Cholecystokinin Excites Interneurons in Rat Basolateral Amygdala

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Cholecystokinin excites interneurons in rat basolateral amygdala. J Neurophysiol 102: 272–284, 2009. First published April 22, 2009; doi:10.1152/jn.90769.2008. The amygdala formation is implicated in generation of emotional states such as anxiety and fear. Many substances that modulate neuronal activity in the amygdala alter anxiety. Cholecystokinin (CCK) is an endogenous neuropeptide that induces anxiety states in behavioral studies in both animals and humans. Using a brain slice preparation, we found that application of CCK increases inhibitory synaptic transmission measured in projection neurons of the basolateral amygdala. To determine the source of the increased inhibition we examined the direct effect of CCK on local interneurons in this region. CCK most strongly depolarized fast-spiking interneurons. Burst-firing and regular-firing interneurons were also depolarized, although to a lesser degree. However, another distinct group of interneurons was unaffected by CCK. These effects were mediated by the CCKB receptor subtype. The excitatory effect of CCK appeared to be mediated by both a nonselective cation and a K* current.

INTRODUCTION

The amygdala formation is a brain structure involved in the generation of emotional states (LeDoux 2000; Phelps and LeDoux 2005). Experimental manipulations of amygdala activity by lesions or by electrical or chemical stimulation alter behavioral states associated with fear or anxiety (Davis 2000). Manipulations that increase fear or anxiety are generally associated with increased neuronal activity in the basolateral amygdala (BLA) (Davis et al. 1994; Sajdyk and Shehkar 1997). In general, manipulations that increase excitability of the BLA tend to be anxiogenic, whereas those decreasing excitability tend to be anxiolytic (Davis et al. 1994). For example, enhancing GABAergic inhibitory transmission by local injection of benzodiazepines, γ-aminobutyric acid (GABA), or GABA receptor agonists is anxiolytic, whereas injection of the GABA receptor antagonist bicuculline is anxiogenic (Hodges et al. 1987; Sajdyk and Shekhar 1997; Scheel-Kruger and Petersen 1982). Blocking excitatory glutamatergic transmission by N-methyl-D-aspartate (NMDA) or non-NMDA receptor antagonists attenuates behavioral measures for fear and anxiety (Kim et al. 1993; Misерendino et al. 1990).

Cholecystokinin (CCK) is a neuropeptide first isolated in the gut, then later localized in widespread brain areas (Ivy and Oldberg 1928; Vanderhaeghen et al. 1975). Two receptor subtypes have been described: CCKA (CCK1) and CCKB (CCK2) (Wank 1995). Using either systemic or intracerebroventricular (icv) injections, CCK increases anxiety-like behaviors; this effect is blocked by CCKA antagonists (Rotzinger and Vaccarino 2003). CCK and its receptors are abundant in the amygdala formation (Noble et al. 1999). The CCKB agonist pentagastrin injected into the amygdala potentiates the acoustic startle response in rats, whereas a CCKB antagonist injected into the amygdala attenuates the startle potentiation induced by icv infusion of pentagastrin (Frankland et al. 1997). Thus it appears likely that the amygdala is a part of the neural circuit for CCK-induced anxiety. Based on these studies, CCK would be expected to increase excitability in the amygdala. However, our recent work shows that CCK increases GABAergic inhibitory synaptic transmission onto projection cells in the basolateral nucleus (Chung and Moore 2007b). Because this effect is blocked by tetrodotoxin (TTX), we assumed that CCK directly activated local interneurons.

Diverse interneuron types are present in the amygdala, similar to a diversity of types in the neocortex and hippocampus (Ascoli et al. 2008; Freund and Buzsáki 1996; Markram 2004). BLA interneurons can be grouped into subsets according to morphology, calcium binding proteins, peptide content, or serotonin receptors (Mascagni and McDonald 2003, 2007; McDonald 1984; McDonald and Mascagni 2001; Rainnie et al. 2006; Woodruff and Sah 2007a). In a previous study in the lateral amygdala, CCK depolarized two types of nonpyramidal cells (Sugita et al. 1993). However, the specificity of the CCK effect on interneuron types and the ionic mechanism was not further characterized in detail. In this study, we examined how the effect of CCK differs among interneuron subtypes. We also investigated the ionic mechanism mediating these effects. A preliminary report of this work was previously presented in abstract form (Chung and Moore 2007a).

