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Effects of Diadenosine Triphosphate and Diadenosine Tetraphosphate on Rat Liver Cells

DIFFERENCES AND SIMILARITIES WITH ADP AND ATP

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ABSTRACT. Liver cells possess multiple types of purinoceptors that mediate the effects of extracellular nucleotides. Like ADP and ATP, the dinucleotides diadenosine triphosphate (Ap_3A) and diadenosine tetraphosphate (Ap_4A) fully activated glycogen phosphorylase, with ED_{50} values of 0.31 μM and 1.3 μM, respectively. At variance with ATP, neither the dinucleotides nor ADP significantly increased the levels of IP_3. Ap_4A (and also ADP) moderately increased IP_3 (±72%) whereas Ap_3A was completely ineffective. Like ATP, Ap_3A, Ap_4A, and ADP inhibited the cAMP increase after glucagon. Phorbol-12-myristate-13-acetate (PMA) pretreatment of the hepatocytes clearly inhibited the glycogenolytic potency of Ap_3A and ADP, but had only a minor effect on the potency of Ap_4A or ATP. It is concluded that, depending upon the effect studied (glycogenolytic effect with or without PMA, increasing IP_3 potency, or inhibition of cAMP increase), different analogies between the agonists studied emerged, indicating the complexity of the interaction of ATP and its analogues with liver purinoceptors and/or of the transduction mechanism(s) initiated by the different nucleotides. BIOCHEM PHARMACOL 52;3:441–445, 1996.

KEY WORDS. purinoceptors; rat liver; Ap_3A; Ap_4A

Liver cells possess purinoceptors mediating the glycogenolytic effect of ATP and ATP-analogues, but also of UTP and GTP. The nature of the purinoceptors involved in this process is still a matter of discussion. The rank order of potency with which the ATP analogues activate glycogen phosphorylase (see [1] for references) suggested the presence of purinoceptors of the P_{2Y} subclass [2]. The situation turned out to be much more complex. Indeed, 1. only a few ATP analogues increased the level of IP_3 [1, 3, 4], 2. ATP and UTP probably use a common receptor [5, 6], 3. different calcium increase patterns were observed after ATP, on the one hand, and ADP [7] and 2-methylthioATP [8] on the other. Furthermore, adenosine 5′[αβ-methylene]-triphosphate only potentiated the oscillatory cytosolic Ca^{2+} responses of hepatocytes to ATP (and not to ADP [9] or to 2-methylthioATP [8]). The presence of multiple receptors and/or a nucleotide receptor on liver hepatocytes has been proposed [1, 6, 8, 10].

The ATP analogues Ap_3A† and Ap_4A account for up to 5% of all adenine nucleotides stored in the platelets and, because they have a relatively long half-life (compared to other nucleotides), it was suggested that they could, more than ATP, act as messenger molecules (see [11] for references). Apart from their effect on platelet aggregation, Ap_3A and Ap_4A have, indeed, been shown to exert vasomotor effects *via* a direct interaction with cell surface receptors [11]. Direct effects of Ap_4A in the brain have also been reported [12]. Hilderman *et al.* [13] presented evidence for the presence of a unique membrane receptor for Ap_4A in several mouse tissues.

In liver, Ap_4A was reported to bind to specific receptors [13] and Ap_3A and Ap_4A have been shown to activate glycogen phosphorylase [14, 15]. Indeed, Busshardt *et al.* [14], in perfused liver, and Craik *et al.* [15], using isolated hepatocytes, reported a glycogenolytic effect of Ap_3A and Ap_4A very similar to that of ATP. Ap_3A and Ap_4A have also been shown to increase cytosolic calcium in single rat hepatocytes [16, 17].

Ap_4A and Ap_3A, despite the minor structural difference, show clear-cut functional differences. It has, indeed, been reported that only Ap_3A (and not Ap_4A) activated platelet aggregation. Ap_4A, rather, inhibits ADP-dependent aggregation and even disaggregates clots. In smooth muscles (arteries) from which the endothelium was removed, only Ap_3A induced vasodilatation, whereas Ap_4A induced a pronounced contraction [11]. In liver, differences between the two dinucleotides also exist. Indeed, the calcium increase pattern observed after Ap_3A resembled that detected after ADP, whereas Ap_4A resembled that found after ATP [16, 17].

