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Bacterial lipopolysaccharide hyperpolarizes the membrane potential and is antagonized by the K2p channel blocker doxapram

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ABSTRACT

Exposure of *Drosophila* skeletal muscle to bacterial lipopolysaccharides (LPS) rapidly and transiently hyperpolarizes membrane potential. However, the mechanism responsible for hyperpolarization remains unclear. The resting membrane potential of the cells is maintained through multiple mechanisms. This study investigated the possibility of LPS activating calcium-activated potassium channels (K_{Ca}) and/or K2p channels. 2-Aminoethyl diphenylborinate (2-APB), blocks uptake of Ca^{2+} into the endoplasmic reticulum (ER); thus, limiting release from ryanodine-sensitive internal stores to reduce the function of K_{Ca} channels. Exposure to 2-APB produces waves of hyperpolarization even during desensitization of the response to LPS and in the presence of doxapram. This finding in this study suggests that doxapram blocked the acid-sensitive K2p tandem-pore channel subtype known in mammals. Doxapram blocked LPS-induced hyperpolarization and depolarized the muscles as well as induced motor neurons to produce evoked excitatory junction potentials (EJPs). This was induced by depolarizing motor neurons, similar to the increase in extracellular K^+ concentration. The hyperpolarizing effect of LPS was not blocked by decreased extracellular Ca^{2+}or the presence of Cd^{2+}. LPS appears to transiently activate doxapram sensitive K2p channels independently of K_{Ca} channels in hyperpolarizing the muscle. Septicemia induced by gram-negative bacteria results in an increase in inflammatory cytokines, primarily induced by bacterial LPS. Currently, blockers of LPS receptors in mammals are unknown; further research on doxapram and other K2p channels is warranted. (220 words).

1. Introduction

Understanding the regulation of resting membrane potential of cells is key to grasping how cells behave in relation to stimulation and inhibition of cellular responses as well as the transmission of signals to other cells. Many types of ion channels, pumps, and exchangers play a key role in maintaining a membrane potential. Pharmacological regulation of cell excitability and inhibition is a hallmark of many clinical treatments for cardiac and neural control. Additionally, understanding how to combat and treat pathogens which affect the cellular membrane potential is essential.

Interestingly, the larval *Drosophila* body wall muscle and skeletal muscle of crayfish results in rapid hyperpolarization followed by a return of the membrane potential to resting levels in the next few minutes when exposed to the endotoxin of gram-negative bacteria (i.e., lipopolysaccharides; LPS). The mechanism behind this rapid hyperpolarization has yet to be determined. The hyperpolarization of the muscle with LPS exposure still occurs in the presence of Cd^{2+} or Gd^{3+}. The known blockers of voltage gated Ca^{2+} channel blockers, Cd^{2+} and Gd^{3+}, used in previous studies confirmed LPS was not activating a Ca^{2+} influx in the muscle to activate calcium-activated potassium channels (K_{Ca}) (Cooper and Krall, 2022; Potter et al., 2021; Vacassenno et al., 2023). In addition, when no Ca^{2+} was added to the bathing saline, LPS still managed to hyperpolarize the membrane potential. Muscle contractions and depolarization of larval *Drosophila* body wall muscle occurs in part from Ca^{2+} entry from extracellular fluid across the plasma membrane and internal release of Ca^{2+} from sarcoplasmic reticulum (SER). Incubation with tetraethylammonium (TEA) (20 mM) for 20 min, which blocks K_{Ca} channels in *Drosophila*, did not dampen the LPS effect. However, the mechanism behind the larval body wall muscle still contracting when extracellular Ca^{2+} was substituted for Cd^{2+} is unclear in earlier studies, in which LPS was applied. This result led to the proposal

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that an internal release of Ca^{2+} from the SER occurs. Thus, if activating Ca^{2+} release from ryanodine-sensitive internal stores is possible then this could be an indirect means to stimulate K_{Ca} channels while the plasma membrane voltage-gated Ca^{2+} channels are blocked by Cd^{2+}. 2-aminoethyl diphenylborinate (2-APB) was used in this study since it is known to block the sarco/endoplasmic reticulum Ca^{2+}-ATPase pump (SERCA) and thus, reduce the release of Ca^{2+} via a ryanodine receptor (RyR) (Ma et al., 2000, 2002; Prakriya and Lewis, 2001;Goto et al., 2010).

Previous reports on adult *Drosophila* and moth muscles have indicated that the equilibrium potential for K^+ may exceed −90 mV (Ikeda et al., 1976; Salkoff and Wyman, 1983). Therefore, the outflux of K^+ could be key to hyperpolarization with LPS. The possibility of Cl^- ion influx can be ruled out, as the chloride equilibrium potential is more depolarized above the resting membrane potential in body wall muscles of larval *Drosophila* (Rose et al., 2007; Stanley et al., 2019). Thoughts of acutely enhancing the action of the Na—K ATPase pump were considered; however, no precedence for such a possibility exists, and the use of ouabain (10 mM) did not block the rapid onset of hyperpolarization by LPS (Potter et al., 2021). To explain the acute hyperpolarization of the muscle by LPS we proposed that two-pore domain potassium (K2p) channels are stimulated by LPS. The acute nature in the hyperpolarization may occur due to desensitization to account for the rapid hyperpolarization for 2 min followed by repolarization. With longer exposure times to LPS the cells depolarize until there is no electrical potential.

The K2p channels are responsible for maintaining resting membrane potential. These channels are generally referred to as leak channels because they are constitutively open and drive the membrane potential toward the potassium equilibrium potential. Various subtypes of K2p channels have different pharmacological profiles. The *Drosophila* genome has 11 known subtypes, and their distribution in expression within tissues is yet to be elucidated (Adams et al., 2000; Littleton and Ganetzky, 2000). The family of channels is divided into six subfamilies (tandem-pore weak inward rectifying K^+ [TWIK], TREK, TASK, TALK, THIK, and TRESK) based on sequence similarity and functional resemblance (Plant and Goldstein, 2015). The nomenclature of the K2p subtypes is based on pharmacology and sensitivity to pH. Some of the classifications have been modified over the years (Duprat et al., 1997; Kamuene et al., 2021; Kim, 2005; Kim et al., 2000; Plant and Goldstein, 2015; Rajan et al., 2000).