METHODS

Brain slice

All procedures were approved by the Duke University and Durham VAMC Animal Care and Use Committees. Male Sprague–Dawley rats (14 to 24 days old) were decapitated under isoflurane anesthesia. Brains were quickly removed and placed into chilled solution containing (in mM) 2.5 KCl, 1.25 NaH2PO4, 10.0 MgSO4, 0.5 CaCl2, 26.0 NaHCO3, 11.0 glucose, and 234.0 sucrose bubbled with 95% O2–5% CO2. We cut coronal slices (300 μm) containing the amygdala region using a vibrating tissue slicer (Vibratome 1000 Plus, Vibratome). The slices were incubated for 30 min at 35°C in artificial cerebrospinal fluid (ACSF) containing (in mM) 126.0 NaCl, 2.5 KCl, 1.25 NaH2PO4, 1.0 MgSO4, 2.0 CaCl2, 26.0 NaHCO3, and 10.0 glucose bubbled with 95% O2–5% CO2. After another 1 h in room temperature, we transferred a single slice to the recording chamber. Inside the chamber, ACSF was continuously superfused at the rate of 2 ± 0.2 ml/min. For lanthanum experiments, HEPES buffer–based ACSF was used containing (in mM) 149.0 NaCl, 2.5 KCl, 1.0 MgSO4, 2.0 CaCl2, 10 HEPES, and 10.0 glucose bubbled with 100% O2 (pH adjusted to 7.4 with NaOH).
Whole cell recording

We visualized individual cells using an upright Axioskop fixed-stage microscope (Axioskop2, Zeiss). We obtained whole cell recordings in voltage- and current-clamp mode using glass micropipettes (1.5 mm OD, WPI) containing (in mM) 130 K-glucuronate, 10 KCl, 10 HEPES, 2 Mg-ATP, 0.2 Tris-GTP, and 0.3% biocytin. The temperature in the recording chamber was maintained at 30°C. Signals were amplified with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), filtered at 2 kHz (−3 dB), digitized at 10 kHz using Clampex (Axon Instruments), and recorded onto an IBM-compatible computer. Cells with small and round soma were targeted as likely interneurons. A fluorescent, Alexa Fluor 568 or 488 hydrazide (75 μM), was routinely added to the pipette solution. The calculated junction potential, 14 mV (pClamp software), was not compensated in the reported membrane potential. The criterion for healthy cells was a stable membrane potential below −55 mV without junction potential compensation (−69 mV with junction potential compensation) shortly after membrane rupture.

Morphology

At the end of recording, the overall morphology was examined, including the presence or absence of spines and beads (Ascoli et al. 2008; Sosulina et al. 2006) in the recording chamber using a fluorescence light source (X-Cite, Burleigh), 546-nm excitation and 590-nm emission filter for Alexa Fluor 568, or 470-nm excitation and 546-nm emission for Alexa Fluor 488. Slices were then fixed overnight in 4% paraformaldehyde phosphate-buffered saline (PBS). After rinsing with 0.1 M PBS, we incubated slices for 1 h in Alexa 488–conjugated streptavidin (1:1,000, Molecular Probes), then coverslipped with mounting medium (SlowFade, Molecular Probes). Photomicrographs were taken with a confocal microscope (Leica TCS SP-5).

Drugs

All agents were added directly to the ACSF. CCK (CCK Octapeptide, sulfated, CCK8S) was obtained from Tocris. 2-Aminoethoxydi-phenylborate (2-APB) and SKF96365 were obtained from Calbiochem and all other drugs from Sigma.

Statistical analysis

Statistical tests used were Wilcoxon signed-rank test for one sample and Mann–Whitney test for two samples. In Table 1, a nonparametric one-way ANOVA (Kruskal–Wallis test) was used with Dunn’s post hoc test (Prizm 4.0, GraphPad). Statistical significance was set at P < 0.05. The group values were expressed as means ± SE.

