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Tramadol produces outward currents by activating μ-opioid receptors in adult rat substantia gelatinosa neurones

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1 An action of a tramadol metabolite, mono-*O*-dimethyl-tramadol (M1), on substantia gelatinosa (SG) neurones in adult rat spinal cord slices was examined by using the whole-cell patch-clamp technique.

2 In 41% of the neurones examined, superfusing M1 produced an outward current at −70 mV; this response reversed at a potential close to the equilibrium potential for K^+. M1 current hardly declined and persisted for >30 min after its washout.

3 M1 current correlated in amplitude with current produced by μ-opioid receptor agonist DAMGO in the same neurone, and largely reduced in amplitude in the presence of μ-opioid receptor antagonist CTAP but not α2-adrenoceptor antagonist yohimbine. In a neurone where M1 had no effect on holding currents, noradrenaline produced an outward current at −70 mV.

4 The amplitude of the M1 response, relative to that of the DAMGO response, exhibited an EC_{50} value of 300 μM.

5 We conclude that M1 produces a persistent hyperpolarization by activating μ-opioid receptors in adult rat SG neurones. This could contribute to at least a part of pain alleviation produced by tramadol.

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Abbreviations: M1, mono-*O*-dimethyl-tramadol; SG, substantia gelatinosa; tramadol, (1*RS*; 2*RS*)-2-[(dimetylamino) methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride; V_H, holding potential

Introduction

Tramadol, (1*RS*; 2*RS*)-2-[(dimetylamino) methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride, is a clinically used, orally active drug that is considered to act as an analgesic in the CNS (for a review see Klotz, 2003). Behavioural studies in rats have demonstrated an inhibition of nociceptive responses by intravenously or subcutaneously administered tramadol (Hennies *et al*., 1988; Raffa *et al*., 1992). Among mechanisms for its antinociceptive action, there are (1) μ-opioid receptor activation (Hennies *et al*., 1988; Raffa *et al*., 1992) and (2) uptake inhibition of monoamines including noradrenaline (Raffa *et al*., 1992; Driessen *et al*., 1993; Reimann & Hennies, 1994). The former mechanism has been revealed from the experimental results of a μ-opioid receptor binding of tramadol (Frink *et al*., 1996) and its [^{35}S]GTP-γ-S-binding stimulation (Gillen *et al*., 2000). To our knowledge, it has not been examined yet whether tramadol produces membrane responses by activating μ-opioid receptors.

The substantia gelatinosa (SG; lamina II) of the spinal dorsal horn is thought to play an important role in modulating nociceptive transmission from the periphery to the CNS (Willis & Coggeshall, 1991). It is possible that at least a part of the antinociception produced by tramadol is due to a modulation of synaptic transmission in the SG. This idea is supported by an observation that opioids administrated into the SG in anaesthetized cats inhibit an excitation of dorsal horn neurones caused by noxious peripheral stimuli (Duggan *et al*., 1977). The μ-opioid receptors have been found in superficial layers of the spinal cord, particularly the SG in rats (Besse *et al*., 1990; Stevens *et al*., 1991; Rahman *et al*., 1998) and in humans (Faull & Villiger, 1987). Opioids are well known to open one or more K^+ channels by activating μ-opioid receptors and thus hyperpolarize membranes, resulting in an inhibition of nociceptive transmission in rat superficial dorsal horn neurones (Yoshimura & North, 1983; Grudt & Williams, 1994).

Tramadol is metabolized to various compounds including mono-*O*-dimethyl-tramadol (M1) *via N*- and *O*-dimethylation in humans and animals (Lintz *et al*., 1981). M1 has the highest affinity for the cloned human μ-opioid receptors among the metabolites (Gillen *et al*., 2000) and is more effective in antinociception than tramadol according to a behavioural study in rats (Hennies *et al*., 1988). As a first step to reveal a role of M1 in antinociception, the present study examined an effect of M1 on membranes of SG neurones in adult rat spinal cord slices by use of the whole-cell patch-clamp technique.