The TWIK-1, TASK-1, and tandem-pore acid-sensing K^+ (TASK-3) channels are sensitive to protonation, which is relevant to the larval *Drosophila* and crayfish muscle as the membranes of these tissues depolarize at lower pH levels (Badre et al., 2005; Bierbower and Cooper, 2010, 2013). Interestingly, TASK-1 is activated only by halothane and isoflurane (Patel et al., 1999) and can lead to the hyperpolarization of cells. Moreover, the pharmacology of the K2p family of channels and their expression profiles in pathological conditions has clinical relevance (Lee et al., 2021).

Doxapram (stimulex or respiram) which is a blocker of some acid sensitive subtypes of K2p channels, and used in clinical applications, results in depolarization of larval *Drosophila* skeletal muscle (Vacassenno et al., 2023). Thus, in this study doxapram is used as a pharmacological approach to determine if the hyperpolarization induced by LPS can be blocked. The *Drosophila* model has served a vital role addressing the actions of the immune response induced by LPS which later led to a better understanding in the mechanisms of action in mammals and ultimately led to a few obtaining a Nobel Prize (i.e., Hoffmann and Beutler). The use of *Drosophila* continues to serve as a model, as a proof of concept, for many physiological studies related to other animals (Buckingham et al., 2005; Ugur et al., 2016; Yamaguchi and Yoshida, 2018).

K2p channels are known to function from yeast to humans with a wide range of subtypes in different organisms and their roles remain to be fully elucidated. The type of K2p channels expressed in skeletal muscle of *Drosophila* has yet to be identified. Even between rodents and humans, sensitivities to the enantiomers of isoflurane, an agonist of some K2p channels, are different. Additionally, regional differences exist in the central nervous system (CNS), including the spinal cord, of rodents and humans to the anesthetic bupivacaine and lidocaine, which are known to activate K2p channels (Keshavaprasad et al., 2005). Thus, examining further the function and pharmacology of K2p channels in various organisms will lead to an increase in the overall understanding of these unique channels (Buckingham et al., 2005).

Activation of K2p and/or K_{Ca} channels could result in hyperpolarization of the plasma membrane. 2-APB was used to further investigate the possibility of the Ca^{2+} release from SER, as loading of the SER should be blocked by this compound. Doxapram was used to examine if depolarization would occur upon exposure and if it could potentially block the hyperpolarization induced by LPS.

There are potential clinical reasons to determine the receptor subtypes for LPS that induce cytokine release, but also the potential for activation of K2p channels by LPS. However, whether LPS exerts an action on the K2p channels in mammals remains unknown. If so, recognizing that the receptors to LPS which could be blocked prior to a bolus of antibiotics to treat severe septicemia caused by gram-negative bacteria, would be advantageous in preventing a cytokine storm (Bone, 1991; Lin and Lowry, 1998). To date, blockers of the assumed LPS receptor complex, which is known as the CD14/TLR4/MD2 complex, remain unidentified in mammals (Du et al., 2000; Meng et al., 2010; Shimazu et al., 1999; Wright et al., 1990). Septicemia still causes high mortality worldwide, and in many cases, it is due to gram-negative bacterial strains and related endotoxins (CDC Statistics, 2022). Thus, understanding the potential action of LPS and developing models to examine the mechanisms of action are necessary.

2. Methods

Similar experimental conditions previously used to examine the possible mechanism of action of LPS in larval *Drosophila* muscles were used in this study (Bernard et al., 2020; Ballinger-Boone et al., 2020; Cooper et al., 2019; Istas et al., 2020; Potter et al., 2021; Vacassenno et al., 2023). The same procedures and *Drosophila* species as described by Vacassenno et al. (2023) were used in this study.

2.1. Animals

Drosophila melanogaster of the Canton S (CS) strain were used in all physiological assays. These strains have been isogenic in the laboratory for several years and were originally obtained from the Bloomington Drosophila Stock Center. All the animals were maintained in vials partially filled with cornmeal–agar–dextrose–yeast medium.

2.2. Neuromuscular junctions of larval Drosophila

The effects of doxapram and 2-APB on membrane potential, occurrence of evoked excitatory junction potentials (EJPs), and occurrence spontaneous quantal events (miniature excitatory junction potentials, mEJPs) were examined. All the experiments were performed at room temperature (20–21 °C). Early third-instar larvae of *D. melanogaster* were dissected in physiological saline. The segmental nerves were cut and sucked into a suction electrode filled with saline and stimulated. Segmental nerves were stimulated at 0.5 Hz (S88 Stimulator, Astro-Med Inc., Grass Co., West Warwick, RI, USA). To monitor the transmembrane potentials of the body wall muscle (m6) of the third instar larvae, sharp intracellular electrodes (30–40 M resistance) filled with 3 M KCl impaled the fibers. An axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA, USA) and a 1× LU head stage were used. The preparation was bathed in physiological saline (de Castro et al., 2014; Stewart et al., 1994): (NaCl 70 mM, KCl 5 mM, 20 mM $MgCl_2 \cdot 6H_2O$, $NaHCO_3$ 10 mM, Trehalose 5 mM, sucrose 115 mM, BES 25 mM, and 1 mM $CaCl_2 \cdot 2H_2O$,

pH 7.1). Doxapram powder was added directly to saline to obtain a concentration of 10 mM and placed on a vortex (high setting) for 5 min. However, the saline solution remained opaque. 2-APB powder was placed directly in saline and dissolved. In cases where no Ca^{2+} was added to the saline, the same compounds were used. In saline containing Cd^{2+}, $CdCl_2$ was used. LPS from *Serratia marcescens* was dissolved in physiological saline on the day of experimentation. A high concentration of LPS (250 μg/mL) was used for comparison with previous studies to best address the mechanisms of action (Potter et al., 2021). The lethal dose for 50 % survival of *S. marcescens* is 650 μg/mL (i.e., 6 × 106 colony-forming units) for rodents (Iwaya et al., 2005). This was the justification for using a relatively high concentration for *Drosophila*, as they are likely exposed to high levels of gram-negative bacterial strains in their native environment. All the chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

The exchanging of the media resulted in small deflections in the recordings in some cases, although these were generally only during the exchange and can be noted easily in the recording as artifactual. However, using an agar bridge with a chlorided silver wire for the ground in the bath minimized the electrical defects from changing the media. A 1 % agar plug was constructed within a 200 μL Eppendorf pipette tip made with saline. In most cases, saline without $CaCl_2$ was used to avoid the possibility of Ca^{2+} diffusing out when testing the Ca^{2+}-free media. The plastic pipette tip prevented a DC offset from varying when changing the media.

