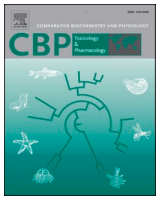
Comparative Biochemistry and Physiology, Part C 285 (2024) 110004

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc



Acute lipopolysaccharide (LPS)-induced cell membrane hyperpolarization is independent of voltage gated and calcium activated potassium channels

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ARTICLE INFO

Edited by Martin Grosell

Keywords:
Drosophila
Lipopolysaccharides
Membrane potential
Pharmacology
Potassium channel

ABSTRACT

The gram-negative toxin lipopolysaccharides (LPS) are known to trigger inflammatory cytokines in mammals, which can result in pathological responses. Upon treatment of bacterial sepsis with antibiotics, the lysing bacteria can present a surge in LPS, inducing a cytokine storm. However, LPS can also have direct cellular effects, including transient rapid hyperpolarizing of the membrane potential, blocking glutamate receptors and even promoting release of glutamate. The detailed mechanism of action for these immediate responses is still unresolved. In addressing the membrane hyperpolarization, voltage gated K^+ channel blockers 4-aminopyridine (4-AP, 3 mM), quinidine hydrochloride monohydrate (0.1 mM) and tetraethylammonium (TEA, 20 mM) were examined along with RNAi knockdowns of potential calcium activated K^+ channels. The immediate responses of LPS were not blocked. Even in the presence of glutamate, the membrane still hyperpolarizes with LPS. When the driving gradient for the ionotropic glutamate receptors is enhanced during hyperpolarization, spontaneous quantal responses are dampened in amplitude. Thus, glutamate receptors are blocked, and the mechanism of hyperpolarization remains unresolved. The larval *Drosophila* glutamatergic neuromuscular junction is used as a model synaptic preparation to address the direct rapid actions by LPS.

1. Introduction

Sepsis is a life-threatening hyperimmune response commonly induced by gram-negative bacterial toxin lipopolysaccharides (LPS). LPS triggers a rise in circulating cytokines (Tang et al., 2021; Gupta et al., 2020; Mangalmurti and Hunter, 2020) that can generate deleterious abnormal neural and cardiac function (Eidelman et al., 1996; Wilson and Young, 2003; Friedrich et al., 2015; Tong and Zhou, 2017). More specifically, skeletal and cardiac muscle are impacted by immune response to endotoxins through the secondary release of proinflammatory cytokines (TNF-alpha, IL-1, or IL-6; Costamagna et al., 2015). Muscle function not only can be severely impacted due to cytokines, but muscles themselves produce cytokines following exposure to LPS (Al-Nassan and Fujino, 2018). In cultured muscle, when NF-κB expression is reduced there is a slowing of the normally associated atrophy by NF-κB (Hahn et al., 2020). Therapeutic treatments for bacterial sepsis in part use antibiotics to kill bacteria; however, the lysis of bacteria can result in a cytokine storm due to the surge in the release of LPS.

One potential therapeutic approach to limit cytokine release in sepsis would be to block the receptors for LPS on cells. The LPS receptor in mammals is a complex known as CD14/TLR4/MD2 (Yoshida et al., 1996; Steiner, 2004). The TLR4 is noted to be conserved from insects to mammals (Levin and Malik, 2017). Unfortunately, an effective therapeutic is yet to be developed as there are no currently available specific blockers of these receptors for LPS.

Even though the Toll receptors were first characterized in *Drosophila melanogaster*, the immune response is not fully mediated by these receptors. The immune deficiency (Imd) signaling pathway appears to be the major receptor-mediated response to LPS exposure (Coscia et al., 2011; Loker et al., 2004; see review by Kleino and Silverman, 2014). The peptidoglycan layer in gram-negative bacteria along with LPS then trigger the Imd receptors (Gottar et al., 2002; Takehana et al., 2002; Leclerc and Reichhart, 2004; Werner et al., 2000). However, the distribution on various tissues and regulation in expression levels of the Imd receptors in insects and other arthropods (i.e. crustaceans) have not been fully determined.

Recently it was demonstrated through using RNAi approaches (Perkins et al., 2015) to the known Imd receptors, PGRC-LC and PGRC-

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https://doi.org/10.1016/j.cbpc.2024.110004
Received 13 June 2024; Received in revised form 29 July 2024; Accepted 15 August 2024
Available online 16 August 2024
1532-0456/Published by Elsevier Inc.

LE in *Drosophila*, that LPS responded in the same manner as with Canton-S lines in the acute actions on body wall muscle or motor neurons (Ballinger-Boone et al., 2020). Thus, it appears PGRP-LC and PGRP-LE receptors are not responsible for the rapid direct action by LPS. As in mammals and *D. melanogaster*, the acute and direct action of LPS on tissues has not yet been well studied. Within a second of LPS exposure to body wall muscle of *Drosophila*, as well as crustaceans (Brock and Cooper, 2023; Cooper and Krall, 2022; Elliott et al., 2024), the membrane potential transiently hyperpolarizes. Interestingly, it appears that for *Drosophila* muscle, LPS also blocks the glutamate receptors at the neuromuscular junction (NMJ). The evoked excitatory potentials (EJPs) and single quantal responses gradually decrease in amplitude with 250 μg/ml and both rapidly decrease with 500 μg/ml LPS exposure of the LPS forms from *Serratia marcescens* or *Pseudomonas aeruginosa*. However, this is not the case for the crayfish muscle as the evoked EJPs increase in amplitude and quantal responses remain present (Cooper and Krall, 2022; Elliott et al., 2024). The mechanisms of how the membrane hyperpolarizes, changes in EJP amplitudes, and responses to glutamate at NMJ have not yet been fully elucidated despite several studies directly addressing the potential mechanisms (Ballinger-Boone et al., 2020; Bernard et al., 2020a, 2020b; Cooper et al., 2019; Greenhalgh et al., 2021; Istas et al., 2019, 2020; Saelinger et al., 2019; Potter et al., 2021).

The action of LPS results in a multitude of independent and different effects at the neuromuscular junctions for crayfish and *Drosophila*. While hyperpolarization is similar, the desensitization to glutamate is unique for *Drosophila* preparation. Since it appears that LPS blocks or potentially desensitizes the glutamate receptors involved in the nerve-evoked glutamate release on the larval *Drosophila* muscle, it was of interest to know whether muscle would depolarize to exogenously applied glutamate after muscle is exposed to LPS. It was also of interest to know if exposure to LPS still resulted in hyperpolarization when the glutamate receptors were desensitized before exposure to LPS. Conducting these experimental paradigms help to examine the independence of the responses induced by LPS. The glutamate receptors on muscle of larval *Drosophila* are readily desensitized by 3 mM exogenously applied glutamate and can recover when glutamate is removed (Ball et al., 2003; Chen et al., 2009).

The various observations of different tissues following LPS exposure have been as follows: (1) Rapidly enhancing synaptic transmission at the crayfish NMJ; (2) Altering heart rate in larval *Drosophila* by speeding up and then slowing down the rate and eventually leading to cessation; (3) Erratic bursts of spontaneous quantal events occurring at the *Drosophila* NMJ; (4) Hyperpolarization of the muscle in crayfish and *Drosophila*. The hyperpolarization of the muscle has led to the postulation that Ca^{2+} flux might be a key factor in activating a calcium-activated potassium channel ($K_{(Ca)}$) (Anyagaligbo et al., 2019; Ballinger-Boone et al., 2020; Potter et al., 2021). There is potential that LPS promotes Ca^{2+} influx into the pre-synaptic terminals of the motor neurons at the crayfish and *Drosophila* NMJs and that a Ca^{2+} loading of the larval heart could speed up and overload the heart, causing it to slow down and eventually stop. A rationale for the transient hyperpolarization of the body wall muscle in *Drosophila* and crayfish is potentially activating a $K_{(Ca)}$ current. These various mechanisms were explored in past studies. It does appear feasible that there could be an enhanced influx of Ca^{2+} in the presynaptic motor nerve terminals. Even the possibility that the Na-K-ATPase pump may be hyperexcited and therefore producing a more negative membrane potential was considered. The use of the pump blocker ouabain did depolarize the muscle but did not prevent the rapid hyperpolarization with LPS (even at 1 mM and 10 mM of ouabain). Furthermore, it is established that the equilibrium potential for Cl^- ions in larval *Drosophila* muscle is more depolarized than the resting membrane potential (Rose et al., 2007; Stanley et al., 2019); thus, the transient hyperpolarization is not due to a Cl^- ion flux.

