with NaOH. The patch-pipette solution for nystatin perforated patch recordings consisted of (mM): KCl 75, K-gluconate 60 and HEPES 10. The pH was adjusted to 7.2 with KOH. Nystatin dissolved in methanol (10 mg ml^{-1}) was diluted with the internal solution just before use. The final concentration of nystatin was 100-200 μg/ml. The internal solution for the conventional whole-cell patch recording mode had the following ionic composition (mM): KCl 70, K-gluconate 70, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 4, $MgCl_2$ 5, Na_2-ATP 4, guanosine 5'-o-(3-thiotriphosphate) (GTPγS) 0.1 and HEPES 10. The pH was adjusted to 7.2 with KOH.

Electrical measurements

Membrane currents were recorded with an Axopatch 1D amplifier (Axon Instruments, CA, U.S.A.) and acquired with Axoscope data acquisition software (Axon Instruments) after digitization with a Digidata 1200B (Axon Instruments). Signals were filtered at 1 kHz using a facility of the amplifier and sampled at 3.3 kHz. The resistance of the patch pipette filled with the internal solution and the reference electrode was 6–8 MΩ. The HEPES-buffered external solutions containing drugs or nothing (washing solution) were applied by the Y-tube microperfusion system

(Murase *et al.*, 1990). With this technique, the external solution surrounding a neurone could be exchanged within 30 ms. Data analysis was done with Excel 2000 (Microsoft, Redmond, WA, USA) or Origin 5 (Microcal, Northampton, MA, USA). Average results are given as mean ± S.E.M. A Student's paired or unpaired t-test was used for statistical analysis. $P<0.05$ was considered as significant.

Materials

(R)+Baclofen, cloperastine HCl, dextromethorphan HBr, GABA, GTPγS, Na_2-ATP, naltriben methanesulfonate, nystatin and thermolysin were purchased from Sigma (St Louis, MO, U.S.A.). EGTA and HEPES were purchased from Dojin (Kumamoto, Japan). CGP34358 (3-aminopropyl)(diethoxymethyl)phosphinic acid) and Pronase® were purchased from Calbiochem (San Diego, CA, U.S.A.) and Tocris Cookson (Bristol, UK), respectively. Picrotoxin and pertussis toxin were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Seikagaku Corporation (Tokyo, Japan).

Results

$GABA_B$ receptor mediated responses in the DRN neurons

With the nystatin-perforated patch-clamp recording configuration, 3×10^{-5} M GABA induced inward currents in the solution containing 20 mM K^+ and 3×10^{-3} M picrotoxin, a $GABA_A$ receptor antagonist at a holding potential (V_H) of −80 mV. The current was mimicked by 3×10^{-5} M baclofen. Baclofen-induced current was antagonized by 3×10^{-4} M CGP34358, a specific $GABA_B$ receptor antagonist and showed strong inward rectification (Fig. 4). Reversal potential of it (−53.3 mV) was close to the equilibrium potential (−49.0 mV) for K^+ calculated by Nernst equation from the given intra- and extracellular K^+ concentration. EC_{50} of baclofen-induced current was 6.66×10^{-6} M and the Hill coefficient was 0.80 (Fig. 3). Pertussis toxin inhibited baclofen-induced current. Baclofen induced irreversible inward current in the presence of intracellular GTPγS (Fig. 5C).

Inhibitory effect of DM on the baclofen-induced currents

Fig. 1 shows an inhibitory effect of DM on the baclofen-induced current (I_{Bac}) at a V_H of −80 mV. DM suppressed the peak I_{Bac} more effectively when a mixture containing baclofen and DM was applied with a pre-exposure to DM. When we challenged at different pretreatment time with 10^{-5} M DM before the mixture

containing baclofen and DM was applied, a 30-s pre-exposure to DM was found to be sufficient to produce a maximal inhibition (Fig. 1A and B).

Fig. 2 shows the concentration-dependent inhibitory effect of DM on I_{Bac} elicited by 3×10^{-5} M baclofen. DM exerted a slight inhibition of I_{Bac} at a concentration of 3×10^{-6} M. Further increases in concentration reduced I_{Bac} in a concentration-dependent manner. The concentration–inhibition relationship could be fitted well by the logistic equation (1) with the IC_{50} of 8.32×10^{-6} M and the Hill coefficient of 1.09.

$$I = \frac{(IC_{50})^{n_H}}{C^{n_H} + (IC_{50})^{n_H}} \quad \text{.................................} \quad (1)$$

where I is the relative inward current amplitude in the presence of DM, C the concentration of DM, IC_{50} the concentration which induces the half-maximal inhibition, and n_H the Hill coefficient.

