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# Levamisole receptor phosphorylation: effects of kinase antagonists on membrane potential responses in *Ascaris suum* suggest that CaM kinase and tyrosine kinase regulate sensitivity to levamisole

Sasa M. Trailovic, Alan P. Robertson, Cheryl L. Clark and Richard J. Martin*

*Department of Biomedical Sciences, Iowa State University, Ames, Iowa, IA 50011, USA*

*Author for correspondence (e-mail: rjmartin@iastate.edu)

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## Summary

**A two-micropipette current-clamp technique was used to record electrophysiological responses from the somatic muscle of *Ascaris suum*. Levamisole and acetylcholine were applied to the bag region of the muscle using a microperfusion system. Depolarizations produced by 10 s applications of 10 μmol $l^{-1}$ levamisole or 20 s applications of 10 μmol $l^{-1}$ acetylcholine were recorded. The effect on the peak membrane potential change of the kinase antagonists H-7, staurosporine, KN-93 and genistein was observed. H-7 (30 μmol $l^{-1}$), a non-selective antagonist of protein kinases A, C and G but which has little effect on $Ca^{2+}$/calmodulin-dependent kinase II (CaM kinase II), did not produce a significant effect on the peak response to levamisole or acetylcholine. Staurosporine (1 μmol $l^{-1}$), a non-selective kinase antagonist that has effects on protein kinases A, C and G, CaM kinase and tyrosine kinase, reduced the mean peak membrane potential response to levamisole from 6.8 mV to 3.9 mV ($P$<0.0001) and the mean response to acetylcholine from 5.5 mV to 2.8 mV ($P$=0.0016). The difference between the effects of H-7 and staurosporine suggested the involvement of CaM kinase II and/or tyrosine kinase. KN-93, a selective CaM kinase II antagonist, reduced the mean peak response to levamisole from 6.2 mV to 2.7 mV ($P$=0.035) and the mean peak response of acetylcholine from 4.7 mV to 2.0 mV ($P$=0.0004). The effects indicated the involvement of CaM kinase II in the phosphorylation of levamisole and acetylcholine receptors. The effect of extracellular $Ca^{2+}$ on the response to levamisole was assessed by comparing responses to levamisole in normal and in low-$Ca^{2+}$ bathing solutions. The response to levamisole was greater in the presence of $Ca^{2+}$, an effect that may be explained by stimulation of CaM kinase II. Genistein (90 μmol $l^{-1}$), a selective tyrosine kinase antagonist, reduced peak membrane potential responses to levamisole from a mean of 6.4 mV to 3.3 mV ($P$=0.001). This effect indicated the involvement of tyrosine kinase in maintaining the receptor.**

Key words: levamisole, kinase antagonist, H-7, KN-93, genistein, staurosporine, *Ascaris summ*, nematode, CaM kinase, tyrosine kinase, membrane potential response.

## Introduction

Since the era of modern chemotherapy began (Ehrlich and Shiga, 1904), it has been recognized that the development of resistance to therapeutic drugs can limit their efficacy. Although this was first established for trypanosome parasites with resistance to trypan red (Browning, 1907), we now know that the development of resistance occurs to antibacterial, antiviral and anticancer drugs as well. There is a Darwinian struggle between the pathogen causing the disease and the drug used to treat it. If the disease organism is to survive in the presence of the drug, it has to find a way to overcome its therapeutic effects. A pessimistic view is that development of resistance is inevitable if we continue to use drugs; and a pragmatic approach is to explore ways of counteracting or delaying the onset of resistance so that we preserve the efficacy of these drugs.

We are interested in the mechanisms by which parasitic nematodes become resistant to anthelmintic drugs. The mechanisms that underlie resistance may be more complex in helminths than in viruses and bacteria, in part because of the larger genome of these pathogens. We have focused our attention on the drug levamisole, which acts as a selective agonist on a sub-set of nicotinic acetylcholine receptors (nAChRs) in nematodes (Robertson et al., 1999; Richmond and Jorgensen, 1999). At therapeutic concentrations, levamisole produces depolarization and contraction of nematode somatic muscle, which leads to paralysis and elimination of the parasite without affecting the host nicotinic receptors. We have observed levamisole-activated single-channel currents in levamisole-sensitive (SENS) and levamisole-resistant (LEV-R) nematode isolates (Robertson et al., 1999). One of the striking differences between resistant and sensitive nAChR receptors is that, in LEV-R, the average

probability of the channel being in the open state, $P_{open}$, is some ten times less than in SENS, while the average channel conductance is the same. We have also observed in SENS and LEV-R isolates that the $P_{open}$ for individual channels varies dramatically between patches. For example, in the presence of 30 μmol $l^{-1}$ levamisole, $P_{open}$ values in SENS varied between 0.090 and 0.003 at a holding potential of –50 mV, which is a 30-fold difference between the highest and lowest $P_{open}$ value. These observations give rise to the question of what causes the wide variation in $P_{open}$ values: could it be that opening of nAChRs in nematodes is regulated?

The levamisole receptors of nematodes, like those in vertebrates, are understood to be composed of five subunits that surround a central non-selective cation pore. In *Caenorhabditis elegans*, the subunits that have been described include UNC-38 (an α-subunit), UNC-29 and LEV-1 (β-subunits; Fleming et al., 1997). It is anticipated that other subunits are also involved in forming nAChRs on nematode muscle. A common feature of all signal-transduction proteins, including ion channels, is regulation of their activity by kinase enzymes. The addition of a bulky phosphate group to a serine, threonine or tyrosine residue in the protein introduces a highly charged group into a region that was only moderately polar before and, in the case of an ion channel, affects its opening. We have examined the amino acid sequence of the α-subunit of the *Ascaris* levamisole receptor, ASAR-1 (GenBank Accession No. AJ011382), and have identified consensus phosphorylation sites (Kennely and Krebs, 1991) for protein kinase C (PKC), protein kinase A (PKA), protein kinase G (PKG), $Ca^{2+}$/calmodulin-dependent (CaM) kinase and tyrosine kinase. These sites are all on the cytoplasmic domain of the subunit between the transmembrane regions TM3 and TM4 (Fig. 1A). Some of these sites are overlapping, suggesting that differing kinases could produce the same effects. Phosphorylation of the nAChRs in nematodes, as in mammalian receptors, is expected to lead to changes in $P_{open}$ (Colledge and Froehner, 1997; Hopfield et al., 1988; Reuhl et al., 1992) and may contribute to the variation in values we have observed in single-channel experiments.