### TABLE 1. Interneuron properties

<table>
<thead>
<tr>
<th>Property</th>
<th>FS1 (n = 98)</th>
<th>FS2 (n = 17)</th>
<th>Burst-Firing (n = 11)</th>
<th>Regular-Firing (n = 57)</th>
<th>Projection (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vm, mV</td>
<td>−57.2 ± 0.5*4</td>
<td>−59.0 ± 0.9*2</td>
<td>−52.9 ± 2.0</td>
<td>−53.7 ± 0.7</td>
<td>−54.1 ± 0.8</td>
</tr>
<tr>
<td>Rm, MΩ</td>
<td>136 ± 5*3</td>
<td>153 ± 12*4</td>
<td>254 ± 35*5</td>
<td>316 ± 21</td>
<td>137 ± 16</td>
</tr>
<tr>
<td>M(t), ms</td>
<td>14 ± 0.6*6</td>
<td>17 ± 1*9</td>
<td>36 ± 7</td>
<td>45 ± 3</td>
<td>35 ± 3</td>
</tr>
<tr>
<td>AP threshold, mV</td>
<td>−33.5 ± 0.3*8</td>
<td>−33.5 ± 0.6</td>
<td>−34.8 ± 1.5</td>
<td>−33.9 ± 0.5*9</td>
<td>−36.7 ± 0.7</td>
</tr>
<tr>
<td>AP amplitude, mV</td>
<td>51.4 ± 0.8*10</td>
<td>64.0 ± 1.1</td>
<td>56.7 ± 3.5*11</td>
<td>63.6 ± 1.5*12</td>
<td>79.4 ± 2.2</td>
</tr>
<tr>
<td>AP half-width, ms</td>
<td>0.61 ± 0.01*13</td>
<td>0.90 ± 0.03</td>
<td>0.72 ± 0.03*14</td>
<td>0.89 ± 0.03</td>
<td>1.12 ± 0.04</td>
</tr>
<tr>
<td>AP rise rate, V/s</td>
<td>189 ± 4</td>
<td>171 ± 8</td>
<td>181 ± 14</td>
<td>171 ± 5</td>
<td>198 ± 13</td>
</tr>
<tr>
<td>AP fall rate, V/s</td>
<td>−103 ± 2*15</td>
<td>−85 ± 4</td>
<td>−97 ± 6</td>
<td>−86 ± 3</td>
<td>−79 ± 5</td>
</tr>
<tr>
<td>AHP, mV</td>
<td>−20.6 ± 0.3*16</td>
<td>−20.7 ± 0.9*17</td>
<td>−20.6 ± 1.3*18</td>
<td>−20.0 ± 0.7*19</td>
<td>−5.4 ± 1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. (Note: Cells recorded in HEPES-based ACSF were not included in this table.) Superscripts indicate groups with significant differences in pairwise comparisons. *1: regular-firing; *2: regular-firing, projection; *3: burst-firing, regular-firing, *4: regular-firing; *5: projection; *6: burst-firing, regular-firing, projection; *7: regular-firing, projection; *8: projection; *9: projection; *10: FS2, regular-firing, projection; *11: projection; *12: projection; *13: FS2, regular-firing, projection; *14: projection; *15: FS2, regular-firing, projection; *16: projection; *17: projection; *18: projection; *19: projection. Nonparametric one-way ANOVA (Kruskal–Wallis test) was used with Dunn’s post hoc test. Significance level was set at P < 0.05.

Basic electrophysiological properties

Input resistance (Rm, MΩ) was calculated from the slope of linear fit to the voltage response to small hyperpolarizing currents (0 to −50 pA, 5-pA steps). Membrane time constants [M(t), ms] were obtained from fitting the hyperpolarizing voltage change with a single-exponential function (Halabisky et al. 2006). We examined action potential (AP)–related parameters using the first AP generated from depolarizing current steps (5 nA). AP threshold (mV) was measured at the voltage of the first flexion before overshooting (Gittis and du Lac 2007). AP amplitude (mV) was the height from the threshold to the peak. AP half-width (ms) was the time between two points at the midpoint of the peak amplitude from the AP threshold. AP rise rate (V/s) and AP fall rate (V/s) were the greatest rising and falling slope. Afterhyperpolarization amplitude (AHP, mV) was the potential difference from the AP threshold and peak repolarization. Clampfit 10 was used for curve fitting and measurements.

RESULTS

Interneuron classification

Interneurons in the BLA have previously been grouped according to electrophysiological characteristics, morphology, and chemical markers (e.g., see Rainnie et al. 2006). In this study we used electrophysiological and morphological criteria to categorize them. The groups were fast-spiking type 1 (FS1), fast-spiking type 2 (FS2), burst-firing, and regular-firing (Table 1).