Methods

Preparation of spinal cord slices

All animal experiments were approved by the Animal Use and Care Committee of Saga University. The methods used for

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obtaining an adult rat spinal cord slice and for patch-clamp recordings from SG neurones have been described elsewhere (Luo *et al*., 2001; Liu *et al*., 2004). Briefly, male adult Sprague–Dawley rats (6–8 weeks old) were anaesthetized with urethane ($1.2\,g\,kg^{-1}$, intraperitoneal), and then a laminectomy was performed to extract a lumbosacral spinal cord (L1–S3) segment. The spinal cord was placed in preoxygenated Krebs solution at 1–3°C; the rats were then killed by exsanguination. After cutting all of ventral and dorsal roots, the pia-arachnoid membrane was removed. The spinal cord was mounted on a vibrating microslicer (DTK-1000, Dousaka, Kyoto, Japan) and then a 650-μm-thick transverse slice was cut. The slice was placed on a nylon mesh in the recording chamber (volume: 0.5 ml), and then perfused at a rate of $15–18\,ml\,min^{-1}$ with Krebs solution bubbled with 95% O_2 and 5% CO_2, and maintained at 36 ± 1°C. The Krebs solution contained NaCl, 117; KCl, 3.6; $CaCl_2$, 2.5; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 11 (in mM; pH = 7.4 when saturated with the gas).

Whole-cell patch-clamp recordings from SG neurones

SG neurones were identified by their location, as reported previously (Luo *et al*., 2001; Liu *et al*., 2004). Under a binocular stereomicroscope and with transmitted illumination, the SG was clearly discernible as a relatively translucent band across the dorsal horn. Blind whole-cell patch-clamp recordings were made from the SG neurones with patch-pipette electrodes having a resistance of 8–15 MΩ. Patch-pipette solution had the following composition (in mM): K-gluconate, 135; KCl, 5; $CaCl_2$, 0.5; $MgCl_2$, 2; EGTA, 5; HEPES, 5; and Mg-ATP, 5 (pH = 7.2). The patch electrode was inserted at the centre of the SG to avoid recordings from lamina I or III neurones. To examine a change in membrane conductance during M1 currents, a voltage step (duration: 100 ms) from a holding potential (V_H) of −70 mV to potentials ranging from −150 to −40 mV was given to SG neurones in the absence and presence of M1. The current–voltage relationships were plotted after membrane potentials were corrected for liquid junction potentials existing between the patch-pipette solution and Krebs solutions having 1.3, 3.6 and 10 mM K^+, which were 12.3, 12.4 and 10.6 mV, respectively. Signals were gained using an AxoPatch 200B amplifier (Axon Instruments, Foster City, CA, U.S.A.), filtered at 3 kHz and digitized at 333 kHz with an A/D converter (Digidata 1200, Axon Instruments). Data were stored and analysed with a personal computer using the pCLAMP6 and AxoGraph data acquisition programs (Axon Instruments).

Application of drugs

Drugs were applied by superfusion with a change in solutions in the recording chamber, being completed within 20 s. Drugs used were (±)-M1 (given kindly by Grünenthal GmbH, Aachen, Germany), yohimbine hydrochloride (Wako, Osaka, Japan), [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]enkephalin (DAMGO), D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP; Sigma, St Louis, MO, U.S.A.) and (±)-noradrenaline (Aldrich Chemical Co., Milwaukee, WI, U.S.A.). These drugs were first dissolved in distilled water at 250–1000 times the concentrations to be used, and then diluted to the desired concentrations in Krebs solution immediately before use. When M1 was repeatedly applied in a slice preparation, an interval between its application was more than 1 h; DAMGO response was examined before M1 superfusion. The osmotic pressure of Krebs solutions having various K^+ concentrations was adjusted by changing the Na^+ concentration.

Statistical analysis

All numerical data were expressed as the mean ± s.e.m. Statistical significance was determined as $P<0.05$ using the paired Student's *t*-test. In all cases, *n* refers to the number of neurones studied.

