Inosine-5’-Monophosphate Dehydrogenase Is Required for Mitogenic Competence of Transformed Pancreatic β Cells*

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ABSTRACT

The relation of inosine-5’-monophosphate dehydrogenase (IMPDH; the rate-limiting enzyme in GTP synthesis) to mitogenesis was studied by enzymatic assay, immunoblots, and RT-PCR in several dissimilar transformed pancreatic β-cell lines, using intact cells. Both of the two isoforms of IMPDH (constitutive type 1 and inducible type 2) were identified using RT-PCR in transformed β cells or in intact islets. IMPDH 2 messenger RNA (mRNA) and IMPDH protein were both regulated reciprocally by changes in levels of their end-products. Flux through IMPDH was greatest in rapidly growing cells, due mostly to increased uptake of precursor. Glucose (but not 3-O-methylglucose, l-glucose, or fructose) further augmented substrate uptake and also increased IMPDH enzymatic activity after either 4 or 21 h of stimulation. Serum or ketoisocaproate also increased IMPDH activity (but not uptake). Two selective IMPDH inhibitors (mycophenolic acid and mizoribine) reduced IMPDH activity in all cell lines, and, with virtually identical concentration-response curves, inhibited DNA synthesis (assessed as bromodeoxyuridine incorporation) in response to glucose, serum, or ketoisocaproate. Inhibition of DNA synthesis was reversible, completely prevented by repletion of cellular guanine (but not adenine) nucleotides, and could not be attributed to toxic effects. Despite the fact that modulation of IMPDH expression by guanine nucleotides was readily detectable, glucose and/or serum failed to alter IMPDH mRNA or protein, indicating that their effects on IMPDH activity were largely at the enzyme level. Precursors of guanine nucleotides failed, by themselves, to induce mitogenesis. Thus, adequate IMPDH activity (and thereby, availability of GTP) is a critical requirement for β-cell proliferation. Although it is unlikely that further increases in GTP can, by themselves, initiate DNA synthesis, such increments may be needed to sustain mitogenesis. (Endocrinology 142: 193–204, 2001)

A LTHOUGH β cells in pancreatic islets of adult animals normally replicate extremely slowly, this proliferative capacity can be increased by different stimuli, such as a reduction in β-cell mass (1), hyperglycemia (2), or pregnancy (3). Abnormalities in the proliferation of β cells may be important to the pathogenesis of various diabetic states. In several animal models of type 2 diabetes, there is evidence that the balance between β-cell death and compensatory proliferation is deleteriously shifted toward the former; consequently, the resultant decline in β cell mass over time may lead to a progressive deterioration of insulin secretion (4–6). In human type 2 diabetes as well, the β-cell mass is reduced by about 50% (7, 8). Additionally, the growth (and/or survival) of β cells within islets transplanted into patients with type 1 diabetes, may also be compromised under certain conditions such as hyperglycemia or increased metabolic demand (9). Therefore, it is important to understand the factors required for the replication (and differentiation) of β cells.

We have presented evidence (10, 11) that a deficiency of intracellular content of guanine nucleotides (GNs; especially GTP) acutely modulates β cell function by impairing insulin release. In preliminary studies, DNA synthesis was also markedly reduced (12–14), and eventually, apoptotic cell death ensued (12). Therefore, we proposed (15) that GTP starvation first inhibits the secretory ability and mitogenesis of β cells, rendering them both quiescent (probably in the G0/G1 phase of the cell cycle) and also functionally incompetent. In the continued presence of stimulation (e.g. by high glucose), the effete β cell, unable to respond to growth signals, is removed from the cell pool by programmed cell death.

The rate-limiting enzyme in the synthesis of new GNs is inosine-5’-monophosphate dehydrogenase (IMPDH), a soluble enzyme which converts IMP (derived via either the salvage or de novo synthetic pathways) to xanthosine monophosphate and from there to GNs. Studies in nondocrine cells suggest that alterations in IMPDH and/or GTP content modify proliferation or differentiation and that there is an increase in IMPDH activity in rapidly growing and/or malignant cells. Mitogenic stimulation may even require the induction of a specific isoform of IMPDH [summarized in (15, 16)] That isoform (IMPDH 2) seems to be growth-related, whereas IMPDH 1 is constitutively active. IMPDH can be inhibited by mycophenolic acid (MPA) or other structurally—and mechanistically—dissimilar agents such as mizoribine [MZ; Ref. (17)]. These agents also block DNA synthesis
and cell proliferation and conversely, may induce cell quiescence and differentiation (18–24). Such pharmacologic studies appear to have physiologic relevance because MPA not only has exceptional selectivity against IMPDH, in contrast to other enzymes of purine metabolism, but it also seems remarkably free of nonspecific effects on signal transduction in β cells (12, 15, 18, 25, 26).