2.3. Statistical methods

In general, when normality was assumed, Shapiro–Wilk tests were used to validate the assumptions to determine the use of a *t*-test or Wilcoxon signed rank test. Significance was set at $P < 0.05$.

3. Results

3.1. Effects of LPS and 2-APB

Exposure to LPS immediately resulted in hyperpolarization of the membrane potential. When the segmental motor nerve was electrically stimulated, the evoked EJPs dampened in amplitude quickly along with mEJPs (Fig. 1). Rapid hyperpolarization occurred every time after exposure to LPS ($N = 6$; $P < 0.05$ sign test). Considering that the reversal potential for the ionotropic glutamate receptors on the larval *Drosophila* muscle is close to −10 mV to 0 mV, the amplitude of the EJPs and mEJPs should have increased with hyperpolarization owing to the increased driving grade. Upon flushing the LPS off the preparation by quickly exchanging the bathing media, the evoked and spontaneous events started to reappear. In previous studies, glutamate was applied while the muscle was exposed to LPS, and the muscle was insensitive to glutamate. Thus, glutamate receptors may be blocked by LPS while simultaneously hyperpolarizing the membrane potential. This result implies two different modes of action for LPS. This study focused on the mechanism of hyperpolarizing the membrane potential of the muscles.

2-APB is a known blocker of SERCA and indirectly reduces the function of K_{Ca} channels by reducing the free intracellular Ca^{2+} concentration. This compound had yet to be investigated for its potential effect on larval *Drosophila* muscle. Since the potential effects on this preparation were unknown, three different concentrations were examined for acute effects. The effects of 2-APB had been reported to occur

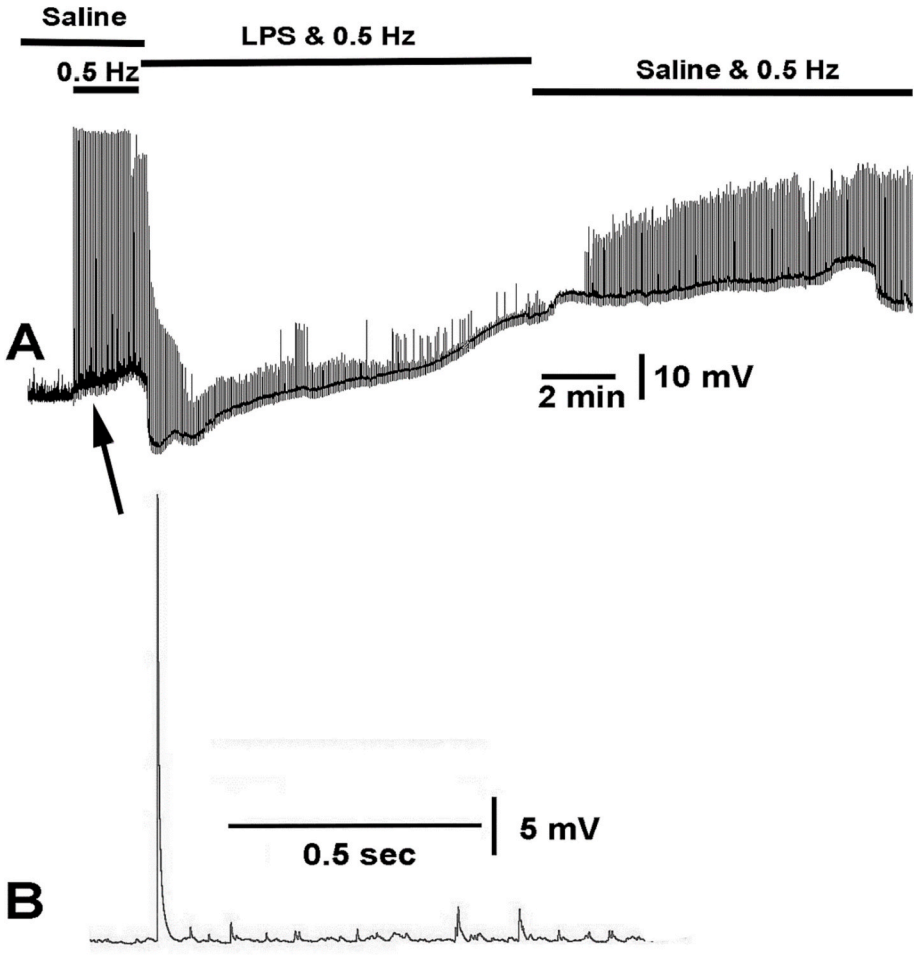

Fig. 1. LPS hyperpolarized the membrane potential in larval *Drosophila* muscle and blocked postsynaptic glutamate receptors. (A) Evoked excitatory junction potentials (EJPs) were induced every 2 s by stimulating the segmental nerve during saline and LPS exposure. The membrane hyperpolarized and increased the driving gradient for EJPs and spontaneous quantal events (mEJPs); however, the amplitudes were dampened gradually by LPS exposure and started to regain their amplitude during removal of LPS. (B) Enlarged view of evoked EJP along with mEJPs while bathed in saline.

rapidly in MDA-MB-231 breast cancer cells and mast cells (Chen et al., 2017; Schild et al., 2020), so we assessed the effects over a few minutes. Exposure to 5 μM 2-APB tended to depolarize the muscle by 2 to 3 mV within 2 min without any hyperpolarizing events (Fig. 2A and B; $N = 6$; $P < 0.05$ in the sign test). However, 50 and 500 μM both resulted in multiple hyperpolarizing events (Fig. 2B and C; N = 6; P < 0.05 in the sign test). In all cases (7/7 trials), the twitching of the muscle was significantly pronounced with 500 μM, which made it difficult to maintain and record a membrane potential. The 50-μM exposure also resulted in contractions within 3 min in many cases, which would dislodge the intracellular electrode (Fig. 2A). The hyperpolarizing pulses could reach 30 mV, although they varied in frequency and amount of electrical changes in each preparation. Two different preparations are illustrated in Figs. 2A and B to illustrate the type of variation. Moreover, upon exchanging the media with normal saline, hyperpolarizing events would still occur (Fig. 2B). Thus, removing the compound from the tissue by exchanging the bathing media may be difficult.

