Depression of cell metabolism and proliferation by membrane-permeable and -impermeable modulators: role for AMP-to-ATP ratio

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Menze, Michael A., Matthew J. Clavenna, and Steven C. Hand. Depression of cell metabolism and proliferation by membrane-permeable and impermeable modulators: role for AMP-to-ATP ratio. Am J Physiol Regul Integr Comp Physiol 288: R501–R510, 2005. First published September 30, 2004; doi:10.1152/ajpregu.00490.2004.—The metabolic and developmental depression commonly observed during natural states of dormancy, such as diapause and quiescence, is typically accompanied by an increase in the intracellular ratio of AMP to ATP. We investigated the impact of artificially increasing the AMP-to-ATP ratio in mouse macrophages. Evidence is presented here that the P2X7 receptor channel can be used as an effective means to load cells with membrane-impermeable compounds. Intracellular loading of adenosine-5′-O-thionomophosphate (AMPS), a nonhydrolyzable analog of 5′-AMP and potent activator of AMP-activated protein kinase, significantly depresses metabolism and proliferation of macrophages. The intracellular effective AMP-to-ATP ratio obtained (the sum of AMPS plus endogenous 5′-AMP) was 0.073, well above that reported to activate AMP-activated protein kinase in vitro. Optimizing both the conditions under which the P2X7 receptor channel is opened and the duration of opening facilitates high analog uptake and ~98% survivorship. An advantage to AMPS is its minimal impact on other components of the nucleotide pool, most notably the unchanged concentration of ADP. An alternative way to shift the effective AMP-to-ATP ratio is by incubation with the membrane-permeable compound 5-aminoimidazole-4-carboxamidine-1-β-D-ribofuranoside (AICAR), which is phosphorylated intracellulary to form the 5′-AMP analog ZMP. Despite a rapid intracellular accumulation of AICAR, conversion to ZMP was slow and inefficient. Furthermore, AICAR incubation increased cellular ADP, and, although cell proliferation was depressed, the overall cellular energy flow was unchanged. The rapid action of AMPS avoids upregulation of compensatory metabolic pathways and may provide a viable approach for promoting cell stasis.

P2X7 receptor channel; macrophages; AMP-activated protein kinase; cellular stasis

Numerous animals are able to arrest metabolism and development prior to or in response to severe environmental conditions like hypoxia, desiccation, or freezing (11, 12, 14, 16). These natural states of dormancy occur in phylogenetically diverse species such as the annual killifish Austrofundulus limnaeus (53), various tardigrade species (7, 54), the brine shrimp Artemia franciscana (3), the nematode worm Aphelechus avenae (8), and the fresh water sponge Eunapius fragilis (29). The length of survival during dormancy is directly proportional to the degree of metabolic depression (15). Therefore, a coordinated downregulation of metabolic processes may be a crucial part of adaptation to survive severe environmental conditions. Metabolic downregulation seems to be accompanied by a shift in the cellular ratio of AMP to ATP in species thus far investigated in this respect. During the entrance into diapause or quiescence, a rise in intracellular AMP combined with either no change or decrease in ATP concentration promotes a rise in the AMP-to-ATP ratio (32, 33). In this study, we evaluated the metabolic impact of utilizing analogs of AMP to artificially elevate the AMP-to-ATP ratio in a mammalian cell line (macrophages, J774.A1).

Interestingly, recent discoveries suggest that an enzyme sensitive to AMP/ATP serves as a metabolic sensor of eukaryotic cells. The AMP-activated protein kinase (AMPK; EC 2.7.1.37.CAMK) is part of an ultrasensitive system for monitoring cellular energy changes and can be interpreted as a metabolic “fuel gauge” (18, 20). Under optimal physiological conditions, the AMP-to-ATP ratio is maintained at a level of ~0.01 (19). An increase in AMP/ATP activates the system by four different mechanisms: allosteric activation of the enzyme by binding to AMP, allosteric activation of the upstream AMPK kinase, which increases the AMPK activity by phosphorylation of AMPK, a decreased affinity of the liganded AMPK for the protein phosphatase, and an increased affinity of the liganded AMPK to AMPK kinase (20). The activation of the AMPK cascade leads to several metabolic responses: an inhibition of anabolic pathways such as fatty acid synthesis, lipogenesis, and cell proliferation and conversely to an activation of selected catabolic pathways such as fatty acid oxidation and glucose uptake. Furthermore, increased AMPK activity seems to decrease apoptosis (36, 38).

These findings support the hypothesis that stimulation of the AMPK cascade could be part of the metabolic depression and cell stasis observed in some naturally occurring states of latency and the associated tolerance to severe environments. Furthermore, an in vivo activation of the AMPK cascade could be a useful tool to prepare mammalian cells for dehydration and freezing and to improve viability under such storage conditions. One approach to activate the AMPK cascade is by incubating cells with the membrane-permeable compound 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR).1 After transport into the cell,
the precursor AICAR is phosphorylated in a reaction catalyzed by adenosine kinase (EC 2.7.1.20) to the membrane-impermeable compound ZMP (35), which enters the nucleotide pool before the IMP branch point (45). ZMP mimics the multiple effects of AMP on AMPK by allosteric activation as well as by activation of the upstream AMPK kinase (4, 21, 30, 42, 43).

