Synaptic excitation is regulated by the postsynaptic dSK channel at the *Drosophila* larval NMJ

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Gertner DM, Desai S, Lnenicka GA. Synaptic excitation is regulated by the postsynaptic dSK channel at the *Drosophila* larval NMJ. *J Neurophysiol* 111: 2533–2543, 2014. First published March 26, 2014; doi:10.1152/jn.00903.2013.—In the mammalian central nervous system, the postsynaptic small-conductance Ca\(^{2+}\)-dependent K\(^+\) (SK) channel has been shown to reduce postsynaptic depolarization and limit Ca\(^{2+}\) influx through N-methyl-D-aspartate receptors. To examine further the role of the postsynaptic SK channel in synaptic transmission, we studied its action at the *Drosophila* larval neuromuscular junction (NMJ). Repetitive synaptic stimulation produced an increase in postsynaptic membrane conductance leading to depression of excitatory postsynaptic potential amplitude and hyperpolarization of the resting membrane potential (RMP). This reduction in synaptic excitation was due to the postsynaptic SK channel (dSK) channel; synaptic depression, increased membrane conductance and RMP hyperpolarization were reduced in dSK mutants or after expressing a Ca\(^{2+}\) buffer in the muscle. Ca\(^{2+}\) entering at the postsynaptic membrane was sufficient to activate dSK channels based upon studies in which the muscle membrane was voltage clamped to prevent opening voltage-dependent Ca\(^{2+}\) channels. Increasing external Ca\(^{2+}\) produced an increase in resting membrane conductance and RMP that was not seen in dSK mutants or after adding the glutamate-receptor blocker philanthotoxin. Thus it appeared that dSK channels were also activated by spontaneous transmitter release and played a role in setting membrane conductance and RMP. In mammals, dephosphorylation by protein phosphatase 2A (PP2A) increased the Ca\(^{2+}\) influx through NMDA receptors by reducing Ca\(^{2+}\)-methyl-D-aspartate (NMDA) receptor and specifically acted to limit Ca\(^{2+}\) influx through NMDA receptors by reducing postsynaptic depolarization.

It remained unclear whether postsynaptic SK channels played a more general role in regulating synaptic excitation. To examine this, we studied the effect of the postsynaptic *Drosophila* SK channel (dSK) in regulating synaptic excitation at the larval neuromuscular junction (NMJ). The *Drosophila* NMJ has become a popular model system for the study of synapses, and these identified synapses are particularly good to study the regulation of synaptic strength (Keshishian et al. 1996). Here synaptic strength must be precisely regulated since synaptic depolarization grades muscle fiber contraction, and very few fibers are used to produce movement. The larval muscle fibers have non-NMDA glutamate receptors that admit Ca\(^{2+}\), and the muscle also appeared to contain a dSK channel (Abou Tayoun et al. 2011; Chang et al. 1994). In addition, we had observed that experimental increases in postsynaptic Ca\(^{2+}\), produced reduced synaptic excitation due to activation of a Ca\(^{2+}\)-dependent K\(^+\) conductance (g\(_{KCa}\)). In the current experiments, we studied whether transmitter release activated the postsynaptic dSK channel. We found that Ca\(^{2+}\) entry at the postsynaptic membrane during spontaneous and evoked transmitter release activated the dSK channel to hyperpolarize the membrane and reduce EPSP amplitude. We propose that the postsynaptic SK channel acts generally as a control mechanism to limit synaptic excitation and stabilize synapses.

**MATERIALS AND METHODS**

Experiments were performed on muscle fiber 6 in segments 3 and 4 of *Drosophila* wandering third-instar larvae. The following *Drosophila* stocks were used: wild type, Canton-S (CS); dSK\(^{-}\), which contains a deletion in the *Drosophila* SK gene (Abou Tayoun et al. 2011); slo\(^{2}\) (Bloomington stock 4587), which eliminates g\(_{CV}\) in larval muscle; P\(^{GawB}\)how24B (Bloomington stock 1767), expresses GAL4 in all embryonic and larval somatic muscles; and UAS-dSK\(^{dn}\)myc, which contains a dominant-negative dSK subunit (Abou Tayoun et al. 2011). P\(^{GawB}\)how24B and UAS-dSK\(^{dn}\)myc were crossed to express the dominant-negative dSK subunit in only muscle fibers (Brand and Perrimon 1993). After an incision through the dorsal body wall, the larvae were pinned out in a physiology chamber, and the internal organs were removed to expose the body-wall muscles. In our initial studies, the preparation was bathed in HL3 saline (Stewart et al. 1994)
containing 1 mM Ca\(^{2+}\), and in later studies, we used HL3.1 saline (Feng et al. 2004) with 0 or 1.5 mM Ca\(^{2+}\).

**Electrophysiology.** To evoke synaptic responses, the cut end of the segmental nerve was stimulated with a suction electrode connected to a S11 stimulator (Grass-Telefactor, West Warwick, RI). Both axons were stimulated to record the compound EPSPs or excitatory post-synaptic currents (EPSCs) (referred to as simply EPSPs or EPSCs). EPSPs or EPSCs were recorded using sharp microelectrodes (20–30 M\(\Omega\) filled with 3 M KCl) connected to Axoclamp 2A or GeneClamp 500 (Molecular Devices, Sunnyvale, CA). Data were acquired (sampling rate 5–10 kHz) and analyzed using a Digidata 1440A digitizer (Molecular Devices) and pCLAMP 10.3 software (Molecular Devices). For voltage clamping, a grounded shield was placed between the electrodes to reduce capacitive coupling, and the holding potential was set at −60 mV. Input conductance (\(G_{\text{m}}\)) was measured in current clamp with a single electrode by passing 5 nA of hyperpolarizing current; the bridge was balanced or the electrode resistance was digitally subtracted. During voltage clamp, \(G_{\text{m}}\) was measured with −20 mV, 0.4-s voltage steps. For all experiments, the initial EPSP amplitudes represent the mean of 10 responses evoked at 0.1 Hz. To inhibit protein phosphatase 2A (PP2A), we added 100 nM calyculin A (Aldrich, St. Louis, MO) to the saline.