To examine whether 2-APB resulted in a Ca^{2+} influx to activate K_{Ca} channels, a medium in which no Ca^{2+} was added but contained Cd^{2+} (1 mM). Cd^{2+} blocks voltage gated Ca^{2+} channels. In addition, Ca^{2+} was unlikely to influx into the cell as the media was free of Ca^{2+}. This altered media with 2-APB (50 μM) prevented the hyperpolarizing pulses from appearing over a three minute period (Fig. 3; $N = 6$; $P < 0.05$ in the sign test). Longer time periods were not examined. After exchanging the media containing 2-APB with fresh saline containing Ca^{2+} and free of Cd^{2+}, hyperpolarizing pulses started to occur. This also reinforces the notion that entry of Ca^{2+} may have a stimulatory effect on a transient effect on K_{Ca} channels.

Similar to that of 2-APB, in examining the potential of LPS indirectly activating K_{Ca} channels, a similar paradigm was utilized, in which the bathing media was exchanged for one without added Ca^{2+} and another with added Cd^{2+} (1 mM). This was followed by an exchange for the same type of media, but with LPS. The switching of the initial normal media rapidly stopped an evoked synaptic transmission, as no Ca^{2+} was able to enter the presynaptic nerve terminal. Subsequently, with LPS exposure, a rapid hyperpolarization was observed. Upon flushing the bath with normal saline, the evoked events and initial membrane potential returned (Fig. 4; $N = 6$; $P < 0.05$, sign test).

Since doxapram is known to block an acid-sensitive subtype of K2p channels in mammals and considering that the larval muscle depolarizes at a lower pH, the muscle was examined for sensitivity to doxapram. Immediately upon exposure to doxapram, the muscle depolarized along with an increase in varied amplitudes of mEJPs and what appeared to be evoked EJPs in the absence of electrically stimulating the segmental

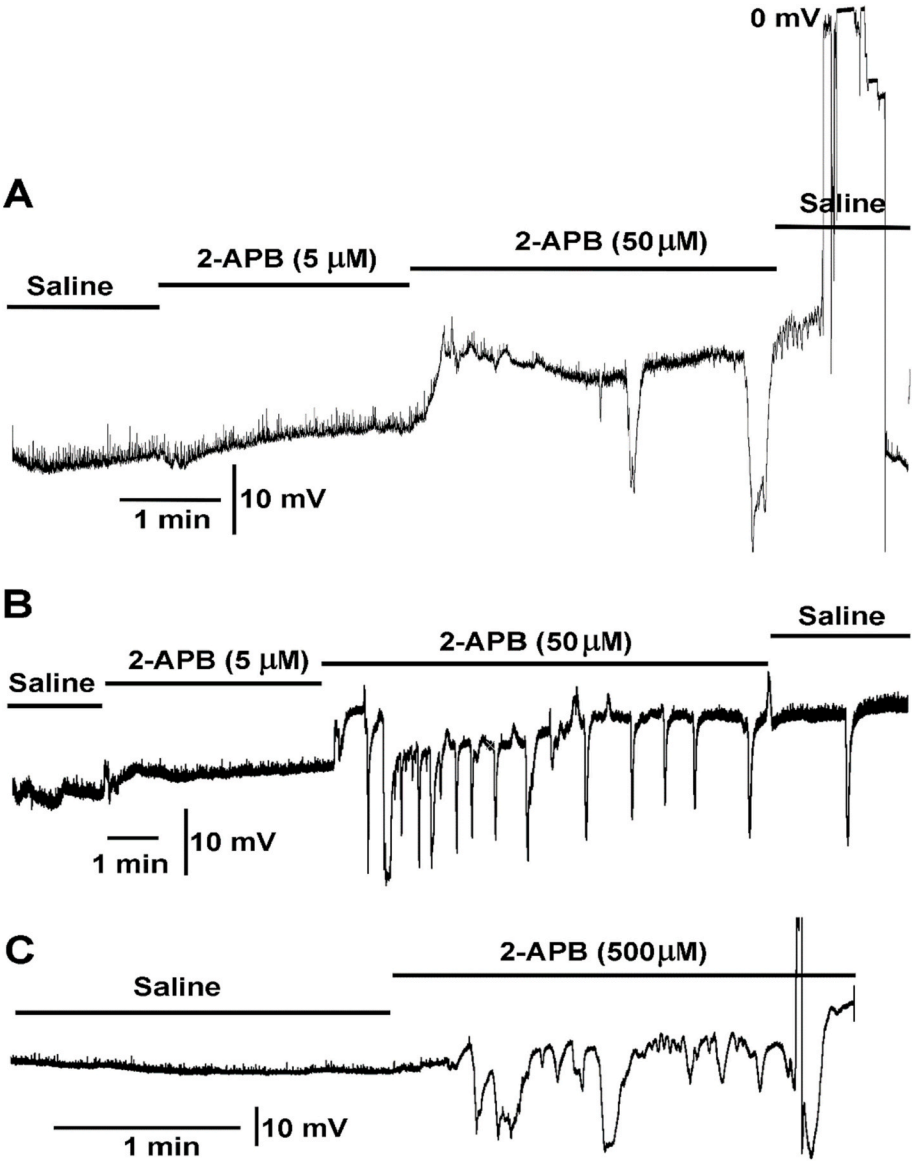

Fig. 2. The effect of 2-aminoethyl diphenylborinate (2-APB). (A) A representative response of 5 and 50 μM to 2-APB. Note the multiple pulses of hyperpolarization pulses. The muscle twitched in time which dislodged the intracellular electrode as noted by the potential going to 0 mV. (B) Another representative response of 5 and 50 μM to 2-APB. Note the multiple pulses of hyperpolarization pulses. (C) A representative response of 500 μM to 2-APB. Note the multiple pulses of hyperpolarization events. This concentration always resulted in strong twitching and damage of muscle fibers while attempting to record the intracellular electrodes (7 of 7 trials).

