Effects of H-89, an inhibitor of protein kinase A, on the acetylcholine release from myenteric plexus of guinea pig ileum

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Abstract

In order to clarify the involvement of cyclic AMP-dependent protein kinase (protein kinase A) in acetylcholine (ACh) release from myenteric plexus of guinea pig ileum, the effect of H-89, a specific inhibitor of protein kinase A, on the ACh release was investigated. H-89 (0.1-10 μM) inhibited the spontaneous and nicotine-induced release of ACh in a concentration dependent manner. It at 1 μM decreased both kinds of release of ACh to almost half of the control, but it did not affect the ACh release evoked by electrical field stimulation and by 5-hydroxytryptamine. H-89 had no significant effect on the indomethacin (IND), an inhibitor of PG synthesis, -insensitive component of the spontaneous and nicotine-induced release of ACh. OP-41483, an analog of PGI₂ and forskolin, an activator of adenylate cyclase, reversed the inhibitory effect of IND on the ACh release. H-89 at 1 μM completely inhibited the reverse effects of OP-41483 and forskolin. These results suggest that activation of protein kinase A is essential for modulation of the nicotine-induced and spontaneous ACh release from myenteric plexus of guinea pig ileum and the activity of protein kinase A is regulated by endogenous PGs via intracellular cyclic AMP level.

Key words: H-89; Protein kinase A; ACh release; Prostaglandins; Myenteric plexus

Introduction

In myenteric plexus of guinea pig ileum, forskolin, an activator of adenylate cyclase, and isobutylmethylxanthine, an inhibitor of phosphodiesterase, increased the spontaneous and the stimuli-induced release of acetylcholine (ACh) (Seamon et al., 1981; Daly et al., 1982). Membrane-permeable analogs of adenosine 3', 5'-cyclic monophosphate (cyclic AMP), 8-bromo and dibutyl cyclic AMP also increased the ACh release (Yau et al., 1987). Adenosine and 2', 5'-dideoxyadenosine, inhibitors of adenylate cyclase, inhibited the release of ACh induced by nicotinic agonist, forskolin and agonists which were increased a content of cyclic AMP (Reese and Cooper, 1982; Wiley and Owyang, 1987). These results suggest that changes in cyclic
AMP level in enteric neurons regulate the ACh release.

Indomethacin (IND), an inhibitor of prostaglandin (PG) synthesis, inhibited nicotine- and substance P-induced release of ACh from myenteric plexus of guinea pig ileum (Yagasaki et al., 1984; Takeuchi et al., 1991). PGI₂ and PGE₂ reversed the inhibitory effects of IND on the ACh release (Takeuchi et al., 1991; Fukunaga et al., 1993). PGs were reported to increase cyclic AMP level in various tissues including the peripheral neuronal tissues (Harris et al., 1979; Kalix, 1979). Some other agents which are known to increase the cyclic AMP level also reversed the inhibitory effect of IND (Takeuchi et al., 1992). These findings suggest that endogenous PGs, especially PGI₂ and PGE₂, modulate the ACh release induced by activation of nicotinic and tachykinin receptors, and that cyclic AMP closely relates to the modulation by PGs.

Cyclic AMP-dependent protein kinase (protein kinase A) is present in most tissues, including the myenteric neurons of guinea pigs (Jeitner et al., 1991; Cohen, 1992). Protein kinase A modulates the signal transduction by phosphorylating a variety of receptors and ion channels in the nervous tissues (Walaas and Greengard, 1991). However, no direct evidence indicates that protein kinase A is involved in the ACh release from cholinergic neurons. Since the activity of protein kinase A is regulated by cyclic AMP (Taylor et al., 1988), it is interesting to know whether protein kinase A is involved in the regulation of ACh release from myenteric plexus of guinea pig ileum. H-89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide, is a selective inhibitor of protein kinase A. It inhibits the activity of protein kinase A more strongly than those of protein kinase C, G and Ca²⁺/calmodulin kinase (Chijiwa et al., 1990; Geilen et al., 1992). In the present study, we investigate the effect of H-89 on the ACh release induced by various stimuli to determine involvement of protein kinase A within the intracellular signal pathway from activation of receptors to release of ACh in the myenteric plexus of guinea pig ileum.

