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CO_2 per se activates carbon dioxide receptors

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Abstract

Carbon dioxide has been used in traps for more than six decades to monitor mosquito populations and help make informed vector management decisions. CO_2 is sensed by gustatory receptors (GRs) housed in neurons in the maxillary palps. CO_2-sensitive GRs have been identified from the vinegar fly and mosquitoes, but it remains to be resolved whether these receptors respond to CO_2 or bicarbonate. As opposed to the vinegar fly, mosquitoes have three GR subunits, but it is assumed that subunits GR1 and GR3 form functional receptors. In our attempt to identify the chemical species that bind these receptors, we discovered that GR2 and GR3 are essential for receptor function and that GR1 appears to function as a modulator. While *Xenopus* oocytes coexpressing *Culex quinquefasciatus* subunits CquiGR1/3 and CquiGR1/2 were not activated, CquiGR2/3 gave robust responses to sodium bicarbonate. Interestingly, CquiGR1/2/3-coexpressing oocytes gave significantly lower responses. That the ternary combination is markedly less sensitive than the GR2/GR3 combination was also observed with orthologs from the yellow fever and the malaria mosquito. By comparing responses of CquiGR2/CquiGR3-co-expressing oocytes to sodium bicarbonate samples (with or without acidification) and measuring the concentration of aqueous CO_2, we showed that there is a direct correlation between dissolved CO_2 and receptor response. We then concluded that subunits GR2 and GR3 are essential for these carbon dioxide-sensitive receptors and that they are activated by CO_2 per se, not bicarbonate.

Keywords

Culex quinquefasciatus; *Anopheles gambiae*; *Aedes aegypti*; CquiGR1; CquiGR2; CquiGR3

1. Introduction

Carbon dioxide is the oldest known and the most powerful mosquito attractant (Van Thiel and Weurman, 1947). It has been widely used for decades for trapping host-seeking female

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Author contributions

P.X., X.W., and W.S.L. performed research; W.S.L. designed research; P.X. and WS.L. analyzed the data; W.S.L. wrote the paper; all authors read and approved the final manuscript.

Competing financial interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.103284.

mosquitoes (Reeves, 1951, 1953) to monitoring populations and to assist in integrated vector management programs. In addition to being an attractant sensu stricto, CO_2 gates mosquito perception of other sensory modalities (Gillies, 1980; Liu and Vosshall, 2019; McMeniman et al., 2014). In the vinegar fly, *Drosophila melanogaster,* CO_2 elicits through different pathways attraction and aversion behaviors (van Breugel et al., 2018). Mosquitoes sense CO_2 with olfactory receptor neurons (ORNs) (Grant et al., 1995; Majeed et al., 2017; Syed and Leal, 2007) housed in peg sensilla (= capitate pegs) in the maxillary palps (McIver and Charlton, 1970). It has been unambiguously demonstrated that CO_2 is sensed by the gustatory receptors DmelGR21a and DmelGR63a, housed in antennal neuron ab1C of the vinegar fly (Jones et al., 2007; Kwon et al., 2007; Suh et al., 2004), but it remains to be resolved whether carbon dioxide receptors are activated by CO_2 per se or bicarbonate (Jones et al., 2007; Kwon et al., 2007; Ning et al., 2016; Xu and Anderson, 2015).

Mosquitoes have three closely related homologs of DmelGR21a and DmelGR63a (Robertson and Kent, 2009), GR1, GR2, and GR3. Because they were identified before the new nomenclature for these GR genes was proposed (Robertson and Kent, 2009), GR1–3 in the malaria mosquito, *Anopheles gambiae,* are still named AgamGR22, AgamGR23, and AgamGR24, respectively (Hill et al., 2002). While it has been clearly demonstrated that GR3 is essential for CO_2 reception in the yellow fever mosquito, *Aedes aegypti* (McMeniman et al., 2014), it is not yet known whether GR1 and GR2 are functionally redundant (McMeniman et al., 2014). While no response to CO_2 was recorded when both AgamGR22 and AgamGR24 (one copy of each) were coexpressed in the empty neuron system of the vinegar fly (Hallem et al., 2004), by increasing the dosage of both transgenes by two-fold led to significant response (Lu et al., 2007). Additionally, flies carrying one copy of each of the three subunits showed a significant response to CO_2, albeit not as strong as responses recorded from the flies carrying two-fold of AgamGR22 and 24 (Lu et al., 2007). Thus, AgamGR23 was implicated in CO_2 reception in the malaria mosquito, but its role was not clarified. On the other hand, by using RNAi combined with behavioral measurements, it has been suggested that AaegGR2 had no role in CO_2 reception by the yellow fever mosquito (Erdelyan et al., 2012). In summary, it remains to be determined whether all three GR subunits are functionally required for CO_2 detection in mosquitoes or whether GR1 and GR2 are functionally redundant (McMeniman et al., 2014).

We envisioned that having a functional carbon dioxide-detecting system in an aqueous environment as in the *Xenopus* oocyte recording system would allow us to address with the Le Chatelier's principle the long-standing question whether carbon dioxide receptors are activated by CO_2 per se or by bicarbonate (Jones et al., 2007; Kwon et al., 2007; Ning et al., 2016; Xu and Anderson, 2015). Because dissolved CO_2 forms an equilibrium with bicarbonate in water $\left(CO_2(aq) \rightleftarrows H^+ + HCO_3^-\right)$, a shift of the equilibrium towards dissolved CO_2 would increase a receptor response if CO_2 per se binds carbon dioxide receptors, whereas a reduced response would indicate activation by bicarbonate. Here, we show that CO_2 per se, not bicarbonate, activates the receptors. While preparing to address this question, we discovered that the gustatory receptor CquiGR2 from the southern house mosquito, *Culex quinquefasciatus,* is essential for function, whereas CquiGR1 appears to be a modulator. Additionally, we show a similar GR1 effect in the *Ae. aegypti* and *An. gambiae*

CO_2-sensing system. Specifically, CO_2 elicited weaker responses in the ternary receptor systems than in the respective binary counterparts devoid of GR1 (GR22 in the case of the malaria mosquito receptors).