Given that LPS can induce the resting membrane potential to change from −60 mV to −80 mV in a second suggests the mechanism of action is on ion channels. This could potentially be due to activation of a K^+ channel (a K2P subtype) or even a $K_{(Ca)}$ channel. To activate a $K_{(Ca)}$ in the absence of Ca^{2+} in the bath would indicate possible release of Ca^{2+} from internal stores within the muscle. If a flux of K^+ ions were occurring, it would indicate that the equilibrium potential for potassium is more hyperpolarized than the resting membrane potential. This is feasible considering that experiments in muscle of adult *Drosophila* and moth estimated the equilibrium potential for K^+ may as large as −90 mV (Ikeda et al., 1976; Salkoff and Wyman, 1983).

In addition, attempts using a pharmacological approach were used to address the possibility of LPS activating a K^+ current. Earlier studies showed that a voltage-gated K^+ channel on *Drosophila* body wall muscle was blocked by TEA (Gho and Mallart, 1986), and given that TEA can block $K_{(Ca)}$ currents it was examined for the possibility of also blocking the LPS-induced response. Even with 10 min of incubation in TEA (20 mM) and then exposure to LPS, the membrane still hyperpolarized (Cooper et al., 2019). It could be possible then that the TEA was not able to incubate long enough to block the $K_{(Ca)}$ from the cytoplasmic side as suggested for its action. It is also likely that the TEA sensitive delayed K^+ channel, on the plasma membrane, is not the target for LPS but some other K^+ channels subtypes.

To investigate these possibilities, we used body wall muscle-specific RNA interference (RNAi) lines and a mutant for the $K_{(Ca)}$ channel in *Drosophila* to examine the responses to LPS exposure. In addition, we examined broad and specific blockers to various voltage gated K^+ channels to determine whether LPS is potentially using one or both mechanisms in hyperpolarizing the muscle membrane. In addition, we addressed whether desensitization of the glutamate receptors would still allow the muscle to demonstrate hyperpolarization upon LPS exposure.

2. Materials and methods

2.1. Drosophila lines

Flies genetically knocked-down by RNAi for potassium channel genes specifically in mesodermal tissues were generated by crossing 24B-Gal4 (III) (Bloomington Drosophila Stock Center # 1767) females to males of two different lines bearing UAS-dsRNA constructs for the *Drosophila* $K_{(Ca)}$ gene *slowpoke* (*slo*):$slo^{JF02146}$ (BDSC 26247) and $slo^{JF01470}$ (BDSC 31677). Additionally, the slo^{1} loss-of-function mutation was used (BDSC 4587; Singh and Wu, 1990).

2.2. Physiological recordings

Standard saline was HL3 (in mM): 1.0 $CaCl_2{\cdot}2H_2O$, 70 NaCl, 20 $MgCl_2$, 5 KCl, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, 25 5*N*, *N*-bis(2-hydoxyethyl)-2-aminoethanesulfonic acid (BES) at pH of 7.1 (Stewart et al., 1994; de Castro et al., 2014). The pharmacological agents used were 4-aminopyridine (4-AP, 3 mM) and quinidine hydrochloride monohydrate (0.1 mM) to block potassium channels. Glutamic acid (glutamate hydrochloride, 3 mM) was mixed with the saline to desensitize the glutamate receptors and to examine whether the muscle responded when exposed to LPS. All chemicals were obtained from Sigma-Aldrich, St. Louis, MO, USA.

LPS from *Serratia marcescens* (S. m.) was dissolved in physiological saline the day of experimentation. A high concentration of LPS (500 μg/mL) was used to compare with previous studies to best address the mechanisms of action (Potter et al., 2021). The lethal dose for 50 % of survival (LD50) in rodents for LPS from *S. marcescens* is 650 μg/mL (i.e., 6 × 106 CFU colony-forming units) (Iwaya et al., 2005). This was the justification to use a relatively high concentration for *Drosophila*, since they are likely exposed to high levels of Gram-negative bacterial strains in their native environment. Glutamate alone or mixed with LPS was used in some of the experimental paradigms.

The technique of dissecting larvae and measuring membrane potentials was described in Istas et al. (2019), with the exception that all segmental nerves were transected close to the larval brain to prevent

Table 1
Paradigms used for assessment the responses to LPS with varying treatments.

Paradigm type	conditions used
Paradigm 1	Std. saline/glutamate/glutamate/Std. saline
Paradigm 2	Std. saline/LPS/LPS + glutamate/Std. saline
Paradigm 3	Std. saline/glutamate/glutamate + LPS/Std. saline
Paradigm 4	Mutation $K_{(Ca)}$ Std. saline/LPS/Std. saline
Paradigm 5	RNAi 26247 Std. saline/LPS/Std. saline
Paradigm 6	RNAi 31677 Std. saline/LPS/Std. saline
Paradigm 7	Std. saline/4-AP/4-AP $^{+}$ LPS/Std. saline
Paradigm 8	Std. saline/quinidine/quinidine + LPS/Std. saline

spontaneous evoked contractions induced from the CNS of the larvae. The dissections were performed by pinning the larvae down on a dish and then making a longitudinal dorsal midline cut. The internal organs were removed to expose the body wall muscle on the ventral side and bathed in the modified HL3 saline as used for physiological measures.

The membrane potential of muscle m6 was measured in standard saline for at least 1 min before the bath was exchanged to a saline with other compounds as detailed in the figures. The total volume of the chamber was <0.5 mL and was generally exchanged three times with each bathing solution except for the addition of LPS. LPS saline was readily exchanged over the dissected preparations during the recording of membrane potential. The change in membrane potential is so rapid with the LPS addition that only one exchange in the bathing media was required (Potter et al., 2021).

All experiments were performed at room temperature (20–21 °C). The membrane potentials were measured by intracellular recordings with a sharp glass electrode (3 M KCl) and AxoClamp-2 B amplifier (Molecular Devices, LLC. 1311 Orleans Drive, Sunnyvale, CA, USA). Preparations were used immediately after dissection. Electrical signals were recorded online to a computer via a PowerLab/4 s interface (ADI Instruments, Colorado Springs, CO, USA).

The paradigms used are presented in Table 1 and highlighted with each representative trace of the changes in the membrane potentials for the paradigm as well as percent change from the initial saline for each compound examined in the Results section.

2.3. Statistics

Statistical analysis was performed as a paired *t*-test for changes in membrane potential. Since some data sets were used to examine a direct change in the membrane potential, the non-parametric Sign test was used to compare percent changes from initial saline to the particular time in the paradigm. It was not of interest to compare conditions to saline wash out as this procedure was just to examine if the preparations were still functional in having a membrane potential. A significant difference is considered as $p < 0.05$.