Results

In 41% of SG neurones examined ($n=92$), M1 (1 mM) superfused for 2 min induced an outward current having a peak amplitude of 14.8 ± 1.9 pA ($n=38$) at −70 mV (Figure 1a). This current persisted after its washout. As seen in Figure 1a, a neurone sensitive to M1 responded to a μ-opioid receptor agonist DAMGO (1 μM). At this concentration of DAMGO, a maximal μ-opioid receptor response was induced, because a ratio (1.07 ± 0.09; $n=3$) of the peak amplitude of DAMGO (3 μM) current to that (35.1 ± 8.7 pA) at 1 μM was not significantly different from one ($P>0.05$; see Grudt & Williams, 1994, for a similar result in spinal trigeminal SG neurones). Figure 1b illustrates a relationship between the peak amplitudes of currents produced by DAMGO (1 μM) and M1 at 1 mM or 20 μM. These amplitudes had a good correlation with each other. All of neurones that responded to DAMGO (1 μM) were responsive to M1 at concentrations higher than 10 μM. When DAMGO (instead of M1) was superfused once again after the application of DAMGO, a current response produced by the second application of DAMGO was almost comparable in amplitude to that of the first application; ratio of their amplitudes was 0.96 ± 0.04 ($n=3$), a value not significantly different from one ($P>0.05$). When superfused for a long time such as 8 min, M1 at 1 mM produced a response that did not decline with time (Figure 1c; $n=3$).

We next investigated what kinds of channels mediate the M1 current by examining membrane currents in response to voltage pulses in the absence and presence of M1, as shown in Figure 2a. Figure 2b demonstrates current–voltage relationships between the step voltage and steady current at the end of its pulse, which are obtained in the absence and presence of M1 (1 mM). A net of M1 current, estimated from a difference between the two currents, exhibited a clear reversal (Figure 2b). This reversal potential averaged to be -98.0 ± 2.2 mV ($n=3$). This potential was quite close to the equilibrium potential (−97.3 mV) for K^+, as calculated from the Nernst equation using K^+ concentrations (3.6 and 140 mM, respectively) of these solutions. Changing extracellular K^+ concentration from 3.6 mM to 1.3 or 10 mM resulted in altering the reversal potential for M1 currents. Figure 2c demonstrates a dependence of the reversal potential on extracellular K^+ concentration. This relationship well corresponded to a straight line obtained from the Nernst equation, as shown in Figure 2c.

In order to know whether the M1 current is mediated by the μ-opioid receptor, we examined how the M1 current is affected

