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INHIBITION OF MAST CELL MEDIATOR RELEASE BY 5-AMINO-4-IMIDAZOLECARBOXAMIDE RIBOSIDE*

DIANA L. MARQUARDT† and HARRY E. GRUBER
Department of Medicine, University of California School of Medicine, San Diego, CA 92103, U.S.A.

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Abstract—Stimulated mast cells produce and release adenosine, and the release of mast cell mediators is potentiated by adenosine, yet very little is known regarding mast cell purine metabolism. Because 5-amino-4-imidazolecarboxamide riboside (AICA riboside) has been shown to alter adenosine metabolism and accelerate the repletion of ATP pools in other tissues, its effects on mast cell function were examined. Neither simultaneous addition of A23187 and AICA riboside nor a 1-hr preincubation with AICA riboside altered mast cell β-hexosaminidase release to an appreciable degree. However, mouse bone marrow-derived mast cells cultured for 2 or more days in the presence of 1–100 μM AICA riboside exhibited a markedly attenuated mediator release response to A23187 compared to control cells with or without the additional presence of adenosine. IgE-mediated leukotriene C_4 generation from AICA riboside-exposed mast cells was even more profoundly inhibited without affecting cell viability or resting mediator content. An unusual ribonucleotide triphosphate previously identified in folate-depleted cells, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-triphosphate (ZTP), has been identified in AICA riboside-treated mast cells as well. Although the mechanism of this global inhibition of mast cell mediator release by chronic AICA riboside treatment is not clear, alterations in mast cell purine metabolism may prove to be important in the treatment of allergic diseases.

Stimulated mast cells break down ATP‡ and release adenosine within 60 sec after antigen or A23187 challenge [1]. Adenosine itself markedly potentiates mast cell granule-associated mediator release [2, 3] and augments intracellular cyclic AMP accumulation in these cells [4]. Neither the adenosine deaminase inhibitor, deoxycoformycin, nor the adenosine transport inhibitor, dipyridamole [2], alters rat serosal mast cell mediator release to a measurable degree, but beyond this, little is known regarding mast cell purine metabolism.

5-Amino-4-imidazolecarboxamide riboside (AICA riboside) is an interesting purine analog which has been shown to accelerate the repletion of ATP pools in ischemic canine myocardium and other tissues [5]. Its triphosphate form, AICA riboside triphosphate or ZTP, has been identified as a regulatory molecule in folate-depleted cells [6]. We chose to study immediate and longer-term effects of AICA riboside on mast cells to ascertain whether potential changes in cellular purine content or metabolism could change cellular function.

Mouse bone marrow-derived mast cells have proven useful in studies of pharmacologic manipulation of these cells in tissue culture [7, 8]. Considering this property and the fact that these cells produce both leukotrienes and prostaglandins after stimulation [9], these "mucosal-like" mast cells were chosen as an *in vitro* model for the study of altered purine metabolism effects on immediate hypersensitivity reactions.

MATERIALS AND METHODS

Chemicals. The following were purchased from the manufacturers indicated: adenosine, concanavalin A, *N*-acetyl-β-D-glucosaminide, 2-mercaptoethanol, 5-amino-4-imidazolecarboxamide (AICA), 5-amino-4-imidazolecarboxamide-1-β-D-ribofuranoside (AICA riboside), and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-triphosphate (ZTP) (Sigma Chemical Company, St. Louis, MO); RPMI-1640, penicillin/streptomycin, minimal essential medium nonessential amino acids, fetal calf serum, and L-glutamine (GIBCO, Grand Island, NY); calcium ionophore A23187 (Calbiochem, La Jolla, CA); Freon (Eastman Kodak, Rochester, NY); alamine (Henkel, Kankakee, IL); and a [^{3}H]LTC_4 radioimmunoassay kit (New England Nuclear, Boston, MA).