Unfortunately, ZMP is a weak activator of AMPK with a $K_m$ value ~20-fold higher than that for 5'-AMP. Furthermore, ZMP enters the pathway of purine synthesis, which might affect the concentration of several purine and pyrimidine precursors (42, 45). Thus another approach would be the use of a more potent activator of AMPK that cannot be transphosphorylated. Murray and Atkinson (31) described in 1968 a 5'-AMP analog with promising features. The 5'-AMP analog adenosine-5'-O-thionomonophosphate (AMPS) mimics the action of AMP but is resistant to modifications such as transphosphorylation in the presence of kinases and exhibits a $K_m$ value approximately sixfold lower than the natural effector 5'-AMP (31, 43). However, the use of AMPS is limited because the compound is membrane impermeable due to the charged phosphorothioate group (-CH$_2$-O-PSO$_2$ $^-$). Therefore, to study the influence of AMPS as an activator of the AMPK cascade in vivo, techniques are required to enable AMPS to cross the membrane barrier.

An elegant way to introduce membrane-impermeable compounds into the cytosol of certain cell lines was first described by Steinberg and Silverstein (40) for the mouse macrophage cell line J774.A1. In this work, it was shown that J774.A1 mouse macrophages exhibit an ATP$^3$-sensitive membrane channel receptor, which allows passage of molecules up to an apparent molecular mass of 830 Da (39, 40, 44). This naturally occurring membrane channel was later identified as a member of the P$_2$-purinergic receptor family and classified as P2X$_7$ (34, 44).

Several specific questions were addressed in this work. Can the P2X$_7$ receptor channel be used as a tool to introduce a membrane-impermeable effector such as AMPS into cells, ultimately for activating the AMPK cascade? What impact does the poration event have on the metabolic activity of the cell? What loading conditions lead to maximum uptake of AMPS with minimal cell mortality? Does the introduction of AMPS into the cells promote the depression of metabolism and cell proliferation? What advantages might AMPS offer, compared with AICAR, for elevating the effective AMP-to-ATP ratio?

**MATERIALS AND METHODS**

**Chemicals.** Tetrabutylammonium bisulfate was purchased from Sigma-Aldrich (St. Louis, MO). Chemicals for the HPLC analysis, including acetoni-trile, potassium phosphate monobasic, and potassium phosphate dibasic, were obtained from Fisher Scientific (Lafawrn, NJ). The AMP-deaminase inhibitor cofomycin [5'-AMP-hydro-lase, EC 3.5.4.6 (47)] was purchased from Calbiochem-Novabiochem, (La Jolla, CA), and $\alpha$-$\alpha$-trehalose dihydrate came from Ferro Pfanstiehl Laboratories (Waukegan, IL). Water for solution preparation was purified with a Milli-Q reagent water system (Millipore, Grand Island, NY). Cells were maintained in a humidified atmosphere of 10% CO$_2$-90% air at 37°C. After materials were dislodged with a cell scraper, the number of cells per flask was determined by counting via a hemocytometer (Hauser and Son, Philadelphia, PA). To obtain sufficient quantities of cells for the actual experiments, ~5 x 10$^6$ cells were transferred to a Pyrex spinner flask (Fisher Scientific, Houston, TX) containing 100–250 ml of standard culture medium. The spinner flask cultures were allowed to grow to a maximum cell density of ~4–8 x 10$^5$ cells/ml with frequent exchange of the medium. To ensure that the cell cultures were mycoplasma free, a mycoplasma detection kit (version 2.0 from ATCC) was used periodically. We followed the procedure supplied with the kit, which prescribed the use of 7,000 cells, a two-stage PCR amplification of DNA, and gel electrophoresis to test for two species of mycoplasma.

**ATP-dependent cell poration via the P2X$_7$ receptor channel.** To introduce membrane-impermeable chemicals into the cytoplasm, J774-A1 cells were centrifuged at 175 g and resuspended in buffer 1 [50 mM K$_2$HPO$_4$/KH$_2$PO$_4$ (pH 7.0, 365 mmosmol/kgH$_2$O), 250 mM potassium acetate, 1 mM MgSO$_4$, 4 mM glucose, supplemented with basal Eagle’s vitamin solution and MEM amino acids solution (Invitrogen) both diluted to 1 x]. ATP stock solution (50 mM) was prepared in PBS (Invitrogen), adjusted to pH 7.4 with 6 M KOH, and stored at ~80°C. Depending on the experiment, 5 x 10$^5$ to 6 x 10$^7$ cells were resuspended in 900 lJ of buffer 1 to which 100 lJ of ATP stock solution were added. The cells were incubated for 15 or 30 min at 37°C in sealed microtubes. Alternatively, the P2X$_7$ receptor channel was opened at 37°C for 5 min, and cells were transferred thereafter to an ice-water bath for 1 h. To close the P2X$_7$ receptor channel, cells were diluted ~100-fold with standard culture medium (37°C) equilibrated with 10% CO$_2$-90% air.