In experiments on the large parasitic nematode of swine, *Ascaris suum*, Trim et al. (1999) described inhibitory effects of tamoxifen on the membrane potential responses to levamisole and acetylcholine and suggested the involvement of PKC in modulating the activity of nAChRs. However, a more selective PKC antagonist, chelerythine had no effect. In view of the difficulty in interpreting the effects of tamoxifen, we have examined the effects of a number of other protein kinase inhibitors on *A. suum* muscle, to test the hypothesis that kinase activity is required to support the activity of nAChRs and is involved in regulating the electrical response to the

A

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  1 ARAKNYGGVS VLYVPYEMIW VPDIVLYNNA DSNYNITIST KATLHYSGEV TWEPPAIFKS

 61 MCQIDVRWFP FDEQKCHLKF GSWTYSEDLL VLELLDGEPH YELETNEFGE VDNITIVDDG

                                                 ------TM1------------
121 IDLSDYYPSV EWDIMSRVAI RRTKNYPSCC PQSDAYIDIM YYLELRRKPL FYTVNLVFPC

      -----TM1------      ---------TM2-------                -----TM3--
181 VGISFLTILV FYLPSDSGEK_VTLCISILVA LTVFFLLLTE IIPATSISLP LIGKYLLFTM

    -TM3------------     C, CaM                                    T  C,A,G,CA
241 VMVTLSVVVT VISLNLHFRT PTTHRMPEWV KWLFLKFLPK VLFMRRPLAD TDDTYRRVSQ

                        C        CaM
301 RRGDNCEKVA INYHEHRVSR DIERALSTSP VDERIQKLYY SPAVVKAFEN VCFIAELLKK

                        --------TM4---------
361 KDRDDKVDED WKYVAMVLDR LFLLLFSFAC FIGTVTILLQ APTLYDRREA NDLQYRPANI

421 STPVTQ
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B

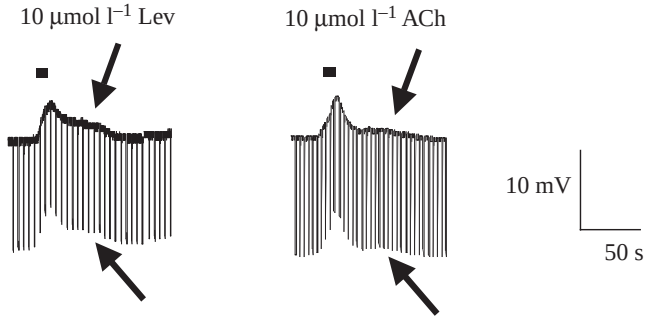


Fig. 1. (A) Acetylcholine (nicotinic) receptor α-subunit of *Ascaris suum* (ASAR-1 GenBank Accession No. AJ011382) and protein kinase phosphorylation sites. The α-subunit combines with other subunits to form the pentameric receptor ion-channel membrane protein. The regions TM1, TM2, TM3 and TM4 are transmembrane regions. The region between TM3 and TM4 is exposed to the cytoplasm and contains consensus serine (S), threonine (T) and tyrosine (Y) phosphorylation sites. The structural motifs S, T or Y are consensus sites recognized by kinases: C, protein kinase C; A, protein kinase A; G, protein kinase G; T, tyrosine kinase; and CaM, $Ca^{2+}$/calmodulin kinase. (B) Representative responses to levamisole (Lev) and acetylcholine (ACh). The amplitude of the first peak was measured for experimental purposes. The secondary component or slow-phase responses to levamisole and acetylcholine (arrows) were not always present but were seen in approximately one-third of the responses; they were more labile than the first component and may have arisen as a secondary effect of calcium entry following stimulation of the nicotinic receptors, or following stimulation of a metabotrophic (muscarinic) receptor (Hwang et al., 1999).

anthelmintic levamisole and acetylcholine. We tested the effects of both levamisole and acetylcholine, because they may select for a different subset of nAChRs in *A. suum*, and found that responses to these compounds are labile and inhibited by CaM kinase II antagonists and a tyrosine kinase antagonist. These observations suggest that the responses to acetylcholine and levamisole are not fixed but vary, and this plasticity depends on the phosphorylation state of the nAChRs. They also suggest that resistance to the anthelmintic levamisole may involve changes in the phosphorylation state of the receptor and may not require amino acid changes at the drug's binding site. The work has relevance to the understanding of mechanisms of anthelmintic resistance.

## Materials and methods

### *Ascaris suum*

Adult *Ascaris suum* (Chitwood and Chitwood, 1950) worms were collected from the abattoir in Storm Lake City, Iowa and maintained in Locke's solution [NaCl (155 mmol $l^{-1}$), KCl (5 mmol $l^{-1}$), $CaCl_2$ (2 mmol $l^{-1}$), $NaHCO_3$ (1.5 mmol $l^{-1}$) and glucose (5 mmol $l^{-1}$)] at 35°C in the dark. Parasites were used within 5 days of collection.

### *Preparation*

A body-wall muscle-flap preparation was prepared from a 1 cm section, 3 cm caudal to the head of adult *Ascaris* and pinned, cuticle-side down, onto a Sylgard™-lined chamber, where the intestine was removed. The preparation was microperfused continuously with *Ascaris* peri-enteric fluid [APF: NaCl (23 mmol $l^{-1}$), Na acetate (110 mmol $l^{-1}$), KCl (24 mmol $l^{-1}$), $CaCl_2$ (6 mmol $l^{-1}$), $MgCl_2$ (5 mmol $l^{-1}$), glucose (11 mmol $l^{-1}$) and Hepes (5 mmol $l^{-1}$), pH 7.6 with NaOH]. In experiments to test the effect of low-$Ca^{2+}$, APF was prepared by replacing $Ca^{2+}$ in APF on an equimolar basis with $Mg^{2+}$. Application of the perfusate was *via* a fine microtube placed with a micromanipulator (approximately 500 μm) over the muscle cell bag. The rate of perfusion was 1.5 ml $min^{-1}$ and this allowed rapid change of the solution bathing the cell being recorded from. The temperature in the chamber was maintained at 32–33°C.

