J Exp Biol Advance Online Articles. First posted online on 13 February 2014 as doi:10.1242/jeb.096941
Access the most recent version at http://jeb.biologists.org/lookup/doi/10.1242/jeb.096941

Mode of Action of a *Drosophila* FMRFamide in Inducing Muscle Contraction

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Short title: *Drosophila* FMRFamide and Contraction

Key words: FMRFamide, G-protein coupled receptor, cAMP, cGMP, CaMKII, neuropeptide

ABSTRACT

Drosophila melanogaster is a model system for examining mechanisms of action of neuropeptides. DPKQDFMRFamide was previously shown to induce contractions in *Drosophila* body wall muscle fibers in a Ca^{2+} -dependent manner. The present study examined the possible involvement of a G-protein coupled receptor and second messengers in mediating this myotropic effect after removing the central nervous system. DPKQDFMRFamide-induced contractions were reduced by 70% and 90%, respectively, in larvae with reduced expression of the *Drosophila Fmrf* Receptor (FR) either ubiquitously or specifically in muscle tissue, compared to response in control larvae in which expression was not manipulated. No such effect occurred in larvae with reduced expression of this gene only in neurons. The myogenic effects of DPKQDFMRFamide do not appear to be mediated through either of the two *Drosphila* myosuppressin receptors (*DmsR-1* and *DmsR-2*). DPKQDFMRFamide-induced contractions were not reduced in Ala1 transgenic flies lacking activity of calcium/calmodulin-dependent protein kinase (CamKII), and were not affected by the CaMKII inhibitor, KN-93. Peptide-induced contractions in the mutants of the phosholipase C-β (*PLC*β) gene (*norpA* larvae) and in IP_3 receptor mutants were similar to contractions elicited in control larvae. The peptide failed to increase cAMP and cGMP levels in *Drosophila* body wall muscles. Peptide-induced contractions were not potentiated by 3-Isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, and were not antagonized by inhibitors of cAMP-dependent or cGMP-dependent protein kinases. Additionally, exogenous application of arachidonic acid failed to induce myogenic contractions. Thus, DPKQDFMRFamide induces contractions via a G-protein coupled FMRFamide receptor in muscle cells but does not appear to act via cAMP, cGMP, IP_3, PLC, CaMKII, or arachidonic acid.

INTRODUCTION

Neuropeptides usually elicit physiological responses via G-protein coupled receptors (GPCRs) which, in turn, act through second messenger pathways (Mercier et al., 2007; Meeusen et al., 2003; Vauquelin & von Mentzer, 2007). Many "RFamide" peptides (i.e. containing Arg-Phe-NH_2 at the carboxyl terminus) have been shown to act via GPCRs (e.g. Van Tol-Steye et al., 1999; Volterra & Siegelbaum., 1988; Wang et al., 1995), although molluscs contain a sodium channel that is directly gated by FMRFamide (Cottrell, 1997; Linguelia et al., 1995). There is considerable interest in identifying the GPCRs that mediate effects of RFamide peptides and the intracellular messengers that mediate their physiological effects.

Early studies demonstrated involvement of GPCRs using pharmacological evidence, such as sensitivity to non-hydrolyzable GTP analogs or to pertussis and cholera toxins, but more recent studies have identified peptide-activated GPCRs using genetic approaches, comparing genomes and transcriptomes with sequences encoding known GPCRs. The *Drosophila* genome contains approximately 44 genes encoding for peptide-binding GPCRs (Hewes & Taghert, 2001, Brody & Cravchik, 2000), and several of these have been functionally characterized in cell cultures, usually Chinese hamster ovary cells. Four of these genes encode for GPCRs that are activated by RFamide peptides. Only one of these GPCRs, referred to as "FR" (encoded in gene *CG2114*), was reported to bind with high affinity to *Drosophila* peptides containing the sequence "FMRFamide," and this gene product was less sensitive to other RFamide peptides (Meeusen et al., 2002; Cazzamali & Grimmelikhuijzen, 2002). This GPCR is a good candidate for mediating the physiological effects of *Drosophila* FMRFamides, and its involvement in mediating physiological responses needs to be tested more thoroughly using *Drosophila* cells or tissues.

DPKQDFMRFamide is the most abundant of eight peptides encoded in the *dFMRFamide* gene (Nambu et al., 1988; Schneider & Taghert, 1988) and is localized in neurohemal sites in fly

larvae (Wegener et al., 2006). This peptide enhances nerve-evoked muscle contractions and synaptic transmission at neuromuscular junctions (Hewes et al., 1998, Dunn & Mercier, 2005). DPKQDFMRFamide increases the amount of transmitter released from nerve terminals (Hewes et al., 1998; Klose et al., 2010), and it also increases the amplitude of nerve-evoked calcium signals via release of calcium from intracellular stores (Klose et al., 2010). The enhancement of synaptic transmission requires calcium/calmodulin dependent protein kinase (CaMKII; Dunn & Mercier, 2005) and involves the FR GPCR (Klose et al., 2010). The intracellular pathways mediating the peptide-induced contractions, however, have not been studied, and possible involvement of the FR GPCR has not been investigated.

Earlier work demonstrate that DPKQDFMRFamide also elicits contractions in muscle fibres of the *Drosophila* larval body wall in the absence of the CNS (Clark et al., 2008). This effect appeared to involve a direct action on muscle fibers, since the peptide induced muscle contractions even after glutamate receptors on the muscle fibres were desensitized, which would prevent activation by increasing spontaneous release of glutamate from presynaptic terminals (Clark et al., 2008). However, other neurotransmitters, such as pituitary adenylate-cyclase activating polypeptide (PACAP), octopamine, proctolin and insulin-like peptide (ILP), are present in synaptic boutons on the larval body wall muscles (Anderson et al., 1988; Gorczyca et al., 1993; Monastirioti et al., 1995; Zhong and Pena, 1995). Thus, a possible presynaptic action of the peptide, through release of these other substances, was not ruled out.

The present investigation was aimed at identifying the receptor and second messenger pathways mediating DPKQDFMRFamide-induced contractions in *Drosophila* larval body wall muscles and at determining whether the receptor was localized presynaptically or postsynaptically. We used RNA interference to test the hypothesis that DPKQDFMRFamide acts via FR to elicit contractions. Our data indicate that the peptide-induced contractions require the

presence of the FR in muscle fibers. We also used genetic and pharmacological methods to investigate second messengers underlying peptide-induced contractions.

RESULTS

As in previous experiments (Clark et al, 2008), DPKQDFMRFamide induced slow contractions lasting up to 5 min (Fig. 1). To quantify this effect (an increase in muscle tonus), the tonus during a 5-min pre-peptide period (basal tonus) was subtracted from the maximal contraction observed during 5 min of peptide application. Responses are reported as mean tonus change ± standard error of mean (SEM). We also observed spontaneous phasic contractions both in the presence and absence of DPKQDFMRFamide, as reported previously (Clark et al., 2008). Since our earlier studies indicated that the frequency and amplitude of such phasic contractions do not correlate with peptide concentration (Clark et al., 2008), we did not examine these contractions further.

FMRFamide G-protein coupled receptor (FR)

To investigate whether the GPCR encoded by the *Drosophila* FR gene plays a role in mediating the effects of DPKQDFMRFamide on muscle tonus, RNA interference was used to reduce expression of this gene in *Drosophila* larvae (Dietzl et al., 2007). We obtained homozygous transgenic flies carrying an FR inverted repeat (*FR-IR*) under the control of an upstream activating sequence (UAS). To express the RNAi and knockdown *FR* expression, these flies were crossed with flies that exhibit ubiquitous expression of GAL4 homozygously under the control of the tubulin promoter (*tubPGAL4*). The progeny (*UAS-FR RNAi/+; tubP-GAL4/+*) exhibited a significantly lower increase in muscle tonus in response to 1μM DPKQDFMRFamide compared to control lines (Fig. 1). Muscle tonus increased by only 9.86 ± 3.45 μN in these

larvae, which was approximately 30% of the response elicited in both parental control lines (*tubP-GAL4/+* genotype and *UAS-FR RNAi/+* genotype; one-way ANOVA, Tamhane's post hoc, $p<0.01$) and only 26% of the response in Canton-S larvae (one-way ANOVA, Tamhane's post hoc, $p<0.05$). No difference was observed between DPKQDFMRFamide-induced contractions of the two parental control lines (Tamhane's post hoc, $p>0.05$), nor between the parental control lines and Canton-S larvae (Tamhane's post hoc, $p>0.05$), in which expression of the *FR* gene was not manipulated. Thus, combining the UAS-*FR* RNAi line with GAL4 to ubiquitously disrupt function of the *FR* was successful at inhibiting the peptide's ability to elicit contraction. Quantitative polymerase chain reaction (qPCR) showed that the ubiquitous *FR* knockdown line exhibited the most dramatic reduction, approximately 90% reduction in transcript levels compared to Canton S and outcross controls (Fig. 2). These results suggest that the *FR* is at least partially responsible for mediating the myostimulatory effect of peptide on larval body wall muscles.

To reduce FR expression specifically in muscle cells, *UAS-FR-RNAi* flies were crossed with *24B-GAL4* flies, which express Gal4 in all embryonic and larval somatic muscles (Schuster et al., 1996). This generated a heterozygous F1 generation, *UAS-FR RNAi/+; 24B-GAL4/+*. To control for potential effects of P-element insertions present in all larvae, two control lines were used: a) heterozygous *UAS-FR-RNAi* flies with a non-active *UAS-FR*-IR construct and b) heterozygous *24B-GAL4* lines containing a Gal4 vector only. Peptide-induced contractions in larvae with RNAi-regulated expression of the *FR* gene (*UAS-FR RNAi/+; 24B-GAL4/+*) in muscle cells were only 11% and 15% of the amplitude observed in *UAS-FR* RNA and *24B-GAL4* controls, respectively (Fig. 1, Tamhane's post hoc, $p<0.05$ and $p<0.01$), and only 9% of amplitude observed in Canton-S larvae (Fig 1, Tamhane's post hoc, $p<0.05$). Thus, reducing *FR* expression in muscle cells reduced responsiveness to the peptide by approximately 85-90%.

There was no difference between the peptide-induced body wall contraction of Canton-S, *UAS-FR* RNA and *24-GAL4* control larvae (Tamhane's post hoc, $p>0.05$). qPCR data from the muscle knockdown line demonstrated a substantial reduction in transcript levels, roughly 75% knockdown compared to controls. The results suggest that expression of *FR* on muscle cells is necessary for most if not all of the peptide-induced contraction. These results also corroborate previously reported evidence that DPKQDFMRFamide induces contractions via a postsynaptic action (Clark et al., 2008).