**RESULTS**

During repetitive synaptic activity, there was an increase in \(G_{\text{m}}\) and resting membrane potential (RMP) due to an increase in postsynaptic [Ca\(^{2+}\)]\(_i\). We found that increasing the muscle fiber [Ca\(^{2+}\)]\(_i\), either by blocking the plasma membrane Ca\(^{2+}\) ATPase or direct Ca\(^{2+}\) injection resulted in membrane hyperpolarization and a reduction in EPSP amplitude. This was due to activation of a \(g_{\text{K}^+}\), and was consistent with previous studies showing a fast, inactivating \(g_{\text{K}^+}\) (\(g_{\text{CF}}\)) and a slow \(g_{\text{K}^+}\) (\(g_{\text{CS}}\)) in larval muscle fibers (Gho and Mallart 1986; Salkoff 1983; Singh and Wu 1989; Wu et al. 1983).

We determined whether synaptic activity produced an increase in the RMP and membrane conductance (\(G_{\text{m}}\)) that was consistent with activation of \(g_{\text{K}^+}\). Both motor axons innervating muscle fiber 6 were stimulated at 20 Hz for 60 s, and we measured \(G_{\text{m}}\) and RMP before and after stimulation. We used HL3 with reduced Ca\(^{2+}\) (1 mM), since 20-Hz stimulation in this saline produced minimal contraction and allowed stable intracellular recordings. During stimulation, there was a decrease in EPSP amplitude and hyperpolarization of the RMP, which combined to reduce synaptic excitation (Fig. 1A, left). In addition, there was an increase in \(G_{\text{m}}\) as a result of 20-Hz stimulation (Fig. 1A, left). We used the EPSP peak potential as a measure of synaptic excitation, since this reflected both the decrease in EPSP amplitude and RMP hyperpolarization. The combined data (Fig. 1B) showed that the final EPSP peak (−56.7 ± 1.9 mV) was significantly more negative than the initial one (−24.7 ± 0.9 mV). This resulted from depression of EPSP amplitude by −22 mV and hyperpolarization of the RMP by −10 mV (Fig. 1C). The increase in \(G_{\text{m}}\) during the train was 0.81 ± 0.11 \(\mu\)S (Fig. 1D); this would have produced about one-third of the decrease in EPSP amplitude seen during 20-Hz stimulation (see below). Surprisingly, there was a small increase in \(G_{\text{m}}\) that persisted for almost 10 min after the train, which is likely after the postsynaptic [Ca\(^{2+}\)]\(_i\) had returned to baseline.

We examined whether the increase in \(G_{\text{m}}\) and RMP was due to an increase in postsynaptic [Ca\(^{2+}\)] by expressing the Ca\(^{2+}\)-buffer, parvalbumin (PV) in the muscle. Flies with UAS-PV transgenes on both the second and third chromosomes (Harrisingh et al. 2007) were crossed to \(P\text{(GawB)how24B}\) flies to produce 24B/PV larvae expressing PV in the muscles. The UAS-PV transgene contained a myc epitope tag, and expression of PV in the muscle was confirmed using an antibody to myc (data not shown). During 20-Hz stimulation, PV expression appeared to eliminate the change in the EPSP peak and \(G_{\text{m}}\) that was seen in CS larvae (Fig. 1A, right). For 24B/PV larvae, the combined data showed no significant change in the EPSP peak, EPSP amplitude or RMP during 20-Hz stimulation (Fig. 1, B and C). The UAS-PV larvae also served as a control and showed a reduction in EPSP amplitude and an increase in RMP and \(G_{\text{m}}\) that was similar to CS larvae. The 24B/PV larvae showed a significant increase in \(G_{\text{m}}\), but the increase was significantly smaller (\(P < 0.05\), ANOVA, Dunn test) than seen for either of the controls (Fig. 1D).

PV expression appears to have resulted in a reduction in transmitter release since the basal EPSP was smaller, and this was not due to a change in resting \(G_{\text{m}}\). The reduction in basal transmitter release would result in less postsynaptic Ca\(^{2+}\) influx, and this could contribute to the reduced change in RMP and \(G_{\text{m}}\) in 24B/PV larvae. (However, there may have also been less depression of transmitter release during 20-Hz stimulation, which would have the opposite effect.) To examine this further, we selected CS larvae with the smallest initial EPSPs (not significantly different from the 24B/PV larvae; \(P > 0.05\), t-test) and compared them to the 24B/PV larvae. These CS larvae (\(n = 5\)) had a significantly greater increase in RMP (−9.0 ± 3.1 mV) and \(G_{\text{m}}\) (0.34 ± 0.11 \(\mu\)S) than 24B/PV larvae (2.3 ± 2.7 mV; \(P < 0.05\) and 0.04 ± 0.01 \(\mu\)S; \(P < .001\), t-test); this argues that the postsynaptic Ca\(^{2+}\) buffering prevented the increase in \(G_{\text{m}}\) and RMP.