The following were donated: deoxycoformycin (Developmental Therapeutics Program, Chemotherapy Division, NCI, Bethesda, MD); and mouse hybridoma anti-dinitrophenol (DNP) IgE antibody

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† Address correspondence to: Diana Marquardt, M.D., c/o UCSD Medical Center, H-811-G, 225 Dickinson St., San Diego, CA 92103.

‡ Abbreviations: ATP, adenosine triphosphate; AICA, 5-amino-4-imidazolecarboxamide; ZTP, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-triphosphate; LTC_4, leukotriene C_4; DNP, dinitrophenol; BSA, bovine serum albumin; and ado, adenosine.

and DNP-conjugated bovine serum albumin (DNP-BSA) antigen (F. Liu and D. Katz, La Jolla, CA).

Mouse bone marrow-derived mast cell culture media and conditions. Femoral bone marrow cells from BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were isolated as described elsewhere [10] and placed into medium containing 50% RPMI-1640 and 50% conditioned medium, produced by co-culture of concanavalin A-stimulated C57B1/6J and C3H mice splenocytes. Tissue culture components and conditions are detailed in a separate publication [11]. Cells were cultured for a minimum of 15 days before use with weekly passaging, resulting in more than 90% purity and 95% viability as assessed by Trypan blue exclusion. Cells exposed to AICA or AICA riboside in culture were washed free of the drug three times prior to use in experiments. Parallel cultures of cells grown in medium alone were used as controls for pharmacologically-manipulated mast cells.

Mast cell β-hexosaminidase release. Mouse bone marrow-derived mast cells in tissue culture were centrifuged at 200 *g* for 5 min and washed three times with Tyrode's buffer lacking divalent cations. In some experiments cells were sensitized with anti-DNP IgE (1 μg/10^6 cells) for 30 min at 37° and then challenged with either DNP-BSA antigen (175 ng/3×10^5 cells) or A23187 (1 μg/ml/3×10^5 cells) for 10 min in 400 μl of complete Tyrode's buffer. The reaction mixtures were centrifuged at 200 *g* for 10 min, and the β-hexosaminidase in supernatant and pellet fractions are assayed by the hydrolysis of *p*-nitrophenyl-β-D-glucosamide as described elsewhere [12]. The net percentage of β-hexosaminidase released is defined as follows:

$$\text{Net \% } \beta\text{-hex release} = \frac{\text{supernatant } (\beta\text{-hex})}{\text{supernatant } (\beta\text{-hex}) + \text{pellet } (\beta\text{-hex}) - \text{spontaneous } (\beta\text{-hex}) \text{ released}}$$

In short-term exposure experiments, AICA, AICA riboside, or adenosine was added simultaneously with the secretagogue or was present in a 1-hr preincubation at 37° before challenge.

Assessment of leukotriene C_4 production in mast cells. To quantitate mast cell supernatant LTC_4 concentrations, cells were prepared and challenged as described above for β-hexosaminidase release except that the reactions were allowed to proceed for 20 min before centrifugation. The resulting supernatant fractions were removed and stored at −20° for up to 4 weeks. Leukotriene C_4 production in control and AICA riboside-exposed mast cells was assessed utilizing a [^{3}H]LTC_4 radioimmunoassay kit which identifies LTC_4, some LTD_4, and a smaller percentage of LTE_4. Mouse bone marrow-derived mast cells have been reported to produce LTC_4 almost exclusively, however [9]. Aliquots (10 μl) of each sample were used in the assay which is accurate between 0.025 and 1.6 ng LTC_4/100 μl.

Assessment of purine nucleotides and nucleosides. Resting and stimulated mast cells grown in medium alone or AICA riboside-containing medium were assayed for the presence of adenosine, ATP, and ZTP by high performance liquid chromatography. Reactions were carried out in the presence of 1 μM deoxycoformycin to inhibit adenosine deaminase and were allowed to proceed for 60 sec after challenge. Cell pellets and supernatant fractions were separated for 15 sec in a Beckman microfuge, and each was mixed with perchloric acid, neutralized with an alamine/Freon solution, and stored at −20° for up to 4 weeks [1]. Supernatant adenosine concentrations were quantitated on a C18 μBondapak reversed-phase column. Pellet ATP and ZTP concentrations were quantitated on a Whatman Partisil-10 SAX column [1]. Identities of peaks were confirmed by sensitivity to adenosine deaminase and/or coelution with appropriate standards. Neither A23187, AICA, nor AICA riboside interfered with chromatographic analysis.

