Excitation and Inhibition via Adenosine Receptors of the Twitch Response to Electrical Stimulation in Isolated Guinea Pig Ileum

Atsushi Tomaru, Yasuhiro Ina, Nobuyuki Kishibayashi and Akira Karasawa
Department of Pharmacology, Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd.,
1188 Shimotogari, Nagatsumi-cho, Sunto-gun, Shizuoka 411, Japan

ABSTRACT—The effects of adenosine and its related compounds on the cholinergic twitch response were examined in electrically stimulated guinea pig ileum. Adenosine $(3 \times 10^{-7} - 10^{-5} \text{M})$ and an adenosine A1-receptor agonist $N^6$-cyclohexyladenosine (CHA, $10^{-8} - 10^{-6} \text{M}$) suppressed the twitch. Conversely, the A2a-receptor agonist 2-[p-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680, $10^{-9} - 10^{-7} \text{M}$) potentiated the twitch in half the preparations examined. The A1-antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), which per se did not affect the twitch, recovered the attenuated twitch caused by CHA $(10^{-7} \text{M})$ or adenosine $(10^{-6} \text{M})$ and converted it into a potentiated twitch. These results suggest the presence of adenosine A1- and A2a-receptors coupled negatively and positively, respectively, to acetylcholine release in the preparation.

Keywords: Adenosine receptor, Twitch response, Ileum

Adenosine functions as a neuromodulator. Activation of adenosine A1- and A2a-receptors inhibits and facilitates, respectively, the release of acetylcholine from striatal synaptosomes and hippocampal slices (1, 2) and that of excitatory amino acid from ischemic cerebral cortex (3). As with the central nervous system, Correia-de-Sá and Ribeiro (4) have recently documented the presence of both the inhibitory A1- and the facilitatory A2a-receptors coupled to acetylcholine release from rat phrenic motor nerve endings. In the gastrointestinal tract, however, the presence of adenosine A2a-receptors positively coupled to transmitter release has not been reported to date, although the activation of adenosine A1-receptors is known to suppress the release of principal contractile substances like acetylcholine and tachykinins from guinea pig myenteric nerve endings (5, 6).

Recently, Barajas-Lopez et al. (7) and Christofi et al. (8) reported that the activation of adenosine A2a-receptors depolarized S/Type 1 submucosal neurons and a subset of AH/Type 2 myenteric neurons, respectively, in the guinea pig ileum. Moreover, our previous work showed that an adenosine A2a-agonist, 2-[p-(2-carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680), elicited colonic contractions (9) and increased feces (10) in rats. From these observations, we assumed that the activation of adenosine A2a-receptors might facilitate the neuronal release of contractile substances and thereby potentiate the mechanical response in intestinal smooth muscle preparations.

The present study examined the effects of adenosine and its related compounds on the cholinergic twitch response evoked by nerve stimulation in isolated whole ileum preparations of guinea pigs. The aim of the present study was to investigate the possible presence of the facilitatory A2a-receptors in addition to the inhibitory A1-receptors reported previously (5).

Male Hartley strain guinea pigs (Japan Shizuoka Laboratory Animal Center, Hamamatsu), weighing 200–400 g, were used. Test compounds were the following (K or IC50 values were from Refs. 11 and 12, if given): adenosine (IC50: A1 73 nM, A2a 150 nM, A2b 15,400 nM, A3 6,500 nM); the adenosine A1-receptor agonist N6-cyclohexyladenosine (CHA) (K: A1 0.85 nM, A2a 19 nM, A2b negligible, A3 > 100,000 nM); the adenosine A1-receptor antagonist N6-cyclohexyladenosine (CHA) (K: A1 0.85 nM, A2a 460 nM, A2b 160,000 nM); the adenosine A2a-receptor agonist CGS 21680 (K: A1 1,800 nM, A2a 19 nM, A2b negligible, A3 140 nM); the adenosine A1-receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (K: A1 0.9 nM, A2a 470 nM, A3 > 100,000 nM); and the adenosine A2-receptor antagonist...
antagonist 4-amino-8-chloro-1-phenyl[1,2,4]triazolo[4,3-
$a$]quinoxaline (CP-66,713) (IC$_{50}$: A$_1$ 270 nM, A$_2$ 21 nM). CHA, DPCPX and CP-66,713 were synthesized in our Research Laboratories (Sunto-gun, Shizuoka). Commercially obtained compounds were: CGS 21680 (Research Biochemicals International, Natick, MA, USA), adenosine and tetrodotoxin (Sigma, St. Louis, MO, USA), atropine (sulfate; Nacalai Tesque, Kyoto), hexamethonium (bromide; Yamanouchi, Tokyo), and guanethidine (Takeda, Osaka). Atropine and tetrodotoxin were dissolved in distilled water. The stock solutions (5 mM) of adenosine, CHA, CGS 21680 and CP-66,713 were each prepared in dimethylsulphoxide (DMSO), and that for DPCPX in DMSO and 5 N NaOH (19:1); then the stock solutions were diluted with distilled water to the appropriate concentrations. These vehicles had no significant effect on the response of the preparation at the maximal final concentration of DMSO (0.2%) and NaOH (50 μN).

Animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p., Nembutal®; Dinabot, Osaka) and then exsanguinated. After laparotomy, a 2-cm segment of the ileum was excised from the region about 10 cm proximal to the ileocecal junction. The ileum preparation was suspended in an organ bath filled with 20 ml of Tyrode solution (pH 7.4) containing guanethidine (2 μM) and hexamethonium (50 μM). Tyrode solution had the following composition: 136 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl$_2$, 1.0 mM MgCl$_2$, 0.4 mM NaH$_2$PO$_4$, 11.9 mM NaHCO$_3$ and 5.6 mM glucose. The bath fluid was maintained at 37°C and continuously gassed with 95% O$_2$ / 5% CO$_2$.

Electrical transmural stimulation of the preparation was achieved by placing a straight electrode into the luminal side and another spiral one around the serosal side of the preparation. A load of 1.0 g was applied to the preparation throughout the experiment. Responses were obtained with an isotonic transducer (TD-112S; Nihon Kohden, Tokyo) and recorded on a pen-recorder (LR4210; Yokogawa, Tokyo). The electrical stimulation was applied at 0.1 Hz using a train of rectangular pulses of 0.5-msec duration and submaximal voltage from a stimulator (SEN-3301, Nihon Kohden). The submaximal voltage (40–80 V) was selected to evoke 70%–80% of the maximal twitch response. Preparations were equilibrated at least 1 hr without the electrical stimulation. The preliminary study showed that the electrically stimulated twitch response was completely suppressed by either atropine (10$^{-8}$ M) or tetrodotoxin (10$^{-7}$ M). Since hexamethonium was present in the organ bath, it seems that the twitch response was elicited via activation of post-ganglionic cholinergic neurons.

The evaluation of test compounds was performed after the preparation was electrically stimulated for more than 15 min. To establish the concentration-response relationship, the compound was non-cumulatively added to the organ bath in ascending order of concentration. In the first series of experiments, the effects of the test compounds per se were examined. In the second series of experiments, immediately after CHA (10$^{-7}$ M) or adenosine (10$^{-6}$ M) produced the maximal suppression of the twitch response, DPCPX or its vehicle was applied to the preparation. In the third series of experiments, we examined the effects of adenosine (3×10$^{-8}$ M) on the twitch response of the preparation pretreated with DPCPX (10$^{-8}$ M) or its vehicle for 2 min. In the second and third series of experiments, DPCPX or its vehicle was alternately applied to the preparation. The volume of the compounds added to the organ bath was either 12 or 40 μl, so that the maximal added volume was 80 μl in the present study.

Data were obtained from 4–5 different preparations. The effects of the test compounds are expressed as a percentage of the amplitude of the twitch response just prior to the addition of the test compounds in the first series of experiments, just prior to the addition of adenosine or CHA in the second series of experiments, and just prior to the addition of adenosine in the third series of experiments. Values are reported as means ± S.E.M.

Figure 1 summarizes the concentration-response curves showing the effects of adenosine and its related compounds on the twitch response. The adenosine A$_1$-agonist
CHA and adenosine markedly suppressed the twitch response. The suppression seems to be caused by the activation of adenosine A₁-receptors since the suppression occurred at nanomolar concentrations of CHA (Fig. 1), and additionally, the suppression by CHA was attenuated after the addition of the adenosine A₁-receptor antagonist DPCPX (Figs. 2A and 3A). The attenuated suppression by adenosine after the addition of DPCPX (Figs. 2B and 3A) also favors the presence of adenosine A₁-receptors that mediate the suppression of the twitch response. The earlier studies (5) have already indicated that in the guinea pig ileum, the suppressed twitch response was due to the activation of presynaptic adenosine A₁-receptors.

