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Suppression of cAMP formation by adenosine in myenteric ganglia from guinea-pig small intestine

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Abstract

Effects of the adenosine receptor agonist 2-chloro-N^6-cyclopentyl-adenosine (CCPA) on stimulation of cAMP formation by histamine, 5-hydroxytryptamine, substance P and forskolin were determined for enzymatically dissociated ganglia from the myenteric plexus of guinea-pig small intestine. Each of the 4 substances stimulated cAMP production. CCPA blocked the stimulation of cAMP by histamine, but not by 5-hydroxytryptamine or substance P. CCPA marginally suppressed stimulation by forskolin. CCPA alone suppressed basal levels of cAMP. The adenosine receptor antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPT) reversed the inhibitory action of CCPA on stimulation of cAMP formation by histamine. Exposure to adenosine deaminase or CPT increased cAMP in the ganglia. The results are consistent with a hypothesis that stimulation of adenylate cyclase and elevation of intraneuronal cAMP in enteric neurons are steps in the signal transduction cascade for the excitatory actions of 5-hydroxytryptamine, substance P and histamine. They are consistent also with an original hypothesis from electrophysiologic studies which states that stimulation of adenosine A_1 receptors suppresses cAMP formation and thereby slow synaptic excitation in response to histamine, but not to 5-hydroxytryptamine or substance P. The results support evidence from intracellular microelectrode studies which suggested that endogenous adenosine accumulates to levels sufficient for tonic suppression of cAMP formation in myenteric ganglia in vitro.

Keywords: Intestine; Ganglion; Enteric nervous system; Myenteric plexus; Histamine; cAMP; Adenylate cyclase; Adenosine; 5-HT (5-hydroxytryptamine, serotonin); Substance P; Signal transduction

1. Introduction

Slow synaptic excitation (slow EPSP) is a prolonged state of heightened excitability lasting for seconds to minutes in AH/Type 2 (after-hyperpolarization/Dogiel Type II morphology) enteric neurons (Wood and Mayer, 1978, 1979a; Johnson et al., 1980). Slowly activating depolarization, decreased conductance in Ca^{2+}-activated K^+ channels, suppression of hyperpolarizing afterpotentials and extraordinary enhancement of excitability are characteristics of the response (reviewed by Wood, 1989, 1994). Several putative neurotransmitters and paracrine/endocrine mediators mimic the slow EPSP when applied to the neurons. Among these are 5-hydroxytryptamine (Wood and Mayer, 1979b), substance P (Katayama and North, 1978), and histamine (Nemeth et al., 1984). Adenosine acts to suppress initiation of slow EPSPs and to abort the responses after they are evoked (Palmer et al., 1987a; Christofi and Wood, 1993a, 1994). Block of the ongoing EPSP is evidence that a component of the inhibitory action of adenosine is at the postsynaptic neuron and not entirely due to presynaptic inhibition of transmitter release.

Electrophysiologic results suggest that signal transduction for the slow EPSP in AH/Type 2 enteric neurons involves receptor-mediated activation of adenylate cyclase and second messenger function of cAMP. Activation of adenylate cyclase by forskolin or application of membrane permeant analogs of cAMP or intraneuronal injection of cAMP evokes slow EPSP-like responses (Nemeth et al., 1986; Palmer et al., 1986a; Tack and Wood, 1992; Frieling et al., 1991). Elevation of intraneuronal cAMP mimics the slow EPSP-like actions of histamine, 5-hydroxytryptamine, substance P and other slow EPSP mimetics including calcitonin gene-related peptide (Palmer et al., 1986b), vasoactive intestinal peptide and cholecystokinin (Zafirov et al., 1985) and pituitary adenylate cyclase activating peptide (Christofi and Wood, 1993b).