### *Electrophysiology*

A two-microelectrode current-clamp technique was used for measuring the membrane potential and input conductance changes of the *Ascaris* muscle cell bags. Micropipettes made from borosilicate glass (Clarke Electromedical, Reading, UK) with resistances in the range of 20–40 MΩ when filled with 2 mol $l^{-1}$ potassium acetate were used for recording. Two microelectrodes were carefully inserted into one muscle cell bag with minimum damage. An Axoclamp 2B amplifier, 1320A Digidata Interface, pClamp 8.0 software (all from Axon Instruments, Union City, CA, USA) and Pentium III PC were used to display, record and analyze the membrane potential and injected current. One micropipette was used for recording of membrane potential, while the second was used for injection of current pulses (hyperpolarizing: 40 nA, 500 ms filtered at 0.3 kHz).

In the present study, we measured the first fast peak membrane potential change response to acetylcholine or levamisole as the nicotinic response to these drugs. In approximately one-third of the responses, we also observed a second component or tail to the response (arrows, Fig. 1B). This component has not been described previously, perhaps because it is more labile than the first component. It may have arisen as a secondary effect of entry of $Ca^{2+}$ following stimulation of the nicotinic receptors, or following stimulation of metabotrophic (muscarinic) receptors (Hwang et al., 1999). In the present study, we focused exclusively on the effects of the kinase antagonists on the peak of the first component because it results from activation of extrasynaptic nicotinic receptors on the bag region of the muscle (Martin, 1982), and these receptors are accessible to direct stimulation by perfused drugs and to recording by standard two-electrode current-clamp technique.

### *Drug solutions*

H-7, staurosporine, genistein and KN-93 were purchased from Calbiochem (San Diego, CA, USA). Levamisole hydrochloride and acetylcholine chloride were purchased from Sigma (St Louis, MO, USA). Stock solutions for staurosporine, genistein and KN-93 were prepared in dimethyl sulfoxide (DMSO) and frozen for use following dilution in APF. The maximum concentration of DMSO used (0.1%) did not affect membrane potential or responses to levamisole and acetylcholine. Stock solutions of H-7 and levamisole were prepared each week in APF and stored at 4°C. Stock and working solutions of acetylcholine were prepared freshly every day in APF.

### *Application of drugs*

In all experiments, acetylcholine was added to the bag region of the cell *via* the microcatheter in the perfusate for 20 s; levamisole was applied for 10 s. Peak membrane potential changes and input conductance were measured. All kinase antagonists were added to the preparation in the perfusate for at least 2 min before the application of acetylcholine or levamisole.

### *Statistics*

All results are presented as means ± S.E.M. Significance was tested using paired *t*-test (for antagonist influences) or one-way analysis of variance (ANOVA; for influence of $Ca^{2+}$ on the levamisole concentration–response relationship). The influences of $Ca^{2+}$ on the effects of increasing levamisole concentrations on membrane potential were estimated using the Hill equation: % response = $1/(1+[EC_{50}/X_A]^n)$, where $EC_{50}$ is the concentration of agonist ($X_A$) producing 50% of the maximum response, and $n$ is the Hill coefficient (slope). Prism Pad software (Version 3.0, San Diego, CA, USA) was used for all statistical determinations.

## Results

### *The levamisole and acetylcholine response*

We made recordings from muscle cell bags with stable membrane potentials more negative than –20 mV and input conductances of <4.0 μS. Recordings were rejected for analysis if there was a non-drug-induced change in resting input conductance of >0.2 μS or a resting membrane potential change of >4 mV during the recording.

Fig. 2A shows a representative example of the effect of a 10 s superfusion of the bag region of the muscle with 10 μmol $l^{-1}$ levamisole. The first response recorded was a 7 mV depolarization peak associated with a change in input conductance from 2.26 μS to 2.86 μS. The membrane potential recovery after the levamisole pulse was slow, with a $t_{1/2}$ of 94 s. Fig. 2B shows a representative trace from another preparation of the effect of 20 s of 10 μmol $l^{-1}$ acetylcholine at a resting membrane potential of –23.8 mV. The peak depolarization of the first response was 3.4 mV associated with a conductance change of 0.31 μS. Note, however, that the recovery from acetylcholine was faster ($t_{1/2}$=13 s) than with levamisole. The slower recovery time of levamisole, when compared with acetylcholine, was a consistent feature, making the measurement of the effect of kinase antagonists on levamisole responses more difficult to determine because of the longer stable recording conditions that were required. Several factors may have contributed to the slow recovery with levamisole, including the more lipophilic nature of levamisole (Robertson and Martin, 1993), its resistance to degradation by cholinesterase (Eyre, 1970) or its ability to antagonize phosphatases (Schnieden, 1981). However, we did not investigate this phenomenon further.