3. Results

In order to examine the effect of LPS on muscle when the glutamate receptors are desensitized, the response of the muscle to glutamate alone was tested. With application of 3 mM glutamate, the evoked and spontaneous quantal responses were blocked and the muscle rapidly depolarized (Fig. 1A). The glutamate containing bath was exchanged to the same concentration again of glutamate to examine the effect of exchanging the bath and a repeated exposure to glutamate. The effect of the glutamate exposure was reversable upon flushing the preparation with fresh saline. However, if glutamate exposure remained on the preparation for 10 min or more the membrane potential struggled to return to initial values. The membrane potential showed an increase in noise in the baseline upon exposure to glutamate which suggests ionic flow off and on, likely because glutamate receptors randomly opened and closed during the exposure to glutamate. The membrane potential was obtained in the initial saline, right after exchanging the bath, and at

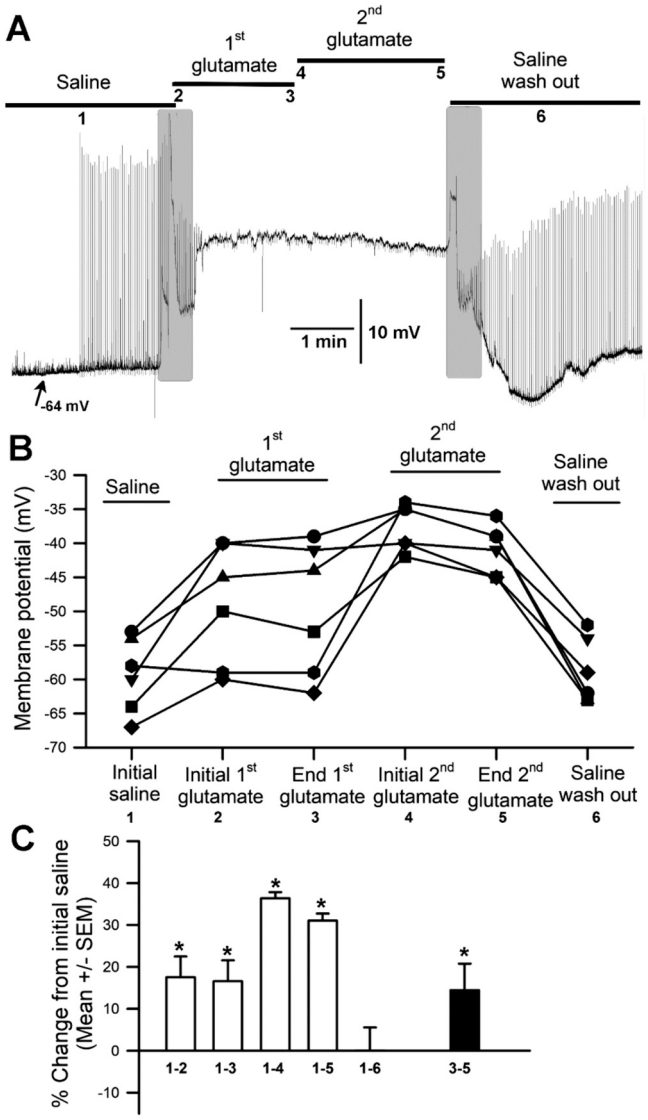

Fig. 1. Examining the effect of glutamate on membrane potential. (A) Glutamate (3 mM) depolarized a muscle fiber immediately upon application, and also desensitized the postsynaptic glutamate receptors to evoked and spontaneous transmitter release. When the preparation was flushed with saline after glutamate exposure, the resting potential returned to normal and the evoked EJPs returned. The shaded boxes indicate the switching out solutions on the preparation. (B) Each symbol indicates a separate *Drosophila* preparation. The bars at the top of the figure allow one to see which study condition was being examined, as both the initial and end membrane potential of each condition were collected. The first glutamate exposure caused depolarization of most preparations, and the second glutamate exposure caused additional depolarization. Saline washout caused the membrane potential to return towards normal (as determined by the starting potential). (C) Histogram shows the change from membrane potential during initial saline before the start of paradigm (1) and each data collection point. For example, 1–2 was the change between membrane potential during initial saline before the start of paradigm (1) and the membrane potential when the first glutamate was first applied (2). The positive bar for each change indicates depolarization for all glutamate conditions in comparison to saline, as well as depolarization between the two applications of glutamate. The numbers indicated are labeled in both (A) and (B) for reader convenience. The stars above each bar indicate a statistically significant change ($p < 0.05$) between study conditions.

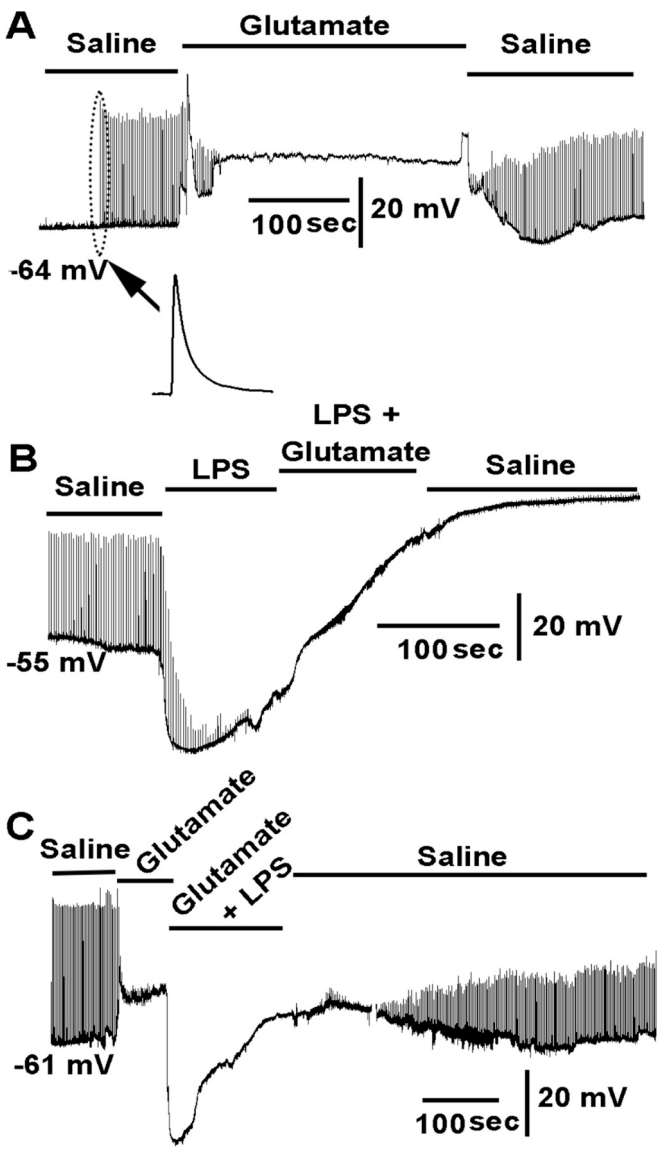

Fig. 2. LPS effect on glutamate sensitivity. (A) Glutamate (3 mM) rapidly depolarized a muscle fiber and desensitized the postsynaptic glutamate receptors to evoked and spontaneous transmitter release. The effects were reversable with acute exposure and flushing with fresh saline without glutamate. The inset highlights the evoke excitatory junction potentials in the trace. (B) Exposure to LPS alone rapidly hyperpolarized the muscle fiber and desensitized the glutamate receptors. Both evoked and spontaneous quantal events gradually decreased in amplitude despite the larger driving gradient for ionic flow through the ionotropic glutamate receptors. The combined exposure to LPS and glutamate did not produce a rapid depolarization as observed for glutamate exposure alone. The effects are not as readily reversible when flushing the preparation with saline. (C) Upon rapid depolarization of the muscle and desensitization of the glutamate receptors with exposure to glutamate (3 mM) the addition of LPS combined with glutamate (3 mM) still rapidly hyperpolarized the membrane potential. With acute exposures the effects were partially reversed by flushing the preparation with fresh saline.

the start and end of each exposure as marked by numbers (Fig. 1A). Responses for each preparation are illustrated (Fig. 1B). The percent change for each measurement was determined to the initial values in the saline at the start of experimentation independently for each preparation and the mean (±SEM) was determined for all the preparations (Fig. 1C). A positive percent change indicates a depolarization.