Repetitive synaptic stimulation activated dSK channels to reduce synaptic excitation. We assumed that the increase in \(G_{\text{m}}\) resulted from \(g_{\text{CS}}\), since \(g_{\text{CF}}\) shows rapid inactivation. It was proposed that \(g_{\text{CS}}\) in larval muscle was produced by the *Drosophila* homologue of the mammalian SK (dSK) channel (Abou Tayoun et al. 2011). Thus we examined the effects of repetitive synaptic activity on dSK− larvae with a deletion in the gene for the dSK channel or *P(GawB)how24B/UAS-dSKDNmyc* larvae (24B/dSKDN) in which the dSK dominant-negative subunit was expressed in the muscle. We used Western blots to confirm that the dSK protein isoform seen in muscle was missing in dSK−. Using an antibody generated against the predicted protein isoforms (Abou Tayoun et al. 2011), our Western blots showed a single band in larval brain and muscle, which was absent in the mutant (Fig. 2A). Also, we
confirmed expression of dSK^DN in the muscle using an antibody to myc (data not shown).

We found that during 20-Hz stimulation of dSK^- larvae the EPSP peak potential remained constant, and there was no increase in G_in (Fig. 2B). The combined data clearly showed that, for both dSK^- and 24B/dSK^DN, there was no significant change in the EPSP peak during 20-Hz stimulation (Fig. 2C). Furthermore, the combined results showed that eliminating a functional dSK channel prevented the changes in EPSP amplitude, RMP and Gin that were previously seen in CS larvae (Fig. 2D). Similar to the PV-expressing larvae, it appeared that dSK^- and 24B/dSK^DN had reduced basal transmitter release since the EPSP was smaller, especially for 24B/dSK^DN. To increase transmitter release for 24B/dSK^DN, we repeated the synaptic stimulation in HL3.1 (1.5 mM Ca^{2+}); HL3.1 has a lower Mg^{2+} concentration (4 mM) than HL3 (20 mM) and produces greater presynaptic Ca^{2+} influx than HL3 (Desai and Lnenicka 2011). Under these conditions, we still did not see an effect of repetitive stimulation on EPSP amplitude, RMP or G_in (Fig. 2, C and D), providing strong evidence that synaptic activity activates dSK.

The synaptic activation of the dSK conductance (g_Sk), and not g_CF, was supported by repeating this experiment using slo^1 larvae, which lack g_CF in their muscle fibers (Singh and Wu 1989). The experiments with slo^1 larvae gave results similar to those using CS larvae. During 20-Hz stimulation, the EPSP peak became more negative (−19.3 ± 3.2 mV to −49.6 ± 2.0 mV), EPSP amplitude was reduced (30.0 ± 3.6 mV to 11.1 ± 5.1 mV), the RMP hyperpolarized (−49.3 ± 0.8 mV to −63.6 ± 2.4 mV) and G_in increased (0.34 ± 0.08 μS to 0.80 ± 0.07 μS; all P < 0.05; paired t-tests, n = 4).

Ca^{2+} entry at the postsynaptic membrane was sufficient to activate the dSK channel. During synaptic activity, the dSK channel could be activated by Ca^{2+} entering through glutamate-activated channels at the postsynaptic membrane (Desai and Lnenicka 2011; Guerrero et al. 2005) and/or voltage-dependent Ca^{2+} channels, which are presumably distributed throughout the muscle membrane (Chang et al. 1994; Wu et al.
To determine whether Ca\(^{2+}\) entry at the postsynaptic membrane was sufficient, we stimulated the synapses at 20 Hz for 60 s while voltage clamping the muscle fiber at \(-60\) mV to prevent the voltage-dependent Ca\(^{2+}\) channels from opening (Ren et al. 1998). We used the same saline as in the previous current-clamp experiments (HL3, 1 mM Ca\(^{2+}\)). During synaptic stimulation, the leakage current was measured during a step hyperpolarization every 10 s to determine \(G_{\text{in}}\) (Fig. 3A). We found that \(G_{\text{in}}\) continually increased during stimulation, and the increase reached 3.0 ± 0.3 \(\mu\)S at the end of stimulation (Fig.
This increase in $G_{in}$ was greater than that seen during 20-Hz stimulation in current clamp (Fig. 1), presumably because there was a greater synaptic current during voltage clamp (see below) and greater Ca$^{2+}$ influx. During the voltage-clamp, Ca$^{2+}$ influx should have been restricted to the postsynaptic membrane; therefore, Ca$^{2+}$ entry at the postsynaptic membrane was sufficient to increase $G_{in}$. We also stimulated the nerve for 60 s at lower frequencies, 5 and 10 Hz, and measured the increase in $G_{in}$ at the end of the stimulation. The results show that these lower stimulation frequencies produced a smaller increase in $G_{in}$, and the relationship between stimulation frequency and $g_{SK}$ appeared to be nonlinear (Fig. 3B).