**Cell viability assays.** To assess the effect of ATP-induced permeabilization on viability, cells were grown in suspension after activation of the P2X$_7$ receptor. Aliquots of cell suspension were removed from the spinner flask at various intervals. The number of viable cells at the various time points was determined by counting with a hemocytometer after 1:1 dilution of the sample with 0.4% trypan blue solution (Sigma). Alternatively, cell viability was assessed by following the metabolic activity of the cells, monitored with alamarBlue (BioSource International, Camarillo, CA). alamarBlue stock solution was added to a final concentration of 10% (vol/vol) in standard culture medium without phenol red. Of the working solution, 2 ml were added to each well of a 12-well Nunclun multidish (Fischer Scientific). After 20,000–40,000 cells were added to each well, the changes in the absorbance at wavelengths of 570 and 600 nm were measured with a microplate reader (SpectraMax Plus, Molecular Devices, Sunnyvale, CA) at various time intervals. The percentage of reduced alamarBlue (RA%) was calculated as follows: RA% = (A$_{570}$ - A$_{600}$ x R$_0$) / 100, where A$_{570}$ and A$_{600}$ are the absorbances at wavelengths of 570 and 600 nm, respectively, after the absorbance of standard medium without alamarBlue is subtracted and R$_0$ is the ratio of A$_{570}$ to A$_{600}$ of phenol red-free standard medium containing 10% alamarBlue.

**HPLC of nucleotides and analogs.** Acid-soluble extracts of cell suspensions were prepared by adding ice-cold 70% perchloric acid to a final concentration of 7% (wt/vol). Acid-insoluble fractions were removed by centrifugation for 10 min at 13,000 g at 4°C (Eppendorf centrifuge 5417R, Fischer Scientific). Extracts were neutralized with ice-cold 5 M K$_2$CO$_3$ and stored at ~80°C until analyzed. Before the HPLC analysis, the potassium perchlorate precipitate was removed by centrifugation as above. Analyses were performed with a Dionex HPLC system (Dionex, Sunnyvale, CA), which included a PDA-100 photodiode array detector, GP 50 gradient pump, AS50 autosampler, and AS50 thermal compartment. Samples were kept at 4°C, and aliquots of the supernatant were applied to a 4.6 mm x 25-cm reversed-phase column (Synergy 4u Hydro RP 80A, Phenomenex, Torrance, CA). The samples were eluted isocratically for 15 min with
a starting buffer of 50 mM K2HPO4/KH2PO4 (pH 6.2) containing 10 mM tetrabutylammonium bisulfate at a flow rate of 1 ml/min at 30°C. A linear gradient from 0% to 25% acetonitrile was then applied over 40 min. The absorbance of the effluent was monitored with a photo-diode array detector at wavelengths from 190 to 390 nm. Peaks were identified by comparison with retention times of standards, as well as by analysis of the given peak spectrum from a recorded three-dimensional field with Chromelone software (Dionex). Concentration of nucleotides was determined from a measurement of peak area at 260-nm wavelength. Calibration curves were linear over the range assayed.

**Calorimetric measurements.** To assess the influences of various treatments on the total energy flow of J774.A1 cells, calorimetric measurements were performed (10, 13). The LKB 2277 thermal activity monitor (LKB, Bromma, Sweden) was used to measure the heat dissipation of J774.A1 cells in suspension. A 4-ml static ampoule charged with 2.7 ml of water was used as a reference vessel. Static and dynamic calibrations against water in both ampoules were performed before the heat signal was measured. For the actual experiments, the sample ampoule was charged with 2.7 ml of cell suspension. An aliquot of the spinner flask cell culture was diluted with standard culture medium equilibrated with 10% CO2-90% air to give between 130,000 and 250,000 cells in 2.7 ml. All calorimetric measurements were performed at 37°C. A 2-h period for thermal equilibration was allowed after the ampoule was lowered into the calorimeter, and then the power signal (μW, μW/s) was recorded for 20 h. The heat signal increased continuously during some of the measurement because of ongoing cell proliferation. The heat signal was then corrected for this increase by extrapolation back to time zero.

**Purification of AMPS.** To remove lithium, methanol, and acetonitrile from commercially available AMPS (Sigma-Aldrich), the lithium cation was exchanged against potassium and the resulting solution was vacuum dried. A 0.5 M AMPS solution was supplemented with 1 M KF (Sigma-Aldrich), yielding a 1-to-1 stoichiometry between lithium and fluoride ions. The precipitating salt, LiF, was removed by centrifugation for 10 min at 13,000 g and 4°C. The supernatant was vacuum dried at room temperature (SPD 111 V, Thermo Savant, Holbrook, NY). The purity of AMPS was determined with HPLC; a single AMPS peak was detected. Because of the low solubility constant of lithium fluoride [0.0501 M (51)], ~95% of the lithium contained in the original AMPS sample was exchanged against potassium by this procedure.

**AMPS uptake in J774.A1 cells.** Cells grown in 75-cm2 Corning cell culture dishes were incubated for 15 min with 5 mM ATP and 10 mM AMPS in buffer 1. The culture dishes were then washed four times with 25 ml of ice-cold PBS (Invitrogen), and an aliquot of the final wash solution was analyzed for residual AMPS by HPLC. No AMPS was found in the final wash solution. The cell culture plates were placed on ice, and cells were removed with a cell scraper. Acid-soluble extracts were prepared as previously described under HPLC of nucleotides and analogs.