Methods

Male guinea pigs, weighing 300 to 700 g, were lightly anesthetized with ether and then stunned and bled. The longitudinal muscle of the ileum with the myenteric plexus attached was prepared as previously described (Yagasaki et al., 1981). The preparation was mounted in an organ bath containing 3 ml of Tyrode solution of the following composition (mM): 136.9 NaCl, 2.7 KCl, 1.8 CaCl₂, 1.05 MgCl₂, 11.9 NaHCO₃, 0.4 NaH₂PO₄, 5.6 glucose, Physostigmine salicylate (5 μM) and choline chloride (1 μM) were added to Tyrode solution. The bath fluid was maintained at 37°C and bubbled with 95% O₂ and 5% CO₂.

Release of ACh induced by various stimuli: ACh released from tissue was collected in accordance with the method described by Takeuchi et al. (1991). The preparations were equilibrated for 15 min superfusion with Tyrode solution at a rate of 1-2 ml/min. Then, the perfusion was stopped and the bathing medium was replaced by 3 ml of fresh Tyrode solution at intervals of 1 min. After two consecutive samples were collected for measurement of spontaneous ACh release (Rₛ), the strips were stimulated for 1 min by addition of nicotine (6.16 μM), 5-hydroxytryptamine (5-HT, 2.6 μM) or by electrical field stimulation (EFS, 10 Hz, 0.5
Protein kinase A and ACh release

Each stimulus was applied twice with an interval of 19 min between stimuli. The first stimulation \( (S_1) \) was carried out in the absence of the tested drugs, and the second \( (S_2) \) in their presence. For the EFS-induced ACh release, the stimulation was performed in trains of 20 s, and bathing fluid was collected after a further 40 s resting period. The drugs were applied to the bathing fluid 17 min before \( S_2 \) and continued to be present until the end of \( S_2 \). All samples collected were kept on ice until assayed (within 3 hr). At the end of the experiment, the strips were blotted and weighed. The amount of ACh released per g tissue in response to each stimulation was calculated by subtracting the release in the preceding resting period of 1 min from the total output during the period of stimulation. ACh release due to agonist stimulation \( (S_1', S_2') \) was calculated by subtracting the spontaneous release \( (R_1, R_2) \) from the total output during the periods of stimulation \( (S_1, S_2) \). Results are shown as relative ACh release defined as \( R_2/R_1 \times 100 \) and \( S_2'/S_1' \times 100 \).

Assay of released ACh: The samples collected were assayed for ACh using isolated strips of longitudinal muscle obtained as described elsewhere (Takeuchi et al., 1991). For removal of the added nicotine or PGs and endogenous PGs, the samples were gently shaken with one tenth volume of Amberlite XAD-2 for 15 min and filtered (Yagasaki et al., 1984). To eliminate the possibility of interference by the tested drugs present in the samples, we compared the responses to the bathing fluids collected as assay sample with those of a standard solution of ACh supplemented with the tested drugs at the appropriate final concentration.

The active substance in the samples was identified as ACh by demonstration that it was destroyed by boiling in alkali and that its action was antagonized by atropine and potentiated by phystostigmine. In some experiments, we confirmed that the release of ACh measured by bioassay was similar to that determined by high-performance liquid chromatography (data not shown).

Statistical analysis: All data in the text are expressed as means±S.E. The statistical significance of the results was determined by Student's \( t \)-test.

Drugs: The following drugs were used; acetylcholine chloride (ACh), 5-hydroxytryptamine (5-HT), indomethacin (IND), phystostigmine salicylate and forskolin (all from Sigma, USA), choline chloride (from Aldrich, USA), H-89 (from Seikagaku kogyo, Japan). OP-41483, a prostaglandin I\(_2\) analog was gifts from Ono Pharmaceutical Co.

