Strychnine-induced potassium current in CA1 pyramidal neurones of the rat hippocampus

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1 Direct actions of strychnine (Str) and brucine (Bru) on the dissociated hippocampal CA1 neurones of the rat have been investigated with the whole-cell mode of the patch-clamp technique.

2 At a holding potential (V_H) of -20 mV, both Str and Bru elicited outward current at concentrations over 10^{-5} M. The reversal potential of Str-induced current (E_{Str}) was -77.8 mV, which was close to the K^+ equilibrium potential ($E_K = -80.3$ mV). The change in E_{Str} for a ten fold change in extracellular K^+ concentration was 58 mV, indicating that the membrane behaves like a K^+ electrode in the presence of Str.

3 The concentration-response curves for Str and Bru were bell-shaped, and nearly maximum response occurred at 10^{-4} M for Str and 3×10^{-4} M for Bru. The maximum current amplitude induced by Bru was about 80% of that induced by Str. A transient 'hump' current appeared immediately after the wash-out of external solutions containing Str and Bru at concentrations higher than 10^{-4} and 3×10^{-4} M, respectively.

4 The Str-induced current (I_{Str}) was antagonized by K^+ channel blockers such as Ba^{2+} , tetraethylammonium (TEA)-chloride, and 4-aminopyridine (4-AP) in a concentration-dependent manner. I_{Str} was insensitive to glibenclamide, a blocker of ATP-sensitive K^+ channels.

5 Internal perfusion with 10 mM BAPTA did not affect the Str-induced I_K . Depletion of the intracellular Ca^{2+} store by caffeine had no effect, indicating that intracellular Ca^{2+} does not mediate the Str-induced activation of K^+ conductance.

6 Both guanosine-5'-O-3-thiotriphosphate (GTP γ S) and guanosine-5'-O-thiodiphosphate (GDP β S) suppressed the Str-induced I_K , the former action appearing more rapidly than the latter. The results suggest that the GTP binding proteins are involved in this Str response.

7 When neurones were loaded with cholera toxin (CTX) or pertussis toxin (PTX) through a patch pipette, PTX suppressed the Str response whereas CTX did not, suggesting that G_i and/or G_o might be involved in the Str-induced I_K .

Keywords: Hippocampal CA1 neurone in rat; strychnine; brucine; K^+ current; G protein

Introduction

Strychnine (Str) is prepared from dried ripe seeds of *Strychnos nux-vomica* that contains 1.1 to 1.4% Str and a similar amount of closely related alkaloid, brucine (Bru). The Str is known as a selective and competitive antagonist of the glycine receptor Cl^- channel complex, and Str has been used as a valuable tool in physiological and pharmacological studies on glycine receptors. However, many studies also showed that Str inhibits not only the glycine-induced Cl^- current (I_{Cl}) but also the γ -aminobutyric acid (GABA)-induced I_{Cl} in a competitive manner in a concentration range between $0.1 \mu M$ and $100 \mu M$ (Aibara *et al.*, 1991; Shirasaki *et al.*, 1991). Moreover, at the higher concentration, Str inhibits the responses induced by acetylcholine (Phillis & York, 1967; Faber & Klee, 1974), noradrenaline (Phillis & York, 1967), 5-hydroxytryptamine (Phillis & York, 1967; Faber & Klee, 1974), histamine (Groul & Weinreich, 1979), dopamine (Faber & Klee, 1974) or N-methyl-D-aspartate (NMDA) (Bertolino & Stefano, 1988). In addition, voltage-dependent Na^{2+} (Shapiro, 1977), K^+ (Shapiro, 1977) and Ca^{2+} (Oyama *et al.*, 1988) channels are also blocked by Str. Recently, we also found that Str itself induces the K^+ current in rat dorsal root ganglion (DRG) cells (Aibara *et al.*, 1991). These results suggest that Str has multiple pharmacological actions in the concentrations used. In this study, we investigated whether Str could also induce K^+ outward currents in mammalian CNS neurones as well as in DRG neurones. In addition, we studied the intracellular mechanisms of this response.

