A novel GABAergic action mediated by functional coupling between GABA<sub>B</sub>-like receptor and two different high-conductance K<sup>+</sup> channels in cricket Kenyon cells

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Nakamura A, Yoshino M. A novel GABAergic action mediated by functional coupling between GABA<sub>B</sub>-like receptor and two different high-conductance K<sup>+</sup> channels in cricket Kenyon cells. J Neurophysiol 109: 1735–1745, 2013. First published January 9, 2013; doi:10.1152/jn.00915.2012.—The GABA<sub>B</sub>-like receptor has been shown to attenuate high-voltage-activated Ca<sup>2+</sup> currents and enhance voltage-dependent or inwardly rectifying K<sup>+</sup> currents in a variety of neurons. In this study, we report a novel coupling of GABA<sub>B</sub>-like receptor with two different high-conductance K<sup>+</sup> channels, Na<sup>+</sup>-activated K<sup>+</sup> (K<sub>Na</sub>) channel and Ca<sup>2+</sup>-activated K<sup>+</sup> (K<sub>Ca</sub>) channel, in Kenyon cells isolated from the mushroom body of the cricket brain. Single-channel activities of K<sub>Na</sub> and K<sub>Ca</sub> channels in response to bath applications of GABA and the GABA<sub>B</sub>-specific agonist SKF97541 were recorded with the cell-attached patch configuration. The open probability (P<sub>o</sub>) of both K<sub>Na</sub> and K<sub>Ca</sub> channels was found to be increased by bath application of GABA, and this increase in P<sub>o</sub> was antagonized by coapplication of the GABA<sub>B</sub> antagonist CGP54626, suggesting that GABA<sub>B</sub>-like receptors mediate these actions. Similarly, GABA<sub>B</sub>-specific agonist SKF97541 increased the P<sub>o</sub> of both K<sub>Na</sub> and K<sub>Ca</sub> channels. Perforated-patch recordings using β-escin further revealed that SKF97541 increased the amplitude of the outward currents elicited by step depolarizations. Under current-clamp conditions, SKF97541 decreased the firing frequency of spontaneous action potential (AP) and changed the AP waveform. The amplitude and duration of AP were decreased, whereas the afterhyperpolarization of AP was increased. Resting membrane potential, however, was not significantly altered by SKF97541. Taken together, these results suggest that GABA<sub>B</sub>-like receptor is functionally coupled with both K<sub>Na</sub> and K<sub>Ca</sub> channels and this coupling mechanism may serve to prevent AP formation and limit excitatory synaptic input.

GABA; GABA<sub>B</sub> receptor; Na<sup>+</sup>-activated K<sup>+</sup> channel; Ca<sup>2+</sup>-activated K<sup>+</sup> channel; mushroom body; cricket; Kenyon cell

IT HAS BEEN REVEALED that GABA activates two classes of receptors, the ionotropic GABA<sub>A</sub> and GABA<sub>C</sub> receptors and the metabotropic GABA<sub>B</sub> receptors. The metabotropic GABA<sub>B</sub> receptor has been shown to be a G protein-coupled receptor that is found both in pre- and postsynaptic membrane and mediates slow, long-term inhibition. This inhibition has been shown to be mediated by a decrease in presynaptic Ca<sup>2+</sup> influx resulting from inhibition of presynaptic voltage-gated Ca<sup>2+</sup> channels (Dittman and Regehr 1996; Lambert and Wilson 1994; Mintz and Bean 1993; Pfrieger and Martone 1994; Takahashi et al. 1998; Wu and Saggau 1995; Zhang et al. 1999). Another inhibitory action has been shown to be mediated by an increase in postsynaptic K<sup>+</sup> efflux resulting from activation of postsynaptic inward-rectifier K<sup>+</sup> channel and voltage-dependent K<sup>+</sup> channel (Bowler et al. 2002; Gahwiler and Brown 1985; Greif et al. 2000; Kaupmann et al. 1998; Labouebe et al. 2007; Li and Guyenet 1996; Slesinger et al. 1997; Slugg et al. 2003; Sodiickson and Bean 1996; Takeda et al. 2004). In addition to this well-documented coupling of GABA<sub>B</sub> receptors, Ca<sup>2+</sup>-activated K<sup>+</sup> (K<sub>Ca</sub>) channels (Blaxter et al. 1986; Koninch and Mody 1997) and TREK-2 channels (Deng et al. 2009) have also been shown to be coupled with GABA<sub>B</sub> receptor. Recently, Breton and Stuart (2012) have reported that somatic GABA<sub>B</sub> receptors are coupled to GIRK K<sup>+</sup> channels whereas dendritic GABA<sub>B</sub> receptors act to downregulate dendritic calcium electrogenesis in cortical layer 5 pyramidal neurons.

The mushroom body (MB) of the insect brain has been shown to be critical to associative memory formation. Intrinsic neurons within the MB called Kenyon cells receive sensory signals from input neurons in the calyces. The calyces of the insect MB receive wide branches of central neurons that exhibit GABA-like immunoreactivity (Liu and Davis 2009; Nishino and Mizunami 1998; Papadopoulou et al. 2011); and these GABA-like immunoreactive neurons have been considered to be inhibitory feedback neurons, and their function has been considered to be a facilitation of the acquisition of sensory signals by disinhibition when the animal is in an arousal state (Nishino and Mizunami 1998), a suppression of olfactory learning (Liu and Davis 2009), and a regulation of Kenyon cell excitability adaptively a function required to maintain the sparseness of odor representations (Papadopoulou et al. 2011). The presence of GABA<sub>B</sub>-like receptors in insect central neurons including Kenyon cells has also been suggested (Bai and Sattelle 1995; Enell et al. 2007; Mezler et al. 2001; Okada et al. 2009; Sattelle et al. 2000; Wilson and Laurent 2005). Enell et al. (2007) have provided evidence that localization of the GABA<sub>B</sub>R2 receptor is correlated with the distribution of GABA<sub>A</sub> receptor subunit RDL (for resistance to dieldrin). CGP54626, a potent and selective antagonist of vertebrate GABA<sub>B</sub> receptor, has also been shown to be effective in honeybees and other insect species (Root et al. 2008; Rotte et al. 2009; Wilson and Laurent 2005). Demmer and Kloppeburg (2009) have recently shown that CGP54626 significantly increased the input resistance and depolarized the resting potential in Kenyon cells of cockroaches Periplaneta americana. Their results implicate the possible existence of GABA<sub>B</sub>-mediated resting membrane K<sup>+</sup> conductance in Kenyon cells.

