Metabotropic Glutamate Receptor Antagonists Selectively Enhance Responses of Slowly Adapting Type I Mechanoreceptors

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ABSTRACT There is evidence that glutamate may participate as a transmitter at the junction between Merkel cells and the nerve terminals of slowly adapting type I (St I) units. We recorded extracellularly from the deep vibrissal nerve of an isolated rat vibrissa preparation in vitro. Five second trapezoid stimulus ramp deflections of the hair shaft were used to evoke responses. We bath-applied two compounds, which we planned would interfere with glutamatergic transmission. (2*S*)-2-Amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495) was used at concentrations up to 100 μM to block all known metabotropic glutamate (mGlu) receptors. The racemic mixture (*RS*)-4-carboxy-3-hydroxyphenylglycine ((*RS*)-4C3HPG) was used up to 100 μM to block ionotropic and Group I metabotropic glutamate receptors, and as an agonist at Group II mGlu receptors. Unexpectedly, both compounds had rapid onset excitatory effects on mechanically-evoked responses. (*RS*)-4C3HPG increased responses, with a mean 146% of control ($P < 0.05$) in a concentration-dependent manner. LY341495 increased responses, with a mean 128% of control ($P < 0.05$). With (*RS*)-4C3HPG in particular, it was noted that the static component (the firing during the last 1 s plateau) was preferentially enhanced relative to the dynamic component (firing during the first 0.5 s). Rapid recovery was seen after wash. Slowly adapting type II units, which have no junctional transmission, were completely unaffected by these compounds up to 200 μM. These results suggest that mGlu receptors play a role in Merkel cell-neurite complex mechanotransduction, although other explanations are considered. **Synapse 59:235–242, 2006.** © 2005 Wiley-Liss, Inc.

INTRODUCTION

There are two types of slowly adapting mechanoreceptors in the skin of vertebrates: type I and type II (Iggo, 1968). Slowly adapting type I units are largely responsible for the detailed spatial resolution, as required for the detection of edges during tactile exploration, and their nerve fibers densely innervate the glabrous skin of the hand (Vallbo and Johansson, 1984). It is generally believed that type I nerve endings terminate against Merkel cells in the basal layer of the epidermis. This intimate contact was conclusively shown in the touch domes of cat and monkey hairy skin, where morphology and physiology were directly correlated (Iggo and Muir, 1969). The same is believed to be true in other animals and other tissues (e.g., the rat sinus hair follicle), although final proof has yet to be obtained. Merkel cells are particularly abundant in the glabrous skin of human finger tips and in rodent sinus hair follicles. Merkel cells are almost spherical measuring 10–15 μm diameter, and exhibit spike-like protrusions, which interdigitate

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with adjacent keratinocytes. Within the cytoplasm of each Merkel cell is present dense core granule vesicles, which cluster near synapse-like junctions made with a single expanded nerve terminal (Iggo and Muir, 1969). The particular role of Merkel cells in the function of type I nerve endings has been disputed for almost 25 years, and has been adequately reviewed elsewhere (Halata et al., 2003; Ogawa, 1996; Tachibana, 1995).

The present study stems from work intended to test whether glutamate was the excitatory transmitter used by Merkel cells to signal to nerve terminals. Earlier work showed that the broad spectrum ionotropic glutamate receptor antagonist, kynurenate, was able to selectively reduce mechanically evoked responses from sinus hair slowly adapting type I (St I) units (Fagan and Cahusac, 2001). Sinus hair slowly adapting type II (St II) units (which do not exhibit synapse-like junctions with epidermal cells) were unaffected by the antagonist. Histological work had shown the presence of NR2A/B subtype NMDA receptors (Senok et al., 2003), as well as metabotropic glutamate (mGlu) receptors on Merkel cells (Tachibana and Nawa, 2003). Further pharmacological experiments revealed that only certain NMDA receptor antagonists were effective in blocking responses of St I units (Cahusac et al., 2005).