The $dSK$ channel influenced resting $G_{m}$ and the RMP; it was apparently activated by spontaneous transmitter release. Previous studies have reported that increases in external Ca$^{2+}$ produced hyperpolarization of the RMP (Jan and Jan 1976; Krans et al. 2010); it was proposed that this resulted from greater membrane-to-electrode sealing and a reduction in the electrode shunt conductance (Jan and Jan 1976). Alternatively, the greater RMP seen in high external Ca$^{2+}$ could result from greater activation of $dSK$ channels. To distinguish between these possibilities, we altered Ca$^{2+}$ in HL3.1 saline and measured the RMP and $G_{in}$ (Fig. 4A). Measurements were made from muscle fiber 6 in segments 3 and 4 from one side of the animal, and then external Ca$^{2+}$ was changed, and we remeasured the original fibers and also made measurements from the contralateral fibers. In the end, we combined the values from the two sides since they were not significantly different; i.e., the prior electrode penetration of the muscle fiber did not affect a subsequent measurement of RMP and $G_{in}$. We either increased external Ca$^{2+}$ from nominally 0 to 1.5 mM and or reduced it from 1.5 mM to 0, and, since both experiments gave similar values, these data were also combined. For CS larvae, these within-animal comparisons clearly showed that physiological Ca$^{2+}$ (1.5 mM) gave a higher $G_{in}$ and greater RMP than 0 Ca$^{2+}$ (Fig. 4, A and B); this is consistent with activation of $g_{SK}$. A decrease in the electrode shunt conductance in 1.5 mM Ca$^{2+}$ would have given a lower $G_{in}$, and this was not seen.

To determine whether the effects of altering external Ca$^{2+}$ were dependent on $dSK$ channels, we repeated the experiments in $dSK^{-}$ larvae. Both the $G_{in}$ and RMP in $dSK^{-}$ larvae were insensitive to changes in external Ca$^{2+}$ (Fig. 4, A and B). This confirmed that external Ca$^{2+}$ influences the resting $G_{in}$ and RMP by acting on $dSK$ channels.

It appeared that the leakage conductance ($g_{L}$) for $dSK^{-}$ larvae was greater than that seen in CS larvae, since $G_{in}$ in 0 Ca$^{2+}$ was significantly greater for the $dSK^{-}$ fibers (0.31 ± 0.03 µS) compared with the CS fibers (0.16 ± 0.02 µS; $P < 0.001$; t-test). We assumed that in 0 Ca$^{2+}$ $g_{SK}$ was not active so that $G_{in}$ results entirely from $g_{L}$. This thus appears that in $dSK^{-}$ larvae there was an increase in $g_{L}$, presumably to compensate for the lack of $dSK$ channels.

What was responsible for activation of $dSK$ channels at rest? One possibility is that the postsynaptic Ca$^{2+}$ transients produced by spontaneous transmitter release activated these $dSK$ channels (Desai and Lnenicka 2011). To test this, we blocked glutamate receptors with 4 µM philanthotoxin (PhTX) and again increased external Ca$^{2+}$ from 0 to 1.5 mM (Fig. 4, A and B). Note we found that 4 µM PhTX produced a 65% decrease in miniature EPSP (minEPSP) amplitude, which was similar to previously reported values (Frank et al. 2006). PhTX application blocked the increase in $G_{in}$ and RMP normally seen when Ca$^{2+}$ was increased to 1.5 mM. Thus activation of glutamate receptors is necessary for the effects of external Ca$^{2+}$, and presumably Ca$^{2+}$ enters through glutamate receptors during spontaneous transmitter release to activate the $dSK$ channels.

Although PhTX blocked the increase in RMP when Ca$^{2+}$ was increased, it appeared to have an independent effect on the RMP, since the RMP measured in 0 Ca$^{2+}$ in CS larvae (Fig. 4B) was greater in the presence of PhTX (−63.6 ± 1.6 mV) than in the absence of PhTX (−52.1 ± 1.1 mV; $P < 0.001$; t-test). To confirm these results, we performed within-animal comparisons in 0 Ca$^{2+}$ before and after adding PhTX. Adding PhTX produced a significant increase in the RMP from −54.0 ± 1.2 mV to −63.8 ± 2.1 mV ($n = 7$; $P < 0.001$, paired t-test) and a small decrease in $G_{in}$, which was not significant (0.16 ± 0.02 µS to 0.14 ± 0.02 µS; $P > 0.1$, paired t-test). This confirmed that PhTX produced about a 10-mV hyperpolarization of the RMP that was apparently independent of a significant change in $G_{in}$. We further examined the effect of PhTX by adding it to muscle fibers bathed in 1.5 mM Ca$^{2+}$. Adding PhTX resulted in a significant reduction in $G_{in}$ (0.33 ± 0.09 µS).
to 0.18 ± 0.04 μS, n = 11; P < 0.001, paired t-test) as expected, since it would reduce activation of the dSK channels. There was no significant change in the RMP (∆65.7 ± 1.3 mV to ∆64.7 ± 1.1 mV; P > 0.1, paired t-test), which is consistent with PhTX having dual opposing effects on RMP. One possible explanation for the independent effect of PhTX on the RMP is that the glutamate receptors are activated at rest, possibly due to nonvesicular glutamate release, as has been proposed (Featherstone et al. 2002). In this scenario, the resting activation of glutamate receptors depolarizes the membrane by activating a synaptic conductance (Gsyn), but the density of open glutamate receptors would be too low to produce Ca²⁺ signals large enough to activate dSK channels. Given a reversal potential of −1 mV (Jan and Jan 1976), the 10-mV hyperpolarization could occur with very little change in Gsyn. In fact, if the small decrease in Gsyn seen when adding PhTX to 0 Ca²⁺ saline resulted from a decrease in Gsyn, then the new RMP would be calculated to be ∆64.4 mV, which is very close to that seen experimentally (∆63.8 mV).