It has been reported that cricket (Acheta domesticus) Kenyon cells do not contain GABA itself but GABA and GABA
receptors show a similar distribution in MB (Strambi et al. 1998). In this species, physiological action of GABA<sub>B</sub>-like receptor on Kenyon cells has also been reported, where activation of the GABA<sub>B</sub> agonist 3-APPA induced a sustained decrease in intracellular free Ca<sup>2+</sup> (Cayre et al. 1999). Little is known, however, about the properties of GABA<sub>B</sub>-like receptors in Kenyon cells of the insect, especially the coupling mechanisms with ionic channels, although functional coupling between GABA<sub>B</sub>-like receptor and several kinds of K<sup>+</sup> channels has been suggested in cockroach motor neuron (Bai and Satelle 1995) and giant interneurons (Hue 1991).

In the present study, we investigated the properties of GABA<sub>B</sub>-like receptors of Kenyon cells by using the cricket Gryllus bimaculatus. This animal has recently been used as an excellent model to study neural mechanisms underlying associative learning because it is relatively easier to access neuropharmacological studies (Matsumoto and Mizunami 2000, 2002a, 2002b, 2004; Matsumoto et al. 2003, 2006; Unoki et al. 2005, 2006), electrophysiological studies in normally behaving animals originally applied to cockroaches (Mizunami et al. 1998; Okada et al. 1999), RNAi analysis (Takahashi et al. 2009), and patch-clamp studies of ionic channels in freshly dispersed cells (Aoki et al. 2008; Kossakai et al. 2008; Terazima and Yoshino 2010). We hypothesized that GABA<sub>B</sub>-like receptors are coupled with two different high-conductance K<sup>+</sup> channels, Na<sup>+</sup>-activated K<sup>+</sup> (K<sub>Na</sub>) and Ca<sup>2+</sup>-activated K<sup>+</sup> (K<sub>Ca</sub>) channels, that are most frequently recorded in cell-attached membrane patches in these cells. The present results indicate that GABA<sub>B</sub>-like receptor is functionally coupled with both K<sub>Na</sub> and K<sub>Ca</sub> channels. The functional significance of this novel coupling mechanism is discussed in relation to inhibitory GABA action on Kenyon cells, which are known as a higher-order central neuron in insect olfactory associative learning and memory.

**METHODS**

**Animals.** Experiments were carried out on adult male crickets (G. bimaculatus), which were maintained in a colony in the Department of Biology at 25–30°C with a relative humidity of 65–85% under a 12:12-h light-dark photoperiod. Crickets were fed an artificial insect diet (Oriental Yeast) and supplied with water. Adult male crickets were anesthetized with CO<sub>2</sub> before dissection.

**Cell isolation.** The brain was carefully removed from the head capsule, taking care not to tear the alimentary tract. MBs were then dissected out of the brain and placed in a silicone chamber (volume of 3 ml) filled with Ca<sup>2+</sup>-free normal saline and incubated for 15 min. MBs were then transferred to the vial tube containing dissociation solution (Sumitomo nerve cell culture medium, Sumitomo Bakelite). The isolated MBs were incubated in this culture medium for 30 min at 25°C. After incubation, the pooled MBs were rinsed with normal saline and dissociated by gentle trituration through a fire-polished pipette with an inner diameter of ~100 µm. Freshly dispersed cells were allowed to settle on the flat glass bottom of a silicone chamber mounted on the stage of an inverted microscope (Nikon Diaphot).

**Patch-clamp experiments.** Patch electrodes were pulled from capillary tubes (G1.5, Narishige) with a two-stage pipette puller (PP83, Narishige). The patch electrode was positioned on the cell surface with a three-dimensional hydraulic micromanipulator (MHW-3, Narishige). The reference electrode was an Ag-AgCl wire connected to the bath solution through a 100 mM KCl-agar bridge. Single-channel recordings using the cell-attached configuration and whole cell recordings using the perforated-patch configuration of the patch-clamp technique were performed. Single K<sup>+</sup> channel currents were measured in cell-attached patches by filling the patch pipettes (~5–6 MΩ) with high-K<sup>+</sup> solution containing (in mM) 140 KCl and 5 HEPES, buffered to pH 7.4 with Tris-Cl. Afterward, the bath was exchanged with 100 mM KCl, 5 HEPES, and 50 µM 3-APPA, buffered to pH 7.4 with Tris-Cl. Voltage across the patch was controlled by setting the cell membrane potential to 0 mV with a high-K<sup>+</sup> extracellular solution containing (in mM) 140 KCl, 44 glucose, and HEPES, buffered to pH 7.4 with Tris-Cl. For selective recordings of K<sub>Na</sub> and K<sub>Ca</sub> channel currents, we further added 10 mM NaCl and 3 mM CaCl<sub>2</sub>, respectively, into a high-K<sup>+</sup> extracellular bath solution. Currents were filtered at 2 kHz and digitized at 10 kHz. In all