In the present study, we used two compounds that we believed would further elucidate glutamate's role in excitatory synaptic transmission. The broad spectrum mGlu receptor antagonist LY341495 is reputed to block all known mGlu receptor types (Schoepp et al., 1999; Wright et al., 2000). If mGlu receptors were actively involved in mediating mechanosensory responses from Merkel cells, or if release of glutamate was regulated by these receptors (Schoepp, 2001) on Merkel cells, then blockade would have predictable effects on evoked responses. The other drug, (*RS*)-4C3HPG, is from a class of phenylglycine derivatives, compounds that have proved useful in the study of glutamatergic transmission (Watkins and Collingridge, 1994). (*RS*)-4C3HPG is a racemic mixture of *R*- and *S*- isomers. The *R*-isomer acts as a selective and potent NMDA receptor antagonist, as well as a weaker AMPA/kainate receptor antagonist (Birse et al., 1993). If ionotropic glutamate receptors were involved in mediating transmission, then this isomer should depress responses. The *S*-isomer was believed to be a potent Group I mGlu receptor antagonist as well as an effective Group II mGlu receptor agonist (Schoepp et al., 1999), so that excitatory transmission mediated by Group I mGlu receptors would be blocked as well as preventing the release of glutamate through an action at presynaptically located Group II mGlu receptors (Schoepp, 2001). Thus, by using the racemic mixture, we hoped to effectively block all types of excitatory glutamatergic binding or transmission at this junction. In fact our results were unexpected but interesting, and indicate further complexities in the generation of mechanotransduction at Merkel cell-neurite complexes. The results may also indicate that these compounds, which are routinely used to study glutamatergic transmission, may have other as yet uncharacterized actions at central and peripheral synapses.

METHODS

The University of Stirling Department of Psychology's Ethics Committee approved the use of animals for this study. All experimental procedures followed UK Home Office regulations as well as the European Directive 86/609/EEC, and consistent with the Declaration of Helsinki and the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. The minimum number of animals necessary to demonstrate scientifically important effects was used.

Recordings were made from single axons of the deep vibrissal nerve, using an isolated vibrissa preparation as previously described (Baumann et al., 1996). Thirteen adult male Wistar rats were used. Each animal was deeply anesthetized with urethane (25% w/v, 4 ml/kg, i.p.) and then sacrificed with a further 2 ml/kg by intracardiac administration. Whisker pads were quickly removed and placed in synthetic interstitial fluid (SIF (Bretag, 1969)) bubbled with medical 95% O_2/5% CO_2 gas, which was maintained at a pH of 7.4. Individual sinus hairs were microdissected out from whisker pads with the distal end of the deep vibrissal nerve attached. While immersed in SIF, the capsule of the hair bulb was slit open longitudinally to release blood from the sinus, and to allow easy access of drugs to the mechanoreceptors inside. The sinus hair was then mounted on a Silguard platform in a custom-made organ bath (kindly provided by Professor K.I. Baumann, Hamburg). Insect pins held the sinus capsule in place, with the shaft of the hair trimmed to 10 mm exposed length and accessible to mechanical stimulation. The nerve bundle was stripped of its outer sheath and fine strands of nerve attached to a silver recording wire situated in a layer of Fluorinert (FC-40, Sigma, Poole, UK) at the bottom of the bath. An indifferent electrode was situated in the SIF floating above. Differential recordings were used with the signal amplified (Neurolog, Digitimer, UK) and monitored visually on oscilloscopes, and aurally on a loud speaker. Pulse output from a spike window discriminator (WPI) went to a laboratory interface (1401+, CED, Cambridge, UK) and computer. The analog neural signal was also fed to the computer via the interface, which allowed multi-spike discrimination from spike shapes. Bath temperature maintained at $31 \pm 1°C$ was recorded via the interface. All inputs and outputs were managed by a custom written SPIKE2 software script (CED).

