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Modulation of nicotinic ACh-, $GABA_A$- and 5-HT_3-receptor functions by external H-7, a protein kinase inhibitor, in rat sensory neurones

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1 The effects of external H-7, a potent protein kinase inhibitor, on the responses mediated by γ-aminobutyric acid A type ($GABA_A$)-, nicotinic acetylcholine (nicotinic ACh)-, ionotropic 5-hydroxytryptamine (5-HT_3)-, adenosine 5′-triphosphate (ATP)-, N-methyl-D-aspartate (NMDA)- and kainate (KA)-receptors were studied in freshly dissociated rat dorsal root ganglion neurone by use of whole cell patch-clamp technique.

2 External H-7 (1–1000 μM) produced a reversible, dose-dependent inhibition of whole cell currents activated by GABA, ACh and 5-HT.

3 Whole-cell currents evoked by ATP, 2-methylthio-ATP, NMDA and KA were insensitive to external H-7.

4 External H-7 shifted the dose-response curve of GABA-activated currents downward without changing the EC_{50} significantly (from 15.0 ± 4.0 μM to 18.0 ± 5.0 μM). The maximum response to GABA was depressed by 34.0 ± 5.3%. This inhibitory action of H-7 was voltage-independent.

5 Intracellular application of H-7 (20 μM), cyclic AMP (1 mM) and BAPTA (10 mM) could not reverse the H-7 inhibition of GABA-activated currents.

6 The results suggest that external H-7 selectively and allosterically modulates the functions of $GABA_A$-, nicotine ACh- and 5-HT_3 receptors via a common conserved site in the external domain of these receptors.

Keywords: Nicotinic receptor superfamily; P2X receptor; modulation; glutamate receptors; H-7; whole cell patch clamp recording; dorsal root ganglion

Introduction

Based on the findings of molecular cloning, functional expression and structural diversity of ligand-gated ion channel (LGIC) receptors, three groups of LGIC superfamily of receptors have been proposed: the nicotinic acetylcholine (ACh) receptor family (Schofield *et al*., 1987; Grenningloh *et al*., 1987; Sontheimer *et al*., 1989; Barnard, 1992; Devillers-Thiery *et al*., 1993; Unwin, 1993; Ortells & Lunt, 1995); the glutamate (Glu) receptor family (Hollmann & Heinemann, 1993; McBain & Mayer, 1994) and the adenosine 5′-triphosphate (ATP, P2X) receptor family (Valera *et al*., 1994; Brake *et al*., 1995). The nicotinic acetylcholine receptor family, which encompasses nicotinic AChR, ionotropic 5-hydroxytryptamine receptor (5-HT_3R), γ-aminobutyric $acid_A$ receptor ($GABA_A$R) and glycine receptor (GlyR), constitutes a subset of the fast-acting LGICs superfamily. A number of structural and functional similarities have been revealed between them and they are considered to be a gene superfamily with a common ancestor (Ortells & Lunt, 1995). These three groups of the LGIC superfamily of receptors share some common functional characteristics despite their lack of overall homology: (i) the control of an ionic channel by activation of them does not need intracellular transduction pathways, in other words, binding of agonists to the bindings sites results in opening of channels by means of conformational changes; (ii) besides the bindings sites for agonists or corresponding competitive antagonists, there exist several other binding sites in the LGIC receptors, such as that for allosteric modulatory agents, for example, benzodiazepines and barbiturates binding sites in $GABA_A$R (Sieghart, 1995; Smith & Olsen, 1995); glycine binding site in *N*-methyl-D-aspartate (NMDA) receptors (McBain & Mayer, 1994); zinc and proton binding sites in ATPR (Li *et al*., 1993; 1996); (iii) all receptors exhibit a fast conductance change upon binding of agonists, the ion permeation of LGIC receptor can be distinguished into two groups, one is the excitatory transmitter-gated receptors which are non-selectively permeable to cations in general, e.g. nicotinic AChR, NMDAR, 5-HT_3R, ATPR, another is permeable to anions and regarded as the inhibitory transmitter-gated receptors, e.g., GlyR, $GABA_A$R. Although it has been shown that a few ligands exert cross effects among these receptors (Bijak *et al*., 1991; Zhang & Feltz, 1991; Brake *et al*., 1994), no information is available about one chemical that can affect an entire group of the receptor superfamily. In addition, experiments have suggested that some second messenger system modulators could allosterically regulate LGIC receptor functions independently of cytoplasmic protein phosphorylation in the central nervous system (Harrison & Lambert, 1989; Lambert & Harrison, 1989; Leidenheimer *et al*., 1990; 1991). In the present study, we showed that extracellular application of 1-(5-isoquinoline-sulphonyl)-2-methyl-piperazine dihydro-chloride (H-7, Figure 1), a protein kinase inhibitor (Hidaka & Hagiwara, 1987; Corradetti *et al*., 1989) selectively inhibited whole cell currents mediated by receptors in the nicotinic acetylcholine receptor family in acutely isolated dorsal root ganglion (DRG) neurones of the rat.

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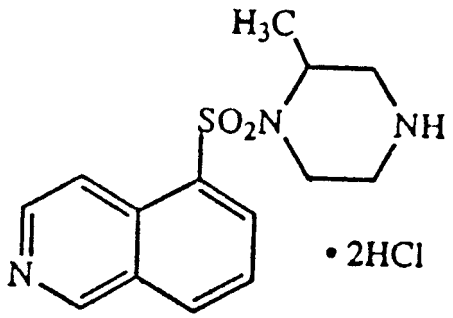

Figure 1 Chemical structure of H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride.

