Inosine released after hypoxia activates hepatic glucose liberation through A3 adenosine receptors

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Guinzberg, Raquel, Daniel Cortés, Antonio Díaz-Cruz, Héctor Riveros-Rosas, Rafael Villalobos-Molina, and Enrique Piña. Inosine released after hypoxia activates hepatic glucose liberation through A3 adenosine receptors. Am J Physiol Endocrinol Metab 290: E940–E951, 2006. First published December 13, 2005; doi:10.1152/ajpendo.00173.2005.—Inosine, an endogenous nucleoside, has recently been shown to exert potent effects on the immune, neural, and cardiovascular systems. This work addresses modulation of intermediary metabolism by inosine through adenosine receptors (ARs) in isolated rat hepatocytes. We conducted an in silico search in the GenBank and complete genomic sequence databases for additional adenosine/inosine receptors and for a feasible physiological role of inosine in homeostasis. Inosine stimulated glycogenolysis (∼40%, EC50 4.2 × 10–9 M), gluconeogenesis (∼40%, EC50 7.8 × 10–9 M), and ureagenesis (∼130%, EC50 7.0 × 10–8 M) compared with basal values; these effects were blunted by the selective A3 AR antagonist 9-chloro-2-(2-furanyl)-5-[(phenylacetyl)amino][1,2,4]-triazolo[1,5-c]quinazoline (MRS 1220) but not by selective A1, A2A, and A2B AR antagonists. In addition, MRS 1220 antagonized inosine-induced transient increase (40%) in cytosolic Ca2+ and enhanced (90%) glycogen phosphorylase activity. Inosine-induced Ca2+ mobilization was desensitized by adenosine; in a reciprocal manner, inosine desensitized adenosine action. Inosine decreased the cAMP pool in hepatocytes when A1, A2A, and A2B AR were blocked by a mixture of selective antagonists. Inosine-promoted metabolic changes were unrelated to cAMP decrease but were Ca2+ dependent because they were absent in hepatocytes incubated in EGTA- or BAPTA-AM-supplemented Ca2+-free medium. After in silico analysis, no additional cognate adenosine/inosine receptors were found in human, mouse, and rat. In both perfused rat liver and isolated hepatocytes, hypoxia/reoxygenation produced an increase in inosine, adenosine, and glucose release; these actions were quantitatively greater in perfused rat liver than in isolated cells. Moreover, all of these effects were impaired by the antagonist MRS 1220. On the basis of results obtained, known higher extracellular inosine levels under ischemic conditions, and inosine’s higher sensitivity for stimulating hepatic gluconeogenesis, it is suggested that, after tissular ischemia, inosine contributes to the maintenance of homeostasis by releasing glucose from the liver through stimulation of A3 ARs.

INOSINE IS A NATURALLY OCCURRING PURINE NUCLEOSIDE formed by adenosine deamination. Its normal interstitial concentrations in rat plasma and serum have been reported in the range of 0.5–20 μM (51, 61), and inosine accumulates to even higher levels (>100 μM) than adenosine does in ischemic tissues (34, 41, 50, 51, 56). Our laboratory was the first to describe a stimulatory action of inosine on ureagenesis and gluconeogenesis in isolated hepatocytes (23, 68). However, over the last decade several reports (e.g., Refs. 19, 32, 59) appeared regarding the role of inosine in regulating the immunologic and cardiovascular systems. Although in the majority of cases inosine binds to A3 adenosine receptors (ARs) to promote its effects (19, 32, 59), there are reports in which A2AR (19) or even an AR-independent G protein-coupled receptor (GPCR) pathway (27) were involved.

To date, four AR subtypes have been cloned (A1, A2A, A2B, and A3), each with unique tissue distributions, ligand affinity, and signal-transducing mechanism (for a review, see Ref. 49). All four AR subtypes are present in isolated hepatocytes, where they stimulate glycogenolysis, gluconeogenesis, and ureagenesis (49). Signal transduction systems for obtaining these increases were via adenyl cyclase for A2A and A2B AR, whereas A1 and A3 AR involved changes in cytosolic Ca2+ (20–22, 60, 66). The purpose of this work included the following: 1) to define the receptor type involved in inosine responses in isolated hepatocytes; 2) to identify the signal transduction pathway mediating these inosine responses; 3) to explore the possibility of finding additional adenosine/inosine receptors; and 4) to obtain insight into the physiological meaning of these inosine actions.

MATERIALS AND METHODS

Selective AR agonists and antagonists used in this work are included in Table 1 and are listed in alphabetical order of their abbreviations. Full chemical names, the receptor-binding constant for AR agonist, reported data on the Kᵢ for AR antagonists, and pertinent references are additionally included. All of these compounds were purchased from Sigma RBI.

All animal experiments were conducted in accordance with the Federal Guidelines for the Care and Use of Animals (NOM-062- ZOO-1999, Ministry of Agriculture, Mexico) and were approved by the Institutional Ethics Committee of the National Autonomous University of Mexico’s Faculty of Medicine (FM-UNAM).