Similar studies of the effects of GTP synthesis on cell proliferation have not been carried out in endocrine cells. Studies of the role of IMPDH in proliferation of the endocrine pancreas require the use of intact β cells, since glucose (which acts via generation of intracellular messengers) is not only a major mitogen per se, but is also required for the expression of the mitogenic effects of other receptor-mediated factors such as insulin-like growth factor (IGF)-1 (27). Herein, we use an approach with which to assay inosine monophosphate dehydrogenase (IMPDH) activity in intact β cells provided with tritiated inosine or hypoxanthine. It takes advantage of the observation (28) that if the purine ring of inosine (or its degradation product hypoxanthine) is labeled with tritium in the 2- and 8-positions, the action of IMPDH detritiates the degradation product hypoxanthine) is labeled with tritium in the observation (28) that if the purine ring of inosine (or its

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[2,8-^3\text{H}]\text{IMP} + \text{NAD} + \text{H}_2\text{O} \rightarrow [8-^3\text{H}]\text{XMP} + \text{NADH} + ^3\text{H}^+ \downarrow \text{GNs}
\]

The expression of IMPDH is also assessed using RT-PCR and immunoblotting. Using these techniques, we compare the effects of MPA and MZ, and physiologic agonists, on IMPDH activity and expression, to their effects on DNA synthesis, chiefly measured as bromodeoxyuridine (BrdU) incorporation. We provide strong evidence that IMPDH is, indeed, a competency factor for β-cell proliferation.

**a. Materials**

Inosine [2,8-^3\text{H}] (28–40 Ci/mm) and hypoxanthine [2,8-^3\text{H}] (37 Ci/mm) were from Moravek (Brea, CA). Mycophenolic acid and MZ were from Sigma (St. Louis, MO); they were diluted in dimethylsulfoxide (DMSO) and water, respectively. The vehicles for nucleosides or nucleobases (from Sigma) were as follows: inosine, in water; guanine, in 1N NaOH to form a concentrated stock solution; guanosine, in RPMI 1640 medium; and adenine, in a stock using 1.0N NaOH. IGF-1 (from Calbiochem; San Diego, CA) was made into a 10 mM stock using acetic acid in 0.1% BSA. Tetracycline, leucine, and ketoisocaproate were from Sigma. All other reagents were of the highest grade available. Whenever a test agent was present during a culture or incubation period, an identical amount of its vehicle was added to all control plates. FCS was purchased from HyClone Laboratories, Inc. (Logan, UT). Glass-polyethyleneimine (PEI)-cellulose TLC plates, with fluorescent indicator (20 × 20 cm), were manufactured by Merck KGaA (Darmstadt, Germany).

**b. Cells**

β-TC6 (29), β-TC-13 (30), and HIT-T15 (31, 32) cells were obtained from Dr. Paul Robertson (Seattle, WA) from stocks originally provided by Drs. A. E. Boyd III and D. Hanahan, respectively. They were used at passage numbers 42–56, 15–26, and 76–82, respectively (numbered following their receipt in Seattle). INS-1 cells (33) were a gift of Dr. Claes Wollheim (Geneva, Switzerland), and were used mostly at passages 50–53. β-TC-tet cells (34, 35) were a generous gift of Dr. Shimom Efrat (Tel Aviv, Israel). These cells are a conditionally transformed cell line under the control of the bacterial tet resistance operon which modulates the expression of SV40 large T antigen (Tag) expression; addition of tetracycline (tet) to the growth medium blocks Tag expression and cell growth. In the studies below, tetracycline, when present, was provided, at 1 μg/ml for 7–10 days of culture. Cell growth was markedly inhibited by the presence of tet, as indicated by a marked reduction in DNA content/well [2.28 ± 0.34 vs. 6.44 ± 1.34 μg DNA/well, tet vs. control; n = 5–6 each] as well as in doubling times (0.5 ± 0.7 doublings over 6–8 days vs. 2.7 ± 0.6 in the presence and absence of tetracycline, respectively; x ± s.e.; n = 11, both (also cf. Table 1, below). These cell lines were generally propagated as described in previous publications, as cited above, with these minor changes in some studies: β-TC-tet cells (34, 35) were cultured in a lower concentration of glucose (11.1 mM), rather than 25 mM, without discernible differences in responses. βHC-13 cells were cultured in RPMI 1640, 11.1–16.0 mM glucose, and 10–15% FCS. Cells were generally fed every 2 days and passed every 7 days. Cells were subcultured for study in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES in a humidified atmosphere of air:CO₂ (95%:5%).