To rule out the possibility that the tonus change might be mediated by presynaptic actions of the peptide, we crossed *UAS-FR RNAi* flies with flies harbouring pan-neuronal GAL4 expression of the *elav-Gal4* driver. The effects of 1 μM DPKQDFMRFamide on the progeny (*UAS-FR RNAi /+; elav-GAL4/+*) were compared to effects on two control progeny lines (*UAS-FR RNAi/+* and *elav-GAL4/+*). The nerve-specific driver was able to reduce transcript levels by ~60% relative to controls. The peptide's effect in larvae with *FR*-RNAi and pan-neuronal expression of Gal4 (*UAS-FR RNAi /+; elav-GAL4/+*) was similar to its effect in heterozygous control *elav-GAL4* (Fig. 1; Tamhane's post hoc, $p>0.05$) and *UAS-FR-IR* larvae (Fig. 1; Tamhane's post hoc, $p>0.05$) as well as to the effect in Canton-S larvae (Fig. 1; Tamhane's post hoc, $p>0.05$). Thus, Pan-neuronal expression of the *FR*-IR construct had no effect on the ability of peptide to increase muscle tonus.

Dromyosuppressin G-protein coupled receptors (DmsR-1 and DmsR-2)

Presynaptic effects of DPKQDFMRFamide are mediated by both FR and one of the two dromyosuppressin receptors (Dms-R2 but not Dms-R1; Klose et al., 2010). To investigate the potential involvement of the *Drosophila* myosuppressin receptors, homozygous transgenic flies carrying an inverted repeat for *DmsR-1* and *DmsR-2* under the control of UAS (*UAS-DmsR-1*

RNAi and *UAS-DmsR-2* RNAi) were crossed the same three drivers as *FR* (*tubPGal4*, *24B-Gal4* and *elavGal4*) as well as the appropriate out-cross controls. Responses of larval progeny of these genetic crosses to 1 μM DPKQDFMRFamide did not display any significant deviations from control lines (Fig. 3). We confirmed the reduction of expression for both myosuppressin receptors using qPCR. Data for *DmsR-1* are as follows: *UAS-DMSR-1 RNAi/+; 24B-Gal4/+* had 46% expression relative to wild-type, *UAS-DMSR-1 RNAi/+; elav-Gal4/+* had 61% expression relative to wild-type, and *UAS-DMSR-1 RNAi/+; tubP-Gal4/+* had 38% expression relative to wild-type. Data for expression of *DmsR-2* relative to wild type are as follows: *UAS-DMSR-2 RNAi/+; 24B-Gal4/+* 51%, *UAS-DMSR-2 RNAi/+; elav-Gal4/+* 65%, and *UAS-DMSR-2 RNAi/+; tubP-Gal4/+* 38%.

CaMKII

The results presented above strongly suggest that the effect of DPKQDFMRFamide on muscle tonus is mediated via *FR* GPCRs localized in muscle fibers. Since the ability of DPKQDFMRFamide to increase neurotransmitter release from synaptic terminals involves *FR* GPCRs in neurons (Klose et al., 2010) and requires CaMKII activity (Dunn and Mercier, 2005), we sought to determine whether or not peptide-induced contractions are also mediated by CaMKII. This question was addressed using pharmacological agents known to reduce CaMKII activity and a transgenic *Drosophila* line (ala1) which increases expression of the inhibitory peptide of CaMKII in response to heat shock (Griffith et al., 1993). We also used an additional transgenic line (UAS-ala) which enabled us to drive the expression of the inhibitory peptide of CaMKII using a muscle specific driver (24B), independently of heat shock.

Contractions of body wall muscles were compared between ala1 larvae and a control line (UAS-ala, which contains the inserted gene for the inhibitory peptide but under control of Gal4

rather than heat shock). In the absence of heat shock, ala1 and control larvae showed similar increases in muscle tonus in response to 1 μM DPKQDFMRFamide (Fig. 4; t-test, $p>0.05$). Hence, constitutive expression of the CaMKII inhibitory protein in ala1 larvae had no effect on the DPKQDFMRFamide-induced tonus change. Griffith et al., (1993) reported some expression of the alanine inhibitory peptide in ala1 flies at 25°C(1.9-3.9 μM) and there is probably some expression at room temperature (22°C), but well below at IC_{50} (13 μM Drosophila). A 1-hour heat-shock (37°C) increases expression of the inhibitory peptide and decreases CaMKII activity by 90-100%. Exposing ala1 flies to an hour-long heat shock (37°C, which has been shown to decrease CaMKII activity by 70-100%; Griffith et al., 1993) did not reduce peptide-induced contractions in ala1 larvae but enhanced them by approximately 150% compared to non heat-shocked ala1 larvae (t- test, $p<0.05$). Heat shock also increased peptide-induced contractions in control larvae (UAS-ala) by approximately 100% (t-test, $p<0.05$). There was no significant difference between the responses of heat-shocked ala1 and heat-shocked UAS-ala larvae (t-test, $p>0.05$). Driving the expression of the inhibitory peptide of CaMKII in muscle cells did not alter muscle responsiveness to exogenously applied DPKQDFMRFamide (Fig 4; UAS-ala/ 24B-Gal4: 23.9 ± 2.3, UAS-ala/+: 24.5 ± 5.4, $P>0.05$). These results suggest that enhanced responsiveness to the peptide is attributable to the heat shock treatment rather than to the CaMKII inhibition, and that CaMKII inhibition does not antagonize the peptide's ability to induce muscle contraction.

A cell permeable inhibitor of CaMKII activity, KN-93, was applied at a concentration of 1 μM for 20 minutes, and immediately thereafter the preparation was exposed to a solution containing both 1 μM DPKQDFMRFamide and 1 μM KN-93. There was no significant difference between contractions elicited by DPKQDFMRFamide alone (29.1 ± 9.6 μN) or in presence of KN-93 (25.8 ± 3.3 μN; t-test, $p>0.05$).

IP_3 receptor and phospholipase C

Excitatory effects of RFamide peptides have been linked to phospholipase C and generation of IP_3 in *Lymnea stagnalis* (Willoughby et al., 1999) and *Helix aspersa* (Falconer et al., 1993). If DPKQDFMRFamide induces contractions via the PLC-IP_3 pathway, it would be predicted that such contractions would be attenuated or abolished by disrupting either PLC or IP_3 receptor (IP_3-R) activity. To test this, we took advantage of *Drosophila* fly lines in which either PLC or IP_3 receptor functionality are disturbed with mutations in genes (*norpA* and *Plc21C*) that encode for PLCβ or in the single gene known to encode for the IP_3 receptor, *itpr*, respectively.

Heterozygous IP_3 receptor mutant larvae (*Itp-r83A*^{05616}) exhibit approximately 50% reduction in *IP3-R* transcript levels (Klose et al., 2010). Fig. 5A shows the averaged change in tonus of heterozygous IP_3 receptor mutants (*Itp-r83A*^{05616}) and wild type larvae in response to DPKQDFMRFamide. The mutant larvae responded to the peptide with an average muscle tonus increase of 27.74 ± 2.87 μN, which was not significantly lower than the response elicited in wild type larvae (t-test, $p > 0.05$).

Several mutant lines with deficits in PLC activity were used. Each of the mutants, *w* norpA*^{33}, *w* norpA*^{36} *and norpA*^{7}, carries a point mutation in the *norpA* gene induced by ethyl methanesulfonate mutagenesis on the Oregon-R line. PLC activity is reduced to approximately 1-1.5 % of wild type levels in *norpA*^{33} and *norpA*^{36} mutants (Pearn et al., 1996), and PLC activity in *norpA*^{7} mutants is reduced to about 2–3% of normal levels (Inoue et al., 1988). We also used the *Plc21C* gene mutant, *y*^{1} *w*^{1118}*; Plc21C*^{A24}, which was produced using P element insertion mutagenesis (Bellen et al., 2004). It has been reported that the *plc21C* gene encodes for two transcripts, of which one is expressed in the adult head only, and the other is expressed in adult head and body tissue throughout development (Shortridge *et al.*, 1991), but exact levels of their

expression in $y^1\ w^{1118}$; $Plc21C^{A24}$ mutant flies have not been reported. All the PLC mutants we examined showed an increase in muscle tonus in response to 1 μM DPKQDFMRFamide (Fig 5B). Peptide-induce contractions in $w^*\ norpA^{36}$, $w^*\ norpA^{33}$ and $norpA^7$ larvae were not significantly different from contractions in control (Oregon R) larvae and were not significantly different from each other (one-way ANOVA, p>o.o5). Peptide-induced contractions were not significantly different between $y^1\ w^{1118}$; $Plc21C^{A24}$ mutant larvae and *y*,*w* control larvae (t-test, p> 0.05). These data do not support the hypothesis that either PLC or IP_3 is involved in mediating myostimulatory effects of DPKQDFMRFamide.

cAMP and cGMP

In other arthropods cAMP mediates peptide-induced muscle contractions by modulating K^+ channels (Erxleben et al., 1995) and Ca^{2+} channels (Bishop et al., 1991,1987). Since the ability of DPKQDFMRFamide to induce contractions requires L-type Ca^{2+} channels (Clark et al, 2008), it seemed plausible that the peptide might activate Ca^{2+}-channels via the cAMP pathway. To investigate whether or not cyclic nucleotide monophosphates are involved in mediating peptide-induced contractions, cAMP and cGMP levels in body wall muscles were determined with enzyme immunoassay after incubation in various peptide concentrations in the presence of 3-isobutyl-1-methylxanthine (IBMX), a phoshodiesterase inhibitor that slows the breakdown of cAMP and cGMP (Beavo et al., 1970, Beavo & Reifsnyder 1990; Goy, 1990).