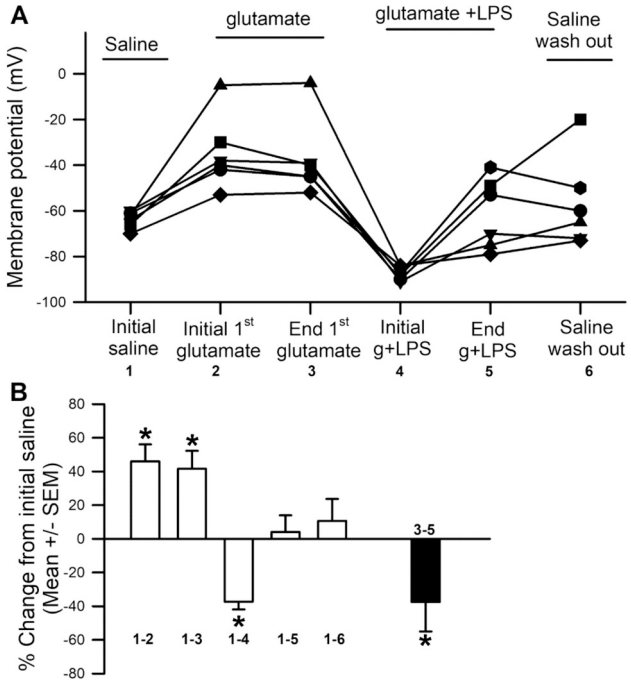

Fig. 3. Comparing membrane potential response to glutamate followed by glutamate and LPS mixture. (A) Each symbol indicates a separate *Drosophila* preparation. The bars at the top of the figure allow one to see which study condition was being examined, as both the initial and end membrane potential of each condition was collected. The glutamate exposure caused depolarization of each preparation. Applying the glutamate and LPS mixture next resulted in rapid hyperpolarization in all six preparations. Saline washout caused the membrane potential to increase again towards starting potential. (B) The positive histogram values for 1–2 and 1–3 indicate depolarization with application of glutamate. The negative value for 1–4 indicates hyperpolarization upon application of glutamate and LPS. The negative value for 3–5 indicates hyperpolarization between the end of glutamate exposure versus the end of glutamate and LPS combined exposure. The stars above each bar indicate a statistically significant change ($p < 0.05$) between study conditions.

3.1. Paradigm 1: the effect of glutamate on membrane potential

3.2. Paradigms 2 & 3: the effect of glutamate or LPS or glutamate followed by LPS combined with glutamate

For ease in comparing the responses to LPS with glutamate, a representative trace to glutamate exposure is shown (Fig. 2A). With the combination of LPS and glutamate after prior LPS exposure, the membrane did not rapidly depolarize which would indicate that the muscle was not sensitive to exogenously applied glutamate (Fig. 2B). In addition, the evoked EJPs and spontaneous quantal events gradually decreased in amplitude despite the larger ionic gradient for Na^+ ion through the ionotropic glutamate receptor (Fig. 2B). This is also in support of LPS desensitizing the postsynaptic receptors to the glutamate released by the nerve terminal. Upon initially desensitizing the glutamate receptors with glutamate and then applying LPS, there still was a dramatic hyperpolarization of the muscle fibers induced by LPS (Fig. 2C).

These same trends were observed in all 6 preparations examined for both paradigms (non-parametric Sign test $P < 0.05$; $N = 6$). Fig. 3A illustrates the responses to glutamate (3 mM) followed by the combination of glutamate and LPS for each preparation. Fig. 3B depicts the mean (±SEM) percent changed from the initial saline for the measurements, determined for each individual preparation. Fig. 4A illustrates the

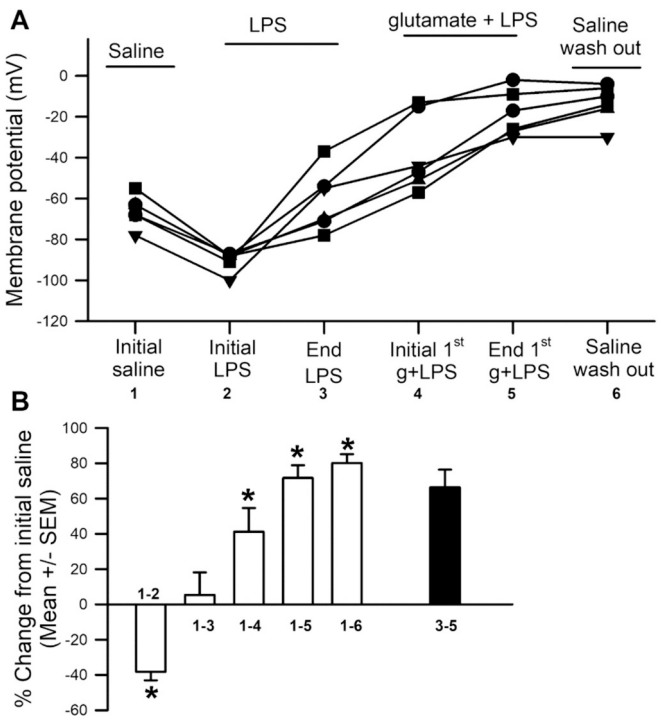

Fig. 4. Comparing membrane potential response to LPS followed by glutamate and LPS mixture. (A) Each symbol indicates a separate *Drosophila* preparation. The LPS exposure caused hyperpolarization of each preparation. However, the potential began to rise again by the end of the LPS condition. Applying the glutamate and LPS mixture next resulted in slight depolarization. Saline washout caused the membrane potential to increase even further, surpassing initial saline membrane potential. (B) The negative histogram value for the 1–2 change indicates hyperpolarization with application of LPS. The positive values for all other change comparisons indicates that the membrane was depolarized for the rest of the time points. The stars above each bar indicate a statistically significant change ($p < 0.05$) between study conditions.

responses LPS followed by the combination of LPS and glutamate for each preparation. Fig. 4B depicts the mean (±SEM) percent changed from the initial saline for the measurements, determined for each individual preparation.

3.3. Paradigms 4, 5 & 6: the effect of LPS on the strains with a mutation for the $K_{(Ca)}$ channel and RNAi knockdowns of the $K_{(Ca)}$ channel

To address the possibility of LPS activating a $K_{(Ca)}$ channel on the muscle membrane, a strain bearing a loss-of-function mutation in the $K_{(Ca)}$ channel gene *slo* was examined. This may have resulted in a global effect on gene function, in both muscle and neurons. Exposure to LPS still produced a rapid hyperpolarization of the muscle (Fig. 5A). To aid in confirming that LPS does not activate a $K_{(Ca)}$ channel, two different RNAi strains were used which selectively expressed RNAi for the $K_{(Ca)}$ channel in mesoderm. This was expected to reduce the expression in the heart as well as body wall muscles (i.e., skeletal muscle). Both RNAi strains showed similar responses (Fig. 5B and C).

This same trend in hyperpolarization was observed in all 6 preparations examined for each strain (non-parametric Sign test $P < 0.05$; $N = 6$; $N = 18$ for all lines combined). However, as illustrated in Fig. 6A, by the end of the minute of LPS exposure the membrane potential was already depolarizing while for the two RNAi strains (Figs. 7A, 8A) the membrane did not depolarize as quickly for those preparations (though RNAi 31677 began to depolarize more quickly than RNAi 26247).