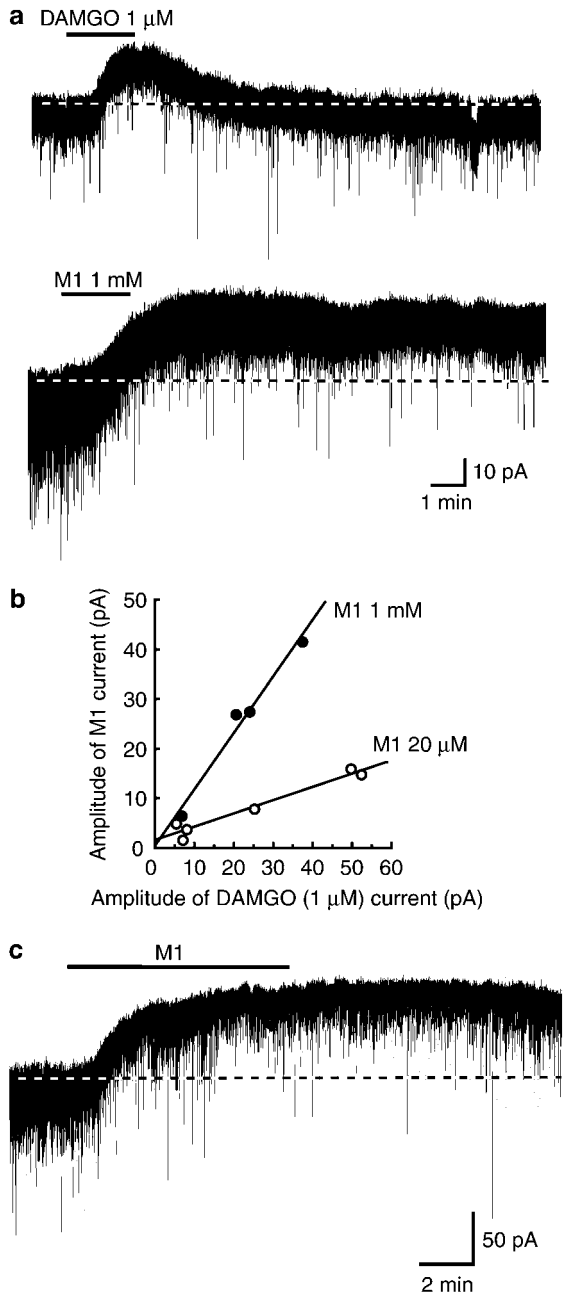


Figure 1 M1 produced a persistent outward current, the amplitude of which correlated well with that of an outward current produced by μ-opioid receptor agonist DAMGO (1 μM) in the same SG neurone. (a) Outward currents produced by superfusing DAMGO and M1 (1 mM) for 2 min in the same neurone. (b) Peak amplitude of M1 (20 μM or 1 mM) current, which was plotted against the peak amplitude of DAMGO current, which was observed in the same neurone. Correlation coefficients in the cases of the M1 concentrations of 20 μM and 1 mM: 0.97 and 0.98, respectively. (c) Outward current produced by superfusing M1 (1 mM) for a long time such as 8 min. In this and subsequent figures, holding current level in the control is denoted by a horizontal dotted line. Holding potential (V_H) = −70 mV.

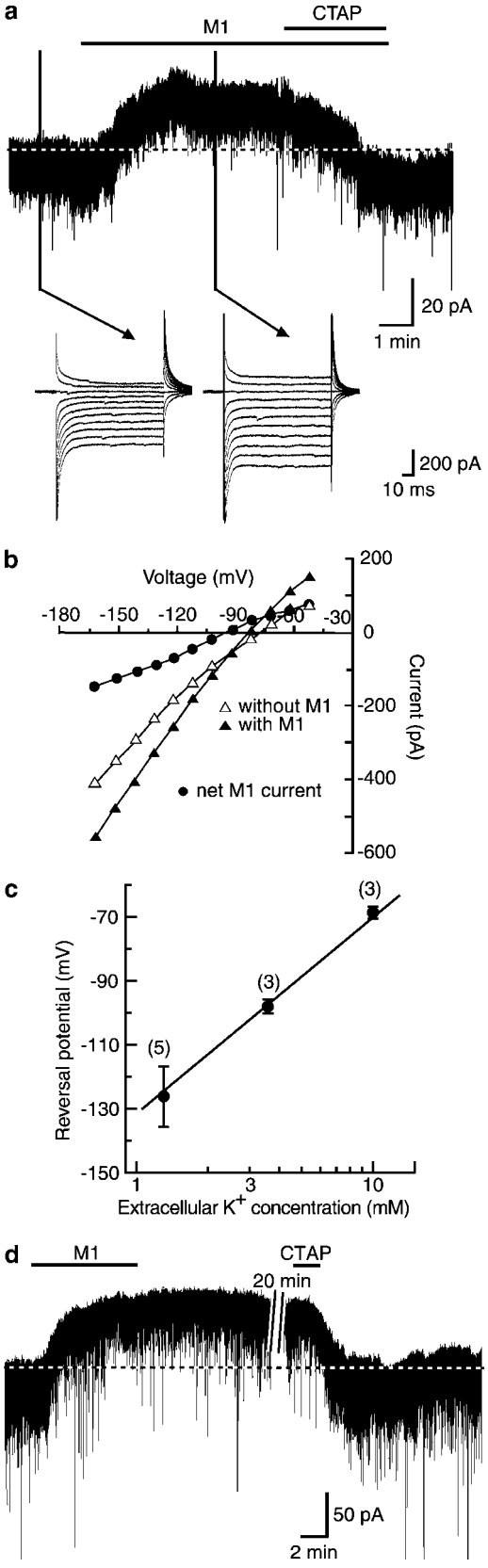
by its receptor antagonist CTAP. At 6 min after the beginning of M1 (1 mM) superfusion, when M1 current persisted (see Figure 1c), adding CTAP (1 μM) for 3 min to the M1-containing Krebs solution resulted in reducing the amplitude of the outward current (Figure 2a). A ratio of M1 current amplitude (30.7 ± 8.3 pA; $n = 7$) at the beginning of CTAP superfusion to that at the end of its superfusion was 0.34 ± 0.20 ($P < 0.05$). On the other hand, 30 min after washout of M1 (500 μM) superfusing CTAP (1 μM) alone recovered the holding current to control, as shown in Figure 2d. Similar results were obtained from other four neurones. Since CTAP itself does not change holding currents under the condition where M1 is not superfused or more than 1 h after washout of M1 ($n = 9$), such an observation indicates that M1 current responses persist for > 30 min after its washout. When superfused for 3 min in the presence of M1, unlike CTAP, an α2-adrenoceptor antagonist yohimbine at 4 μM did not affect the M1 current (Figure 3a); this yohimbine concentration was enough to attenuate an outward current produced by noradrenaline in SG neurons (see Sonohata *et al*., 2004). A ratio of M1 current amplitude (10.0 ± 1.8 pA; $n = 6$) at the beginning of yohimbine superfusion to that at the end of its superfusion was 0.97 ± 0.04, a value being significantly not different from one ($P > 0.05$). After washout of them, the M1 current persisted, as seen in currents produced by M1 only. In a neurone where M1 (1 mM) had no effect on holding currents, noradrenaline (20 μM) produced an outward current at −70 mV, as seen in Figure 3b. Such no response of M1 was observed in six neurones where noradrenaline-induced outward currents had the peak amplitude of 11.0 ± 3.2 pA.