Methods

Cell preparation

Freshly isolated neurones from DRG were prepared as described previously (Li *et al*., 1993; Hu & Li, 1996; Hu *et al*., 1997). Two to three week-old Sprague-Dawley rats were decapitated, and DRG in the thoracic and lumbar segments were isolated rapidly and transferred to a petri dish containing Dulbecco's modified Eagle's medium (DMEM, Sigma) and cut into pieces. The ganglion fragments then were placed in a flask containing 5 ml of DMEM, in which trypsin (type III, Sigma) 0.5 mg ml^{-1}, collagenase (type I, Sigma) 1 mg ml^{-1} and DNase (type IV, Sigma) 0.1 mg ml^{-1} had been dissolved, and incubated at 35°C for 35–40 min in a water bath shaker. Soybean trypsin inhibitor (type II-S, Sigma) 1.25 mg ml^{-1} was then added to stop trypsin digestion. Neurones were placed into a 35 mm culture dish and kept for at least another 30 min before electrophysiological recording. The neurones selected in this investigation were 15–60 μm in diameter (Hu *et al*., 1997).

Electrophysiological recordings

Whole cell patch-clamp recordings were carried out at room temperature using a PC-II patch clamp amplifier (Huazhong Univ. of Sci. & Tech., Wuhan, China). Patch-pipettes were filled with an intracellular solution containing (in mM): CsCl 140, $CaCl_2$ 1, $MgCl_2$ 2.5, HEPES 10, EGTA 11 and ATP 5; pH was buffered to 7.3 with CsOH and osmolarity was adjusted to 310 mosmol with sucrose. The resistance between the recording pipette filled with internal solution and the reference electrode was 2–4 MΩ. Cells were continuously superfused at 1–2 ml min^{-1} with an extracellular medium containing (in mM): NaCl 150, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2, HEPES 10 and D-glucose 10; pH was buffered to 7.3 with NaOH and osmolarity was adjusted to 340 mosmol. Membrane currents were filtered at 1 KHz, data were stored and analysed on a laboratory computer with a data acquisition software and hardware system (Huazhong Univ. of Sci. & Tech., Wuhan, China) or recorded by a pen recorder (Nihon Kohden). Membrane potential was usually held at −60 mV, except where indicated.

Drug application

Drugs used in the experiments were GABA (Sigma), bicuculline (Sigma), acetylcholine (Sigma), hexamethonium (hex) (Sigma), (+)-tubocurarine (+-Tc) (Sigma), ATP (Sigma), Suramin (RBI), 5-hydroxytryptamine (Sigma), 3-tropanyl- indole-3-carboxylate hydrochloride (ICS 205 930 (RBI), *N*-methyl-D-aspartate (NMDA) (Sigma), (±)-2-amino-5-phosphonopentanoic acid (AP-5) (Sigma), kainate (KA) (Sigma), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (RBI), 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydro-chloride (H-7) (RBI), *bis* (2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate (BAPTA; Sigma), aden-osine 3′:5′-cyclic monophosphate (cyclic AMP) (Chinese academy of Sciences), 2-methylthio-ATP (RBI). All drugs were dissolved daily in the external solution just before use and delivered by gravity flow from a linear barrel array consisting of fused silica tubes (o.d./i.d. = 500/200 μm) connected to a series of independent reservoirs. The distance from the end of the tubes to the cell examined was around 100 μm. This rapid solution exchange system was manipulated by shifting the tubes horizontally with a micromanipulator. The interval between GABA applications was at least 4 min in order to avoid desensitization. H-7 was preapplied externally for 30–90 s. In some experiments the drugs (H-7, BAPTA, cyclic AMP) needed to be applied intracellularly were dissolved in the internal solution.

Statistics

Data were compared statistically by Student's *t* test or one-way ANOVA, as appropriate. Data are presented as means ± s.e. mean.

Results

Effects of H-7 on GABA-, ACh-, 5-HT-, ATP-, NMDA- and KA-activated currents

The majority of the cells examined responded to the external application of GABA (1–1000 μM) (124/126), ATP (1–100 μM) (118/126), ACh (10–100 μM) (107/126) and NMDA (10–1000 μM) (34/48), about half were sensitive to 5-HT (10–100 μM) (64/126) and KA (10–1000 μM) (26/48). Responses