Methods

Preparations

The method used to dissociate rat hippocampal CA1 pyramidal neurones has been described in a previous paper (Ito *et al.*, 1991). Briefly, 1- to 2-week-old Wistar rats were decapitated under ether anaesthesia and $350 \mu m$ transverse slices of the brains were prepared with a microslicer (D.S.K., DTK-1000). The brain slices were incubated in Krebs solution containing 0.01% pronase [Calbiochem] for 15 to 20 min at $31^\circ C$, and subsequently treated with 0.01% thermolysin [Sigma]. The CA1 region of the hippocampus was micro-punched out of the slices, then dissociated mechanically with fine glass pipettes. Dissociated hippocampal CA1 pyramidal neurones adhered to the bottom of the culture dish within 30 min. In the present experiment, we used neurones that maintained their original morphological features, such as the dendritic processes.

Solutions

The normal external solution contained (in mM): NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10 and HEPES 10, buffered to pH 7.4 with Tris-base. The Ca^{2+} -free 2 mM EGTA solution contained (in mM): NaCl 150, KCl 5, $MgCl_2$ 1, EGTA 2, glucose 10 and HEPES 10, buffered to pH 7.4 with Tris-base. In the conventional patch-clamp study, the normal internal solution contained (in mM): KCl 50, K-gluconate 70, NaCl 20, $CaCl_2$ 0.25, $MgCl_2$ 6, Na_2ATP 5,

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EGTA 5 and HEPES 10, buffered to pH 7.2 with Tris-base. In the perforated patch-clamp study, nystatin was added at a final concentration of $150 \mu\text{g ml}^{-1}$ to the normal internal solution, the composition of which was (in mM): KCl 150 and HEPES 10. The pH was adjusted to 7.2 with Tris-base.

Electrical recording

Two types of voltage-clamp recordings were made in the whole-cell mode, either by use of a conventional patch-clamp (Hamill *et al.*, 1981) or by a nystatin perforated patch-clamp technique (Horn & Marty, 1988). In both recordings, the resistance between the glass pipette filled with internal solution and the reference electrode was 4 to 8 M Ω . The current and voltage were measured with a patch-clamp amplifier (List Medical, EPC-7), monitored on a storage oscilloscope (Toshiba, 10M63) and a pen-recorder (Sanei, RECTI-HORIZ-8K), and recorded on video tapes after digitization with a PCM processor (Sony, PCM-501ES). All experiments were carried out at room temperature (20 to 23°C).

Drugs

The following drugs were used: strychnine hydrochloride (Str), kainic acid (KA), 10,11-dimethoxystrychnine, glibenclamide, *bis*(γ -aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), guanosine-5'-O-3-thiotriphosphate (GTP γ S), guanosine-5'-O-thiophosphate (GDP β S), cholera toxin (CTX) [Sigma], 4-aminopyridine (4-AP), tetraethylammonium (TEA)-chloride [Tokyo Kasei], caffeine [Ishizu] and pertussis toxin (PTX) [Seikagaku Corporation]. All drugs were dissolved in the external or internal solution just before use. External solutions were exchanged by a rapid application technique termed the 'Y-tube' method, which allows the exchange of solutions surrounding a neurone within 10 to 20 ms (Akaike *et al.*, 1989; Murase *et al.*, 1989).