Since there were a variety of M1 current amplitudes among SG neurones (see Figure 1b), which was possibly due to a difference in the density of μ-opioid receptors expressed there, we examined a dose–response curve for M1-current amplitudes as a value relative to that of DAMGO response, which was examined in the same neurone (see Figure 4A). Analysis of the curve based on the Hill equation gave 300 μM for the effective concentration of M1 for producing half-maximal response (EC_{50}; Figure 4B).

Discussion

The present study demonstrated that a metabolite of tramadol, M1, induces an outward current at −70 mV in adult rat SG neurones. This was possibly due to the activation of μ-opioid receptors, because the M1 response correlated well in amplitude with a response, which was produced by the μ-opioid agonist DAMGO in the same neurone and was largely reduced in amplitude by the μ-opioid antagonist CTAP. Although laminae I–II contain not only μ- but also δ- and κ-receptors, the latter two are fewer than the first (μ: 63% or more; δ: 23% or less; κ: 15% or less; Besse *et al*., 1990; Stevens *et al*., 1991; Rahman *et al*., 1998), and M1 exhibits a higher affinity to μ receptors than δ and κ receptors according to binding experiments (Frink *et al*., 1996). Like responses produced by endogenous μ-opioid agonists endomorphins (see Fujita *et al*., 2003), the M1 response was reversed at a potential being close to the equilibrium potential for K^+. Since tramadol reportedly inhibits noradrenaline uptake in the spinal dorsal horn (Reimann & Hennies, 1994), M1 may have increased the concentration of noradrenaline near SG neurones, resulting in producing outward currents mediated by K^+ channels as a result of the activation of α2-adrenoceptors in SG neurones (see Sonohata *et al*., 2004). However, this was not the case in the present study, because the M1 current was

unaffected by yohimbine. Noradrenaline produced a significant outward current in a neurone that did not respond to M1. The amplitude of the M1 response, which was estimated as a value relative to that of DAMGO response, had an EC_{50} value of 300 μM. The M1 response at 1 mM (a concentration exhibiting a maximal response; see Figure 4B) was observed in 41% of the SG neurones examined; this percentage value was similar to a proportion (50%) of adult rat SG neurones having a hyperpolarizing response by enkephalin (Yoshimura & North, 1983). M1 at high concentrations such as 0.5–1 mM produced outward currents that did not decline in the presence of M1 and persisted for >30 min after its washout. Such a persistent activation was not induced by DAMGO at 1 μM, a concentration maximally activating μ-opioid receptors. Superfusing CTAP during the production of M1 current accelerated a recovery of holding currents to control (see Figure 2a and d).