A 10 min exposure of body wall muscles to DPKQDFMRFamide at concentrations ranging from 10 nM to 1μM in the presence of 0.5 mM IBMX did not significantly increase cAMP levels above the control level measured in IBMX alone (Fig. 6A, One-Way ANOVA, Tamhane's post hoc, p>0.05). There was also no significant difference between cAMP levels following the exposure of larval muscles to 0.5 mM IBMX alone and saline alone (Fig. 6A, one-

247 way ANOVA, Tamhane's post hoc, $p>0.05$), which raised concern that this concentration of
248 IBMX might not be sufficient to slow the breakdown of cAMP in *Drosophila* larval muscles.
249 This same concentration of IBMX, however, potentiated the increases in cAMP induced by
250 forskolin, an adenylate cyclase activator (Fig. 6B). Forskolin increased cAMP levels by
251 approximately 230% compared to saline controls (one-way ANOVA, Tamhane's post hoc,
252 $p<0.01$), but it increased cAMP by 650% in the presence of 0.5 mM IBMX, representing a 2.3-
253 fold increase in forskolin's effectiveness (one-way ANOVA, Tamhane's post hoc, $p < 0.01$).
254 Thus, the concentration of IBMX should have been sufficient to act synergistically with other
255 cAMP-elevating compounds to increase cAMP levels.
256 The effect of the peptide on cGMP levels was also determined (Fig. 7). One-way
257 ANOVA revealed a significant difference in cGMP levels between treatment groups ($p<0.01$).
258 IBMX (0.5 mM) increased cGMP levels by 260 % compared to saline (one-way ANOVA,
259 Tamhane's post hoc, $p<0.01$). Treatment with 10 nM, 100 nM and 1 μM DPKQDFMRFamide in
260 the presence of IBMX, however, did not change cGMP from the level observed in IBMX alone
261 (Tamhane's post hoc, $p>0.05$). These data suggest that cGMP does not play a role in mediating
262 the peptide's effects on muscle tonus.
263 To further investigate a possible role of cyclic nucleotide signal transduction pathways,
264 we determined whether or not selective pharmacological agents would mimic the ability of
265 DPKQDFMRFamide to induce contractions. Treatment with IBMX for 10 min had no
266 significant effect on muscle tonus (1.1 ± 7.3 μN, n=7). Treatment with 50 μM forskolin for 10
267 min appeared to cause a small change in tonus (11.6 ± 4.7 μN, n=7), but this change was just at
268 the threshold level for discernable effects reported previously (approximately 10 μN; Clark et al.,
269 2008). This apparent effect of forskolin was substantially weaker than the effect of 1 μM

DPKQDFMRFamide (Fig. 8A), despite the fact that forskolin increased cAMP levels at this concentration and DPKQDFMRFamide did not (Fig. 6).

If the peptide were to act by increasing cAMP or cGMP levels, its effectiveness should be enhanced by slowing hydrolysis of the cyclic nucleotide monophosphates with IBMX, particularly at threshold concentrations of the peptide, where cAMP and cGMP have not reached maximal levels. When DPKQDFMRFamide was applied at the threshold concentration for eliciting contractions (approximately 0.01 μM, Clark et al, 2008), its effectiveness was not significantly increased by the presence of 0.5 mM IBMX (Fig 6A, t-test $p>0.05$). Inhibitors of the cAMP-dependent protein kinase (Rp-cAMPs) and the cGMP-dependent protein kinase (Rp-8-pCPT-cGMPS) did not significantly alter the amplitude of contractions elicited by 1 μM DPKQDFMRFamide (Fig. 8B, t-test, $p>0.05$). Thus, the results do not support a role for cAMP, cGMP or their target kinase enzymes in mediating peptide-induced contractions.

Arachidonic acid

To determine if the myogenic effects of DPKQDFMRFamide might result from the involvement of signaling pathways mediated by arachidonic acid, we perfused third instar larvae with arachidonic acid at concentrations ranging from 10^{-10} to 10^{-4} M and looked for changes in muscle tonus that might mimic the effect of the peptide. No significant change in tonus was observed at any of the concentrations tested (n=7-10 preparations for each concentration; 9.3 $\pm$ 11.8 μN at 10^{-4}M, n=8).

Pertussis toxin

After determining that the various second messengers above are not involved in mediating the effects of DPKQDFMRFamide on muscle fibers, one possible explanation is a direct G protein interaction. To examine this possibility, we incubated dissected third-instar tissue with an

inhibitor of GPCR subunit uncoupling, pertussis toxin (PTX). Incubation with 500ng/mL PTX for 20min resulted in a significant reduction in muscle responsiveness to exogenously applied DPKQDFMRFamide (PTX: 4.6 ± 0.5, heat-inactivated PTX: 19.3 ± 3.3, Mann-Whitney rank sum, P=0.002).

Membrane potential

To determine if DPKQDFMRFamide application leads to changes in membrane potential, we assessed over 300 intracellular recordings and found that the peptide does not produce any significant change in membrane voltage (Control: -41.3 ± 1.3mV, Peptide: -43.6 ± 1.9mV, P>0.05).

DISCUSSION

The present investigation provides evidence that the *Drosophila* FMRFamide, DPKQDFMRFamide, elicits contractions in larval muscle fibers via the GPCR, FR. Peptide-induced contractions were reduced by 70-90% in heterozygous larvae in which RNA interference reduces expression of the *FR* gene either in all cells or specifically in muscle cells. No reduction in peptide-induced contractions, however, was observed in larvae with reduced *FR* expression only in neurons. These observations confirm earlier work (Clark et al., 2008) indicating that DPKQDFMRFamide induces contractions via a direct action on larval muscle fibers.

Although peptide-induced contractions were reduced dramatically in larvae with reduced *FR* expression ubiquitously or in muscle, such contractions were not completely abolished. At least two possibilities could account for this result. First, the larvae used for physiological recordings were heterozygous for the *GAL4* and *UAS-RNAi* element, which would make it plausible that larval cells retained sufficient expression of the dFMRFamide receptor to mediate a

weak response to the peptide. We assessed this possibility through qPCR. In ubiquitous *FR* knock-down lines a 70% reduction in responsiveness to DPKQDFMRFamide corresponded to a 90% reduction in expression, and in muscle-specific *FR knock-down lines an* 85% reduction in peptide responsiveness corresponded to a 70% reduction in expression. However, qPCR results are not a direct measure of protein expression levels, which we did not assess. Thus, in both cases the lack of a complete reduction in responsiveness to exogenous application of DPKQDFMRFamide correlates with incomplete inhibition of *FR* expression. An alternative possibility is that DPKQDFMRFamide may act via targets other than the FMRFamide receptor. Johnson et al. (2003) showed that 100 nM DPKQDFMRFamide elicits effects that are mediated by both the FMRFamide receptor and a myosuppressin receptor when expressed in Human Embryonic Kidney (HEK) cells. The FMRFamide receptor (*FR*) and the myosuppressin receptor (*DmsR-2*) are also necessary for DPKQDFMRFamide to enhance transmitter release from synaptic terminals (Klose et al., 2010). None of the *DmsR* knock-down lines, however, showed any change in responsiveness to DPKQDFMRFa compared to controls (Fig. 3) despite reductions in gene expression, which was as high as a 62% reduction in lines where the RNAi was expressed ubiquitously. Thus, it is unlikely that the myogenic effects of DPKQDFMRFamide are mediated through the myosuppressin receptors.

Although earlier work demonstrated a role for CaMKII in modulating transmitter release in *Drosophila* larvae by DPKQDFMRFamide (Dunn and Mercier, 2005; Klose et al., 2010), data reported here indicate that CaMKII activity is not necessary for the peptide to induce muscle contraction. The requirement of different intracellular pathways for these two physiological responses indicates that they are distinct and strengthens the interpretation that the peptide's ability to induce contractions represents a direct effect on muscle fibers rather than some presynaptic effect, such as an increase in spontaneous release of neurotransmitters. In the context

of earlier work, the current findings indicate that postsynaptic and presynaptic effects of one modulatory substance may be mediated by different intracellular signalling pathways and by different receptor combinations.

Heat-shock treatment potentiated contractions elicited by DPKQDFMRFamide in ala1 and UAS-ala larvae by approximately 150 and 100 %, respectively. The reason for such potentiation is not clear but probably involves an increase in intracellular calcium levels in the muscle fibers. Warming *Drosophila* larval salivary gland cells to 35°C increases intracellular Ca^{2+} concentration 10-fold, with a slow recovery of $[Ca^{2+}]_i$, starting approximately 45 min after the temperature had cooled to 25°C (Drummond et al., 1986). If heat shock has a similar effect in muscle cells, an increase in intracellular Ca^{2+} might last long enough to augment the response to the peptide. Alternatively, a substantial rise in $[Ca^{2+}]_i$, might activate intracellular processes that potentiate peptide-induced contractions even after Ca^{2+} levels have subsided.

Our observation that DPKQDFMRFamide-induced contractions were not impaired in the IP_3 receptor mutant (*Itp-r83A05616/+*) suggests that this receptor may not be required for the increase in muscle tonus. There is only one IP_3 receptor encoding gene in *Drosophila*, and larvae of this particular mutant were shown to exhibit a 50% reduction in *itpr-83* mRNA levels compared to wild type larvae (Klose et al. 2010). Such a reduction in expression was sufficient to abolish the ability of DPKQDFMRFamide to enhance peak Ca^{2+} levels in presynaptic terminals and EJP amplitude. The present observations indicate that postsynaptic induction of muscle contraction does not exhibit the same dependence on the IP_3 receptor as the presynaptic effects for this neuropeptide. Since the IP_3 receptor mutant still expresses 50% of the normal level of *itpr-83* mRNA, we cannot completely rule out the involvement of this receptor.

DPKQDFMRFamide-induced contractions were neither impaired nor potentiated in four PLC mutant fly lines, each containing a different mutation. Although there are several PLC

364 types, only PLC-β is activated by a G protein coupled receptor (Vauquelin & von Mentzer,
365 2007). In *D. melanogaster* two genes, *norpA* and *plc-21c*, encode for PLC- βγ. The *norpA*
366 mutants exhibit 97-99 % reduction in PLC activity compared to control flies, as determined by *in*
367 *vitro* PLC assays of eye and whole head homogenates (Inoue et al., 1988, Pearn et al., 1996).
368 Unfortunately, the level of PLC activity in tissues of *Plc-21C* mutants is unknown. The lack of
369 impairment in peptide-induced contractions in any of the *norpA* mutants, however, suggests that
370 PLC is probably not required for the postsynaptic response. This would suggest that
371 DPKQDFMRFamide does not initiate muscle contractions by PLC-dependent mechanisms, such
372 as generation of diacylglycerol and IP_3.
373 Several lines of evidence presented in this study suggest that cAMP, cGMP and their
374 respective protein kinase enzymes are not required for DPKQDFMRFamide to increase muscle
375 tonus in *Drosophila* larvae. First, the peptide did not increase cAMP or cGMP levels in
376 *Drosophila* larval muscles in the presence of the phosphodiesterase inhibitor, IBMX. Second,
377 IBMX failed to potentiate the peptide's effect on muscle contraction. Third, neither IBMX nor
378 forskolin mimicked the peptide's effect on muscle tonus very well. IBMX had no effect on
379 muscle contraction, despite its ability to increase cGMP levels by 260%. The contractions
380 elicited by 50 μM forskolin were much smaller than those elicited by DPKQDFMRFamide,
381 despite the fact that forskolin increased cAMP levels by 230% and the peptide did not alter
382 cAMP significantly. Fourth, inhibitors of cAMP-dependent and cGMP-dependent protein
383 kinases failed to reduce peptide-induced contractions. These observations suggest very strongly
384 that changes in the concentration of cAMP or cGMP are not necessary to mediate the peptide's
385 ability to increase muscle tonus. This would differentiate the peptide-induced contractions in
386 *Drosophila* larval muscles from effects of several neuropeptides (Bishop et al., 1991,1987;

Erxleben et al., 1995; Nykamp & Lange, 2000; Trim et al., 1998) and biogenic amines (Clark & Lange, 2003; Knotz & Mercier, 1995; Nykamp & Lange, 2000) which are reported to modulate contraction of invertebrate muscles via cAMP.