2. Material and methods

2.1. RNA extraction, DNA synthesis, and cloning

Three hundred pairs of palps from 4- to 6-d-old female *Culex* mosquitoes were dissected under a stereomicroscope (Zeiss, Stemi DR 1663) and collected in 75% (vol/vol) ethanol diluted in diethylpyrocarbonate (DEPC)-treated water on ice. The samples were centrifuged, and ethanol was removed before total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized from 1 μg of total RNA using GoScript™ Reverse Transcription kit according to the manufacturer's instructions (Promega). For *An. gambiae* and *Ae. aegypti,* gene sequences of AgamGR22, 23, 24 and AaegGR1, 2, 3 from Genbank were synthesized by GenScript USA Inc. (Piscataway, NJ). Full-length gene-specific cloning primers were designed based on the sequences from Genbank and added 15-bp In-Fusion cloning adapters.

CqGR1-Fw GATCAATTCCCCGGGaccATGATTCACAGTCAGATGGAGGACG

CqGR1-Rv CAAGCTTGCTCTAGACTATTGTGGGTTCGTTTTGGCCG.

CqGR2-Fw GATCAATTCCCCGGGaccATGGTCATCAAGGACAGTGACTTTGAC

CqGR2-Rv CAAGCTTGCTCTAGATTAGTGAGCGTGAGCTTTCTGTAAATTCTC.

CqGR3-Fw GATCAATTCCCCGGGaccATGAGCATATTTCCGGATACTCTGCG

CqGR3-Rv CAAGCTTGCTCTAGATCAATGCGCTGCCGTCG.

AgGR22-Fw GATCAATTCCCCGGGaccATGATTCACACACAGATGGAAG

AgGR22-Rv CAAGCTTGCTCTAGATTAGGTGTTCACTTTGTCTGC.

AgGR23-Fw GATCAATTCCCCGGGaccATGGTTATCAAGGAAAGTGAGTTC

AgGR23-Rv CAAGCTTGCTCTAGATTACTGTTTGTGTAGCAGCTTAACA.

AgGR24-Fw GATCAATTCCCCGGGaccATGAGTCTCTACTTCAACGCGG.

AgGR24-Rv CAAGCTTGCTCTAGACTAAGAATGAGACGAATTACTGTGC.

AaGR1-Fw GATCAATTCCCCGGGaccATGATTCACAGCCAGATGGAAG

AaGR1-Rv CAAGCTTGCTCTAGACTAGTTCTCCTTCAGCTTAGTTAGA.

AaGR2-Fw GATCAATTCCCCGGGaccATGGTCATCAAAGACAGTGAGT

AaGR2-Rv CAAGCTTGCTCTAGACTATCCCTTATGACTGTGCTTGATT.

AaGR3-Fw GATCAATTCCCCGGGaccATGAATCTCAACCAAGACCCCA

AaGR3-Rv CAAGCTTGCTCTAGACTACTCGCGATATGAACCCGTCATA.

Adapter sequences are underlined, and lower case acc is Kozak consensus. PCR products were purified by Monarch® DNA Gel Extraction Kit (NEBioLab) and directly inserted to linearized destination vector-pGEMHE by In-Fusion reaction (Clontech). The colonies were picked, and vectors were extracted using Monarch® Plasmid Miniprep Kit. Inserts were submitted to DNA Sequencing Facility, UC Berkeley for verification of sequences.

2.2. Oocytes preparations and two-electrode voltage clamp recordings

The vectors carrying GR genes were linearized with restriction endonuclease *NheI,* except for AgamGR22, which was cut by *Sph*I. The linearized vectors with gene inserts were used as templates to transcribe capped cRNAs with poly(A) using an mMESSAGE mMACHINE T7 kit (Ambion) following the manufacturer's protocol. The cRNAs were dissolved in RNase-free water and adjusted at a concentration of 200 μg/mL by UV spectrophotometry (NanoDrop™ Lite Spectrophotometer, ThermoFisher). 9.2 nl of each cRNA samples were microinjected into stage V or VI *Xenopus* oocytes (purchased from EcoCyte Bioscience, Austin, TX). Then injected oocytes were incubated at 18 °C for 3–7 days in modified Barth's solution [in mM: 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 10 HEPES, pH 7.4] supplemented with 10 μg/mL of gentamycin, 10 μg/mL of streptomycin, and 1.8 mM sodium pyruvate. For two-electrode voltage clamp (TEVC) recordings, as previously done (Xu et al., 2019), oocytes were placed in a perfusion chamber and challenged with test compounds. Compound-induced currents were amplified with an OC-725C amplifier (Warner Instruments, Hamden, CT), the voltage held at −80 mV, low-pass filtered at 50 Hz and digitized at 1 kHz. Data acquisition and analysis were carried out with Digidata 1440 A and pClamp10 software (Molecular Devices, LLC, Sunnyvale, CA).

2.3. Sample preparations and CO_2 measurement

Sodium chloride (Fisher Scientific, > 99%) and sodium bicarbonate (Sigma-Aldrich, 99.7%) were used to prepare fresh samples by dissolving the appropriate amounts of these salts in perfusion Ringer buffer (NaCl 96 mM, KCl 2 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, HEPES 5 mM, pH 7.6) to make 0.5 M solutions. Then, the desired concentrations were prepared by diluting with perfusion Ringer buffer. CO_2 samples were prepared by bubbling MediPure™ CO_2 (Praxair, Danbury, CT) directly into perfusion buffer for 5 s, and subsequently adjusting the pH.

