of -60 mV. Cell input resistance was determined with

180   series of small hyperpolarizing current pulses (20 pA - 80 pA; 20 pA increments) and the

181   action potential waveform was analyzed during depolarizing current pulses (30 pA – 210 pA;

182   30 pA increments). Action potential related parameters were compared from selected spike

183   trains, which had similar instantaneous frequencies (16Hz - 18Hz) before and during blocker

184   application. Blocker effects on excitability (latency to first spike and number of spikes) were

185   analyzed during current pulses (with the same amplitude before and during blocker

186   application) with a 'control latency' of at least 100 ms.

187

188   *Current isolation*

189        $I_{K(Ca)}$ was isolated using a combination of pharmacological blockers, appropriate

190   holding potential, and digital current subtraction protocols, based on protocols that have been

191   effective in insect preparations (Heidel and Pflüger 2006; Husch et al. 2009; Kloppenburg

192   and Hörner 1998; Kloppenburg et al. 1999b; Lee et al. 2014; Mercer et al. 1995, 1996; Pym

193   et al. 2006; Ryglewski and Duch 2009; Schäfer et al. 1994). Voltage-activated transient Na^+

194   currents were blocked by tetrodotoxin (10^{-6} M, TTX, T-550, Alomone, Jerusalem, Israel). The

195   transient K^+ current (I_A) was blocked with 4-aminopyridine (4×10^{-3} M, 4-AP, A78403, Sigma-

196   Aldrich). Ca^{2+} currents were blocked by $CdCl_2$ (5×10^{-4} M). $I_{K(Ca)}$ could be eliminated indirectly

197   by blocking the Ca^{2+} currents by $CdCl_2$. To compensate for changes in osmolarity, the

198   glucose concentration was appropriately adjusted. Details of recording solutions and voltage

199   protocols for each set of experiments are given in the Results.

200

201   *Recordings from dopaminergic neurons of the mouse substantia nigra*

202   All animal procedures were conducted in compliance with protocols approved by local

203   government authorities (Bezirksregierung Köln, Cologne, Germany) and were in accordance

204   with National Institutes of Health guidelines. Experiments were performed on brain slices

205   from 12 weeks old female and male C57/Bl6 mice. Dopaminergic neurons of the substantia

206   nigra were recorded in the whole-cell configuration following previously described

207   experimental procedures (Hess et al. 2013; Könner et al. 2011). The extracellular saline

208   contained (in mM): 125 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 10 HEPES, 2 $MgCl_2$, 5 glucose, 21

209     $NaHCO_3$, 2 $CaCl_2$, adjusted to pH 7.2 with NaOH resulting in an osmolarity of ~310 mOsm.

210   The patch pipettes were filled with (in mM): 128 K-D-gluconat, 10 KCl, 10 HEPES, 2 $MgCl_2$,

211   0.1 EGTA, 3 ATP, 0.3 GTP, adjusted to pH 7.3 with KOH resulting in an osmolarity of ~300

212   mOsm.

213

214   *Data analysis*

215   Calculated values are given as mean ± standard deviation. For IC_{50} values the 95%

216   confidence intervals are presented in parentheses. When comparing significance between

217   two groups of data a paired two-tailed *t* test was used, when comparing three groups of data

218   ANOVA was used with a Tukey's *post-hoc* test. A significance level of 0.05 was accepted for

219   all tests. Statistical significance is represented by one, two, or three asterisks displayed

220   between groups of data in the figures, which represent a *p*-value ≤ 0.05, ≤ 0.01, and ≤ 0.001,

221 respectively. We used the software Igor Pro 6.0.1 (Wavemetrics, including the Patcher's

222 PowerTools plug-in) for analysis of electrophysiological data and GraphPad Prism 5

223 (GraphPad Software, San Diego, CA) for statistical analysis.

224

225 *Single cell labeling*

226 To label individual cells, 1% (w/v) biocytin (B4261, Sigma-Aldrich, Taufkirchen,

227 Germany) was added to the pipette solution. After the electrophysiological recordings, the

228 brains were fixed in Roti-Histofix (2-3 h, TR; P0873, Carl Roth, Karlsruhe, Germany) at room

229 temperature (RT). Subsequently the brains were rinsed in phosphate buffered saline (PBS, 3

230 x 20 min, RT). All brains were processed as whole-mounts. To facilitate the streptavidin

231 penetration, the brains were treated with a commercially available collagenase/dispase

232 mixture (1 mg $\cdot$ ml^{-1}, 269638, Roche Diagnostics, Mannheim, Germany) and hyaluronidase (1

233 mg $\cdot$ ml^{-1}, H3506, Sigma-Aldrich, Taufkirchen, Germany) in PBS (20 min, 37°C), rinsed in

234 PBS (3 x 10 min, 4°C), incubated in PBS containing 1% (w/v) Triton X-100 (A1388,

235 Applichem, Darmstadt, Germany) and 10% normal goat serum (40 min, RT, S-1000, Linaris,

236 Wertheim, Germany) and rinsed again in PBS (3 x 10 min, 4°C). Afterwards, the brains were

237 incubated in Alexa Fluor 633 (Alexa 633) conjugated streptavidin (1:400, 1-2 days, 4°C,

238 S21375, Life technologies, Darmstadt, Germany) that was dissolved in PBS containing 10%

239 (v/v) normal goat serum. Brains were rinsed in PBS (3 x 10 min, 4°C), dehydrated, and

240 cleared and mounted in methylsalicylate (M6752, Sigma-Aldrich, Taufkirchen, Germany).

