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Inhibition of glycine currents by dextromethorphan in neurones dissociated from the guinea-pig nucleus tractus solitarii

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1 The effect of dextromethorphan (DM) on the current induced by glycine in acutely dissociated nucleus tractus solitarii (NTS) neurones of guinea-pigs was studied by use of the whole-cell patch clamp technique. The effect of DM on γ-aminobutyric acid (GABA)-induced currents (I_{GABA}) was also examined.

2 DM inhibited 30 μM glycine-induced current (I_{Gly}), without affecting the current caused by 30 μM GABA. The action of DM was concentration-dependent, with a maximum effect at 100 μM, and reversible. The half-maximum inhibitory concentration (IC_{50}) of DM was 3.3 μM, about 85 times higher than that of strychnine.

3 DM 3 μM shifted the concentration-response curve for glycine to the right without affecting the maximum value. DM 10 μM shifted the curve even more to the right, although it was not a parallel shift. Strychnine at a concentration of 0.1 μM shifted the curve for glycine in a nearly parallel fashion.

4 The effect of 10 μM DM was slightly weak voltage-dependency, but the lower concentration of DM, 3 μM, inhibited I_{Gly} equally at −50 mV and +50 mV. The effect of 3 μM DM on I_{Gly} showed no use-dependence. Blockade by strychnine 0.1 μM showed no voltage- or use-dependence.

5 The results indicate that DM inhibits I_{Gly} in single neurones of NTS, and further suggest that DM at a low concentration may act on the glycine receptor-ionophore complex, but not on the Cl^- channel of the complex. However, a relatively high concentration of DM may at least partly affect the Cl^- channel of the complex.

Keywords: Dextromethorphan; glycine; nucleus tractus solitarii; whole-cell recording; dissociated neurones; guinea-pig; antitussive

Introduction

Glycine and γ-aminobutyric acid (GABA) are generally considered to be inhibitory neurotransmitters in the mammalian central nervous system (CNS). Although findings about the pharmacological significance of GABA receptor-ionophore complexes in the brain have accumulated (Schult & MacDonald, 1981; Olsen *et al*., 1982; Ticku & Rastogi, 1986; Yakushiji *et al*., 1989), much remains unknown about the pharmacological and pathophysiological significance of the glycinergic transmission in the CNS. We found that the intensity of cough responses was strengthened in guinea-pigs, when a minute amount of glycine was injected directly into the nucleus tractus solitarii (NTS) and the adjacent region where the cough reflex pathway is located (Honda *et al*., 1990). Furthermore, toxic doses of antitussives often facilitate convulsions in experimental animals (Takahama *et al*., 1978), possibly due to blockade of the receptors of inhibitory neurotransmitters such as glycine and GABA. Therefore, it seems of interest to determine whether antitussives influence the glycine-mediated response in the CNS.

In this study, we investigated the effect of an antitussive, dextromethorphan (DM), on I_{Gly} in single neurones acutely dissociated from the NTS of guinea-pigs; strychnine was used as a reference drug. Since it has also been shown that GABAergic mechanisms participate in the effect of antitussives acting on the cough centre (Bolser *et al*., 1994), the effect of DM on GABA-induced current (I_{GABA}) in the NTS neurones was also examined.

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Methods

Preparation

Single neurones of NTS were acutely dissociated from 7- to 10-day-old Hartley guinea-pigs according to the method previously described (Huguenard & Alger, 1986). The guinea-pigs were decapitated under ether anaesthesia. The brain was quickly removed and dissected into slices 400 μm in thickness by a microslicer (DTK-1000, D.S.K.). The slices were pre-incubated in external solution bubbled with 100% O_2 at 37°C for 30 min. Thereafter, the NTS region was dissected out by punching the slices with a small hand-held needle. The small tissue chunks including the NTS were stirred in external solution containing 1.8 mg ml^{-1} trypsin for 30–60 min at 37°C with constant oxygenation, then triturated with a fire-polished micro-pipette.