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Adenosine or adenosine A_1 receptor agonists suppress slow EPSPs and the slow EPSP-like actions of both forskolin and histamine, but do not suppress the responses to 5-hydroxytryptamine, calcitonin gene-related peptide or substance P (Palmer et al., 1987b; Christofi and Wood, 1993a). In fact, adenosine enhances the responses to 5-hydroxytryptamine, calcitonin gene-related peptide and substance P (Palmer et al., 1987b). Slow EPSP-like responses to intraneuronal injection of cAMP or application of membrane permeant analogs of cAMP are unaffected by adenosine. Failure of adenosine to suppress slow EPSP-like effects of elevated intraneuronal cAMP in these cases suggests that the action of adenosine is inhibition of adenylate cyclase rather than inhibition at a step further along the cAMP signal transduction cascade. Blockade of the inhibitory action of adenosine by preincubation of the neurons with pertussis toxin suggests G-protein coupling of the inhibitory adenosine receptor to the enzyme (Tamura et al., 1995).

The ionic mechanisms responsible for the slow EPSP-like responses are the same for all the putative messengers. This leads to the issue of how 5-hydroxytryptamine, substance P and calcitonin gene-related peptide evoke the same kind of response as histamine and forskolin and yet, unlike histamine and forskolin, are insensitive to the blocking action of adenosine or adenosine A_1 receptor agonists in the same neurons. One possibility is that the transduction mechanism for 5-hydroxytryptamine, calcitonin gene-related peptide and substance P may not involve adenylate cyclase and second messenger function of cAMP. On the other hand, the transduction mechanism for 5-hydroxytryptamine, calcitonin gene-related peptide and substance P may be the same as for other slow EPSP mimetics and involve activation of adenylate cyclase, but to differ by not having inhibitory adenosine receptors linked to the adenylate cyclase and therefore being insensitive to inhibition by adenosine. Standard methods of intracellular microelectrode recording in enteric neurons cannot effectively distinguish these possibilities.

The general aim of the present study was to expand the insight already gained from the electrophysiologic studies of signal transduction for slow synaptic excitation in enteric neurons. The work was organized to test three specific hypotheses that emerged from the electrophysiologic studies. First was to test the hypothesis that 5-hydroxytryptamine and substance P, as well as histamine, stimulate formation of cAMP when applied to myenteric ganglia. Second was to test whether application of an adenosine A_1 receptor agonist, that was known to inhibit slow EPSPs, will suppress stimulation of formation of cAMP by histamine and forskolin, but not by 5-hydroxytryptamine or substance P. Third was to test a hypothesis that endogenous adenosine accumulates to levels sufficient for tonic suppression of slow synaptic excitation in myenteric ganglia in vitro. Protocols that directly measured changes in cAMP levels in isolated myenteric ganglia were used to test the three hypotheses. Preliminary accounts of the study have been published in abstract form (Xia et al., 1993a,b, 1994a,b).

2. Materials and methods

Myenteric ganglia were obtained from the small intestine of male albino guinea-pigs (300–400 g) that were killed by stunning and exsanguination. This method of killing was approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee. Enzymatic digestion was used to dissociate the ganglia from the muscle and connective tissue of longitudinal muscle-myenteric plexus preparations. Approximately 600 ganglia were obtained from each animal. Thirty freshly dissociated ganglia were placed in each incubation tube. The ganglia were incubated at 37°C in Krebs solution gassed with 95% O_2/5% CO_2 and buffered at pH 7.4. Putative agonists and antagonists were added to the incubation medium for a predetermined time before the experiment was terminated by addition of trichloroacetic acid. The drugs were dissolved in Krebs solution and added as a concentrated stock solution to yield the desired concentration in the incubation tubes. cAMP from each incubation was measured by radioimmunoassay. Specific details of the methods of dissection of longitudinal muscle-myenteric plexus preparations, enzymatic dissociation and harvest of ganglia from the preparations and the assay for cAMP are described in detail in earlier reports (Xia et al., 1991; Baidan et al., 1992).

The cAMP content was normalized to the number of ganglia. An earlier report (Xia et al., 1991) validated expression of cAMP levels in terms of single ganglia rather than normalizing to amounts of protein or DNA. Data are normalized to basal levels in the absence of drugs and expressed as means $\pm$ standard errors with each mean representing the number of incubation tubes each of which contained 30 ganglia. Statistical differences among means were determined by one-way analysis of variance with $P < 0.05$ considered significant.