Effect of DM on the concentration-response relationship for baclofen

To study the mechanism of the inhibitory action of DM on I_{Bac}, the effect of DM on the concentration–response relationship for baclofen was investigated. As shown in Fig. 3A, DM caused a suppression of the maximum response, indicating a

non-competitive inhibition of the baclofen response. The values of EC_{50} estimated from the concentration–response curves (equation 2) were 8.12×10^{-6} M in the presence of 10^{-5} M DM. When current amplitudes were normalized to the peak current evoked by 3×10^{-5} M baclofen alone, the maximum response changed from 1.27 for control to 0.77 in the presence of 10^{-5} M DM. The Lineweaver-Burk plot also confirms a non-competitive mode of the inhibitory action (Fig. 3B).

$$I = I_{\max}\frac{C^{n_H}}{C^{n_H} + (EC_{50})^{n_H}} \quad \cdots\cdots\cdots\cdots \quad (2)$$

where I is the relative inward current amplitude in the presence or absence of DM, $I_{\max}$ maximal response, C concentration of baclofen, EC_{50} concentration that induces the half-maximal response, and n_H the Hill coefficient.

Effect of DM on the current–voltage relationship for the baclofen response

The inhibitory action of DM was examined in neurones held at various V_Hs. Fig. 4A shows the current–voltage (I–V) relationships for the baclofen response with or without 10^{-5} M DM. In the presence of DM, the I–V relationship showed the inward rectification, and I_{Bac} reversed the current direction at −57.6 mV, which is almost identical to the K^+ equilibrium potential (-49 mV).

Fig. 4B shows the effect of DM on I_{Bac} at various V_Hs. The baclofen response was depressed by about 40 % at every V_H, and there was no significant difference among inhibition ratios at various V_Hs. The result indicates that the blockade of $I_{5\text{-HT}}$ by DM is voltage-independent.

Effects of cloperastine and naltriben on baclofen-induced currents

Recently, we reported that δ-opioid receptor antagonists having antitussive effect inhibit 5-HT_{1A} receptor mediated GIRK channel currents in acutely dissociated DRN neurons of rat. As shown in Fig. 5A, 3×10^{-5} M naltriben inhibited baclofen induced currents in a concentration dependent manner. The IC_{50} of naltriben estimated by the equation (1) was 4.36×10^{-5} M and the Hill coefficient was 0.89 (Fig. 5B).

Our recent results also indicate that cloperastine, another centrally acting, non-narcotic antitussive inhibits 5-HT_{1A} receptor mediated GIRK channel currents. It inhibited baclofen-induced currents in a concentration dependent manner (Fig. 5B). The IC_{50} and the Hill coefficient were 1.36×10^{-6} M and 1.14, respectively.

Effects of CAATs on the irreversibly activated inward current by baclofen in the presence of intracellular GTPγS

Present results indicate that the activation of $GABA_B$ receptors in the DRN neurons involves PTX-sensitive G-protein in the transduction pathway. Beta/gamma subunit of PTX-sensitive Gi protein directly opens the GIRK channel (Mark *et al.*, 2000). To determine the site of action of DM, the effect of DM on baclofen-induced K^+ currents in neurons intracellular perfusion with the nonhydrolysable GTP analog GTPγS was investigated in the conventional whole-cell recording mode. When GTPγS (0.1 mM) was included in the pipette solution, a small inward current began to appear about 1 min after rupture of the patch membrane. Under these conditions, brief application of baclofen resulted in an almost irreversible and continuous activation of K^+ current (Fig. 5C). DM (3×10^{-5} M) remarkably inhibited the GTPγS-activated currents by 77.7 ± 9.7 % (n=3) in the absence of 5-HT, thus suggesting that the inhibitory effect of DM may be due to a blockade of the K^+ channels coupled with $GABA_B$ receptor via G_i-protein.