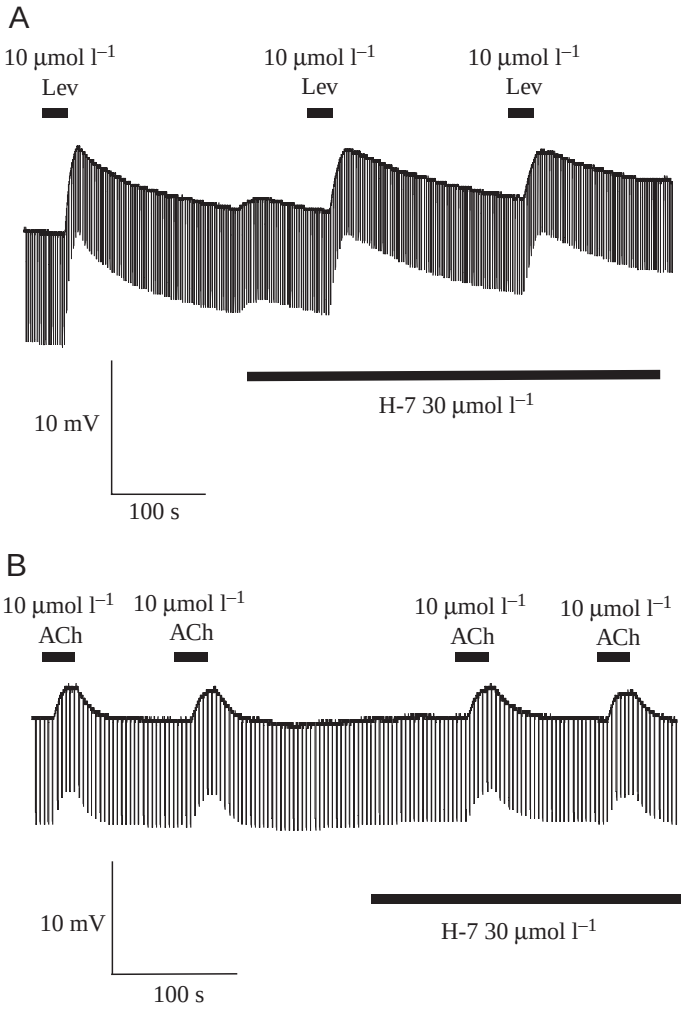


Fig. 2. Effects of H-7. (A) Control levamisole (Lev) response and responses in the presence of 30 μmol $l^{-1}$ H-7. Notice that there is a transient response following the application of H-7 and that the levamisole responses in the presence of H-7 are only a slightly lower amplitude than the control. (B) Effect of H-7 on the membrane potential response to 10 μmol $l^{-1}$ acetylcholine (ACh). Representative recording, control acetylcholine response and responses in the presence of 10 μmol $l^{-1}$ H-7 are shown. Notice that the acetylcholine responses in the presence of H-7 are the same size as the control responses.

### *Effect of H-7 on levamisole and acetylcholine responses*

H-7 is a broad-spectrum serine–threonine kinase inhibitor with activities against the AGC kinase group of PKA, PKC and PKG, and inhibitor constants ($K_i$s) in the range of 0.1–6.0 μmol $l^{-1}$ (Kawamoto and Hidaka, 1984; Boulis and Davis, 1990; Hoffman and Newlands, 1991; Li et al., 1992; Reuhl et al., 1992; Hu and Li, 1997; Ku et al., 1997; Calbiochem 1998 data sheet CN: 371955). We superfused H-7 at a concentration of 30 μmol $l^{-1}$ over the bag region of the muscle cell to determine the effect of H-7 on responses to 10 μmol $l^{-1}$ levamisole and 10 μmol $l^{-1}$ acetylcholine (Figs 2A,B). The mean control peak levamisole response was 7.6±2.0 mV. In the presence of 30 μmol $l^{-1}$ H-7, the mean

Table 1. *Summary of the effects of kinase antagonists on peak membrane potential responses to levamisole and acetylcholine*

| Agonist | Antagonist | *N* | Mean response ± S.E.M. (mV) | Inhibition (%) | *P* (paired *t*-test) |
|---|---|---|---|---|---|
| Levamisole | Control | 5 | 7.6±2.0 | | |
| Levamisole | H-7 | | 6.0±1.0 | 21 | NS |
| Acetylcholine | Control | 5 | 4.1±0.3 | | |
| Acetylcholine | H-7 | | 3.5±0.6 | 15 | NS |
| Levamisole | Control | 6 | 6.8±1.9 | | |
| Levamisole | Staurosporine | | 3.9±1.1 | 43 | 0.0001 |
| Acetylcholine | Control | 7 | 5.4±0.6 | | |
| Acetylcholine | Staurosporine | | 2.8±0.3 | 49 | 0.0016 |
| Levamisole | Control | 8 | 6.2±1.0 | | |
| Levamisole | KN-93 | | 2.7±0.3 | 56 | 0.035 |
| Acetylcholine | Control | 9 | 4.7±0.6 | | |
| Acetylcholine | KN-93 | | 2.0±0.4 | 47 | 0.0004 |
| Levamisole | Control | 8 | 6.4±0.7 | | |
| Levamisole | Genistein | | 3.3±0.3 | 49 | 0.001 |
| Acetylcholine | Control | 6 | 5.6±0.8 | | |
| Acetylcholine | Genistein | | 5.0±0.6 | 11 | 0.03 |

response was 6.0±1.0 mV ($N$=5) or 79% of the control. The mean control peak acetylcholine response was 4.1±0.3 mV and, in the presence of 30 μmol $l^{-1}$ H-7, it was 3.5±0.6 mV ($N$=5) or 85% of the control. H-7 did not induce a statistically significant effect on the response to either compound (Table 1). These observations suggest that PKA, PKC and PKG do not have a modulatory effect on nematode nAChRs.

### *Effect of staurosporine on levamisole and acetylcholine responses*

Staurosporine is a broad-spectrum kinase inhibitor with activities against PKA, PKG, PKC, myosin light chain kinase (MLCK), CaM kinase and tyrosine kinase and $K_i$s in the range of 0.7–70 nmol $l^{-1}$ (Bergstrand et al., 1992; Coultrap et al., 1999; Li et al., 1992; Nishimura and Simpson, 1994; Calbiochem 1999 data sheet CN: 569397). It combines with the ATP-binding site of the kinase enzyme to inhibit its function. We perfused 1 μmol $l^{-1}$ staurosporine over the muscle bag to determine its effect on responses to levamisole and acetylcholine. Fig. 3A shows the inhibitory effect of staurosporine on representative recordings of levamisole responses. Table 1 shows that the mean control peak levamisole depolarization was 6.8±1.9 mV ($N$=6), and, in the presence of 1 μmol $l^{-1}$ staurosporine, the mean peak levamisole response was 3.9±1.1 mV ($N$=6) or 57% of the control. The difference was statistically significant ($P$<0.0001).

Fig. 3B shows the effect of staurosporine on representative recordings of acetylcholine responses. Table 1 shows that, as with levamisole, there was a depressant effect on the acetylcholine responses. The mean control acetylcholine response was 5.4±0.6 mV ($N$=7) and this was reduced in the presence of 1 μmol $l^{-1}$ staurosporine to 2.8±0.3 mV ($N$=7) or to 51% of the control ($P$=0.0016).

As we observed a significant effect with the broad-spectrum kinase antagonist staurosporine, but not with H-7, we can suggest that the difference is due to the effect of staurosporine on a kinase that is not affected by H-7. One of the protein kinase enzymes not affected by H-7, but affected by staurosporine, is CaM kinase II. A selective CaM kinase II antagonist was therefore used to investigate the role of this enzyme.