On the other hand, the A₂ₐ-receptor agonist CGS 21680 potentiated the twitch response markedly. As shown in Fig. 3B, the potentiation by CGS 21680 of the twitch response was concentration-dependent. CGS 21680 induced immediate potentiation of the twitch response, followed by the gradual decline of the potentiation. In the preliminary study, however, we recognized that the potentiation was hardly observed in some preparations. In other words, the potentiated response to CGS 21680 varied greatly from preparation to preparation. So, in the present study, we first performed an additional evaluation of CGS 21680 at 30 nM to determine if the preparation could show a prominent response to this compound. As a result, 4 out of 8 preparations showed negligible or slight potentiation (100%, 100%, 100%, 111%) in response to CGS 21680. In contrast, the other 4 preparations exhibited marked potentiation (139%, 174%, 177%, 185%), for which preparations the concentration-response curve of CGS 21680 was constructed (Fig. 1). Since CGS 21680 potentiated the twitch response at low nanomolar concentrations, the adenosine receptors involved is probably of the A₂ₐ-subtype. It is known that the postsynaptic activation of the A₁- and A₂-receptors generally results in contraction and relaxation, respectively, of the smooth muscle preparation (13), although it has not been extensively studied which subtype of the A₂-receptors, A₂ₐ or A₂β, is involved in the relaxation. Broad and Cook (5) reported that CGS 21680 did not affect the contractile response to exogenously applied acetylcholine in the longitudinal muscle of guinea pig ileum. Furthermore, in our preliminary study using guinea pig whole ileum without the electrical stimulation, CGS 21680 (10⁻⁹–10⁻⁷ M) affected neither the basal tonus nor the contractile response to acetylcholine (3 × 10⁻⁹ M) (data not shown). Taken together, the potentiated twitch by CGS 21680 in the present study might be attributed to the presynaptic action of the drug.

The reason for the preparation-dependent difference in the response to CGS 21680 remains obscure in the present study. Recently, Correia-de-Sá and Ribeiro (4) have reported that in rat phrenic nerve-hemidiaphragm preparations, the increase by CGS 21680 of acetylcholine release results from the activation of the A₂ₐ-receptors positively linked to the adenylate cyclase/cAMP system. Thus, the preparation-dependent difference in response to
CGS 21680 might be attributable to a different activated level of the adenylate cyclase/cAMP system; otherwise, a possible difference in the density of the A2a-receptors might have contributed to it. These must be resolved by further work to elucidate the mechanism underlying the preparation-dependent difference.

As shown in Figs. 2 and 3A, DPCPX not only counteracted the suppression by either CHA (10^{-7} M) or adenosine (10^{-6} M), but also converted it into a potentiation. This potentiation was observed in 4 out of 4 preparations for CHA (DPCPX, 10^{-7} M) and adenosine (DPCPX, 10^{-7} M) and in 3 out of 4 for adenosine (DPCPX, 3 \times 10^{-7} M). It is likely that the adenosine A1-receptor antagonist DPCPX counteracted the A1-receptor-mediated inhibitory action of CHA and adenosine and thereby unveiled the A2a-receptor-mediated facilitatory action of these compounds. There seems to be a tendency that the above combined treatment of DPCPX with CHA or adenosine potentiates the twitch response more frequently than CGS 21680 alone. The implication of this, however, remains to be determined.

In the preparation pretreated with DPCPX (10^{-8} M) or its vehicle, adenosine (3 \times 10^{-8} M) potentiated (106.8 \pm 6.6\%) or suppressed (80.8 \pm 1.8\%) the twitch response, respectively (n=5). In the presence of DPCPX, a slight but unequivocal potentiation of the twitch response was observed in 4 out of 5 preparations, while in the absence of DPCPX, prominent suppression was observed in all the preparations (see representative tracings in Fig. 3C). This finding favors the idea that the facilitatory A2a-
receptors are involved in the potentiation of the twitch response. The involvement of adenosine A2 receptors or A3 receptors in the potentiation could probably be excluded, since adenosine, having low affinity for the A2 receptors and A3 receptors (IC50: A2, 15,400 nM; A3, 6,500 nM) (11), produced potentiation at a concentration as low as 3 x 10^-8 M in the presence of DPCPX. These observations, together with the notion in the preliminary study that the twitch response is elicited via activation of postganglionic cholinergic nerves, suggest that the activation of adenosine A2 receptors facilitates acetylcholine release from postganglionic cholinergic nerve endings in guinea pig ileum.

In the present study, neither the A1-receptor antagonist DPCPX nor the A2-receptor antagonist CP-66,713 per se influenced the twitch response (Fig. 1), suggesting that endogenous adenosine was not substantially released in response to the electrical stimulation. If a substantial amount of endogenous adenosine was released, DPCPX should have counteracted the tonic suppression by endogenous adenosine via the A1-receptors, leading to the potentiated twitch response. Conversely, CP-66,713 should have counteracted the tonic potentiation by endogenous adenosine via the A2 receptors, leading to the suppressed twitch response.