The concentration of dissolved CO_2, ie, CO_2 (aq), was calculated by equation (1):

$$\left[co_2(aq)\right] = \frac{10^{pK_{overall} - pH} \times \left[HCO_3^-\right]_{nominal}}{1 + 10^{pK_{overall} - pH}} \quad (1)$$

$K_{overall}$, which is sometimes referred to as K_a, is a constant, which incorporates the constant of hydrolysis of CO_2 and the first dissociation constant of carbonic acid, K_{a1}, ie, $K_{overall} = K_h \times K_{a1}$. pKa is 6.3 (https://pubchem.ncbi.nlm.nih.gov/compound/Sodium-

bicarbonate#section=pKa). The molar concentration of dissolved CO_2 is, therefore, related to the pH of the solution and the nominal concentration of sodium bicarbonate. Thus, the concentration of dissolved CO_2 in a 10 mM $NaHCO_3$ solution at pH 7.73 is approximately 0.358 mM or 15.76 ppm. At pH 7.32, the calculated concentration of dissolved CO_2 is 0.872 mM or 38.36 ppm.

pH and carbon dioxide were measured with an Orion Star™ A214 pH/ISE Benchtop Meter (ThermoFisher Scientific, Waltham, MA 02451). A carbon dioxide electrode was used for CO_2 measurements. The internal filling solution of this electrode is separated from the sample solution with a gas-permeable membrane. CO_2 dissolved in a sample solution diffuses through this membrane until an equilibrium between CO_2 in the electrode filling and the sample solution is reached. The influx of CO_2 causes a change in hydrogen ion concentration in the electrode filling solution, which is measured by a sensing element behind the membrane. The system was calibrated before measurements with freshly prepared 22, 55 and 110 ppm solutions. Standard solutions were sealed with parafilm before measurements. To minimize losses of carbon dioxide during measurements, we used 50 mL Falcon tubes to house sample solutions. A hole was opened in the tube cap, and the electrode was inserted and kept in place with two O-ring seals below and two O-ring seals on the top of the cap. Then the cap was sealed with Teflon tapes and finally covered with electrical tape. Fifty milliliter Falcon tubes housing test samples were attached to the electrode and fastened tightly. The concentrations of dissolved CO_2 in bicarbonate solutions were measured by preparing 10, 25, 50, 100, 150, 200, 250, and 300 mM solutions in perfusion Ringer buffer, with four replicates for each sample. To compare the concentrations of dissolved CO_2 without adjustment and after acidification, 7 mL aliquots of 10 mM sodium bicarbonate buffers were prepared in pairs. One of the two aliquots in a pair was used to determine CO_2 concentration without pH change. To the other pair, 150 μl of 0.1 N HCl was added and immediately used for CO_2 measurement, and then the pH was recorded. To compare oocyte response, samples were similarly prepared by using smaller aliquots (700 μl) in V-vials. In this case, 0.1 N HCl was injected through the Teflon liner of the open-top caps. After that, an aliquot was collected to be applied to the *Xenopus* oocyte recording system, and the remainder was used for pH measurement.

2.4. Statistical analysis and graphical preparations

Prism 8.2.0 from GraphPad Software (La Joya, CA) was used for both statistical analysis and graphical preparations. A dataset that passed the Shapiro-Wilk normality test was analyzed by t-test; otherwise, data were analyzed by Wilcoxon matched-pairs signed-rank test. All data are presented as mean ± SEM.

3. Results and discussion

3.1. Cx. quinquefasciatus gustatory receptor GR2/GR3 is sensitive to carbon dioxide

We cloned three gustatory receptors from the southern house mosquito, specifically CquiGR1 (VectorBase, CPIJ006622), CquiGR2 (GenBak, MN428502), and CquiGR3 (MN428503), which are likely to be involved in CO_2 reception. Initially, we coexpressed combinations of these three GRs in the *Xenopus laevis* oocytes and recorded their responses

to sodium bicarbonate. Of note, the application of sodium bicarbonate increases the sodium concentration in the perfusion Ringer, thus causing inward currents through endogenous oocyte channels due to an increase in the extracellular concentration of Na^+ (Fig. 1A, Fig. S1). We, therefore, applied equivalent doses of NaCl and $NaHCO_3$ in all experiments to control for the Na^+ response. Equal responses to NaCl and $NaHCO_3$ at the same dose, as in the case of naked oocytes (Fig. 1A), indicate that the currents were generated by Na^+ ions. Surprisingly, oocytes coexpressing CquiGR1 and CquiGR3 did not respond to bicarbonate as the response to sodium bicarbonate did not differ from the response to sodium chloride (Fig. 1B, Fig. S1). A lack of specific response to sodium bicarbonate was also observed with CquiGR1/CquiGR2-coexpressing oocytes (Fig. 1C, Fig. S1). By contrast, CquiGR2/CquiGR3-expressing oocytes gave robust dose-dependent responses to sodium bicarbonate (Figs. 1D and 2, Fig. S1). Although it is not surprising that injections of sodium chloride generated dose-dependent currents, they were significantly smaller (*t*-test, $P = 0.0008$) than those generated by sodium bicarbonate at the same dose (Fig. 2).

We then compared CquiGR2/CquiGR3 and CquiGR1/CquiGR2/CquiGR3 (Fig. 3). The ternary receptor system showed a remarkably lower (*t*-test, $P = 0.0320$) response to sodium bicarbonate than the binary receptor (Fig. 3).