### *Effect of KN-93 on levamisole and acetylcholine responses*

To follow up the above studies, we tested KN-93, a selective CaM kinase II inhibitor with a $K_i$ of 0.4 μmol $l^{-1}$ (Mamiya et al., 1993; Ishida et al., 1995; Anderson et al., 1998; Ledoux et al., 1999; Nakayama et al., 2001; Calbiochem 2001 data sheet CN: 422708) on levamisole and acetylcholine responses (Fig. 4). 10 μmol $l^{-1}$ KN-93 reduced the control mean peak levamisole response from 6.2±1.0 mV ($N$=8) to 2.7±0.3 mV ($N$=8) or 44% of the control (Table 1; $P$=0.035). An inhibitory effect on the acetylcholine responses was also observed; the control response of 4.7±0.6 mV ($N$=9) was reduced to 2.0±0.4 mV ($N$=9) by 10 μmol $l^{-1}$ KN-93 (Table 1, $P$=0.0004). The effect of KN-93 on levamisole and acetylcholine responses suggests that CaM kinase II is involved in phosphorylating nAChRs on the muscle bag and promoting the opening of the ion-channel receptor.

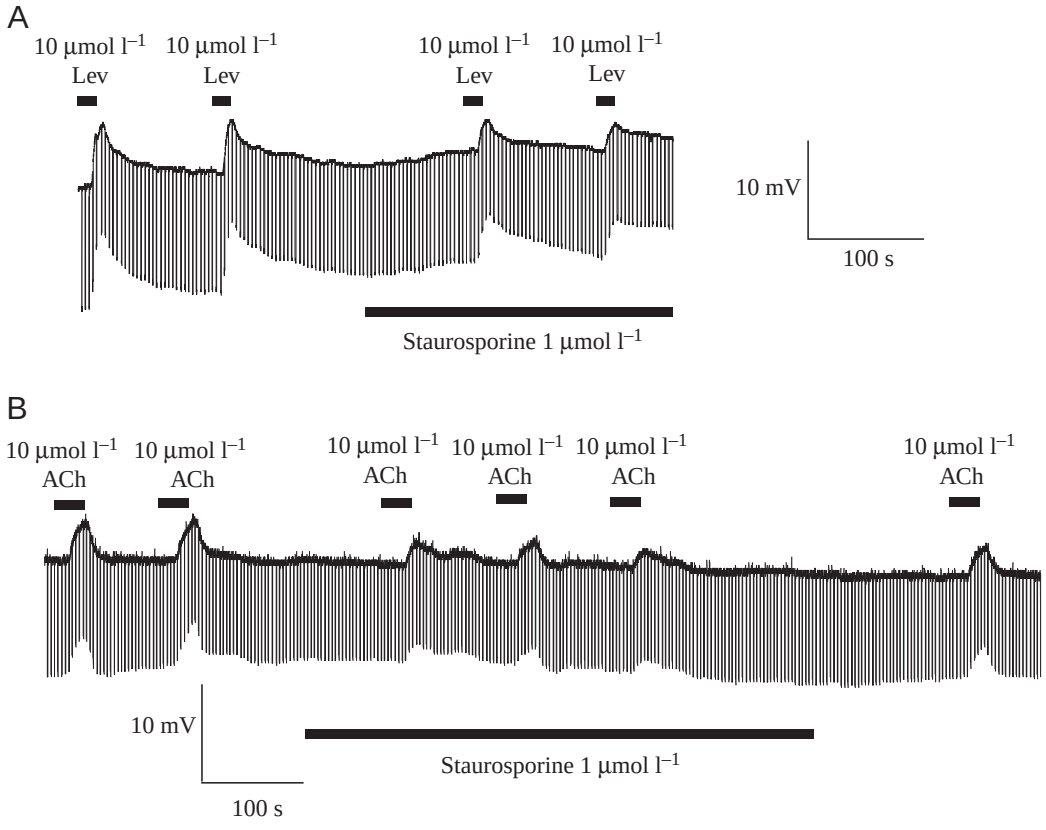


Fig. 3. Effects of staurosporine. (A) Two control levamisole (Lev) responses and two smaller responses in the presence of 1 μmol $l^{-1}$ staurosporine. (B) Control acetylcholine (ACh; 10 μmol $l^{-1}$) response and responses in the presence of 1 μmol $l^{-1}$ staurosporine. Acetylcholine responses in the presence of staurosporine are smaller than the control responses.

*Reversal on washing*

We tried routinely to wash off the effects of the kinase inhibitors, but reversal of the effects of the antagonists was not always apparent. This was probably due to the lipophilic nature of the antagonists and the large size of the bag muscle region. However, we did occasionally observe reversal with staurosporine and KN-93 when we used acetylcholine as an agonist. Fig. 3B illustrates the reversal on washing with acetylcholine as the agonist and staurosporine as the kinase antagonist. Fig. 4B illustrates reversal of the effect of KN-93 on washing: here, 20 min was required to observe definitive recovery towards the control response values.

*Levamisole responses are sensitive to $Ca^{2+}$*

An effect of CaM kinase II in modulating levamisole receptors implies that the response will also be sensitive to $Ca^{2+}$. We tested the effect of levamisole at different concentrations in preparations bathed for longer than 30 min in low-$Ca^{2+}$ APF and in normal $Ca^{2+}$ APF. Elimination of $Ca^{2+}$ from the bathing solution is known to reduce cytoplasmic $Ca^{2+}$ concentrations because low-$Ca^{2+}$ extracellular solutions reduce the opening of Ca-activated Cl-channels (Thorn and Martin, 1987). In normal $Ca^{2+}$ APF, the resting membrane potential was –22.9±3.5 mV and input conductance was 2.63±0.38 μS ($N$=4). These values are similar to those recorded in low-$Ca^{2+}$ APF, where membrane potential was –21.7±0.8 mV and input conductance was 2.16±0.12 μS ($N$=4, $P$=0.11, $t$-test).

Fig. 5 shows the effect of different concentrations of levamisole on a preparation bathed in normal $Ca^{2+}$ APF (Fig. 5A) and one bathed in low-$Ca^{2+}$ APF solution (Fig. 5B). Notice that the responses to levamisole at lower concentrations are bigger in the presence of $Ca^{2+}$. Fig. 5C shows a plot of the mean ($N$=4) peak depolarizations in the presence and absence of $Ca^{2+}$. The best fit to the observed responses in the presence of $Ca^{2+}$ had an $EC_{50}$ of 1.2 μmol $l^{-1}$, with a Hill slope of 1.21 and a maximum response of 7.1 mV. In low-$Ca^{2+}$ APF, the best-fit $EC_{50}$ was 3.1 μmol $l^{-1}$, the Hill slope was 3.9 and the maximum response was 6.8 mV. Note again that the presence of $Ca^{2+}$ is associated with an increase in the size of the response ($P$=0.045, ANOVA).