Discussion

The present results demonstrate that two centrally acting antitussives, DM and cloperastine, and naltriben, which has antitussive activity, potently inhibit baclofen-induced K^+ currents in rat DRN neurones by the use of nystatin-perforated and conventional whole-cell patch-clamp recording configurations. In this study, these produced a concentration-dependent inhibition of the baclofen response in the DRN neurones. The IC_{50} values were 8.32×10^{-6} M for DM, 1.36×10^{-6} M for cloperastine and 4.36×10^{-5} M for naltriben. These IC_{50} values were almost close to those on 5-HT_{1A} receptor-mediated currents ($I_{5\text{-}HT}$) in the DRN neurons (1.43×10^{-5} M for DM, 8.6×10^{-7} M for cloperastine and 1.28×10^{-5} M for naltriben) (Ishibashi *et al.*, 2000; Kuwano *et al.*, 2000; Shirasaki *et al.*, 2004).

Previous study demonstrated that DM and naltrindole, a δ-antagonist, inhibited $I_{5\text{-}HT}$ in noncompetitive and voltage independent manners in the DRN neurons (Ishibashi *et al.*, 2000; Shirasaki *et al.*, 2004). In the present study, we revealed the non-competitive type of inhibitory effect of DM on the response for baclofen (Fig. 3), and the action of DM on I_{Bac} was voltage-independent (Fig. 4). On the other hand, DM inhibits the strychnine-sensitive glycine-induced currents in competitive and voltage-independent manner in NTS neurones of guinea-pigs (Takahama *et al.*,

1997) These suggest that the mechanism of inhibitory effect of DM on I_{Bac} is similar to that on $I_{5\text{-}HT}$ in the DRN neurons but not on the glycine-induced Cl^- current.

Previous intracellular recording study in the rat brain slice preparation has been demonstrated that both 5-HT and baclofen inhibit 5-HT neurons in the DRN by inducing a hyperpolarization of membrane potential and a decrease in apparent input resistance (Innis *et al.*, 1988). 5-HT- and baclofen-mediated inhibition of 5-HT neurons showed an apparent reversal potential of approximately -90 mV, consistent with mediation by K channels. In slices from rats that had previously received a local injection of PTX (0.5 microgram) immediately rostral to the DRN, there was a virtually complete blockade of inhibition induced by both the 5-HT autoreceptor and the $GABA_B$-receptor. Intracellular injection of GTPγS mimicked the actions of both 5-HT and baclofen. The inhibitory actions of GTPγS were not additive with those of either 5-HT or baclofen, suggesting they share some common effector system. 8-Bromo-adenosine-3',5'-cyclic monophosphate (8-Br-cAMP), the stable cAMP analog, had no effect on membrane potential or apparent input resistance and did not block the inhibitory actions mediated by 5-HT or baclofen. PTX-mediated ADP-ribosylation of G proteins in membranes prepared from the DRN negatively correlated with sensitivities to 5-HT and baclofen. From these results, the role of a G

protein(s) has been suggested in the mediation of the cAMP-independent increase in K^+ conductance in 5-HT neurons of the DRN induced by both 5-HT_{1A}- and $GABA_B$-receptors.

In the present study, we demonstrated that baclofen induced strongly inward rectifier K^+ current (Fig. 4) and CGP34358 and PTX inhibited it using the nystatin perforated whole cell patch clamp technique. In addition, baclofen triggered the induction of irreversibly activated inward current in neurons internally perfused with GTPγS by conventional whole cell patch clamp technique (Fig. 5C). The activation kinetics of I_{Bac} was as fast as that of $I_{5\text{-}HT}$ and I_{Bac} induced by 3×10^{-5} M baclofen was completely occluded by $I_{5\text{-}HT}$ induced by 10^{-7} M 5-HT. These results were well agreed with previous intracellular recording study in the brain slice preparation. Since $G_{\beta\gamma}$ subunit of $G_{i/o}$ protein, especially G_{i2} and G_{i3} proteins, directly activate GIRK channels (Fernandez-Fernandez *et al.*, 2001; Ivanina *et al.*, 2004; Kurachi, 1995), it was suggested that the activation of $GABA_B$ receptor opens the GIRK channel via G_i protein directory in the DRN neurons.

As shown in Fig. 5C, three drugs used in the present study inhibited the irreversibly activated inward currents by baclofen in the presence of intracellular GTPγS. This property is the same as that on the irreversibly activated inward

currents activated by 5-HT (Ishibashi *et al.*, 2000; Kuwano *et al.*, 2000; Shirasaki *et al.*, 2004). Thus, two possibilities are considered as likely to be responsible for the inhibitory action of CAATs on the GTPγS-activated currents. One is due to the blocking of the GIRK channel itself. The other is due to the blocking of the interaction between $G_{\beta\gamma}$ subunit and the GIRK channel. To elucidate these possibilities, further studies including biochemical studies such as GST pull-down assay are needed.