Isolation of hepatocytes. Male Wistar rats (150–200 g) were anesthetized with ether, and cells were isolated by the method of Berry and Friend (7) as modified by Guinzberg et al. (23). Hepatocytes were used when viability was at least 95%, as assayed by the trypan blue exclusion method. Experiments were conducted by duplicate with 30–40 mg wet wt hepatocytes.

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Table 1. Specific agonists and antagonists for ARs used in this work

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Chemical Name</th>
<th>Receptor</th>
<th>Action</th>
<th>Receptor-Binding Value</th>
<th>$K_i$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADSPX</td>
<td>1-allyl-3,7-dimethyl-8-p-sulfophenylxanthine</td>
<td>A$_{2B}$</td>
<td>Antagonist</td>
<td>0.6 nM</td>
<td></td>
<td>(28)</td>
</tr>
<tr>
<td>Alloxazine</td>
<td>Benzyl[3H]pyridine 2,4-(H,3H)-dione</td>
<td>A$_1$</td>
<td>Agonist</td>
<td>13 nM</td>
<td></td>
<td>(40)</td>
</tr>
<tr>
<td>CCPA</td>
<td>2-chloro-N$^\mathsf{C}$-cyclopyrenadinosine</td>
<td>A$_1$</td>
<td>Antagonist</td>
<td>0.4 nM</td>
<td></td>
<td>(43)</td>
</tr>
<tr>
<td>CGS-15943</td>
<td>9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazoline-5-amine</td>
<td>A$_1$</td>
<td>Antagonist</td>
<td>4 nM</td>
<td></td>
<td>(31)</td>
</tr>
<tr>
<td>CGS-21680</td>
<td>2-P(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine</td>
<td>A$_{2A}$</td>
<td>Agonist</td>
<td>15 nM</td>
<td></td>
<td>(30)</td>
</tr>
<tr>
<td>CSC</td>
<td>1,3,7-trimethyl-8-(3-chloroethyl) xanthine</td>
<td>A$_{2A}$</td>
<td>Antagonist</td>
<td>54 nM</td>
<td></td>
<td>(29)</td>
</tr>
<tr>
<td>DPCPX</td>
<td>8-cyclopentyl-1,3-dipropylxanthine</td>
<td>A$_1$</td>
<td>Agonist</td>
<td>0.69 nM</td>
<td></td>
<td>(25)</td>
</tr>
<tr>
<td>IB-MECA</td>
<td>1-deoxy-1-[6-[(3-iodophenyl)methyl]amino]-9H-purin-9-yl-N-methyl-$\beta$-D-ribofuranuronamide</td>
<td>A$_1$</td>
<td>Agonist</td>
<td>1.1 nM</td>
<td></td>
<td>(64)</td>
</tr>
<tr>
<td>MRS 1220</td>
<td>9-chloro-2-(2-furanyl)-5-(phenylacetyl) amino-[1,2,4]triazolo[1,5-c]quinazoline</td>
<td>A$_1$</td>
<td>Antagonist</td>
<td>14 nM</td>
<td></td>
<td>(36)</td>
</tr>
<tr>
<td>NECA</td>
<td>5'-N-ethylcarboxamidoadenosine</td>
<td>A$<em>1$, A$</em>{2B}$</td>
<td>Agonist</td>
<td>$A_1$ = 11 nM, $A_{2B}$ = 16 nM</td>
<td></td>
<td>(9)</td>
</tr>
</tbody>
</table>

AR, adenosine receptor.

Ureagenesis. Hepatocytes from 24-h-starved rats were incubated for 1 h at 37°C in an atmosphere of O$_2$-CO$_2$ (95%-5%) for 60 min in a gyratory water bath in Krebs-Ringer buffer (KRB) containing 10 mM glucose, 5 mM (NH$_4$)$_2$CO$_3$, and 3 mM ornithine. Urea synthesis was assayed after 60 min (24).

Gluconeogenesis. Hepatocytes from 24-h-starved rats were incubated for 1 h in KRB containing 10 mM lactate. Glucose synthesis was measured in the supernatant of cells by the glucose oxidase method (18).

Glycogenolysis. Hepatocytes from rats fed ad libitum were incubated for 45 min in KRB without lactate or any other substrate. Glucose release was measured (18).

Glycogen phosphorylase activity. This activity was assayed by measuring the incorporation of [U-$^14$C]glucose 1-phosphate into glycogen, as described by Starke et al. (57). Hepatocytes were exposed to the agents, and aliquots were withdrawn at time intervals and placed in 0.2 ml of ice-cold medium containing 10 mM MES, 20 mM NaF, 25 mM glycerophosphate, 10 mM EDTA, and 0.8 mM digitonin. Hepatocyte extracts (25 µl) were mixed with an equal volume of phosphorylase assay medium containing 50 mM NaF, 4.8 mM caffeine, 86 mM glucose 1-phosphate, 2% glycogen, and 8.5 μCi of [U-$^{14}$C]glucose 1-phosphate and incubated at 37°C. The reaction was stopped after 30 min by the addition of 25 µl of glacial acetic acid. A 50-µl sample was spotted onto filter paper and washed twice with 66% ethanol, washed with acetone, and placed in a cocktail for liquid scintillation counting.

cAMP accumulation. Hepatocytes from fed rats were incubated at 37°C for 2 min in KRB. cAMP was measured using the Amersham kit TRK-4312.