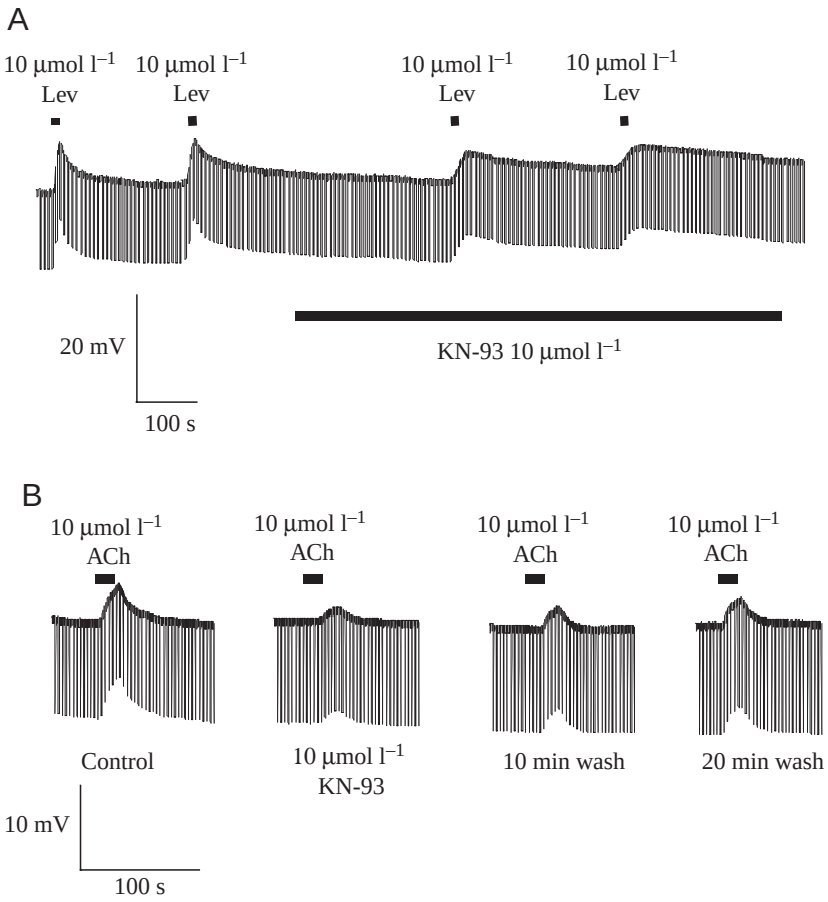


Fig. 4. Effects of KN-93, a selective $Ca^{2+}$/calmodulin kinase II (CaM kinase II) antagonist. (A) Two control responses to 10 μmol $l^{-1}$ levamisole (Lev) and two responses in the presence of 10 μmol $l^{-1}$ KN-93. Notice that the peak responses in the presence of KN-93 are smaller than the control responses. (B) Effect of KN-93 on acetylcholine (ACh) is slowly reversible. The figure illustrates the antagonistic effect of 10 μmol $l^{-1}$ KN-93 on the peak membrane potential responses to 10 μmol $l^{-1}$ acetylcholine. Notice that 20 min was required in this preparation before the response to acetylcholine was clearly returning towards the control level. This may reflect the lipophilic nature of KN-93 and the long time required to wash the drug from the preparation.

*Effect of genistein*

We pointed out earlier that staurosporine, but not H-7, has significant effects on the amplitude of the response to levamisole and acetylcholine. As staurosporine at higher concentrations may also antagonize the effects of tyrosine kinases, and there are consensus tyrosine kinase sites present on the ASAR-1 α-subunit of *A. suum* (Fig. 1), we tested the effects of genistein on the levamisole and acetylcholine response. Genistein competes with ATP non-competitively, as a substrate for tyrosine kinases, to inhibit phosphorylation, with $K_i$s of ≤1 μmol $l^{-1}$ (Huang et al., 1992; Young et al., 1993; Wang et al., 1997; Saxena and Henderson, 1997; Neye and Verspohl, 1998; Calbiochem 2001 data sheet, CN: 345834). We tested the effect of 90 μmol $l^{-1}$ genistein on the levamisole and acetylcholine responses (Fig. 6).

Genistein reduced the amplitude of the levamisole response (Fig. 6). Table 1 shows that the mean control peak levamisole response was 6.4±0.7 mV ($N$=8), and the mean response in the presence of 90 μmol $l^{-1}$ genistein was 3.3±0.3 mV ($N$=8) or 51% of the control ($P$=0.001).