We examined whether the normal variability in the RMP resulted from variability in the activation of gSK. If this is the case, there should be a positive correlation between Gsyn and the RMP; i.e., greater activation of gSK should result in a larger Gsyn and RMP. We examined Gsyn and the RMP for the data in Fig. 1 (before 20-Hz stimulation) where we had the largest number of experiments performed in the same saline. In these experiments, there was approximately a fourfold range of resting Gsyn values, and the RMP had a range of about 20 mV. We found a significant correlation between Gsyn and the RMP (Fig. 4C). We performed the same analysis on dSK mutants using the data from Fig. 2 and found no significant correlation between Gsyn and the RMP (r = 0.13, n = 32, P > 0.10). Thus the dSK channel plays a role in setting the RMP, and normal variability in gSK contributes to variability in the RMP.

**Activation of the dSK channel is reduced by phosphatase 2A inhibitors.** In mammals, the Ca²⁺ sensitivity of the SK channel is increased by dephosphorylation by PP2A; it appears that an increase in [Ca²⁺]ᵢ favors dephosphorylation and increased Ca²⁺ sensitivity (Allen et al. 2007). Activation of the dSK channels by increased [Ca²⁺]ᵢ, seen here, could involve the action of PP2A. To examine this, we repeated experiments involving 20-Hz stimulation (Fig. 1) and increasing external Ca²⁺ (Fig. 4) in the presence of PP2A inhibitors. We utilized two phosphatase inhibitors: a specific PP2A inhibitor (calyculin A), and a general phosphatase inhibitor (okadaic acid). These inhibitors have been used to block PP2A function in Drosophila Schneider cells, and we used 100 nM concentrations for both inhibitors (Banreti et al. 2012; Itagaki et al. 2004; Sathyanarayanan et al. 2004). We repeated the 20-Hz stimulation experiments in the presence of the inhibitors, which were added 10 min prior to stimulation, and compared the results to the control (CS) values from Fig. 1. Overall the changes in

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**Fig. 4.** Resting Gsyn and RMP were influenced by dSK channels and spontaneous transmitter release. The effect of external Ca²⁺ on Gsyn and RMP was examined for muscle fiber 6 in segments 3 and 4. A: representative measurements of Gsyn in single fibers when the external Ca²⁺ was changed from 0 to 1.5 mM. V traces for wild-type larvae (CS), dSK− larvae and CS larvae after adding philanthotoxin (CS PhTX) are shown. The I trace was the same for all. Calibration: V = 15 mV, 200 ms, I = 3 nA. B: combined data showed the change in Gsyn and RMP when external Ca²⁺ was increased from 0 to 1.5 mM. For each animal, multiple muscle fibers were sampled, and the average Gsyn and RMP values were determined. For CS larvae, there was a significant increase in Gsyn and RMP as Ca²⁺ increased; however, dSK− larvae and CS PhTX larvae did not show a significant change in Gsyn or RMP. Values are means ± SE; n are listed in parentheses. ***P < 0.001, paired t-test. C: measurements of resting Gsyn and RMP from the CS larvae in Fig. 1. For individual fibers, there was a significant correlation between Gsyn and RMP.
The EPSP amplitude after applying calyculin A was 30.3 ± 1.7 mV, which was significantly smaller than controls (34.5 ± 0.7 mV; \( P < 0.05 \), t-test). If the smaller EPSP was due to a decrease in transmitter release, then there would less activation of glutamate receptors, and this could result in less activation of the dSK channel. To rule out this possibility, we repeated the calyculin A experiments in HL3.1 (1.5 mM Ca\(^{2+}\)) to increase transmitter release as in Fig. 2. Under these conditions, the EPSP amplitude was larger (37.1 ± 2.1, \( n = 8 \)), and the EPSP decrease (33.8 5.3%), RMP change (−2.1 ± 1.7 mV) and \( G_{\text{in}} \) increase (0.05 0.01 \( \mu \)S) remained significantly less (\( P < 0.01 \) for all, t-test) compared with controls. Thus we conclude that calyculin A is acting postsynaptically to produce its effect.

To examine further the role of PP2A, we repeated the Ca\(^{2+}\) exchange experiments described in Fig. 4 in the presence of the inhibitors and compared the results to the previous data. The inhibitor was added to HL3.1 (0 Ca\(^{2+}\)) 10 min before measuring RMP and \( G_{\text{in}} \), and the saline was replaced with HL3.1 (1.5 mM Ca\(^{2+}\)) also containing the inhibitor. For both inhibitors, the increase in RMP and \( G_{\text{in}} \) was significantly less than for controls (Fig. 5B). These results suggest that an increase in postsynaptic [Ca\(^{2+}\)] produces an increase in the Ca\(^{2+}\) sensitivity of the dSK channel involving PP2A.

**Modeling the role of \( g_{\text{CS}} \) in regulating synaptic strength.** To examine the effect of \( g_{\text{SK}} \) on EPSPs, we modeled the larval NMJ. Muscle fiber 6 has been found to be virtually isopotential (Jan and Jan 1976) and can be treated as a sphere rather than a cable; the specific \( G_{\text{m}} \) was estimated by the \( G_{\text{in}} \times \text{surface area} \) (SA) of the muscle fiber. Measurements of muscle fiber 6 (\( n = 99 \) fibers) in segments 3 and 4 gave a mean dorsal SA of 54,804 ± 472 \( \mu \)m\(^2\), a perimeter of 1,297 ± 5 \( \mu \)m and a thickness of 19.2 ± 0.1 \( \mu \)m. Assuming a curved edge with a radius of 9.6 \( \mu \)m, the total apparent SA would be 122,187 \( \mu \)m\(^2\). Measurements of the specific \( G_{\text{m}} \) and time constant gave a specific membrane capacitance of 3.9 \( \mu \)F/cm\(^2\). These measurements will overestimate the membrane capacitance and specific \( G_{\text{m}} \) due to the contribution of the t-tubules; however, these apparent values can be used in calculating the electrical response of the membrane to synaptic currents (Gage 1976).