Ca$^{2+}$ measurement in fura 2-AM loaded hepatocytes. This was performed as described by Llopis et al. (42). Briefly, isolated hepatocytes from fed rats were diluted in KRB to a final concentration of 40 mg wet wt/ml and incubated for 10 min in a constantly flow rate of 16 ml/min. Hepatic venous effluents were obtained via a cannula in the vena cava.

Adenosine and inosine release quantification. Nucleosides were measured by enzymatic assay in double-beam spectrophotometer by the method described by Olsson (47).

Statistical methods. Values are reported as means ± SE. Student’s t-test was applied to assess differences between groups. Statistical significance was set at $P < 0.05$.

Identification of cognate ARs on protein databases. Initially, sequences of known ARs from the rhodopsin superfamily were retrieved from the Swiss-Prot protein database at http://caebi.cambio.org/ (3). The amino acid sequence from each of these known ARs was used as bait for BLASTP (1) searches at the National Center for Biotechnology Information GenBank nonredundant protein database (6). To determine the number of sequences encoding ARs in animals with complete genome sequence, we repeated the BLAST search with the tBLASTn program (1), using amino acid sequences of characterized adenosine GPCRs as queries against whole genomic DNA sequences or the high-throughput genomic sequence database from human, mouse, rat, zebra fish, Japanese puffer fish (International Fugu Genome Consortium, assembly version 3.0); http://genome.jgi-psf.org/fug6/fug6.home.html, and the ascidian Ciona intestinalis (assembly version 1.0; http://genome.jgi-psf.org/ciona4/ciona4.home.html). Ab initio gene predictions were performed with the GeneComber system (54), which provides increased gene recognition accuracy by combining predictions from the gene-finding Genscan (10) and HMMGene (37) programs. GeneComber-predicted exons were verified by multiple alignments with amino acid sequences from adenosine GPCRs to gather additional support for constructing gene models.

Multiple sequence alignment and phylogenetic analysis. Multiple sequence alignments were performed by using ClustalX v1.81 (58) and corrected according to gapped BLASTP results (1). Phylogenetic analyses were carried out with MEGA v2.1 (38) software, using both the maximum parsimony and distance-based methods UPGMA (unweighted pair group method with arithmetic mean) and neighbor-joining, along with the Poisson correction distance method, and gaps were treated by pairwise deletion. Accuracy of reconstructed trees was examined by the bootstrap test with 1,000 replications. Phylogenetic trees were rooted with the bovine rhodopsin sequence. Complete names of organisms included in the phylogenetic analysis are as follows: ANOGA, Anopheles gambiae (Arthropoda, insecta); ASTMI, Asterina miniata (starfish; Echinodermata); BOVIN, Bos taurus (Chordata, vertebrata, mammalia); CAEBR, Caenorhabditis briggsae (Nematoda); CAMEL, Caenorhabditis elegans (Nematoda); CANFA, Canis familiaris (Chordata, ver-
tebrata, mammalia); CAVPO, Cavia porcellus (domestic guinea pig; Chordata, vertebrata, mammalia); CHICK, Gallus gallus (Chordata, vertebrata, mammalia); CIOIN, Ciona intestinalis (Chordate, urochordata, ascidiacea); DANRE, Danio rerio (zebra fish; Chordata, vertebrata, teleostei); DROME, Drosophila melanogaster (Arthropoda, insecta); FUGRU, Fugu rubripes (Japanese puffer fish; Chordata, vertebrata, teleostei); HORSE, Equus caballus (Chordata, vertebrata, mammalia); HUMAN, Homo sapiens (Chordata, vertebrata, mammalia); MOUSE, Mus musculus (Chordata, vertebrata, mammalia); RABBIT, Oryctolagus cuniculus (Chordata, vertebrata, mammalia); RAT, Rattus norvegicus (Chordata, vertebrata, mammalia); SHEEP, Ovis aries (Chordata, vertebrata, mammalia); XENLA, Xenopus laevis (Chordata, vertebrata, mammalia), and XENLA, Xenopus laevis (Chordata, vertebrata, mammalia).

RESULTS

Inosine stimulates glycogenolysis, gluconeogenesis, and ureagenesis in hepatocytes via A3 AR. Adenosine and inosine concentration-response curves to stimulate glycogenolysis, gluconeogenesis, and ureagenesis rates are presented in Fig. 1. Effective concentration (EC50) values of adenosine and inosine were calculated, along with ratios for (adenosine EC50 value)/ (inosine EC50 value) in each activated pathway (Table 2). These data indicated that gluconeogenesis and ureagenesis might be activated at lower concentrations of inosine than of adenosine. The stimulating effect of 1 μM inosine on glycogenolysis, gluconeogenesis, and ureagenesis was blunted specifically with the selective A3 AR antagonist 9-chloro-2-(2-furanyl)-5-[(phenylacetyl)amino]-[1,2,4]triazolo[1,5-c]quinazoline (MRS 1220) but was not modified when inosine was simultaneously incubated with 9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS-15943), 1,3,7-trimethyl-8-(3-chlorostyryl)xanthine (CSC), and 1-allyl-3,7-dimethyl-8-p-sulfophenylxanthine (ADSPX), or alloxazine, selective antagonists for A1, A2A, and A2B AR, respectively (Fig. 2); i.e., inosine stimulated these three metabolic routes in isolated rat liver cells only if A3 AR was not blocked. Two selective A2B AR antagonists were used in these experiments because the required ADSPX solvent [A2B antagonists with lower receptor-binding constant (Table 1)] is dimethyl sulfoxide, which, when used at a concentration of 1 mM to quantify urea, interfered with the assay (results not shown) (24). Thus, in this case, ADSPX was substituted for a water-soluble selective A2B AR antagonist such as alloxazine.