**Incubation with AICAR and the formation of ZMP.** AMPK can be stimulated by the intracellular formation of 5-aminimidazole-4-carboxamide-1β-D-ribofuranosyl-5′-mono-phosphate (ZMP), which can be promoted in at least some cell types by incubation with AICAR (4, 42). J774.A1 cells were exposed to multiple concentrations of AICAR for various periods. As with AMPS incubations, cells grown in culture dishes were washed four times with 25 ml of ice-cold PBS, and analysis of the final wash solution by HPLC confirmed the absence of residual AICAR. The cells were removed with a cell scraper, and acid-soluble extracts were prepared as previously described under HPLC of nucleotides and analogs. The intracellular concentrations of AICAR and ZMP were measured by HPLC.

**Statistical analyses.** Significant data were evaluated by performing the t-test, *P* = 0.05 (56), with SigmaPlot 2001 for Windows version 7.101 (SPSS, Chicago, IL), or were analyzed with one-way ANOVA and Fisher’s least significant difference for pairwise comparison, *P* = 0.05, with Minitab 1998 (Minitab, State Collage, PA).

**RESULTS**

The mouse macrophage cell line J774.A1 is a logical system for testing whether a rise in the effective AMP-to-ATP ratio promotes a depression in metabolic activity and cell growth, as seen in natural states of animal dormancy. The reason is that the P2X7 receptor channel is intrinsically abundant in the plasma membrane of these cells. We predict that this channel, reversibly toggled open and closed by extracellular ATP, might serve as a means to introduce AMPS, a membrane-impermeant analog of AMP, into the cytoplasm.

**ATP-dependent poration of J774.A1 cells with high viability.** We developed a poration medium (buffer 1) that fosters ATP-induced permeabilization with high cell survival, despite evidence that under some conditions ATP activation of the P2X7 receptor can lead to apoptosis and cell lysis (25, 48). Uptake of ethidium bromide was followed to verify cell poration via the P2X7 receptor channel. Loading of ethidium bromide was observed in ~99% of cells in buffer 1 containing 5 mM ATP, and virtually no uptake was seen without ATP (Fig. 1). The survivorship of the cells was ~98% as measured by trypsin blue exclusion 24 h after a 15-min cycle of ATP-induced poration at 37°C. The metabolic influence of ATP-dependent poration as a function of time posttreatment was monitored with two different approaches. Heat dissipation served as a measure of overall cellular energy flow (aerobic plus anaerobic contributions; Fig. 2), and results indicated a transient depression of heat dissipation when normalized per unit number of cells. The typical heat production of J774.A1 cells under optimal conditions (control) was ~50.7 ± 4.2 (SD) μW/10⁶ cells (*n* = 7 experiments). Opening the P2X7 receptor channel

2 The term “effective AMP-to-ATP ratio” refers to the sum of the concentrations of the introduced AMP analog and the naturally occurring 5′-AMP, divided by the ATP concentration.
by addition of 5 mM ATP to buffer 1 for 15 min, followed by a 3-h recovery period in standard culture medium, caused a statistically significant decrease in heat dissipation to $-36.8 \pm 6.3$ (SD) $\mu W/10^6$ cells ($n = 7; P < 0.01$). If the cells were allowed to recover from the poration event for 24 h in standard culture medium, the heat dissipation returned to a value statistically indistinguishable from untreated cells [$-49.2 \pm 1.2$ (SD) $\mu W/10^6$ cells; $n = 3$].

alarBlue reduction was used as a second measure of metabolic activity (Fig. 3). Cells were incubated for 15 min in buffer 1 or in buffer 1 containing 5 mM ATP and then diluted into standard culture medium containing alamarBlue. Incubation in buffer 1 alone led to a small but significant depression of metabolic activity of $-13\%$ measured at the first recovery time (16 h). However, ATP-treated cells displayed a larger decrease in metabolic activity of $-50\%$. It is appropriate to note that equal quantities of cells were added to the incubation wells at the start of the recovery period. Thus, as recovery proceeds, cell proliferation was occurring in the control wells, which resulted in a continuously increasing signal. However, this increase was clearly blunted for at least 24 h in the cells that experienced poration. If the poration time was extended from 15 to 30 min, alamarBlue reduction decreased by 70% measured 16 h posttreatment and by 85% measured 40 h posttreatment, compared with control values (Fig. 3). Cells did not recover from this prolonged pore opening, which suggested to us pronounced cytotoxicity (see Discussion). Consequently, poration at 37°C was limited to 15 min for subsequent experiments.

Importantly, the impact of a 15-min poration on alamarBlue reduction was only transient. Cells fully recovered from this treatment. It is noteworthy that trypan blue exclusion indicated membrane integrity was not compromised postporation. In principle, the decrease in alamarBlue reduction after poration could be due two events: a stop in cell proliferation or a decrease in cell viability and/or metabolism. To address whether the alamarBlue result was due to cell death or to a temporary stop in cell proliferation, cell counts were performed at different time points after a 15-min poration event (Fig. 4). During the first 20 h after the poration event, the number of cells remained constant, but this was then followed by an increase in total cells by a factor of $2.34 \pm 0.5$ (SD) ($n = 4$) during the next 28 h. Thus the depression of alamarBlue reduction in J774.A1 cells after a 15-min poration event was explained by an arrest of cell proliferation for $\sim 20$ h, after which cell growth resumed.