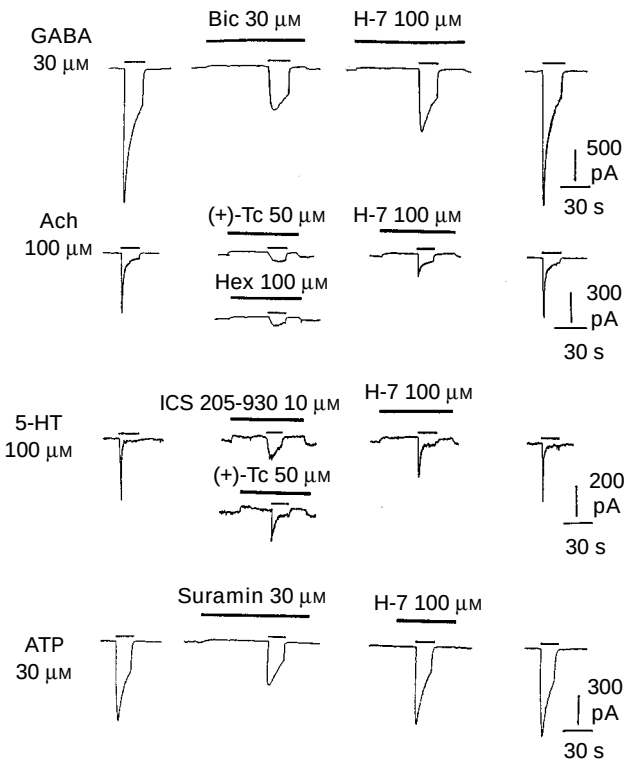

Figure 2 Modulation by H-7 of responses mediated by nicotinic AChR, $GABA_AR$ and $5\text{-}HT_3R$ in the same rat freshly isolated DRG neurone. The cell was voltage-clamped at −60 mV. The inward current traces show GABA-, ACh-, 5-HT- and ATP-activated currents, which were blocked by their corresponding receptor antagonists, bicuculline (Bic), (+)-tubocurarine ((+)-Tc)/hexamethonium (Hex), ICS 205 930/(+)-tubocurarine and suramin (shown in the second column from left to right), respectively. After the currents had recovered fully to their control value (omitted in the figure), they were pretreated with H-7 for 30–90 s before the application of the receptor agonists mentioned above. H-7 (100 μM) exerted a marked and reversible inhibition of GABA (30 μM)-, ACh (100 μM)-, 5-HT (100 μM)-activated currents, while it did not affect ATP (30 μM)-activated currents.

activated by GABA, ATP, ACh and 5-HT were inward currents with apparent desensitization especially at high concentrations. NMDA and KA activated small inward currents with no obvious desensitization while sometimes a transient peak current was seen in KA-activated currents. However, in all DRG neurones tested, no responses could be evoked by glycine (1 mM) which also belongs to the nicotinic receptor superfamily (Grenningloh *et al.*, 1987; Ortells & Lunt, 1995). External application of H-7 (1–1000 μM) itself induced an inward (11.1%) current with an average magnitude of 128.5±4.5 pA, outward (14.3%) current with an average magnitude of 108.4±5.2 pA or no responses (74.6%) in the cells examined ($n=126$). The ionic mechanism underlying these responses is beyond the scope of this paper. Preapplied H-7 depressed GABA-, ACh- and 5-HT-activated currents markedly and reversibly in all neurones tested ($n=126$), there was no relationship between H-7-induced membrane currents and the inhibition of nicotinic receptor superfamily functions by H-7. Figure 2 shows GABA (30 μM), 5-HT (100 μM), ACh (100 μM) and ATP (30 μM) activated inward currents in a DRG cell and the inhibitory effects of their corresponding receptor antagonists. External H-7 (100 μM) depressed GABA (30 μM)- ACh (100 μM)- and 5-HT (100 μM)-activated currents markedly and reversibly without affecting ATP (30 μM)-activated current. Figure 3 shows that H-7 (30 μM) inhibited GABA (100 μM)-activated current while 2-methylthio-ATP (10 μM)-, NMDA (100 μM)- and KA (100 μM)-activated currents were not affected by H-7. Figure 4 shows that H-7 (30 μM) inhibited responses mediated by any of the nicotinic acetylcholine receptor family, activated by 100 μM agonist, to the same extent, i.e. 61.6±4.7% for nicotinic AChR, 60.6±3.6% for $GABA_AR$ and 63.8±5.8% (means±s.e.mean)

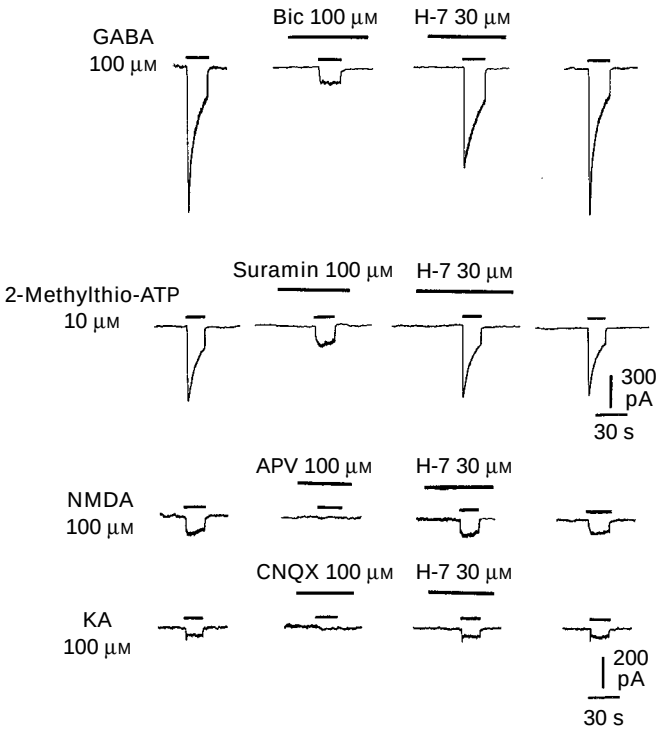

Figure 3 Effects of extracellular H-7 (30 μM) on GABA (100 μM)-, 2-methylthio-ATP (10 μM)-, NMDA (100 μM)- and KA (100 μM)-activated currents. Current traces were obtained from a DRG cell voltage clamped at −50 mV. The GABA (100 μM)-, 2-methylthio-ATP (10 μM)-, NMDA (100 μM)- and KA (100 μM)-activated currents were blocked by their corresponding receptor antagonists, bicuculline (100 μM), suramin (100 μM), AP-5 (100 μM) and CNQX (100 μM), respectively. H-7 (30 μM) depressed the GABA-activated current, while no inhibition was observed for 2-methylthio-ATP-, NMDA- and KA-activated currents. The calibration bar (300 pA, 30 s) was for GABA- and 2-methylthio-ATP-activated currents and the other one (200 pA, 30 s) was for NMDA- and KA-activated currents.