Agents used and sources were: (1) IBMX (3-isobutyl-1-methylxanthine), histamine, 5-hydroxytryptamine creatinine sulfate and substance P all obtained from Sigma (St. Louis, MO, USA); (2) forskolin (H_2O-soluble form) was obtained from Calbiochem (La Jolla, CA, USA); CCPA (2-chloro-N^6-cyclopentyl-adenosine), DMPX (3,7,-dimethyl-1-propargyl-xanthine) and CPT (8-cyclopentyl-1,3-dimethylxanthine) were obtained from Research Biochemicals International (Natick, MA, USA).

3. Results

3.1. Effects of histamine and CCPA on cAMP formation

The inhibitory adenosine receptor agonist CCPA was used instead of adenosine because the antibodies used in

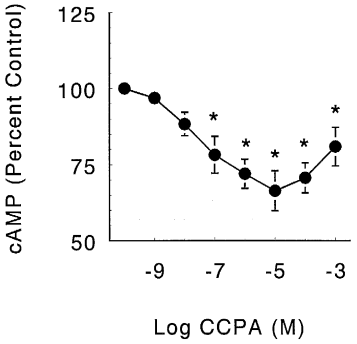

Fig. 1. Concentration dependence of suppression of cAMP formation by the inhibitory adenosine receptor agonist CCPA in myenteric ganglia from guinea-pig small intestine. Data are means ± S.E. for 9 experiments done in duplicate. * $P < 0.05$.

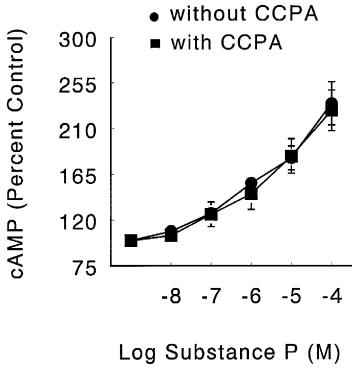

Fig. 3. Effects of the inhibitory adenosine receptor agonist CCPA on concentration–response relation for stimulation of cAMP formation by substance P in myenteric ganglia from guinea-pig small intestine. (●) Without CCPA; (■) with CCPA. Data are means ± S.E. for 9 experiments done in duplicate.

the cAMP radioimmunoassays were found to cross-react with adenosine to an unacceptable extent. CCPA had minimal cross-reactivity in the assay. Electrophysiologic evidence from guinea-pig myenteric ganglia suggests that CCPA is selective for the adenosine A_1 receptor that is responsible for inhibition of slow synaptic excitation (Christofi and Wood, 1993a, 1994).

CCPA alone in 7 incremented concentrations over a range of 1 nM to 1 mM suppressed the levels of cAMP dose dependently (Fig. 1). The threshold concentration was in the range of 1 nM with an EC_{50} between 50 and 100 nM.

Application of histamine in the incubation medium elevated the levels of cAMP in the ganglia (Fig. 2). Concentration–response relations were determined by incrementing the concentration of histamine in 6 steps over a range of 1.0 nM to 100 μM. The results showed the threshold concentration to be in the 1 nM range with the EC_{50} occurring between 5 and 20 nM.

Addition of 1 μM CCPA to the incubation medium 10 min prior to application of histamine suppressed the stimulatory action of histamine on cAMP levels (Figs. 2 and 6). The histamine concentration–response curve was flattened below baseline levels over the full range of histamine concentrations between 1 nM and 100 μM. Inclusion of the selective adenosine A_1 receptor antagonist CPT (10 μM) with CCPA (1 μM) prevented suppression of the histamine response (Fig. 2). The presence of CPT with CCPA restored histamine stimulation of cAMP to levels greater than found with histamine alone. Unlike CPT, the selective adenosine A_2 receptor antagonist DMPX (10 μM) did not prevent suppression of the histamine response by CCPA.