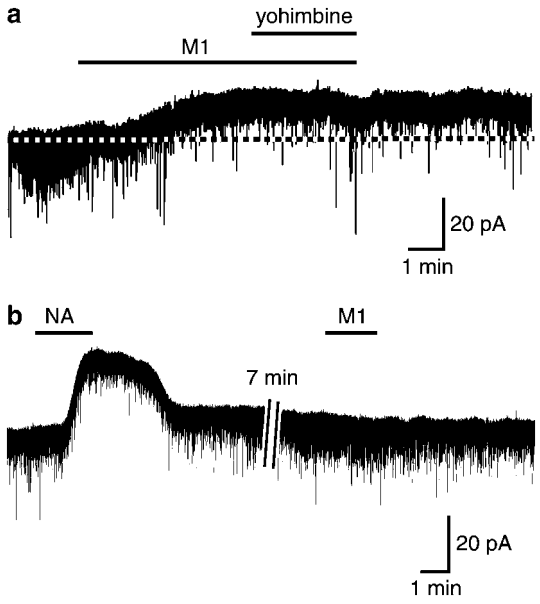


Figure 3 M1 current was not mediated by noradrenaline in SG neurones. (a) Outward current induced by M1 (1 mM) was unchanged following superfusion of the α2-adrenoceptor antagonist yohimbine (4 μM). (b) Noradrenaline (NA; 20 μM) produced an outward current while M1 (1 mM) had no effect on holding currents. $V_H = -70$ mV.

Figure 2 Outward current produced by M1 in SG neurones was reduced in amplitude by μ-opioid receptor antagonist CTAP (1 μM) and was due to the activation of K^+ channels. (a) Outward current induced by M1 (1 mM), where voltage pulses (duration: 100 ms) were given in the absence and presence of M1, which disappeared following superfusion of CTAP (1 μM). Two lower records: membrane currents in response to voltage pulses from the V_H of −70 mV in the control (left) and under the action of M1 (right). (b) Amplitudes of the membrane currents, measured at the end of voltage pulses, which were plotted against voltages; these were obtained in the absence and presence of M1. The current–voltage relationship for a net of M1 current, estimated from a difference between the current responses in the absence and presence of M1, is also shown. (a) and (b) were obtained from the same neurone. (c) Reversal potential for the M1 current, which was plotted against the logarithm of extracellular K^+ concentration. The straight line was drawn according to the Nernst equation using K^+ concentrations of the extracellular Krebs and patch-pipette solutions. In this and subsequent figures, each point with vertical bar represents the mean value and s.e.m., and the number of neurones examined is indicated in parentheses. (d) A persistent outward current at 30 min after washout of M1 (500 μM) was completely reduced in amplitude by CTAP (1 μM). $V_H = -70$ mV.