**c. Assessment of IMPDH activity**

Following culture, cells (1–2 × 10⁶, in 24-well plates) were washed and studied during a 21 h experimental incubation period (except where indicated otherwise) in 1 ml of RPMI 1640 medium; where present, unlabeled inosine and MPA or MZ, or their diluents, were added for the final 4 h of this incubation period. Labeled substrate (1H-inosine; 8 μCi/well in 250 μl of RPMI 1640 medium) was added for the final 3 h, except where indicated. For studies of intact islets, incubation times were extended to 16 h, with MPA and 1H-inosine present throughout, to reproduce the conditions of our previous studies (e.g. Refs. 10 and 11) and to assure adequate permeation of label to the β-cell enriched inner core of cells. Intact, Sprague Dawley rat islets were isolated from the pancreas and purified by hand picking as described, with the recent modifications in (36). They were cultured overnight in 60 × 15 mm Petri dishes in 2.5 ml of RPMI 1640 medium), with the addition of 0.4 μm radiolabeled inosine plus unlabeled inosine to bring the final concentration to 6–16 μM. These studies were approved by the Animal Care Committee of the University of Wisconsin-Madison.

Following incubation, the cellular medium was then mixed with 625 μl of activated charcoal (100 mg/ml) to precipitate any unincorporated ³H-inosine, and the charcoal pellets were washed by gravity; 250 μl of media was then mixed with 625 μl of charcoal, vortexed, and centrifuged (1100 × g × 10 min). Radioactivity remaining in the medium was then

**TABLE 1. IMPDH activity and flux in relation to the rate of growth of the major cell types studied**

| Cell Type          | Uptake of label (μCi/μg DNA × 10⁻⁶) | Absolute uptake (μCi/μg DNA × 10⁻⁶) | Net flux through IMPDH (μCi/μg DNA × 10⁻⁶) | True IMPDH activity (% of uptake) | Doubling times |
|--------------------|-----------------------------------|------------------------------------|------------------------------------------|-----------------------------------|----------------|
| INS-1              | 18 ± 4% (5)                       | 0.31 ± 0.052 (5)                   | 0.715 ± 0.18 (5)                         | 15 ± 1% (14)                      | 56 ± 2 h (20)  |
| β-TC-tet (proliferating) | 27.3 ± 1% (5)                 | 0.44 ± 0.094 (5)                   | 0.94 ± 0.199 (5)                         | 16 ± 0.5% (22)                    | 66 ± 5 h (20)  |
| β-TC-tet (quiescent) | 7.6 ± 1% (7)                      | 0.11 ± 0.025 (3)                   | 0.401 ± 0.079 (3)                        | 17 ± 0.4% (20)                    | 287 ± 157 h (20) |

_a See Data presentation for calculations. Values are mean ± SEM from n (independent experiments, in which extracellular inosine was 0.8 μM.
_b Uncorrected for uptake of label._
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counted by scintillation spectroscopy. Values for IMPDH activity (i.e. cpm in charcoal-treated media) were always corrected for cell-free blanks (i.e. charcoal). Radioactive counts present in wells incubated exactly as described above (except for the absence of cells). These values ranged from 1.3 to 2.8% of total cpm added. In some studies, 3H-hypoxanthine was replaced by 3H-hypoxanthine to eliminate potential variations in the rate of phosphorolysis of inosine to hypoxanthine, as well as to study a shorter period of stimulation (4 h of incubation, as vs. 21 h with inosine). For these studies, experimental perturbations were only present during the last four hours of the study. Each value in all studies represents the means of triplicate determinations.