**Fig. 1.** Recording of Na<sup>+</sup>-activated K<sup>+</sup> (K<sub>Na</sub>) and Ca<sup>2+</sup>-activated K<sup>+</sup> (K<sub>Ca</sub>) channel currents from cell-attached patch membranes of isolated Kenyon cells. A: activities of K<sub>Na</sub> channels were recorded in a bath containing a K<sup>+</sup>-rich solution with 10 mM Na<sup>+</sup>. P<sub>o</sub> open probability; TTX, tetrodotoxin. B: activities of K<sub>Ca</sub> channels. The bath contained a K<sup>+</sup>-rich solution with high Ca<sup>2+</sup> (3 mM). C: average single-channel current-voltage (I-V) relationship for K<sub>Na</sub> (left) and K<sub>Ca</sub> (right) channels. D: average relative P<sub>o</sub> of K<sub>Na</sub> channel currents in control (1.0), 1 µM TTX (0.96 ± 0.01, n = 3), 1 mM Cd<sup>2+</sup> (0.19 ± 0.06, n = 7), after washout of Cd<sup>2+</sup> (0.95 ± 0.25, n = 7), 100 µM verapamil (0.03 ± 0.009, n = 3), and after washout of verapamil (0.98 ± 0.26, n = 3). *P < 0.05.
experiments voltage clamp and voltage pulse generation were controlled with a List EPC7 patch-clamp amplifier.

For perforated-patch experiments, β-escin was prepared as a 50 mM stock solution in water and was added into the pipette solution to a final concentration of 50 μM (Fan and Palade 1998). All pipette solutions were back-filled into the pipette. The composition of the pipette solution was as follows (in mM): 140 KCl, 5 EGTA, and 2 HEPES, buffered to pH 7.4 with Tris-Cl. The extracellular solution was normal cricket Ringer solution containing (in mM) 140 NaCl, 10 KCl, 1.6 CaCl₂, 2 MgCl₂, 2 HEPES, and 44 glucose, buffered to pH 7.4 with Tris-Cl. The experiments were performed at room temperature (20–25°C).

Analysis of single-channel currents. Analysis of the unitary currents was performed by fitting all-point amplitude histograms based on the analysis of current amplitudes for 1–n channels present in the patch. The open probability \( P_o \) of single-channel currents was expressed as \( N P_o = (A_1 + 2A_2 + 3A_3 + \ldots + nA_n)/(A_0 + A_1 + A_2 + \ldots A_n) \), where \( A_0 \) is the area under the curve of an all-point amplitude histogram corresponding to the current in the closed state and \( A_1-A_n \) represents the histogram area reflecting the different open-state current levels for 1–n channels present in the patch. Histogram parameters were obtained from multiple least-squares Gaussian fits of the data with Clampfit 9.2 software (Axon Instruments). The single \( K_{Na} \) channel currents recorded from the cell-attached patch membrane solutions often showed subconductance levels of various amplitudes between the closed and full-open levels. Therefore, we focused only on the \( K_{Na} \) channel activity with the full-open level.

Drugs. The following drugs were used: tetrodotoxin (TTX) (Wako), GABA_{A}/GABA_{C} antagonist picrotoxin (PTX) (Calbiochem), GABA_{B} agonist 3-aminopropyl(methyl)phosphinic acid (SKF97541) (Tocris). GABA_{B} antagonist [S-(R*,S*)]-[3-[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl)phosphinic acid (CGP54626) (Tocris) was prepared as a 250 mM stock in DMSO, and the final concentration of DMSO in the bath was less than 1:1,000. All drug solutions were prepared freshly before use and were continuously perfused at a rate of ~5 ml/min.

Statistical analysis. Values are given as means ± SE, with \( n \) representing the number of cells. For comparisons between two groups, Student’s t-test, paired or unpaired, was used for statistical analysis, with \( P \) values < 0.05 considered statistically significant.

RESULTS

Selective recordings of \( K_{Na} \) and \( K_{Ca} \) channel currents. In this study we recorded two types of high-conductance \( K^+ \) channel currents, \( K_{Na} \) channel and \( K_{Ca} \) channel currents, with cell-attached patch-clamp techniques (Fig. 1). Single \( K_{Na} \)
channel activities were recorded in a high-K\(^+\) (140 mM) bath solution with addition of 10 mM NaCl as described previously (Aoki et al. 2008) (Fig. 1A). To demonstrate whether the single-channel activity obtained under this condition is indeed mediated by K\(_{Na}\) channels, we examined the effect of bath-applied TTX (1 \(\mu\)M) and nonspecific Ca\(^{2+}\) channel blocker Cd\(^{2+}\) (1 mM) on channel activity. \(P_o\) of this channel was reduced from 0.501 to 0.039 by addition of TTX into a bath solution, whereas little effect was observed with addition of Cd\(^{2+}\) (Fig. 1A). These results indicated that this channel activity is due to K\(_{Na}\) channels, which are activated by sodium ions crossing the cell membrane through TTX-sensitive Na\(^+\) channels.