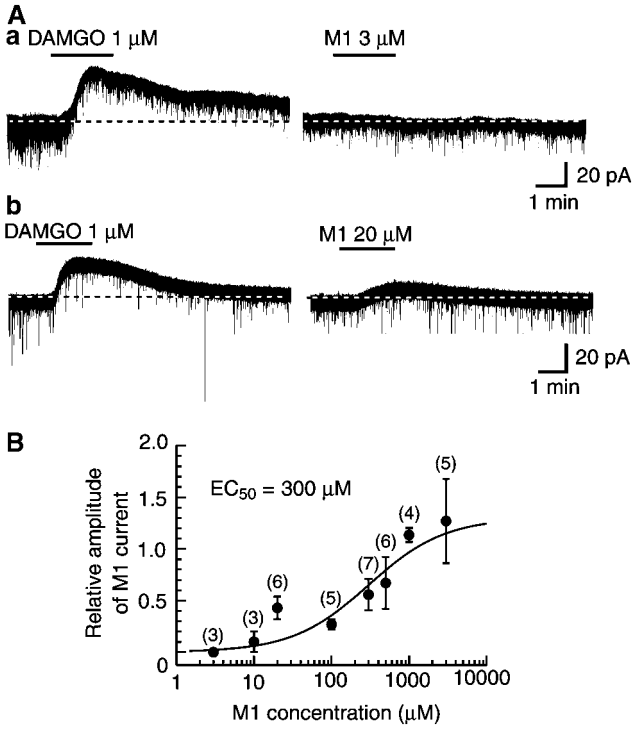


Figure 4 M1 produced an outward current in a dose-dependent manner in SG neurones. (A) Current responses produced by superfusing DAMGO (1 μM) and M1 (a and b: 3 and 20 μM, respectively) for 2 min in the same neurone. (B) Relative amplitudes of currents elicited by M1 at various concentrations to those (23.7 ± 2.2 pA, $n = 39$) by DAMGO (1 μM) in the same neurone, which were plotted against the logarithm of M1 concentration. The continuous curve is drawn according to the following Hill equation: $y = 1.3/\{1 + (EC_{50}/x)^b\}$, where x is M1 concentration in μM; EC_{50} is 300 μM and Hill coefficient (b) is 0.88. $V_H = -70$ mV.

After CTAP washout, the effect of M1 recovered (data not shown). As seen in Figures 1–4, the M1 current was slow in rising phase. Such a slow offset and onset of the M1 current may be due to a slow rate of diffusion of M1 into and out of spinal cord slices because of an unknown reason. This issue remains to be examined.

The present study demonstrated for the first time that M1 produces a membrane response by activating μ-opioid receptors in native neurones, although it had been demonstrated that M1 acts on cloned μ-opioid receptors in a heterogeneous system (Gillen *et al*., 2000). There was a difference between the native and cloned receptors in that EC_{50} value for M1 in the former was much larger than those (0.86 and >0.050 μM, respectively, for (+)-M1 and (−)-M1) in the latter, and that M1 was less effective than DAMGO in the cloned receptor by at least 10-fold (Gillen *et al*., 2000), an extent much smaller than that in the native receptor (about 1000-fold; see Figures 1b and 4B). Such a large value of the EC_{50} value for M1 in the present compared to previous study may have been due to a rundown in μ-opioid receptor activation by DAMGO that was bath-applied before the application of M1, because the patch-pipette solution used here did not contain GTP, the dialysis of which under the condition of whole-cell patch-clamp configuration resulted in a decrease with time in μ-opioid receptor responses in SG neurones (Schneider *et al*., 1998). This is, however, unlikely in the present study, because a repetitive application of DAMGO produces current responses having almost the same amplitudes at a time interval when M1 responses are examined following DAMGO superfusion. The difference in potency between native and cloned μ-opioid receptors may be due to a distinction in estimating the activation of the receptors, such that Gillen *et al*. (2000) measure [^{35}S]GTP-γ-S-binding stimulation, while we examine membrane responses. A possibility cannot be ruled out that the EC_{50} value for M1 in the present study may have been underestimated because M1 is not superfused until a steady current is reached, as seen in Figures 1 and 2. This issue about the discrepancy in potency of M1 remains to be addressed.

Substances, thought to exhibit an antinociceptive action in the spinal dorsal horn, such as nociceptin (Luo *et al*., 2001), noradrenaline (Sonohata *et al*., 2004), adenosine (Liu *et al*., 2004) and endomorphins (Fujita *et al*., 2003), induced an outward current (hyperpolarization) in adult rat SG neurones. The present study demonstrated that M1 also hyperpolarizes membranes by activating μ-opioid receptors. This action of a metabolite of tramadol, M1, might contribute to at least a part of antinociception produced by tramadol.

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