Next, we examined whether variants of CquiGR1 would have different effects. We cloned five CquiGR1 variants, one of them showed an amino acid sequence identical to the sequence appearing in VectorBase (CPIJ 006622). We named this variant the wild type form. Three other variants differed in one amino acid residue each, specifically CquiGR1K456 N (GenBank, MN418391), CquiGR1M365 V (MN418394), and CquiGR1L246 F (MN418393). Lastly, one variant differed in two amino acid residues, ie, CquiGR1D143G; R434K (MN418392). We then expressed variants along with CquiGR2 + CquiGR3 in the *Xenopus* oocyte recording system and compared their responses to those obtained with CquiGR2/CquiGR3-coexpressing oocytes when stimulated with the same dose of sodium bicarbonate (Fig. S2). None of the five ternary receptor systems showed a stronger response than those recorded from CquiGR2/CquiGR3-coexpressing oocytes (Fig. 4). Responses recorded from CquiGR1D143G; R434K were not significantly different from those measured from the binary receptor system (n = 4, unpaired *t*-test, $P = 0.0710$). All other variants, including the wild type, generated significantly lower responses than those recorded from CquiGR2/CquiGR3-coexpressing oocytes (Fig. 4). These findings suggest that CquiGR1 may modulate the receptor system response to sodium bicarbonate.

3.2. Gustatory receptors are activated by carbon dioxide per se, not bicarbonate

To address this question, we used a carbon dioxide electrode, which was designed to measure the total amount of dissolved carbon dioxide in solution. The solution in the internal filling of the electrode is separated from the analytical sample by a CO_2 permeable membrane. Typically, a carbon dioxide buffer is added to the analytical sample to lower the pH to 4.8–5.2 thus shifting the CO_2/bicarbonate equilibrium $\left(CO_2(aq) \rightleftarrows H^+ + HCO_3^-\right)$ towards free CO_2. Carbon dioxide diffuses through the membrane, and an equilibrium is reached between the concentration of CO_2 in the analyte and the internal filling. The increase of CO_2 in the internal filling causes an increase in hydrogen ion concentration,

which is ultimately measured by the pH electrode. To measure the actual (equilibrium concentration) rather than the total concentration of carbon dioxide, we used this electrode in a sealed system and without the carbon dioxide buffer. To calibrate our measurement system, we compared the calculated concentrations of CO_2 in sodium bicarbonate buffers with those obtained by direct measurements with the carbon dioxide electrode (Fig. 5). The results showed a faithful relationship (two-tailed *t*-test, $P = 0.1037$) between calculated and measured CO_2 concentrations (Fig. 5). We then used this system to measure the concentrations of dissolved CO_2 in samples obtained by bubbling CO_2 in perfusion Ringer buffer. The higher the concentration of CO_2, the larger the currents recorded from CquiGR2/CquiGR3-coexpressing oocytes (Fig. S3). Of note, currents recorded from sodium bicarbonate is the summation of carbon dioxide and Na^+ responses. Although we adjusted the pH of the tested bubbled CO_2 samples (Fig. S3) to rule out the possible effect of hydrogen ion concentrations on oocyte responses, these recordings did not unambiguously determine whether the receptor system is responding to carbon dioxide per se or bicarbonate.

The next experiment further examined the possible role of pH on recorded currents. We injected Ringer and 50 mM sodium chloride samples with their pH adjusted to that of the perfusion Ringer buffer, as well as to 7, 6.5, 6, and 5.5. To make certain that the CquiGR2/CquiGR3-coexpressing oocyte system was functional throughout the tests, we injected Ringer, sodium chloride, and sodium bicarbonate at the beginning and the end of each run (Fig. S4). These experiments (n = 6) further demonstrated that hydrogen ion concentration at the pH range of 7.94 to 5.5 had little or no effect on sodium bicarbonate-elicited response (Fig. S4).

Having developed tools to measure the amount of free carbon dioxide in solution, CO_2 (aq), and demonstrated that pH change had minimal or no effect on oocyte responses, we next tested whether sifting the equilibrium of CO_2/bicarbonate buffers would cause an increase or a decrease in the receptor response. We prepared in duplicates aliquots of 10 mM sodium bicarbonate buffers and placed them in sealed V-vials with Teflon liner, open-top caps. One of the experimenters took a sample from one of a pair of vials, applied to a CquiGR2/CquiGR3-coexpressing oocyte, and recorded the elicited current. Meanwhile, the other experimenter injected an aliquot of 0.1 N HCl to the second pair of the same sample, vortexed, and provided the sample to the other experimenter to apply immediately to the oocyte preparation (Fig. S5). The pairs of unadjusted and pH adjusted samples were kept sealed until their pH values were recorded. As expected, the initial pH was nearly the same (pH = 7.73) because they were aliquots from the same 10 mM sodium bicarbonate sample. The final pH of the tested samples was 7.39 ± 0.05.

Quantification of the recorded currents from paired samples showed a significant increase (two-tailed *t*-test, n = 12, p = 0.0005) in the lower pH samples (Fig. 6A). Similar experiments were performed to measure the actual concentration of dissolved CO_2 in paired samples with initial and lowered pH. The initial and final pH values of these samples (n = 4) were 7.73 and 7.32 ± 0.03, respectively. As expected, the quantification of CO_2 (aq) showed an increase in dissolved CO_2 at lower pH (Fig. 6B). The measured CO_2 concentrations in the samples without adjustment ($pH_{initial}$) and with pH adjusted ($pH_{lowered}$) were 17.90 ± 1.55

ppm and 32.40 ± 2.40 ppm, whereas the calculated CO_2 concentrations were approximately 15.8 and 38.4 ppm, respectively. An increase in dissolved CO_2 concentration by lowering pH is predicted by Le Chatelier's Principle, given that an increase in H + concentration shifts the equilibrium towards CO_2 (aq). Because the increased oocyte response (Fig. 6A) resulted from the increase of in situ CO_2 concentration (Fig. 6B), we concluded that CO_2 per se, not bicarbonate, activates the *Cx. quinquefasciatus* gustatory receptor system when expressed in *Xenopus* oocytes. It has also been reported that the BAG neurons of the nematode *Caenorhabditis elegans* are activated by molecular CO_2, although they can be activated by acid stimuli (Smith et al., 2013). We found no evidence for the activation of the *Culex* mosquito GR system by hydrogen ions.