Inosine-induced Ca2+ mobilization to stimulate glycogenolysis, gluconeogenesis, and ureagenesis. A common action of adenosine and an AR-specific agonist is to increase [Ca2+]; i.e, in isolated hepatocytes (22). Results in Table 3 show that inosine shares in this action. It is noteworthy that stimulation with either inosine or the individual AR agonists employed resulted in a rise in Ca2+ similar to the rise obtained with adenosine, which might activate all four ARs. We performed three series of experiments to investigate the role of calcium in liver metabolic pathway inosine-mediated activation. In the first series, Ca2+ was eliminated from KRB; in the second series, EGTA was included in Ca2+-free KRB to chelate extracellular Ca2+; and in the third series, BAPTA-AM was added to Ca2+-free KRB to chelate intracellular Ca2+. Inosine elicited a lesser stimulation in studied metabolic pathway rates when cells were incubated in Ca2+-free KRB. In addition, these pathways were not stimulated at all by the nucleoside when either of the used chelating agents was present (Fig. 3).

To identify AR involved in the transient inosine-mediated increase of free Ca2+, we conducted the experiment presented in Fig. 4. Inosine alone produced a temporary increase in Ca2+ (Fig. 4A) that was not modified by A1, A2A, and A2B AR-selective antagonists (Fig. 4, B–D) but was blunted by A3 AR antagonist (Fig. 4E).

Table 2. EC50 values for adenosine and inosine to stimulate glycogenolysis, gluconeogenesis, and ureagenesis in isolated rat hepatocytes

<table>
<thead>
<tr>
<th>Pathway</th>
<th>EC50 Values for Adenosine</th>
<th>EC50 Values for Inosine</th>
<th>Ratio, EC50 Adenosine to EC50 Inosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogenolysis</td>
<td>3.8 × 10^-7 M</td>
<td>4.2 × 10^-7 M</td>
<td>0.90</td>
</tr>
<tr>
<td>Gluconeogenesis</td>
<td>1.7 × 10^-6 M</td>
<td>7.8 × 10^-6 M</td>
<td>2.2</td>
</tr>
<tr>
<td>Ureagenesis</td>
<td>1.8 × 10^-7 M</td>
<td>7.0 × 10^-7 M</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Data obtained from experiments in Fig. 1. EC50, effective concentration.
Fig. 2. Effect of inosine in the absence or presence of adenosine receptor (AR)-selective antagonists on the rate of glycogenolysis (A), gluconeogenesis (B), and ureagenesis (C) in hepatocytes. Cells were incubated as detailed in MATERIALS AND METHODS. To stimulate A2B AR alone, an AR agonist for A2B and A1, such as NECA (Table 1), was mixed with DPCPX, a selective antagonist for A1 AR. Nucleosides, agonists, and antagonists were used at a 1-μM final concentration. Statistical significance, nucleoside or agonist vs. control; *P < 0.001 in all cases.

Desensitization experiments were conducted to test whether inosine acted through GPCR. Isolated hepatocytes were stimulated with 1 μM adenosine or inosine, and Ca^2+ transient rises were monitored. After recovery to initial values in ~2 min, cells were stimulated again. Under this protocol, adenosine failed to reinitiate cell activation independently of whether first activation was produced by adenosine (Fig. 5A) or by inosine (Fig. 5B). Similarly, inosine failed to reinitiate cell activation independently of whether initial activation was obtained with adenosine (Fig. 5B) or inosine (Fig. 5C).

**Table 3.** [Ca^{2+}]i, in isolated hepatocytes treated with adenosine, inosine, or selective AR agonists

<table>
<thead>
<tr>
<th>Additions</th>
<th>AR-Stimulated</th>
<th>[Ca^{2+}]i, nmol/l</th>
<th>Values, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>195 ± 6.3</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Adenosine</td>
<td>All 4</td>
<td>284 ± 7.3</td>
<td>146</td>
</tr>
<tr>
<td>Inosine</td>
<td>?</td>
<td>274 ± 6.3</td>
<td>141</td>
</tr>
<tr>
<td>CCPA</td>
<td>A1</td>
<td>301 ± 6.9</td>
<td>154</td>
</tr>
<tr>
<td>CGS-21680</td>
<td>A1</td>
<td>281 ± 6.9</td>
<td>144</td>
</tr>
<tr>
<td>NECA plus DPCPX</td>
<td>A2B</td>
<td>298 ± 7.9</td>
<td>153</td>
</tr>
<tr>
<td>IB-MECA</td>
<td>A3</td>
<td>279 ± 7.9</td>
<td>143</td>
</tr>
</tbody>
</table>

Numbers are means ± SE of duplicates from 4 independent cell preparations. [Ca^{2+}]i, cytosolic Ca^{2+} concentration. Experimental conditions as in MATERIALS AND METHODS. To stimulate A2B AR alone, an AR agonist for A2B and A1, such as NECA (Table 1), was mixed with DPCPX, a selective antagonist for A1 AR. Nucleosides, agonists, and antagonists were used at a 1-μM final concentration. Statistical significance, nucleoside or agonist vs. control; *P < 0.001 in all cases.