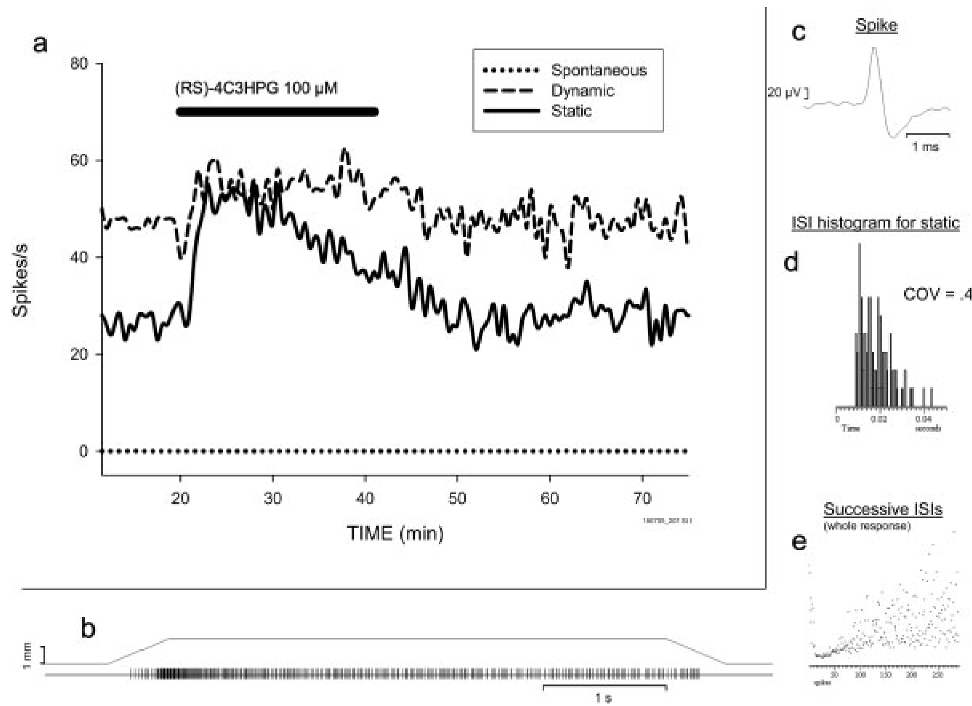


Fig. 1. The excitatory effect of (*RS*)-4C3HPG on the responses of a single St I unit. **a:** The application of 100 µM (*RS*)-4C3HPG shown by the bar increased the responses to both static (continuous line) and dynamic (dashed line) components. There was a particularly marked effect on the static component. There was no spontaneous firing of this unit. Some desensitization of the static component enhancement occurred during the drug application. **b:** Shows a sample response of this unit (spikes are shown as vertical lines) to the stimulus ramp (shown earlier). Note the irregular firing during the last 1 s of the plateau (marked by the calibration bar, representing the static component). **c:** A sample extracellularly recorded spike of this unit. **d:** A sample interspike interval (ISI) histogram for the static component (the COV is indicated, and is a measure of ISI variability-see text). Note the positively skewed distribution. **e:** A sample plot of successive ISIs shown in b plotted in their order of arrival. This plot proved to be most useful in discriminating between St I and St II units on-line.

Responsiveness of units to mechanical stimuli was informally tested by lateral displacements of the hair shaft, using fine forceps. Once an appropriate response was obtained, the probe of a feedback-controlled mechanical transducer was attached to the hair shaft 5–10 mm from the capsule. Stimulus parameters that evoked ~75% maximal response using displacements of 0.2–1.5 mm were used. In some experiments, pushing the shaft tip towards the capsule was also used. Each stimulus consisted of a 5-s trapezoid ramp, 0.5-s onset, 4-s plateau, and 0.5-s offset. Stimulus ramps were repeated at 30-s intervals.

St I units were characterized by their irregular firing during the static phase of the response. In contrast, St II units were highly regular. These differences were quantified on-line using the coefficient of variation (COV) for the interspike intervals during the 1 s static phase during the last second of the plateau stimulus phase. Specifically, a COV > 0.1 was considered to represent activity from a St I unit, while a COV < 0.1 was considered to represent activity from a St II unit. The mean COV of 12 St I units was 0.48 ± 14 (±SD), while the mean COV for 6 St II units was 0.068 ± 012. Plots of interspike intervals against successive spikes (Iggo and Muir, 1969) were done on-line and proved to be a useful indicator of the type of unit (see Figures 1e and 4e). It was also possible to confirm their identity by their characteristic response to 5–10 mM caffeine (Senok and Baumann, 1997) and to 3–10 mM tetraethylammonium chloride (TEA) (Senok et al., 2001).