We used virtual cell software (Schaff et al. 1997; Slepchenko et al. 2003) to model the generation of the EPSP in muscle fiber 6. The time course of the \( G_{\text{syn}} \) was simulated using an alpha function (Johnston and Wu 1995), and a value was chosen for alpha that best replicated the waveform of the EPSCs (Fig. 3). Our model included two parallel conductances: the \( g_{\text{c}} \), in series with the leakage potential and \( g_{\text{sk}} \) in series with the \( K^+ \) equilibrium potential (\( E_K \)). (This virtual cell model, epsp_gcs, is available in the public domain at http://www.vcell.org/ under the shared username gregl.) Values for \( g_{\text{in}} \) and leakage potential were based on \( G_{\text{in}} \) and RMP in the absence of \( g_{\text{SK}} \), which should be given by measurements made in 0 external [Ca\(^{2+}\)]; these values from Fig. 4B were 0.16 ± 0.01 \( \mu \)S and −53.4 ± 0.8 mV, respectively. \( E_K \) was calculated from the Nernst equation to be −79.0 mV, assuming that the internal \( K^+ \) concentration was 115 mM, as measured in larval blowfly muscle (Dawson and Djamgoz 1988). One obtains a similar value for \( E_K \) (−77.5 mV) when it is calculated based on the change in RMP and \( G_{\text{in}} \) that we observed when increasing external [Ca\(^{2+}\)] from 0 to 1.5 mM.

We modeled the effect of increasing \( g_{\text{SK}} \) up to 2 \( \mu \)S on synaptic excitation; this covered much of the range in \( g_{\text{SK}} \) we
observed when stimulating the synapse (Fig. 1). We used a peak $G_{\text{syn}}$ of 250 nS, 500 nS or 1,000 nS, since this spanned most of the values obtained from the compound EPSCs (Fig. 3); typically, the single EPSCs would fall between 250 nS and 500 nS. Of course, these measurements were made in HL3 with reduced Ca$^{2+}$, and the $G_{\text{syn}}$ would be larger if measured in HL3.1 with 1.5 mM Ca$^{2+}$. Increasing $g_{\text{SK}}$ clearly reduced synaptic excitation by hyperpolarizing the RMP and reducing EPSP amplitude (Fig. 6A). Small increases in $g_{\text{SK}}$ produced a large hyperpolarization of the RMP (Fig. 6B); this effect was seen at the resting $g_{\text{SK}}$, particularly in HL3.1, consistent with the experimental results. The EPSP amplitude did not decrease during small increases in $g_{\text{CS}}$, since the synaptic current ($I_{\text{syn}}$) increased; e.g., the simulation predicted that the peak $I_{\text{syn}}$ would increase from 36 nA to 50 nA ($G_{\text{syn}} = 1,000$ nS) for an increase in $g_{\text{SK}}$ from 0 to 0.5 $\mu$S. [Note that the peak $I_{\text{syn}}$ values were larger (59 nA) when the membrane potential was clamped $-60$ mV.] As $g_{\text{SK}}$ exceeded 0.5 $\mu$S, further increases in $g_{\text{SK}}$ produced less RMP hyperpolarization and a greater decrease in EPSP amplitude (Fig. 6, B and C).

The results of Fig. 1 provided evidence that an increase in $g_{\text{SK}}$ contributed to synaptic depression during 20-Hz stimulation. The modeling supported this conclusion. For example, during 20-Hz stimulation, the $g_{\text{SK}}$ increased from its resting level (0.04 $\mu$S) to 0.85 $\mu$S, and, according to Fig. 6C, this should reduce EPSP amplitude by about one-third. Alternatively, we used simulations to determine the amplitude of the final EPSP, assuming $g_{\text{SK}}$ remained constant during stimulation. At the end of 20-Hz stimulation, the EPSP amplitude was 12.7 mV, and, based upon the final $g_{\text{SK}}$ (0.85 $\mu$S), the simulation gave a final $G_{\text{syn}}$ of 260 nS. If $g_{\text{SK}}$ did not increase during stimulation, then the final $G_{\text{syn}}$ would give a 20.2-mV EPSP. Thus activation of $g_{\text{SK}}$ contributed to synaptic depression by reducing the final EPSP by 37%.

**DISCUSSION**

Ca$^{2+}$ entering at the postsynaptic membrane activates postsynaptic $d$SK channels to reduce synaptic excitation. Stimulation of the neuromuscular synapses at 20 Hz resulted in an increase in resting $G_{\text{in}}$, and a decrease in synaptic excitation due to membrane hyperpolarization and a decrease in EPSP amplitude. Our simulations predicted that the increase in resting $G_{\text{in}}$ during stimulation accounted for about one-third of the depression of EPSP amplitude due to shunting of the synaptic current. Synaptic depression is generally held to be due to a reduction in transmitter release (Zucker and Regehr 2002); thus the remaining decrease in EPSP amplitude may have resulted from a decline in transmitter release. Nonetheless, we provide evidence for a change in postsynaptic conductance contributing to synaptic depression. The increase in $G_{\text{in}}$ and RMP was consistent with activation of a $g_{\text{Ca}}$; this was further supported by the reduced increase in $G_{\text{in}}$ and RMP seen after expressing PV in the muscle.