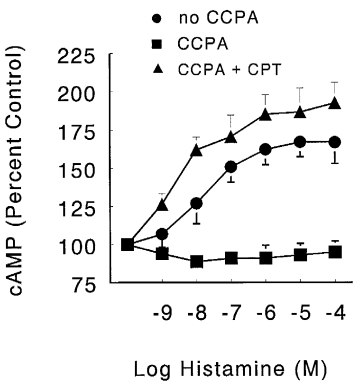

Fig. 2. Suppression by the inhibitory adenosine receptor agonist CCPA of the stimulating action of histamine on cAMP formation in myenteric ganglia from guinea-pig small intestine. The adenosine A_1 receptor antagonist CPT reversed the inhibitory action of CCPA on histaminergic stimulation of cAMP. (●) Concentration–response relation for stimulation of cAMP production by histamine. (■) Concentration–response relation for histamine in the presence of 1 μM CCPA. (▲) Concentration-response relation for histamine in the presence of 1 μM CCPA and 10 μM CPT. Formation of cAMP was restored to levels higher than those in the absence of CCPA. Data are means ± S.E. for 7 experiments done in duplicate.

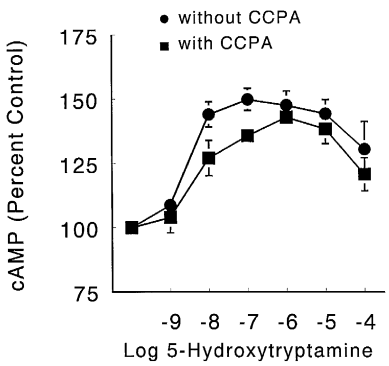

Fig. 4. Effects of the inhibitory adenosine receptor agonist CCPA on concentration–response relation for stimulation of cAMP formation by 5-hydroxytryptamine in myenteric ganglia from guinea-pig small intestine. (●) Without CCPA; (■) with CCPA. Data are means ± S.E. for 10 or 11 experiments done in duplicate.

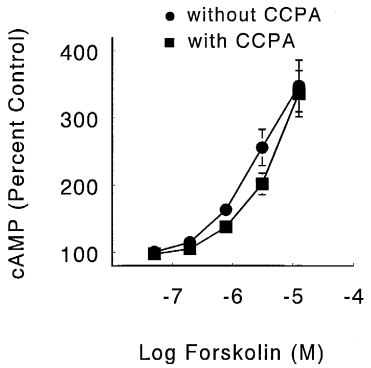

Fig. 5. Effects of the inhibitory adenosine receptor agonist CCPA on concentration–response relation for stimulation of cAMP formation by forskolin in myenteric ganglia from guinea-pig small intestine. (●) Without CCPA; (■) with CCPA. Data are means ± S.E. for 6–14 experiments done in duplicate.

3.2. Effects of substance P and CCPA on cAMP formation

Substance P stimulated the formation of cAMP in the ganglia. Concentration–response relations were determined by incrementing the concentration of substance P in 5 steps over a range of 10.0 nM to 100 μM (Fig. 3). The threshold concentration was in the 10 nM range with the EC_{50} occurring between 1 and 10 μM. This action of substance P confirmed findings reported earlier (Baidan et al., 1992).

The presence of 1 μM CCPA in the incubation medium did not alter the concentration–response relation for substance P (Figs. 3 and 6). A downward shift in the concentration–response curve was apparent at 1 μM; however, the change was minimal and statistically insignificant.

3.3. Effects of 5-hydroxytryptamine and CCPA on cAMP formation

Application of 5-HT in the incubation medium elevated the levels of cAMP in the ganglia (Fig. 4). Concentration–response relations were determined by incrementing the concentration of 5-HT in 6 steps over a range of 1.0 nM to 10 μM. The threshold concentration was between 1 and 5 nM with the EC_{50} in the range of 5 to 10 nM. This action of 5-hydroxytryptamine confirmed earlier findings (Fioricahowells et al., 1993; Xia et al., 1994a).

The presence of 1 μM CCPA suppressed the 5-HT concentration–response curve by a maximum of 20% (Figs. 4 and 6). This was equivalent to that found for CCPA alone and appeared to reflect suppression of basal levels of cAMP formation by CCPA rather than direct suppression of the stimulatory action of 5-HT.

3.4. Effects of forskolin and CCPA on cAMP formation

Forskolin stimulated the formation of cAMP in the ganglia. Concentration–response relations were determined by incrementing the concentration of forskolin in 4 steps over a range of 0.1 to 100 μM (Figs. 5 and 6). The threshold concentration was in the 10 nM range with the EC_{50} occurring between 1 and 10 μM. This action of forskolin confirmed findings reported earlier (Xia et al., 1991).