241 The fluorescence images were captured with a confocal microscope (LSM 510, Carl

242 Zeiss, Göttingen, Germany) equipped with Plan-Neofluar 10x (0.3 NA), Plan-Apochromat 20x

243 (0.75 NA), and Plan-Apochromat 63x (1.4 NA Oil) objectives. Streptavidin-Alexa 633 was

244 excited with a He-Ne Laser at 633 nm. Emission of Alexa 633 was collected through a 650

245 nm long-pass filter. Scaling, contrast and brightness adjustment, and z-projections were

246 performed with ImageJ (version 1.47v) and the WCIF plugin bundle

247 (www.uhnresearch.ca/facilities/wcif/). For overview images overlapping image stacks were

248   merged in Photoshop CS5 (Adobe Systems Incorporated, San Jose, CA). The final figures

249   were prepared with Photoshop CS5 and Illustrator CS5 (Adobe Systems).

250

251   **RESULTS**

252   In most neurons Ca^{2+} dependent K^+ currents are crucial in shaping their intrinsic firing

253   properties. Here we aimed to characterize $I_{K(Ca)}$ in uPNs, which are key components of the

254   insect olfactory system. The study was performed in two steps. First we characterized the

255   functional properties of $I_{K(Ca)}$ in uPNs with whole-cell voltage clamp recordings in an intact

256   brain preparation of male *P. americana*. Second, we used occlusion experiments, in which

257   portions of $I_{K(Ca)}$ were pharmacologically blocked, to better understand the functional role of

258    $I_{K(Ca)}$ in shaping the electrophysiological properties of uPNs.

259   The recordings were performed under visual control from cell bodies in the ventral

260   portion of the ventrolateral somata group (VSG) (Distler 1990; Fusca et al. 2013, 2015;

261   Husch et al. 2009) in an intact brain preparation. These somata belong mostly to a relatively

262   homogeneous population of uPNs. During the recordings the neurons were labelled with

263   biocytin/streptavidin to confirm the identity of the recorded neurons. Each of the recorded

264   uPNs had arborizations in a single glomerulus and sent its axon through the medial antennal

265   lobe tract (mALT) to the protocerebrum (Malun et al. 1993; Nishino et al. 2003, 2010;

266   Watanabe et al. 2010, 2012), innervating the mushroom body calyces and the lateral horn

267   (Fig. 1 *A*1-*A*4). Since we did not find any systematic differences in the electrophysiological

268   properties that would justify a separation into distinct uPN classes, we pooled the data for the

269   analysis. It has yet to be shown if other PN types have similar properties.

270

271   *Isolation of $I_{K(Ca)}$.*

272          $I_{K(Ca)}$ was isolated using a combination of pharmacological tools, appropriate holding

273   potentials, and current subtraction protocols (Fig. 1 *B-E*). The preparation was superfused

274   with saline containing 10^{-6} M TTX and 4 x 10^{-3} 4-AP. If not stated otherwise the Ca^{2+}

275   concentration during the voltage clamp experiments was 1 mM and the neurons were held at

276   -60 mV between voltage protocols. As a result of these current isolation protocols the current

277   profiles were clearly dominated by $I_{K(Ca)}$, but they may still have included small residuals of

278   other currents. The current waveforms did not indicate significant voltage control problems

279   (e.g. no delay of current onset, no jumps in the voltage-dependence), suggesting that they

280   originated mostly from well voltage-clamped regions. Given the long, thin primary neurite, we

281   assume that a major portion or all of the currents we measured originated from the cell

282   bodies.

283   To determine the current-voltage (*I-V*) relation, two series of 300 ms voltage pulses

284   were delivered in 10 mV increments between -60 and +60 mV (Fig. 1 *B*). During the second

285   series the extracellular saline additionally contained 5×10^{-4} M $CdCl_2$ (Fig. 1 *C*), which

286   completely abolished voltage-activated Ca^{2+} currents (Husch et al. 2008). Accordingly, under

287   Cd^{2+} the peak current was drastically reduced and the inverted U-shape of the *I-V* relation

288   was eliminated. The difference between the 'untreated' and the 'Cd^{2+}-treated' current series

289   was defined as $I_{K(Ca)}$ (Fig. 1 *D*). When defined like this, $I_{K(Ca)}$ contains I_{Ca}. Since the inward I_{Ca}

290   is very small compared to the whole current (~5%; Fig. 7) it does not affect the conclusions of

291   the experiments. $I_{K(Ca)}$ activated with voltage steps more depolarized than -40 mV and

292   consisted of a fast and slowly decaying component. This decay kinetics during a sustained

293   voltage pulse reflected the inactivation of I_{Ca} (Husch et al. 2009). $I_{K(Ca)}$ had a pronounced

294   inverted U-shaped *I-V* relation (Fig. 1 *E*; Fig. 2 *A-C*) which mirrored the *I-V* relation of I_{Ca}

295   (Husch et al. 2009). On average the maximal peak current was 21.3 ± 6.1 nA (n=7). Given a

296   mean whole-cell capacitance of 17.9 ± 3.7 pF, this corresponded to a mean current density

297   of 1.23 ± 0.46 nA · pF^{-1}. On average the maximal peak current was reached at 17 ± 11 mV

298   (n=7) and decreased at more depolarized voltage steps as the driving force for Ca^{2+} declined.