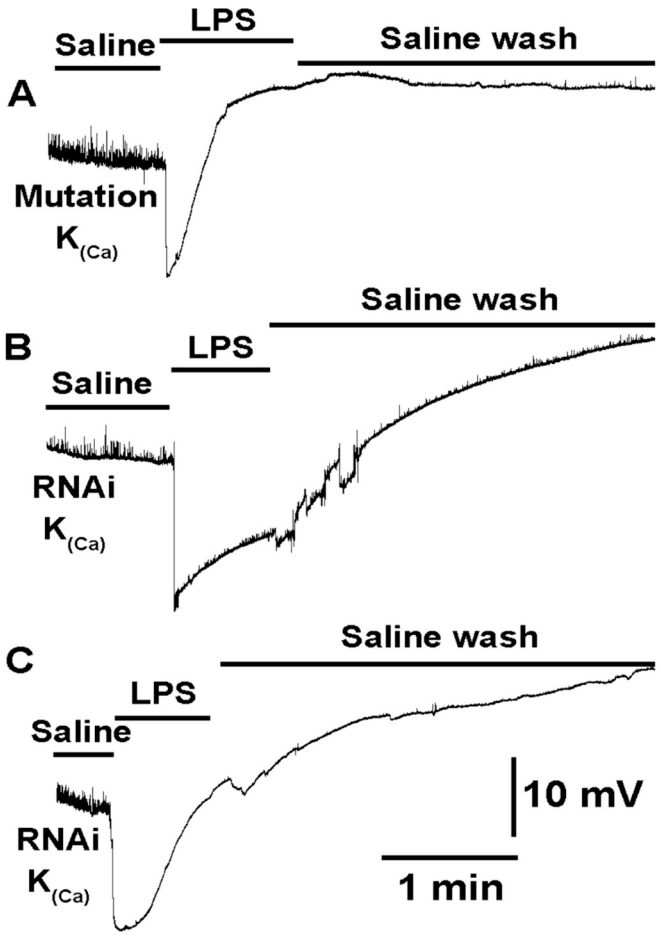

Fig. 5. Examining the effect of LPS without functional $K_{(Ca)}$ channels present. (A) Muscles without a functional gene to express the $K_{(Ca)}$ channel (*slo*^{1}) still responded to LPS by hyperpolarizing and causing desensitizing to glutamate from spontaneous quantal events. (B and C) Two different RNAi lines, for altering mRNA expression of functional $K_{(Ca)}$ channels, also produced rapid hyperpolarization upon LPS exposure, and desensitization to glutamate from spontaneous quantal events. The genotype shown in B is 24B-Gal4; UAS-ds-slo^{JF02146} and C is 24B-Gal4; UAS-ds-slo^{JF01470}.

3.4. Paradigms 7 & 8: the effect of 4-AP and quinidine on the actions of LPS

4-AP slightly depolarized the muscle fibers and upon LPS exposure a rapid hyperpolarization occurred despite 4-AP being applied before and during LPS exposure (Fig. 9A). 4-AP was used to block the A type potassium channels. Spontaneous quantal events were observed in saline before and during 4-AP application (Fig. 9A, B, C) as well as after a flushing of the preparation with saline after LPS and 4-AP exposure (Fig. 9E). However, quantal events are not able to be detected during the LPS and 4-AP exposure due to the desensitization of the evoked release of glutamate by LPS (Fig. 9D). During 4-AP exposure, the muscle showed rapid depolarization similar to an evoked EJP but with an even more rapid upstroke and decay. This suggests the muscle produced spontaneous action potentials in the presence of 4-AP. This is also supported by the occurrence of these depolarizations while the glutamate receptors are highly desensitized by LPS exposure (Fig. 9A). In a few cases, the muscles produced rapid contractions resulting in the loss of the recording which were not able to be followed for the experimental paradigm. Since LPS desensitizes the muscle to evoked glutamate release it is most likely that 4-AP causes the muscle to randomly produce action potentials.

These same responses to 4-AP and the combination of 4-AP + LPS

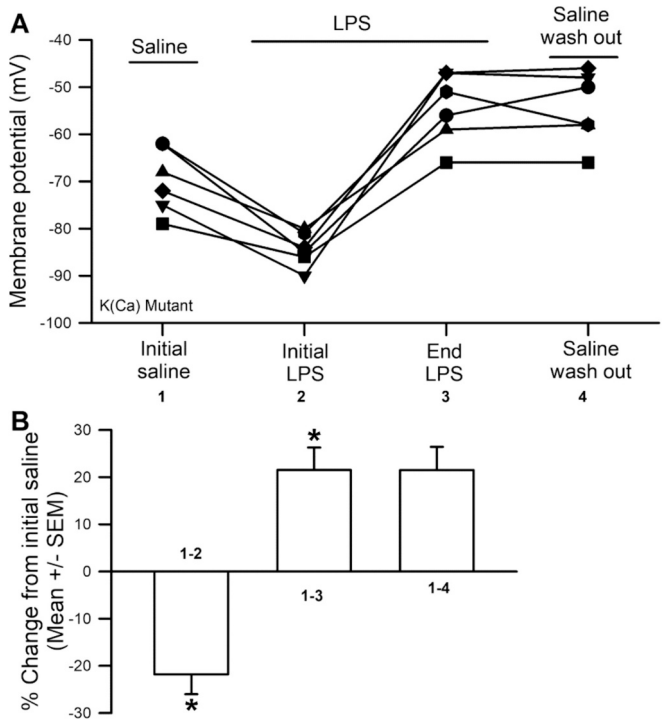

Fig. 6. Examining the effect of LPS on membrane potential without functional $K_{(Ca)}$ channels present (*slo*^{1}). (A) LPS exposure caused hyperpolarization of each preparation, even in the absence of functional $K_{(Ca)}$ channels. During LPS conditions, each membrane potential depolarized past the initial membrane potential prior to saline washout. Furthermore, saline washout did not cause a significant change in potential in comparison to the end of the exposure to LPS. (B) The negative percent change graphed for 1–2 indicates hyperpolarization upon application of LPS, even without functional $K_{(Ca)}$ channels. The positive histogram values for 1–3 and 1–4 indicate depolarization after extended LPS exposure as well as with saline washout.

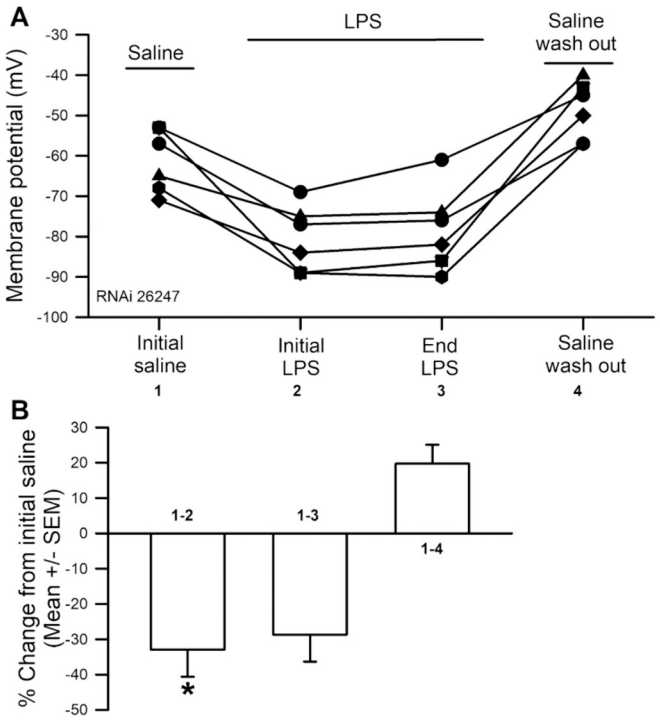

Fig. 7. Examining the effect of LPS on membrane potential following $K_{(Ca)}$ knock down (24B-GAL; UAS-ds-slo^{JF02146}). (A) LPS exposure again caused hyperpolarization of each preparation. The membrane potential did not depolarize as greatly by end of exposure period to LPS as seen in the first $K_{(Ca)}$ mutation. However, saline washout caused depolarization in comparison to the initial values in saline. (B) The negative percent change graphed for 1–2 and 1–3 indicates hyperpolarization upon application of LPS, throughout LPS exposure. The positive histogram value for 1–4 indicates depolarization with saline washout.

were observed in all 6 preparations examined (Fig. 10A, non-parametric Sign test $P < 0.05$; $N = 6$). The response to LPS, even combined with 4-AP, produced a pronounced hyperpolarization (Fig. 10B).