Results

Strychnine- and brucine-induced currents

Experiments were carried out at a holding potential (V_H) of -20 mV in the conventional whole-cell mode. Str and Bru were applied every 2 min, at which interval constant responses could be repeatedly evoked for 1 h or more. The threshold concentrations for both the Str- and Bru-induced current (I_{Str} and I_{Bru} , respectively) were approximately 10^{-5} M. The I_{Str} increased with increasing Str concentration in the range 10^{-5} to 10^{-4} M. The I_{Bru} also increased as Bru concentration increased in the range 10^{-5} to 3×10^{-4} M (Figure 1). The amplitude of the maximum I_{Bru} was about 80% of that of the maximum I_{Str} . Further increase in Str or Bru concentration beyond that which induced the maximum response decreased the peak current during a continuous application of these drugs. The 'wash-out' of external solution containing Str or Bru at concentrations higher than 10^{-4} M for the former and 3×10^{-4} M for the latter always induced a transient outward 'hump' current (Figure 1a). Figure 1b summarizes the Str- and Bru-induced peak currents plotted as functions of Str and Bru concentrations. When all responses were normalized to the peak current induced by 10^{-4} M Str, the concentration-response curves for Str and Bru showed a bell-shaped pattern.

Current-voltage (*I-V*) relationship for Str response

Figure 2a shows the currents induced by 10^{-4} M Str at various V_H s. In the *I-V* relationship, the responses at various V_H s were normalized to the peak response induced by Str at a V_H of -50 mV (*). The reversal potential of I_{Str} (E_{Str}) estimated from the intersection on the voltage axis in the *I-V* curve was -77.8 ± 1.3 mV (mean $\pm$ s.e.mean, $n = 5$). The

Figure 1 (a) Strychnine (upper panel) - and brucine (lower panel)-induced outward currents in dissociated hippocampal CA1 neurones. V_H was -20 mV. The neurones were exposed to various concentrations of strychnine (Str) or brucine (Bru) during the periods indicated by the horizontal bar below each response. (b) Concentration-response curves for Str (●) and Bru (○). Each point shows the average of 5–7 experiments and the vertical bars show $\pm$ s.e.mean. All responses were normalized to the peak current induced by 10^{-4} M Str (see symbol marked with asterisk). The continuous curves were drawn by eye.

Figure 2 Strychnine (Str)-induced currents: (a) 10^{-4} M Str-induced currents at various V_H s in the same cell; (b) current-voltage (*I-V*) relationship of 10^{-4} M Str-induced responses. All responses were normalized to the peak response induced by Str at a V_H of -50 mV (see symbol marked with asterisk). The vertical bars show $\pm$ s.e.mean. (c) Effect of $[K^+]_o$ on the E_{Str} . Dissociated hippocampal CA1 neurones were perfused with external solutions containing various concentrations of K^+ . The $[K^+]_i$ was 120 mM throughout this experiment. Each point is the average of 4–5 experiments. A straight line shows E_K . The vertical bars represent $\pm$ s.e.mean.

value was quite close to the K^+ equilibrium potential (E_K) of -80.3 mV calculated with the Nernst equation for the given extra- and intracellular K^+ concentrations ($[K^+]_o$ and $[K^+]_i$, respectively) (Figure 2b). Moreover, the 'hump' current induced by the removal of high concentrations of Str also reversed at nearly E_K (data not shown). The results indicate that I_{Str} and the 'hump' current are carried by K^+ ions.

When the dissociated neurones were perfused with the normal internal solution containing 120 mM K^+ and the external solutions containing various K^+ concentrations ($[K^+]_o = 5, 10, 30$ and 60 mM), the measured E_{Str} values are

-77.4 ± 1.3 mV ($n = 5$), -52.0 ± 4.2 mV ($n = 4$), -24.5 ± 1.8 mV ($n = 5$), -10.6 ± 0.8 mV ($n = 4$) for 5, 10, 30 and 60 mM [K⁺]_o, respectively (Figure 2c). The change in E_{Str} for a ten fold change in [K⁺]_o was 58 mV, indicating that the cell membrane behaves like a K⁺ electrode in the presence of Str.