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<tr>
<td>None</td>
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<tr>
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<td>A1</td>
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<td>154</td>
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<tr>
<td>CGS-21680</td>
<td>A1</td>
<td>281 ± 6.9</td>
<td>144</td>
</tr>
<tr>
<td>NECA plus DPCPX</td>
<td>A2B</td>
<td>298 ± 7.9</td>
<td>153</td>
</tr>
<tr>
<td>IB-MECA</td>
<td>A3</td>
<td>279 ± 7.9</td>
<td>143</td>
</tr>
</tbody>
</table>
hepatocytes (Fig. 7). Next, hepatocytes were incubated with AR antagonist-supplemented inosine. Thus selective antagonists for each of the four ARs, CGS-15943 for A1, CSC for A2A, ADSPX for A2B, and MRS 1220 for A3, were used so that different mixtures of three of these antagonists added to hepatocytes would maintain three of the four ARs blocked, leaving only one AR able to be activated, which might or might not be stimulated by inosine. Only in experiments in which A3 AR was not antagonized by the adequate mixture of AR agents did inosine decrease the cAMP cellular pool (Fig. 8), similarly to IB-MECA, an A3 AR agonist, whereas, if inosine was added to cells in which A1, A2A, or A2B AR were not antagonized by adequate AR blocker mixtures, cAMP values remained unmodified (Fig. 8). Additional experiments are required to understand why inosine alone did not modify the cAMP cellular pool (Fig. 7), whereas inosine did indeed decrease the cAMP pool if A1, A2A, and A2B AR were blocked by their selective antagonists (Fig. 8).

Fig. 3. Calcium participation in inosine-mediated stimulation of glycogenolysis (A), gluconeogenesis (B), and ureagenesis (C) in hepatocytes. Cells were placed under 4 different conditions: 1) complete Krebs-Ringer buffer (KRB) containing 1.2 mM Ca2+; 2) Ca2+-free KRB; 3) cells were preincubated for 15 min in Ca2+-free KRB supplemented with 1.2 mM EGTA; and 4) cells were preincubated for 20 min in Ca2+-free KRB supplemented with 10 μM BAPTA-AM. Control cells at left (filled bars) of each experimental condition and hepatocytes were supplemented with 1 × 10⁻⁶ M inosine at the right (open bars) of each experimental condition. Each datum in the figure corresponds to mean ± SE of duplicate incubations from 4 to 6 independent cell preparations. *Statistical significance for cells incubated with 1) KRB with Ca2+ vs 2) Ca2+-free KRB + inosine, P < 0.01; ** Statistical significance for cells incubated with 1) KRB with Ca2+ + inosine vs. 2) Ca2+-free KRB + inosine, P < 0.01; ** Statistical significance for cells incubated with 1) KRB with Ca2+ + inosine and 4) Ca2+-free-KRB with BAPTA-AM + inosine, both P < 0.001.

Fig. 4. Effect of inosine in the absence or presence of AR-selective antagonists on cytosolic Ca²⁺ concentration ([Ca²⁺]ᵢ) in hepatocytes. Cells were labeled with fura 2-AM and stimulated with 10⁻⁶ M inosine alone or supplemented with 10⁻⁶ M for each AR-selective antagonist indicated. Experiment was repeated 3 times with identical results.

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In any event, cAMP does not appear to be involved in inosine-activated metabolic routes in hepatocytes.

Phylogenetic analyses ruled out the existence of an additional GPCR homologous to ARs in mammals. The results in this paper, as well as those of other authors, clearly demonstrate that GPCR, mainly through A1 AR, mediates some inosine effects. However, this does not discard the possibility that other adenosine-related GPCRs might exist, including a cognate inosine GPCR. To explore the latter possibility, we conducted an extensive search for homologous protein sequences to the adenosine/inosine receptor in whole genomic DNA sequences from human, mouse, rat, zebra fish (D. rerio), Japanese puffer fish (F. rubripes), and the ascidian C. intestinalis. Subsequently, we conducted a phylogenetic analysis for retrieved adenosine/inosine receptor sequences. We found no additional cognate adenosine/inosine receptors in addition to the four known adenosine GPCRs in human, mouse, and rat. Unexpectedly, however, we did find three additional AR-homologous protein sequences in puffer fish and one in zebra fish. Recently, a similar observation was reported with α2-adrenoceptors, because the zebra fish possesses five α2-adrenoceptors instead of the three found in mammals and the puffer fish possesses eight α2-adrenoceptors (11, 52, 53). Figure 9 shows a phylogenetic tree constructed with a total of 46 full-length protein sequences identified as ARs (all belonging exclusively to animals). It can be observed that all AR protein sequences in mammals belong to one of the four known AR types. No additional AR types were found in mammals; however, in the puffer fish, two distinct A1 AR were found (designated provisionally as A1A and A1B) along with one additional A2 AR (provisionally denominated A2C). On the other hand, in the complete genome of C. intestinalis (a nonvertebrate chordate that diverged very early from other chordates, including vertebrates) we identified only three AR-homologous protein sequences, although none resulted orthologous (same gene in different species) to the four AR types known in mammals. These three C. intestinalis ARs are grouped with other AR sequences found in zebra fish, puffer fish, starfish (echinodermata), arthropoda, and nematoda; these sequences probably comprise a fifth AR type. Within this group, only the AR from the starfish A. miniata has been experimentally demonstrated as an AR coupled to a G1-linked protein (35).