Analyses of adenine nucleotides and analogs in J774.A1 cells. To measure induced changes in the cellular AMP/ATP as well as to assess the uptake of AMPS and the conversion from AICAR to ZMP, we used reversed-phase ion-exchange chromatography with tetrabutylammonium bisulfate as an ion-pair reagent. All compounds of interest exhibited baseline separation and a signal-to-noise ratio of $>20:1$ (Fig. 5). This chromatography procedure separates both charged and uncharged species. Thus one can quantify AICAR and ZMP in the same sample. Figure 5A shows a representative chromatogram of an
extract from macrophages that were exposed to 0.5 mM AICAR for 24 h. A high accumulation of AICAR and small accumulation of ZMP were observed. Figure 5B shows the uptake of AMPS achieved by opening the P2X7 receptor channel for 15 min (5 mM ATP in buffer J) in the presence of 10 mM adenosine-5'-O-thiophosphosphate (AMPS; B). mAU, milliabsorbance units.

of the natural adenylate 5'-AMP occurred under these conditions.

Surprisingly, a correlation between cell density and adenylate concentration was found in these immortalized mouse macrophage cells. Cells were plated at different densities and allowed to grow attached to culture dishes for 18 h (Fig. 6). Cells that were plated at 0.6–1.5 × 10^6 cells per 75-cm² culture dish (next-day confluence of 10–30%) had a significantly higher content of total adenylates than cells plated at 3–9 × 10^6 cells per culture dish (next-day confluence of 40–95%) (one-way ANOVA; P < 0.05). However, because the concentrations of AMP and ADP increased proportionally to that observed for ATP (Fig. 6), the AMP-to-ATP ratio was independent of the cell density [AMP/ATP = 0.023 ± 0.006 (SD); n = 31]. The adenylate levels of cells at confluences of 40–85% were not statistically different from each other (Fig. 6; one-way ANOVA, P > 0.05). Therefore, data obtained at these densities were combined and used as control values for other treatment groups. Subsequent experiments were carried out at a plate density of 50–85% confluence (~5 × 10^6 cells were plated 18 h before the experiment). The ATP, ADP, and AMP concentrations in mouse macrophages under control conditions were as follows: 7.90 ± 1.83, 0.89 ± 0.15, and

Table 1. Quantities of adenylates and adenylate analogs in J774.A1 cells after different treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[AMP]</th>
<th>[ADP]</th>
<th>[ATP]</th>
<th>[AMPS]</th>
<th>[ZMP]</th>
<th>[AICAR]</th>
<th>Effective Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.16±0.04</td>
<td>0.89±0.15</td>
<td>7.90±1.83</td>
<td>0.74±0.12</td>
<td>0.22±0.15</td>
<td>1.43±0.46</td>
<td>0.021±0.005</td>
</tr>
<tr>
<td>+ATP +COF</td>
<td>0.48±0.29*</td>
<td>2.09±1.36</td>
<td>9.72±3.4*</td>
<td>0.19±0.19</td>
<td>4.68±1.29*</td>
<td>0.052±0.030*</td>
<td>0.057±0.030</td>
</tr>
<tr>
<td>+ATP +AMP +COF</td>
<td>0.98±0.52*</td>
<td>3.28±2.62*</td>
<td>11.34±2.74*</td>
<td>0.23±0.07</td>
<td>6.07±2.24*</td>
<td>0.030±0.014</td>
<td></td>
</tr>
<tr>
<td>+ATP +AMPS +COF</td>
<td>0.44±0.06*</td>
<td>1.59±0.34</td>
<td>12.09±1.43*</td>
<td>0.09±0.01</td>
<td>5.81±0.90*</td>
<td>2.24±0.69*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD of 6–15 experiments. All concentrations (indicated by brackets) are in nmol/10⁶ cells. Effective ratio is [AMP]/[ATP] if no analog was introduced and ([AMP] + [analog])/[ATP] if an analog was introduced. Control values are an average of cells extracted at densities of 40–85% confluence (n = 15). AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPS, adenosine-5'-O-thiophosphosphate; COF, coformycin. *Values differ significantly from control (one-way ANOVA; P < 0.05). †Values differ significantly from each other (one-way ANOVA; P < 0.05). ‡Under this condition, accumulations of [ZDP] (6.07±2.24, n = 11) and [ZTP] (4.25±1.29, n = 11) were observed.
0.16 ± 0.04 (SD) nmol/10⁶ cells, respectively (Table 1; n = 15). With the use of imaging software (MetaMorph 6.1; Molecular Devices, Downingtown, PA), the diameter of suspended J774.A1 cells was estimated to be 15.23 ± 1.59 (SD) μm (n = 13). Assuming a spherical cell form and a water content of 70% gave a calculated water content of 1.295 μl/10⁶ cells. Under these assumptions, the intracellular concentrations for ATP, ADP, and AMP are 6.11 ± 1.54, 0.68 ± 0.13, and 0.12 ± 0.03 mM, respectively.