3.3. CquiGR1 orthologs in An. gambiae and Ae. aegypti are putative modulators

Given the unexpected results that CquiGR1 was not necessary for receptor function, but rather attenuated the responses of the CquiGR2/CquiGR3 receptor system to CO_2, we asked whether this would be a common feature of mosquito biology. We prepared oocytes to coexpress the three GR subunits from the malaria mosquito, specifically AgamGR22, AgamGR23, and AgamGR24. Using the same batch of oocytes, we prepared a binary combination of these receptors devoid of AgamGR22, ie, AgamGR23/AgamGR24-expressing oocytes. Then we compared the responses of these oocytes to the same concentrations of sodium chloride and sodium bicarbonate. Specifically, we challenged each oocyte preparation of the same age and the same batch of oocytes with 50, 100, 200, and 300 mM solutions of NaCl and $NaHCO_3$. AgamGR23/AgamGR24-coexpressing oocytes gave stronger, concentration-dependent responses to sodium bicarbonate (Fig. 7A) than the oocytes coexpressing a ternary receptor system, AgamGR22/AgamGR23/AgamGR24 (Fig. 7B). These experiments were replicated with another batch of oocytes (Fig. 7C and D). These results suggest that the CquiGR1 ortholog from the malaria mosquito, ie, AgamGR22, functions as a modulator.

Next, we prepared oocytes coexpressing the equivalent binary and ternary receptor systems with GRs from the yellow fever mosquito. Specifically, we compared the responses of AaegGR2/AaegGR3-coexpressing oocytes to oocytes of the same batch and the same age that coexpressed AaegGR1, AaegGR2, and AaegGR3. Again, the receptor system devoid of AaegGR1 generated stronger, dose-dependent responses to bicarbonate (Fig. S6A) than the ternary system (Fig. S6B), thus implicating AaegGR1 in a modulatory role.

3.4. Overall conclusions

Our research plans to address a long-standing question regarding CO_2 reception led to unexpected results. Because it has been well-established in the literature that the reception of CO_2 in the vinegar fly antennae is mediated by DmelGR21a and DmelGR63a (Jones et al., 2007; Kwon et al., 2007; Suh et al., 2004), it has been assumed that CO_2 is detected by GR1+GR3 in mosquitoes. This notion has been supported by RNAi-based experimental data suggesting that GR2 was not involved in CO_2 reception in *Ae. aegypti* (and *Cx. quinquefasciatus)* (Erdelyan et al., 2012). On the other hand, AgamGR23, the CquiGR2 ortholog from the malaria mosquito *An. gambiae,* was implicated in CO_2 response (Lu et al., 2007), but the role of AgamGR22 is yet to be unraveled. Our findings suggest that the three

subunits might be involved, with GR1 (GR22 in the case of *An. gambiae*) being a putative modulator. Future studies will determine whether GR2 (or GR23) knock out mosquitoes are indeed insensitive to CO_2, whereas GR1 (or GR22) lines will allow us to get a better understanding of the mechanism(s) of modulation. It is conceivable that only the reception of CO_2 at high doses might be different in GR1 (or GR22) knock out mosquitoes. With a functional carbon dioxide-detecting receptor system in an aqueous environment, we applied a fundamental principle in chemistry to interrogate which of two species in equilibrium (CO_2 or HCO_3^-) might activate the receptors. We compared receptor responses as well as CO_2 concentrations in paired samples without acidification and after acidification and showed that an increase in the concentrations of dissolved CO_2 was associated with an increase in receptor response. We then concluded that CO_2 per se, neither HCO_3^- nor protons, activates the carbon dioxide-detecting system in mosquitoes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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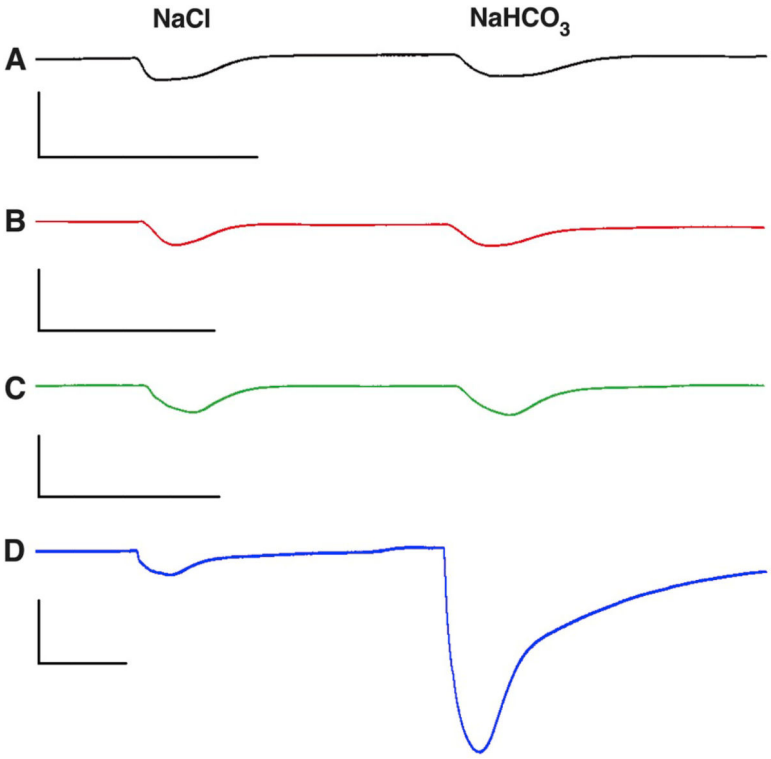

Fig. 1. Responses of Oocytes to Sodium Chloride and Sodium Bicarbonate

Representative traces obtained with the following types of oocytes from the same batch and age:

(A) Intact (naked) *Xenopus* oocytes. (B) Oocyte coexpressing CquiGR1 and CquiGR3 (C) Oocyte coexpressing CquiGR1 and CquiGR2 (D) Oocyte coexpressing CquiGR2 and CquiGR3. All scales are 200 nA and 0.5 min. Oocytes were first challenged with 200 mM NaCl and then 200 mM $NaHCO_3$.