Larval muscle fibers contain two types of Ca$^{2+}$-dependent K$^+$ channels producing $g_{\text{CF}}$ and $g_{\text{CS}}$. $g_{\text{CF}}$ was found to result from the slowpoke channel, a BK-type channel showing voltage- and Ca$^{2+}$-dependent activation and voltage-dependent inactivation (Elkins et al. 1986; Komatsu et al. 1990; Salkoff et al. 2006), and the $d$SK channel was proposed to produce $g_{\text{CS}}$ (Abou Tayoun et al. 2011). Our Western blots showed a single $d$SK isoform in larval brain and muscle; a Western blot of adult fly brains showed two closely spaced bands, which could have represented two $d$SK isoforms or a single isoform along with its posttranslationally altered form (Abou Tayoun et al. 2011). SK channels are not voltage-gated, and calmodulin acts as their Ca$^{2+}$ sensor, resulting in a relatively high Ca$^{2+}$ sensitivity (Xia et al. 1998). Repetitive synaptic stimulation activated the postsynaptic $d$SK channels, since the increase in $G_{\text{in}}$ and RMP was eliminated in $d$SK$^{-}$ or 24B/dSK$^{DN}$ larvae, but not in slo$^{l}$ larva. As expected, there was reduced synaptic depression in...
dSK mutants and PV-expressing larvae; however, it was surprising that synaptic depression was completely eliminated. This suggests that there was also less depression of transmitter release in these larvae.

We assume that the dSK channel is activated during evoked transmitter release in vivo, since the 20-Hz stimulation applied here likely falls within the physiological range of activity. The Ib synapses on muscle fiber 6 fire on average 20–30 Hz, and the Is terminals fire less than 10 Hz in dissected larvae (Chouhan et al. 2010, 2012); however, these firing frequencies may be greater in vivo since the waves of contraction are slow in dissected larvae, probably due to lack of appropriate sensory input (Song et al. 2007). Also, since we used HL3 with low Ca²⁺ for our stimulation procedure, the postsynaptic Ca²⁺ influx was less than would occur under physiological conditions.

Ca²⁺ entering at the postsynaptic membrane was sufficient to activate dSK channels, since evoked transmitter release produced an increase in Gₛₐ even when the muscle was voltage clamped at −60 mV. Under these conditions, we expect that Ca²⁺ entry was limited to glutamate receptors, since voltage-dependent Ca²⁺ channels in larval muscle open at membrane potentials more positive than −30 mV (Ren et al. 1998). However, we cannot rule out the possibility that there was a voltage drop across the subsynaptic reticulum (SSR), such that voltage-dependent Ca²⁺ channels near the glutamate receptors remained unclamped. In this case, both the glutamate receptors and nearby Ca²⁺ channels could have been the source of Ca²⁺ that activated the dSK channel. In fact, dSK channels in larval muscle can be activated by Ca²⁺ entering through voltage-dependent Ca²⁺ channels since depolarization of the muscle in the absence of synaptic activity produced an increase in Gₛₐ (Gho and Mallart 1986). Additional evidence that the glutamate receptor can act as the Ca²⁺ source comes from the activation of the dSK channel by spontaneous transmitter release; here, it seems unlikely that the SSR resistance would be great enough for quantal currents to open voltage-dependent Ca²⁺ channels. It appears that dSK channels exist at the postsynaptic membrane near the glutamate receptors, and this is consistent with findings in mammals, where the SK channel has been shown to be closely coupled to postsynaptic nicotinic acetylcholine receptors and NMDA receptors (Ngo-Anh et al. 2005; Oliver et al. 2000).

The postsynaptic dSK channels were activated by spontaneous transmitter release. The dSK channels were activated at rest and were responsible for the previously reported dependence of the RMP on external Ca²⁺ (Jan and Jun 1976; Krans et al. 2010). This was based upon our finding that the increase in Gₛₐ and RMP typically seen when increasing external Ca²⁺ from 0 to 1.5 mM was not seen in dSK− larvae. In fact, it appeared that activation of the dSK channel is responsible for much of the normal variability in the RMP, since resting Gₛₐ and the RMP were positively correlated in wild-type larvae, but not in dSK− larvae. Spontaneous transmitter release apparently activates dSK channels to set the resting Gₛₐ and RMP, since blocking the glutamate receptors prevented the rise in Gₛₐ and RMP produced by increasing external Ca²⁺. This is a novel function for spontaneous transmitter release, but not entirely unexpected, since spontaneous transmitter release produced postsynaptic Ca²⁺ transients similar in amplitude to evoked release (Desai and Lnenicka 2011). The spontaneous Ca²⁺ transients must have produced an increase in resting Gₛₐ that outlasted the Ca²⁺ signal, since the frequency of spontaneous events in this muscle is usually about 1 Hz, and the spontaneous Ca²⁺ transients had a decay time constant of ~50 ms (Desai and Lnenicka 2011). The increase in Gₛₐ produced by repetitive synaptic stimulation also appeared to outlast the increase in [Ca²⁺], since it persisted for about 10 min after stimulation. We previously found that after 5 s of 10-Hz stimulation, postsynaptic [Ca²⁺] decayed with a time constant of about 100 ms, and this decay was largely due to the plasma membrane Ca²⁺ ATPase, which appeared to be highly enriched in the SSR (Desai and Lnenicka 2011). The SSR may limit Ca²⁺ diffusion to enhance the amplitude of postsynaptic Ca²⁺ transients; however, it seems likely that it also reduces their duration by providing efficient postsynaptic Ca²⁺ extrusion. Thus it seems improbable that postsynaptic [Ca²⁺] remained elevated for 10 min after the stimulation train.