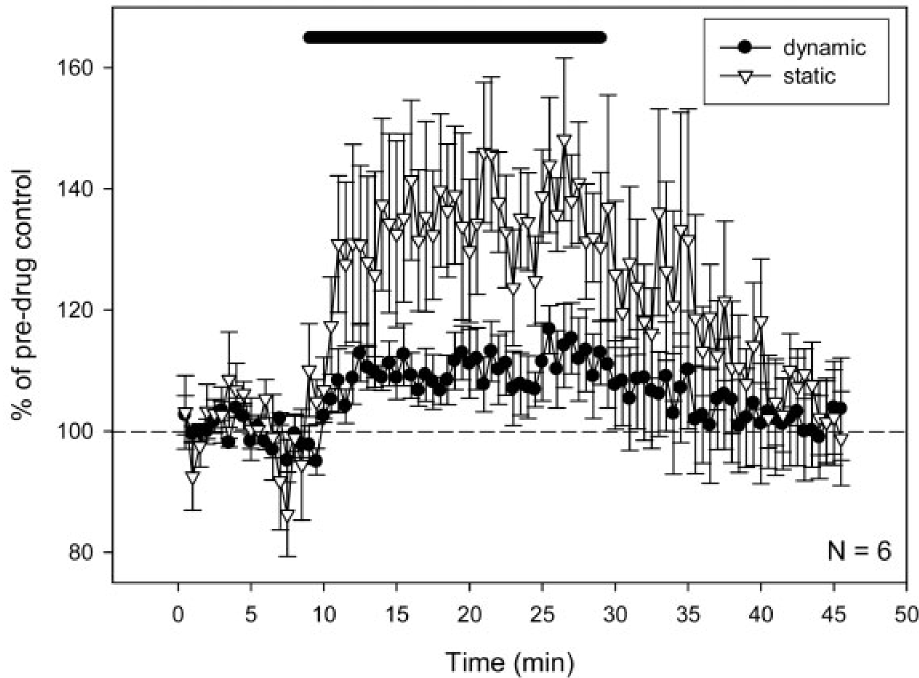
Caffeine and TEA were obtained from Sigma (Poole, UK). (2*S*)-2-Amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495) and (*RS*)-4-carboxy-3-hydroxyphenylglycine ((*RS*)-4C3HPG) were obtained from Tocris (Bristol, UK). Drugs were dissolved in 20 ml of SIF at pH 7.4 and were introduced into the bath at a rate of 1 ml/min.

Discriminated spikes were counted in the dynamic phase of the response during the first 0.5 s, the static phase of the response during the last 1 s of the plateau, and the spontaneous rate during the 25 s interstimulus interval. Data were stored in spreadsheets, and *t*-tests were used to determine statistical significance.

RESULTS

Effects of (*RS*)-4C3HPG on St I Units

The responses of St I units were enhanced by (*RS*)-4C3HPG applied between 10 and 100 µM, see exam-



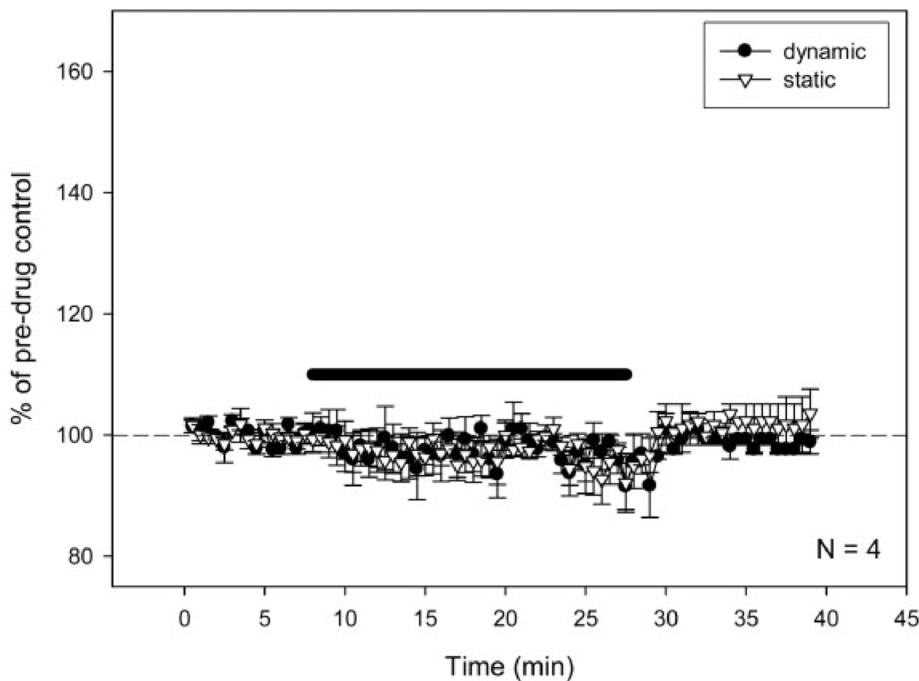


Fig. 2. The selective excitatory effect of (*RS*)-4C3HPG on the responses of St I units. **a:** Shows grouped data from 6 St I units using 30–100 μM (*RS*)-4C3HPG. Dynamic (filled circles) and static (triangles) components were rapidly increased after application of the drug. **b:** Shows grouped data from 4 St II units, using 100–200 μM (*RS*)-4C3HPG. No effect on response components was seen. Error bars represent standard errors of the mean.