299   Note that these values differed from the maxima of the mean *I-V* and of the *current density-V*

300   plots, since the membrane potential at which the maximal currents occurred varied between

301   cells (Fig. 2 *A-C*). Assuming that the main charge carrier is K^+ this corresponds to a

302   conductance density of 11 ± 4.7 nS · pF^{-1} (110 ± 47 pS · μm^{-2}). Increasing the extracellular

11

303   Ca^{2+} concentration from 1 mM to 6 mM altered the *I-V* relation from an inverted U-shaped to

304   a more linearly increasing *I-V* relation (Fig. 2 *D*).

305

306   *Calcium dependence of $I_{K(Ca)}$.*

307   Our first series of experiments demonstrated that $I_{K(Ca)}$ is voltage and Ca^{2+} dependent.

308   However, the exact time course and amplitude of this outward current are complex functions

309   of membrane potential, intracellular Ca^{2+} concentration at the K_{Ca} channel, voltage and Ca^{2+}

310   dependence of I_{Ca}, and the Ca^{2+} dependence of the underlying K_{Ca} channels. Accordingly,

311   only limited conclusions about the intrinsic activation and inactivation properties can be made

312   from the shape of whole-cell currents activated by simple depolarizing voltage steps. To

313   further study the Ca^{2+} dependence of $I_{K(Ca)}$ we used two-step voltage protocols (Solaro et al.

314   1995). A test pulse depolarized the membrane to +60 mV, where virtually no voltage

315   activated Ca^{2+} influx occurs, since the membrane voltage approaches the Ca^{2+} equilibrium

316   potential. The +60 mV test pulse was preceded by depolarizing voltage pulses (Ca^{2+} loading-

317   steps) of varying amplitude (Fig. 3 *A*) or duration (Fig.3 *B*) which modified the Ca^{2+} influx and

318   thereby the intracellular Ca^{2+} concentration in the vicinity of the K_{Ca} channel. Thus, during the

319   +60 mV test pulse, $I_{K(Ca)}$ was activated a) by the strongly depolarized membrane potential

320   and b) by the Ca^{2+} resting level plus the varying amount of Ca^{2+} delivered by I_{Ca} during the

321   loading-pulse. Taking into account that I_{Ca} inactivates during a sustained voltage pulse, the

322   instantaneous Ca^{2+} influx during the loading-pulse depended a) on the potential of the

323   loading-pulse and b) on the duration of I_{Ca} activation.

324   In a first set of experiments we used 200 ms loading-steps from -60 mV to +60 mV

325   with 10 mV increments. This voltage range covered the complete voltage operating range of

326   I_{Ca} in uPNs (Husch et al. 2009). During the loading-steps $I_{K(Ca)}$ was activated (Fig. 3 *A,B*) as

327   shown already in Fig. 1. Figure 3 *A1* shows the current in response to a +60 mV test pulse

328   that was preceded by loading-pulses to +10 mV (large Ca^{2+} influx) and to +60 mV (virtually

329   no Ca^{2+} influx) (compare with Fig. 2 *A-C*). The Ca^{2+} influx that is induced by the +10 mV

330   pulse is terminated at the start of the +60 mV test pulse. Accordingly, during the +60 mV test

331   pulse $I_{K(Ca)}$ was activated by the depolarized membrane potential and the residual Ca^{2+} from

332   the +10 mV loading-pulse. Thus, $I_{K(Ca)}$ that was activated during the test pulse is transient

333   with fast deactivation (Fig. 3 *A1*), whereby the time course of the deactivation is strongly

334   dependent on the decaying residual intracellular Ca^{2+} concentration. The whole experiment

335   with the complete set of loading-pulses is shown in Figure 3 *A2-A3*. Plotting the transient

336   peak $I_{K(Ca)}$ during the test pulses over the potentials of the loading-pulses (Fig. 3 *A4*) revealed

337   inverted U-shaped relations with an average maximum of 11.7 ± 13.3 mV (n = 6), which is

338   within the voltage range where I_{Ca} has its maximum (Husch et al. 2009). The mirrored *I-V*

339   relations of I_{Ca} and $I_{K(Ca)}$ clearly demonstrated the strong Ca^{2+} dependence of $I_{K(Ca)}$.

340   In a second set of experiments the +60 mV test pulse was preceded by loading-steps

341   with increasing duration from 5 ms to 400 ms (Fig. 3 *B*). For each experiment the loading-

342   step voltage was set to the value at which we observed the maximal current during the +60

343   mV test pulse in the first part of the experiment (previous paragraph). Due to the inactivation

344   of I_{Ca} during a sustained depolarization (Husch et al. 2009) the instantaneous Ca^{2+} influx at

345   the end of each loading-pulse was decreasing with increasing duration of the loading-pulses.

346   Therefore, the amplitude of the transient peak $I_{K(Ca)}$ during the +60 mV test pulses decreased

347   with increasing loading-pulse duration (Fig. 3 *B1-B2*), again demonstrating the strong Ca^{2+}

348   dependence of $I_{K(Ca)}$.