In order to help determine if another form of a K^+ channel was being activated by LPS, a broad spectrum K^+ channel blocker was used (quinidine hydrochloride monohydrate at 0.1 mM). The actions of quinidine was different than 4-AP and previous treatments with TEA, in that an increase in spontaneous quantal events occurred rapidly upon exposure (Fig. 11A). A slight depolarization was observed which could be due to a rapid increase in vesicular fusion events (compare Fig. 11B and C). Subsequent exposure to LPS along with quinidine still produced a rapid hyperpolarization (Fig. 11A); however, LPS was not able to completely desensitize the nerve released glutamate response as the spontaneous quantal events were still present, but with a reduced amplitude as compared to when exposed to saline only or with quinidine only. This suggests some desensitization along with an increase in the frequency of occurrences of the spontaneous events (Fig. 11A and D). These same trends were observed in all 6 preparations examined (Fig. 12A, non-parametric Sign test $P < 0.05$; $N = 9$). As for 4-AP prior exposure and the exposure to LPS combined with 4-AP, the LPS combined with quinidine showed a pronounced hyperpolarization as compared to quinidine alone (Fig. 12B).

4. Discussion

Exposure to LPS from *Serratia marcescens* produced a rapid hyperpolarization in the larval body wall muscle for two RNAi lines of a $K_{(Ca)}$ channel expressed in muscle, as well as a genetic mutant line for the $K_{(Ca)}$ channel. The synaptic glutamate receptors also showed a rapid desensitization in all of these lines. When glutamate receptors were desensitized with glutamate, the membrane was able to hyperpolarize rapidly with LPS exposure, suggesting two different actions of LPS. One action would be hyperpolarizing the membrane, and the other would be blocking or desensitizing the ionotropic glutamate receptors activated by glutamate released from the nerve terminals.

To address whether other types of potassium channels besides the $K_{(Ca)}$ might be responsible for the hyperpolarization induced by LPS, 4-AP and quinidine hydrochloride monohydrate were also examined. LPS was able to still hyperpolarize the membrane with these potassium channel blockers present. Thus, these channels do not appear to be a viable mechanism contributing to the hyperpolarization of the membrane potential by LPS.

It was shown recently that pre-incubation with tetraethylammonium (TEA) at 20 mM and exposure to LPS mixed with TEA still produced a pronounced hyperpolarization which was not different than without exposure to TEA. So, considering TEA blocks delayed voltage-gated potassium channels and is known to even block K(Ca) (Gho and Mallart, 1986), it does not seem feasible that a TEA sensitive potassium channel would be responsible for the hyperpolarization or contribute to the effect. Recently, the possibility of the Na-K-ATPase pump being hyperactive by LPS was put to rest due to the pump blocker (i.e., ouabain at 10 mM) not altering the response to LPS (Potter et al., 2021). These findings led us to want to examine different subtypes of potassium channels, thus our rational for trying 4-AP and quinidine hydrochloride monohydrate.

When the A-type potassium channels and non-inactivating currents were blocked by 4-AP, LPS was still able to hyperpolarize the muscle. Likewise, the use of quinidine hydrochloride monohydrate, which is a broad potassium channel blocker (Gorczyca and Wu, 1991; Gho and

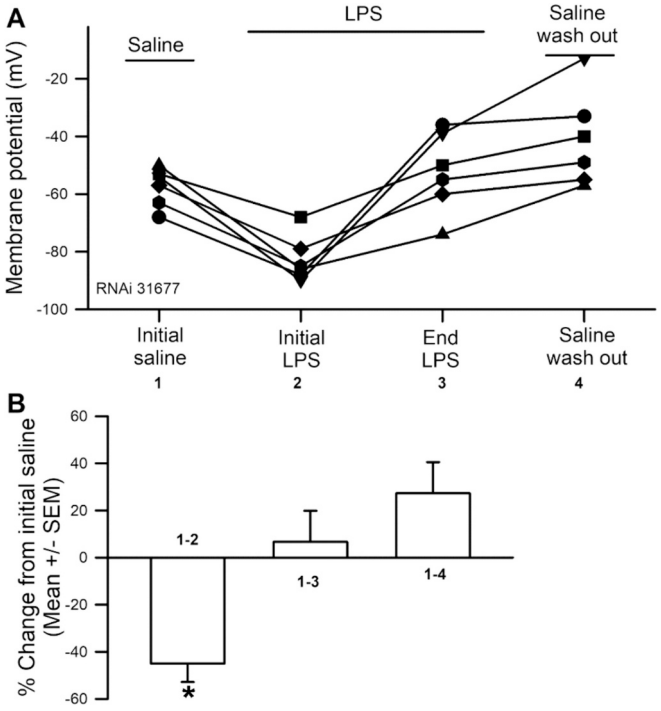

Fig. 8. Examining the effect of LPS on membrane potential in a second RNAi line (24B-GAL; UAS-ds-slo^{JF01470}) which altered mRNA expression of functional $K_{(Ca)}$ channels. (A) LPS exposure again caused hyperpolarization of each preparation. The membrane potential began to depolarize by the end of LPS exposure, though the amount varied between preparations. Saline washout then caused depolarization of the preparations in comparison to beginning saline values, but not necessarily in comparison to the end LPS membrane potential. (B) The negative percent change graphed for 1–2 indicates hyperpolarization upon application of LPS. However, the positive percent change for 1–3 indicates the preparations began to depolarize while LPS was still applied. The positive histogram value for 1–4 indicates further depolarization with saline washout.

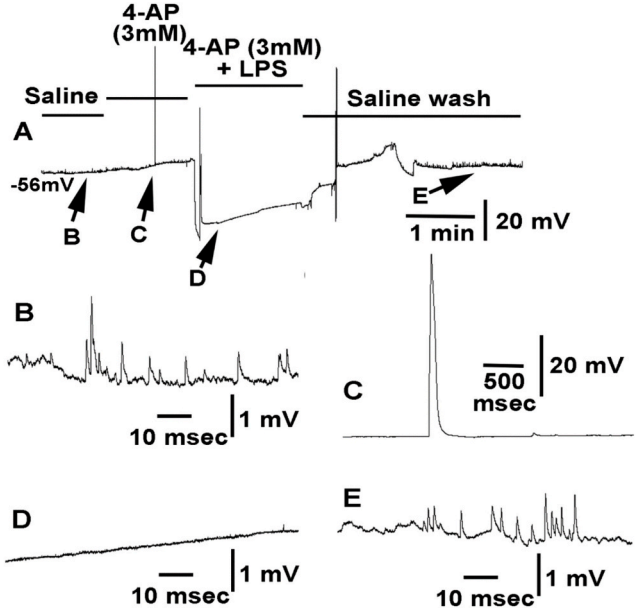

Fig. 9. The effect of LPS in the presence of 4-aminopyridine (4-AP at 3 mM). (A) Blocking the 4-AP sensitive K^+ channels prior to exposure of LPS still resulted in a rapid hyperpolarization of the muscle in the presence of LPS combined with 4-AP (3 mM). Upon exposure to 4-AP, the muscle showed random evoked EJPs or random action potentials as highlighted in C and in the trace shown in A. (B and C) Spontaneous quantal events were present before and during 4-AP exposure. (D) LPS in the presence of 4-AP desensitized the muscle to the glutamate from spontaneous quantal events. (E) Removal of 4-AP and LPS by flushing fresh saline on the preparation recovered the sensitivity to glutamate from spontaneous quantal events. The large rapid depolarizations in the prolonged trace in A were due to the 4-AP exposure provoking either the motor neuron to randomly fire or the muscle fiber to produce an action potential.