Fig. 6B shows a representative trace of the effect of

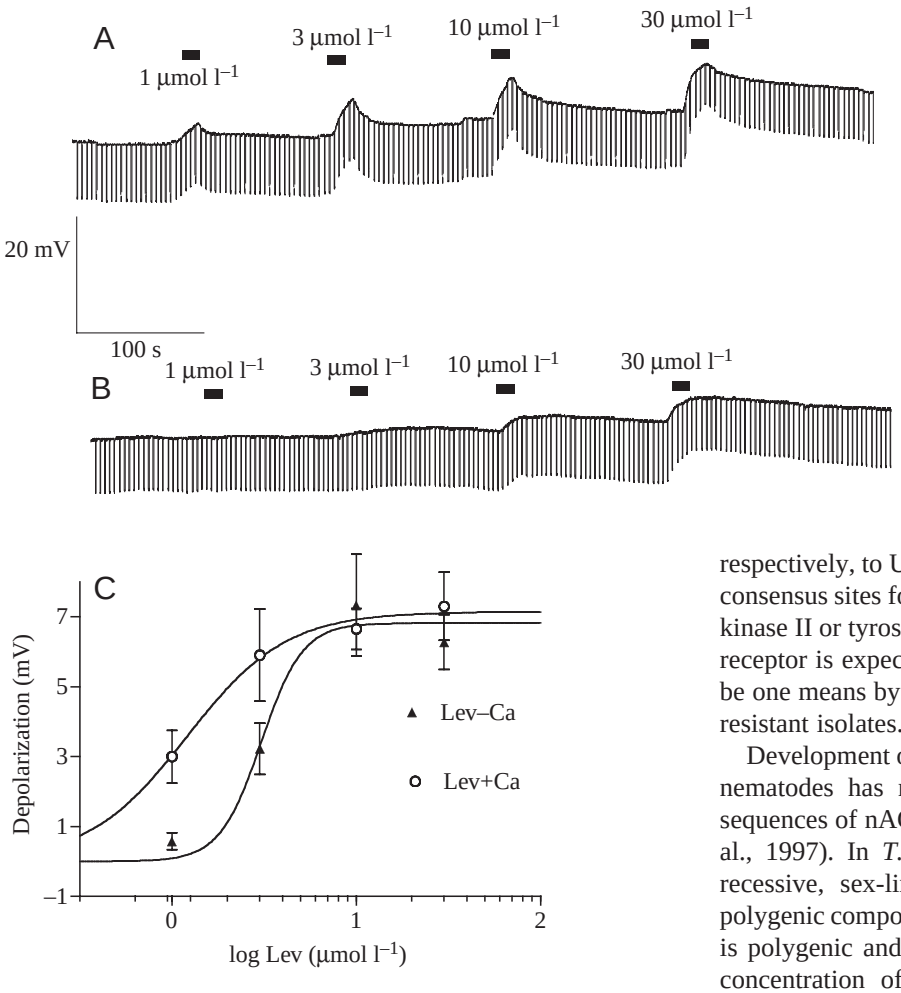


Fig. 5. Potentiating effect of $Ca^{2+}$ on the levamisole concentration–response relationship. (A) Effect of different concentrations of levamisole applied in *Ascaris* peri-enteric fluid (APF) bathing solution (6 mmol $l^{-1}$ $Ca^{2+}$). (B) Effect of different concentrations of levamisole in low-$Ca^{2+}$ APF ($Ca^{2+}$ replaced with $Mg^{2+}$). (C) Concentration–response plots of the effect of levamisole peak membrane potential responses in normal APF (Lev+Ca) and low-Ca APF (Lev–Ca). Best fit to response plot in the presence of $Ca^{2+}$ in normal APF had an $EC_{50}$ of 1.2 μmol $l^{-1}$, a Hill slope of 1.21 and a maximum response of 7.1 mV. In low-$Ca^{2+}$, the best-fit $EC_{50}$ was 3.1 μmol $l^{-1}$, the Hill slope was 3.9 and the maximum response was 6.8 mV. Note that the presence of $Ca^{2+}$ is associated with an increase in the size of the response. The effect of $Ca^{2+}$ on the response was statistically significant ($P$=0.045, analysis of variance).

90 μmol $l^{-1}$ genistein on the response to acetylcholine. The effect was small (Table 1), with the mean control acetylcholine peak response reaching 5.6±0.8 mV ($N$=6) and the acetylcholine response in the presence of 90 μmol $l^{-1}$ genistein reaching 5.0±0.6 mV ($N$=6) or 89% of the control. Although the change in response was small, it did reach statistical significance ($P$=0.03). The effect of genistein on the levamisole and acetylcholine responses suggests that tyrosine kinase is involved in maintaining the nematode nicotinic receptor.

## Discussion

### *Phosphorylation of nematode nAChRs and levamisole resistance*

*Caenorhabditis elegans* is a soil nematode that has been subjected to detailed genetic analysis. Genes encoding nAChR subunits in *C. elegans* have been described (Fleming et al., 1997), including *unc-38*, *unc-63*, *unc-29* and *lev-1*. Both *unc-38* and *unc-63* encode α-like subunits, while *unc-29* and *lev-1* encode non-α-subunits. Homologues in *Ascaris suum* (ASAR-1, GenBank Accession No. AJ011382), *Trichostrongylus colubriformis* (Wiley et al., 1996) and *Haemonchus contortus* (Hoekstra et al., 1997) have 78%, 84% and 83% identity, respectively, to UNC-38. All of these nAChR subunits possess consensus sites for phosphorylation by PKA, PKC, PKG, CaM kinase II or tyrosine kinase. Phosphorylation of the levamisole receptor is expected to alter the activity of receptors and may be one means by which sensitivity to levamisole is affected in resistant isolates.

Development of resistance to levamisole therapy in parasitic nematodes has not been linked to changes in amino acid sequences of nAChR subunits (Wiley et al., 1997; Hoekstra et al., 1997). In *T. colubriformis*, levamisole resistance has a recessive, sex-linked single gene component and a small polygenic component on autosomes. In *H. contortus*, resistance is polygenic and associated with a 4–25-fold increase in the concentration of levamisole required to produce paralysis (Sangster et al., 1991, 1998).

### *Role for CaM kinase II and tyrosine kinase in modulation of the nAChRs*

In the present study, we examined the effects of selected kinase antagonists to test the hypothesis that phosphorylation of nAChRs is involved in regulating the response to levamisole. We observed no significant effects on levamisole and acetylcholine responses with H-7, an antagonist of PKA, PKC and PKG (Kawamoto and Hidaka, 1984; Boulis and Davis, 1990; Hoffman and Newlands, 1991; Li et al., 1992; Reuhl et al., 1992; Hu and Li, 1997; Ku et al., 1997). The broad-spectrum antagonist staurosporine, which inhibits PKA, PKC, PKG, CaM kinase and tyrosine kinase (Bergstrand et al., 1992; Coultrap et al., 1999; Li et al., 1992; Nishimura and Simpson, 1994), reduced the response to both acetylcholine and levamisole in *A. suum* muscle. Reductions in response were also recorded in the presence of KN-93, a selective CaM kinase II antagonist (Mamiya et al., 1993; Ishida et al., 1995; Anderson et al., 1998; Ledoux et al., 1999; Nakayama et al., 2001), and genistein, a selective tyrosine kinase antagonist (Huang et al., 1992; Young et al., 1993; Wang et al., 1997; Saxena and Henderson, 1997; Neye and Verspohl, 1998). These effects suggest the involvement of CaM kinase II and tyrosine kinase in supporting the opening of the ion channels.

The lack of effect of H-7, a protein kinase antagonist that is not active on CaM kinase II or tyrosine kinase, and the antagonistic effects of staurosporine, a broad-spectrum protein kinase antagonist, support this interpretation. The potentiating effects of $Ca^{2+}$ on the levamisole responses may be explained by the involvement of CaM kinase II. Observations with H-7 and staurosporine, which depress muscle contraction to acetylcholine in the filarid *Acanthocheilonema viteae* (Minardi et al., 1995), are consistent with the view that CaM kinase II regulates the response to acetylcholine in parasitic nematodes.