349

350   *Voltage dependence of $I_{K(Ca)}$*

351   Next we studied the steady state voltage dependence, the voltage inactivation during

352   a sustained depolarization, and the Ca^{2+} dependence directly and independently of each

353   other. A series of voltage pulses was applied in 10 mV increments between -60 mV and +60

354   mV, while the cytosolic Ca^{2+} concentration was clamped at different concentrations using an

355   EDTA or EGTA - Ca^{2+} buffering system and by blocking I_{Ca} with 5 x 10^{-4} M $CdCl_2$. The

356   concentration of free cytosolic Ca^{2+} $[Ca^{2+}]_i$ was clamped at 56 μM, 143 μM, mM, 540 μM, or

357   1800 μM. These values were measured using a Ca^{2+} electrode. Under clamped $[Ca^{2+}]_i$ $I_{K(Ca)}$

358   did not inactivate during a sustained voltage pulse (Fig. 4 *A,B*), showing directly that the

359   decay of $I_{K(Ca)}$ as shown in Figure 1 depends on the I_{Ca} kinetics. At all cytosolic Ca^{2+}

360   concentrations $I_{K(Ca)}$ increased with increasing depolarization showing a clear voltage

361   dependence for steady state activation (Fig. 4). Comparing conductance-voltage (*G-V*)

362   relations for the different $[Ca^{2+}]_i$ showed that increasing Ca^{2+} concentrations lowered the

363   voltage threshold for activation of $I_{K(Ca)}$ and the voltage for half-maximal activation ($V_{0.5,act}$)

364   (Fig 4 *C,D*). For example: $V_{0.5,act}$ was 125±12 mV (n = 5) at $[Ca^{2+}]_i$ = 56 µM and 50±14 mV (n

365   = 6) at $[Ca^{2+}]_i$ = 1800 µM (p < 0.02; unpaired test). We did not observe a 'Ca^{2+} block' at high

366   $[Ca^{2+}]_i$.

367

368   *Effects of apamin, charybdotoxin and iberiotoxin on $I_{K(Ca)}$.*

369   In principle depolarizing voltage steps with the resulting Ca^{2+} influx can activate both

370   SK and BK channels. Here we tested the effect of apamin, charybdotoxin (CTX) and

371   iberiotoxin (IbTX) on $I_{K(Ca)}$, which are toxins that have been shown to block specific

372   components of $I_{K(Ca)}$ in vertebrates (Bennett et al. 2000; Blatz and Magleby 1986; Faber and

373   Sah 2003; Fioretti et al. 2004; Galvez et al. 1990; Ghatta et al. 2006; Giangiacomo et al.

374   1992; Kaczorowski et al. 1996; Pineda et al. 1992; Wolfart et al. 2001). Apamin blocks SK

375   (K_{Ca} 2.1, K_{Ca} 2.2, K_{Ca} 2.3) channels and iberiotoxin blocks BK (K_{Ca} 1.1) channels.

376   Charybdotoxin not only blocks BK (K_{Ca} 1.1) channels but has also been shown to block IK

377   (K_{Ca} 3.1) and K_V (K_V 1.2; K_V 1.3) channels. Ideally such blockers could be used a) for

378   occlusion experiments during current clamp recordings to analyze the role of $I_{K(Ca)}$ in shaping

379   the intrinsic firing properties of uPNs and b) to get information about the molecular identity of

380   the channels that mediate $I_{K(Ca)}$ in uPNs.

381   Both ChTX and IbTX reduced $I_{K(Ca)}$ in a concentration dependent way (Fig. 5 *A,B*)

382   while apamin had no effect (data not shown). The concentration-response relations were

383   determined with concentrations ranging from 50 pM to 100 nM and were well fit with the

384   sigmoidal relation

385   $$F(x) = Bottom + \frac{(Top - Bottom)}{(1 + 10^{(x-logIC50)})}$$ (1)

14

386   (Fig. 5 *B*). CTX started to block $I_{K(Ca)}$ at concentrations higher than ~100 pM and completely

387   abolished $I_{K(Ca)}$ at concentrations higher than ~30 nM. The concentration response relation

388   had an IC_{50} of 2.7 nM (1.8 nM – 3.8 nM). IbTX started to block $I_{K(Ca)}$ at concentrations around

389   100 pM and blocked maximally ~60% of $I_{K(Ca)}$ at concentrations of ~10 nM and higher. The

390   concentration response relation had an IC_{50} of 157 pM (53.7 pM to 460 pM). Apamin was

391   applied in concentrations up to 1 μM with no detectable effect on $I_{K(Ca)}$. Since previous work

392   had shown that I_{SK} in insect neurons can be insensitive to apamin (Abou Tayoun et al. 2011;

393   Wicher et al. 2001), we used voltage clamp protocols, which have been used to evoke I_{SK} in

394   rodents (Chen et al. 2014; Wolfart et al. 2001), to probe for SK channels in uPNs. In these

395   experiments neurons were hold at -60 mV. To elicit I_{SK} a 100 ms depolarizing voltage pulse

396   (Ca^{2+} loading-pulse) to 0 mV was applied, that was preceded by a 1 s hyperpolarizing pulse

397   to -80 mV. In dopaminergic substantia nigra neurons of mice this protocol evoked an apamin

398   (100 nM) sensitive I_{SK} (Fig. 6 *A*). Using the same protocol, we did not detect I_{SK} in uPNs (Fig.

399   6 *B*). To ensure the 'right parameter space' of the loading-pulse, its amplitude (-40 mV to +50

400   mV) and duration (10 ms to 200 ms) were varied.

401   Since CTX (100 nM) completely blocked $I_{K(Ca)}$, we compared the residual currents

402   after the direct CTX block of $I_{K(Ca)}$ with the residual currents after the indirect block of $I_{K(Ca)}$

403   using Cd^{2+} (Fig. 7). As expected, in contrast to the Cd^{2+} block, the residual current after CTX

404   block still contained I_{Ca} (Fig. 7E).