Statistical analysis. Statistical significance was assessed utilizing the paired, two-tailed Student's *t*-test. Results are expressed as means ± SE unless otherwise indicated.

RESULTS

Effects of AICA and AICA riboside on mouse bone marrow mast cell β-hexosaminidase release. To identify the effects of acute and chronic AICA or AICA riboside exposure on resting and stimulated mast cell mediator release, cells were incubated with selected concentrations of AICA or AICA riboside for various periods of time, challenged with either A23187 or DNP-BSA antigen for 10 min, and assayed for cell and supernatant β-hexosaminidase concentrations. When either AICA or AICA riboside (100 μM) was added simultaneously with a secretagogue, there were no consistent differences in β-hexosaminidase release observed compared to control cells (Fig. 1). The additional presence of exogenous adenosine augmented stimulated mediator release, but this was not altered by AICA or AICA riboside. Both agents after 10 min produced a mild increase in resting mast cell β-hexosaminidase release which was not statistically significant. If mast cells were preincubated in 100 μM AICA or AICA riboside for 1 hr followed by A23187 challenge, β-hexosaminidase release was again nearly identical to that seen in control cells with or without adenosine (Fig. 1), though spontaneous β-hexosaminidase release was somewhat higher than control values after AICA riboside incubations. The 100 μM concentration was chosen because of the dose–response data described below.

Of greater interest is the fact that, when mouse bone marrow-derived mast cells were cultured for 6 days in medium containing 100 μM AICA riboside, a profound inhibition of A23187-induced β-hexosaminidase release resulted. Inhibition of β-hexosaminidase release was seen both with and without the addition of exogenous adenosine. This inhibition was approximately 50% of control values; a small elevation in spontaneous β-hexosaminidase release

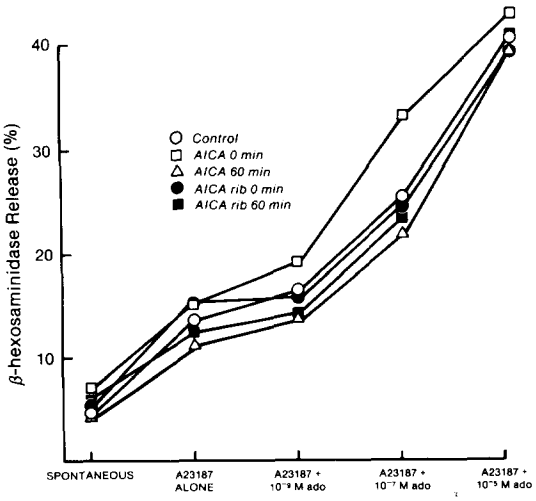

Fig. 1. Effects of simultaneous addition and 1-hr incubations with AICA or AICA riboside and secretagogue on mast cell β-hexosaminidase release. Buffer alone, 100 μM AICA or 100 μM AICA riboside was added to mast cells at the same time as, or 1 hr before, A23187, with or without exogenous adenosine, and total cell β-hexosaminidase release was assessed. Means of β-hexosaminidase releases from control cells (○), cells receiving AICA and secretagogue simultaneously (□), cells preincubated with AICA for 1 hr (△), cells receiving AICA riboside and secretagogue simultaneously (●), and cells preincubated with AICA riboside for 1 hr (■) are shown. Values from three to six experiments performed in duplicate were not statistically different from each other ($P > 0.05$ in each case).

in cells grown in AICA riboside was also noted (Fig. 2). AICA exposure produced data essentially the same as that seen in control cells.