The presence of 1 μM CCPA significantly suppressed the forskolin concentration–response curve by a maximum of 15% at forskolin concentrations less than 10 μM (Fig. 5). At concentrations of forskolin greater than 10 μM, CCPA (1 μM) did not suppress stimulation of cAMP.

3.5. Effects of CCPA and adenosine deaminase on cAMP formation

The effect of CPT to restore cAMP formation to levels greater than basal when added to the incubation medium with histamine and CCPA (Fig. 2) suggested that basal levels of cAMP may be tonically suppressed by endoge-

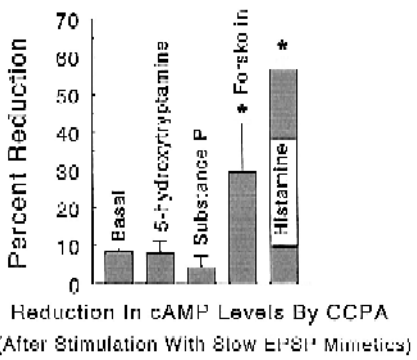

Fig. 6. Summary of mean percent reduction in cAMP formation produced by the inhibitory adenosine receptor agonist CCPA when cAMP was stimulated by 5-hydroxytryptamine, substance P, forskolin, or histamine in myenteric ganglia from guinea-pig small intestine. The mean percent reduction in basal levels of cAMP by CCPA are included for comparison. * $P < 0.05$.

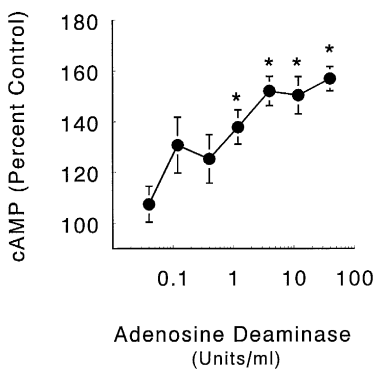

Fig. 7. Concentration dependence of increased cAMP formation by the degradative enzyme adenosine deaminase in myenteric ganglia from guinea-pig small intestine. Data are means ± S.E. for 6 experiments done in duplicate. * $P < 0.05$.

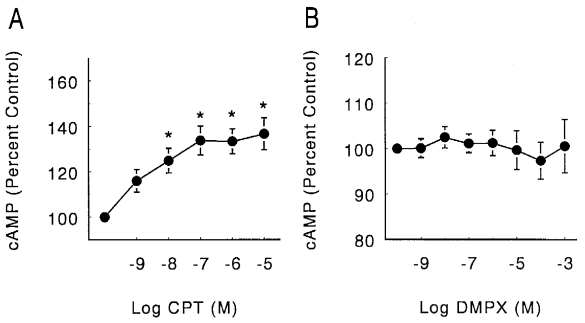

Fig. 8. Concentration dependence of effects of the putative adenosine A_1 receptor antagonist CPT (A) and the adenosine A_2 receptor antagonist DMPX (B) on cAMP formation. Data for CPT are means ± S.E. for 11 experiments done in duplicate; data for DMPX are for 12 experiments done in duplicate. * $P < 0.05$.

nous levels of adenosine in the ganglia. Therefore, we tested a hypothesis that overshoot of cAMP levels found in histamine-stimulated ganglia after application of the adenosine A_1 antagonist CPT (Fig. 2) was a reflection of blockade of spontaneous accumulation of adenosine and its inhibitory action on adenylate cyclase. This was done by treating ganglia with either adenosine deaminase, CPT or the selective adenosine A_2 antagonist, DMPX and measuring effects on cAMP levels.

Degradation of endogenous adenosine by incubating the ganglia with adenosine deaminase increased the levels of cAMP (Fig. 7). This action was concentration dependent and resulted in a maximal increase of 46% relative to basal levels in the ganglia.