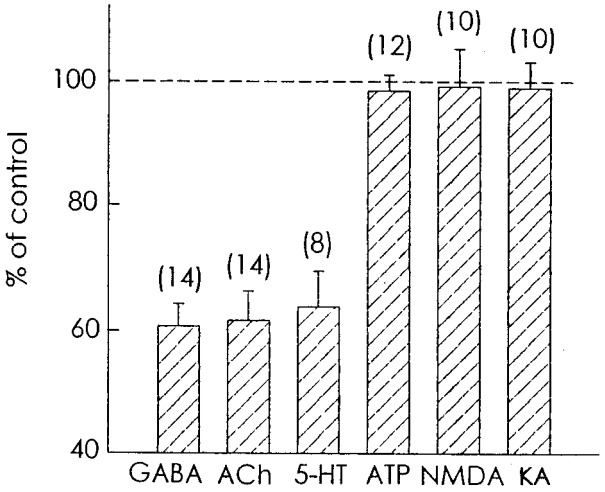

Figure 4 Effects of extracellular H-7 (30 μM) on currents activated by ACh (100 μM), GABA (100 μM), 5-HT (100 μM), ATP (100 μM), NMDA (100 μM) and KA (100 μM). The modulatory effects of external pretreatment with H-7 (30 μM) for 30 to 90 s on ACh (100 μM)-, GABA (100 μM)- and 5-HT (100 μM)-activated currents are shown, the inhibitory potency was not significantly different between the groups. However, H-7 did not affect ATP (100 μM)-, NMDA (100 μM)- and KA (100 μM)-activated currents.

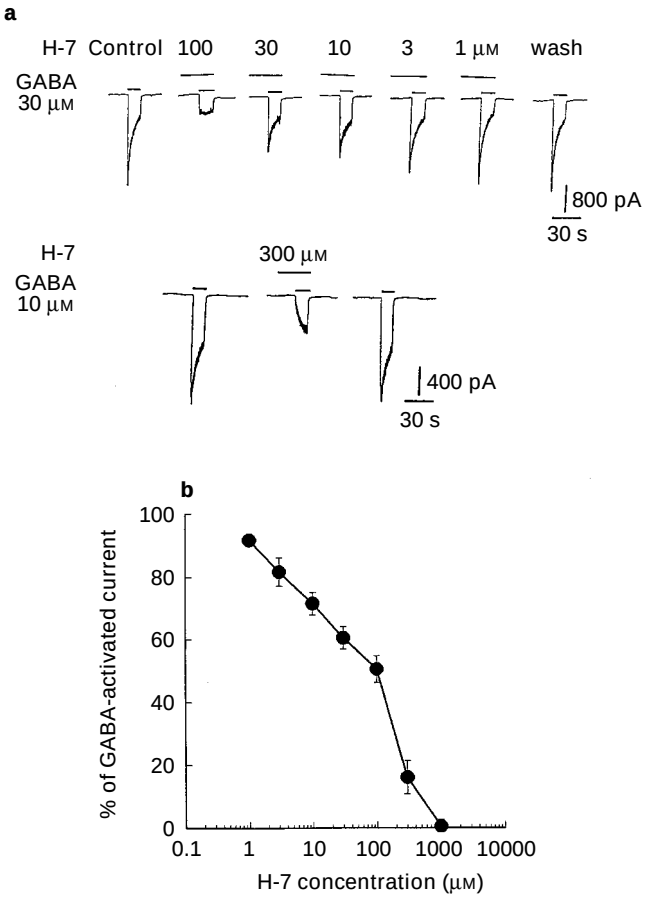

Figure 5 Dose-dependent inhibition of external H-7 on GABA-activated currents. (a) Current traces (top) show that H-7 (1–100 μM) inhibited GABA (30 μM)-activated current in a concentration-dependent manner. The lower current traces show that 300 μM H-7 almost completely inhibited the peak current activated by 10 μM GABA. Data were obtained from a DRG cell voltage-clamped at −60 mV (b) Demonstrates the dose-response curve for H-7 (1–1000 μM) inhibition of GABA (30 μM)-activated currents. Each point represents the mean of 7–24 neurones (except $n=4$ for 1000 μM); vertical lines show s.e.mean.

for 5-HT_3R, while no inhibition was observed of currents mediated by ATPR, NMDAR and KAR.

For further analysis of the inhibition by H-7 of the functions of these receptors in the nicotinic acetylcholine receptor family in detail, the $GABA_A$R was selected as a paradigm and the results obtained are as follows. Similar results were also obtained in nicotinic AChR and 5-HT_3R (not shown). Figure 5a illustrates that the modulatory effect of H-7 on GABA-activated current (I_{GABA}) was concentration-dependent and reversible. In all the neurones examined 1, 3, 10, 30, 100, 300 and 1000 μM H-7 depressed I_{GABA} to 91.7±1.9%, 81.6±4.5%, 71.5±3.6%, 60.5±3.6%, 50.5±4.3%, 16.1±5.3% and 0.5±2.3%, respectively (means±s.e.mean) (Figure 5b).