Adenosine, inosine, and glucose are released by the liver under hypoxia/reoxygenation conditions. Once we defined which AR was involved in inosine action in liver, the signal transduction pathway mediating inosine action, and the absence of an additional adenosine/inosine receptor participating in these responses, we focused on the physiological meaning of inosine-mediated action in liver. It is known that adenosine and inosine can be released by different organs, e.g., brain (39, 65), heart (34, 41, 56), eye (50), lung (45), kidney, and liver (51). Furthermore, release of these nucleosides is induced under hypoxic conditions (34, 41). Isolated rat hepatocytes also release adenosine under hypoxic conditions (5); however, the metabolic effect of endogenous adenosine and inosine release in liver has not been tested. Thereafter, we subjected both perfused rat liver and isolated hepatocytes to hypoxia/reoxygenation conditions and measured inosine, adenosine, and glucose release. During hypoxic incubation, isolated hepatocytes accumulated inosine, adenosine, and glucose in extracellular volume (Table 4). Both nucleosides and glucose accumulation were observed additionally under conditions of hypoxia/reoxygenation. The selective antagonist for A1 AR, MRS 1220, impaired liberation of glucose from intracellular sources; but interestingly, it also impaired inosine and adenosine release from hepatocytes.

We obtained similar results in perfused rat livers that were subjected to hypoxia and hypoxia/reoxygenation conditions (Fig. 10). Once experimental conditions were set, inosine, adenosine, and glucose release began after an initial lag of 2.5 min. Inosine reached a plateau after 10 min and adenosine after 5 min, but glucose increased progressively during the following 30 min (Fig. 10).
Under hypoxia and hypoxia/reoxygenation conditions, gluconeogenesis and ureagenesis activities were assayed in rat hepatocytes that were isolated from fasted rats (16 h). Both ATP-dependent glucose and urea production diminished by 50% in isolated hepatocytes incubated under hypoxia or hypoxia/reoxygenation conditions (data not shown). These latter results can be explained because under low oxygen tension, insufficient ATP production precludes flux through anabolic pathways (8).

Fig. 6. Effect of inosine and adenosine of glycogen phosphorylase activity in hepatocytes from fed rats. Isolated hepatocytes (20 μg/110 μg of protein) were incubated in 5 ml of Krebs-Ringer bicarbonate containing 1.2 mM CaCl₂. Glycogen phosphorylase activity was measured as detailed in MATERIALS AND METHODS. A: samples were incubated with 10⁻⁶ M inosine alone (○), 10⁻⁶ M inosine + 10⁻⁶ M MRS 1220 (□), or 10⁻⁶ M inosine + 10 μM BAPTA-AM (▲). Values are means ± SE of 3 independent experiments by duplicate. Statistical significance: P < 0.001 by comparing inosine at 0 min vs. inosine at 2.5, 5, and 10 min; P < 0.001 by comparing inosine alone vs. inosine + MRS 1220 or inosine + BAPTA-AM; P < 0.01 (at least) by comparing adenosine at 0 min vs. adenosine at 2.5, 5, and 10 min; P < 0.01 (at least) by comparing adenosine alone vs. adenosine + MRS 1220 or adenosine + BAPTA-AM.