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† *Abbreviations:* Ap_3A, diadenosine triphosphate; Ap_4A, diadenosine tetraphosphate; IP_3, inositol 1,4,5 triphosphate; PMA, Phorbol-12-myristate-13-acetate; 2MeSATP, 2-methylthio adenosine triphosphate; TCA, trichloroacetic acid

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This study further characterizes the effects of Ap_3A and Ap_4A on rat liver cells. In freshly isolated hepatocytes, they both activated glycogen phosphorylase without significantly increasing IP_3 (a moderate increase is observed only after Ap_4A and ADP). PMA treatment, especially, inhibited the Ap_3A (and ADP) effect on phosphorylase activation and had only a minor effect on Ap_4A and ADP. No difference between the diadenosines was observed in their effectiveness in antagonizing the effect of glucagon on cAMP levels. The relation with the effects of ATP and ADP is discussed.

MATERIALS AND METHODS

We used male Wistar albino rats (200–250 g body wt) that were fed *ad lib*. IP_3 and cAMP assay kits and [^{3}H]IP_3 ([^{3}H]-inositol 1,4,5 trisphosphate) were from Amersham International, Amersham, Bucks U.K.; ATP was from Boehringer, Mannheim, Germany or from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ADP, Ap_3A, Ap_4A and PMA were from Sigma; glucagon was from Novo Laboratories, Copenhagen, Denmark. Fig.P was from Fig.P Corporation, distributed by Biosoft, Cambridge, U.K.

Liver cells were isolated and incubated in a Krebs-Henseleit bicarbonate buffer equilibrated with O_2/CO_2 (19:1, v/v) as previously described [18]. Briefly, the liver was perfused at 37°C for about 10 min with a Krebs-Henseleit bicarbonate buffer without calcium, followed by a 25–30-min perfusion with collagenase (30 mg/100 mL of perfusion buffer) and calcium (2.5 mM). The hepatocytes were harvested and incubated at 37°C in a Krebs-Henseleit buffer containing 10 mM glucose, in closed plastic vials saturated with 95% O_2, 5% CO_2 (v/v). These cells were, then, treated with the different agonists and samples were taken at the indicated times for the determination of IP_3 [5] and for glycogen phosphorylase [19]. Samples for IP_3 determination were mixed with TCA (14% final concentration)