In our efforts to illuminate the putative second messenger cascade initiated by the activation of the *Drosophila FR*, we also examined effects of arachidonic acid, which failed to elicit changes in tonus at concentrations ranging from 10^{-10} to 10^{-4} M. This is not surprising since two previous reports provide evidence that arachidonic acid may not be present or utilized in *Drosophila* (Chyb et al, 1999; Yoshioka et al 1985). Thus, it is unlikely that DPKQDFMRFamide mediates its effects through pathways utilizing arachidonic acid.

Overall, the current results confirm that DPKQDFMRFamide increases muscle tonus in *Drosophila* larval muscles by acting directly on the muscle fibers via at least one G-protein coupled receptor. The mechanisms underlying this modulatory effect are still not known but do not appear to involve CaMKII, PLC, cAMP, cGMP, or arachidonic acid. We have not completely ruled out involvement of the IP_3 receptor, but the data presented here suggest this to be unlikely. Other second messengers, such as linoleic acid, have not been examined. The ability of PTX to reduce peptide-induced contractions (Figure 9) suggests that the FR acts on a G-protein of $G_{i/o}$ type (Li et al, 1995). G-protein sub-units have been reported to act directly without the involvement of second messengers in other systems (e.g. Dascal et al., 2001, Jiang and Bajpayee, 2009; Soejima & Noma, 1984; Wickman et al., 1994). Since DPKQDFMRFamide-induced contractions are completely abolished by nifedipine and nicardipine and by lowering extracellular Ca^{2+} concentration (Clark et al., 2008), they appear to require Ca^{2+} influx through L-type channels associated with the sarcolemma, which have been shown to be present in body wall muscles of *Drosophila* larvae (Gielow et al., 1995). A similar dependence on extracellular Ca^{2+} and L-type channels has been reported for peptide-induced

contractions in other arthropod muscles (Donini & Lange, 2002; Kravitz et al., 1980; Lange et al., 1987; Quigley & Mercier, 1997; Wilcox & Lange, 1995). One possible model is that DPKQDFMRFamide activates the PTX-sensitive G-protein, whose subunits act directly on the L-type channels to elicit Ca^{2+} influx, possibly shifting their activation voltage. Peptide-induced Ca^{2+} influx would induce contraction via calcium-induced release of calcium from the sarcoplasmic reticulum (Sullivan et al., 2000; Peron et al., 2009; Ushio et al., 1993). $G_{\beta\gamma}$ subunits have been shown to act directly on N, P/Q, R and T-type calcium channels (Herlitze et al., 1996; Ikeda, 1996; Wolfe et al., 2003) but they inhibit these channels rather than excite them. Furthermore, there is no evidence that L-type channels are modulated directly by G-protein subunits. Alternatively, the peptide might act downstream of calcium, possibly by reducing its re-uptake into the SR or changing the sensitivity of the contractile proteins to calcium.

METHODS

Fly stocks

Canton S (CS) flies, obtained from Bloomington *Drosophila* Stock Centre (BDSC) were used for the experiments unless otherwise indicated.

The IP_3 receptor mutant, Itp-r83A^{05616} {P{PZ}Itp-r83A^{05616} Nmdar1^{05616} ry^{506}/TM3, ry^{RK}Sb^{1}Ser^{1}} (Spradling et al., 1999), was obtained from BDSC. Since this mutation was balanced over a Sb^{1}Ser^{1} dominantly marked balancer chromosome, a genetic cross scheme was performed to remove the balancer. First, a cross between the IP_3 mutant receptor line and a Ser^{1} balancer line (BDSC) was performed. The progeny line was further crossed with w ^{1118} flies (BDSC) to remove the marked balancer chromosome.

Four fly lines with mutations in two genes encoding for PLCβ were used. Three were mutants of the *norpA* (*no receptor potential*) gene: w* norpA^{33}, w*norpA^{36} and norpA^{7} (BDSC). Since the *norpA* mutants originated from OregonR fly stocks, wild type OregonR was used as a control. The *Plc21C* gene mutation line and its control line were from BDSC.

To investigate the role of CaMKII, we used an ala 1 transgenic line containing a synthetically generated alanine inhibitory peptide gene on the first chromosome under the control of a heat shock promoter (Griffith et al, 1993). The UAS-ala line contains an alanine inhibitory peptide gene inserted downstream of UAS and was used as a control for ala1 line. Both transgenic lines were gifts from Dr. Leslie Griffith (Brandeis University, Waltham, MA). Additionally, we used a commercially available ala line containing the inhibitory peptide for CaMKII under UAS control which we crossed with the muscle specific driver (24B).

A transgenic line containing FMRFamide receptor inverted repeat (*FR-IR*) downstream UAS was obtained from the Vienna *Drosophila* RNAi Center (VDRC #9594). The generation of this line (UAS-*FR RNAi*) was described by Dietzl et al. (2007). Briefly, a FMRFamide receptor gene fragment was cloned as a 301bp long inverted repeat (IR) in antisense-sense orientation into a modified pUAST vector, pMF3 with multiple UAS sites. This cloned construct was then inserted on the second chromosome of an isogenic w^{1118} host, generating a homozygous viable *UAS-FR* RNAi line. Two transgene lines containing inverted repeats downstream of UAS for the *Drosophila* myosuppressin receptors 1 and 2 (*DmsR-1* and *DmsR-2*) were obtained from Viennna Drosophila RNAi center (VDRC #9369 and 49952).

The following driver lines were used to express the RNAi for the *FR*, *DmsR-1* and *DmsR-2*: *elav-GAL4* (BDSC) and *24B-GAL4* (BDSC) and *tubP-GAL4* (BDSC). We used *elav-GAL4* for pan-neuronal expression of the *UAS-FR*-IR transgene (Luo et al, 1994, Sink et al., 2001). *24B-*

GAL4 (Luo et al., 1994, Brand and Perrimon, 1993) was used to express *UAS-FR*-IR in all larval somatic muscles (Schuster et al.,1996). *tubP-GAL4* is an insert on the third chromosome that is balanced over TM3, *Sb* and allows for ubiquitous expression of Gal4 (Lee and Luo, 1999).

All flies were raised on a cornmeal-based medium (Boreal Laboratories Ltd., St. Catharines, Ontario, Canada), supplemented with dry yeast, at 21 °C on a 12:12 light–dark cycle.

Fly crosses

Tissue-specific expression of the *UAS-FR* IR construct was driven using the UAS/GAL4 system as described by Brand and Perrimon (1993). To express the *UAS-FR* IR construct ubiquitously, homozygous *UAS-FR* RNAi virgin females were crossed to *tubP-GAL4* males that were previously balanced. In addition, balanced *tubP-GAL4* males were crossed with w^{1118} virgin females to remove the balancer chromosome and generate a heterozygous control line (*+/+; tubP-GAL4/+*). Expression of the *UAS-FR* IR construct in muscle and neuronal tissues was accomplished by crossing homozygous *UAS-FR* RNAi virgin females to homozygous *24B*-GAL4 and *elav-GAL4* males, respectively. Heterozygous larvae of the F1 generation were used for the experiments. To generate appropriate heterozygous Gal4 controls, homozygous *elav-GAL4* and *24B-GAL4* males were crossed to w^{1118} virgin female flies. To generate appropriate control larvae with the non-activated *UAS-FR* construct, homozygous *UAS-FR* RNAi virgin females were mated to w^{1118} virgin female flies. The same approach was used for the expression of *UAS-DmsR-1* IR and UAS *DmsR-2* IR. All progeny and parent lines were kept at 27°C on a 12:12 h. light-dark cycle.

Reverse Transcriptase Qualitative polymerase chain reaction (RT-qPCR)

Knockdown of RNAi-lines were assessed by RT-qPCR. Total RNA was isolated using Norgen's Total RNA Purification Kit (St. Catharines, Ontario, Canada) and 500 ng of total RNA were reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). For

real-time qPCR, SYBR Green qPCR Supermix (Invitrogen) was added to cDNA and primers. Each sample was amplified for 40 cycles in a thermocycler (Bio-Rad): 5 min at 95°C, 15 sec at 95°C, 90 sec at 58°C and 30 sec at 72°C. The delta delta Ct ($2^{-\Delta\Delta Ct}$) method was used for data analysis, with rp49 as a house keeping gene for data normalization. Primers used in RT-qPCR reactions were as follows:

rp49 Forward:	**5' GATCGTGAAGAAGCGCAC 3'**
rp49 Reverse:	5' CGCTCGACAATCTCCTTG 3'
FR Forward:	5` TACGGTGGACCCATCAGC 3`
FR Reverse:	5`ATGCCGAGGACACCAACG 3`
DmsR-1 Forward:	5' AGCACTCACCATTCTGTCGG 3'
DmsR-1 Reverse:	5' TTGCTATTTTTCCGGGGCCT 3'
DmsR-2 Forward:	5' TGGCTGAGAAAGCTGCGTAA 3'
DmsR-2 Reverse:	5' GTTCACTGACCAAAACCGCC 3'

Heat-shock

Ala 1 and UAS-ala flies were placed in empty glass vials and placed in a dry incubator for 1 hour at 37 °C. After the heat-shock, flies were allowed to recover at room temperature (21°C) for approximately two hours before muscle contractions were recorded.