ple of one such experiment in Figure 1. The drug effects had an unusually short latency of onset (within 2 min from the start of drug application). The average time-course of the action of the drug can be seen in the averaged plot in Figure 2a for 6 units. The offset was also very fast, with a mean recovery time of 11 min (range 5–30 min). The rapid offset may in part be due to the desensitization apparent at higher concentrations (100 μM), which would mask the true duration of the effect. In Figure 1, the desensitization was particularly evident for the static component, where there was a fall-off of responses half

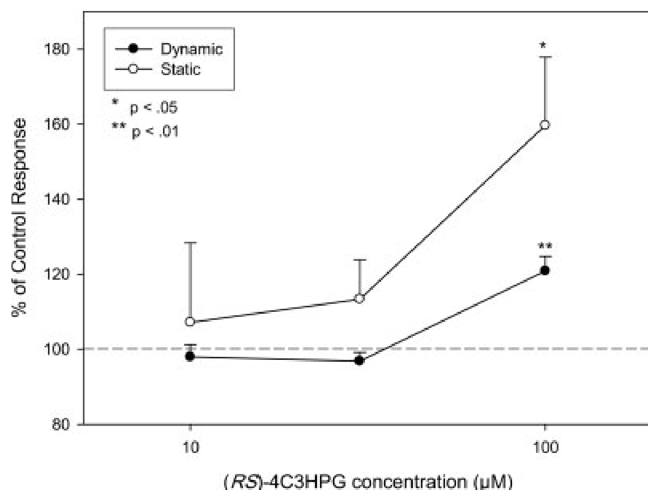


Fig. 3. Dose-response curve for the effect of (*RS*)-4C3HPG on St I units. Percentage of control response was plotted against the concentration of (*RS*)-4C3HPG (plotted logarithmically). Separate lines are shown for static (circles) and dynamic (filled circles) components. Even at lower doses (10–30 μM), enhanced effects on the static component were evident. At 100 μM, both components were significantly enhanced. Error bars represent standard errors of the mean.

way through the 20 min drug administration. When spontaneous activity was present, it was often but not always, enhanced as well.

A particularly notable feature of the effect of (*RS*)-4C3HPG was that the static component was preferentially enhanced relative to the dynamic component. This was particularly apparent in the single experiment shown in Figure 1a, but is also clear in Figure 2a, Figure 3, and the summary Figure 5. However, this apparent difference was marginally nonsignificant for the summary data shown in Figure 5 ($t(6) = 2.33$, $P = 0.058$).

A dose-response plot is shown in Figure 3. There is evidence for some effect on the static component at 10 and 30 μM, although these effects were not statistically significant. At 100 μM, the enhancement was very clear and was statistically significant ($t(4) = 3.28$, $P = 0.03$). The static component was characterized by high variability compared with the dynamic component. The latter (dynamic) component at 100 μM, even though of lower amplitude than the static, was more significantly different from control ($t(4) = 5.40$, $P = 0.006$).

Effects of LY341495 on St I Units

Responses of St I units were also enhanced by LY341495 applied at 10–100 μM. However, the effects were more variable and less clear than the effects seen with (*RS*)-4C3HPG. They showed a similar short latency of onset and offset. Unlike the effects seen with (*RS*)-4C3HPG, there was no difference between static and dynamic components, see summary Figure 5, although there was an indication that there was a difference, since the static component alone was significantly different from control ($t(6) = 2.79$, $P = 0.032$).

Effects of (*RS*)-4C3HPG and LY341495 on St II Units

Both drugs were tested over 10–200 μM range of concentrations. No consistent effects were seen. A typical example from one experiment is shown in Figure 4 using 200 μM (*RS*)-4C3HPG. An averaged plot for (*RS*)-4C3HPG for all concentrations used is shown in Figure 2b. In the summary Figure 5, the data for St II units are shown for both drugs combined (since there were no effects nor differences between the drugs and various concentrations used). The overall mean % of control for the static and dynamic components was close to 100%.