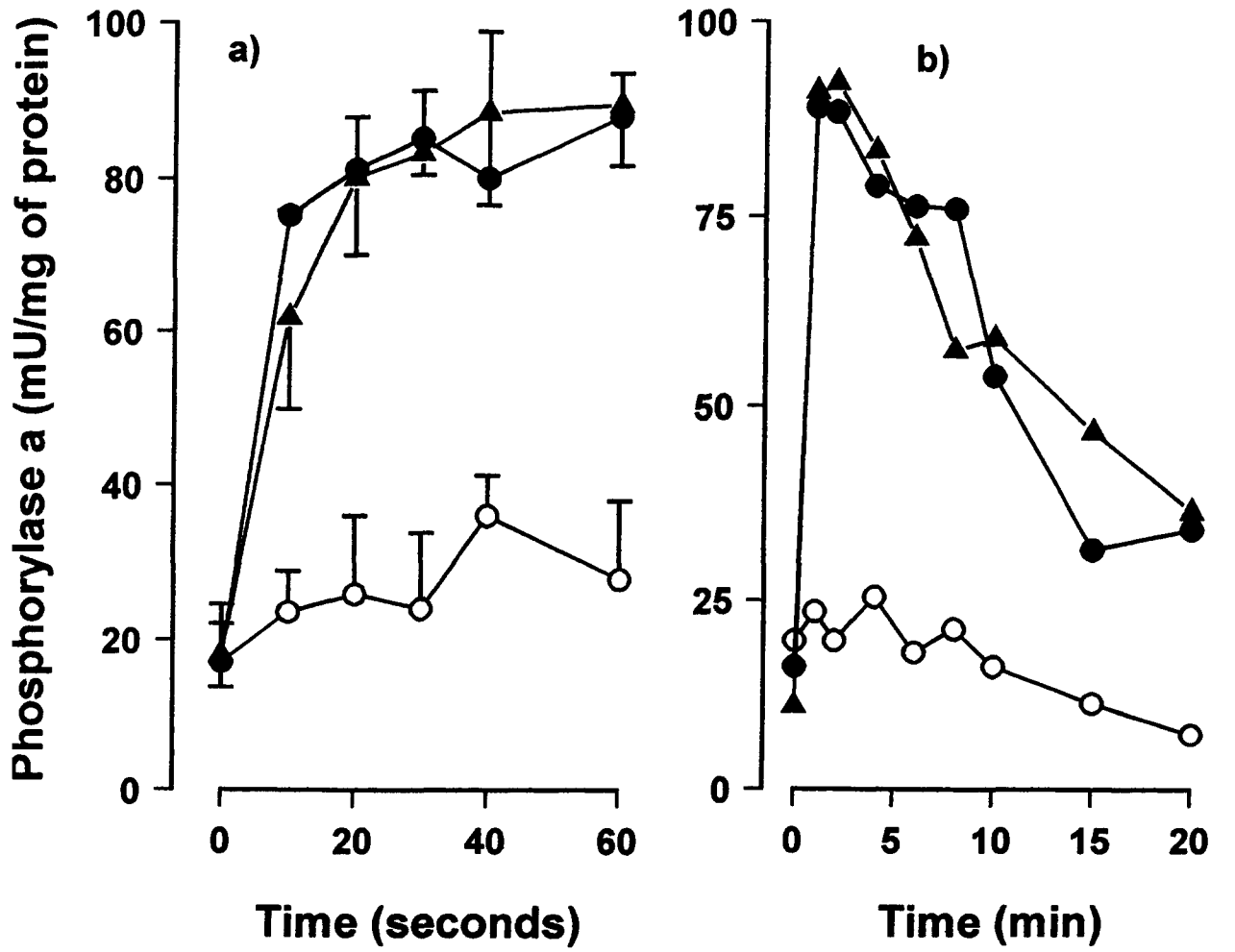

FIG. 1. Time-dependent activation of glycogen phosphorylase by Ap_3A and Ap_4A in hepatocytes. Rat hepatocytes were preincubated at 37°C for 25 min with 10 mM glucose. Control (○), 50 μM of Ap_3A (●), or of Ap_4A (▲) was then added. Enzyme activity was determined at the indicated time points. (a) Short-time activation: data shown are means ± SEM from 4 independent experiments. (b) Long-term activation pattern: data shown from one experiment, representative of several similar (with different time-points) experiments.

and EDTA (5 mM final concentration). After extraction of the TCA and neutralization of the samples (pH 7.4), IP_3 was determined with a competitive protein-binding technique based on a procedure described by Bredt *et al.* [20] with slight modifications. Cerebellum plasma membranes, containing the IP_3 binding protein, were purified from rabbit brain as described [5]. The incubation medium contained about 50 μg of these membranes, 1 nM [^{3}H]IP_3 and 10 μL cell extract in a final volume of 100 μL. Bound and free ligand were separated after 10 min of incubation at 4°C by filtration through Whatman GF/A filters, which were washed 3 times with 7 mL 50 mM Tris-HCl (pH 8.4) and 1 mM EDTA. Radioactivity of the digested filters was determined by liquid scintillation counting. Occasionally, IP_3 was also measured using the IP_3 assay kit from Amersham. cAMP was assayed using the cAMP-assay kit from Amersham. Sampling of hepatocytes and assaying of glycogen phosphorylase activity was as described [18].