Trim et al. (1999) have observed inhibitory effects of tamoxifen on the depolarizing responses to levamisole and acetylcholine and suggested that a PKC-dependent phosphorylation of *A. suum* nAChRs is necessary to maintain the sensitivity of the receptor to acetylcholine. In this model, blockade of PKC results in inhibition and reduction of the response. However, Trim et al. (1999) also tested the selective PKC antagonist chelerythrine and did not observe an effect on the membrane-potential responses to acetylcholine. In our experiments, we did not observe a significant effect with H-7, another kinase antagonist that has inhibitory effects on PKC. As tamoxifen also has an inhibitory effect on calmodulin (Edwards, 2002), we can explain the inhibitory effects of tamoxifen by inhibition of calmodulin and depression of the activity of CaM kinase II. A role for CaM kinase II in regulating nematode body wall muscle activity has been demonstrated in *C. elegans*, where a single gene, *unc-43*, codes for CaM kinase II (Reiner et al., 1999). The *unc-43* gene was also shown to interact with *unc-103*, a gene encoding a voltage-gated $K^+$ channel, so a clear connection between phosphorylation of ion channels, muscle excitability and CaM kinase II in nematodes has been demonstrated. CaM kinase II may mediate cell excitability through modulation of channel activity or modeling of the synapse, as demonstrated in the vertebrate central nervous system (Rongo and Kaplan, 1999). We do not rule out the possibility of PKC involvement, but our evidence with H-7 suggests that, over the time-span of the experiment (approximately 3 h), it is less important than CaM kinase II. Over a longer time span (12–24 h), where receptor synthesis can play a role, evidence from *C. elegans* does indicate a role for PKC (*tpa-1*) in long-term adaptation of nAChRs (Waggoner et al., 2000). In mammalian muscle, PKC and CaM kinase II can regulate nAChR expression, but it is CaM kinase II rather than PKC that is required for $Ca^{2+}$ or activity-dependent control of nAChR gene expression (Macpherson et al., 2002).

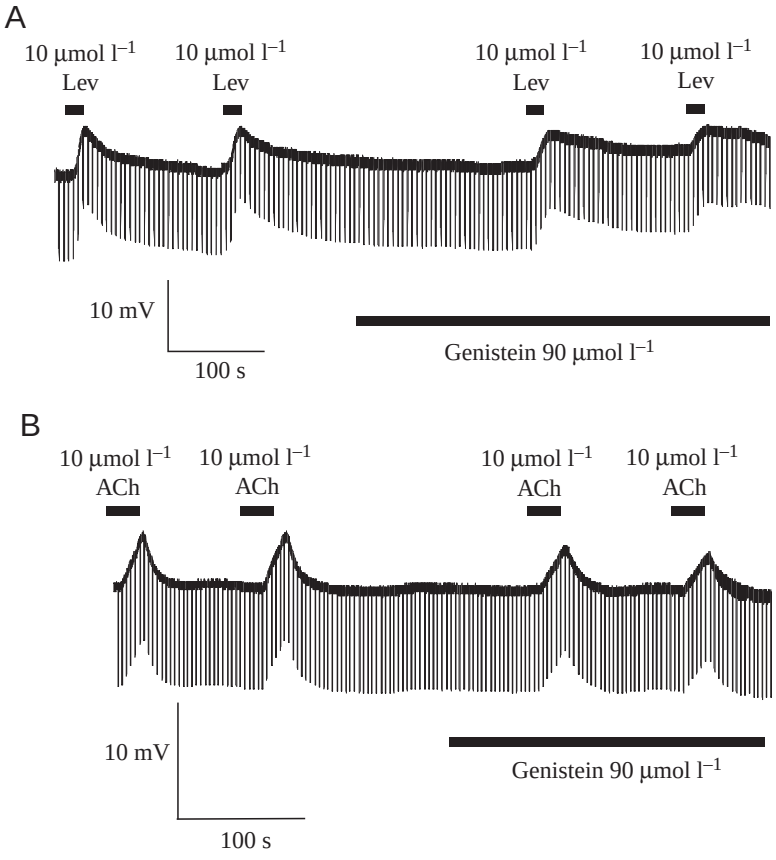


Fig. 6. Effects of genistein, a selective tyrosine kinase antagonist. (A) Two control responses to 10 μmol $l^{-1}$ levamisole (Lev) and two responses in the presence of 90 μmol $l^{-1}$ genistein. Notice that the responses in the presence of genistein are smaller than the control responses. (B) Two control responses to 10 μmol $l^{-1}$ acetylcholine (ACh) and two responses in the presence of 90 μmol $l^{-1}$ genistein. The effect is a small, statistically significant reduction in the size of the peak (see Table 1).

In summary, our studies indicate that CaM kinase II and tyrosine kinases are involved in supporting the opening of acetylcholine/levamisole receptors on *A. suum* somatic muscle. In previous publications, we have observed differences in opening of nAChRs in SENS and LEV-R isolates of the parasitic nematode *Oesophagostomum dentatum* and hypothesized that phosphorylation may contribute to the drug-resistant phenotype by modulating the opening of levamisole receptors. In the present study, we have used selective kinase antagonists to test the importance of the kinases and phosphorylation process in modulating the receptors. These observations demonstrate that the responses to acetylcholine and the anthelmintic levamisole are not fixed but vary, and this plasticity depends on the phosphorylation state of the nAChRs. The observations emphasize the possibility that resistance to the anthelmintic levamisole does not necessarily involve amino acid changes at the levamisole-binding site but may involve regulation of the activity to the levamisole receptor *via* changes in its phosphorylation state.

The mechanisms used by nematodes to modulate their responses to acetylcholine and nicotinic anthelmintics is worthy of further study; it seems increasingly likely that modulation of the receptors may contribute to at least some of the observed drug resistance. Further study necessitates the use of *Ascaris* because it is the only readily available nematode parasite suitable for electrophysiological study. To date, there are no anthelmintic-resistant

isolates of *Ascaris*, but information derived from *Ascaris* will inform work on parasitic nematodes where anthelmintic isolates are available.

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