Fig. 7. Effect of adenosine, inosine, and AR-selective agonists on cAMP production in hepatocytes. Cells were incubated for 2 min in KRB with adenosine (○), inosine (□), and the following AR selective agonists: 2-chloro-N⁶-cyclopentyladenosine for A₁ (▲); 2-P(2-carboxyethyl)phenethyl-amino-5'-N-ethylcarboxamidoadenosine for A₂A (△), and 1-deoxy-1-[6-[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-β-d-ribofuranuronamide for A₃ (▼); to stimulate A₂B AR (▪), a mixture of 5'-N-ethylcarboxamido-adenosine and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was used. Results are expressed as %basal value, which was 0.74 ± 0.03 pmol of cAMP formed in 2 min/mg (wet wt). Each value represents means ± SE of 4 independent experiments, each performed in duplicate. Statistical significance vs. basal is indicated: *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 8. Effect of inosine on cAMP values of hepatocytes, to which 3 of 4 ARs were inhibited by mixtures of selective AR antagonists as detailed in the text. Inosine (1 μM, final concentration) was added to each tube in which the AR noninhibited remnant AR was contained: A₁ (▲) when mixing 1 μM (final concentration) CSC + 1 μM ADSPX + 1 μM MRS 1220; A₂A (△) when mixing 1 μM DPCPX + 1 μM ADSPX + 1 μM MRS 1220; A₃(▼) when mixing 1 μM DPCPX + 1 μM CSC + 1 μM MRS 1220; and A₂B (▪) when mixing 1 μM DPCPX + 1 μM CSC + 1 μM ADSPX. Cells were incubated in KRB with the indicated additions. Results are expressed as %basal value, which was 0.74 ± 0.03 pmol of cAMP formed in 2 min/mg (wet wt). Each value represents means ± SE of 4 independent experiments, each performed in duplicate. Statistical significance vs. basal is indicated: *P < 0.001.
Fig. 9. Phylogenetic analysis of ARs. Phylogenetic tree constructed with available protein sequences belonging to the AR subfamily by using minimum evolution method. Trees were calculated using MEGA 2.1 (38). Dotted bars indicate nodes supported in >70% (open), >80% (gray), or >90% (filled) of 1,000 random bootstrap replicates of all UPGMA (unweighted pair group method with arithmetic mean), neighbor-joining, minimum-evolution, and maximum-parsimony trees. Scale bar represents 0.2 amino acid substitutions per site. Obtained trees were rooted by use of bovine rhodopsin. Thick vertical bars indicate the taxonomic group to which the protein sequence belongs and fine vertical bars the type of AR to which the protein sequence belongs. Sequence names are indicated according to a Swiss-Prot-like identifier (gene organism) followed by the database accession number (GenBank, PIR, Swiss-Prot, etc.) and protein amino acid length. AR sequences deduced from genomic sequences were obtained from the following sources: the Danio rerio Sequencing Group at the Sanger Institute (http://www.sanger.ac.uk/Projects/D_rerio/), the Fugu rubripes Genome Project v3.0 (2), and the Ciona intestinalis Genome Project v1.0 (16), the last 2 both at the US Department of Energy Joint Genome Institute (http://genome.jgi-psf.org/fugu6/fugu6.home.html and http://genome.jgi-psf.org/ciona4/ciona4.home.html). Experimentally characterized ARs are underlined. A full list of organism names included in the tree is provided in MATERIALS AND METHODS.
Main metabolic pathway stimulation in liver by inosine is absolutely dependent on an increase in free [Ca^{2+}]. (Fig. 3). Thus incubation of cells in Ca^{2+}-free KRB supplemented with the intracellular chelant BAPTA-AM impaired any inosine-mediated activation in glycogenolysis, gluconeogenesis, and ureagenesis rates (Fig. 3). Nonetheless, when hepatocytes were incubated in Ca^{2+}-free KRB in the absence of chelant agents, inosine produced minor stimulation in the metabolic pathway rates that we studied compared with stimulation observed in KRB containing 1.2 mM Ca^{2+} (Fig. 3). All these data point to a relevant role of extracellular Ca^{2+} in inosine-mediated transduction actions in liver and to a minor contribution of intracellular Ca^{2+} storage compartments to drive the same actions. Unpublished experiments (Guinzenberg R and Piña E) using isolated hepatocytes, incubated in KRB with 1.2 mM Ca^{2+} and challenged with MRS 1220, an A3 AR agonist, are confirmatory. Thapsigargin, an inhibitor of Ca^{2+} release from intracellular storage compartments, decreases stimulation of urea synthesis by nearly 40%.

The following experiment presents another property of the inosine-sensitive AR. This nucleoside desensitizes AR toward adenosine (Fig. 4); a lower concentration of serum adenosine will be quantitatively less important compared with inosine to promote further metabolic responses in liver. In addition, these data support that a GPCR is involved in inosine-mediated actions in liver. Intracellular Ca^{2+} increase has been shown to stimulate glycogenolysis (33, 63). In particular, two Ca^{2+}-mobilizing agents, namely epinephrine and ionophore A-23187, promoted hepatocyte glycogen phosphorylase activation that led to an increase in cell glucose release (62). Thereafter, a [Ca^{2+}], rise by A3 AR stimulation in hepatocytes by any of the studied nucleosides (Fig. 4) in turn activated glycogen phosphorylase to a greater extent with inosine than with adenosine (Fig. 6). With the information recorded to this point in this work, we could anticipate a blockade in nucleoside-mediated phosphorylase activation with the use of either a selective A3 AR antagonist (Fig. 4) or an intracellular chelating agent (Fig. 3). In fact, both inhibitory actions were recorded (Fig. 6).

Two additional experiments analyzing the role of cAMP as a signal transduction pathway for inosine-mediated metabolic actions gave negative results. Inosine alone, as well as adenosine alone, did not modify cAMP pool in liver cells (Fig. 7). In another set of experiments with isolated hepatocytes (Fig. 8),...
insoluble lowered the cAMP pool and behaved similarly to the selective A₁ AR agonist IB-MECA (Fig. 7) but only when selective A₁, A₂A, and A₂B AR antagonists were supplemented in the incubation mixture (Fig. 8). The significance of these experiments remains to be evaluated but is inconsistent with any participation of cAMP in inosine-mediated activation of metabolic pathways in liver.