Dose-response curves for GABA-activated currents before and after pretreatment with H-7

H-7 may have reduced GABA-activated responses by decreasing the binding of the agonist to the receptor. In order to determine if H-7 reduced the affinity of the receptor for GABA, the dose-response curves for GABA-activated currents were examined in the presence and absence of H-7. Control responses were first obtained by applying various concentrations of GABA (1–1000 μM). Cells were then perfused for 30 to 90 s with H-7 (30 μM) and GABA was subsequently re-applied in the continued presence of H-7. Individual dose-response curves were constructed from the control responses for each cell and the EC_{50} values were determined from the fitted curves. All GABA responses at various concentrations were normalized to the peak response induced by 1000 μM GABA. H-7 (30 μM) produced an obvious downward shift of the GABA concentration-response curve. The maximum value of the GABA concentration-response curve was depressed by 34.0±5.3% ($n=5$) while the threshold values in both curves were the same (about 1 μM). The EC_{50} values of the two curves were very close (15.0±4.0 μM and 18.0±5.0 μM). Therefore, H-7 did not substantially modulate the apparent affinity of the receptor for GABA, suggesting a non-competitive mechanism of action. H-7 (30 μM) also did not alter the slope factor (1.3±0.2 and 1.1±0.3 for GABA alone and GABA plus H-7, respectively) (Figure 6a).

Effect of voltage on H-7 inhibition of GABA-activated current

Figure 6b shows the current-voltage (*I*-V) relationships for I_{GABA} in the presence and absence of 30 μM H-7. The reversal potential for I_{GABA} in the present study was around −4 mV, a value close to the Cl^- equilibrium potential (E_{Cl}) of −2.2 mV, which was calculated from the Nernst equation according to