The ionic composition of external solution was (in mM): NaCl 140, KCl 5, $CaCl_2$ 1, $MgC1_2$ 1, N-2-hydroxyethyl-piperazine-N′-2-ethanesulphonic acid (HEPES) 10 and D-glucose 24. The pH was adjusted to 7.4 with tris(hydroxymethyl) aminomethane (Tris-OH). The patch-pipette solution contained (in mM): KCl 140, $CaCl_2$ 1, ATP-Mg 2, ethylene glycol-bis (β-aminoethyl ether)-,N,N,N′,N′-tetraacetic acid (EGTA) 10 and HEPES 10. The pH was adjusted to 7.2 with Tris-OH. In the current-voltage relationship experiment, K^+ in the pipette solution was replaced with equimolar Cs^+, to suppress the voltage-dependent K^+-currents.

Electrical measurements

Electrical measurements were made with the whole-cell configuration mode of the patch clamp technique (Hamill *et al*., 1981). The resistance between the recording electrode filled with the patch-pipette solution and the reference electrode in the external solution was 4–6 MΩ. Currents were measured

with a patch-clamp amplifier (Axopatch 1D, Axon Instrument), and monitored on both an oscilloscope (MS-5021, Iwatsu) and a pen recorder (Model 3021, Yokogawa). Data were filtered at 1 kHz, digitized, and stored on microcomputer disk for subsequent analysis by use of the pClamp system (Axon Instruments).

Drugs

Drugs dissolved in the external solution were applied by the 'Y-tube' technique that allowed complete exchange of the solution within 20 ms as described by Murase *et al*. (1989). The drugs and chemicals used in the present experiments were from commercial sources.

Statistical analysis

Experimental values are given as mean ± s.e.mean. Unpaired Student's *t* test was used to assess the significance of difference when necessary, and a *P* value of less than 0.05 was considered to be significant.

Results

Effect on glycine- and GABA-induced current

In freshly dissociated NTS neurones, which were voltage-clamped to a holding potential (V_H) of -50 mV, glycine induced an inward current in a concentration-dependent manner at concentrations of 3 μM to 3000 μM. The glycine-induced current (I_{Gly}) gradually desensitized during a continuous application of glycine lasting 20 s. Glycine was applied every 1 min, at which interval no desensitization of the peak current occurred. The I_{Gly} induced by glycine 30 μM in the NTS neurones of guinea-pigs was completely depressed by strychnine 1 μM but not by bicuculline 10 μM at all (data not shown).

DM at a concentration of 100 μM produced no response in the NTS neurones. But it depressed the current induced by glycine 30 μM (Figure 1a). The depressive effect was concentration-dependent over the range 0.1 μM to 100 μM (Figure 1b), with complete depression at 100 μM. The half-maximum inhibitory concentration (IC_{50}) of DM on the current induced by 30 μM glycine was 3.3 μM. On the other hand, strychnine strongly blocked I_{Gly} at 0.001 μM to 1 μM, with an IC_{50} of 0.039 μM, about 85 times lower than that for DM. DM at 100 μM had no effect on the current induced by GABA 30 μM (I_{GABA}) (Figure 1a) in 5 neurones examined, while I_{GABA} was depressed by bicuculline 10 μM (data not shown).

Effect on concentration-response curve for glycine

To study the mechanism of the inhibitory action of DM on I_{Gly}, the effect of DM on the concentration-response relationship for glycine was examined. As shown in Figure 2, DM 3 μM slightly shifted the concentration-response curve for glycine to the right without depressing the maximal response of glycine. DM 10 μM shifted the curve even more to the right, although it appeared not to be a parallel shift. The EC_{50} values in the presence and absence of DM 3 μM were 67 μM and 39 μM, respectively, and the value in the presence of DM 10 μM was 120 μM. Strychnine also shifted the curve to the right in a nearly parallel fashion. The EC_{50} in the presence of 0.1 μM strychnine was 200 μM.