Effects of various K⁺ channel blockers on strychnine response

In order to elucidate pharmacological properties of the K⁺ channel activated by Str, the effects of K⁺ channel blockers on I_{Str} were tested at a V_H of -20 mV. The neurones were perfused with normal internal and external solutions. In these experiments, glibenclamide and BaCl₂ were directly dissolved in the standard external solution. The external solution containing 4-AP or TEA was made by replacement of NaCl in the external solution. Ba²⁺, 4-AP and TEA suppressed I_{Str} in a concentration-dependent manner (Figure 3). The inhibitory potency was in the order Ba²⁺ > 4-AP > TEA. Glibenclamide, an ATP-sensitive K⁺ channel blocker, had no effect on I_{Str} at concentrations of 10 to 100 μ M.

Effects of intracellular Ca²⁺ depletion on I_{Str}

To find whether a rise in intracellular free Ca²⁺ is necessary for the Str-induced response, the following two types of study were performed. In the first study, we chelated the intracellular Ca²⁺ by an internal solution containing 10 mM BAPTA. In this experiment, the current recordings were made in the nystatin perforated-patch mode and successively in the conventional whole-cell mode by rupture of the cell membrane. After the I_{Str} was recorded at a V_H of -20 mV using the nystatin perforated patch-clamp technique, the patched membrane was ruptured by applying negative pressure. Successive intracellular perfusions of BAPTA had little effects on the amplitude and kinetics of the I_{Str} in all cells

tested ($n = 4$) (Figure 4a). The next study was performed with the nystatin perforated patch-clamp technique. Caffeine (10⁻² M), which is known to release Ca²⁺ from a caffeine-sensitive Ca²⁺ (CICR) pool in excitable cells (Endo *et al.*, 1970), induced an outward current, and the caffeine response completely recovered within 4 min from a previous exposure to caffeine. In the absence of extracellular Ca²⁺, the first application of caffeine induced an almost identical response. However, the 2nd application after 4 min induced only a small response. Even in this condition of Ca²⁺ depletion of the CICR pool (Akaike & Sadoshima, 1989), Str could induce I_{Str} having a current amplitude ($n = 4$) similar to that in the normal external solution (Figure 4b). These results suggest that the I_{Str} is induced by a mechanism different from that of the caffeine-induced K⁺ current: i.e., the Str response does not result in activation of K⁺ conductance by intracellular Ca²⁺.

Effects of guanosine derivatives on I_{Str}

In order to examine the possible involvement of guanine nucleotide binding proteins (G proteins), the effects of intracellular perfusion with GTP γ S or GDP β S on I_{Str} were examined with the conventional whole-cell patch-clamp technique. The Str was applied every 2 min between each application of kainate (KA), which constantly activates the receptor-channel complex (Wang *et al.*, 1991). When neurones were loaded with 100 μ M GTP γ S through the pipettes at a V_H of -20 mV, I_{Str} was abolished within 12 min whereas the KA-induced inward current was little affected. Intracellular perfusion with 100 μ M GDP β S also suppressed I_{Str} within 30 min. In contrast, during perfusion with the normal internal solution (control), almost the same responses were maintained for more than 30 min (Figure 5). The results suggest that I_{Str} is mediated by G proteins.

Effects of cholera toxin and pertussis toxin on I_{Str}

We examined the type of G proteins involved in I_{Str} . When a conventional whole-cell patch was made with a patch-pipette containing pertussis toxin (PTX) 1 μ g ml⁻¹, the A protomer of islet-activating protein (IAP), together with 1 mM nicotinamide-adenine dinucleotide (NAD), I_{Str} gradually declined to about 35% of its initial amplitude within 30 min

Figure 3 Effects of various K⁺ channel blockers on the I_{Str} . The neurones were pretreated for 1 min with normal external solution containing various K⁺ channel blockers and 10⁻⁴ M (Str) together with one of the blockers was applied, in succession. V_H was -20 mV. (a) All recordings were obtained from the same cell. Open and closed horizontal bars indicate the application of the blockers and Str, respectively. (b) All responses induced by a mixture of 10⁻⁴ M Str and one of the blockers at various concentrations were normalized to the peak current induced by 10⁻⁴ M Str alone. Abscissa scale shows the concentration of blockers: (Δ) Ba²⁺; ($\bullet$) 4-aminopyridine (4-AP); ($\circ$) tetraethylammonium (TEA). Each point shows the mean of 4–6 experiments and vertical bars show $\pm$ s.e.mean.