RESULTS AND DISCUSSION

Figure 1 (a and b) shows the time-dependent activation of glycogen phosphorylase by Ap_3A and Ap_4A. The degree of activation and the pattern of the activation are similar to what we, and others, observed after ATP or ADP [19, 21]. Indeed, like ATP or ADP, both dinucleotides completely (to a similar degree as after glucagon) activated glycogen phosphorylase. Maximal effect was obtained within 20–30 sec (Fig. 1a) and then declined (Fig. 1b). The transient nature of the activation of phosphorylase was probably not due to a desensitisation of the hepatocytes. Indeed, a sec-

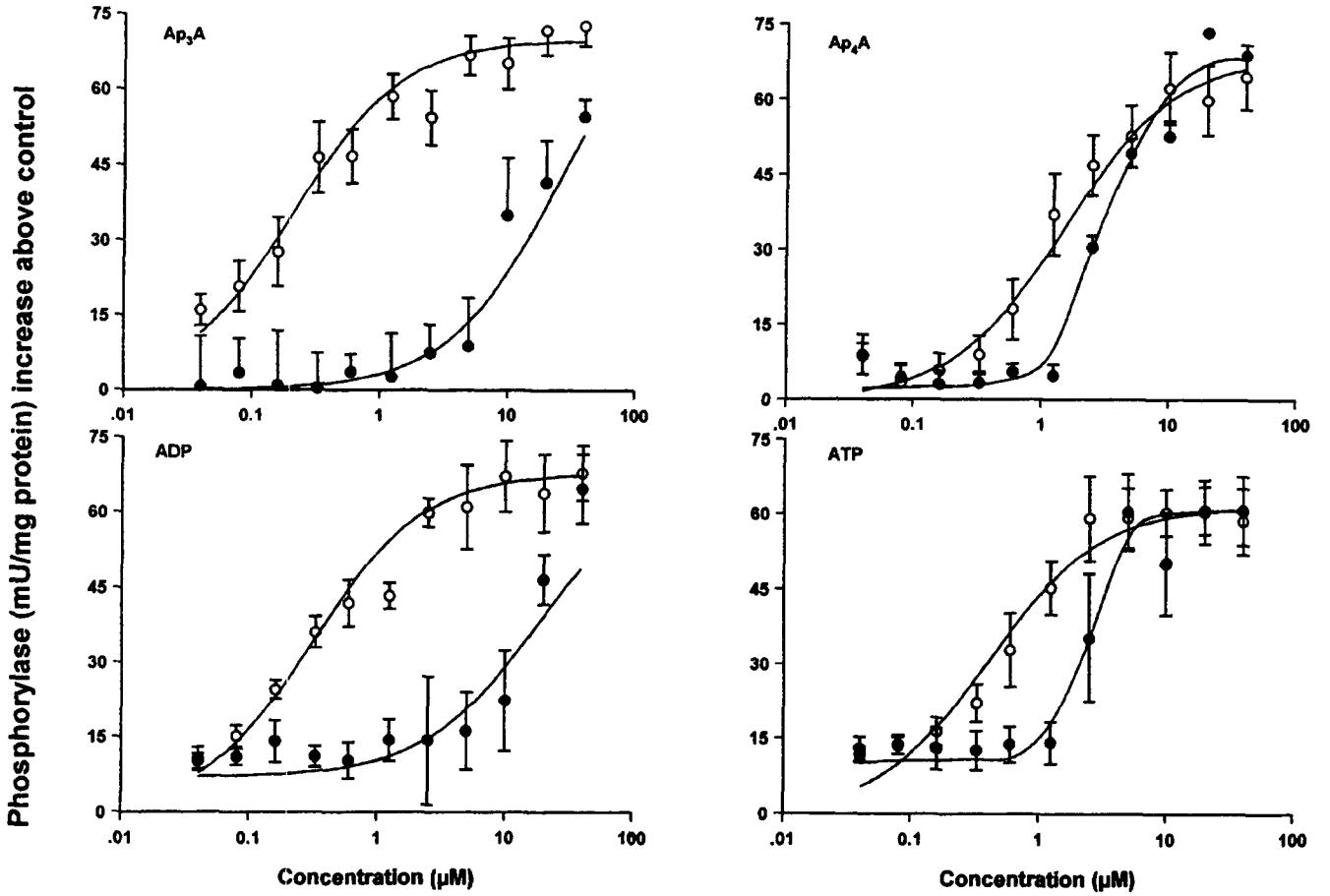

FIG. 2. Effect of PMA treatment of hepatocytes on the activation of glycogen phosphorylase by Ap_3A, Ap_4A, ADP, and ATP. Hepatocytes were incubated for 10 min (after the preincubation period) either with 1% DMSO (○) or with PMA dissolved in DMSO (●) (final concentration is 1.6 μM PMA and 1% DMSO). Afterwards, increasing concentrations of Ap_3A, Ap_4A, ADP, or ATP were added; 30 sec later, samples were taken and phosphorylase was assayed. Phosphorylase activity is expressed as mU/mg of protein above control. Basal levels of phosphorylase ranged from 10–20 mU/mg of protein. Data shown are means ± SEM from at least 3 independent experiments (different cell preparations). Lines drawn were either computer-generated using a Michaelis-Menten type of equation and the fitting facilities of the FigP program, or hand-drawn if proper fitting was not possible. FigP is a nonlinear curve fitting (parameter estimation) program. It uses the compilation and recursive nonlinear least squares techniques to find a set of specific values for the parameters. Control conditions (open symbols): The fit is within 95% expected limits for a correct model (Michaelis-Menten model) for all agonists ($r^2 > 0.95$). Conditions with PMA (closed symbols). Ap_3A and ADP: for these agonists the fit is also within the 95% expected limits for a correct model ($r^2 > 0.95$). To fit these data, we assumed that the maximal effect obtained in the presence of PMA was the same as without PMA. Ap_4A and ATP: these data could not significantly be fitted with the Michaelis-Menten model. The curves are hand-drawn.

TABLE 1. Effect of Ap_3A, Ap_4A, ADP, and ATP on IP_3levels in hepatocytes

	IP_3(pmoles/mg of protein)
Control	36.07 ± 3.74
ATP	95.23 ± 18.33*
ADP	50.37 ± 15.31
Ap_3A	33.46 ± 5.54