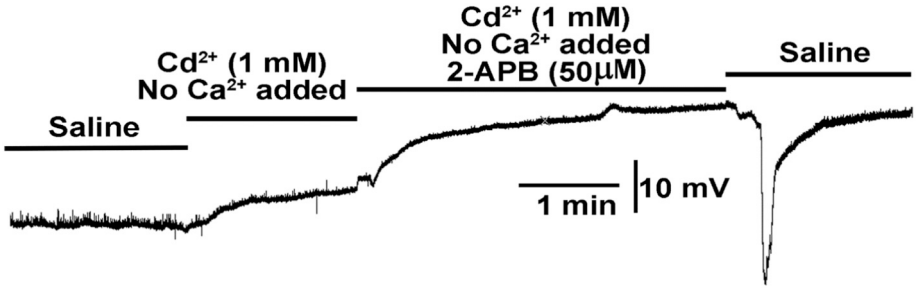

Fig. 3. Representative effect of exposure to Cd^{2+} in a saline without Ca^{2+} added to saline while exposed to 2-aminoethyl diphenylborinate (2-APB) (50 μM). Note the effect that low Ca^{2+} resulted in depolarization of the membrane potential, and no rapid hyperpolarization events occurred with exposure to 2-APB and Cd^{2+}. However, upon exposure to normal saline, a rapid hyperpolarization occurred due to the presence of Ca^{2+}.

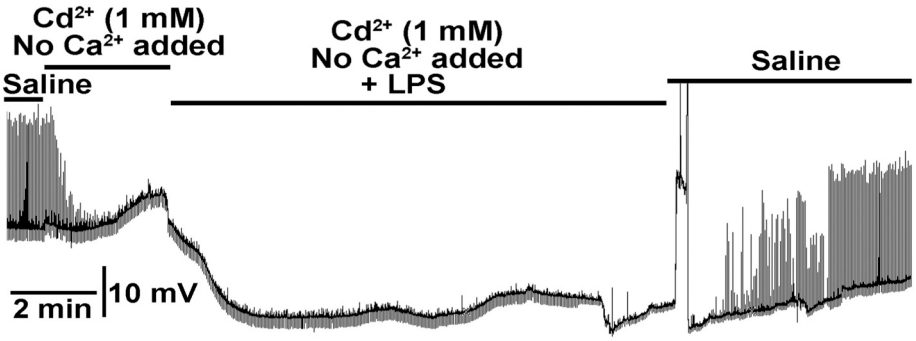

Fig. 4. Representative recording in response to exposure of LPS in a saline without Ca^{2+} added and Cd^{2+} (1 mM) present. Note the membrane potential rapidly hyperpolarized when exposed to LPS even without Ca^{2+} and with Ca^{2+} channels blocked by Cd^{2+}. Evoked EJPs are blocked when saline was exchanged with saline containing Cd^{2+} and no added Ca^{2+}. Upon re-exposure to normal saline, the evoked EJPs returned, and the membrane potential trended to the initial membrane potential.

nerve (Fig. 5; $N = 12$; $p < 0.05$ in the sign test). The muscles throughout the larval preparation had waves of contraction within 3 to 5 min. Maintaining an intracellular recording became difficult. Since the segmental nerves were cut, and the CNS was removed, the CNS did not induce EJPs. The motor neurons may likely have been depolarized from the doxapram and reached a threshold to produce action potentials resulting in the nerve terminal to be depolarized. The same phenomenon in depolarizing the muscle and inducing large evoked-like EJPs occurs when the extracellular media with 50 mM K^{+} was exposed to the preparation as revealed in a previous report (Vacassenno et al., 2023).

It was unexpected that 2-APB would produce a series of hyperpolarizing responses. If by chance 2-APB and LPS are using a similar mechanism in producing hyperpolarizing events, then when the membrane is showing some desensitization to LPS over time, the application of 2-APB would not likely have an effect if added along with LPS during this desensitizing period. However, 2-APB produced hyperpolarizing effects when applied along with LPS during the state in which the membrane demonstrated a reduced effect from LPS exposure (Fig. 6; $N = 6$; $P < 0.05$ in the sign test). Additionally, the muscle started to exhibit contractions with exposure to 2-APB, which normally did not occur with LPS exposure alone.

To examine whether doxapram would block the effect of LPS, doxapram was first exposed to the preparation for 2 min followed by a bath exchange containing doxapram and LPS. The short exposure to doxapram was intended to be able to exchange the media before the muscle started to have large contractions. With the combination and prior exposure to doxapram, the effect of LPS was substantially dampened and, in some cases, no hyperpolarization in the traces were detected (Fig. 7; $N = 6$; $p < 0.05$ in the sign test). In Fig. 7, a slight 2 mV deflection is observed during the exchange, although no pronounced hyperpolarization occurred. In all cases, a continual depolarization resulted in spontaneous muscle contractions over time.