**Influence of AICAR on adenylate levels and cell proliferation.** A 2-h exposure of cells to 0.5 or 2 mM AICAR led to a significant increase in the ADP and ATP levels of mouse macrophages (Table 1; one-way ANOVA, P < 0.05, n = 11). Although an uptake of AICAR into cells was found after 2-h incubations (1.21 ± 0.23 nmol/10⁶ cells with 0.5 mM AICAR; 4.68 ± 1.29 nmol/10⁶ cells with 2 mM AICAR), only a small conversion of AICAR to ZMP was observed (Table 1). The ZMP concentration that was found after 2-h incubation with 0.5 mM AICAR (0.11 ± 0.07 nmol/10⁶ cells) was not significantly different from the concentration after 2 h with 2 mM AICAR (0.21 ± 0.19 nmol/10⁶ cells). Furthermore, the effective AMP-to-ATP ratios were not influenced by the 2-h treatments with AICAR and not statistically different from those of control cells (Table 1). After 24-h incubation with 2 mM AICAR, a large accumulation of ZMP (16.20 ± 2.92 nmol/10⁶ cells) was observed, which led to a statistically significant change in the effective AMP/ATP ratio. However, substantial accumulations of the diphasorlated [ZDP; 6.07 ± 2.24 (SD) nmol/10⁶ cells, n = 11] and triphosphorylated [ZTP; 4.25 ± 1.29 (SD) nmol/10⁶ cells, n = 11] forms of AICAR were also found (Table 1). The extension of the incubation time to 24 h promoted a return of the ADP and ATP concentrations to control values, but a modest elevation in the AMP concentration remained (Table 1).

Incubation of mouse macrophages with AICAR led to a significant decrease in cell proliferation. Starting at the same initial densities, plated cells showed a lower next-day confluence when incubated with 2 mM AICAR for 24 h (Fig. 7). The proliferation rate in response to incubation with 0.5 mM AICAR decreased only slightly, whereas 2 mM led to a complete stop in proliferation. After 24-h incubation with 2 mM AICAR, cell counts showed no increase in cell number relative to the quantity originally plated at time zero [79.9 ± 32.5% (SD) present after 24 h (n = 4)].

**Loading of AMP and AMPS via the P2X₇ receptor channel.** Activation of the P2X₇ receptor channel by incubation for 15 min in buffer 1 with 5 mM ATP led to a statistically significant increase in the concentrations of AMP and ATP to 0.48 ± 0.29 and 9.72 ± 3.41 (SD) nmol/10⁶ cells, respectively (Table 1, one-way ANOVA, P < 0.05, n = 9). These results were obtained in the presence of 35 μM coformycin because coformycin is essential to prevent AMP-deaminase activity (5′-AMP-hydrolase, EC 3.5.4.6), which can lead to degradation of intracellular AMP and AMPS. If ATP-dependent poration was performed in the presence of 10 mM AMP, the AMP level increased significantly from 0.48 ± 0.29 nmol/10⁶ cells (AMP-free poration) to 0.98 ± 0.52 nmol/10⁶ cells. The entry of exogenous AMP significantly increased the AMP/ATP to 0.085 ± 0.033 (SD) (one-way ANOVA, P < 0.05, n = 9). However, this increase was transient due to rapid transphosphorylation of AMP (data not shown). As expected, the ATP levels were also significantly elevated, similar to results without exogenous AMP. In contrast to AMP-free poration, a significant increase in ADP values were observed with 10 mM AMP (one-way ANOVA, P < 0.05, n = 9). If 10 mM AMPS was substituted for AMP, an uptake of AMPS of 0.46 ± 0.19 (SD) nmol/10⁶ cells (n = 6) was found, and the effective AMP/ATP was significantly elevated above the control value (Table 1). Thus AMPS can be loaded effectively through the P2X₇ channel and used to change the effective AMP-to-ATP ratio in mouse macrophage cells. Importantly, counter to the findings for poration in the presence of AMP, no increase in ADP values was measured with AMPS (Table 1), which demonstrates the inability to transphosphorylate AMPS to ADP or the homologous sulfur analog and thus the greater stability of AMPS under intracellular conditions.

**Heat dissipation and proliferation of macrophages in response to analogs.** As mentioned above, the typical heat production of J774.A1 cells under control conditions was 50.7 ± 4.2 (SD) μW/10⁶ cells (n = 7). A high intracellular accumulation of ZMP and a stop in cell proliferation were only
observed after 24-h incubations with 2 mM AICAR. Thus we investigated the impact of this AICAR exposure on the overall heat flow of macrophages. Despite a high ZMP accumulation and a stop in cell proliferation, the heat dissipation after AICAR (~47.0 ± 5.7 (SD) μW/10⁶ cells, n = 5; Fig. 8) was not statistically different from control cells (Fig. 2, one-way ANOVA, P > 0.05).

When a nonpermeable analog (i.e., AMPS) is loaded into cells by poration, the P2X7 receptor channel is toggled closed by dilution of cells 100-fold into culture medium to chelate extracellular ATP and reduce its concentration. Due to the purification procedure of AMPS, ~170 μM LiF was present in solution when cells were porated in the presence of 10 mM AMPS. Furthermore, as indicated earlier, coformycin was present to prevent AMP-deaminase activity. Therefore, to obtain control values for the influence of LiF and coformycin during poration, as well as extracellular AMPS after pore closure, on heat dissipation postporation, cells were porated in the presence of 35 μM coformycin and 170 μM LiF and then reclosed by diluting the cells into medium containing 100 μM AMPS and 35 μM coformycin.