Various concentrations of AICA riboside were present in mast cell culture medium for 6 days, and any resultant changes in cell growth, mediator content, and mediator release were examined. Overall, cells grown in 100 μM AICA riboside multiplied to 74% of the number of control cells. LDH release from AICA riboside-exposed cells was not different from controls. Individual cell β-hexosaminidase and histamine contents were similar in the two cell populations. As shown in Fig. 3, the cells exhibited a dose-dependent attenuation of β-hexosaminidase release when grown in 1 to 100 μM AICA riboside for 6 days which remained to a similar degree in the additional presence of adenosine (10^{-5}–10^{-4} M). At concentrations above 100 μM AICA riboside, cell growth was extremely poor, and below 1 μM AICA riboside no inhibition of mediator release could be demonstrated.

Although the AICA riboside inhibition of mast cell secretion was absent at 1 hr and evident at 6 days, some intermediate time points were chosen to more precisely determine the time needed for AICA riboside to affect mast cell function. Culture for 24 hr in AICA riboside-containing medium resulted in β-hexosaminidase release unchanged from control levels, but by 48 hr of exposure cells exhibited a 50% decrease in β-hexosaminidase release by A23187 compared to controls, and this effect persisted for

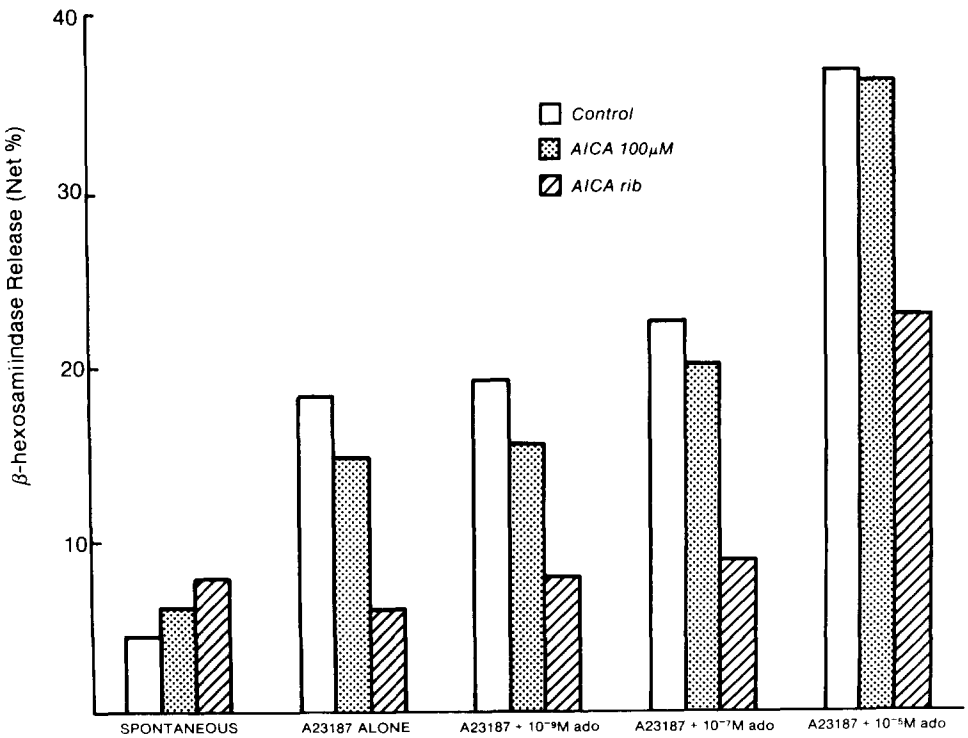

Fig. 2. Effect of 6-day AICA or AICA riboside exposure on mast cells β-hexosaminidase release. Mast cells cultured in the presence of AICA (100 μM, ▨) or AICA riboside (100 μM, ▨) for 4–8 days were washed and challenged with A23187 for 10 min. This representative experiment, one of six similar experiments, demonstrates the inhibition of net β-hexosaminidase release observed in AICA riboside-treated mast cells. All AICA riboside values except spontaneous were statistically different from AICA and control values ($P < 0.01$).