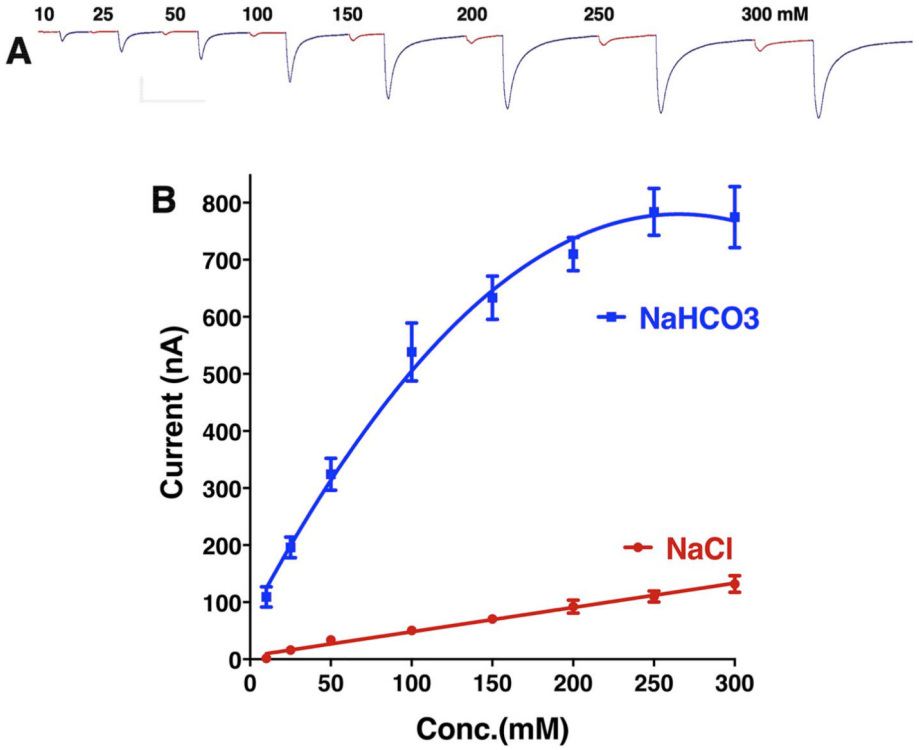

Fig. 2. Responses of CquiGR2 + CquiGR3-coexpressing oocytes to sodium chloride and sodium bicarbonate

(A) Representative trace from a single oocyte preparation stimulated with increasing doses (10–300 mM) of the two compounds. For clarity, responses to sodium chloride and sodium bicarbonate were colored red and blue, respectively. (B) Concentration-dependent curves obtained with five different oocytes coexpressing CquiGR2 and CquiGR3. Data are presented as mean ± SEM. Some error bars for NaCl curve do not appear, because they are shorter than the size of the symbol. From left to right: 0.81, 2.59, 3.24, 5.35, 6.92, 11.24, 9.59, and 14.42. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

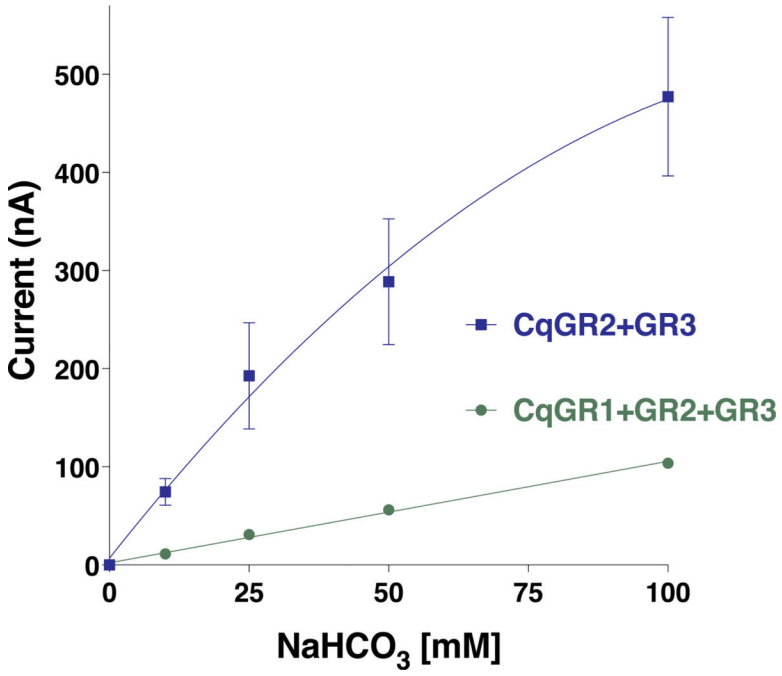

Fig. 3. Comparative concentration-dependent curves for oocytes coexpressing either two or three functional subunits

Results were obtained with four oocytes coexpressing CquiGR2 and CquiGR3 and four oocytes coexpressing the three GRs. For simplicity, in figures, we use two-letter symbols for proteins as opposed to four-letter symbols (eg, Cq = Cqui) used elsewhere. Data are presented as mean ± SEM. Error bars for the green curve do not appear because they are shorter than the size of the symbol. From left to right: 0, 0.63, 3.24, 1.65, and 3.84. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