Effect on current-voltage relationship for glycine

Figure 3 shows the current-voltage (I–V) relationship for glycine in the presence and absence of DM 10 μM. In the absence of DM, I_{Gly} reversed direction at $+10$ mV, and the I-V relationship was almost linear. The reversal potential for I_{Gly} was close to the Cl^- equilibrium potential ($+1.2$ mV) calculated by Nernst equation from the given intra- and extracellular Cl^- concentrations and their Cl^- activities, indicating that the currents induced by glycine were carried by Cl^-. DM depressed the I_{Gly} at different V_H. In the presence of DM 10 μM, the I-V relationship for glycine did not show complete linearity; the I_{Gly} at a V_H of -50 mV was reduced by $71.4 \pm 2.8\%$ while that at a V_H of $+50$ mV by $46.4 \pm 3.3\%$. There was a statistically significant difference between the two values. This result suggests that the inhibitory action of DM 10 μM on I_{Gly} is weakly

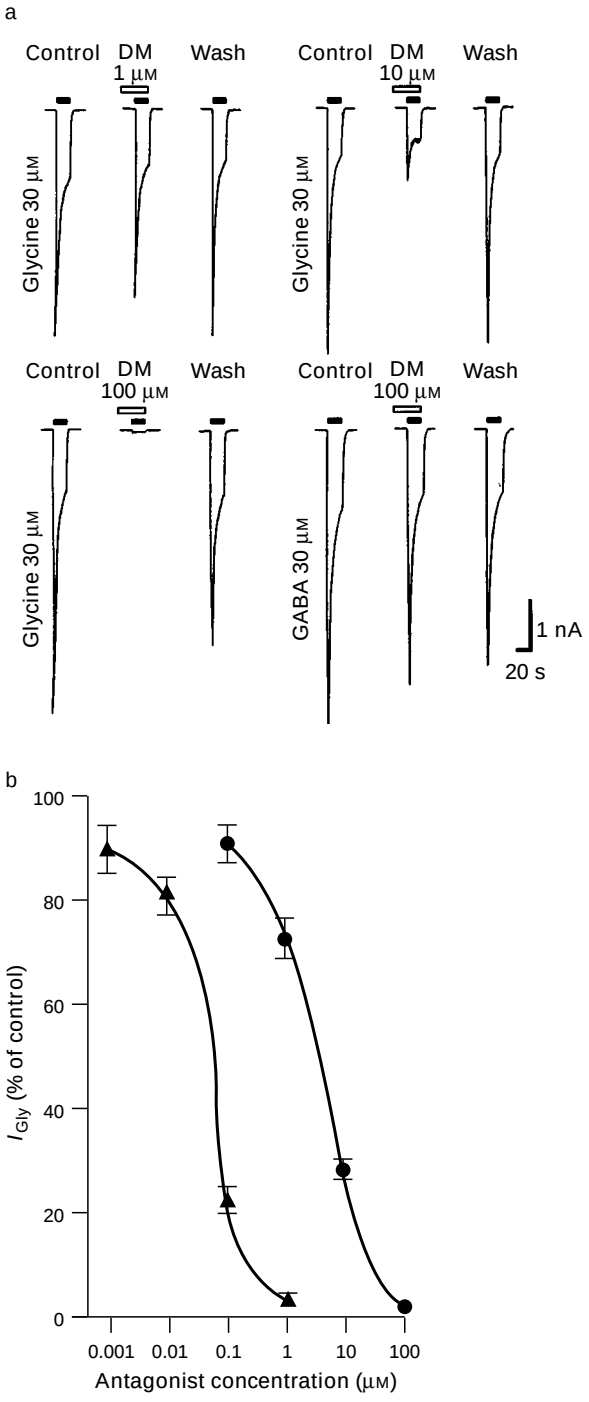

Figure 1 Concentration-dependent inhibitory effect of dextromethorphan (DM) and strychnine in NTS neurones. (a) A neurone was voltage-clamped at -50 mV. DM depressed 30 μM glycine-induced Cl^- current I_{Gly} in a concentration-dependent manner, but did not change the current induced by 30 μM GABA. An apparent depression of the I_{GABA} was due to a run-down of the response. All responses were obtained from the same neurone. (b) Concentration response curve for the inhibitory effects of DM (●) and strychnine (▲) on I_{Gly}. Each point denotes mean from 4–5 experiments; vertical lines show s.e.mean.