All other chemicals were of analytical grade. Stock solutions of indomethacin, OP-41483 and forskolin were prepared in ethanol, and H-85 and H-89 were in DMSO. Other drugs were prepared in distilled water. Before use, these solutions were diluted appropriately with Tyrode solution. Ethanol and DMSO at the concentrations used did not affect the ACh release.

Results

Longitudinal muscle strips obtained from guinea pig ileum were stimulated twice \( (S_1 \text{ and } S_2) \) by agonists or EFS with an interval of 19 min between stimuli as described in Methods. The spontaneous release of ACh was relatively constant; that is, the relative value of spontaneous release collected immediately before the second stimulation to that before the first stimulation was 117.3±7.0% \( (n=13) \). Nicotine \( (6.16 \mu\text{M}) \), 5-HT \( (2.6 \mu\text{M}) \) and EFS significantly
increased the ACh release (Takeuchi et al., 1991), and no significant difference in the relative ACh release was obtained between the first (S₁) and second (S₂) stimulation. The values for relative amounts of ACh released (S₂'/S₁' ×100) were 103.3±5.9 (n=13), 112.7±4.4 (n=4) and 110.8±10.9% (n=3) for nicotine-, EFS- and 5-HT-induced release of ACh, respectively.

Effects of H-89 on the spontaneous and stimuli-induced release of ACh from myenteric plexus of guinea pig ileum

H-89 significantly inhibited the spontaneous and nicotine-induced release of ACh (Fig. 1), and the inhibitory effect was concentration-dependent: H-89 inhibited both kinds of release of ACh to about 80% at 0.1 µM, about 50% at 1 µM (Fig. 1), and induced maximum effect (about 20% of control) at 10 µM (Fig. 2). Its IC₅₀ value for the nicotine-induced release obtained from concentration–response curve was about 1 µM, being a similar value to that for the spontaneous release. However, EFS- and 5-HT–induced release of ACh were not affected by H-89 at 1 µM (Fig. 3). H-85, an inactive derivative of H-89 in inhibiting protein kinase A did not have any significant effect on the spontaneous and nicotine-induced release (Fig. 2).
Effects of H-89 on the restoration of indomethacin-induced inhibition of ACh release by prostaglandin I₂

Previously, we demonstrated that IND inhibited the spontaneous and nicotine–induced release of ACh, but not EFS- and 5-hydroxytryptamine (5-HT)-induced release, from myenteric plexus of guinea pig ileum (Takeuchi et al., 1991). This inhibitory effect of IND was completely reversed by a PGI₂ analog, forskolin and isobutylmethylxanthine (Fukunaga et al., 1993; Takeuchi et al., 1992). Therefore, we next examined whether protein kinase A is involved in the restoration by PGI₂. As shown in our previous report (Takeuchi et al., 1992), IND at 2.8 μM induced the maximal inhibition of the spontaneous and nicotine–induced release of ACh. In the presence of IND at 2.8 μM, H-89 did not show significant inhibitory effect on both kinds of release of ACh (Table 1). OP-41483, a PGI₂ analog reversed the inhibitory effect of IND on nicotine–induced release of ACh (Fig. 4). The same results were obtained in the spontaneous release. The effects of OP-41483 were completely inhibited by addition of H-89 (Fig. 4). Forskolin also reversed the inhibitory effect of IND on nicotine–induced release and this effect of forskolin was also abolished by H-89 (Fig. 5). H-85 did not affect the effects of OP-41483 and forskolin (Figs. 4 and 5).