In our studies, FS1 neurons are fast-spiking classical interneurons with sharp action potentials and little or no “sag” with hyperpolarizing current pulses (Fig. 1A). The firing pattern is typically high frequency with little adaptation. FS2 neurons are similar to FS1 in the lack of “sag” and little adaptation in firing pattern, but differ from FS1 in having a broader AP (P < 0.05, Table 1) (Fig. 1B). The defining feature of the burst-firing type is the presence of spontaneous bursts of APs (Fig. 1Ca, inset), with sharp APs similar to FS1 (Fig. 1C, Table 1). Two other features are the presence of spontaneous frequent excitatory postsynaptic potentials and a “sag” with hyperpolarizing current pulses. Regular-firing interneurons have a firing pattern that typically does not exhibit adaptation, with a broader AP than that of FS1 (P < 0.05, Table 1) and higher input impedance than that of either FS1 or FS2 (P < 0.05, Table 1) (Fig. 1D). Although we attempted to be consistent with previous descriptions of interneurons in rat BLA (Rainnie et al. 2006), there were two differences. First, we combined fast-firing and stutter-firing neurons into one group (FS1) because in our preparation some interneurons appeared to be...
intermediate between the two types. Second, in this region, FS2 interneurons had not previously been described as a separate subtype distinguishable from FS1 by AP width and firing pattern.

**CCK excites interneurons**

In our previous study (Chung and Moore 2007a), CCK increased the frequency of spontaneous inhibitory postsynaptic potentials and currents (sIPSP/sIPSCs) in projection neurons in the BLA; thus we hypothesized that CCK may directly activate GABAergic interneurons. Superfusion of CCK (1 μM, 30 s) depolarized resting membrane potentials (Fig. 2). The appearance of depolarization varied among the neurons. All FS1-type neurons were depolarized (n = 8) and 5 cells fired APs (Fig. 2A). FS2 neurons were not depolarized in 7 of 8 cells (Fig. 2B), whereas one was depolarized by 1.2 mV. Burst-firing neurons were depolarized (n = 4 of 4, Fig. 2C). Eight of 17 regular-firing neurons were depolarized and most of them generated APs (7 of 8, Fig. 2D).

**Effect of CCK in the presence of tetrodotoxin**

To measure the direct effects of CCK we applied tetrodotoxin (TTX, 1 μM) to block APs. TTX was applied after characterization of firing. The mean depolarization in each cell type was 7.0 ± 0.6 mV for FS1 cells (n = 25, Fig. 3A),...
4.0 ± 0.5 mV for burst-firing cells (n = 5, Fig. 3C), and 3.5 ± 0.4 mV for regular-firing cells (n = 13, Fig. 3D). No significant depolarization occurred in any of the FS2 cells (0.1 ± 0.1 mV, n = 9, Fig. 3B), in 6 regular-firing cells (0.3 ± 0.2 mV, n = 6), in 2 burst-firing cells, or in 4 projection neurons (0.5 ± 0.2 mV, n = 4) (Fig. 3E). Amplitude of the depolarization correlated with narrower AP (correlation coefficient = −0.47) and with speed of hyperpolarization (smaller membrane time constant) in response to current pulses (r = −0.44).

**CCK<sub>B</sub> receptor subtype mediates interneuron depolarization**

Two distinct CCK receptor subtypes have previously been described, designated as CCK<sub>A</sub> (CCK<sub>1</sub>) and CCK<sub>B</sub> (CCK<sub>2</sub>) (Noble et al. 1999; Wank 1995). We next attempted to identify which receptor subtype mediated this depolarization. For antagonist experiments, CCK was applied twice at 15-min intervals and the antagonists were applied for ≥10 min before the second CCK application. In TTX (control condition), the magnitude of the effect of the second CCK application effect was about 60% of the first (first CCK, 7.2 ± 1.1 mV; second CCK 4.4 ± 0.7 mV; n = 6, 4 FS1, 2 regular-firing; Fig. 4, A and D). In the presence of the CCK<sub>A</sub> receptor antagonist, lorglumide (1 µM), the second CCK application still depolarized cells (first 7.9 ± 1.7 mV; second, 4.8 ± 1.0 mV; n = 7, 6 FS1, one regular-firing; Fig. 4, B and D). However, application of the CCK<sub>B</sub> receptor antagonist CR2945 (1 µM) significantly attenuated the depolarization (first 7.3 ± 0.9 mV; second 0.9 ± 0.5 mV; n = 11, 9 FS1, one regular-firing, one burst-firing) com-
pared with control and lorglumide condition (Fig. 4, C and D) 
(P < 0.05). In addition, the CCK<sub>R</sub> receptor agonist CCK4 alone (1 μM, 30 s) depolarized interneurons (3.3 ± 0.6 mV; 
n = 5, 3 FS1, 2 regular-firing; Fig. 4E). These results indicate 
that the depolarization is mediated by CCK<sub>R</sub> receptors.