For recording K\(_{Ca}\) channel activity, we added a high concentration of Ca\(^{2+}\) (3 mM) instead of NaCl (10 mM) into a bath solution (Fig. 1B). Single-channel activities recorded under this condition were identified as K\(_{Ca}\) channels for the following reasons: first, \(P_o\) of this channel was little affected by 1 \(\mu\)M TTX (0.069 in control, 0.067 in TTX); second, \(P_o\) of this channel was drastically reduced by 1 mM Cd\(^{2+}\) (0.02 in Cd\(^{2+}\)). We have also revealed that the vertebrate L-type Ca\(^{2+}\) channel blocker verapamil (100 \(\mu\)M) reduced \(P_o\) in another five or six cells (Fig. 1D). The average values of relative \(P_o\) examined from three to seven cells were as follows: 1.0 (control), 0.96 ± 0.01 (TTX), 0.19 ± 0.06 (Cd\(^{2+}\)), 0.95 ± 0.25 (after wash of Cd\(^{2+}\)), 0.03 ± 0.001 (verapamil), and 0.99 ± 0.26 (after wash of verapamil). The average single-channel conductance of K\(_{Na}\) and K\(_{Ca}\) channels was 112 and 122 pS (\(GABA \cdot \text{nA/mV}\)) in control, 0.254; 50 \(\mu\)M: 0.25; 100 \(\mu\)M: 0.25 (after wash of Cd\(^{2+}\)). These results showed that activation of KCa channel by GABA, without PTX may shunt the cell membrane because of an increase in Cl\(^-\) conductance and thereby may affect GABA\(_B\) receptor action indirectly. Application of PTX alone had no significant effect on \(P_o\) of K\(_{Na}\) channels (Fig. 3). \(P_o\) was 0.004 in control and 0.005 in the presence of PTX. Further addition of GABA increased \(P_o\) significantly to 0.01 (GABA + PTX) from 0.005 (PTX). The average values of relative \(P_o\) from three to five cells were as follows: 1.0 (control), 0.98 ± 0.16 (PTX), and 3.31 ± 0.81 (GABA + PTX). These results further confirmed that activation of K\(_{Na}\) channels in response to GABA application is mediated by GABAB-like receptor and activation of GABA\(_A\)/GABA\(_C\) receptors contributes little to K\(_{Na}\) channel activity.

**Effects of GABA\(_B\) antagonist CGP54626 on GABA-induced effect on K\(_{Na}\) channel currents.** The effect of GABA on K\(_{Na}\) channel activity in Kenyon cells was investigated. Figure 2A, left, shows control records in the absence of GABA at a holding potential of +60 mV. When 100 \(\mu\)M GABA was applied to the bath solution, the channel activities increased (Fig. 2A, right). As a result, \(P_o\) of K\(_{Na}\) channel increased from 0.132 (control) to 0.415 (GABA) in this cell. To investigate whether the stimulatory effect of GABA on K\(_{Na}\) channel is due to GABA\(_B\) receptor activation, we examined the effect of GABA\(_B\)-specific antagonist CGP54626 on GABA-induced effect on K\(_{Na}\) channels. As shown in Fig. 2B, addition of CGP54626 (100 \(\mu\)M) reduced \(P_o\) from 0.013 (GABA) to 0.004 (GABA + CGP54626) in this cell. The average values of relative \(P_o\) from five to seven cells were as follows: 1.0 (control), 2.9 ± 0.4 (GABA), 0.99 ± 0.3 (GABA + CGP54626), and 1.06 ± 0.1 (CGP54626) (Fig. 2C). To investigate the dose-dependent effect of CGP54626 on the GABA-induced effect, we applied bath solution containing CGP54626 at concentrations of 10, 50, and 100 \(\mu\)M. As shown in Fig. 2D, CGP54626 dose-dependently decreased \(P_o\) of K\(_{Na}\) channels in the presence of GABA (10 \(\mu\)M: 0.254; 50 \(\mu\)M: 0.016; 100 \(\mu\)M: 0.005) in the same cell. These results further confirmed that activation of K\(_{Na}\) channels by GABA is mediated by GABA\(_B\)-like receptor activation.

We next examined the effect of GABA on K\(_{Ca}\) channels in the condition where GABA\(_A\)/GABA\(_C\) receptors are blocked by their specific antagonist PTX, since bath application of GABA without PTX may shunt the cell membrane because of an increase in Cl\(^-\) conductance and thereby may affect GABA\(_B\) receptor action indirectly. Application of PTX alone had no significant effect on \(P_o\) of K\(_{Na}\) channels (Fig. 3). \(P_o\) was 0.004 in control and 0.005 in the presence of PTX. Further addition of GABA increased \(P_o\) significantly to 0.01 (GABA + PTX) from 0.005 (PTX). The average values of relative \(P_o\) from three to five cells were as follows: 1.0 (control), 0.98 ± 0.16 (PTX), and 3.31 ± 0.81 (GABA + PTX). These results further confirmed that activation of K\(_{Na}\) channels in response to GABA application is mediated by GABA\(_A\)-like receptor and activation of GABA\(_A\)/GABA\(_C\) receptors contributes little to K\(_{Na}\) channel activity.

**Effects of SKF97541 on K\(_{Na}\) and K\(_{Ca}\) channel currents.** The effect of GABA on K\(_{Ca}\) channel activity in Kenyon cells was investigated. Throughout the experiment, PTX was added into a bath solution to exclude a possible contribution of GABA\(_A\)/GABA\(_C\) receptors. Figure 4A, left, shows the control records in the absence of GABA at a holding potential of +60 mV. When 100 \(\mu\)M GABA was applied to the bath solution, the channel activities increased (Fig. 4A, right). As a result, \(P_o\) of K\(_{Ca}\) channels increased from 0.046 (control) to 0.383 (GABA) in this cell. To investigate whether the stimulatory effect of GABA on K\(_{Ca}\) channel is due to GABA\(_B\) receptor activation, the effect of CGP54626 on GABA-induced action was examined. As shown in Fig. 4B, addition of CGP54626 (100 \(\mu\)M) reduced \(P_o\) from 0.137 (GABA) to 0.105 (GABA + CGP54626) in this cell. The average values of relative \(P_o\) examined in five to seven cells were as follows: 1.0 (control), 3.81 ± 1.15 (GABA), 1.77 ± 0.67 (GABA + CGP54626), and 1.03 ± 0.02 (CGP54626). These results showed that activation of K\(_{Ca}\) channel by GABA is mediated by GABA\(_B\)-like receptor.