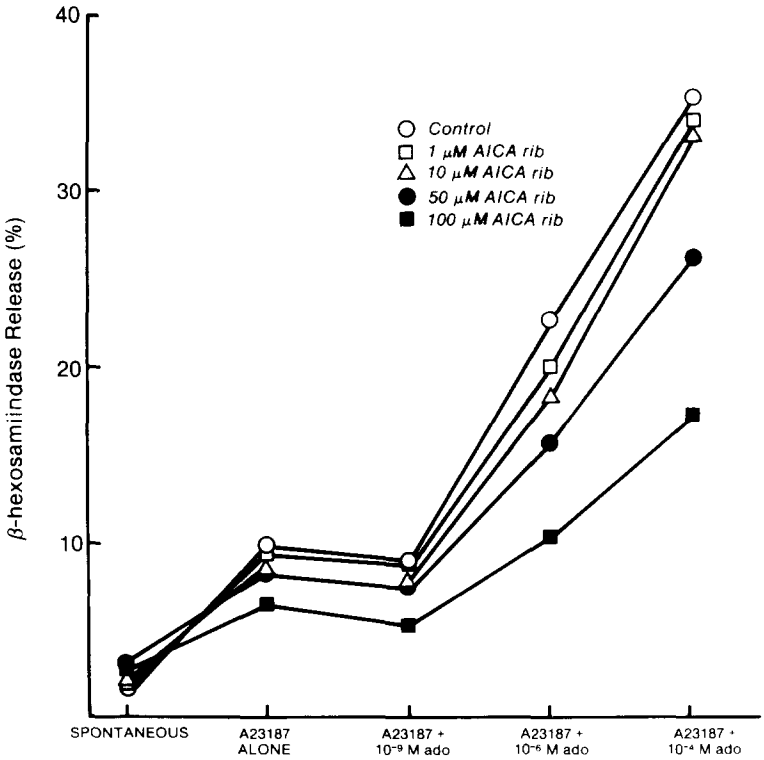

Fig. 3. Dose–response of 6-day AICA riboside exposure on mast cell β-hexosaminidase release. Cells were cultured for 6 days in medium alone (○), 1 μM (□), 10 μM (△), 50 μM (●), or 100 μM (■) AICA riboside, washed, and challenged with A23187 for 10 min. The mean percent (from three experiments) of total cell β-hexosaminidase released spontaneously, with A23187, and with A23187 plus exogenous adenosine is shown. Except for spontaneous release, β-hex releases from 50 and 100 μM AICA riboside-treated cells were statistically significantly different from controls ($P < 0.05$).

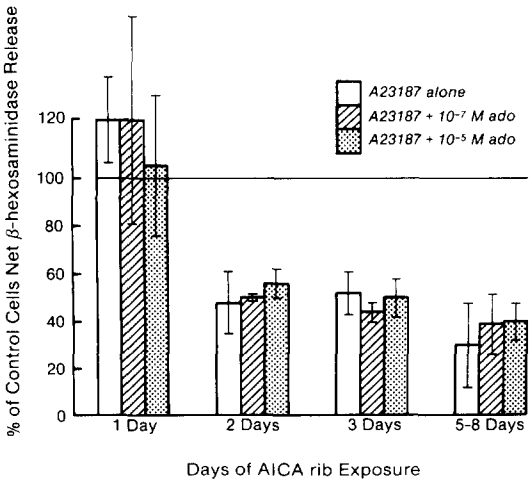

Fig. 4. Time–course of the AICA riboside effect on mast cell mediator release. Cells exposed to 100 μM AICA riboside in tissue culture for 1, 2, 3, or 5–8 days were washed and challenged with A23187 alone (□), A23187 + 10^{-7} M adenosine (▨), or A23187 + 10^{-5} M adenosine (▩). Net β-hexosaminidaase release is expressed as percent of that observed in parallel cells grown in medium alone. Depicted are means ± SE of duplicate determinations from three experiments. All values from days 2 to 8 were statistically different from controls ($P < 0.01$). Spontaneous β-hexosaminidase release in control cells was 4.5 ± 1.2%; A23187-challenged release was 13.7 ± 2.5%.

up to 8 days of continuous AICA riboside treatment (Fig. 4).