The heat dissipation of cells porated in the presence of 10 mM AMPS was statistically reduced from controls (Fig. 8). Heat dissipation dropped from the control value of ~5.4 ± 5.4 (SD) μW/10⁶ cells (n = 4) to ~27.5 ± 2.5 (SD) μW/10⁶ (n = 3). Because poration for 5 min at 37°C followed by transfer to 0°C for 1 h has less impact on cell proliferation than does poration for 15 min at 37°C (data not shown), we used this two-temperature loading technique to assess the effect of AMPS on cell numbers over time. When the cells were transferred to a spinner flask after poration, a significant increase in cell numbers after 48 h was measured for the control group (LiF, coformycin), whereas the AMPS group exhibited no increase in cell number for 72 h (Fig. 9). Thus AMPS had a marked influence on proliferation that was not reversed during this period. Reversal may require the addition of proliferation stimulators or poration-based washout of the analog.

**DISCUSSION**

We have demonstrated in this study that the P2X7 receptor channel can be used as an effective means to load mammalian cells with membrane-impermeable compounds such as the nonhydrolyzable 5′-AMP analog AMPS, a potent activator of AMPK. By applying this technique to mouse macrophages, we can significantly increase the effective AMP/ATP, resulting in both the metabolism and proliferation being depressed. This latter result is fully consistent with the upregulation of AMP/ATP observed during many naturally occurring states of cellular stasis, where depression of energy flow and development is advantageous for survival during energy limitation and/or harsh environmental challenges. Optimizing the conditions under which the P2X7 receptor channel is opened, as well as the duration of this event, permits cells to recover without appreciable mortality. The membrane-permeable drug AICAR, which is a precursor to ZMP, can be readily loaded without poration into macrophages. However, as shown by HPLC analysis of cell extracts, the conversion of AICAR to ZMP is slow and inefficient in mouse macrophages. Given sufficient exposure time to AICAR, we show that macrophages accumulate ZMP, which in turn increases the effective AMP/ATP ratio and reduces cell proliferation, although not overall metabolism.

**P2X7 receptor channel for reversible cell poration.** For cell lines such as macrophages that naturally express the protein, our present data show that the P2X7 receptor channel is a convenient way to introduce high levels of AMP and chemically similar analogs into cells. Alternatively, plasmids containing the mouse, rat, or human P2X7 genes are readily available and can be used to transiently transfet other cell lines as needed when the expression of the pore is low or absent (34). It is appropriate to note that some reports indicate that the opening of the P2X7 receptor channel may lead to cell death under certain conditions (5, 25, 48). Similar to these findings, we also have observed high mortality when ATP-dependent poration is conducted in typical cell culture medium. However, we developed a poration solution that allows the introduction of compounds such as AMPS into cells with-
out observable signs of apoptosis. In our system, the addition of trehalose, a low sodium concentration, and the use of a phosphate buffer system were crucial for obtaining high poration efficiency with minimal cell death. After 15 min of poration at 37°C, decreases in metabolism and cell proliferation were observed, but these were fully reversed 2 h post-poration (Fig. 4). It is noteworthy that increasing the poration time at 37°C to 30 min significantly decreased viability. If one wishes to extend the duration of pore opening to 1 h or more without a substantially increased mortality, the P2X7 channel can be activated at 37°C and then the temperature promptly lowered to 0–4°C for loading without a significant increase in cell mortality (data not shown). This observation suggests that compensatory metabolic processes during pore opening like ion pumping were depressed by the low temperature and perhaps aids the conservation of cellular energy. Thus, by carefully optimizing the permeabilization conditions and the duration of pore opening, one can minimize cytotoxic effects of P2X7-mediated poration.

AMP-to-ATP ratio and natural states of cellular stasis. The ability to increase the effective steady-state ratio of AMP to ATP in cells provides the opportunity to evaluate in detail the impact of this change on the metabolic and proliferation states. In natural states of cellular stasis like diapause (a dormant state entered under normoxia, euthermia, and full hydration), a repeated theme is that AMP/ATP is elevated and tightly correlated with the arrest of metabolism (32, 33). As a result, we predict that activation of the AMPK cascade may contribute to cell stasis. Energy production (e.g., oxidative pathways) and energy consumption (e.g., biosynthesis, transcription, translation, ion pumping) are both depressed during diapause such that cellular energy reserves are not depleted. The normal developmental program of animals is interrupted, and imbalances in cellular processes are minimized so that pathological conditions are avoided (16, 22, 23, 41). Evidence indicates that selected upregulation of specific gene products may contribute to promotion of the diapause state (9). Similarly, our recent selected upregulation of specific gene products may contribute conditions are avoided (16, 22, 23, 41). Evidence indicates that compensatory metabolic processes during pore opening like ion pumping were depressed by the low temperature and perhaps aids the conservation of cellular energy. Thus, by carefully optimizing the permeabilization conditions and the duration of pore opening, one can minimize cytotoxic effects of P2X7-mediated poration.