We found that there was an increase in gₛₐ in dSK− larvae. This is consistent with previous studies showing covariation of ion conductances (MacLean et al. 2003). In particular, reduced activation of Ca²⁺-dependent K⁺ currents in Drosophila cultured neurons resulted in a compensatory upregulation of the transient K⁺ current, apparently due to increased expression of transient K⁺ current channels (Peng and Guo 2007). Although the channels responsible for gₛₐ have not been characterized in larval muscle, they may include ORK1 which was identified as a K⁺ leak channel in Drosophila and appears to be expressed in adult muscle (Goldstein et al. 1996); it is a member of the two-pore domain K⁺ channels identified in mammals (Enyedi and Czirjak 2010). The nonselective cation channel NALCN produces the Na⁺ leak conductance in mammalian neurons (Lu et al. 2007) and its ortholog na is expressed in Drosophila neurons (Nash et al. 2002), raising the possibility that it could also be responsible for the Na⁺ leak conductance in larval muscle.

The dSK channel is modulated by PP2A. In mammals, CK2 and PP2A are constitutively bound to the SK channel and regulate the phosphorylation state of calmodulin, which determines the Ca²⁺ sensitivity and deactivation rate of the SK channel. Dephosphorylation of calmodulin by PP2A can reduce EC₅₀ to 0.3 μM Ca²⁺ and phosphorylation of the SK channel by CK2 can increase EC₅₀ to 2.0 μM (Allen et al. 2007; Bildl et al. 2004). An increase in [Ca²⁺], should shift the balance toward dephosphorylation and produce an increase in Ca²⁺ sensitivity, since CK2 cannot phosphorylate calmodulin when the channel is open (Allen et al. 2007). Our results showed that the dSK channel is also regulated by PP2A, since inhibiting PP2A reduced the increase in Gₛₐ and RMP produced by repetitive stimulation or increasing external Ca²⁺.

During repetitive stimulation, the increase in postsynaptic [Ca²⁺] may have increased the Ca²⁺ sensitivity of the dSK channel. Similarly, it may be that the Ca²⁺ transients produced by spontaneous transmitter release produced an increase in Ca²⁺ sensitivity so that the dSK channel is partially activated by resting [Ca²⁺]. If the Ca²⁺-dependent increase in Ca²⁺ sensitivity persisted, this could explain the lasting increase in Gₛₐ seen after the end of 20-Hz stimulation and after spontaneous Ca²⁺ transients.

The dSK channel acts to regulate synaptic excitation. Our results demonstrate that Ca²⁺ entering at the postsynaptic

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membrane during transmitter release provides negative feedback on synaptic excitation. This could rapidly stabilize the synapse and dampen the effects of changes in impulse activity, transmitter release or postsynaptic sensitivity. The effect of \( g_{SK} \) on synaptic excitation was modeled using a \( G_{syn} \) of 500 nS. The resting \( g_{SK} \) in 1 mM (HL3) and 1.5 mM external \( Ca^{2+} \) (HL3.1) would make the EPSP peak about 3 mV and 14 mV more negative, respectively, mainly due to hyperpolarization of the RMP. An increase in \( g_{SK} \) from 0 to 1 \( \mu \)S (approximately the value seen after 20 Hz stimulation) made the EPSP peak 22 mV more negative due to both RMP hyperpolarization and a decrease in EPSP amplitude. Activation of the dSK channel by evoked transmitter release is consistent with this negative-feedback mechanism, but what is the function of dSK channel activation by spontaneous transmitter release? minEPSP frequency is elevated after the function of dSK channel activation by spontaneous

This action of the postsynaptic dSK channel is similar to that found at mammalian central nervous system synapses, where the SK channel counteracts synaptic changes: experiments blocking SK channels with apamin, or overexpressing them, showed that activation of SK channels increased the threshold for the induction of LTP and impaired learning (Behnisch and Reyman 1998; Faber et al. 2005; Hammond et al. 2006; Ngo-Anh et al. 2005; Stackman et al. 2002). At these synapses, it was proposed that SK channels act specifically to restrict \( Ca^{2+} \) entry through NMDA receptors limiting LTP and learning and memory (Faber and Sah 2007). Our findings show that dSK channels play a more general role in regulating synaptic excitation.

The dSK channel could act in concert with forms of homeostatic synaptic plasticity; these have involved compensatory changes in transmitter release at the NMJ, and most operate over long time scales (DeRosa and Govind 1978; DiAntonio et al. 1999; Lnenicka and Mellon 1983; Petersen et al. 1997). A failure of the dSK channel to maintain appropriate synaptic excitation could result in compensatory changes in transmitter release; this is supported by the apparent decrease in transmitter release seen when dSK channel activity was reduced in dSK mutants or by expressing PV in the muscle. This is consistent with previous findings that expressing additional K+ channels in Drosophila larval muscle resulted in a homeostatic increase in transmitter release (Paradis et al. 2001).

At mammalian central synapses, the SK channel reduced the amplitude of the EPSC; single EPSPs and EPSCs were larger after adding the SK-channel blocker apamin (Faber 2005, 2010; Ngo-Anh et al. 2005). Thus the SK channel was rapidly activated during synaptic transmission so that the resultant EPSC was a composite of the inward current through the postsynaptic receptor and outward current through the SK channel. We have not directly examined whether the dSK channel influences the amplitude of the EPSC; this is made difficult since apamin does not block dSK channels. We have evidence that dSK currents reduced the duration of the EPSC, but further experiments are required to characterize fully the contribution of the dSK current to the EPSC.

**REFERENCES**


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