Ap_4A	52.15 ± 8.54

Hepatocytes were treated for 7 sec with 200 μM of the indicated agonists. Data are means ± SEM from 5 independent experiments (each done in duplicate or triplicate. * significant difference from control (P = 0.01 from Student's t-test). ANOVA analysis of the data reveals significant differences between ATP and control and between ATP and Ap_3A. No other differences emerged from this analysis.

ond addition of the agonist to the cells, again, fully reactivated the enzyme (not shown). We, next, determined the glycogenolytic potency (characterised by the ED_{50} value) of Ap_3A and Ap_4A. Figure 2 shows the activation of phosphorylase assayed 30 sec after addition of different concentrations of Ap_3A or Ap_4A. For comparison, the effects of increasing concentrations of ADP and ATP are also shown (Fig. 2c and d). From these data, an ED_{50} of 0.31 ± 0.03 μM for Ap_3A and of 1.3 ± 0.3 μM for Ap_4A was deduced (see legend to the figure). ED_{50} for ATP was 0.52 μM and for ADP, 0.34 μM ([22], see also [21]). It follows from these data that Ap_3A is more potent than Ap_4A.

We previously used PMA to discriminate between ATP and some ATP analogues, such as 2-methylthio-ATP [23] and ADP-β-S [24]. We, therefore, checked the effect of PMA on the activation of phosphorylase by Ap_3A, Ap_4A, ADP, and ATP. Figure 2 (closed symbols) shows that, in the presence of PMA, much higher doses of Ap_3A and ADP were needed to activate glycogen phosphorylase. The ED_{50} values calculated under this condition were 15.5 ± 3.1 for Ap_3A and 13.2 ± 1.8 for ADP. The glycogenolytic effects of Ap_4A and of ATP seem less influenced by PMA. Moreover, proper statistical analysis of the data obtained with ATP and Ap_4A in the presence of PMA reveals that these data can no longer be fitted with the Michaelis-Menten type of equation that was used to fit the other response curves. The ED_{50} values for ATP (4.6 μM) and for Ap_4A (5.7 μM) are, therefore, estimated values. These data show that Ap_3A and ADP are about equally affected by PMA, clearly differing from the PMA effect on ATP or Ap_4A. These data are, therefore, supportive of the reported differences between Ap_3A (ADP) and Ap_4A (ATP) [16, 17]. The effect of PMA is probably obtained in an indirect way, possibly mediated by a phosphorylation *via* protein kinase of one of the molecules involved in the transduction of the effect of the nucleotides.