DISCUSSION

The results clearly show that these mGlu receptor antagonists (*RS*)-4C3HPG and LY341495 selectively enhance the evoked responses of St I units. There were no effects of these drugs on St II units, even up to 200 μM concentrations. (*RS*)-4C3HPG gave the clearest effects, and may be the more potent drug, since it is likely that the effect was due to the action of only one of the isomers present in the racemic mixture. The enhanced responses had a remarkably rapid onset (within 2 min), and a quick recovery back to control levels of response (mean 11 min). Such rapid onset and offset times have so far only been seen with 10 mM caffeine (Senok and Baumann, 1997), and contrast with (for example) the depressant effect of 100 μM MK801, which had an onset time of 8 min and took more than 2 h to recover (Cahusac et al., 2005). The enhancement seen in the present study was most apparent in the static phase of the response. A "two-receptor-sites" hypothesis proposed, from several lines of evidence, that the nerve endings largely mediate the dynamic response, whereas the Merkel cells largely mediate the static response (Ogawa, 1996;Yamashita and Ogawa, 1991). The current results where the static component showed the greatest enhancement, particularly with (*RS*)-4C3HPG (see Figures 1–3), provide indirect evidence that the excitability of Merkel cells was specifically involved.

To determine which mGlu receptors may be involved, we will need to review what is known about the two mGlu receptor antagonists.

LY341495 is a broad spectrum mGlu receptor antagonist, which at 100 μM is able to block all known mGlu subtypes tested (Johnson et al., 1999; Kingston et al., 1995; Wright et al., 2000). The excitatory effect of LY341495 in this study clearly suggests that this action is via blockade of one or more mGlu receptor types, although an action at quite different receptors remains a possibility.