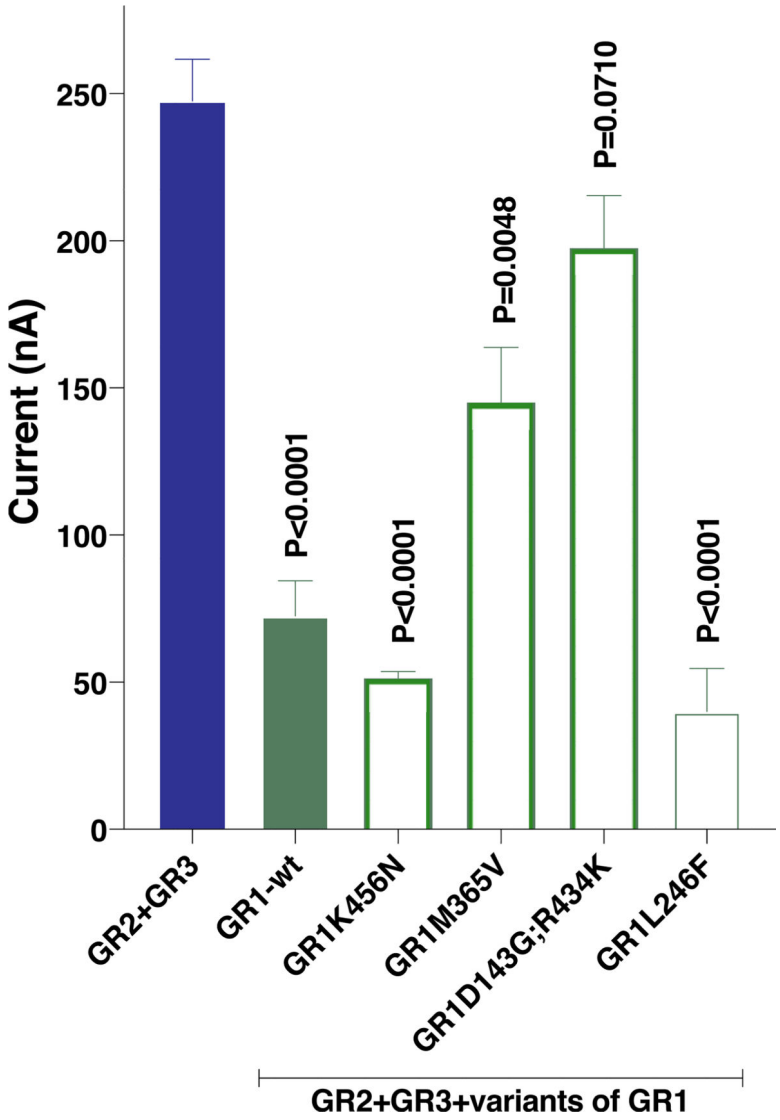

Fig. 4. Effect of different variants of CquiGR1 on the response of CquiGR2 + CquiGR3-coexpressing oocytes

All experiments were performed with four replicates with oocytes from the same batch coexpressing either CquiGR2 + CquiGR3 or CquiGR2 + CquiGR3 plus one of the CquiGR1 variants (K456N, M365V, D143G; R434K, and L246F). The clone with sequence identical to that in VectorBase (CJPI 006622) was named wild type (wt). Responses recorded from CquiGR2 + CquiGR3-coexpressing oocytes and those recorded from oocytes coexpressing CquiGR2 + CquiGR3 plus one CquiGR1 variant after stimulus with 200 mM sodium bicarbonate (n = 4) were compared by unpaired *t*-test. P values are presented on the top of each variant column. Data are presented as mean ± SEM.

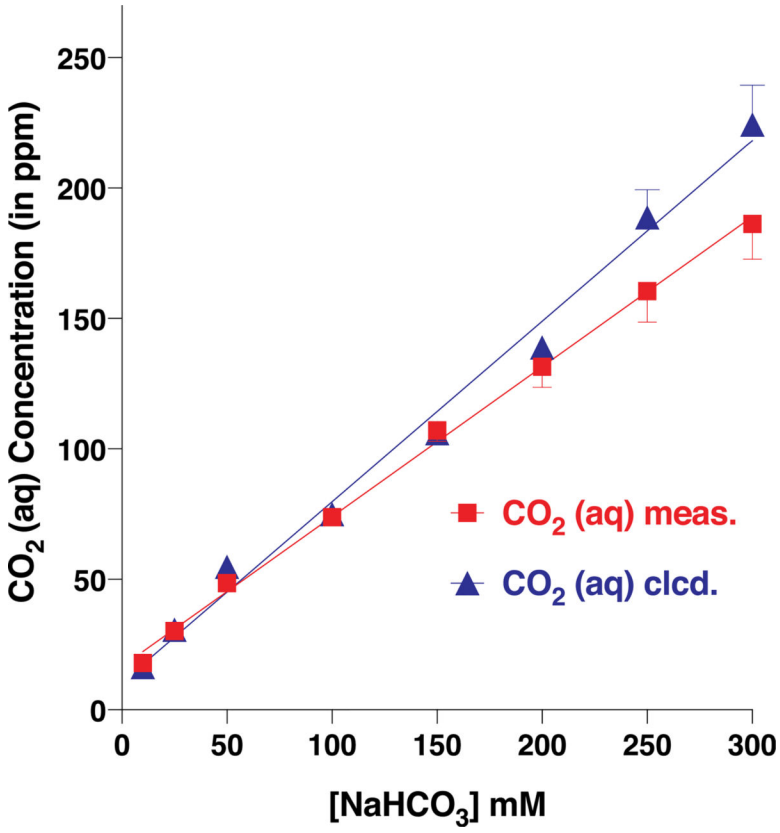

Fig. 5. CO_2 concentrations in solutions of sodium bicarbonate prepared in Ringer buffer

The calculated concentrations based on the final pH of each preparation (n = 4) are shown in red. The actual concentration of CO_2 in each solution was measured with a CO_2 Ion Selective Electrode. Data are presented as mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