Dissection

Wandering stage third-instar larvae were used for all experiments. They were collected from the sides of their culture vials immediately before dissections were made. All dissections were made at room temperature (~21 °C) in modified hemolymph-like (HL6) *Drosophila* physiological saline (Macleod et al, 2002). The saline contained (in mM): 23.7 NaCl, 24.8 KCl, 0.5 $CaCl_2$, 15 $MgCl_2$, 10 $NaHCO_3$, 80 trehalose, 20 Isethionic acid, 5.7 L-alanine, 2.0 L-arginine,

14.5 glycine; 11.0 L-histidine; 1.7 Lmethionine; 13.0 L-proline; 2.3 L-serine; 2.5 L-threonine; 1.4 L-tyrosine; 1.0 L-valine and 5 BES, pH 7.2. The saline was stored at -20 °C in 200 mL aliquots.

Larvae were pinned dorsal side up at the anterior and posterior ends to a dish containing saline. They were stretched slightly longitudinally, and a mid-dorsal cut was made along the length of the animal (Fig 1, top). The viscera were removed, and the segmental nerves were cut near the ventral ganglion, which was subsequently removed along with the brain (Jan and Jan, 1976). These preparations were used for muscle contraction recording or enzyme immunoassay.

Muscle contraction

After dissection, the pin in the anterior end of the larva was removed, and the anterior tip of the larva was hooked to a Grass FT03 tension transducer (Grass Instruments, Quincy, MA) as described previously (Clark et al.,2008). Contractions were amplified using a MOD CP 122A amplifier (Grass Telefactor, W. Warwick, RI) and were recorded on a chart recorder. The recording dish had a volume of approximately 0.2-0.4 ml and was perfused continuously at a rate of 0.7 ml/min. Excess fluid was removed by continuous suction.

cAMP and cGMP enzyme immunoassays

Dissected preparations of larval body wall muscles were placed in cold saline, and pools of 2-9 larval preparations were transferred into 100 µl of desired incubation solution. Individual pools of tissues to be tested for cAMP content were incubated separately in either (a) saline, (b) a non-selective phosphodiesterase inhibitor 5 x 10^{-4} M 1 3-isobutyl-1-methylxanthine (IBMX), (c) 5 x 10^{-4} M IBMX containing either DPKQDFMRFamide at concentrations ranging from 10^{-8} to 10^{-6} M or 5 x 10^{-5} forskolin (an adenylate cyclase activator) (d) 5 x 10^{-4} M IBMX containing 5 x

519 10^{-5} forskolin or (e) 5 x 10^{-5} M forskolin alone. Preparations to be assayed for cGMP were
520 incubated in either (a) saline alone, (b) saline containing 5 x 10^{-4} M IBMX or (c) saline
521 containing 5 x 10^{-4} M IBMX with concentrations of DPKQDFMRFamide ranging from 10^{-8} to
522 10^{-6} M. All incubation solutions contained 0.25 % DMSO with the exception of solutions
523 containing forskolin, which contained 0.65% DMSO. After 10 min of incubation at room
524 temperature, each larval pool was placed into 100 µl of 0.1 M hydrochloric acid (HCl) for 5 min
525 to inhibit endogenous phosphodiesterase activity. To halt the chemical reactions, larvae were
526 frozen on a steel plate cooled over dry ice, and tissue was homogenized in 500 µl of 0.1 M HCl
527 and centrifuged for 12 min at 11,000 x g. The supernatant was stored at -80 °C until ready to
528 assay. The pellet was further submitted to a protein determination assay.

529 Levels of cAMP or cGMP were determined in duplicates from supernatant (100 µl)
530 following acetylation protocols for direct cAMP and cGMP enzyme immunoassay kits (Assay
531 Designs, Ann Arbor, USA). Optical densities were read at 405 nm with a Bio-Tek Synergy™ HT
532 microplate reader (BioTek, Vermont, USA), and the concentrations of cAMP or cGMP
533 (pmol/ml) were estimated using a 4 parameter logistic curve-fitting program provided in the Bio-
534 Tek KC4™ Software. The sensitivities of the acetylated version of the cAMP and cGMP assays
535 were 0.037 pmol/ml and 25 pmol/ml, respectively.

536 Each pellet was dissolved in 100 µl of 1M NaOH and placed for 2 hours in a hot water
537 bath (40 °C). Protein content was measured from 10-15 µl of solubilized pellet with a BioRad
538 Protein Assay kit (based on method of Bradford, 1976), using bovine serum albumin as a protein
539 standard. To account for variations in the number and sizes of larval body wall preparations,
540 cAMP and cGMP levels were expressed as pmol per mg of protein.

541 *Pertussis Toxin*

Pertussis toxin (PTX) was obtained from Cedarlane (Burlington, Canada). Dissected third-instar larval tissue was incubated in 500ng/mL PTX for 20 minutes prior to examination of tonus. For control experiments we heat-inactivated PTX by placing the solution in a water-bath just below boil for 30 minutes.

Chemicals

The *Drosophila* peptide, DPKQDFMRFamide, was synthesized by Cell Essentials (Boston, Mass.) and was 98% pure as determined by reverse-phase High Performance Liquid Chromatography (HPLC). Peptide was stored at -20 ˚C and was dissolved in saline to yield a 10 mM stock solution. IBMX was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). 5 mM IBMX stock solution was made in 0.5 % DMSO containing saline and kept at -20 °C. The DMSO concentration in the final IBMX solution used for physiological recordings did not exceed 0.05%. Forskolin, Rp-cAMPS (adenosine 3′,5′-cyclic monophosphorothioate, Rp-Isomer, triethylammonium salt), Rp-8-pCPT-cGMPS (guanosine 3′,5′-cyclic monophosphorothioate, 8-(4-Chlorophenylthio)-, Rp-Isomer, triethylammonium salt) and KN-93 were obtained from Calbiochem. Forskolin and KN-93 were dissolved in 100 % DMSO and stored at 4 °C as stock solutions (12 and 10 mM, respectively), which were subsequently diluted in saline to yield the desired drug concentration with final DMSO concentrations of 0.4 % and 0.1 %, respectively. 5 µmol of Rp-cAMPS and 1 µmol of Rp-8-pCPT-cGMPS were stored at -20 °C until ready to dilute in saline to yield the desired final concentration. Arachidonic acid sodium salt was purchased from Sigma-Aldrich (Oakville, Ontario, Canada) readily dissolved in saline. All experimental solutions were made fresh on the day of testing.

Statistical analysis

Both one way ANOVA and t-test for independent samples, unless otherwise stated, were used where appropriate to determine statistical significance, and $p<0.05$ was used for acceptance of statistical significance. All data were expressed as means ± standard error of the mean (SEM). The number of preparations is indicated in the parentheses on the graphs (n).

ACKNOWLEDGEMENTS

We thank Dr. Satpal Singh for advice on fly line crosses.

FUNDING

This work was supported by an NSERC Discovery Grant (grant number 46292) to AJM, an NSERC PGS scholarship to KGO and Ontario Government Scholarships to MM and KGO.

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Figure 1. DPKQDFMRFamide modulates larval muscle tonus via the *Drosophila* FMRFamide G-protein coupled receptor (FR). (A) Sample traces of larval body wall muscle contractions in response to application of 1 μM DPKQDFMRFamide. The recordings from different genetic fly lines are shown in this order (from top and left): Canton S, UAS-FR RNAi/+ (a line containing a single copy of non-activated FR inverted repeat construct-FR IR), tubP-Gal4/+ (a line containing a single copy of the ubiquitous Gal4 driver), 24B-Gal4/+ (a line containing a single copy of the muscle-specific Gal4 driver), elav-Gal4/+ (a line containing a single copy of the neuron-specific Gal4 driver), UAS-*FR* RNAi /+; tubP-GAL4/+ (a line containing a single copy of both the ubiquitous Gal4 driver and FR IR construct), UAS-*FR* RNAi/+; 24B- GAL4/+ (a line containing both single copy of the muscle-specific Gal4 driver and FR IR construct), UAS-*FR* RNAi/+; elav-GAL4/+ (a line containing both single copy of the neuron-specific Gal4 driver and FR IR construct). Peptide was applied for 5 min; beginning at the arrow mark and continuing to the end of the sample recording. Upward deflections represent phasic contractions. Incline in the horizontal trace represent change in muscle tonus. (B) Summary of responses to 1 μM DPKQDFMRFamide. Peptide-induced muscle contractions were attenuated in larvae with the ubiquitous expression of FR IR construct, UAS-*FR* RNAi /+; tubP-GAL4/+ larvae, but not in heterozygous parental control lines, (Tamhane's post hoc, $p<0.01$). Similarly, larvae expressing the FR IR construct specifically in muscle tissue showed a reduced response to the peptide (by ~ 85-90%) when compared to two heterozygous parental control lines, (Tamhane's post hoc, $p<0.01$). Pan-neuronal expression of the FR IR construct in *Drosophila* larvae had no effect on the peptide's ability to modulate muscle tonus. No differences were found between the response of UAS-*FR* RNAi/+; elav-GAL4/+ larvae and their parental controls (UAS- One-Way ANOVA, Tamhane's post hoc, $p>0.05$). A triangle indicates significance of $p<0.05$ compared to Canton-S flies. A square represents a significant difference ($p<0.05$) compared to UAS-IR-FR line.

Figure 2. Confirmation of UAS-RNAi –RFamide knockdown using Real time Quantitative polymerase chain reaction. RNA was isolated from whole third-instar larvae tissue from each of the transgenic lines used to knockdown the expression of FR as well as the outcross controls. RNA was reverse-transcribed and subsequently, cDNA levels were quantified using qPCR. The housekeeping gene ribosomal protein 49 was used for data normalization.

Figure 3. Myogenic effects of DPKQDFMRFamide are not mediated through either myosuppressin receptor 1 or 2. (A) Summary of responses to 1 μM DPKQDFMRFamide in UAS-DmSR-1 RNAi lines. Peptide-induced changes in basal tonus were equal-to or greater in force than wild-type controls. (B) Summary of responses to 1 μM DPKQDFMRFamide in UAS-DmSR-2 RNAi lines. Peptide-induced changes in basal tonus were equal-to or greater in force than wild-type controls.