Maximized conditions for the IMPDH assay were first determined. Residual intracellular inosine and hypoxanthine was found (6% of cell totals by barium acetate fractionation and 7% by TLC), indicating that substrate was not depleted during the incubation period. The detritiation of [2,8-3H]inosine was readily demonstrated in all cell preparations. The only other enzyme inducing similar detritiation as IMPDH is xanthine oxidase; however, addition of 50 μM allopurinol (a potent inhibitor of xanthine oxidase) had no effect on tritium release (not shown). Therefore, release of 3H can be specifically related to IMPDH activity. The dependence of IMPDH activity on substrate concentration was then assessed in β cell lines by adding 0–50 μM unlabeled inosine to tracer amounts of labeled inosine. Relative IMPDH activities at various extracellular substrate (inosine) concentrations were obtained by multiplying radioactivity by the dilution factor (i.e. corrected for added unlabeled inosine). Studies to assess the intracellular generation of purine metabolites were carried out similarly to those described above, except that the total extracellular inosine concentration was 1.6 μM, and incubations were extended to 18–21 h to label relatively minor purines (i.e. GTP, GDP, IMP) sufficiently for quantitation by TLC, as well as to reproduce the period assessed during studies of mitogenesis.

To examine the effects of variations in GN availability on IMPDH activity, protein, and gene expression, cells were treated for 12–45 h with MPA (0.3 or 1.0 μM/ml), MZ (50 μg/ml), or guanosine (0 through 300 μM). Cells were then washed five times to remove as much as possible of the residual MPA, MZ, or guanosine, before the level of IMPDH activity and expression was assessed.

d. Expression of IMPDH: mRNA and protein

For assessments of gene expression, real-time, semiquantitative RT-PCR using TaqMan technology was employed, as described in (37). Total RNA was extracted from INS-1 or β-TC-tet cells using RNeasy mini-kit (QiAGEN, Valencia, CA). One-step RT-PCR was carried out essentially as described (37) using the Gold RT-PCR kit from Perkin-Elmer Corp. Biosystems and an ABI Prism 7700 sequence detector equipped with a thermocycler (TaqMan technology) and a cooled CCD camera to detect fluorescence. The TaqMan reaction mixture comprised 2 μl of TaqMan probe and primers, 1 μl of 2x TaqMan Master Mix (Applied Biosystems), and 5 μl of 20 ng total RNA per reaction. Amplification was for 40 cycles with denaturation at 95 C for 15 sec, annealing and extension at 60 C for 1 min. Because a complete curve of amplification is developed over the course of 40 cycles, analyses could be carried out solely within the exponential range of amplification. Comparative analysis was then based upon the cycle number at which a significant increase in the amplification signal above threshold is detected.

Immunoblot for total cellular content of IMPDH were carried out as follows. Whole cell extracts of β-TC-tet cells were prepared by washing cells with PBS, and lysing them in buffer containing 50 mM Tris·HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 1% Igepal (Sigma), 1% glycerol, and 1× complete protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN), using five freeze/thaw cycles in liquid N2/37 degree water bath. Lysates were spun down in a refrigerated microfuge at 15,000 rpm for 15 min and supernatants were transferred to a fresh tube. Protein concentrations were determined using BCA protein assay kit (Pierce Chemical Co., Pittsburgh, PA). Ten or 20 μg of total protein was loaded on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Chicago, IL). Western blot analyses were performed with mouse monoclonal anti-IMPDH at 1 μg/ml (a gift from Dr. F. Collart) and goat anti-rabbit peroxidase conjugate (which precipitates all phosphorylated purines while leaving bases or nucleosides in solution). Extracts (containing 2.5 μg each of unlabelled exogenous standards) were applied to PEI-cellulose plates that had been developed first in 100% MeOH and allowed to dry. The plates were then developed in a gradient of LiCl (pH

packard ScanJet IIXc/T with image analysis using DeskScan II version 2.6.

The oligonucleotide probe and primers used were: IMPDH 1 probe: 6FAM-CCCTCATAACTAGTGTTGGTCACAAGA-TAMRA
Forward primer: CTTTACGAAAAAGCAGCCTACT
Reverse primer: GAAGAGCTCGAGAAACCGCGT
IMPDH 2 probe: 6FAM-CCTCATTGCTATGAAGCCGCTT-TIC-TAMRA
Forward primer: GGAGCTTAAGTTGAGAAGACCT
Reverse primer: AGGTGTGCTGATCCCTTTC
The reporter dye was FAM and the quencher dye, TAMRA.