voltage-dependent. However, in the presence of DM 3 μM, the I_{Gly}s at -50 mV and $+50$ mV V_H were reduced to the same extent (data not shown), and the line through the two values crossed the abscissa scale at $+3.6$ mV (data not shown), close to the calculated Cl^- equilibrium potential. This result suggests that the action of DM at a low concentration might be not voltage-dependent. On the other hand, strychnine at 0.1 μM depressed I_{Gly} by about 65% at different V_Hs of -70 to $+50$ mV, i.e., no distortion of the I-V relationship for glycine was observed. The result indicates that the inhibitory action of strychnine on I_{Gly} is not voltage-dependent.

Effect of DM on response to repeated application of glycine

Further experiments were undertaken to see whether the effect of DM is use-dependent. As shown in Figure 4, the magnitude of the inhibitory action of DM 3 μM was not changed by repeated application of glycine in the continued presence of DM. A similar result was obtained in the presence of a low concentration of strychnine.

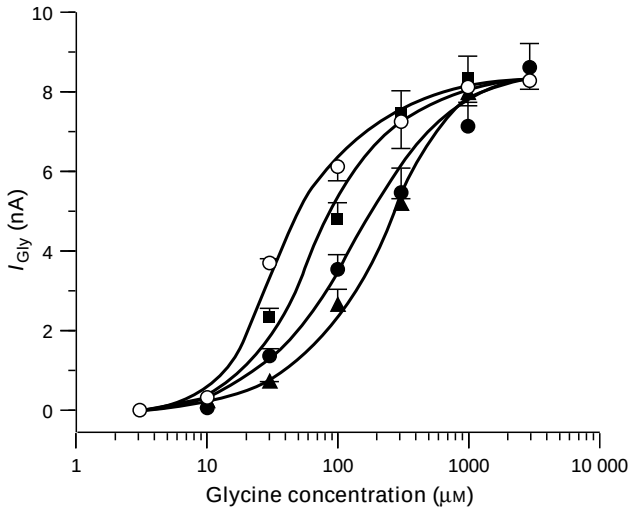

Figure 2 Concentration-response curves for the inhibitory effect of glycine on I_{Gly} in the absence and presence of dextromethorphan (DM) or strychnine. Neurones were voltage-clamped at -50 mV. Each point denotes mean and vertical lines s.e.mean from 4–9 experiments. (○) Effect of glycine alone, (■) with DM 3 μM, (●) with DM 10 μM, (▲) with strychnine 0.1 μM.

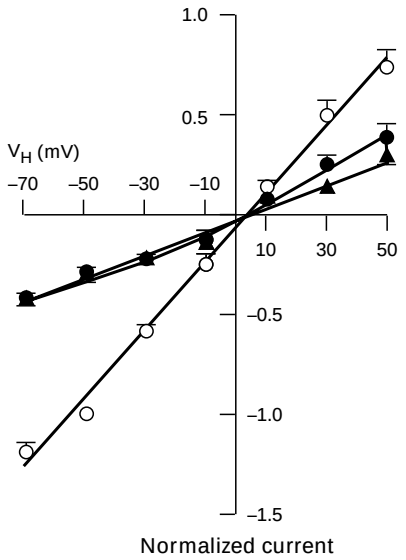

Figure 3 Current-voltage (*I*-V) relationship for I_{Gly} in the absence or presence of dextromethorphan (DM) 10 μM or strychnine 0.1 μM. All responses were normalized to the peak current induced by glycine 30 μM at a V_H of -50 mV. Each point denotes mean and vertical lines s.e.mean from 4–14 experiments. (○) Effect of glycine alone, (●) with DM 10 μM, (▲) with strychnine 0.1 μM.