405

406   *The role of $I_{K(Ca)}$ on spike waveform*

407   Clearly odor responses and tuning profiles of uPNs are determined by their synaptic

408   input and by their intrinsic electrophysiological properties. In synaptically isolated uPNs

409   depolarizing current injections induced trains of action potentials with fairly regular firing

410   patterns. Typically, the spike frequency increased during a prolonged depolarization. We did

411   not observe spike frequency adaptation. To better understand the role of $I_{K(Ca)}$ in mediating

412   the intrinsic electrophysiological properties we performed occlusion experiments under

15

413   current clamp in which $I_{K(Ca)}$ was partially blocked by the $I_{K(Ca)}$ blockers CTX or IbTX. In

414   synaptically connected neurons application of 100 nM CTX, which blocked 100% of $I_{K(Ca)}$,

415   resulted in an unstable membrane potential and spontaneous high frequency bursts of action

416   potentials (data not shown). These drastic effects of completely blocking $I_{K(Ca)}$ indicated the

417   important role of $I_{K(Ca)}$ in shaping the intrinsic electrophysiological and synaptic properties of

418   AL neurons. Based on these experiments the blocker concentration was reduced to block

419   maximally 60% to 80% of $I_{K(Ca)}$, and we also blocked synaptic input pharmacologically (see

420   METHODS). Before, during and after the application of the $I_{K(Ca)}$ blockers, we applied current

421   injection protocols to analyze a set of intrinsic electrophysiological properties. These included

422   resting membrane potential, cell input resistance, action potential repolarization rate, action

423   potential width, action potential afterhyperpolarization, latency to the first action potential

424   upon depolarizing current injection, firing threshold, and number of elicited action potentials.

425   Details of the current clamp protocols are given in METHODS.

426

427   *Charybdotoxin.* 10 nM CTX, which blocked ~80% of $I_{K(Ca)}$ (Fig. 5), changed the action

428   potential waveform significantly (Fig. 8*;* Fig. 9*A*). The maximal rate of action potential

429   repolarization was reduced from -174 ± 16 to -119 ± 10 mV · s^{-1} (p<0.001; n = 10; Fig. 9 *A*1).

430   Accordingly, the spike width at half-maximal amplitude was increased from 505 ± 60 μs to

431   668 ± 40 μs (p<0.001; n = 10; Fig. 9 *A*2) and the action potential afterhyperpolarization was

432   reduced (p<0.001; n = 10; Fig. 9 *A*3). During constant 500 ms depolarizing current pulses,

433   the latency to the first spike decreased from 252 ± 106 ms to 123 ± 46.8 ms (p < 0.01; n =

434   10; Fig. 9 *A4*) and the number of spikes increased from 6 ± 3 to 13.3 ± 2.8 (p < 0.001; n = 10;

435   Fig. 9 *A6*). The threshold for spike initiation (Fig. 9 *A5*), the resting membrane potential and

436   cell input resistance (measured around the resting membrane potential) did not change (data

437   not shown). All of the observed CTX effects were at least in part reversible.

438

439   *Iberiotoxin.* IbTX was applied at concentrations of 100 nM IbTX, where it had its maximal

440   effect and blocked ~60% of $I_{K(Ca)}$ (Fig. 5). Generally, 100 nM IbTX had similar but weaker

441   effects than 10 nM CTX. IbTX decreased the repolarization rate from 194 ± 26 V · s^{-1} to 130

442   ± 34 V · s^{-1} (p<0.001; n = 11; Fig. 9 *B*1), increased spike width from 430 ± 49 μs to 561 ± 125

443   μs (p<0.001, n = 11; Fig. 9 *B*2), and reduced the action potential afterhyperpolarization

444   (p<0.001; n = 11; Fig. 9 *B*3). The latency to the first spike decreased from 146 ± 20 ms to 95

445   ± 53 ms (p < 0.05; n = 7; Fig. 9 *B4*) and the number of spikes increased from 6.5 ± 1.5 to 9.6

446   ± 2 (p < 0.05; n = 7; Fig. 9 *B6*). All these effects were reversible upon wash out. IbTX had no

447   significant effects on firing threshold (Fig. 9 *B*5), cell input resistance (measured around the

448   resting membrane potential), and resting membrane potential (data not shown).

449

450   *Apamin.* In agreement with our voltage clamp experiments, in which apamin had no effect on

451    $I_{K(Ca)}$ and in which we did not detect I_{SK} (Fig. 6), there was no effect of 1 μM apamin on the

452   membrane potential or spike related properties (Fig. 9C).

453

454   **DISCUSSION**

455   The overall goal of this study was to characterize $I_{K(Ca)}$ in uPNs. In the insect brain

456   uPNs form the principal pathway from the AL to the protocerebrum, where centers for

457   multimodal sensory processing and learning, and relays for innate behaviors are located

458   (Davis 2004; Heimbeck et al. 2001; Heisenberg 2003; Stopfer 2014). Ca^{2+}-activated currents

459   are an important link between the intracellular signaling system and the membrane potential

460   of the cell. In most neuron types $I_{K(Ca)}$ is pivotal for shaping intrinsic electrophysiological

461   properties. Accordingly, we performed the study in two steps. First, we characterized the

462   functional properties of $I_{K(Ca)}$. Second, we used occlusion experiments, in which portions of

463    $I_{K(Ca)}$ were pharmacologically blocked, to reveal the role of $I_{K(Ca)}$ in shaping the intrinsic

464   electrophysiological properties of uPNs. For all recordings we used the whole-cell patch

465 clamp configuration combined with single cell labeling in an intact, adult brain preparation of

466 *P. americana.*

467   In general, $I_{K(Ca)}$ in uPNs was similar to Ca^{2+}-activated K^+ currents found in other

468 insect neurons (Derst et al. 2003; Grolleau and Lapied 1996; Grünewald 2003; Heidel and