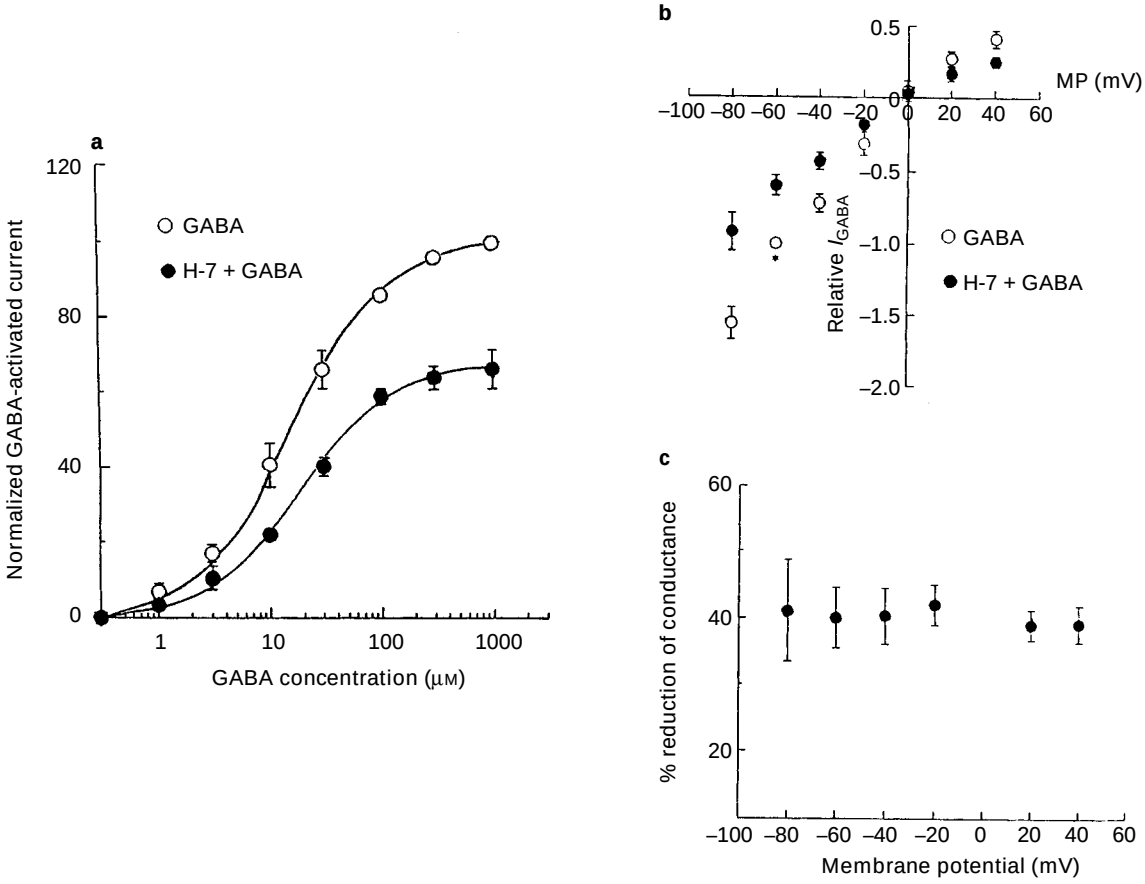

Figure 6 The inhibition by H-7 of GABA-activated currents was noncompetitive and voltage-independent. (a) Dose-response curves for GABA-activated currents before and after pretreatment with H-7. Each point represents the mean GABA-activated current from 8–16 neurones at −60 mV; vertical lines show s.e.mean. All GABA responses at various concentrations were normalized to the peak response induced by 1000 μM GABA. The curves were the best fit of the data to the logistic equation $Y = E_{max}/[1+(K_d/c)^n]$, where C is the concentration of GABA, Y is the fraction of the maximum value, K_d, the dissociation constant of the $GABA_A$ receptor. No significant differences were observed in the absence or presence of 30 μM H-7, for the K_d: 15±4 μM ($n=6$), 18±5 μM ($n=7$), respectively, or the Hill coefficients (n_H): 1.3±0.3 and 1.1±0.2, respectively ($P>0.05$, Student's test). (b) *I*-V relationship of GABA (30 μM)-activated currents before and after pretreatment with H-7 (30 μM). All responses were normalized to the peak response induced by GABA (30 μM) at −60 mV (see symbol marked with asterisk). H-7 attenuated GABA (30 μM)-activated currents at all holding potentials (HP) while the reversal potentials were both near −4 mV. Each point represents the mean from 6–14 dorsal root ganglion neurones; vertical lines show s.e.mean. (c) The percentage of reduction of 100 μM GABA-activated conductance induced by 30 μM H-7 plotted against the membrane holding potential in DRG neurones. Data points are means ($n=6$) and vertical lines show s.e.mean. There was no evident change in conductance when the voltage was altered between −80 and +40 mV (analysis of variance; $P>0.25$; $n=6$).

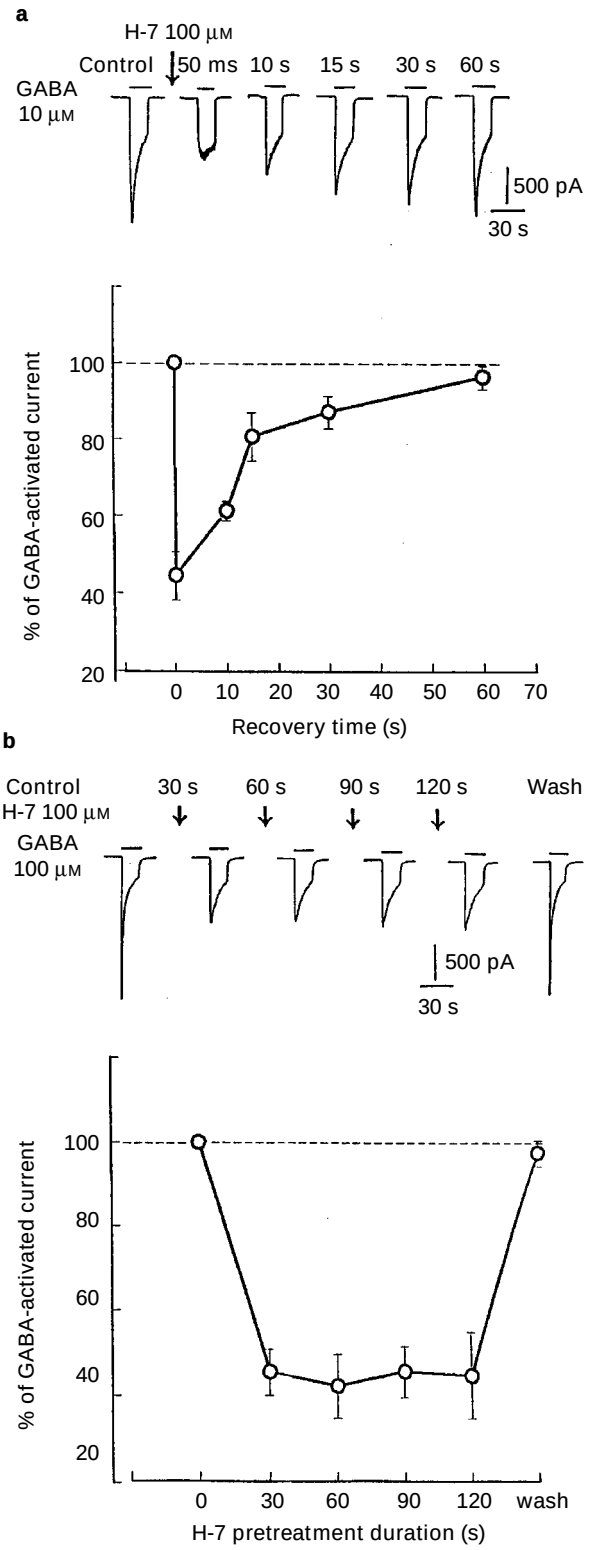

Figure 7 (a) Timecourse of H-7 inhibition of I_{GABA}. Current traces in the upper row illustrate that H-7 inhibition of I_{GABA} was dependent on the intervals between pretreatment of H-7 for 30 s and the application of GABA in a DRG cell. Recovery of I_{GABA} was obtained but not shown in the figure. The graph shows that it took about 60 s for a full recovery from the inhibition by H-7 (100 μM). Data were obtained from ten neurones. The numbers shown over the current traces and on the abssica scale represent the intervals between the treatment with H-7 and the application of GABA (10 μM). (b) Effect of duration of H-7 pretreatment on I_{GABA}. Current traces in the upper panel illustrate the effect of H-7 (100 μM) pretreatment duration on GABA (100 μM)-activated currents in a DRG cell. A 4 min interval was used between each application of H-7 and recovery responses of I_{GABA} were obtained but not shown in the figure. The graph below shows that the inhibitory potency of H-7 (100 μM) was unchanged with an increase in the duration of H-7 pretreatment from 30 to 120 s. Data were obtained from eleven neurones. The numbers over the current traces and on the abssica scale are the duration of H-7 (100 μM) pretreatment.

the conditions of the present study. H-7 depresssed I_{GABA} equally (about 40–45%) at holding potentials (V_H) between −80 and +40 mV. The slope of the *I*-V curve was decreased and the reversal potential was unchanged by the presence of H-7. Figure 6c shows the effect of membrane potential on the conductance of H-7 inhibition of GABA-activated currents. The effect of H-7 on membrane conductance did not differ significantly at membrane holding potentials between −80 and +40 mV. It is evident from the results described above that the inhibitory effect of H-7 on I_{GABA} was voltage-independent. Thus, another possibility that H-7 acted as a channel blocker, which might bind to the MII membrane spanning domain and plug the pore, was ruled out, although voltage-independent, non competitive antagonism might still involve occlusion of the channel.