Recently, the complimentary DNA (cDNA) clones encoding the A2 receptors have been isolated from the brain cDNA library of both rats and guinea pigs (14, 15). The examination of tissue distribution by Northern blot analysis failed to show the presence of the A2 receptors in the gastrointestinal tract including the stomach, small intestine and large intestine in the rodents. The present study, however, suggested the presence of the facilitatory A2 receptors in the ileum. This discrepancy may be explained by the low sensitivity of the Northern blot analysis, which employed the homogenate of the whole tissue. Alternatively, the A2 receptors of the ileum may differ from those of the brain; i.e., another subtype of adenosine A2 receptors may exist.

In conclusion, the present study provided support for the presence of both adenosine A1 and A2 receptors in guinea pig ileum. The activation of the A1 and A2 receptors seems to inhibit and facilitate, respectively, acetylcholine release from cholinergic nerve endings.

REFERENCES

1 Kirkpatrick KA and Richardson PJ: Adenosine receptor-mediated modulation of acetylcholine release from rat striatal synaptosomes. Br J Pharmacol 110, 949 – 954 (1993)
2 Cunha RA, Milusheva E, Vizi ES, Ribeiro JA and Sebastião AM: Excitatory and inhibitory effects of A1 and A2 A receptors on acetylcholine release from enteric nerve endings by 2-[p-(carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamido-adenosine (CGS-21680) and related adenosine analogs. Lack of simple competition by antagonists. J Pharmacol Exp Ther 266, 634 – 641 (1993)
3 Simpson RE, O'Regan MH, Perkins LM and Phillips JW: Excitatory transmitter amino acid release from the ischemic rat cerebral cortex: Effects of adenosine receptor agonists and antagonists. J Neurochem 58, 1683 – 1690 (1992)
4 Correia-de-Sá P and Ribeiro JA: Evidence that the presynaptic A2 adenosine receptor of the rat motor nerve endings is positively coupled to adenylate cyclase. Naunyn Schmiedebergs Arch Pharmacol 350, 514 – 522 (1994)
5 Broad RM and Cook MA: Inhibition of neurotransmitter release from enteric nerve endings by 2-[p-(carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamido-adenosine (CGS-21680) and related adenosine analogs: Lack of simple competition by antagonists. J Pharmacol Exp Ther 266, 634 – 641 (1993)
6 Broad RM, McDonald TJ, Broidin E and Cook MA: Adenosine A1 receptors mediate inhibition of tachykinin release from perfused enteric nerve endings. Am J Physiol 262, G525 – G531 (1992)
7 Barajas-Lopez C, Suprenant A and North RA: Adenosine A1 and adenosine A2 receptors mediate presynaptic inhibition and postsynaptic excitation in guinea pig submucosal neurons. J Pharmacol Exp Ther 258, 490 – 495 (1991)
8 Christofi FL, Baidan LV, Fertel RH and Wood JD: Adenosine A3 receptor-mediated excitation of a subset of AH/Type 2 neurons and elevation of cAMP levels in myenteric ganglia of guinea-pig ileum. Neurogastroenterol Mot 6, 67 – 78 (1994)
9 Tomaru A, Ishii A, Kishibayashi N and Karasawa A: Susceptibility to adenosine agonists of giant migrating contractions induced by glycerol enema in anesthetized rats. Jpn J Pharmacol 65, 361 – 365 (1994)
10 Tomaru A, Ishii A, Kishibayashi N, Shimada J, Suzuki F and Karasawa A: Possible physiological role of endogenous adenosine in defecation in rats. Eur J Pharmacol 264, 91 – 94 (1994)
11 Müller CE and Scior T: Adenosine receptors and their modulators: Dedicated to John William Daly on the occasion of his 60th birthday. Pharm Acta Helv 68, 77 – 111 (1993)
12 van Galen PJM, Stiles GL, Michaels G and Jacobson KA: Adenosine A1 and A2 receptors: Structure-function relationships. Med Res Rev 12, 423 – 471 (1992)
13 Bailey SJ and Hourani SMO: Effects of purines on the longitudinal muscle of the rat colon. Br J Pharmacol 105, 885 – 892 (1992)
14 Stehie JH, Rivkees SA, Lee JJ, Weaver DR, Deeds JD and Reppert SM: Molecular cloning and expression of the cDNA for a novel A2 adenosine receptor subtype. Mol Endocrinol 6, 384 – 393 (1992)
15 Meng F, Xie G, Chalmers D, Morgan C, Watson SJ Jr and Akil H: Cloning and expression of the A2 adenosine receptor from guinea pig brain. Neurochem Res 19, 613 – 621 (1994)