CCK increases a nonselective cation current

The depolarization evoked by CCK was accompanied by a 
significant input resistance increase of 10 ± 2% (n = 26, P < 
0.05; Fig. 2A). This suggested that the depolarization is mediated 
by a reduction in a resting K<sup>+</sup> current. If this were true, the inward 
current should reverse near the K<sup>+</sup>-reversal potential. To test this, 
current–voltage plots were obtained before and at near-peak 
inward current using voltage steps (10 mV from −120 to +10 
mV, 200-ms duration, 2-s interval) in TTX (Fig. 5, A and B). At a 
holding potential of −60 mV, application of CCK to FS1 
interneurons increased an inward current ranging from 18 to 113 
pA (44 ± 5 pA, n = 18; Fig. 5C). In individual FS1 interneurons 
the inward current reversed in three ways: between −80 and 
−117 mV (−100 ± 4 mV, n = 9), between −29 and 0 mV 
(−17 ± 6 mV, n = 4), or two reversals (n = 5) at both 
hyperpolarized (−110 ± 5 mV) and depolarized potentials (−7 ± 
6 mV). In regular-firing interneurons (n = 9), CCK evoked an 
inward current ranging from 8 to 31 pA (15 ± 3 pA, n = 7) or no 
inward current (n = 2). Among the 7 cells showing CCK effects, 
three types of reversals occurred at hyperpolarized (−80, −103, 
and −107 mV; Fig. 5C), depolarized (−18 and −18 mV), or both 
potentials (−90 and −7 mV, −120 and −34 mV). These data 
suggest involvement of two types of current, one reversing at a 
hyperpolarized potential such as K<sup>+</sup> current and another current 
reversing at a depolarized potential.

We next examined effects of CCK in the presence of 
tetraethylammonium (TEA, 10 mM) or cadmium (100 μM) in 
FS1 and regular-firing types to test cell-type–specific effects of 
K<sup>+</sup>- or Ca<sup>2+</sup>-channel blockade. TEA and cadmium 
experiments were intermixed in order. Burst-firing and FS2 types 
were not tested because the burst-firing type was very rare and 
the FS2 type showed no significant CCK effect. In FS1 neu-

FIG. 2. Cholecystokinin (CCK) excites neurons in the basolat-
eral amygdala. A: in the FS1-type interneuron, CCK (1 μM, 30 s) 
depolarized the resting membrane potential over the action potential 
threshold (same cell of Fig. 1A). Hyperpolarizing test pulses 
(−50 pA, 0.5 s, 10-s interval) were given to measure impedance. 
Inset: input resistance was increased during CCK application. 
Three traces were averaged. B: the FS2-type interneuron (same cell as in Fig. 1B) was not depolarized by CCK. C and D: burst-firing 
(C) and regular-firing neurons were depolarized by CCK. Action potentials were truncated at −30 mV.
rons, after slight depolarization by TEA (1.8 ± 0.2 mV, n = 6) and cadmium (0.8 ± 0.8 mV, n = 5), CCK still depolarized the membrane potential in TEA (9.9 ± 1.4 mV, n = 6) and cadmium (4.8 ± 0.8 mV, n = 5) (Fig. 6, A and B). In regular-firing neurons, after the membrane potential change by TEA (4.0 ± 0.8 mV) and cadmium (0.5 ± 0.5 mV, n = 5), CCK evoked little effect in the presence of TEA (0.4 ± 0.3 mV, n = 5), but still depolarized the membrane potential in cadmium (2.2 ± 0.6 mV, n = 5) (Fig. 6, C and D).