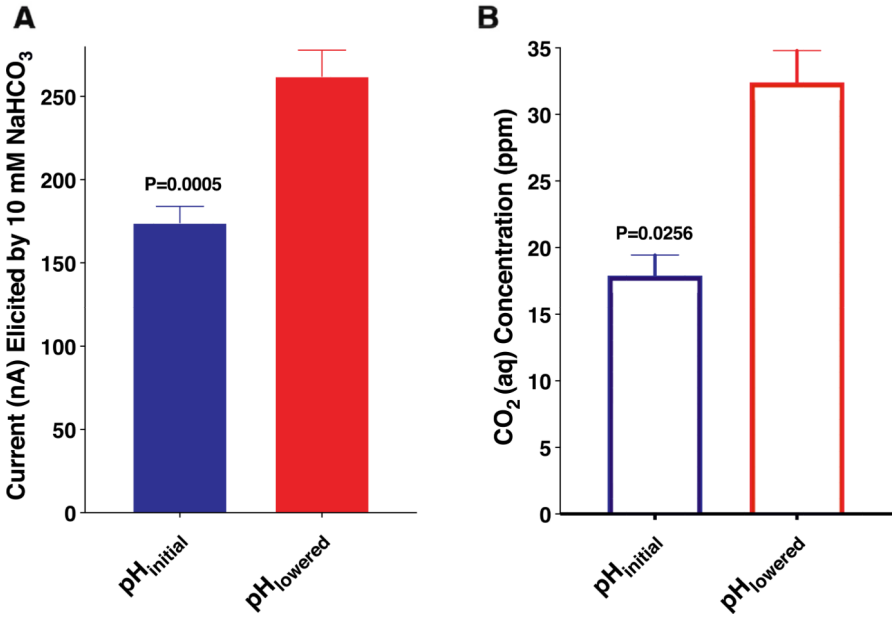

Fig. 6. Effect of pH on the oocyte response and the concentrations of CO_2 in solution

(A) Responses of CquiGR2 + CquiGR3-expressing oocytes to 10 mM of sodium bicarbonate without acidification and after acidification (n = 12). The final pH ($pH_{lowered}$) of the sodium bicarbonate buffer was 7.39 ± 0.05. (B) Quantification of CO_2 in sodium bicarbonate samples (n = 4) without and after pH adjustment (acidification) as measured with a CO_2 Ion Selective Electrode. After acidification ($pH_{lowered}$), the pH of the sodium bicarbonate samples was 7.32 ± 0.03. Data are presented as mean ± SEM.

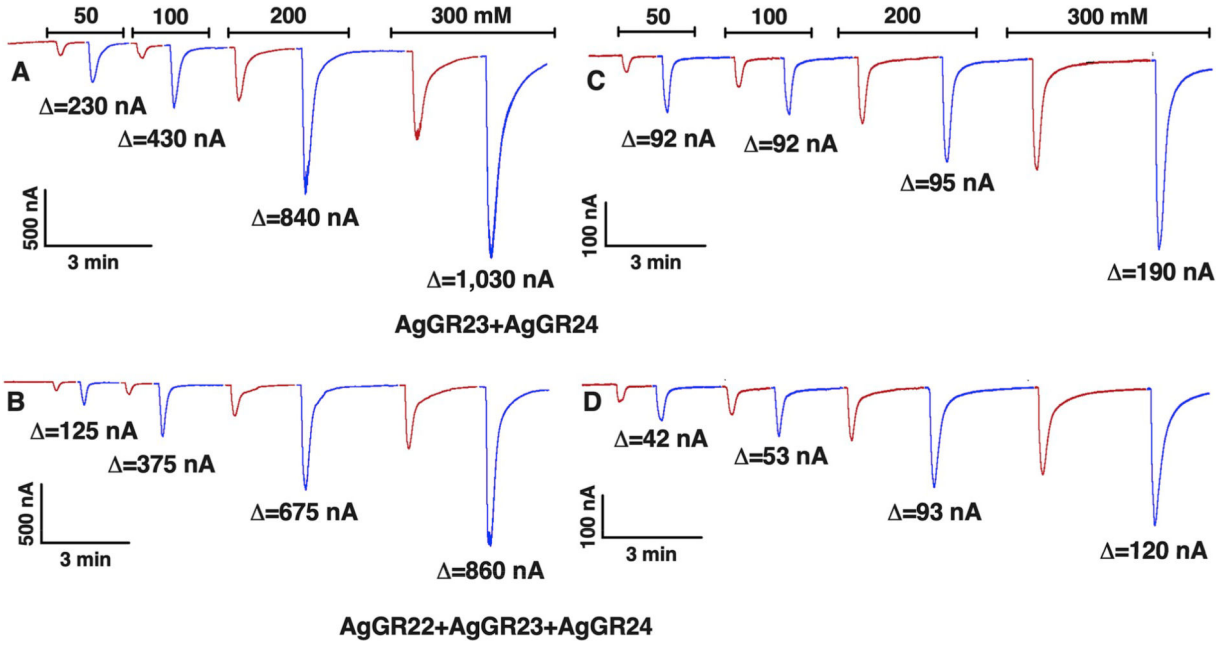

Fig. 7. Responses of *An. gambiae* GRs-expressing oocytes to sodium chloride and sodium bicarbonate

(A) Trace obtained with an oocyte coexpressing AgamGR23 and AgamGR24. (B) Responses of another oocyte (from the same batch) co-expressing the three GRs from *An. gambiae,* GR22, GR23, and GR24. (C) Replication of experiment in (A), but using a different batch of oocytes. (D) Replication of the experiment in (B) with the same batch of oocytes used in (C). Δ values represent responses to sodium bicarbonate subtracted from responses to sodium chloride at the same concentration. In a third replication with a different batch of oocytes the Δ values recorded from a GR23 + GR24-coexpressing oocyte stimulated with 50, 100, 200, and 300 mM were 120, 225, 250, and 780 nA, whereas the respective values recorded from an oocyte from the same batch and coexpressing GR22 + GR23 + GR24 were 23, 89, 193, and 270 nA.