Ganetzky, 1992), did not dampen the effect of LPS. Thus, if the hyperpolarization is due to a K^+ flux, then it is not occurring through a TEA, 4-AP, or even a quinidine hydrochloride sensitive channel. In addition, based on the past blockers of Ca^{2+} channels (i.e., Cd^{2+}, Gd^{3+}; Potter et al., 2021), it does not appear that a Ca^{2+} influx is responsible for activating a $K_{(Ca)}$ to account for the action of LPS. Potentially Ca^{2+} could be induced to be released from internal stores (i.e., SER) but with the use of a mutational $K_{(Ca)}$ line (which does not allow a functional channel) as well as two RNAi lines (which blocked normal expression of $K_{(Ca)}$ channels) it appears $K_{(Ca)}$ is not being activated since muscles these lines still produced a hyperpolarization when exposed to LPS.

The mutation in the $K_{(Ca)}$ line used, *slowpoke* (*slo*). This line is known to eliminate a Ca^{2+}-activated K^+ current in muscles and neurons (Atkinson et al., 1991). The two RNAi lines used should also reduce *slo* gene function (Perkins et al., 2015; FBgn0003429). There was a difference in the response to LPS among the mutant and the RNAi strains used in that the transient effect was briefer in the mutant strain than the RNAi strains. Also, casual observation of the *slo*^{1} adults appeared to be less active than for the RNAi flies but no qualification in the behaviors were performed. These small differences between the mutant and RNAi knockdown genotypes may be due to differences in degree of impairment of the $K_{(Ca)}$. The degree of gene knockdown in muscle for the RNAi genotypes was not measured, although a previous study observed mutant phenotypes associated with one of the UAS-RNAi lines (Overend et al., 2016).

The combined past and present studies would suggest that LPS from *Serratia marcescens* would be activating a K2P potassium channel of an unknown subtype. Since there are 11 genes in *Drosophila* to code for K2P channels (Adams et al., 2000; Littleton and Ganetzky, 2000), there could be various subtypes expressed in the body wall muscles. Thus, even if LPS transiently activated a subtype of K2P channels, it still remains to be determined if LPS is selective to activating all or a select few K2P subtypes. Pharmacological advances are being made to antagonize some forms of K2P channels (Buckingham et al., 2005; Enyedi and Czirják, 2010; Kamuene et al., 2021) and investigations are underway to address if these agents fully block effects of LPS (Elliott et al., 2023, 2024; Vacassenno et al., 2023a, 2023b). It is suggestive that LPS from *S. marcescens* and *P. aeruginosa* transiently activate and then inhibit a form of K2P channels as the membrane hyperpolarizes and then depolarizes and the responses can be blocked by doxapram, a known blocker of a subset of K2P channels (Vacassenno et al., 2023a, 2023b). Doxapram has been established for its capacity to block mammalian, pH-sensitive K2p channels and depolarize larval body wall muscles. It appears LPS activates K2p channels on the body wall muscles, while doxapram dampens that effect. Since doxapram targets mammalian, acid-sensitive K2p TASK channels, it was postulated that it may have similar effects on the cardiac tissue and body wall muscles of larval *Drosophila* (Elliott et al., 2023, 2024; Vacassenno et al., 2023a, 2023b).

Glutamate response from the quantal events desensitized in a similar manner after exposure to both LPS as well as glutamate as the spontaneous quantal events became smaller and were no longer able to be detected. The differences between the two is that exposure to glutamate depolarized the muscle while LPS hyperpolarized the muscle (while also desensitizing the glutamate receptors). Considering that muscle remains depolarized with exposure to glutamate, it is likely that some of the glutamate receptors (i.e. non-synaptic receptors) remain open but not responsive to spontaneous quantal events. And even with the membrane being depolarized in the presence of glutamate, the membrane still had a

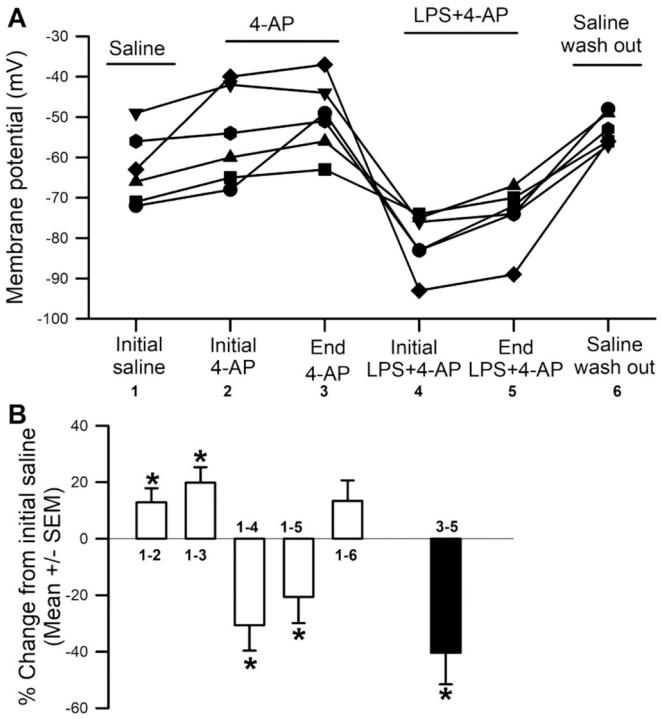

Fig. 10. Comparing membrane potential response to LPS when the preparation was pre-treated with 4-AP (a type of potassium channel blocker). (A) Addition of 4-AP caused the preparations to depolarize, both initially and as time went on. The LPS exposure caused hyperpolarization of each preparation, even with 4-AP pretreatment. However, saline washout caused the membrane potential to begin to depolarize again, often rising above initial starting saline potential. (B) The positive histogram bars for 1–2 and 1–3 indicate depolarization with blocking of 4-AP sensitive potassium channels. The negative bars for 1–4 and 1–5 show that LPS caused hyperpolarization, regardless of 4-AP sensitive channels. The negative bar for 3–5 shows a hyperpolarization of the membrane potential when comparing a 4-AP block alone to a combination of 4-AP block and LPS exposure. The positive value for 1–6 however indicates that the preparation could still be washed out.