We also used a ramp protocol (Fig. 7) since the voltage-step protocol could miss transient current changes between the steps. The command voltage was continuously increased from -100 to +30 mV at 40 mV/s. The current trace before CCK was subtracted from the current trace after CCK to produce the net current change (Fig. 7, A and B, bottom graphs). For this experiment, only the FS1 interneurons were tested because they showed the greatest amplitude of CCK-induced depolarization. We tested whether TEA could block the inward current, as in hippocampal CA1 interneurons in which a CCK-induced current was masked by TEA (Miller et al. 1997). Our data indicate that the CCK effect was greatest in cells with narrow AP widths (FS1 type), which in part is a function of the TEA-sensitive K⁺ current, Kv3 (Rudy and McBain 2001). Application of TEA (10 mM) alone caused a small inward current (13 ± 2 pA, n = 8), but subsequent addition of CCK still induced a significant inward current (37 ± 3 pA, n = 4, P < 0.05; Fig. 7A). The transient current peak appeared between -4 and +4 mV in 3 of 4 cells, suggesting a high-voltage-activated calcium current (Foehring and Scroggs 1994).
In the presence of both CdCl$_2$ (100 μM) and TEA, CCK still caused an inward current of similar amplitude (36 ± 3 pA, n = 4; Fig. 7B). However, no transient current increase appeared. This result suggests that TEA-sensitive potassium currents do not underlie the actions of CCK on FS1 neurons. In addition, although CCK increases a calcium current, calcium may not be the main charge carrier because the CCK-evoked current persisted in the presence of CdCl$_2$.

We next measured the inward current in the presence of TTX (55 ± 8 pA, n = 14; Fig. 8, A and G) to determine the main carrier ion underlying the current. To further test involvement of Ca$^{2+}$ ions, extracellular Ca$^{2+}$ was reduced to a nominal concentration by omitting 2 mM Ca$^{2+}$ from the ACSF. No divalent ion (e.g., Mg$^{2+}$) was substituted (Tsujino et al. 2005). Because the Ca$^{2+}$ current was increased after CCK application in the ramp experiments, we expected that lowering extracellular Ca$^{2+}$ concentration would decrease the inward current. On the contrary, the CCK inward current amplitude was increased (117 ± 20 pA, n = 5; Fig. 8, B and G). Because the other main extracellular cation is Na$^+$, we reduced the extracellular Na$^+$ concentration to 20% by

![Figure 4](http://jn.physiology.org/)

**FIG. 4.** The CCK$_B$ receptor subtype mediates effects of CCK. A and B: under control conditions (A) and in the presence of the CCK$_A$ receptor antagonist lorglumide (1 μM, B), the amplitude of the second depolarization was still 61% that of the first depolarization. C: however, the CCK$_B$ receptor antagonist CR2945 (1 μM) significantly attenuated the 2nd CCK depolarization compared with control condition (P < 0.05). D: ratio of the 2nd to the 1st CCK response in a neuron is plotted in 3 conditions. E: the CCK$_B$ receptor agonist CCK4 (1 μM, 30 s) also depolarized interneurons (n = 5). All examples are FS1 type.
FIG. 5. The CCK-induced current reverses at depolarized (≥ −20 mV) and/or at hyperpolarized (≤ −80 mV) potential. A, left: voltage steps were applied before (Aa) and during (Ab) the slow inward current evoked by CCK. Step pulses (−120 to +20 mV, 200-ms duration, 2-s interval) were given from a holding potential of −60 mV. Right: the time point of current measurement was marked as a filled square and a filled circle. B, left: current–voltage relationship measured from A. Right: the control current was subtracted from CCK current to produce the difference current (I_{diff}). C: summary graphs of reversal patterns. FS1 and regular-firing interneurons showed 3 types of reversals at depolarized, hyperpolarized, and both membrane potentials. For regular-firing interneurons, only one type is plotted as a graph.
replacing extracellular NaCl with choline-Cl in the ACSF. This condition significantly reduced the CCK inward current amplitude (9 ± 4 pA, n = 5; Fig. 8, C and G), indicating that Na⁺ is likely the main carrier ion (P < 0.05).

From these results, we hypothesized that the current is mediated primarily by Na⁺, whereas the small Ca²⁺ current hinders Na⁺ ion flow through certain channels. This current appears similar to the previously described nonselective cation current mediated through the transient receptor potential (TRP) channel (Faber al., 2006; Meis et al. 2007; Ramsey et al. 2006; Strübing et al. 2001; Tsujino et al. 2005). We then tested whether the nonselective cation channel blocker, 2-APB, would block the CCK current. We found that the CCK current amplitude was significantly attenuated in the presence of 2-APB (100 μM, 7 ± 2 pA, n = 5, P < 0.05; Fig. 8, D and G). The TRP channel blocker SKF96365 (100 μM) slightly reduced the inward current amplitude compared with control condition, but the effect was not significant (SKF96365, 43 ± 9 pA, n = 5; Fig. 8, E and G vs. control). Because lanthanum is known to block TRP channels in millimolar concentration (Clapham 2007; Faber et al. 2006), we tested whether lanthanum (1 mM) could change the current by CCK. We used HEPES-based ACSF to prevent precipitation by lanthanum. In this condition, lanthanum significantly attenuated the current amplitude (6 ± 2 pA, n = 5) compared with control condition (23 ± 3 pA, n = 5; Fig. 8, F and H) (P < 0.05).