We, next, measured IP_3 levels after the different agonists. For this, we used a relatively high concentration of 200 μM of the different agonists, because we know from our previous work that, for ATP, an increase in IP_3 can only be observed at concentrations at least 10 times higher than the ED_{50} [1, 3]. Sampling for IP_3 assay was done after 7 sec because maximal effects of ATP are obtained within 5–10 sec and decline afterwards [5]. Table 1 shows that, under these conditions, only ATP (as anticipated) is able to significantly increase IP_3. This suggests either that IP_3 is not involved in the activation of phosphorylase by the other agonists or that a small, not easily detectable, increase is sufficient for the observed effect. These data show that ATP is not only different from ADP and Ap_3A, but also from Ap_4A. Although the effect of the dinucleotides on IP_3 is not statistically different (ANOVA, see Table 1), a moderate increase in IP_3 after Ap_4A compared to control conditions, or after Ap_3A, suggests a certain difference between the two dinucleotides.

None of the nucleotides increased cAMP (not shown), but they are all able to counteract the increase in cAMP after glucagon. A cAMP-lowering effect was already reported by us, and others, for ATP and ADP [19, 4], and Fig. 3 illustrates that Ap_3A and Ap_4A are also able to counteract glucagon. No difference among the dinucleotides themselves or between the dinucleotides and ATP or ADP emerges from these data.

These data show that Ap_3A, Ap_4A, and ADP 1. are full glycogenolytic agonists, 2. do not significantly increase the level of IP_3 (Ap_4A as only a moderate effect on IP_3, Ap_3A is completely inactive), and 3. lower the glucagon increased levels of cAMP. Based on these characteristics it is concluded that the agonists (ADP, Ap_3A, and Ap_4A) behave rather similarly, and are different from ATP. However, our data further show that, in the presence of PMA, other similarities emerge between the dinucleotides ADP and ATP. Indeed, the glycogenolytic effect of ADP and Ap_3A

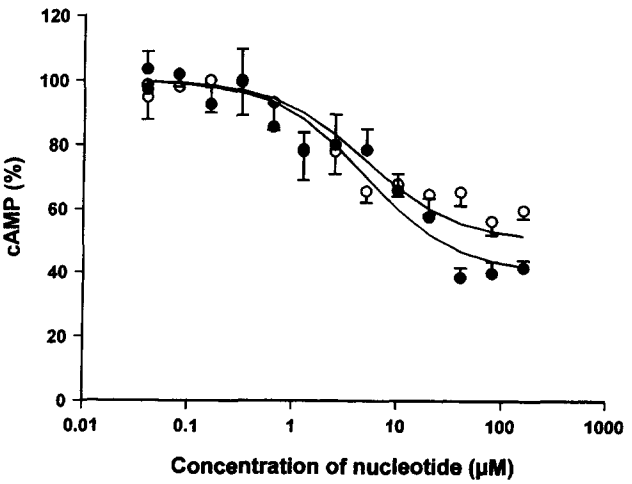

FIG. 3. Dose-dependent inhibition of cAMP increase by Ap_3A and Ap_4A. Cells were treated for one min with glucagon (20 nM) and then for another min with increasing concentrations of Ap_3A (○) or Ap_4A (●). cAMP was, then, assayed and is expressed as % of the control level obtained in the presence of glucagon alone (basal levels of cAMP were around 3 pmoles/mg protein and increased after glucagon to about 15–20 pmoles/mg protein). Data shown are means ± SEM of 3 independent experiments, each done in duplicate. Lines drawn are computer-generated, using the fitting facilities of the FigP program.

is much more sensitive to pretreatment with PMA than that of Ap_4A or ATP. This latter finding corroborates the results of others [7, 16, 17], suggesting that liver cells possess different receptors with different affinities for ATP and ADP and that Ap_3A behaves more like ADP, whereas Ap_4A rather resembles ATP.

Overall, the data suggest the presence of multiple purinoceptors, possibly coupled to different messenger systems and with different affinities for the purinergic agonists.

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