Figure 4. Genetic and pharmacological inhibition of CaMKII does not affect the ability of the DPKQDFMRFamide to induce tonic contracture in *Drosophila* larval body wall muscles. (A) Sample recordings demonstrate the effect of 1 μM DPKQDFMRFamide on spontaneous tonic contractions of body wall muscles in ala1 (top left) and UAS-ala (top middle) larvae without prior heat-shock treatment and ala1 (bottom left) and UAS-ala (bottom middle) after they were submitted to a 1 hour heat-shock treatment at 37°C. Driving expression of ala peptide inhibitor in muscle tissue, independently of heart shock, did not attenuate the effects of DPKQDFMRFamide (top right) or in the controls (bottom right). Peptide was applied at the downward pointing arrows by continuous perfusion. (B) Bottom left: Average muscle tonus change induced by DPKQDFMRFamide in ala1 larvae, and the control, UAS-ala larvae. Larvae of both genetic lines showed similar increases in tonus in response to peptide treatment. Heat shock treatment, which increases the expression of CaMKII inhibitory protein in ala 1 flies, but not in UAS-ala flies, potentiated DPKQDFMRFamide-induced contractions in both ala1 and UAS-ala larvae (t-test for independent samples, $p<0.05$). No significant difference was observed between the responses in heat-shocked ala1 and UAS-ala larvae (t-test for independent samples, $p>0.05$). Bottom middle: Average muscle tonus change induced by DPKQDFMRFamide in Canton-S larvae in the presence of a CaMKII inhibitor. 1 μM KN-93, a CaMKII inhibitor, had no effect on DPKQDFMRFamide-induced muscle contractions (t-test for independent samples, $p>0.05$). Bottom right: Driving the expression of the ala peptide inhibitor selectively in muscle fibers (UAS-ala / 24B-Gal4) did not alter the average muscle tonus change induced by DPKQDFMRFamide or in control lines (UAS-ala/+) (t-test for independent samples, $P>0.05$). (n), the number of preparations.

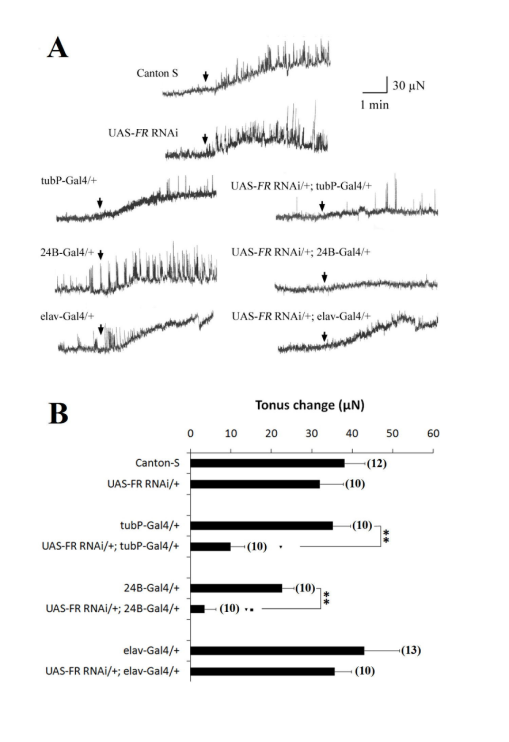
Figure 5. Disruption of PLCβ or IP_3 receptor gene had no effect on the ability of DPKQDFMRFamide to modulate muscle tonus in *Drosophila* larvae. Muscle contraction assays were performed on fly lines carrying mutation on the genes encoding for (A) IP_3 receptor and (B) PLCβ . Muscle contraction assays were performed by perfusing body wall muscles with 1 μM DPKQDFMRFamide for 5 minutes and recording the tonus change. The tonus change was calculated as the difference between the maximal rise in tonus within the 5 minute peptide application period and the pre-peptide tonus level. (A) No significant difference was observed between the effect of the peptide on muscle tonus in heterozygous IP_3 receptor mutants (Itp-r83A^{05616}/+) and wild type larvae (t-test for independent samples, $p>0.05$). Similarly, no significant difference was observed between the effects of the peptide on muscle tonus in each of *norpA* mutants (w* norpA^{33}, w* norpA^{36} and norpA^{7}) and control Oregon R (t-test for independent samples, $p>0.05$) nor between *Plc21C* mutant (y^{1} w^{1118}; Plc21C^{A24}) larvae and their respective controls, y,w (t-test for independent samples, $p>0.05$). (n), the number of preparations.

Figure 6. DPKQDFMRFamide does not affect cAMP levels in *Drosophila* body wall muscles. (A) The effects of DPKQDFMRFamide on cAMP levels in larval body wall muscles in the presence of 0.5 mM IBMX. After a 10 min incubation of *Drosophila* body wall muscles in 10 nM, 100 nM or 1μM DPKQDFMRFamide in the presence of 0.5 mM IBMX, cAMP levels did not exceed the cAMP level observed after incubation in 0.5 mM IBMX alone (one-way ANOVA, Tamhane's post hoc, $p>0.05$) . B) Jointly 0.05 mM forskolin and 0.5 mM IBMX increased larval body wall muscle cAMP concentration by approximately 2.3 times of the level reached with mM forskolin alone (One-Way ANOVA, Tamhane's post hoc, $p < 0.01$). (n), the number of preparations.

Figure 7. DPKQDFMRFamide does not increase cGMP levels in *Drosophila* body wall muscles. The cGMP levels measured in *Drosophila* body wall muscles that were incubated simultaneously in DPKQDFMRFamide (10 nM, 100 nM or 1μM) and 0.5 mM IBMX for 10 min did not exceed the cGMP level observed after the muscles were incubated in 0.5 mM IBMX alone. (n), the number of preparations.

Figure 8. The application of 0.5 mM IBMX, a broad-spectrum phosphodiesterase inhibitor (A) and 100 μM Rp-cAMPS and 10 μM Rp-8-pCPT-cGMPS, selective cAMP and cGMP dependent protein kinase inhibitors (B), had no effect on the ability of peptide to induce contractions in *Drosophila* body wall muscles (t-test for independent samples, $p>0.05$). (n), the number of preparations.

Figure 9: Incubation of third-instar tissue with 500ng/mL PTX, a G-protein couple receptor inhibitor, greatly reduced the ability of DPKQDFMRFamide to induce changes in muscle tonus. Top trace (PTX) shows that the effect of DPKQDFMRFamide on body wall muscle is greatly reduced in the presence of PTX. Bottom trace shows that heat-inactivated PTX does not attenuate the effect of the peptide. Downward arrow indicates when 1 μM DPKQDFMRFamide was added. Average muscle tonus change demonstrates that incubating tissue with PTX significantly reduces the ability of DPKQDFMRFamide to induce changes in muscle tonus (Mann-Whitney rank sum test, $P<0.01$).e


A
Canton S
30 µN
1 min
UAS-FR RNAi
tubP-Gal4/+
UAS-FR RNAi/+; tubP-Gal4/+
24B-Gal4/+
UAS-FR RNAi/+; 24B-Gal4/+
elav-Gal4/+
UAS-FR RNAi/+; elav-Gal4/+
B
Tonus change (µN)
0
10
20
30
40
50
60
Canton-S
(12)
UAS-FR RNAi/+
(10)
tubP-Gal4/+
(10)
**
UAS-FR RNAi/+; tubP-Gal4/+
(10)
*
24B-Gal4/+
(10)
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(10)
**
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(13)
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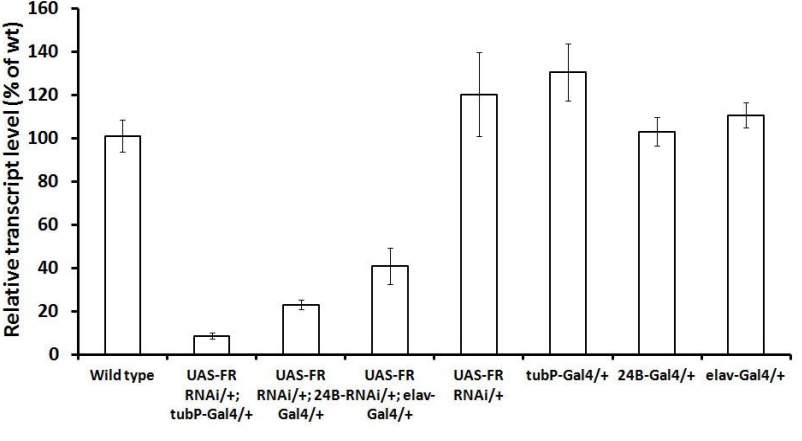
(10)


Relative transcript level (% of wt)
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140
120
100
80
60
40
20
0
Wild type
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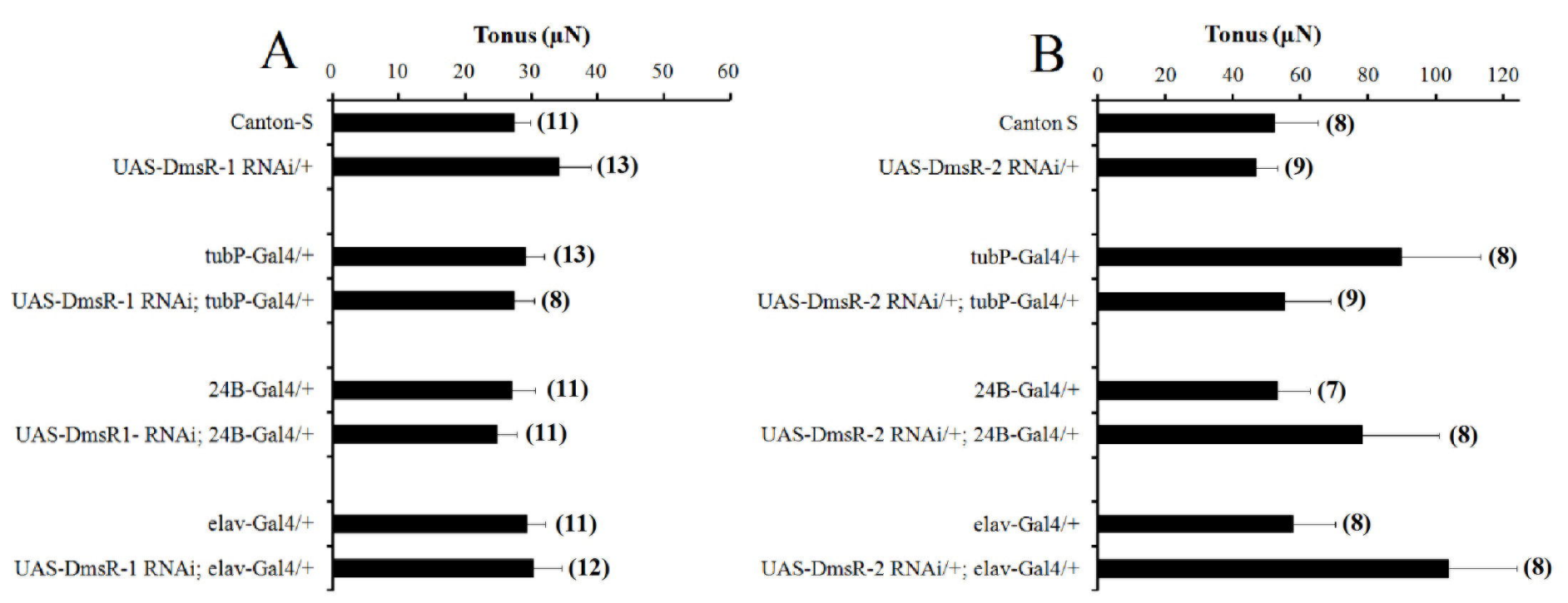
tubP-Gal4/+
24B-Gal4/+
elav-Gal4/+