**DISCUSSION**

**CCK excitation and interneuron specificity**

In a previous study, CCK and pentagastrin were found to excite nonpyramidal neurons in the lateral amygdala, whereas pyramidal neurons were unaffected (Sugita et al. 1993). In our current study we found that CCK excites interneurons to varying degrees according to subtype. The amplitude of depolarization was larger in cells with narrower action potentials. Accordingly, FS1 interneurons were most sensitive to CCK. Our classification is also consistent with cortical fast-spiking interneurons (Kawaguchi 1995; Kawaguchi and Kubota 1996; Miyoshi et al. 2007).

We did not separate fast-firing and stutter-firing types for two reasons. First, a significant discrepancy exists between previous studies. In Rainnie et al. (2006), the majority of stutter-firing neurons (8 of 14) were parvalbumin (PV)-positive, but none of 10 fast-firing was PV-positive in rat BLA. However, in Woolf and Sah (2007a) fast-firing (or fast-spiking) cells were 47% of the recorded PV-positive interneurons in mouse BLA. Stutter-firing neurons were also PV-positive. As yet we are unable to account for this apparent discrepancy. Second, in a recent effort to organize cortical interneuron nomenclature, stutter-firing neurons were included under the category of fast-spiking (FS) type neurons (Ascoli et al. 2008). The firing patterns of FS cells at rheobase were subdivided into three groups: delayed response, a single onset spike, and a stuttering pattern. According to this classification, stutter-firing neurons are considered a subtype of FS cells (Ascoli et al. 2008). For these reasons we decided to include fast-firing and stutter-firing neurons as one group (FS1) until we are able to clearly distinguish between the two types.

We also found a subset of interneurons (FS2) that was insensitive to CCK. Interneurons with similar electrophysiological characteristics have been described as late-spiking interneurons with neurogliaform morphology in the cortex and hippocampus (Ascoli et al. 2008; Chu et al. 2003; Kawaguchi and Kubota 1996; Price et al. 2005). This interneuron type often contains neuropeptide Y (NPY) (Ratzliff and Soltesz 2001; Uematsu et al. 2008). NPY reduces anxiety behaviors.
when injected into the BLA (Sajdyk et al. 2002, 2008) and thus may counteract behavioral actions of CCK.

Currents activated by CCK

The primary current activated by CCK appears to be a nonselective cation current. A similar current has been reported in hippocampus (Dodd and Kelly 1981), mouse lateral amygdala (Meis et al. 2007), mouse lateral hypothalamus orexin neurons (Tsujino et al. 2005), guinea pig gastric smooth muscle cells (Wang and Sims 1998), and mouse pancreatic acinar cells (Thorn and Petersen 1992). In rat neocortex, muscarinic agonists activate a nonselective cation current that is highly voltage dependent and rapidly inactivates at hyperpolarized potentials. This voltage sensitivity results in an agonist-induced increase in apparent input resistance (Haj-Dahmane and Andrade 1996), similar to our observations.

The underlying channel for this current appears to be the TRP channel, which has subunits present in both rat and human amygdala (Faber et al. 2006; Riccio et al. 2002; Strubing et al. 2001). In projection neurons of mouse BLA, CCK induced a nonselective cation current that was completely blocked by 2-APB and the mRNA of TRP channel subunits was present in these cells (Meis et al. 2007). In projection neurons of rat lateral amygdala, a nonselective cation current activated by metabotropic glutamate receptors was significantly attenuated by 2-APB and lanthanum ion and by antibodies for the TRP channel subunits (Faber et al. 2006). These studies support the notion that the nonselective current activated by CCK may be a TRP-like current. However, unlike the two previous studies (Faber et al. 2006; Meis et al. 2007), we recorded from interneurons in rats.