**Effects of SKF97541 on K\(_{Na}\) and K\(_{Ca}\) channel currents.** To further confirm whether GABA action on K\(_{Na}\) and K\(_{Ca}\) chan-
channels is mediated by GABA\textsubscript{B} receptor, we investigated the effects of GABA\textsubscript{B}-specific agonist SKF97541 on K\textsubscript{Na} and K\textsubscript{ca} channel activities in Kenyon cells. Single K\textsubscript{Na} channel currents were recorded in cell-attached patches in a bath with the addition of 10 mM NaCl. $P_\text{o}$ of K\textsubscript{Na} channel was increased from 0.165 to 0.302 (Fig. 5A) by SKF97541 in this cell. The average value of relative increase in $P_\text{o}$ examined in five cells was 5.38 ± 0.88 after addition of 100 µM SKF97541 (Fig. 5B). Single K\textsubscript{Ca} channel currents were also recorded in a bath with the addition of 3 mM CaCl\textsubscript{2}. $P_\text{o}$ of K\textsubscript{Ca} channel was also increased, from 0.127 to 0.782 (Fig. 5C). The average value of relative increase in $P_\text{o}$ examined in six cells was 4.31 ± 1.32 after addition of 100 µM SKF97541 (Fig. 5D). These results appear to further support our hypothesis that GABA\textsubscript{B} receptor is linked to both K\textsubscript{Na} and K\textsubscript{Ca} channels in cricket Kenyon cells.

Whole cell current properties of cricket Kenyon cells and effect of SKF97541 on outward $K^+$ current. The previous observations showed that channel activities of both K\textsubscript{Na} and K\textsubscript{Ca} channels were increased by bath-applied SKF97541. Therefore, it could be considered that SKF97541 increases whole cell outward $K^+$ currents, which are sensitive to Na\textsuperscript{+} and Ca\textsuperscript{2+} influx through voltage-dependent Na\textsuperscript{+} and Ca\textsuperscript{2+} channels, respectively. Therefore, we first examined the basic properties of net inward and outward current in cricket Kenyon cells in normal physiological saline. We applied the perforated-patch-recording technique, using β-escin in order to prevent the loss of signal substances associated with downstream pathway of GABA\textsubscript{B} receptors. Figure 6Aa shows a typical example showing the superimposed current traces obtained by step depolarization to +50 mV from a holding potential of −60 mV with 10-mV increments in isolated Kenyon cells. The net current is composed of the rapidly activating inward and the following rapidly activating and sustained outward current (Fig. 6Aa). When extracellular Na\textsuperscript{+} was removed by substituting the NaCl with choline Cl, the outward current reduced as well as the inward current (Fig. 6Ab). The $I$-$V$ curves for the net inward and outward current in control and Na\textsuperscript{+}-free solution revealed that the amplitude of both inward and outward current was reduced. $P_\text{o}$ of outward current measured at +50 mV reduced to 69% ($N = 5$, $P = 0.05$) from control (100%) (Fig. 6, B and D), indicating that ~30% of the total outward current is mediated by K\textsubscript{Na} channel. Further removal of Ca\textsuperscript{2+} from Na\textsuperscript{+}-free external solution completely abolished the inward current, but the amplitude of outward current was also reduced to 17.91 ± 3.19% ($N = 10$) from control (100%) (Fig. 6, C and D). This result indicated that ~80% of the

Fig. 4. Effect of GABA\textsubscript{B} antagonist CGP54626 on GABA-induced effect on K\textsubscript{Ca} channel activity recorded from isolated Kenyon cells. A, top: representative cell-attached single-channel recordings before (Control) and after (GABA) addition of GABA to bathing solutions containing 3 mM CaCl\textsubscript{2}. Membrane potential was set to +60 mV. Bottom: corresponding all-point amplitude histogram. O\textsubscript{1} and O\textsubscript{2}, 1- and 2-channel opening, respectively. $B$: effect of CGP54626 on GABA induced-effect on K\textsubscript{Ca} channel activity: representative cell-attached single-channel recordings before (Control), after the addition of GABA, and after addition of GABA + CGP54626. All recordings were performed in the presence of PTX. $C$: average relative $P_\text{o}$ of K\textsubscript{Ca} channel currents in control (1.0), GABA (3.81 ± 1.15, $n = 6$), GABA + CGP54626 (1.77 ± 0.67, $n = 4$), and CGP54626 (1.03 ± 0.02, $n = 3$). *$P < 0.05$. 

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The total outward current must be accomplished by both sodium and calcium ions crossing the membrane into the cell. This is consistent with our previous finding that the activities of K\textsubscript{Na} and K\textsubscript{Ca} channels are most frequently recorded in cell-attached patch membranes.

In light of these results, we next examined whether the outward current increases with SKF97541. A step depolarization to 30 mV from a holding potential of 0.03 mV caused a gradual increase in the outward current and its amplitude reached a maximum at 10 min after application of SKF97541. During the drug action an increase in the amplitude of both the transient (peak) and the steady outward current was seen, and washing out the cell with normal saline solution recovered the cell to the original level relatively slowly, in <5 min (Fig. 6F). Figure 6G shows that the average values of relative peak amplitude of the outward current increased significantly from 1 (control) to 1.47 ± 0.2 (SKF97541) and were recovered to the control level (1.02 ± 0.03) by washout of the drug. The slow time course of agonist action on the outward current was similar to that on single K\textsubscript{Na} and K\textsubscript{Ca} channel activities. These results imply that an increase in outward current in the continuous presence of SKF97541 could be explained primarily by activation of both K\textsubscript{Ca} and K\textsubscript{Na} channels.