Discussion

We previously reported that IND inhibited the spontaneous and nicotine–induced ACh release from myenteric plexus of guinea pig ileum, and that PGs, especially PGI₂ reversed the inhibition (Fukunaga et al., 1993). We also reported that the agents, which increase the cyclic

Table 1. Effects of indomethacin (IND) and H-89 on nicotine–induced and spontaneous release of ACh

|                | Control            | IND (2.8 μM)        | IND (2.8 μM) + H-89 (1 μM) |
|----------------|--------------------|---------------------|----------------------------|
| Nicotine (6.16 μM) | 103.3±5.9 (13)     | 39.0±4.3 (8)        | 29.0±9.8 (4)               |
| Spontaneous     | 117.3±7.2 (13)     | 28.3±4.3 (8)        | 20.4±2.0 (4)               |

Relative ACh release was determined in the absence (Control), or presence of 2.8 μM IND without or with 1 μM H-89. The values (S'/S'×100) are means±S.E. Numbers of experiments are shown in parentheses. For further details, see Methods.
AMP level in tissue, reversed the IND-induced inhibition (Takeuchi et al., 1992). Moreover forskolin and membrane-permeable analogs of cyclic AMP stimulated the ACh release from myenteric plexus (Yau et al., 1987). Recently, protein kinase A was reported to be present in isolated myenteric ganglia and to phosphorylate synapsin I which was associated with synaptic vesicles and involved in the regulation of neurotransmitter release (De Camilli and Greengard, 1986; Jeitner et al., 1991). In the present study, H-89, but not H-85, inhibited the spontaneous and the nicotine-induced release of ACh. H-89 was shown to have a potent and selective inhibitory effect against protein kinase A (Chijiwa et al., 1990). In synaptosome preparation prepared from myenteric plexus, the nicotine-induced release of ACh was inhibited by adenosine through inhibition of adenylylate cyclase (Reese and Cooper, 1982; Zafirov et al., 1985). Interestingly, H-89 also inhibited the reverse effects of PGI2 and forskolin on the IND-induced inhibition of ACh release. From these results, it seems that protein kinase A is important in the ACh release from myenteric plexus of guinea pig ileum, and that endogenous PGs regulate protein kinase A via intracellular cyclic AMP level. Essential role of endogenous PGs in maintaining intracellular cyclic AMP level was supported by the following results. The concentration of IND used in the present study decreased release of endogenous PGs from longitudinal muscle preparations to minimal level (Fukunaga et al., 1993) and inhibited maximally the spontaneous and nicotine-induced ACh release (Takeuchi et al., 1992). In the
presence of this concentration of IND, H-89 did not show further inhibitory effect on ACh release (Table 1).

At concentration induced 50% inhibition on the ACh release, H-89 inhibited completely the reverse effect of PGI2. It was demonstrated previously that the treatment of IND decreased many kinds of PGs liberation from guinea pig ileum (Kadlec et al., 1978; Fukunaga et al., 1993). PGE also reversed IND-induced inhibition of ACh release (Yagasaki et al., 1981; 1984). Thus, the ACh release from myenteric plexus may be modulated by various kinds of endogenous PGs. This is reason that the inhibitory effect of H-89 on the ACh release was weak in the absence of IND.

In the present study, EFS- and 5-HT-induced release of ACh were not affected by H-89. Since EFS- and 5-HT-induced release of ACh were not inhibited by IND, we suggested that both kinds-induced release of ACh are not modulated by endogenous PGs (Takeuchi et al., 1991). Thus, the difference among effects of H-89 on nicotine-, EFS- and 5-HT-induced release of ACh may be due to difference in dependency of various stimuli-induced ACh release on endogenous PGs.

Hirsh et al. (1990) showed that phosphorylation by protein kinase A was not important in ACh release from frog motor nerve endings. The difference between their results and ours may be due to inhibitors of protein kinase A used. They used H-7 which is non-selective for various protein kinases and is less potent than H-89 in inhibiting protein kinase A. In fact, H-7 blocked an inhibitory effect of 2-chloroadenosine on ACh release by decreasing the cyclic AMP level (Chen et al., 1989).

In summary, the present findings suggest that activation of protein kinase A by cyclic AMP is involved in the regulatory mechanism of PGs for the ACh release in myenteric plexus of guinea pig ileum.

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