Physiological impacts of AICAR vs. P2X7-mediated uptake of AMPS. Two different routes to alter the effective AMP/ATP ratio were evaluated in this study. The membrane-permeable drug AICAR was used as an indirect means to alter the effective AMP/ATP, whereas P2X7-mediated poration was a more direct approach; i.e., it did not require a cell-dependent conversion to the active molecular species. The effective AMP/ATP obtainable with the direct approach (0.073) is well above that reported to activate the AMPK in vitro (4). However, poration by itself leads to a significant increase in the AMP-to-ATP ratio from 0.021 to 0.052 (Table 1). Therefore, a part of the observed reduction in proliferation after poration could be due to transient AMPK activation by the poration event itself. AMPS is well documented to be a strong activator of the isolated AMPK enzyme in vitro (43), yet there has been no attempt previously to use it in living cells for stimulation of the AMPK cascade due to its impermeability to the cell membrane. The Km of AMPK for AMPS was estimated to be about sixfold lower than for 5′-AMP; therefore, not surprisingly, we found a much greater reduction in overall heat flow when AMPS was present compared with cells porated without the analog (Fig. 8).

The membrane-permeable drug AICAR, which is a precursor to ZMP, can be readily introduced into macrophages without poration. However, as documented by HPLC analysis of cell extracts, the conversion of AICAR to ZMP was slow and inefficient in macrophages. When given prolonged exposure time to AICAR at high concentration (2 mM), we showed that macrophages accumulated ZMP, which in turn reduced cell proliferation (Fig. 7C), although not overall metabolism (Fig. 8). The long AICAR exposures also caused accumulation of ZDP and ZTP, and cells with an elongated morphology were frequently observed (Fig. 7C). For HepG2 cells (26), results showed depression of cell proliferation at 0.5 mM AICAR, along with p53 accumulation and phosphorylation of this protein regulator of the cell cycle. The inhibition of cell growth of NIH-3T3 fibroblasts by AMPK-dependent regulation of the extracellular signal-regulated kinase cascade and Ras activation has also been reported (28). Furthermore, Sprenkle et al. (37) demonstrated the capacity of AMPK to phosphorylate Raf-1, a key intermediate in the transduction of growth factor signals. Thus the AMPK cascade has major impacts on cell signaling pathways that control growth and proliferation.

The influence on metabolism that we measured with AMPS was achieved at much lower intracellular concentrations relative to the level of ZMP. Furthermore, the AMPS effect on metabolism was elicited with an acute exposure, whereas a chronic treatment with AICAR was needed to elevate ZMP.

Baseline adenylate content of J774.A1 cells. Cells that were extracted at low densities had a significantly higher content of total adenylate than cells that were extracted at higher densities (Fig. 6). To our knowledge, this is the first description of an impact of cell density on adenylate content in macrophages. It is well known that senescent cells show a strong reduction in the levels of ribonucleotide triphosphates, including ATP, which are required for nucleotide biosynthesis and hence proliferation (49). This reduction is associated with an increase in the AMP concentration ultimately activating AMPK, which in turn inhibits cell proliferation (57). In our system, despite an increase in the total ATP concentration at low cell densities, the ATP-to-AMP ratio was not influenced because a parallel increase in the AMP concentration was found. This might suggest that at lower cell densities the overall energy demand is increased due to a higher proliferation rate. With increasing cell density, a reduced proliferation rate might decrease the demand for overall ribonucleotide triphosphates.
Consistent with the differences in time course, it is not surprising to us that the metabolic influences measured with the two analogs were different. AICAR exposure for 24 h resulted in no measurable change in heat dissipation, whereas the acute AMPS treatment elicited a significant drop in metabolism. It is possible that the chronic AICAR exposure allowed time for the well-known AMPK-stimulated upregulation of the GLUT-4 transporter and hexokinase activity (24), which would increase glucose-based metabolism and potentially offset any metabolic depression anticipated because of downregulated gene expression. Consequently, it would seem that AMPS might be a preferable approach for promoting cell stasis, in that its rapid impact avoids the upregulation of compensatory metabolic pathways.

Two further advantages to AMPS are noteworthy. First, its loading has minimal impact on other components of the nucleotide pool. Specifically, in contrast to poration in the presence of AMP, the ADP level was unchanged in response to AMPS (Table 1). Increasing the steady-state level of ADP, as occurs with AICAR (see Table 1), presumably stimulates mitochondrial oxidative phosphorylation. Second, the capacity of cells to synthesize the active AMP analog (ZMP) from AICAR varies greatly from one cell type to another. Such variability has been suggested to arise from differences in the levels of adenosine kinase, the enzyme responsible for phosphorylating AICAR to ZMP, and in the rate of conversion of ZMP to IMP (e.g., see Ref. 27). Thus care must be taken to quantify the intracellular ZMP levels promoted by AICAR exposure for a given cell line before any physiological response is ascribed to AMPK activation. Direct loading of AMPS avoids this issue entirely.

In summary, the P2X<sub>7</sub> receptor channel offers a new approach for loading membrane-impermeable compounds into cells. AMP analogs with greater potency and specificity for metabolic modulation can be employed. Loading large quantities of membrane-impermeant sugars like trehalose is readily possible with the P2X<sub>7</sub> channel (G. Elliot, J. Cusick, M. A. Menze, T. Witt, S. C. Hand, and M. Toner; unpublished observation). Trehalose is known to be a powerful cellular protectant against damage incurred during cellular drying and freezing (52).

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REFERENCES