To determine if doxapram blocks the effect of 2-APB in hyperpolarizing the muscle, doxapram was applied first, followed by

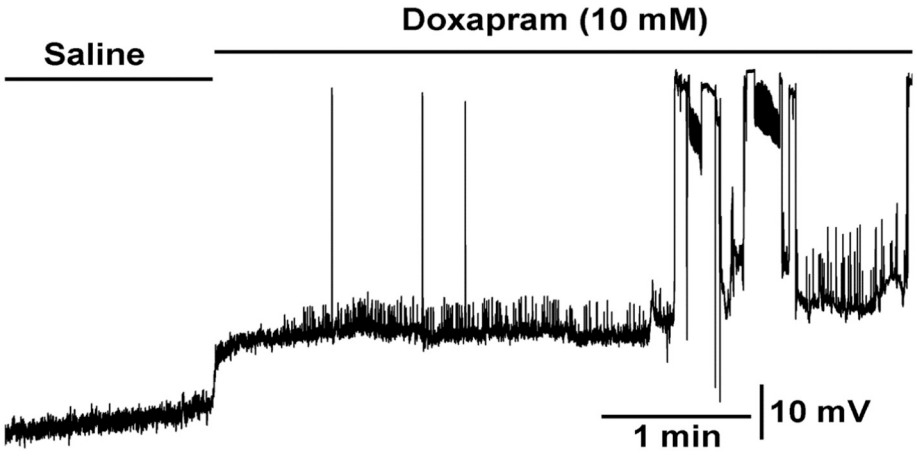

Fig. 5. Representative response to exposure of doxapram (10 mM) to the membrane potential of the larvae muscle and the motor neuron. Doxapram depolarized the muscle membrane and the motor neuron to a level of inducing evoked EJPs. The three large evoked EJPs are depicted along with an increase in frequency of mEJPs. The amplitude of the quantal responses become synchronized in fusion, likely due to increased presynaptic Ca^{2+} loading. In time, exposure of doxapram would result in muscle twitching and dislodging of the intracellular recording electrode.

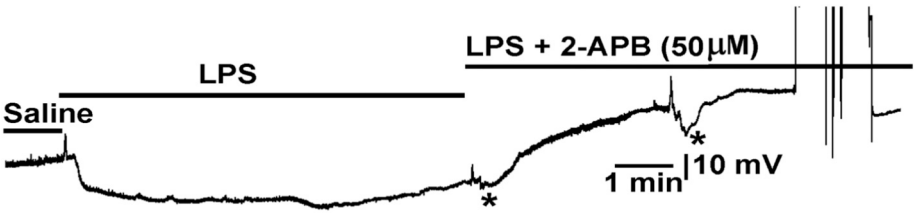

Fig. 6. Representative recording of the effect of lipopolysaccharides (LPS) and LPS in combination of 2-aminoethyl diphenylborinate (2-APB) (50 mM). LPS resulted in rapid hyperpolarization of the muscle. 2-APB still hyperpolarized the membrane (*asterisk) and in time resulted in twitching of the muscle dislodging the intracellular electrode from the muscle. The muscle desensitized to LPS during the application of LPS combined with the 2-APB solution.

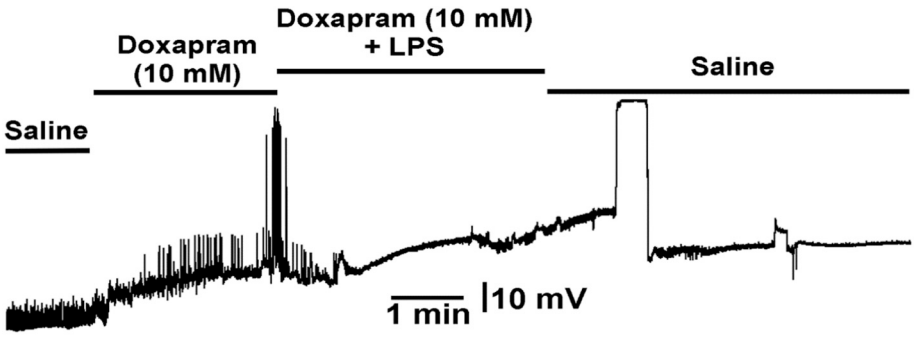

Fig. 7. Doxapram blocks the large hyperpolarization normally induced by lipopolysaccharides (LPS). Application of doxapram resulted in depolarization of the muscle and induced the presynaptic nerve to evoke excitatory junction potentials (EJPs). The EJPs are blocked by the LPS; however, the membrane does not undergo a significant hyperpolarization. LPS still blocked the postsynaptic glutamate receptors even with doxapram present.

doxapram combined with 2-APB. In all six preparations, 2-APB (50 μM) produced pulses of hyperpolarization in the presence of doxapram (Fig. 8; $N = 6$; $p < 0.05$ in the sign test). Two representative recordings are illustrated in Fig. 8A and B. As presented in Fig. 8A, even after switching the media to normal saline, a hyperpolarizing pulse was present. This was also observed when 2-APB was applied alone and