At present, the stoichiometry of functional GIRK channels coupled with 5-HT_{1A} and $GABA_B$ receptors. However, present study together with previous ones suggest that 5-HT_{1A} and $GABA_B$ receptors share the GIRK channels and CAATs inhibits common GIRK channels in the DRN neurons. However, slight differences were observed between the IC_{50}s of DM, cloperastine and naltriben on I_{Bac} and those on $I_{5\text{-HT}}$. Therefore, it is not able to rule out the possibility that 5-HT_{1A} and $GABA_B$ receptors activate different type of GIRK channels at relatively lower concentrations of agonists and CAATs nonselectively inhibit these different subtypes of GIRK channel. Indeed, the activity of CAATs on I_{Bac} did not depend on the day after birth, although the expression pattern of GIRK channel subunits changes during the development (Chen *et al.*, 1997). In addition, developmental change in the

expression pattern of GIRK channel subunits in locus ceruleus differs from that in raphe nuclei. However, CAATs similarly inhibit α_2 adrenoceptor-mediated GIRK channel currents. Therefore, the subtype specificity of CAATs must be studied in the recombinant system in the future.

In conclusion, we demonstrated that CAATs inhibit $GABA_B$ receptor-mediated GIRK channel currents probably at GIRK channel site. There was no significant difference between the inhibitory effects of CAATs on $GABA_B$ receptor- and $5\text{-}HT_{1A}$ receptor-mediated currents. CAATs probably alter not only serotonergic auto regulation via $5\text{-}HT_{1A}$ receptors but also the GABAergic regulation of the 5-HT release from the nerve terminals of 5-HT neurons widely distributed in the brain.

Acknowledgements

This study was supported by Grants-in-Aid for Scientific research from the Ministry of Education, Science and Culture, Japan and Grant-in-Aid for Comprehensive Research on Aging and Health from the Ministry of Health Labour and Welfare.

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Figure Legends

Fig. 1. Inhibitory Effects of DM on I_{Bac} in the DRN neurons. A: Representative current traces illustrating the effects of DM on I_{Bac}. Neurons were pretreated with DM for 30 or 60 sec before simultaneous application with 3×10^{-5} M baclofen at a V_H of −80 mV. B: Relative peak amplitude of I_{Bac} in the presence of 10^{-5} M DM after 30 or 60 sec pretreatment of DM. All current amplitudes were normalized to the current amplitude induced by 3×10^{-5} M baclofen alone. Data are represented as the mean ±S.E.M. (n = 3~7).

Fig. 2. Concentration-dependent inhibition of I_{Bac} by DM. A: Representative current traces illustrating the concentration-dependent inhibition on I_{Bac} by DM. Neurons were pretreated with DM for 30 sec before simultaneous application with 3×10^{-5} M baclofen at a V_H of −80 mV. B: Concentration-inhibition relationship for DM. All current amplitudes were normalized to the current amplitude induced by 3×10^{-5} M baclofen alone. Data are represented as the mean ± S.E.M. (n = 3). The continuous line was drawn according to the equation (1) in the text.

Fig. 3. Non-competitive inhibition of I_{Bac} by DM. A: Concentration-response relationships for baclofen in the presence (○) or absence (●) of 10^{-5} M DM. Neurons were pretreated with DM for 30 sec before simultaneous application with 3×10^{-5} M baclofen at a V_H of −80 mV. All data were normalized to the current amplitude induced by 3×10^{-5} M baclofen alone (*). Data are represented as the mean ± S.E.M. (n = 3~5). The continuous curves were drawn according to the equation (2) in the text. B: The Lineweaver-Burk plot of the data.

Fig. 4. Effects of DM on the current-voltage (*I*–*V*) relationship for I_{Bac}. A: *I*–*V* relationship for the peak amplitude of I_{Bac} induced by 3×10^{-5} M baclofen in the absence (●) or the presence (○) of 10^{-5} M DM. Neurons were pretreated with DM for 30 sec before simultaneous application with 3×10^{-5} M baclofen at each membrane potential. All data were normalized to the currents induced by 3×10^{-5} M baclofen alone at a V_H of −80 mV (*). B: Percent inhibition of I_{Bac} in the presence of 10^{-5} M DM at various holding potentials. Each column represent the mean ± S.E.M. (n = 3~5).