Figure 4 Effects of intracellular Ca²⁺ depletion on I_{Str} . V_H was -20 mV. (a) Effect of intracellular Ca²⁺ chelator, bis (γ-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), on I_{Str} . Strychnine (Str)-induced (10⁻⁴ M) currents before (left) and 6 min after (right) rupture of the patch membrane with a pipette containing 10 mM BAPTA. I_{Str} was not affected by intracellular perfusion of 10 mM BAPTA. Arrow indicates rupture of the patch membrane. The results are typical of four reproducible observations. (b) Effects of depletion of Ca²⁺ in an intracellular Ca²⁺ store by 10 mM caffeine. Str was given to the neurone before and during superfusion with Ca²⁺-free 2 mM EGTA external solution. The results are typical of four reproducible observations.

Figure 5 Effects of guanosine-5'-O-thiodiphosphate (GDP β S) and guanosine-5'-O-3-thiotriphosphate (GTP γ S) on I_{Str} . (a) Representative recordings of I_{Str} s after 3, 15 and 31 min perfusion with normal (upper), 100 μ M GDP β S containing (middle) and 100 μ M GTP γ S containing (lower) internal solutions. The horizontal bars indicate the period of application of 10^{-4} M Str. (b) Relationships between the time of intracellular perfusion and the relative peak amplitude of I_{Str} s. All responses were normalized to the peak amplitude of I_{Str} 1 min after rupture of the patch membrane. (○), Control; (●) GDP β S; (△), GTP γ S. Data are mean $\pm$ s.e.mean (vertical bars) obtained from 4 to 6 experiments. $V_H = -20$ mV.

after the beginning of PTX perfusion. In contrast, intracellular perfusion with cholera toxin (CTX) 1μ g ml $^{-1}$ exerted little effect on I_{Str} (Figure 6). Since PTX is known to inhibit specifically G_i and G_o by ADP-ribosylation (Katada & Ui, 1982; Florio & Sternweis, 1985; Ewald *et al.*, 1988) the present result suggests that G_i and/or G_o may be involved in the I_{Str} .

Discussion

In these experiments we clearly showed that at high concentrations over 10^{-5} M, Str induced K^+ currents in mammalian CNS neurones (hippocampal CA1 pyramidal neurones). The concentration-response relationship, the current-voltage relationship and the pharmacological properties of I_{Str} in hippocampal CA1 pyramidal neurones were almost identical to those in rat dorsal root ganglion (DRG) cells (Aibara *et al.*, 1991). We also showed that I_{Str} does not result from activation of the ATP-sensitive K^+ channel, based on the absence of an inhibitory effect of glibenclamide.

Shapiro (1977) showed that blockade of voltage-dependent K^+ channels by Str resembles that by intracellular application of certain quaternary ammonium (QA) ions, such as tetraethylammonium compounds and demonstrated that the molecular structure of the most potent QA, ϕC_3 (Armstrong, 1971), is nearly superimposed on that of Str. Using *N*-methylstrychnine, he also reported that Str must cross the membrane and block from the cytoplasmic side in the same fashion as QA ions do. In the present experiments, when the

Figure 6 Effects of cholera toxin (CTX) and pertussis toxin (PTX) on I_{Str} . (a) Representative recordings of I_{Str} after 3, 15 and 31 min intracellular perfusion of CTX, 10μ g ml $^{-1}$, containing (upper) or PTX, 1μ g ml $^{-1}$, containing (lower) internal solutions. The horizontal bars indicate the period of application of 10^{-4} M strychnine (Str). $V_H = -20$ mV. (b) Relationship between the time of internal perfusion and the relative peak amplitude of I_{Str} . All responses were normalized to the peak amplitude of I_{Str} 1 min after rupture of the patch membrane. (○) CTX; (●) PTX. Data are mean $\pm$ s.e.mean (vertical bars) obtained from 5 to 7 experiments.