**Fig. 5. Effect of SKF97541 on K\textsubscript{Na} and K\textsubscript{Ca} channel activity recorded from isolated Kenyon cells.** A. **top:** effect of SKF97541 (100 μM) on K\textsubscript{Na} channel activity recorded from isolated Kenyon cells. **Bottom:** corresponding all-point amplitude histogram. B: average relative P\textsubscript{o} of K\textsubscript{Na} channel currents in control (1.0) and SKF97541 (5.38 ± 0.88, n = 5). C. **top:** effect of SKF97541 (100 μM) on K\textsubscript{Ca} channel activity recorded from isolated Kenyon cells. **Bottom:** corresponding all-point amplitude histogram. D: average relative P\textsubscript{o} of K\textsubscript{Ca} channel currents in control (1.0) and SKF97541 (4.31 ± 1.32, n = 6). *P < 0.05.

**Effect of SKF97541 on action potentials in Kenyon cells.** To get insight into the functional significance of the coupling mechanisms between GABA\textsubscript{B}R-like receptor and two different high-conductance K\textsuperscript{+} channels, we next examined the effect of SKF97541 (100 μM) on the spontaneous firing of action potentials (APs) in isolated Kenyon cells. Cells were bathed in normal Ringer solution, and current-clamp recordings were made (Fig. 7A). Approximately 70% of the isolated large Kenyon cells showed a sustained tonic firing of APs with an average frequency of 1.8 ± 0.3 Hz (n = 8). Figure 7A shows a typical example showing the effect of SKF97541 on this spontaneous firing of APs. A small but a significant reduction of the frequency of spontaneous firing of APs was observed during the period of drug application. AP firing frequency reduced to 1.3 ± 0.3 Hz (SKF97541) (Table 1). Next, we compared the AP waveform in the absence and the presence of the drug. The mean amplitude of AP was significantly decreased by SKF97541 from 62.0 ± 2.8 mV to 58.4 ± 2.8 mV (n = 8, P < 0.05; Table 1). The mean duration of AP was also decreased significantly from 10.6 ± 0.7 ms to 9.4 ± 0.6 ms by SKF97541 (Table 1). The magnitude of membrane afterhyperpolarization (AHP) was increased significantly from 6.7 ± 0.4 mV to 7.7 ± 0.7 mV by SKF97541 (Table 1). However, the resting membrane potential (RMP) was not significantly altered by SKF97541 (−49.1 ± 0.5 mV in control and −48.1 ± 0.7 mV in SKF97541; Table 1).
DISCUSSION

The remaining 30% of the cells did not present any spontaneous firing. However, as shown in Fig. 7C, the AP could be induced by application of depolarizing current injection in these cells, as has previously been reported (Terazima and Yoshino 2010). When SKF97541 was applied to such cells, the AP almost completely disappeared in response to depolarizing current injection in control conditions (extracellular [Na\(^+\)] = 140 mM; a) and 5 min after external Na\(^+\) removal (extracellular [Na\(^+\)] = 0 mM; b). a–b: Na\(^+\)-sensitive currents calculated by subtraction of b currents from a currents. B: averaged I-V relationships of net inward and outward currents measured at peak in control and in Na\(^+\)-free solution. Each point represents mean ± SE (n = 5). C: effect of both Na\(^+\) and Ca\(^{2+}\) removal from external solution on net inward and outward current. c and d. Currents under control conditions (extracellular [Na\(^+\)] = 140 mM, [Ca\(^{2+}\)] = 1.6 mM; c) and 5 min after external Na\(^+\) and Ca\(^{2+}\) removal (extracellular [Na\(^+\)] = 0 mM, [Ca\(^{2+}\)] = 0 mM; d). c–d, Na\(^+\)- and Ca\(^{2+}\)-sensitive currents calculated by subtraction of c currents from d currents. D: relative amplitude of net inward and outward currents in control (1.0), inward current with both Na\(^+\) and Ca\(^{2+}\) removal (0), and outward current with both Na\(^+\) and Ca\(^{2+}\) removal (0.2 ± 0.04). E: superimposed current traces elicited by depolarizing cell from a holding potential of −60 mV to 30 mV are shown under control conditions, 5 min after application of SKF97541, 10 min after application of SKF97541, and 5 min after washout of the drug; 50 μM β-escin was included in the patch pipettes. F: effect of SKF97541 on the amplitude of the net inward current and outward current measured at peak and steady levels. Same cell as in E. G: relative peak amplitude of outward current in control (1.0), in 100 μM SKF97541 (1.47 ± 0.2, n = 3), and after washout of SKF97541 (1.02 ± 0.03, n = 3). *P < 0.05.

The present study showed that application of GABA increased \(P_0\) of both \(K_{Na}\) and \(K_{Ca}\) channels, indicating that GABA action on \(K_{Na}\) and \(K_{Ca}\) channels is mediated by GABA\(_B\)-like receptors. A reduction of spontaneous firing frequency of AP and a change in AP waveform elicited by bath application of SKF97541 implicate an important regulatory action of GABA via functional coupling between GABA\(_B\)-like receptors and high-conductance \(K_{Na}\) and \(K_{Ca}\) channels.