Discussion

This manuscript provides the first evidence that DM potently inhibits I_{Gly} in brain neurones. DM at a concentration of 100 μM did not have an effect on the current caused by 30 μM GABA (GABA concentration near the K_D value for NTS neurones of guinea-pig, data not shown).

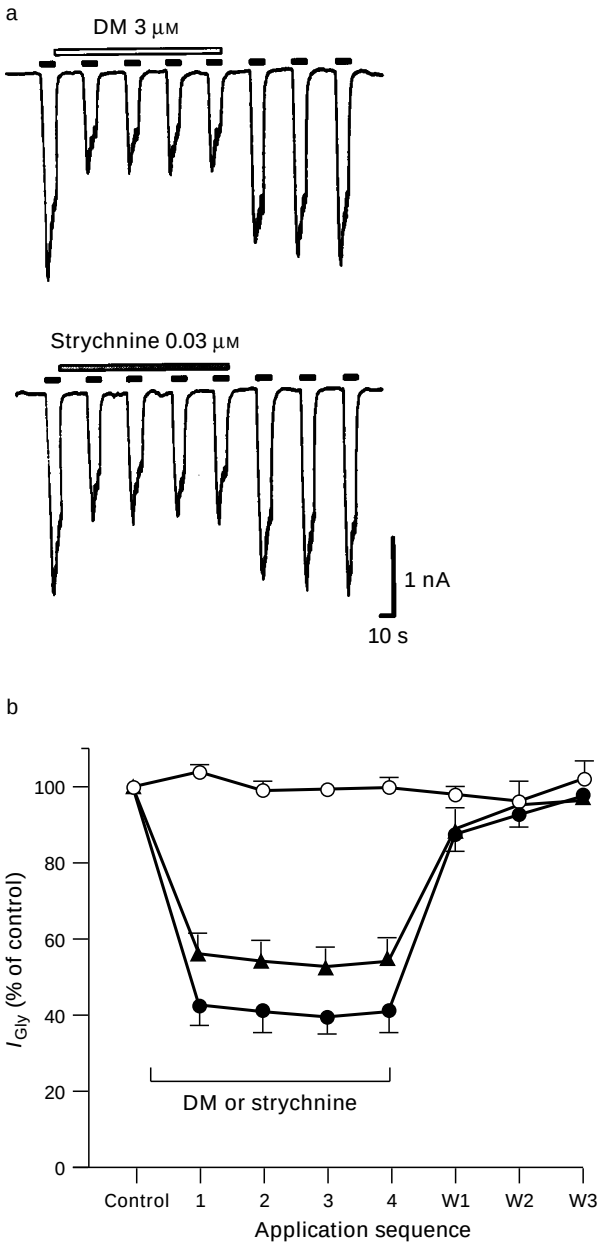

Figure 4 Effects of continuous application of dextromethorphan (DM) and strychnine on I_{Gly}s induced by 4 successive applications of glycine. (a) Typical current record of I_{Gly}s in the continuous presence of DM 3 μM or strychnine 0.03 μM. Glycine 30 μM was applied for 10 s every 30 s. V_H was -50 mV. (b) Summarized data of the effects of continuous presence of DM or strychnine on I_{Gly}s. Concentrations of DM and strychnine, applied for the period indicated by a bar in the figure, were 3 μM and 0.03 μM, respectively. Each point denotes mean and vertical lines s.e.mean ($n=4-6$) of percentages of control response. Effects of (●) DM, (▲) strychnine and (○) vehicle are shown. W1–W3: washing.

DM in the range 10–100 μM inhibits voltage-dependent Ca^{2+} and Na^{+} channels (Tortella *et al*., 1989; Lodge & Johnson, 1990; Netzer *et al*., 1993; Trube & Netzer, 1994). However, it is unlikely that the effect of DM on I_{Gly} is produced through an action on these channels. I_{Gly} is carried by Cl^{-} in various neurones of the CNS (Bormann *et al*., 1987; Ito *et al*., 1991; Uneyama *et al*., 1993); including the NTS neurones of guinea-pigs.