Blockade of adenosine A_1 receptors by the selective antagonist CPT also increased the levels of cAMP (Fig. 8A). The action was concentration dependent over a range from 1 nM to 10 μM and resulted in a maximal increase of 37% relative to basal levels of cAMP measured in the ganglia. Treatment with the adenosine A_2 receptor-preferring antagonist DMPX did not change the levels of cAMP when applied in a range of concentrations from 1 nM to 1 mM (Fig. 8B).

4. Discussion

4.1. Histamine, 5-hydroxytryptamine and substance P

Significant elevation of cAMP was found in dissociated myenteric ganglia when exposed to histamine, substance P, 5-hydroxytryptamine or forskolin. This is consistent with the electrophysiologic evidence for function of cAMP as a second messenger in the signal transduction mechanism for the slow EPSP-like actions of these agents in AH/Type 2 myenteric neurons (Nemeth et al., 1986; Palmer et al., 1986a; Wood, 1987). The results confirm earlier evidence that cAMP levels in guinea-pig myenteric ganglia are increased by 5-hydroxytryptamine (Xia et al., 1994a; Fioricahowells et al., 1993), substance P (Baidan et al., 1992) and forskolin (Xia et al., 1991).

Our finding of suppression of basal production of cAMP by a selective adenosine A_1 receptor agonist (CCPA) is consistent with our earlier electrophysiologic findings that activation of P_1 receptors inhibits excitability in AH/Type 2 myenteric neurons in the guinea-pig. Electrophysiologic results suggest that high affinity adenosine A_1 receptors are negatively linked to adenylate cyclase. When activated by adenosine or selective adenosine receptor agonists, formation of cAMP is suppressed resulting in increased Ca^{2+}-activated K^+ conductance, enhancement of hyperpolarizing after-potentials and suppression of repetitive spike discharge (Christofi and Wood, 1994). The adenosine A_1 receptors appear to be coupled to adenylate cyclase by inhibitory G-proteins because treatment with pertussis toxin removes the inhibitory action of A_1 agonists on the slow EPSP-like effects of forskolin (Tamura et al., 1995).

Aside from the evidence that activation of adenosine A_1 receptors inhibits adenylate cyclase and enzymatic formation of cAMP, an electrophysiologic paradox exists in that adenosine or selective adenosine A_1 receptor agonists do not block the actions of all slow EPSP mimetics. The slow EPSP-like effects of histamine, gastrin releasing peptide, vasoactive intestinal peptide or cholecystokinin, as well as forskolin, are suppressed by adenosine (Palmer et al., 1987b), whereas pre-treatment with adenosine does not suppress the slow EPSP-like actions of substance P, calcitonin gene-related peptide or 5-hydroxytryptamine (Palmer et al., 1987b). In fact, the responses to these substances are enhanced by adenosine. The excitatory component of adenosine action in the electrophysiologic studies reflects activation of high affinity adenosine A_2 receptors that exist on subsets of AH/Type 2 myenteric neurons (Christofi et al., 1994). Measurements of cAMP levels in dissociated myenteric ganglia in parallel with microelectrode recording showed that selective activation of the adenosine A_2 receptor subtype stimulated cAMP formation.

The results with histamine in the present study were consistent with the hypothesis, derived from electrophysiologic results, that inhibition of adenylate cyclase by adenosine can account for its inhibitory actions on histamine-induced excitation of AH/Type 2 neurons. Histamine stimulated cAMP formation and this was inhibited by the putative adenosine A_1 receptor agonist CCPA.

The results with substance P and 5-hydroxytryptamine were consistent with electrophysiologic results in that the adenosine receptor agonist appeared not to suppress stimulation of cAMP formation by the two putative neurotransmitters. There were no significant differences in the concentration–response curves for substance P in the presence and absence of the inhibitory adenosine receptor agonist CCPA. Nevertheless, there was a suggestion of suppression of cAMP formation at 1 μM substance P (Fig. 3). On the other hand, the concentration–response curves for 5-

hydroxytryptamine did show small, but significant suppression by CCPA at concentrations between 10 and 100 nM (Fig. 4). Our data for the action of 1 μM CCPA alone showed suppression of basal cAMP levels by about 25% (Fig. 1) and this may account for the small degree of inhibition seen for both substance P and 5-hydroxytryptamine. Based on this and the relatively greater suppression of the responses to histamine by CCPA (Fig. 6), it seems justified to conclude that the inhibitory adenosine receptor agonist did not suppress the direct stimulatory action of either substance P or 5-hydroxytryptamine.