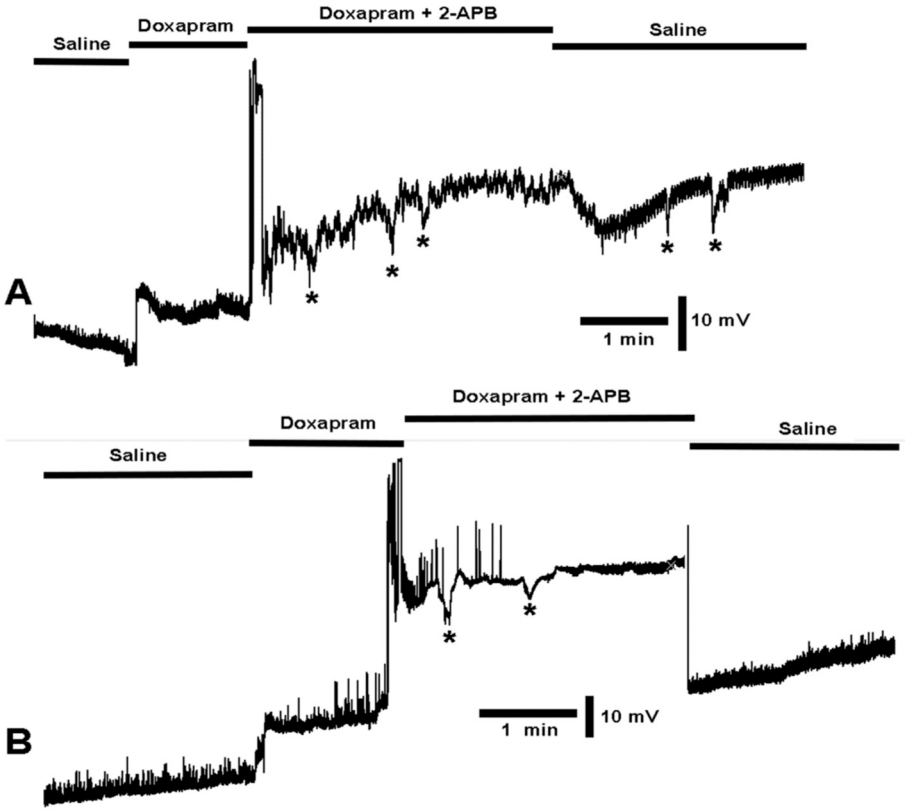

Fig. 8. 2-aminoethyl diphenylborinate (2-APB) produced hyperpolarization while doxapram depolarized the membrane. Doxapram did not block the response induced by 2-APB (*asterisk).

flushed with normal saline (Figs. 2 and 3). There was a wide variation in the amplitude of hyperpolarizing pulses within a preparation and among preparations during the exposure of a few minutes. The amplitude of hyperpolarizing pulses also varied depending on the amount of depolarization induced by doxapram. To quantify if doxapram had a small effect on altering the degree of hyperpolarization induced by 2-APB was problematic. Although a series of hyperpolarizing pulses did still occur in the presence of doxapram. The 2-APB also induced the muscles to contract, which in time resulted in losing the intracellular recording.

4. Discussion

The mechanism of action by which LPS hyperpolarizes the membrane potential had not been resolved previously. A Cl^- channel, or potentially K_{Ca} channels, as well as hyperactivation of the Na—K ATPase pump, were considered to be responsible. However, these suggestions regarding the use of the larval *Drosophila* muscle as a model can now be put to rest. The Cl^- equilibrium potential is more depolarized than the resting membrane potential of these cells (Rose et al., 2007; Stanley et al., 2019). Additionally, the use of low to no Ca^{2+} in the bathing media, along with the use of Cd^{2+} to block Ca^{2+} channels, reduces the feasibility of LPS mediating the K_{Ca} channels via Ca^{2+} influx. In addition, the use of 2-APB reduced the possibility of Ca^{2+} being released from the SER to activate K_{Ca} channels. Previous studies bathing preparations in voltage-gated K^+ channel blockers (i.e., TEA and 4-AP) did not reduce the effect of LPS in rapidly hyperpolarizing the membrane (Ballinger-Boone et al., 2020; Potter et al., 2021). In addition to blocking voltage-gated K^+ channels, TEA is known to block K_{Ca} channels (Gho and Mallart, 1986). Thus, it appears these channels were not activated by LPS. The application of ouabain, which blocks the Na—K ATPase pump, was used in a recent study (Potter et al., 2021), which demonstrated that LPS was still very effective in hyperpolarizing the membrane. An additional mechanism was then determined to account for the action of LPS, such as the activation of K2p channels in the muscle. K2p channels have known agonists, such as some anesthetics (Patel et al., 1999); however, as it was recently demonstrated that a blocker (i.e., doxapram) of K2p channels had an effect on larval *Drosophila* muscle (Vacassenno et al., 2023), analyzing whether doxapram could block the responses induced by LPS seemed logical. As demonstrated herein, doxapram blocked hyperpolarization normally induced by LPS. Additionally, an indirect means to reduce the function K_{Ca} via a reduction on Ca2+ released from RyR, via the ER by 2-APB, demonstrated that LPS still produced hyperpolarization. Thus, activation of K2p channels by LPS resulting in an increase in cytoplasmic Ca^{2+} from internal stores to then activate K_{Ca} channels and hyperpolarize the membrane also seems unlikely. An unexplained action of 2-APB in producing waves of hyperpolarizing events on its own as well as in the presence of doxapram was noted in this study. In speculation, perhaps 2-APB transiently simulates a different subtype of K2p channels than those activated by LPS. How the muscle contracts with exposure to 2-APB and the presence of extracellular Cd^{2+} has not been answered. A possibility is that some Cd^{2+} can enter the muscle through Ca^{2+} channels known to be on the plasma membrane of larval muscle. Cd^{2+} binding to troponin to induce skeletal muscle contraction in larval *Drosophila* may be possible as other studies have reported that Cd^{2+} can bind to troponin (Chao et al., 1990; Drakenberg et al., 1987).

K2p channels have many subtypes in nature, ranging from those expressed in yeast and plants to those expressed in humans. Of the 11 subtypes identified in the *Drosophila* genome, their location and pharmacological profiles have not yet been investigated (Adams et al., 2000; Buckingham et al., 2005; Littleton and Ganetzky, 2000). One subtype, referred to as ORK, which is expressed in the heart of *Drosophila* pupae, is known to affect the rate of the heartbeat (Lalevee et al., 2006), and altering the expression of K2p channels in the CNS of adult flies changes the circadian pattern (Park and Griffith, 2006). Physiological profiling of various K2p channels in *Drosophila* tissues has begun, and more specific tissues need to be investigated. Considering that low pH depolarizes larval muscle and that doxapram also results in depolarization is a good indication that one of the acid sensing K2p subtypes is expressed in the larval muscle. The subtype referred to as the pH sensitive (i.e., TASK) is likely to be expressed in the skeletal muscle as well as in the larval heart as the rate of the heartbeats is highly dependent on extracellular pH.

Since doxapram also causes the motor neurons to depolarize, resulting in possibly evoked EJPs, the neurons may likely express a TASK subtype. It would be of interest to determine whether motor neurons in mammals also express similar doxapram-sensitive subtypes because this compound is also clinically used in therapies primarily related to increasing respiration via activation of sensory neurons (Chen et al., 2020; Cunningham et al., 2020; Feketa and Marrelli, 2015; Komatsu et al., 2005; Song and Lyden, 2012; Vliegenthart et al., 2017; Yost, 2006). The compound may also influence neuromuscular junctions related to muscles used for respiration.