Lack of involvement of second messenger system

Both the onset of, and recovery from, inhibition by H-7 were fast. Figure 7a shows that the inhibitory effect of H-7 decreased with the increased time interval between H-7 pretreatment and GABA application from 50 ms to 1 min, taking about 1 min for a full recovery. The inhibitory effect of H-7 on I_{GABA} did not increase when duration of pretreatment with H-7 was increased from 50 ms to 120 s, as shown in Figure 7b. The inhibition maintained a steady value even after long-term treatment with H-7.

LGIC receptor functions are modulated by intracellular pathways, for example, protein kinases (Swope *et al.*, 1992; Raymond *et al.*, 1993), intracellular calcium ions (Inoue *et al.*, 1986; Marchenko, 1991). In order to explore whether the inhibitory effect of H-7 was mediated by intracellular events activated thorugh PKA, PKC or Ca^{2+}, H-7 (20 μM) was included into the recording pipette. However, the inclusion of H-7 did not affect GABA-activated current (Figure 8a) and it also could not reverse the inhibitory effect of pretreatment with extracellular H-7 (Figure 8c, f). Intracellular application of cyclic AMP (1 mM) and BAPTA (10 mM), a chelator of calcium ions did not affect I_{GABA}; the I_{GABA} remained stable for about 1.5 h (not shown). Also cyclic AMP and BAPTA did not affect inhibition by H-7 of I_{GABA} (Figure 8d, e, f.).

Discussion

H-7 belongs to the broad-spectrum nonspecific protein kinase inhibitors. It has been shown to penetrate cell membranes and to inhibit protein kinase C (PKC) with an IC_{50} of 6 μM (Hidaka & Hagiwara, 1987). In our recent work, we found that intracellular application of H-7 was effective at blocking the facilitatory effect of substance P on ATP-activated currents (Hu & Li, 1996) and the inhibitory effect of adenosine on GABA-activated currents in rat dorsal root ganglion neurones (Hu & Li, 1997). In the present study, when the neurones were pretreated with H-7 for 30–90 s just before GABA application or coapplied with GABA, the I_{GABA} was attenuated markedly. The result was consistent with previous findings that H-7 inhibits muscimol-stimulated $36Cl^-$ uptake in intact microsacs and in EDTA-treated lysed/resealed microsacs (Leidenheimer *et al.*, 1990).

Many studies have suggested that LGIC receptors are regulated by protein phosphorylation of residues in the intracellular loop, which intervenes between MIII and MIV transmembrane domains, and this is considered as one of the primary modes of regulation of cellular processes influencing synaptic plasticity (Swope *et al.*, 1992; Raymond *et al.*, 1993). Phosphorylation of $GABA_A$ receptor itself or of a closely as-