Effects of AICA riboside on mouse bone marrow mast cell LTC_4 generation. Mast cells were cultured in 100 μM AICA riboside for 6 days, challenged for 20 min with A23187 and various concentrations of adenosine, and supernatant fractions were assayed for the presence of LTC_4. Although adenosine produced little augmentation of LTC_4 release, stimulated control cells released 50–60 ng $LTC_4/10^6$ cells, whereas AICA riboside-exposed cells released a maximum of 25 ng $LTC_4/10^6$ cells (Fig. 5). The resting LTC_4 levels were similar in the two cell populations. A time course of the AICA riboside effect on LTC_4 generation induced by antigen (Fig. 6) revealed that the inhibition of LTC_4 was evident at 2 days, similar to the inhibition of β-hexosaminidase release. Thus, chronic AICA-riboside treatment of mast cells blunts their ability to generate LTC_4 after A23187 or antigen stimulation.

Relationship between AICA riboside treatment and mast cell ribonucleotide triphosphate concentrations. After A23187 challenge, mast cell ATP levels have been shown to fall by up to 50% [1]. Mast cells cultured with AICA riboside for 48 hr possessed slightly more intracellular ATP than cells grown in medium alone, and these levels decreased after stimulation by an amount similar to that seen in control cells (Fig. 7). Resting and stimulated super-

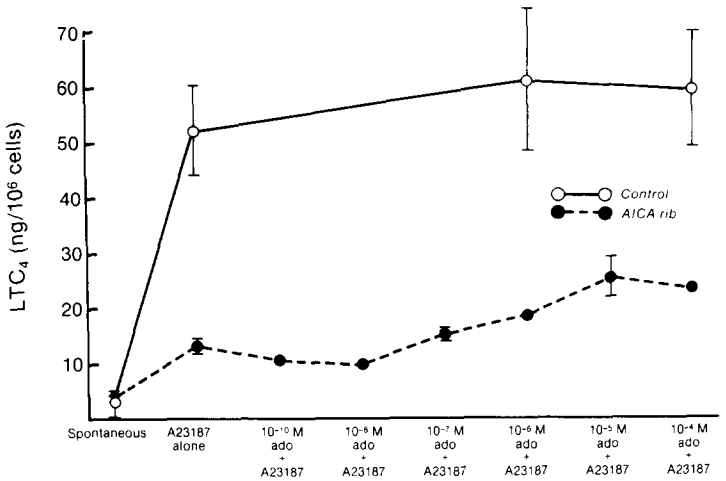

Fig. 5. Leukotriene C_4 release in AICA riboside-treated mast cells. Cells were grown in medium alone (○) or 100 μM AICA riboside (●) for 6 days, washed, and challenged for 20 min with A23187 with or without adenosine; supernatant LTC_4 concentrations were determined by RIA. Depicted are means ± SE of values from four experiments. Except for spontaneous LTC_4 release, control and AICA riboside values were statistically different ($P < 0.01$).

natant adenosine concentrations were also similar in the two cell groups. Of perhaps greater interest is the fact that mast cells exposed to 100 μM AICA riboside produced an unusual nucleotide, AICA riboside triphosphate or ZTP, that was not present in control cells (Fig. 8). ZTP concentrations in these cells averaged 0.168 nmole/10^6 cells after 2 days of AICA riboside exposure; these levels decreased to a mean of 0.127 nmole/10^6 cells after secretagogue challenge. ZTP was not detectable after a 1-hr incubation with AICA riboside.