A
Tonus (µN)
0
10
20
30
40
50
60
Canton-S
(11)
UAS-DmsR-1 RNAi/+
(13)
tubP-Gal4/+
(13)
UAS-DmsR-1 RNAi; tubP-Gal4/+
(8)
24B-Gal4/+
(11)
UAS-DmsR1- RNAi; 24B-Gal4/+
(11)
elav-Gal4/+
(11)
UAS-DmsR-1 RNAi; elav-Gal4/+
(12)
B
Tonus (µN)
0
20
40
60
80
100
120
Canton S
(8)
UAS-DmsR-2 RNAi/+
(9)
tubP-Gal4/+
(8)
UAS-DmsR-2 RNAi/+; tubP-Gal4/+
(9)
24B-Gal4/+
(7)
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elav-Gal4/+
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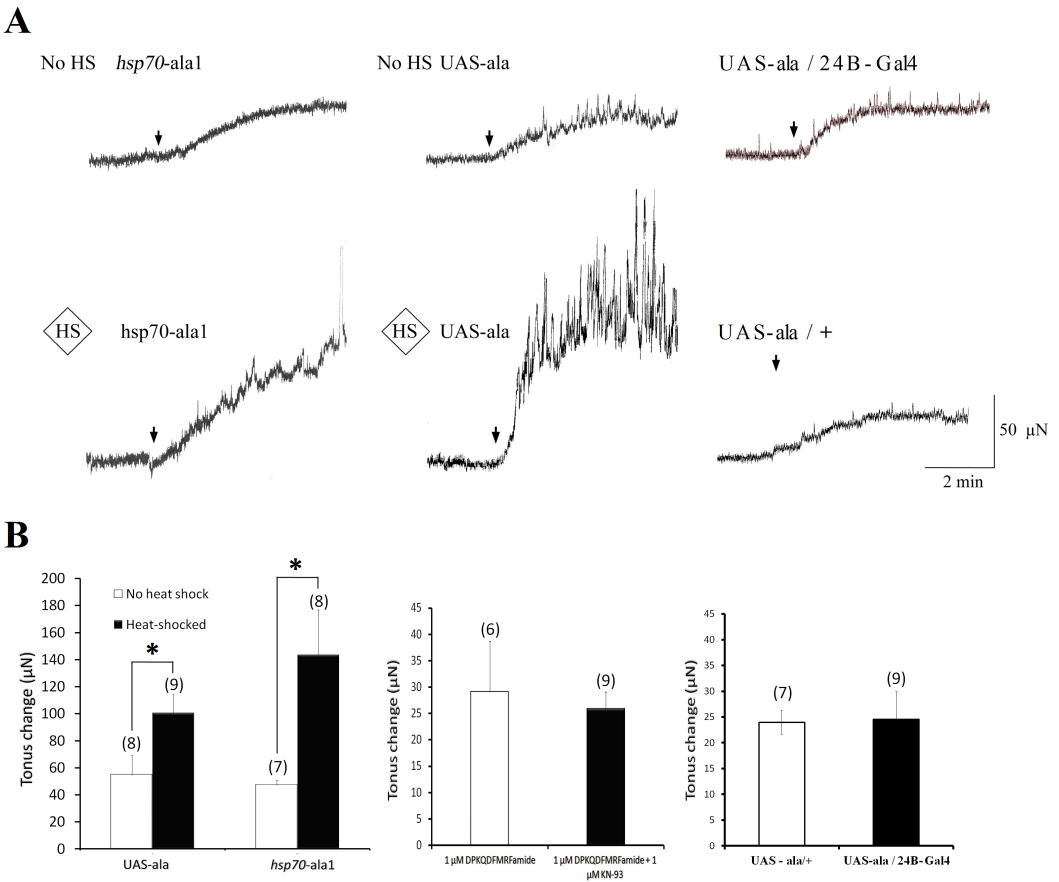
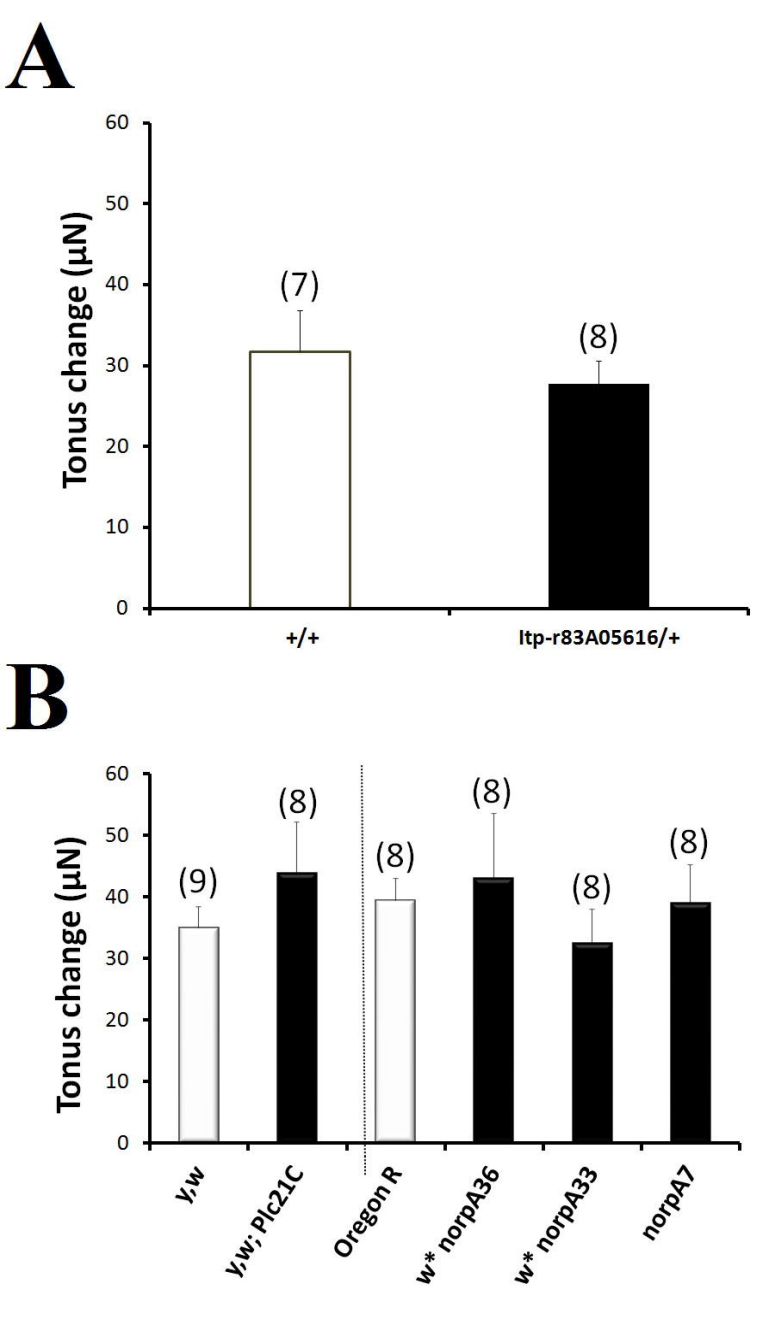
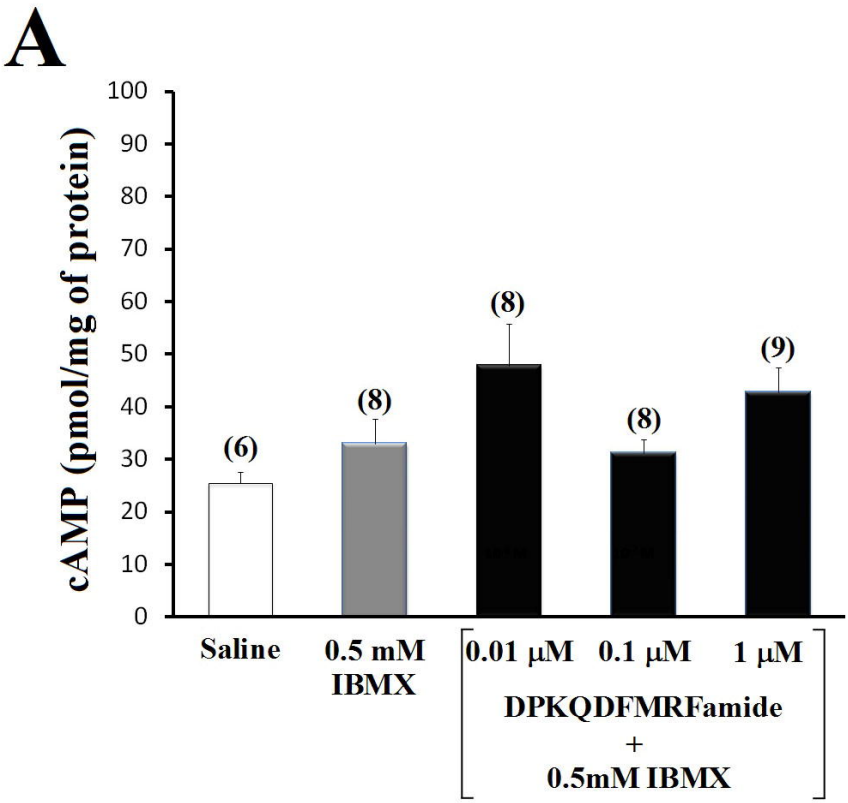
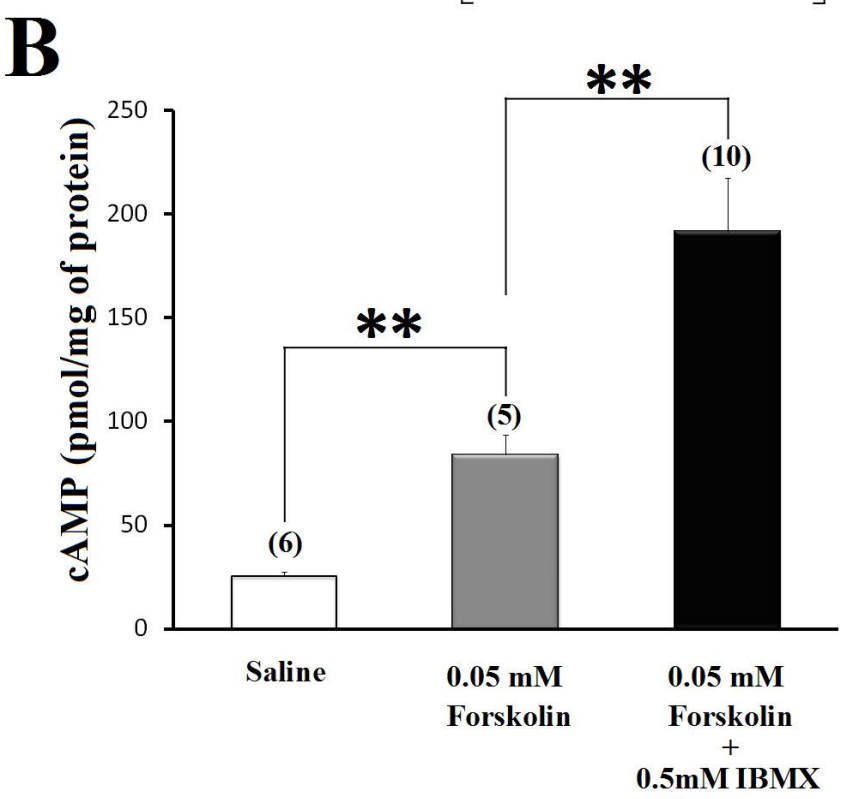
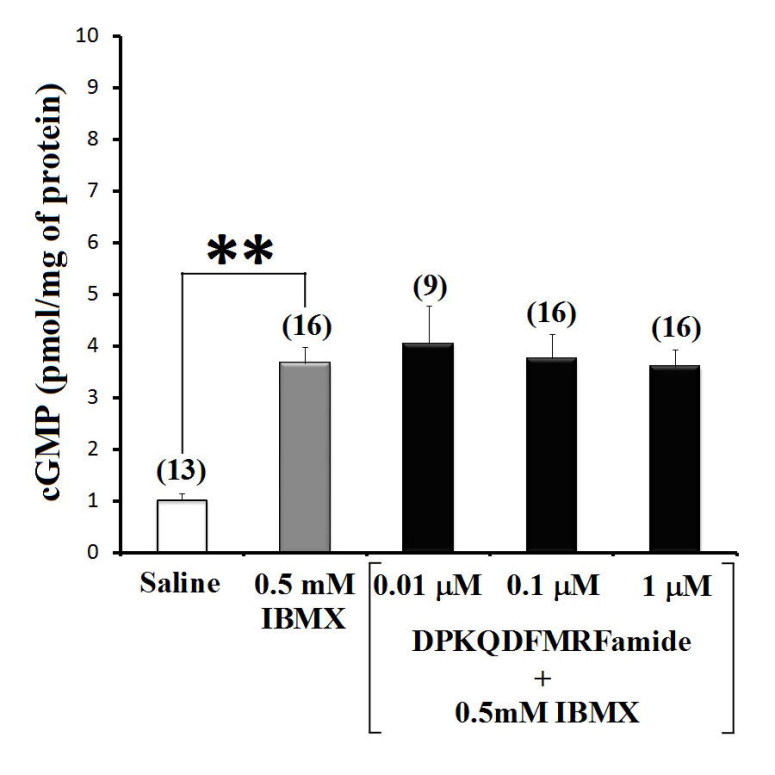
(8)