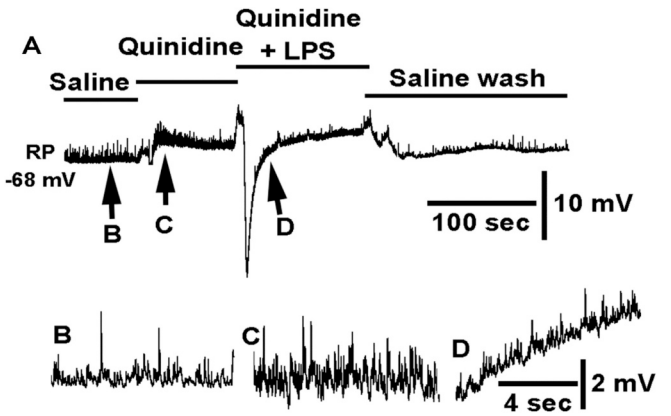

Fig. 11. The effect of LPS in the presence of quinidine hydrochloride monohydrate (0.1 mM). (A) Blocking the quinidine sensitive K^+ channels prior to exposure of LPS still resulted in a rapid hyperpolarization of the muscle in the presence of LPS combined with quinidine (1 mM). Upon exposure to quinidine, the muscle showed an increased frequency of spontaneous quantal events along with a slight depolarization of the muscle. (B) Spontaneous quantal events were present in saline. (C) An increase in the occurrence of spontaneous quantal events was seen with exposure to quinidine. (D) During LPS exposure, spontaneous quantal events were still observed although smaller in amplitude despite the larger driving gradient for ionic flow through the ionotropic glutamate receptors and general effects of LPS desensitization of glutamate receptors.

large enough driving gradient to produce a hyperpolarizing event with LPS. The depolarized state with glutamate indicates desensitization to additional glutamate exposure released from the motor nerve, but the receptors appear not to be closed. However, with LPS exposure, the glutamate receptors are obviously not open due to the large degree of hyperpolarization and the inability to detect evoked or spontaneous depolarizations.

When glutamate is applied while the muscle is bathed in LPS, it does not rapidly depolarize as it does in the absence of LPS exposure. Thus, nerve-evoked glutamate release or application of glutamate does not appear to displace the LPS from the desensitized close state of the receptors or from the hyperpolarized state induced by LPS. Since LPS produces a transit hyperpolarization and then a gradual depolarization, the glutamate exposure was applied quickly while the membrane was still hyperpolarized, which still did not result in a rapid depolarization. It was also obvious that the membrane potential was not rapidly affected by the addition of glutamate in the presence of LPS, and over time the membrane continued to depolarize with constant exposure to LPS.

So, there are still two factors which remain unknown in explaining the mechanisms of LPS's alteration on membrane potential. Not only is the cause of the hyperpolarization not fully known, but the mechanism for the transit response and continual depolarization beyond the resting membrane potential also remains unknown. There does not appear to be a local effect of LPS on Ca^{2+} channels (Cooper et al., 2019; Ballinger-Boone et al., 2020), as the hyperpolarization with LPS still occurred even when Ca^{2+} was not added to the saline (Potter et al., 2021). One might use EGTA or BATA to bind and residual Ca^{2+} but this would also have bound Mg^{2+} in the saline resulting in yet other effects. It also does not appear to be a pH effect as the saline has a stable pH after LPS is added. In wild speculation, it is possible that a transit incorporation of a portion of a negatively charged region of the LPS molecule in the bilipid membrane results in negative polarity of charge. However, since LPS can be reversed quickly if the saline is exchanged, it is hard to image it would be removed from the membrane so readily if tightly interacting with the lipid moieties. As for addressing a negative control in these studies for the effect of LPS, in an earlier study saline containing LPS (from *S. marcescens*) was vigorously boiled for 5 min and corrected for vapor loss by adding water back, the membrane did not produce a significant change in membrane potential (Potter et al., 2021). Current studies underway in our group are comparing various strains of LPS (*Serratia marcescens*, *Pseudomonas aeruginosa*, *E. coli* O111:B4, *Salmonella enterica* Minnesota and Ultra-pure *Salmonella enterica* Minnesota S R595). We hope to be able to address any contamination with commercially obtained LPS that could contain RTX toxin, as well as other peptidoglycans by use of ultra-pure forms of LPS (Hertle, 2000; Ballinger-Boone et al., 2020; Kaneko et al., 2004; Ochoa-Cortes et al., 2010). Only a few studies have addressed LPS which has been carefully purified from other bacterial components. Future studies could be conducted to address whether the membrane is compromised after prolonged LPS exposure. The *Drosophila* model serves as a rapid and inexpensive testing ground for addressing these acute mechanisms of action on cell membranes. *Drosophila* has proven to be very useful in breaking the ground for new discoveries related to understanding physiological and pathological conditions in humans (Yamaguchi and Yoshida, 2018). To continue addressing effects of LPS strains on membranes, this could be done through examining input resistance of the muscle and potentially if the muscle took up markers for damaged or dead cells (i.e., trypan-blue, propidium iodide, acridine orange), or determining if terminal deoxynucleotodyl transferase dUTP nickend labeling (-TUNEL) showed an effect. Examining an equilibrium potential for K^+ ions by altering the potassium concentration in the bath producing a less negative Ek would reduce the driving gradient for an influx of K+ ions, but the nerve may depolarize and produce contractions. This could potentially be avoided by glutamate application; however, it does appear the glutamate

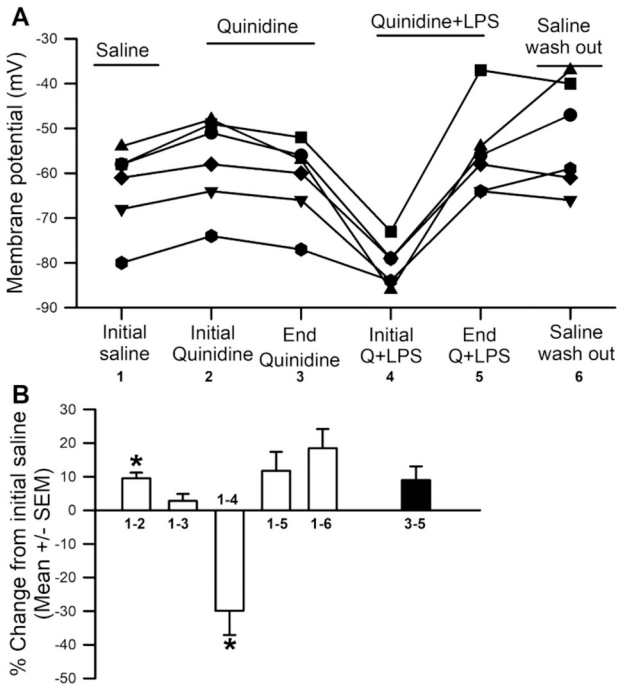

Fig. 12. Comparing membrane potential response to LPS when preparation was pre-treated with a K^+ channel blocker by (quinidine). (A) Addition of quinidine caused the preparations to depolarize initially, though the effect appeared to become less pronounced with time. LPS exposure caused hyperpolarization of each preparation, even though the preparation was pretreated with quinidine block. However, the preparations began to depolarize again by the end of the quinidine and LPS combined exposure, prior to saline washout. Saline washout caused them to continue to depolarize. (B) The positive histogram bar for 1–2 and 1–3 indicate depolarization with blocking of quinidine sensitive channels. The negative bar for 1–4 shows that LPS caused hyperpolarization, but the positive bar for 1–5 shows that this effect did not last throughout the entire application. The positive value for 1–6 indicates that the preparation could still be washed out. Interestingly, there was a positive histogram bar for 3–5, suggesting a depolarized membrane potential when comparing the end timepoint for the quinidine block versus the end timepoint for the LPS combined with quinidine block.

receptors are fully desensitized but are in an open state which would reduce input resistance of the membrane making it difficult to address the effect by LPS alone.

Funding source

Funding was provided by the University of Kentucky Neuroscience Research Priority Area NEURO Research Fellow Grant awarded to S.L.M.

CRediT authorship contribution statement

Shelby McCubbin: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Alexis Meade:** Methodology, Investigation, Formal analysis. **Douglas A. Harrison:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Conceptualization. **Robin L. Cooper:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study.

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