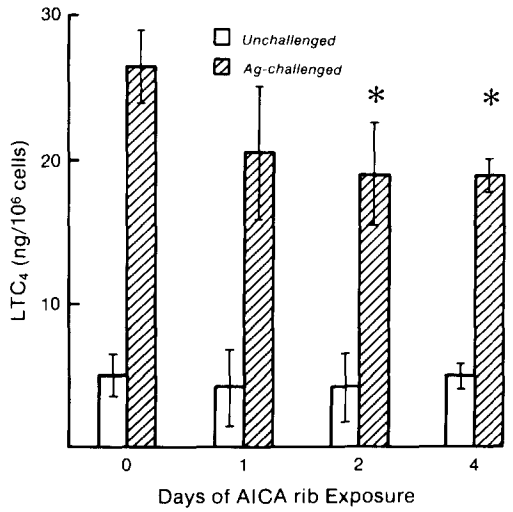

Fig. 6. Time-course of AICA riboside effect on mast cell LTC_4 generation. Resting (□) and antigen-stimulated (▨) mast cell supernatant fractions were assayed for leukotriene C_4 concentrations by radioimmunoassay. Cells were grown in the presence of 100 μM AICA riboside for 0 (control), 1, 2, or 4 days. Depicted as means ± SE of duplicate determinations from four experiments. An asterisk (*) = $P < 0.05$ as compared to stimulated values on day 0.

DISCUSSION

AICA riboside is readily taken up by cells, enters the *de novo* purine synthesis pathway at a distal, relatively unregulated point, and exhibits low toxicity when administered to dogs [13]. It is phosphorylated by the action of adenosine kinase [14]. AICA is metabolized by adenosine phosphoribosyltransferase, a highly regulated enzyme in many cell types. These properties of AICA riboside combined with the growing importance of purine metabolism in mast cell functioning make this compound an interesting one to examine as a potential modulator of mast cell secretion.

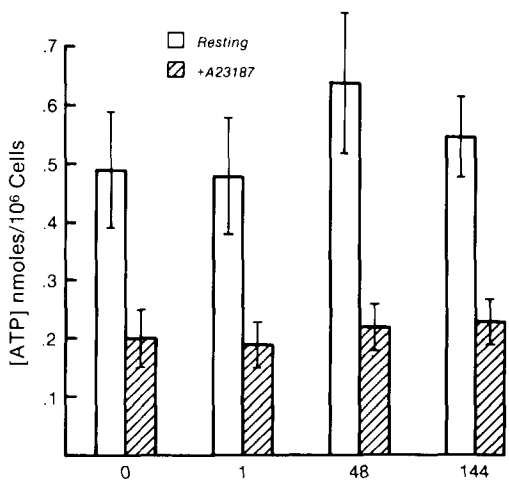

Fig. 7. ATP content of control and AICA riboside-treated mast cells. Resting (□) and A23187-stimulated (▨) mast cell pellets were assayed for ATP content by HPLC. Means ± SE are shown for cells cultured in medium alone (0 hr), and for 1, 48, or 144 hr in 100 μM AICA riboside.