[^{3}H]-DM has at least two distinct binding sites in guinea-pig brains, a high-affinity site (K_D = 13–20 nM) and a low-affinity site (K_D > 200 nM). High affinity [^{3}H]-DM binding was found not to be displaced by various neurotransmitters including glycine and GABA (Craviso & Musacchio, 1983). Furthermore, because the K_D for the high affinity site is in the nM range, far smaller than the the concentration needed to block I_{Gly}, it is unlikely that DM inhibits I_{Gly} through an action on the high affinity site.

Of the two glycine-binding sites in the CNS, one is strychnine insensitive and part of the N-methyl-D-aspartate (NMDA) receptor (Johnson & Ascher, 1987; Harris & Miller, 1989; Kessler *et al*., 1989; Yamada *et al*., 1989; Kemp & Leeson, 1993). Netzer *et al*. (1993) have shown that DM depresses the NMDA-induced current in cultured cortical neurones of the rat. The depression was not due to competitive blockade of the strychnine insensitive glycine binding site but due to blockade of the ion channel activated by NMDA and glycine (Netzer *et al*., 1993). Therefore, DM does not interfere with the strychnine insensitive glycine binding site in a competitive fashion.

There are a few studies that suggest a non-competitive mode of action for strychnine blockade of glycine responses in the brain (Krishtal *et al*., 1988; Akaike & Kaneda, 1989) and in *Xenopus* oocytes injected with rat brain mRNA (Houamed *et al*., 1984). However, many findings have shown that the strychnine antagonism is competitive, especially in neurones of the lower brain stem and spinal cord. In the present study, strychnine 0.1 μM shifted the concentration-response curve for glycine to the right in a nearly parallel fashion without affecting the maximum value. This result suggests that the action of strychnine in NTS neurones of guinea-pigs is probably competitive. DM also shifted the curve for glycine to the right. However, the rightwards shift appeared not to be in a typical parallel fashion, although the maximum I_{Gly} response was not affected. Thus, it seems to be difficult to conclude simply that DM inhibits I_{Gly} in a competitive manner, especially with regard to the action of 10 μM DM.

The block of the NMDA receptor by DM is thought to be voltage- and use-dependent, although direct evidence for this has not been published. With respect to this, the block of the NMDA receptor by dextrorphan, a closely related morphinan, is voltage- and use-dependent (Parsons *et al*., 1993). Therefore, we examined the voltage- and use-dependency of the inhibitory action of DM on I_{Gly}. Interestingly, the effect of a low concentration of DM on I_{Gly} was neither voltage-dependent nor use-dependent, although the action of DM 10 μM showed a very weak voltage-dependency. The results suggest that DM in a low concentration may act on the glycine receptor-ionophore complex, but not on the Cl^{-} channel of the complex. However, a relatively high concentration of DM may at least partly affect the Cl^{-} channel of the complex.

It seems possible that the inhibitory action on I_{Gly} might be associated with antitussive drugs, because our recent work revealed that various antitussives including narcotic antitussives such as codeine inhibit I_{Gly} in NTS neurones without depressing I_{GABA} (Otsuka *et al*., 1996). However, further studies are needed to clarify whether it is the antitussive or the adverse effects of these drugs that is linked to the action on glycine receptors. Bolser *et al*. (1994) suggested that an involvement of $GABA_B$ receptors in the mechanism of action for centrally-acting antitussive drugs.

Finally, the present results may partly explain the mechanism of the ameliorating action of DM on the neurological symptoms of nonketotic hyperglycinemia (Schmitt *et al*., 1993). In this condition, the glycine concentration in plasma and cerebrospinal fluid is elevated (Nyhan, 1989) and the pathophysiological effects of this disease are attributed to the inhibitory action of glycine at the postsynaptic, strychnine-sensitive receptor (Gitzelmann *et al*., 1977).

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