The difference in cell type could be one reason why reversal did not occur within the tested ramp range (−100 to +30 mV) in our study, whereas reversal occurred near −20 mV with a ramp protocol in lateral amygdala projection neurons (Meis et al. 2007). However, with the step protocol reversals appeared in the range of −20 to 0 mV in our study (Fig. 5C). Another possibility is the presence of apparent inward current from inhibition of a K⁺ current that is not
blocked by TEA. In this case, the inward current will interfere with the reversal by offsetting the outward current of the nonselective cation current at depolarized membrane potentials (Zhang et al. 2008).

In addition to the nonselective cation current, we observed an inward current associated with CCK-induced reduction of a $K^+$ current, seen independently or concurrently with the nonselective cation current (Fig. 5). This type of inward current has been reported in thalamic reticular neurons and hippocampal interneurons (Cox et al. 1995; Miller et al. 1997). However, no other currents were reported in those studies. We also observed a possible increase in calcium current (Fig. 7), al-
though further studies are needed to determine whether CCK increases calcium current independently or whether the calcium current is secondary to the depolarization (Meis et al. 2007; Tsujino et al. 2005; Yang et al. 2007).

Functional significance

Projection neurons in the BLA are presumed glutamatergic (McDonald 1996). Stimulation of BLA increases central amygdala (CeA) activity, the main output nucleus of the amygdala complex (Quirk et al. 2003). Because CCK is anxiogenic in behavioral tests, CCK might be expected to increase the activity of BLA projection neurons (Sherrin et al. 2009). Seemingly contrary to this expectation, we did not see membrane potential depolarization in projection cells in our slice preparation. Instead, a slight indirect hyperpolarization occurred due to the combined IPSPs.

How can we explain a presumed increase of BLA projection cell firing from this? One possibility is the rebound excitation in projection cells after the IPSP. For example, in amygdala BLA slice preparation, a single action potential from a PV-positive fast-spiking cell caused a rebound increase in firing probability in a synaptically connected projection cell (Woddruff and Sah 2007b). In the same study, the firing of two projection neurons was more synchronized when GABAergic synaptic transmission was activated by electrical stimulation in the presence of glutamate receptor blockers NBQX and T-APV. In vivo intracellular recordings, a rebound action potential occurred after synaptic inhibition in lateral amygdala projection cells during depolarization (Lang and Paré 1997). In the lateral and basolateral amygdala, spontaneous activity of projection neurons is quite low, presumably due to tonic inhibition by interneuron activity (Paré and Gaudreau 1996). Thus the rebound action potential may not occur in a resting behavioral state. However, a behavioral paradigm invoking anxiety such as auditory startle-reflex may increase the activity level of amygdala projection neurons.

Alternately, it may be too simplistic to assume a generalized inhibitory response to excitation of interneurons; thus in the intact amygdala network the activation of interneurons may serve more to regulate the pattern of projection neuron activity rather than simply decreasing firing frequency. This may be suggested by the observation that although local amygdala injection of direct GABA_A receptor agonists reduces anxiety (for review see Davis et al. 1994), other agents that act more directly on GABAergic interneurons may not have a similar effect. Like CCK, serotonergic agonists also selectively excite amygdala interneurons and indirectly hyperpolarize projection neurons (Rainnie 1999). However, local intramygdala injections of serotonergic agonists generally elicit increased anxiety-like behaviors (for review see Lowry et al. 2005).

Another possible explanation for reconciling anxiogenic effects with the increase in inhibition by CCK in BLA is the potential role of the intercalated (ITC) amygdala neurons. The ITC neurons receive information from BLA and send feedforward inhibition to the central amygdala nucleus (CeA), which may inhibit fear memory (extinction) (Likhitik et al. 2008). In this study, selective lesions of ITC neurons reduced freezing. According to this hypothesis, inhibition of BLA projection cells by CCK could reduce excitatory input to ITC neurons, in turn reducing the inhibition to CeA. This could result in enhanced CeA activity, inducing expression of fear and anxiety.

In summary, CCK preferentially excites fast-spiking interneurons by an action at CCK_B receptors, mediated by a nonselective cation current. We speculate that the increased inhibitory synaptic transmission from PV-positive interneuron activation ultimately increases the projection cell firing by rebound excitation and/or network synchronization of projection neuron firing or, alternatively, has facilitatory effects on CeA activity by reducing excitatory input to ITC neurons.

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References


Hodges H, Green S, Glenn B. Evidence that the amygdala is involved in benzodiazepine and serotonergic effects on punished responding but not on discrimination. Psychopharmacology (Berl) 92: 491–504, 1987.