469 Pflüger 2006; Kulke et al. 2014; Lucas and Shimahara 2002; Perk and Mercer 2006; Pézier

470 et al. 2007; Ryglewski and Duch 2009; Schäfer et al. 1994). $I_{K(Ca)}$ had the typical inverted U-

471 shaped *I-V* relation and $I_{K(Ca)}$ decayed during a sustained depolarizing voltage pulse, but did

472 not inactivate when $[Ca^{2+}]_i$ was clamped to constant values. The strong Ca^{2+} dependence of

473 $I_{K(Ca)}$ is corroborated by the inverted U-shaped *I-V* relation that mirrored the *I-V* relation of I_{Ca}

474 and also the decay kinetics during a sustained voltage pulse, which reflected the inactivation

475 kinetics of I_{Ca} (Husch et al. 2009). The Ca^{2+} dependence is also shown in the loading-pulse

476 experiments where $I_{K(Ca)}$ activated transiently with a very fast decay during the +60 mV test

477 pulse (where virtually no Ca^{2+} is entering the cell). The transient activation and fast decay of

478 $I_{K(Ca)}$ in these experiments indicated a close spatial relation of the Ca^{2+} and K_{Ca} channels

479 (Augustine et al. 2003; Berkefeld et al. 2010) and efficient Ca^{2+} sequestration (Pippow et al.

480 2009) leading to a fast attenuation of the Ca^{2+} microdomain in the vicinity of the K_{Ca} channel.

481 Taken together our data demonstrate that $I_{K(Ca)}$ is directly linked to the voltage-activated Ca^{2+}

482 influx and that it is strongly Ca^{2+} and voltage dependent. Following the classical biophysical

483 classification of K_{Ca} channels our results suggest that $I_{K(Ca)}$ in uPNs is mediated by BK

484 channels, since it required depolarized membrane potential and elevated intracellular Ca^{2+}.

485 This notion is in line with our pharmacological experiments. We found sensitivity of $I_{K(Ca)}$ to

486 IbTX and CTX, but not to apamin. However, these experiments have to be interpreted with

487 caution. IbTX, which is considered a specific BK (K_{Ca} 1.1) channel blocker, reduced $I_{K(Ca)}$ by

488 60%, while the less specific blocker CTX completely abolished $I_{K(Ca)}$. CTX not only blocks BK

489 (K_{Ca}1.1) channels but has also been shown to block IK (K_{Ca} 3.1) and K_V (K_V 1.2; K_V 1.3)

490 channels (Garcia et al. 1995). Accordingly, this might indicate that IK channels also

491 contribute to $I_{K(Ca)}$. Given that our current isolation protocol selected for true Ca^{2+} dependent

492 currents, K_V channels did not contribute to the current that we define as $I_{K(Ca)}$. Our results are

18

493   also in line with previous work from Derst et al. (2003) who cloned pSlo, the BK channel α

494   subunit of *P. americana,* and expressed it in HEK 293 cells. The resulting whole-cell current

495   had similar properties as $I_{K(Ca)}$ in uPNs. It had a clear Ca^{2+} and voltage dependence, and did

496   not inactivate under clamped $[Ca^{2+}]_i$.

497   Apamin, which is considered a specific SK channel (K_{Ca} 2.1, K_{Ca} 2.2, K_{Ca} 2.3) blocker

498   (Adelman et al. 2012), had no effect on $I_{K(Ca)}$. Since previous work has shown that SK

499   channels in insects can be insensitive to apamin (Abou Tayoun et al. 2011; Wicher et al.

500   2001), we used voltage clamp protocols that induce I_{SK} in dopaminergic midbrain neurons of

501   mice. These experiments did not reveal I_{SK} in uPNs and suggest that uPNs of *P. americana*

502   do not express SK channels. While these results seem somewhat unexpected, they are in

503   agreement with in situ hybridization experiments which suggest that SK channels are indeed

504   not expressed in AL neurons of *P. americana* (S. Korsching, V. Zapilko and D. Fusca,

505   personal communication). A straightforward and tempting conclusion is that the lack of I_{SK}

506   makes the resting membrane potential of uPNs relatively independent of the resting $[Ca^{2+}]_i$,

507   since I_{BK} requires high $[Ca^{2+}]_i$ *and* a depolarized membrane potential.

508   The occlusion experiments with high concentrations of CTX, which completely

509   blocked $I_{K(Ca)}$ (I_{BK}), increased synaptic input and excitability of uPNs indicated the significant

510   physiological role of $I_{K(Ca)}$ in neurons of the AL network. To reveal in more detail how $I_{KCa)}$

511   contributes to shaping the intrinsic electrophysiological properties of uPNs, we performed

512   experiments on synaptically isolated uPNs in which $I_{K(Ca)}$ was only moderately blocked. A

513   60% to 80% block of $I_{K(Ca)}$ (I_{BK}) significantly changed the action potential waveform and

514   excitability, which is in agreement with the roles of $I_{K(Ca)}$ that have been observed in other

515   neuron types of different species (Berkefeld et al. 2010; Faber and Sah 2003; Greffrath et al.