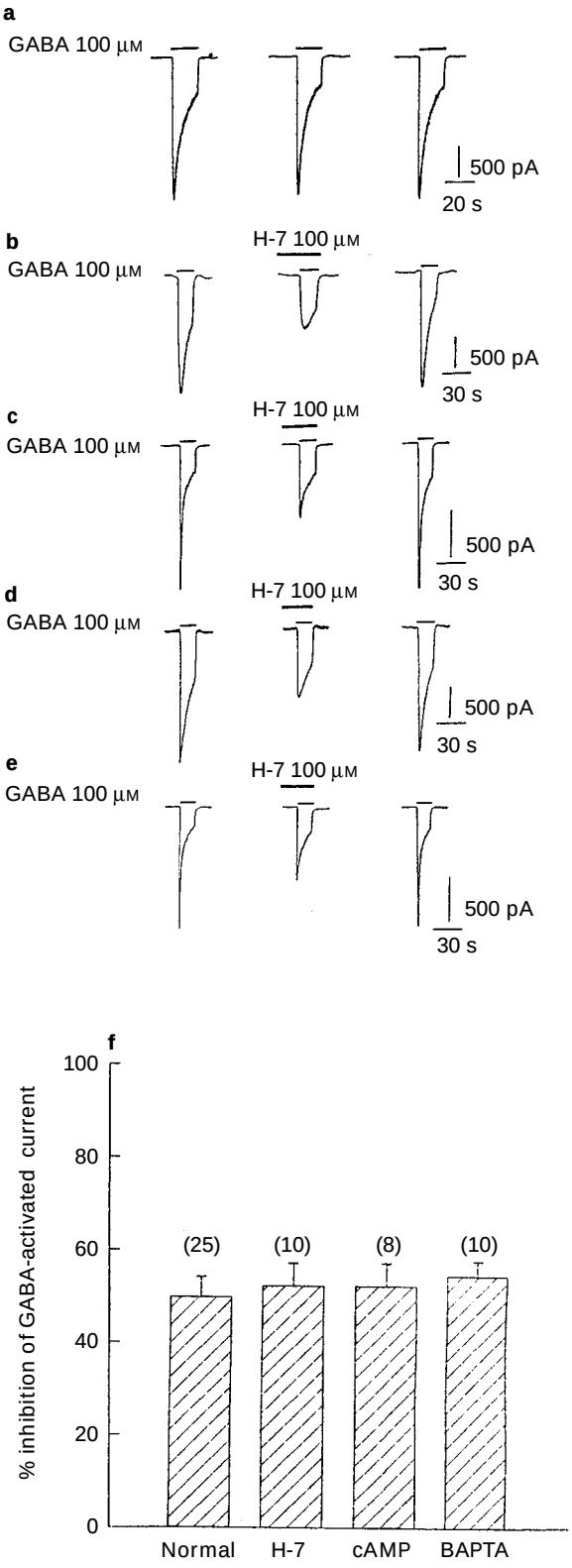

Figure 8 Effects of intracellular application of H-7, cyclic AMP and BAPTA on H-7 inhibition of I_{GABA}. (a) Current traces illustrate that GABA-activated currents kept stable after intracellular application of 20 μM H-7. The left record was taken 1 min after the formation of whole-cell mode, the middle record was taken 30 min later and the right record was taken 90 min later. Similar results were observed in cells dialyzed with cyclic AMP (1 mM) and BAPTA (10 mM) (not shown). Current traces in (b–e) illustrate the effect of normal internal solution (b), intracellular application of H-7 (20 μM) (c), cyclic AMP (1 mM) (d) and BAPTA (10 mM) (e) on H-7 (100 μM) inhibition of GABA (100 μM)-activated currents in four DRG cells, respectively. (f) Shows that intracellular application of H-7 (20 μM), cyclic AMP (1 mM) and BAPTA (10 mM) was unable to reverse the inhibitory effect of extracellular H-7 (100 μM).

sociated protein may be required to maintain receptor function (Stelzer *et al*., 1988; Gyenes *et al*., 1988; Sieghart, 1995). Therefore, the effect of H-7 on the nicotinic acetylcholine receptor superfamily may be due to inhibition of PKC/PKA. However, this possibility could be reasonably excluded: firstly, in our experiment, H-7 was applied externally before the application of GABA and the onset of the H-7 inhibition was rapid (only 50 ms or less); whereas, blockade of PKC/PKA phosphorylation by H-7 needed at least 10–15 min (Corradetti *et al*., 1989). Secondly, the recovery from H-7 inhibition was also quick (about 60 s or less for a fully recovery). Thirdly, the inhibitory effect of H-7 on I_{GABA} was unchanged with long term stimulation by H-7. In contrast, adenosine inhibited GABA-activated current in a cumulative manner, i.e. the inhibitory potency increased with an increase in pretreatment duration and reached its peak at 3 min and this kind of inhibition has been proved to be mediated by protein kinase C (Hu & Li, 1997). Fourthly, intracellular application of H-7, cyclic AMP and BAPTA showed no significant effect on inhibition of I_{GABA} by H-7. Therefore, H-7 might act directly on a binding site located in the extramembrane domain rather than via intracellular transduction pathways.

The subunits of nicotinic AChR, 5-HT_3R, $GABA_A$R and GlyR are all homologous, particularly in their transmembrane segments. However, the subunits of the glutamate-gated channels share no overall homology with these receptors, but do between themselves. All of the glutamate receptor subunits identified to date are two to three times the size of those in the nicotinic receptor superfamily and the most recently proposed model with an extracellular N-terminus, three transmembrane domains and an intracellular C-terminus was also different from those of other LGIC receptors (Roche *et al*., 1996), suggesting the existence of a separate receptor family (Unwin, 1993; McBain & Mayer, 1994). In our experiments, H-7 did not inhibit NMDA- and kainate-activated currents while GABA-activated currents were suppressed markedly. This also suggests functional differences in these two receptor families.

The ATP receptor (P2X receptor) suggested to be $P2X_2$ and/or $P2X_3$ subtypes in rat DRG neurones (Chen *et al*., 1995; Lewis *et al*., 1995), is an ionotropic subtype of ATPR, belonging to the LGIC superfamily and possessing common features of fast acting kinetics similar to other LGIC receptors (Bean, 1992). However, recent studies have revealed unsuspected findings of a novel structural phenotype which is different from other LGIC receptors, not only in the degree of sequence homology, but also in predicted topological structure, such that it has only two membrane spanning domains MI and MII, the N and C terminals being located inside rather than outside the membrane, while a large extracellular loop on which ligand binds intervenes between MI and MII (Valera *et al*., 1994; Brake *et al*., 1994; Surprenant *et al*., 1995). It is also interesting that whole cell currents activated by ATP and 2-methylthio-ATP, which is considered to be the most potent agonist at P2X receptors in peripheral ganglion neurones (Khakh *et al*., 1995), were not affected by H-7, unlike those of nicotinic AChR, $GABA_A$R and 5-HT_3R.

The members of LGIC superfamilies are derived in phylogenetic evolution from their common ancestor. In the long-term evolutionary course, some sequence information of the gene could be conserved in a superfamily. That H-7 allosterically modulates the responses mediated by nicotinic AChR, $GABA_A$R, 5-HT_3R but not ATPR, NMDAR and KAR suggests that there must be a consensus region existing and conserved in the extracellular domain of the former three receptors in the phylogenetic evolutionary course. These results offer a clue to the understanding of the molecular evolution of the LGIC superfamily and further information could be obtained by exploring the relationship between molecular structure and function of LGIC receptors, which could lead to a novel therapeutic target in pharmacology.

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