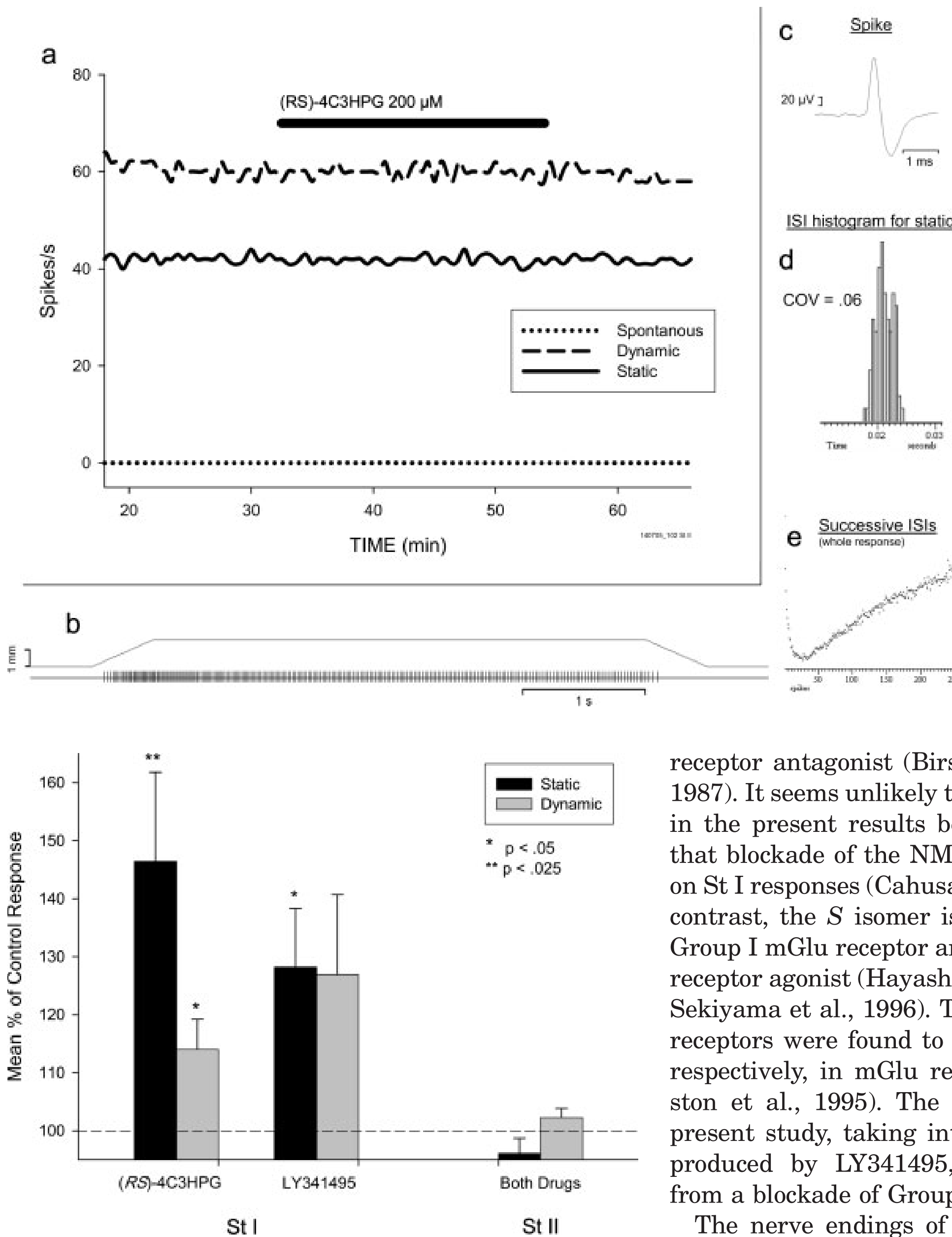


Fig. 4. Lack of effect of (*RS*)-4C3HPG on a single St II unit. Parts of the figure are as already described in Figure 1. **a:** Drug application at 200 μM had no effect on dynamic, static, or spontaneous activity. **b:** Shows spikes in relation to the stimulus ramp. Note the regularity of firing during the static phase (corroborated in **d:** by the symmetrical normal-like distribution) and during the whole response (corroborated in **e:** by the narrow distribution of points).

Fig. 5. Summary of the drug effects on the two response components. The two pairs of bars on the left represents data for St I units, while the single pair of bars at the right represent data for St II units. The effect of (*RS*)-4C3HPG 10–100 μM on St I units is shown by the left pair of bars. The static (black fill) and dynamic (gray fill) components were significantly enhanced, although the static appeared more enhanced than the dynamic; this difference did not reach statistical significance, $P = 0.058$. The effect of LY341495 (10–100 μM) on St I units is shown in the next pair of bars. Both static and dynamic components were significantly enhanced, although only the static component was statistically significant. The data for both drugs (*RS*)-4C3HPG and LY341495 (10–200 μM) on St II units were combined and plotted in the pair of bars on the right. No effect of these drugs was apparent. Error bars represent standard errors of the mean.

(*RS*)-4C3HPG is a racemic mixture of the *R* and *S* isomers. The *R* isomer is reported to be a potent NMDA receptor antagonist and a weaker non-NMDA receptor antagonist (Birse et al., 1993; Watkins et al., 1987). It seems unlikely that this isomer played any role in the present results because other work has shown that blockade of the NMDA receptor site had no effect on St I responses (Cahusac et al., 2005), and see later. In contrast, the *S* isomer is generally considered to be a Group I mGlu receptor antagonist and a Group II mGlu receptor agonist (Hayashi et al., 1994; Nishi et al., 2000; Sekiyama et al., 1996). The IC_{50} for mGlu1 and mGlu5 receptors were found to be 19–50 μM and 53–280 μM, respectively, in mGlu receptor-transfected cells (Kingston et al., 1995). The excitatory effects seen in the present study, taking into account the similar effects produced by LY341495, suggest that they resulted from a blockade of Group I mGlu receptors.

The nerve endings of St I units make synapse-like contacts with Merkel cells (Iggo and Muir, 1969). Although there may be chemical transmission from Merkel cell to nerve terminals, it is now believed that the fast transmitter is unlikely to be glutamate for 2 related reasons: (1) blockade of ionotropic glutamate receptors with classical NMDA and non-NMDA receptor antagonists (e.g., D-AP5, R-CPP, CNQX, and NBQX) had no effect; and (2) while blockade with specific NMDA receptor antagonists that act at the polyamine or ion channel site does depress St I firing, the labeling for the receptor subunit likely to be responsible for this depressant effect (NR2A/B) was only found on Merkel cells and not on the nerve terminals (Cahusac et al., 2005).