Since the channel activator 2-APB has been demonstrated in this study to affect *Drosophila* body wall muscles, the compound may serve the purpose of exploring the role of Ca^{2+} from internal stores in cardiac function in *Drosophila* as well as neurons for future experimental studies. The membrane potential fluctuates in waves of hyperpolarization when exposed to 2-APB which is an unusual response. It appears if there is some resistance to the action of the hyperpolarization pulse induced by 2-APB as the events are with a rapid onset and then repolarize. The mechanism of gradual desensitization of LPS with constant exposure has not yet been addressed. The K2p channels may desensitize to LPS or that compensatory mechanisms of the cell may reestablish the original resting membrane potential. Macrophages from mammals are widely known to downregulate to LPS binding sites over time (Fahmi and Chaby, 1993) but not in minutes, as with the acute exposure of the larval *Drosophila* muscle.

In summarizing the outcome of this study and progress in the current understanding in the actions of LPS on the larval *Drosophila* skeletal muscle, an overview schematic is presented in Fig. 9. This study addressed if doxapram, a K2p channel blocker, could block the actions of the hyperpolarization induced by LPS and secondly if the hyperpolarization could potentially be the result of activating K_{Ca} channels either through Ca^{2+} entry via the plasma membrane or through the release of Ca^{2+} via the SER (Fig. 9A). In previous studies, and in this study, it was demonstrated that LPS blocks the postsynaptic glutamate receptors (Fig. 9B) as evoked and spontaneous quantal events are gradually reduced in amplitude and the muscle is not responsive to applied glutamate (Cooper et al., 2019; Istas et al., 2020). Doxapram blocks the hyperpolarization induced by LPS but does not relieve the blockage of glutamate receptors (Fig. 9B). However, doxapram alone does not block glutamate receptors. It does not appear that an intracellular rise in Ca^{2+} from either Ca^{2+} influx across the plasma membrane or internal release of the SER can account for the potential activation of K_{Ca} as a mechanism of the LPS induced hyperpolarization (Fig. 9B). Doxapram alone is assumed to depolarize the motor neuron to account for the random EJPs. Thus, suggesting doxapram sensitive K2p channels are also present on the motor neurons. Thus, the action of LPS in the larval *Drosophila* neuromuscular junction preparation is twofold: (1) blocking postsynaptic glutamate receptors and (2) hyperpolarizing the muscle due to doxapram sensitive K2p channels.

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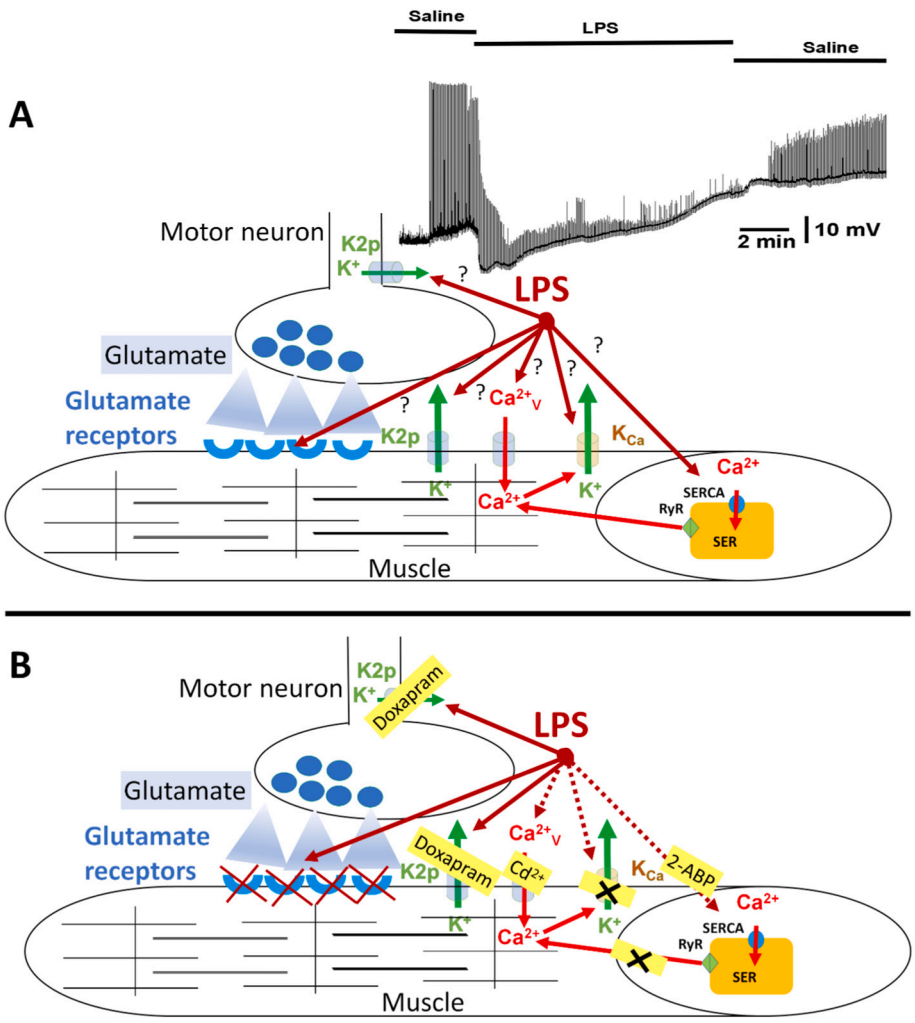

Fig. 9. Schematics in the potential actions of LPS and processes which have been identified or eliminated as mechanisms to explain the observations using the larval *Drosophila* neuromuscular junction. (A) Proposed targets of LPS to account for the observed effects of LPS. (B) LPS blocks glutamate receptors on the muscle and activates the doxapram sensitive K2p channels (solid red lines). LPS is not activating K_{Ca} channels via Ca^{2+} influx or from Ca^{2+} release from SER (dashed red lines). Doxapram depolarizes the resting membrane potential of the muscle and nerve which induces the nerve to produce random post-synaptic potentials.

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.

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