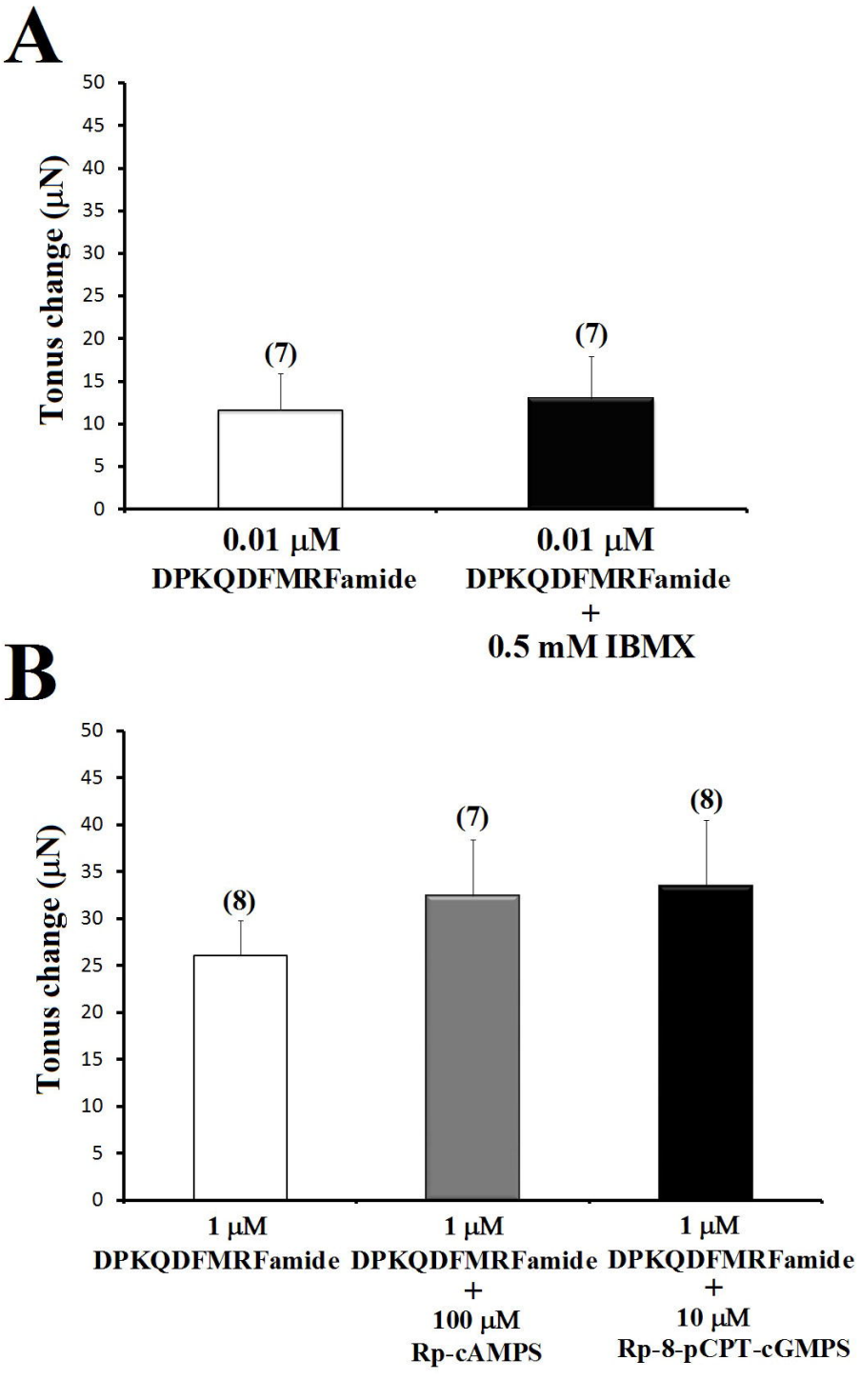

A
No HS hsp70-ala1
No HS UAS-ala
UAS-ala / 24B-Gal4
HS
hsp70-ala1
HS
UAS-ala
UAS-ala / +
50 µN
2 min
B
No heat shock
Heat-shocked
Tonus change (µN)
UAS-ala
hsp70-ala1
1 µM DPKQDFMRFamide
1 µM DPKQDFMRFamide + 1 µM KN-93
UAS - ala/+
UAS-ala / 24B-Gal4


A
Tonus change (µN)
60
50
40
30
20
10
0
(7)
(8)
+/+
ltp-r83A05616/+
B
Tonus change (µN)
60
50
40
30
20
10
0
(9)
(8)
(8)
(8)
(8)
(8)
y,w
y,w; Plc21C
Oregon R
w* norpA36
w* norpA33
norpA7


A
cAMP (pmol/mg of protein)
100
90
80
70
60
50
40
30
20
10
0
(6)
(8)
(8)
(8)
(9)
Saline
0.5 mM IBMX
0.01 µM
0.1 µM
1 µM
DPKQDFMRFamide
+
0.5mM IBMX


B
cAMP (pmol/mg of protein)
250
200
150
100
50
0
**
**
(6)
(5)
(10)
Saline
0.05 mM Forskolin
0.05 mM Forskolin
+
0.5mM IBMX


cGMP (pmol/mg of protein)
10
9
8
7
6
5
4
3
2
1
0
**
(13)
(16)
(9)
(16)
(16)
Saline
0.5 mM IBMX
0.01 μM
0.1 μM
1 μM
DPKQDFMRFamide
+
0.5mM IBMX


A
Tonus change (µN)
50
45
40
35
30
25
20
15
10
5
0
(7)
(7)
0.01 µM
DPKQDFMRFamide
0.01 µM
DPKQDFMRFamide
+
0.5 mM IBMX
B
Tonus change (µN)
50
45
40
35
30
25
20
15
10
5
0
(8)
(7)
(8)
1 µM
DPKQDFMRFamide
1 µM
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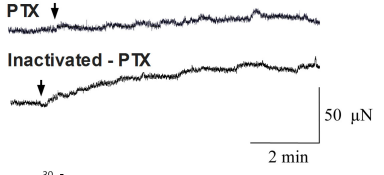
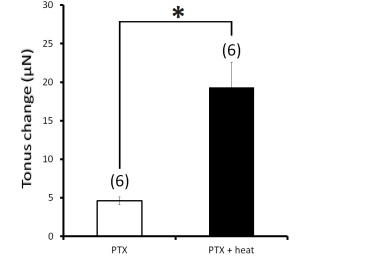
Rp-cAMPS
1 µM
DPKQDFMRFamide
+
10 µM
Rp-8-pCPT-cGMPS


PTX
Inactivated - PTX
50 µN
2 min


Tonus change (µN)
30
25
20
15
10
5
0
*
(6)
(6)
PTX
PTX + heat