Selective recordings from two different big \(K^+\) channels. In this study, we could selectively record \(K_{Na}\) and \(K_{Ca}\) channel activity by simply adding NaCl (10 mM) and CaCl\(_2\) (3 mM), respectively, into a high-\(K^+\) bath solution. To demonstrate whether the single-channel activity recorded from cell-attached patch membrane in the presence of 3 mM CaCl\(_2\) in a bath

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**Fig. 6.** Inward current (Na\(^+\) and Ca\(^{2+}\)) dependence of outward current in cricket Kenyon cells and its modulation by SKF97541. A: a and b, currents under control conditions (extracellular [Na\(^+\)] = 140 mM; a) and 5 min after external Na\(^+\) removal (extracellular [Na\(^+\)] = 0 mM; b). a–b: Na\(^+\)-sensitive currents calculated by subtraction of b currents from a currents. B: averaged I-V relationships of net inward and outward currents measured at peak in control and in Na\(^+\)-free solution. Each point represents mean ± SE (n = 5). C: effect of both Na\(^+\) and Ca\(^{2+}\) removal from external solution on net inward and outward current. c and d. Currents under control conditions (extracellular [Na\(^+\)] = 140 mM, [Ca\(^{2+}\)] = 1.6 mM; c) and 5 min after external Na\(^+\) and Ca\(^{2+}\) removal (extracellular [Na\(^+\)] = 0 mM, [Ca\(^{2+}\)] = 0 mM; d). c–d, Na\(^+\)- and Ca\(^{2+}\)-sensitive currents calculated by subtraction of c currents from d currents. D: relative amplitude of net inward and outward currents in control (1.0), inward current with both Na\(^+\) and Ca\(^{2+}\) removal (0), and outward current with both Na\(^+\) and Ca\(^{2+}\) removal (0.2 ± 0.04). E: superimposed current traces elicited by depolarizing cell from a holding potential of −60 mV to 30 mV are shown under control conditions, 5 min after application of SKF97541, 10 min after application of SKF97541, and 5 min after washout of the drug; 50 μM β-escin was included in the patch pipettes. F: effect of SKF97541 on the amplitude of the net inward current and outward current measured at peak and steady levels. Same cell as in E. G: relative peak amplitude of outward current in control (1.0), in 100 μM SKF97541 (1.47 ± 0.2, n = 3), and after washout of SKF97541 (1.02 ± 0.03, n = 3). *P < 0.05.
solution is indeed due to $K_{Ca}$ channels, we examined the effects of bath-applied $Na^+$ channel blocker TTX (1 μM), organic $Ca^{2+}$ channel blocker verapamil (100 μM), and non-specific $Ca^{2+}$ channel blocker Cd$^{2+}$ (1 mM). The result showed that $P_o$ of single-channel currents was decreased by bath-applied verapamil and Cd$^{2+}$ but was unaffected by TTX. Contrary to this, $P_o$ of single-channel activity recorded from cell-attached patch membrane in the presence of NaCl (10 mM) in a bath solution was unaffected by Cd$^{2+}$ but decreased by TTX (1 μM). From these observations, the high-conductance $K_{Ca}$ channels recorded in the cell-attached configuration with 10 mM NaCl and 3 mM CaCl$_2$ in a bath solution could be identified as $K_{Na}$ and $K_{Ca}$ channels, respectively. Recent studies have shown that voltage-dependent $Ca^{2+}$ channels provide the primary source of $Ca^{2+}$ for activation of $K_{Ca}$ channels (Womack et al. 2004). A selective coupling of $K_{Ca}$ channels to a specific class of voltage-gated $Ca^{2+}$ channels has been shown to be achieved by localization of the $K_{Ca}$ channels near the $Ca^{2+}$ channels within the region in which $Ca^{2+}$ increases locally when the $Ca^{2+}$ channel opens (Womack et al. 2004). Several lines of evidence also have suggested that large-conductance $K_{Ca}$ channels could be activated by the domain of ions entering through closely associated calcium channels (Fakler and Adelman 2008; Gola and Crest 1993; Marrion and Tavalin 1998; Protti and Uchitel 1997; Roberts et al. 1990; Thompson 1994). Our previous studies on cricket Kenyon cells have shown the existence of voltage-dependent L-type $Ca^{2+}$ channels (Kosakai et al. 2008). Therefore, in high-$K^+$ bath solution, these $Ca^{2+}$ channels would be activated and provide the primary source of $Ca^{2+}$ for activation of $K_{Ca}$ channels located in the cell-attached patch membranes of isolated Kenyon cells. A possible functional coupling between $K_{Ca}$ channels and L-type $Ca^{2+}$ channels needs to be investigated in the future.

Similarly, the close association of $K_{Na}$ channel with TTX-sensitive voltage-dependent $Na^+$ channel has been reported (Budelli et al. 2009; Hage and Salkoff 2012). Hage and Salkoff (2012) have provided evidence that $Na^+$ influx through persistent $Na^+$ channels is sufficient for activation of $K_{Na}$ channels, without substantial contribution from the transient $Na^+$ current or bulk [$Na^+$]. In the present study we have shown that relatively ~30% of total outward current in cricket Kenyon cells is due to $K_{Na}$ channels, and our preliminary data indicate the presence of TTX-sensitive persistent $Na^+$ current in isolated cricket Kenyon cells, indicating that persistent $Na^+$ current may also be involved in the activation of $K_{Na}$ channel activity (unpublished observation), although it remains uncertain whether functional coupling between $K_{Na}$ channel and persistent $Na^+$ channel exists in cricket Kenyon cells.

**Physiological implications.** In insects, the calyces of Kenyon cells have been shown to receive synaptic input from cholinergic projection neurons that convey odor information. Axons of projection neurons enter the calyces of the MB and make synaptic connection with Kenyon cells (Nishino and Mizunami 1998; Yasuyama et al. 2002). Released acetylcholine (ACh) then activates nicotinic acetylcholine receptors (nAChRs) and induces fast excitatory synaptic potentials in Kenyon cells (Su and O’Dowd 2003). Many studies on nAChR in Kenyon cells have shown that it is a cation-selective channel having $Na^+$ and $Ca^{2+}$ permeability (Campusano et al. 2007; Goldberg et al. 1999; Oertner et al. 1999, 2001; Single and Borst 2002; Su and O’Dowd 2003). Therefore, it is conceivable that $Na^+$ and $Ca^{2+}$ channels are involved in the regulation of the Kenyon cells.