516   2004; Kadas et al. 2015). By linking free $[Ca^{2+}]_i$ and membrane potential BK channels

517   contribute to regulating the intrinsic electrophysiological properties. BK channels are rapidly

518   activated during the rising phase of the action potential by the depolarizing membrane

519   potential and the voltage dependent Ca^{2+} influx. Once activated, BK channels influence the

520   rate of action potential repolarization and action potential afterhyperpolarization (Berkefeld et

521 al. 2010; Faber and Sah 2003; Greffrath et al. 2004), thus they directly contribute to the

522 control of action potential waveform and spike frequency (Berkefeld et al. 2010; Faber and

523 Sah 2003; Greffrath et al. 2004; Kadas et al. 2015).

524   All the recordings in this study were performed in an intact brain preparation, in which

525 the currents are not altered by any culturing procedure or in vitro development. Given that

526 the complex arborizations are still intact, perfect voltage control across the whole neuron is

527 impossible. The current waveforms usually did not indicate significant voltage control

528 problems (e.g. no delay of current onset, no jumps in the *I-V* relation) suggesting that they

529 originated mostly from well voltage-clamped regions. Since we recorded from the soma,

530 which has a long thin neurite connecting it to the rest of the cell, it is likely that the currents

531 we have measured originate primarily from the cell body. Ionic currents generated by

532 channels selectively located in very distal regions of the neuron may not be detectable by

533 voltage-clamping the soma.

534   Because intrinsic electrophysiological properties of neurons are determined by the

535 expressed ion channel types and by their expression ratio, we consider such a detailed

536 analysis of the biophysical properties an important prerequisite to conductance-based

537 models to gain a clear understanding of how olfactory signals are processed at the single cell

538 and circuit level.

539

547

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754

FIGURE CAPTIONS

756

757 FIG. 1: Morphology of uniglomerular projection neurons and isolation of $I_{K(Ca)}$. *A*: Morphology

758 of the recorded uPN revealed by labeling with biocytin-streptavidin via the patch pipette. *A1*:

759 overview. The neuron innervated a single glomerulus and sent a single axon along the mALT

760 to the mushroom body's calyces (CA) and the lateral horn (LH). The position of the soma,

761 which was lost during processing, is marked (★). *A2-A4:* higher magnification of the framed

762 areas in *A1. A1; A2:* boutons in the calyces (*A2*) and in the LH (*A3*). *A4*: neurites in the single

763 innervated glomerulus. *B-E*: isolation of $I_{K(Ca)}$. The holding potential was -60 mV. *B*: Currents

764 were activated by 300 ms depolarizing steps from -60 mV to 60 mV in 10 mV increments. *C*:

765 Current traces elicited by the same depolarizing steps as in *B*, during the application of a 5 x

766 10^{-4} M Cd^{2+}. *D*: subtraction of the *C* traces from the *B* traces yields $I_{K(Ca)}$. *E*: voltage-

767 dependence for activation of $I_{K(Ca)}$. *I-V* relation and *current density-V* relation of $I_{K(Ca)}$. na,

768 neural anterior; AL, antennal lobe; CA, calyx; GL, glomerulus; mALT, medial antennal lobe

769 tract; l, lateral; LH, lateral horn; ★, soma location.

770

771 FIG. 2: Voltage-dependence of $I_{K(Ca)}$. *A*: *I-V* relation. *B*: *current density-V* relation. Current

772 density was calculated from the ratio between $I_{K(Ca)}$ and the cell capacitance. *C,D*: normalized

773 *I-V* relation ($I_{K(Ca)}$ as fractions of the maximal $I_{K(Ca)}$ for each neuron). *A-C* are based on the

774 same original recordings with $[Ca^{2+}]_o$=1 mM. The recordings in *D* were performed in $[Ca^{2+}]_o$ =

775 6 mM. Gray: individual cells; black: mean ± SD.

776

777 FIG. 3: $I_{K(Ca)}$ is dependent on the amplitude and duration of the "Ca^{2+} loading-pulses". Test

778 pulses depolarized the membrane to +60 mV where virtually no voltage activated Ca^{2+} influx

779 occurs, since the membrane approaches the Ca^{2+} equilibrium potential. The test pulses were

780 preceded by "Ca^{2+} loading-pulses" of varying amplitude (*A1-A4*) or duration (*B1-B3*). *A1-A4*:

781   the +60 mV test pulses were preceded by 200 ms depolarizing Ca^{2+} loading-pulses of

782   varying amplitude (-60 mV to +60 mV; 10 mV increments). *A1*: currents in response to the

783   +60 mV test pulse preceded by loading-pulses to +10 mV (large Ca^{2+} influx) and to +60mV

784   (virtually no Ca^{2+} influx). *A2*: same experiment as in *A1* with the whole series of loading-

785   pulses from -60 mV to +60 mV. *A3*: framed area of *A2* in higher resolution. *A4*: *I-V$_{loading}$*

786   relation of $I_{K(Ca)}$. The *I-V$_{loading}$* relation shows $I_{K(Ca)}$ at the beginning of the +60 mV test pulse

787   plotted as a function of the loading-pulse voltage. *B1-B3*: the +60 mV test pulses were

788   preceded by Ca^{2+} loading-pulses of varying duration (5 ms – 400ms). The loading-pulse

789   amplitude was adjusted for each neuron to values where maximal loading occurred during

790   the first part of the experiments (see *A4*). *B1*: currents in response to the + 60 mV test pulse

791   that were preceded by 10 mV loading-pulses of varying duration. *B2*: $I_{K(Ca)}$ at the beginning of

792   the +60 mV test pulses (normalized to the maximal $I_{K(Ca)}$ of each cell) plotted as a function of

793   the loading-pulse duration. *B3*: The decay time constant of $I_{K(Ca)}$ in dependence of the

794   loading-pulse duration.