peak I_{Str} was suppressed with concentrations of Str over 10^{-4} M, the wash-out of Str always induced a transient 'hump' current (Figure 1a). Such decreases of the peak currents in the presence of Str may be due to blockade of Str-activated K^+ channels by the Str molecule itself, in the same fashion as Str blocks voltage-dependent K^+ channels. The hump immediately after the wash-out of high concentrations of Str is probably due to activation of undensitized receptors by Str, released from channels blocked by Str. Similar humps were observed at the wash-out of high concentrations of acetylcholine in the voltage-clamped frog endplate (Adams, 1975) and pentobarbitone in the frog DRG neurone (Akaike *et al.*, 1987).

Bru is less potent than Str as a convulsant. It produces convulsions only with 40 times the effective dose of Str (Webster, 1930) but in this study, the potency of Bru as an agonist for evoking K^+ current seemed to be almost 10 times as weak as that of Str, judging from the concentration-response relationship (Figure 1b).

A vast array of cellular signal transduction processes arise from combinations of many different types of agonists, receptors, effectors and coupling molecules such as heterotrimeric G proteins or protein kinases that connect the receptors to effectors. In this case, we supposed that Str might activate the simplest multiple signalling pathway that is composed of three elements: receptor, G protein and ion channel. There are three reasons for this: (1) The conventional whole-cell patch configuration cannot preserve cytoplasmic constituents that are necessary to maintain the second messenger system in the dissociated neurones because of 'wash out' (Wakamori *et al.*, 1991). However, I_{Str} was evoked even 30 min after the conventional whole-cell had been initiated. (2) Guanosine derivatives and PTX suppressed the Str res-

ponses. (3) The Str response was not involved in intracellular Ca²⁺.

In GTPγS-loaded cells, *I*_{Str} was suppressed within 15 min, suggesting that inhibition occurred at the level of the G protein, due to its continuous activation. As a result, the activity of the G protein would not be transmitted efficiently to the K⁺ channel. A similar mechanism has been proposed for the muscarinic activation of the anomalous rectification in heart muscle cells (Kurachi *et al.*, 1987). Under normal conditions, however, an apparent desensitization of the Str-induced outward currents was scarcely observed in hippocampal CA1 neurones, even when almost saturated concentrations of the agonists were applied. This result is not compatible with inactivation of the G protein involved. In heart muscle cells, increased concentrations of ACh markedly facilitated inactivation of the muscarinic receptor-activated anomalous rectifying K⁺ current. Our results may suggest that infusion of GTPγS through the patch-pipette spontaneously activates not only a G protein inducing the K⁺ current by Str but also another G protein which may be involved in inactivation of the activated K⁺ channel. It is well known that adenylate cyclase is controlled in opposing

ways by an inhibitory G protein (G_i) and an excitatory G protein (G_s) (reviewed by Gilman, 1987). Therefore, a similar mechanism might function in the Str-activated K⁺ channel. In the present case, the inhibitory G protein system may act on the activated K⁺ channel since in the GTPγS-loaded cells inactivation of the Str response occurred only after the current was produced. Recently, closing of the S-channel produced by 5-hydroxytryptamine (5-HT) was found to be overridden by Phe-Met-Arg-Phe-NH₂ (FMRFamide; Belardetti *et al.*, 1987), probably by a reversal of the phosphorylation of the channel or its closely related protein induced by 5-HT (Sweatt *et al.*, 1989). Moreover, Inoue & Kuriyama (1991) reported that intracellular GTPγS suppressed muscarinic responses in guinea-pig chromaffin cells and they considered the same mechanism in discussing that phenomenon. Therefore, it is of interest that Str induced K⁺ current, probably due to activation of G proteins, particularly G_i and/or G_o, in mammalian CNS.

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