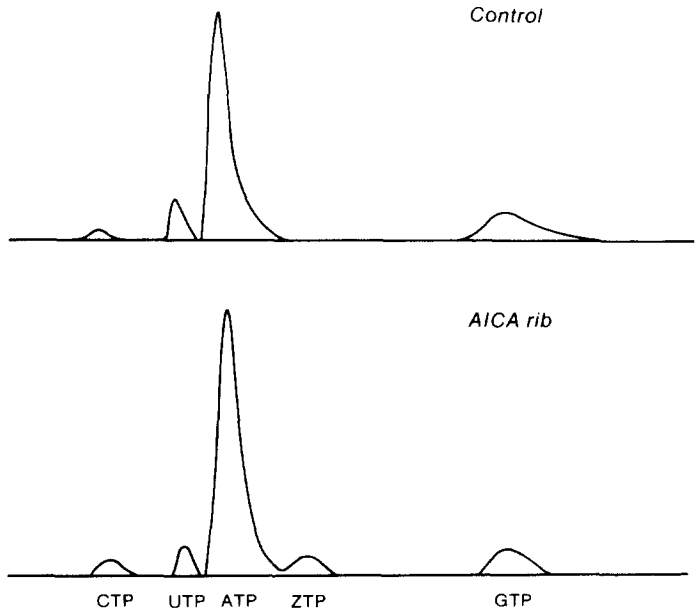

Fig. 8. HPLC tracings of stimulated control and AICA riboside-treated mast cell pellets. Mast cells cultured in medium alone (top panel) or 100 μM AICA riboside (bottom panel) for 6 days were challenged with A23187 for 60 sec and then centrifuged. The cell pellet nucleotide contents were analyzed by HPLC. A ZTP peak was identified in the AICA riboside-exposed cells. X axis indicates time; retention times: ATP, 30 min; ZTP, 35 min; and GTP, 50 min. Y axis indicates absorbance at 254 nm.

Studies of the simultaneous addition of AICA or AICA riboside to resting or A23187-stimulated mast cells revealed little effect on mast cell β-hexosaminidase release, and a 1-hr preincubation with either of these agents was similarly unrevealing (Fig. 1). However, a 50% reduction of A23187-induced mast cell β-hexosaminidase release was observed in cells cultured for 6 days in medium containing 100 μM AICA riboside (Fig. 2). This attenuation was dose-dependent (Fig. 3) and was not based on changes in mast cell mediator content. A 25% inhibition of mast cell growth was observed after 6 days of 100 μM AICA riboside exposure, but cell viability remained greater than 95%. At least 2 days of AICA riboside treatment was required to identify its inhibitory effect (Fig. 4), and the degree of inhibition of stimulated β-hexosaminidase release remained stable for up to 8 days. The AICA riboside effect does not appear to take place via adenosine receptors in that similar inhibition occurred in the absence and presence of exogenous adenosine. Even the extremely potent stimulus of A23187 plus 100 μM adenosine was inhibited approximately 50% by chronic AICA riboside exposure. This effect also does not appear to take place via IgE receptors in that both IgE-mediated and A23187-induced degranulation were inhibited to similar degrees by AICA riboside treatment.

Leukotriene C_4 generation in control and AICA riboside-treated cells paralleled the findings for β-hexosaminidase release with nearly identical basal LTC_4 levels in the two cell populations and a profound inhibition of LTC_4 generation in AICA riboside-exposed cells (Fig. 5). The inhibition of LTC_4 release after IgE-mediated antigen challenge required at least 2 days of AICA-riboside exposure (Fig. 6). The mechanism of this effect as well as the observed inhibition of preformed mediator release is not clear, but the facts that the inhibition was marked, was global, and required time deserve emphasis.

One possible agent in the inhibition of mast cell mediator release by AICA riboside is ZTP, the unusual ribonucleotide identified in folate-starved bacteria [6]. That ZTP is present in the cells after AICA riboside exposure is documented in Fig. 8, but any specific action of ZTP itself on mast cells is not known. It did not alter mast cell ATP levels to a major extent (Fig. 7) and neither caused cell toxicity when added directly to mast cells nor altered mast cell mediator release directly (data not shown). Whether ZTP could compete with ATP at an intracellular site of action is not known at present but warrants consideration.

Other possible modes of action of AICA riboside on mast cells involve the enzyme adenylosuccinate AMPlyase or alterations in folate metabolism. ZMP accumulation has been shown to inhibit adenylosuccinate AMPlyase in Chinese hamster ovary fibroblasts [15] and lead to the accumulation of adenylosuccinate, but this requires AICA riboside concentrations ≥ 450 μM. It is not known what effect adenylosuccinate has on mast cell secretion. Although folate-deficient bacteria produce ZTP, ZTP accumulation does not necessarily indicate folate starvation, and the addition of exogenous folate to mast cells does not reverse the AICA riboside effects.

In any event, alterations in mast cell purine metabolism may invoke alterations in mast cell mediator generation and release, and these changes may be independent of intracellular ATP concentrations or mast cell adenosine responsiveness. Further investigation into the mechanism of action of AICA riboside suppression of mast cell secretion may result in the formulation of new pharmacologic agents to regulate mast cell function and thereby modulate immediate hypersensitivity reactions.

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