Since Merkel cells show a positive immunoreaction to mGlu5 but not mGlu1 receptors (Tachibana et al.,

2001), it is tempting to interpret the effects of our two drugs (LY341495 and (*RS*)-4C3HPG) as a selective effect on this subtype of Group I receptors. Group I mGlu receptors are coupled to a Gq protein and stimulate phospholipase C (PLC) (Fagni et al., 2004) leading to the production of inositol-(1,4,5)-triphosphate (IP3) (Berridge, 1993), which in turn act on IP3 receptors to mobilize calcium from intracellular stores. Indeed, Tachibana et al. in a series of studies have immunolocalized each of the necessary protein substrates in this pathway in Merkel cells, namely Gαq protein, PLC-β1 isozyme, and IP3R-I and IP3R-II receptors (Tachibana et al., 2001, 2003; Tachibana and Nawa, 2003). None of these substrates were found in the Merkel-associated nerve terminals, although Gαo, Gαi, PLC-β2, and PLC-β4 were found there instead. If selective blockade of mGlu5 receptors is responsible for the enhanced responses seen in our study, this suggests that there is tonic activation of these receptors by glutamate and, unusually, that they are negatively coupled with PLC. Their tonic activation would depress release of neurotransmitter at the Merkel cell synapse with nerve terminals, and so blockade by the receptor antagonists would lead to an increase in neurotransmitter release. It is unclear where the glutamate acting on these receptors originates, and one or more could provide a source. (1) Release from the Merkel cells themselves (and acts on mGlu5 autoreceptors), since they contain all the necessary machinery for glutamate release such as glutamatergic vesicle-loading proteins VGLUT1–3 and vesicle recycling proteins (Haeberle et al., 2004; Hitchcock et al., 2004; Nunzi et al., 2004). (2) There may be reciprocal synapses with the nerve endings, since the latter contain small clear vesicles (Mihara et al., 1979) and also express VGLUT1–3 (Nunzi et al., 2004). (3) Glutamate may be released from neighboring basal keratinocytes (Genever et al., 1999; Skerry and Genever, 2001).

There are other possibilities suggested by the variety receptor actions, some contradictory, reported for (*S*)-4C3HPG. (*S*)-4C3HPG has instead been found to be a Group I mGlu agonist in neonatal rat cerebral cortical slices (approximate EC_{50} of 345 μM) (Birse et al., 1993; Eaton et al., 1993), and in neonatal brain slices (approximate EC_{50} of 250 μM), which could be blocked by the Group I antagonist (*S*)-4CPG (Sacaan et al., 1998). There have also been reports that (*S*)-4C3HPG acts at Group III receptors. It was reported to act as an agonist at mGlu6 receptors on retinal ON bipolar cells (IC_{50} of 399 μM) (Thoreson et al., 1995), and as a potent partial agonist at mGlu8 receptors in transfected cells (EC_{50} of 126 μM) (De Colle et al., 2000). All these claimed agonist actions would appear to be inconsistent with our results obtained with LY341495, a universally agreed broad spectrum mGlu receptor antagonist (Schoepp et al., 1999). (*S*)-4C3HPG has been reported to inhibit kynurenate synthesis, where 200 μM resulted in approximately 50% reduction in kynurenate production in rat cortical slices (Battaglia et al., 2000). We previously demonstrated that 5 mM kynurenate preferentially reduced the static response of St I units (Fagan and Cahusac, 2001). Assuming that there is tonic release of kynurenate at the Merkel cell/nerve terminal synapse (for which there is no evidence as yet), the inhibition of kynurenate production by (*S*)-4C3HPG would be expected to enhance responses. It may be relevant that we found quinolinic acid (a neurotransmitter candidate related to kynurenate (Stone, 1993)) at concentrations of 500 μM had no effect on 2 St I units tested. Finally, it is possible that LY341495 and (*RS*)-4C3HPG were having other, as yet uncharacterized, actions; for example, a direct action on mechanogated channels (e.g., (Casado and Ascher, 1998)).

In summary, our results show that mGlu receptor antagonists (*RS*)-4C3HPG and LY341495 selectively enhance St I unit responses. Current histological evidence suggests that this is due to their action at mGlu receptors situated on Merkel cells. If Merkel cells are involved in this process, this further implicates them in mechanotransduction and neurotransmission. Further work using selective receptor agonists and antagonists will be necessary to fully elucidate the receptor subtype(s) or mechanisms involved.

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