795

796   Fɪɢ. 4: Voltage and Ca^{2+} dependence of $I_{K(Ca)}$. To measure the voltage and Ca^{2+} dependence

797   of $I_{K(Ca)}$ independently from the instantaneous Ca^{2+} influx, $I_{K(Ca)}$ was recorded under voltage

798   and $[Ca^{2+}]_i$ clamp. The holding potential was -60 mV and voltage pulses were applied

799   between -60 mV and +90 mV in 10 mV increments. The cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$

800   was clamped at 56 µM (n=5), 143 µM (n=5), mM, 540 µM (n=6) or 1800 µM (n=6) using an

801   EDTA or EGTA - Ca^{2+} buffering system. Voltage activated Ca^{2+} currents were blocked by 5 x

802   10^{-4} M CdCl$_2$. *A*: Under clamped $[Ca^{2+}]_i$ $I_{K(Ca)}$ did not inactivate during a sustained voltage

803   pulse. Increasing Ca^{2+} concentrations increased the $I_{K(Ca)}$ amplitude and at all Ca^{2+}

804   concentrations $I_{K(Ca)}$ increased with increasing depolarization. *B*: Example traces (different

805   neurons) demonstrating the effect of increasing $[Ca^{2+}]_i$ on $I_{K(Ca)}$. *C: G-V* relations for different

806   $[Ca^{2+}]_i$. Error bars are omitted for better visualization. *D*: Voltages for half-maximal activation

807   ($V_{0.5,act}$) for different $[Ca^{2+}]_i$.

808

809 FIG. 5: Charybdotoxin and iberiotoxin reduce $I_{K(Ca)}$. *A*: Example traces (different neurons)

810 demonstrating the effect of increasing concentrations of CTX (left) and IbTX (right) on $I_{K(Ca)}$.

811 *B*: *Concentration - response* relation of CTX and IbTX. The curves are fits to a sigmoidal

812 relation (*Eq. 1*). CTX had an IC_{50} of 2.7 nM (1.8 nM – 3.8 nM) and IbTX had an IC_{50} of 157

813 pM (53.7 pM - 460 pM).

814

815 FIG. 6: Uniglomerular projection neurons do not generate I_{SK}. *A:* In dopaminergic substantia

816 nigra neurons of mice a short depolarizing voltage pulse (Ca^{2+} loading-pulse), evoked Ca^{2+}

817 influx which activated an apamin sensitive I_{SK}. *B:* uPNs did not generate I_{SK}. The 100 ms

818 loading-pulse to 0 mV was preceded by a 1 s hyperpolarizing step to -80 mV. The holding

819 potential was -60 mV. The recordings are only shown for the framed region of the voltage

820 protocol. p/n protocols were not applied. The apamin concentration was 100 nM in *A* and 1

821 µM in *B*.

822

823 FIG. 7: Comparison of direct and indirect "block" of $I_{K(Ca)}$ by CTX and Cd^{2+} respectively. The

824 holding potential in all recordings was -60 mV. Currents were activated by 300 ms

825 depolarizing steps from -60 mV to +60 mV in 10 mV increments. *A*: whole cell currents of a

826 uPN during TTX and 4-AP application. *B,C*: recordings of the same neuron as in *A*, during

827 the additional application of CTX (*B*) and during the application of CTX and 5×10^{-4} M Cd^{2+}

828 (*C*). *D*: Subtraction of the *B* traces from the *A* traces. *E*: Subtraction of the *C* traces from the

829 *B* traces. *F*: *I-V* relation for the current traces shown in *A-E*.

830

831 Fig. 8: Example current clamp recording to demonstrate the CTX effect on action potential

832 waveform.

29

833

834   FIG. 9: Summery and quantification of the charybdotoxin (*A*), iberiotoxin (*B*), and apamin (*C*)

835   effects on electrophysiological properties (rate of repolarization [1], amplitude of

836   afterhyperpolarization [2], width of half maximal amplitude [3], latency to first action potential

837   during a depolarizing voltage step [4], action potential threshold [5], and number of action

838   potentials during 500 ms depolarizing current pulses [6]). The schematics demonstrate how

839   the parameters were measured; for details see METHODS. n values are given in the bars of

840   each graph. * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001. Apa, apamin; CTL, control; CTX, charybdotoxin;

841   IbTX, iberiotoxin; W, wash.

842

843

A1 — A2 (dotted box), CA, A3 (dotted box), LH, mALT, *, AL, GL, A4 (dotted box)

na
m ⊥ l
np

100 μm

A2 — CA, 50 μm

A3 — LH, 50 μm

A4 — GL, 50 μm

B — TTX, 4-AP

C — TTX, 4-AP, Cd²⁺

+60 mV

−60 mV

5 nA

100 ms

D — B-C

E

$I_{K(Ca)}$ (nA) / $I_{K(Ca)}$ / C_M (nA pF⁻¹)

E_M (mV)

A

CTX   control
       100 ms
       5 nA
3 nM
5 nM
10 nM
Cd²⁺

0 mV
-90 mV

IbTX   control
0.5 nM
5 nM   100 ms
50 nM   5 nA
100 nM
Cd²⁺

B

A DA neuron
0 mV *
-60 mV
-80 mV
control
apamin

B uPN
200 pA
50 ms
apamin
control

A

TTX, 4-AP

B

TTX, 4-AP, CTX

C

TTX, 4-AP, CTX, Cd²⁺

100 ms
10 nA

D

(A - B)

E

(B - C)

F

+60 mV
-60 mV

E_M (mV)

I (nA)

□ TTX, 4-AP, CTX
○ TTX, 4-AP
▲ TTX, 4-AP, CTX, Cd²⁺
● A-B

20 mV

4 ms

50 nM CTX

10 nM

control

— — wash