Phylogenetic analysis results excluded the existence of additional cognate adenosine/inosine receptors in mammals, but this analysis leads us to propose that the four AR types observed in mammals, A₁, A₂A, A₂B, and A₃, arose during the evolution of early vertebrates. Their origin is related to genome duplications produced before radiation of jawed vertebrates some 500 million years ago (26, 55). Phylogenetic analysis also suggests the probable existence of a fifth type of AR in invertebrates and lower vertebrates (fishes). This latter finding agrees with previous papers that claim the presence of adenosine GPCR in nonvertebrate animals such as the blowfly Calliphora vicina (44), the bloodsucking bug Rhodnius prolixus (12), the mussels Mytilus californianus (13) and Mytilus edulis (4), and the spiny lobster Panulirus argus (17). Furthermore, one protein within this group (accession no. AAN33001) has been experimentally demonstrated as an AR in the starfish A. miniata (35), reinforcing the idea that this group of proteins probably corresponds to a fifth type of AR. It should be mentioned that Clark et al. (15), after a great effort to identify novel human transmembrane proteins, reported an additional putative AR of 347 amino acid (AA) length (accession no. AAQ89007). However, this novel protein, predicted from isolated full-length cDNA, is a chimeric protein comprising an NH₂-terminal domain identical to the first 119 AA from the A₃ AR and a COOH-terminal domain homologous to single Ig domain receptor (140 – 347 AA) (14). This chimeric protein results from alternative mRNA splicing, fusing the first exon of A₃ AR (ADORA3) gene and ADO26 gene located downstream of ADORA3 gene. However, on the basis of modeling studies of A₃ AR (46) it can be predicted that the adenosine-binding domain in this chimeric protein is disrupted and, therefore, cannot be considered as an AR. In short, the considered exclusion of additional cognate adenosine/inosine receptors in humans and rats (Fig. 9) reinforces the previously suggested central role of hepatic A₃ AR as the physiological receptor for inosine in preference to adenosine.

Fig. 10. Release of inosine, adenosine, and glucose from perfused rat liver under different oxygenation conditions. All experiments were performed after a 30-min equilibration period in which liver was perfused with KRB solution saturated with O₂-CO₂ mixture (19:1). Under control conditions, the same perfusion solution (KRB) saturated with an O₂-CO₂ mixture (19:1) was passed through the liver for an additional 30 min (○). In hypoxia experiments, the perfusion solution was replaced with KRB saturated with an N₂-CO₂ mixture (19:1) and passed through the liver for an additional 30 min (△). In hypoxia/reoxygenation experiments, the perfusion solution was replaced first with KRB solution saturated with N₂-CO₂ mixture (19:1) followed 5 min later with KRB solution saturated with O₂-CO₂ mixture (19:1) for 25 min (●). An additional hypoxia/reoxygenation experiment was performed in identical form, but 10⁻⁶ M MRS 1220 was included in the KRB solutions (□). Liver effluent samples (100 µl) were withdrawn at time intervals. Values are means ± SE for 3 independent and duplicated experiments. Statistical significance for inosine values: P < 0.001 by comparing control vs. the other 3 experimental groups at all tested times; P < 0.001 by comparing hypoxia/reoxygenation vs. hypoxia/reoxygenation + MRS 1220 at all tested times. Statistical significance for adenosine values: P < 0.01 by comparing control vs. hypoxia or hypoxia/reoxygenation; P < 0.05 by comparing control vs. hypoxia/reoxygenation + MRS 1220; P < 0.001 by comparing hypoxia/reoxygenation + MRS 1220 vs. hypoxia or hypoxia/reoxygenation. Statistical significance for glucose values: P < 0.001 by comparing control or hypoxia/reoxygenation + MRS 1220 vs. hypoxia or hypoxia/reoxygenation at all times tested.
(22.7-fold in Fig. 10) followed the release of nucleosides. Because this liberation was blunted with the A3 AR antagonist, we can conclude that glucose release was due to the presence of nucleosides. Relevance of the herein-reported studies on inosine and its physiological role in the liver, stimulating glucose release, is further supported by the documented protective role of inosine for a variety of ischemic and inflammatory injuries, particularly in muscular tissues (67). Therefore, it appears plausible that release of inosine/adenosine under hypoxic conditions in tissues other than liver (34, 41, 50, 51, 56, 61) might promote liberation of glucose from hepatic cells responding to the activation of both nucleosides, preferably inosine. In conclusion, we propose that in situations of tissular ischemia, inosine liberated from different tissues has a physiologic role of paramount importance, i.e., to contribute in maintaining body homeostasis by providing blood glucose from liver glycogen through A3 AR activation. In contrast, although stimulation of specific A1, A2A, and A2B hepatic ARs resulted in glycogenolysis, gluconeogenesis, and ureagenesis from liver glycogen through A3 AR activation, presently, the effective function of these receptors appears plausible that release of inosine/adenosine under hypoxic conditions will promote liberation of glucose from hepatic cells.

REFERENCES

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