Adenosine and adenosine A_1 receptor agonists strongly suppress the excitatory action of forskolin in electrical recordings from AH/Type 2 myenteric neurons (Palmer et al., 1987a). Compared with electrophysiologic results, suppression of the cAMP response to forskolin by CCPA in the dissociated ganglion preparations appeared to be weaker (Fig. 6). Fig. 6, which summarizes the dissociated ganglion results, shows that the forskolin response was inhibited to a lesser extent than the histamine response. Elevation of cAMP formation in enteric ganglia by forskolin is known to reflect stimulation of both neurons and ganglionic glial cells (Christofi et al., 1993). The ratio of glia to neurons in the ganglia is about 5:1 (Christofi et al., 1993). Because the glia outnumber the neurons, levels of cAMP formation, stimulated by forskolin, are expected to be higher than those produced by the neuro-selective mediators. The neurons have receptors for histamine and adenosine, whereas no evidence for existence of these receptors on enteric glial cells has been forthcoming. However, in the absence of conclusive evidence on their expression by enteric glial cells, we cannot rule out that modulation of glial adenylate cyclase may also contribute to the data reported here. Future studies aimed at quantifying the relative contribution of cAMP formation in glia vs. neurons will help clarify this important point.

Stimulation by forskolin could occur in both neurons and glia while inhibition is localized to neurons and this may explain the differences in the apparent degree of suppression of the histamine response relative to forskolin. If it is assumed that histamine and inhibitory adenosine receptors are localized to neurons and absent from glia, a greater degree of suppression by activation of the inhibitory adenosine receptor would be expected for histamine than for forskolin.

4.2. Endogenous synthesis of adenosine

Evidence obtained with intracellular microelectrode recording in myenteric neurons suggests that ongoing synthesis and release of adenosine in the ganglia increases its concentration to levels sufficient for tonic suppression of neuronal excitability and excitatory neurotransmission (Christofi and Wood, 1993a,c). Three observations in the present study are consistent with this: (1) levels of cAMP in the ganglia increased when endogenous adenosine was degraded enzymatically by adenosine deaminase; (2) blockade of adenosine receptors with the adenosine A_1 receptor antagonist CPT allowed cAMP to accumulate in the ganglia; (3) results obtained with DMPX, a moderately selective antagonist of adenosine A_2 receptors, suggest that probably these receptors are not functionally important in modulating cAMP levels in our experimental model.

4.3. Conclusions

A hypothesis that signal transduction for the slow excitatory actions of 5-hydroxytryptamine, substance P and histamine involves stimulation of adenylate cyclase and elevation of cAMP is supported by the present data. Likewise, the results with CCPA and adenosine deaminase on basal levels of cAMP is consisent with a hypothesis that accumulation of endogenously released adenosine tonically inhibits neuronal excitation and synaptic transmission. The cellular mechanisms responsible for the sharp differences in effects of inhibitory adenosine receptor activation on the actions of histamine compared with substance P and 5-hydroxytryptamine cannot be fully explained by the available data. A hypothesis that inhibitory adenosine receptors are only associated with adenylate cyclase when the enzyme is linked to histamine receptors, and not expressed when the enzyme is linked to substance P or 5-hydroxytryptamine receptors, is not supported by findings for forskolin. Forskolin evokes slow EPSP-like behavior in the same neurons where substance P and 5-hydroxytryptamine mimic slow synaptic excitation. Yet, inhibitory adenosine receptor agonists block forskolin responses and spare substance P and 5-hydroxytryptamine responses. If it is assumed that no forms of adenylate cyclase involved in slow EPSP production escape activation by forskolin, then the implication is that adenylate cyclase in the membranes of AH/Type 2 neurons is always associated with inhibitory adenosine receptors. How stimulation of adenylate cyclase by substance P or 5-hydroxytryptamine escapes inhibition by these adenosine receptors remains unresolved.

Acknowledgements

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