**Table 1. Properties of RMP and AP waveform in absence and presence of SKF97541**

<table>
<thead>
<tr>
<th></th>
<th>RMP, mV</th>
<th>Frequency, Hz</th>
<th>Duration, ms</th>
<th>Amplitude, mV</th>
<th>AHP, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-49.1 ± 0.5</td>
<td>1.8 ± 0.3</td>
<td>10.6 ± 0.7</td>
<td>62.0 ± 2.8</td>
<td>6.7 ± 0.4</td>
</tr>
<tr>
<td>SKF97541</td>
<td>-48.1 ± 0.7</td>
<td>1.3 ± 0.3*</td>
<td>9.4 ± 0.6*</td>
<td>58.4 ± 2.8*</td>
<td>7.7 ± 0.7*</td>
</tr>
</tbody>
</table>

Values are means ± SE for $n = 8$ cells. Comparison of the properties of resting membrane potential (RMP) and action potential (AP) waveform in the absence (control) and presence of SKF97541. Statistical significance was assessed by a Student’s $t$-test, paired or unpaired. *$P < 0.05$. 

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influx via nAChR can activate both K\textsubscript{Na} and K\textsubscript{Ca} channels and would result in a reduction of the amplitude of excitatory synaptic potentials. Indeed, it has already been reported that K\textsubscript{Na} channels are colocalized with AMPA receptors and the Na\textsuperscript{+} influx via AMPA receptor activates K\textsubscript{Na} channels and results in a decrease in the amplitude of excitatory synaptic potentials (Nanou and El Manira 2007; Nanou et al. 2008). Similarly, it has been reported that Ca\textsuperscript{2+} influx via NMDA receptor activates small-conductance K\textsubscript{Ca} channels and results in a decrease in the amplitude of excitatory synaptic potentials (Ngo-Anh et al. 2005). Recently Watanabe et al. (2011) have suggested that nAChRs that is specifically antagonized by mecamylamine (Mec) has been shown to play an important role in olfactory conditioning. If selective coupling between Mec-sensitive nAChRs and both K\textsubscript{Na} and K\textsubscript{Ca} channels exists in cricket Kenyon cells, then it may serve as a novel mechanism to reduce excitatory synaptic input impinging upon Kenyon cells from cholinergic projection neurons originated in antennal lobe. Therefore, if GABA is released on Kenyon cells and \( P_0 \) of K\textsubscript{Na} and K\textsubscript{Ca} channels located in postsynaptic membrane increases via activation of GABA\textsubscript{B}-like receptor, it may result in a decrease in the amplitude of excitatory synaptic potentials (subthreshold membrane depolarization) or an acceleration of AP membrane repolarization by enhancing the negative-feedback loops initiated from Na\textsuperscript{+} and Ca\textsuperscript{2+} influx through voltage-dependent Na\textsuperscript{+} and Ca\textsuperscript{2+} channels, respectively (Fig. 8). Further work is required to determine the immunocytochemical localization of GABA\textsubscript{B} receptors and nAChRs in the MB of the cricket G. bimaculatus.

It has been shown that AHP following the AP may underlie the main determinant of spike frequency regulation, and AHP after a single AP is largely due to K\textsubscript{Ca} channels, activated by calcium entering the cell during the AP. In cerebellar Purkinje neurons, BK (big-conductance K\textsubscript{Ca}) channels have been proposed to regulate membrane excitability by contributing to AHPs and by shaping individual APs (Womack and Khodakhah 2002). Similarly, big-conductance K\textsubscript{Ca} channel in cricket Kenyon cells may also participate in local negative-feedback loops by coupling with Ca\textsuperscript{2+} entry through voltage-dependent L-type Ca\textsuperscript{2+} channels as reported previously (Kosakai et al. 2008). A reduction of spontaneous firing frequency of AP and a change in AP waveform (a reduction of AP amplitude, a reduction of AP duration, and an increase in AHP) have also been reported as an action of big-conductance K\textsubscript{Ca} channel openers such as NS1619 and NS11021 (Bentzen et al. 2007; Zhang et al. 2003). These aspects appear to further support our hypothesis that GABA\textsubscript{B}-like receptor is coupled to K\textsubscript{Ca} channels.

In our study, RMP did not show significant change after application of SKF97541. This result implied that K\textsubscript{Na} and K\textsubscript{Ca} channels in cricket Kenyon cells contribute little to the resting membrane conductance when the cell is quiescent. Our preliminary observation indicated that the small-conductance K\textsubscript{Ca} channel-specific blocker apamin depolarized the Kenyon cell membrane potently, whereas the big-conductance K\textsubscript{Ca} channel-specific blocker iberiotoxin had little effect on RMP, indicating that both K\textsubscript{Ca} and K\textsubscript{Na} channels in cricket Kenyon cells are exclusively used for diminishing excitatory synaptic input via enhancing negative-feedback loops initiated by Ca\textsuperscript{2+} and Na\textsuperscript{+} influx through voltage-dependent Ca\textsuperscript{2+} and Na\textsuperscript{+} channels, respectively. It remains to be established whether the small-conductance K\textsubscript{Ca} channel in cricket Kenyon cells is mainly responsible for RMP.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.N. and M.Y. conception and design of research; A.N. and M.Y. performed experiments; A.N. and M.Y. interpreted results of experiments; A.N. and M.Y. prepared figures; A.N. and M.Y. drafted manuscript; A.N. and M.Y. edited and revised manuscript; A.N. and M.Y. approved final version of manuscript.

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