Data are expressed as the ratio between the IMPDH signal and that of a housekeeping gene (actin, or glyceraldehyde-3-phosphate dehydrogenase).

e. Assays for BrdU incorporation and proliferating cell nuclear antigen (PCNA) content

Cells (5–15 × 10⁴ per well) were subcultured in 24-well plates and allowed to grow at least 2 days. They were then synchronized in a quiescent state by a 21–24 h period in low glucose (1.7 or 3.3 mM, except 0.4 mM for HIT-T15 cells) in RPMI 1640 medium containing a reduced concentration of PCS (0.2% or 0.5%). For the experimental period, high serum (10%) and/or high glucose (11.1–16.7 mM, except 5.6 mM for HIT-T15 cells) with or without other test agents were added to the RPMI 1640 medium, for a final incubation period of 21 h (except where indicated). The cells were loaded with BrdU (final concentration = 10 μM) concurrent with the addition of agonist(s) during this final 21 h. For studies of the reversibility of MPA effects, synchronized cells were treated for an initial 24 h with MPA or with its diluent. Cells were then gently washed three times (5 min each) to allow residual drug to exit the cells, and then were reincubated for 21 h with BrdU, in the presence of MPA or its diluent.

BrdU incorporation was quantified using an enzyme-linked immunoassay method from Oncogene Science Products, Inc. (Cambridge, MA), mostly using modifications of the manufacturer’s directions. After washing, denaturation, and fixation, anti-BrdU serum (1:100) was added and incubated for 60 min, followed by three washes of the cells. Second antibody (peroxidase goat antimouse IgG HRP conjugate, 1:1000) was then added and incubated for another 30 min. Following washing, peroxidase substrate (tetramethylbenzidine; 100 μl/well) was added and cells were reincubated in the dark for 15 min. Color production was quantified within 5 min of adding stop solution using a spectrophotometric plate reader at a wavelength of 450 nm, with 490 nm as the reference wavelength. Two controls were assessed: cell-free wells, treated as described above, and one containing cells but no BrdU. These were barely detectable and were essentially identical to values from cells incubated in low glucose and low serum, indicating that the cells had indeed been rendered quiescent. Blank values were subtracted from experimental values. Each condition was assessed by at least quadruplicate determinations within each study. Since true basal values of DNA synthesis in quiescent cells were essentially undetectable, expression of stimulated values as a percentage of basal was considered meaningless.

For measurement of PCNA content, 2–4 × 10⁴ INS-1 cells were plated and synchronized as described above. At the end of a 21-h final incubation in 0.2% serum/1.7 mM glucose, or 10% serum/16.7 mM glucose ± MPA 0.3 μg/ml, PCNA was extracted and measured by ELISA, as described by instructions in the kit (Oncogene, catalog no. QIA 59).

f. Assessments of purine nucleotide content

Following the removal of media used to measure IMPDH activity, the cells were extracted twice using 10% ice-cold trichloroacetic acid (TCA) followed by back-extraction using 50 μl of cell extract; 1.26 ml diethylyther was added, vortexed, and, after phase separation, the top phase was drained off and discarded. This procedure was repeated three more times. The small amount of TCA-insoluble material was not assessed further. The TCA extracts were then either applied to PEI-cellulose TLC plates directly, or coated with goat anti-rabbit peroxidase conjugate (which precipitates all phosphorylated purines while leaving bases or nucleosides in solution). Extracts (containing 2.5 μg each of unlabelled exogenous standards) were applied to PEI-cellulose plates that had been developed first in 100% MeOH and allowed to dry. The plates were then developed in a gradient of LiCl (pH

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The percentage of total radiolabel initially added to the medium, which was taken up and/or metabolized, i.e. uptake, ranged from 8 to 27% and was, in general, correlated with the degree of differentiation and/or growth rate (Table 1). Net flux through IMPDH increased with inosine concentration (not shown) in all but one cell line (HIT-T15 cells; see below); it approached or reached saturation at the highest concentrations of inosine tested (25–50 μM). Assessment of IMPDH activity was unaltered by such changes in extracellular concentrations of inosine (e.g. 18.6 ± 2% of cellular uptake of label was metabolized via IMPDH by INS-1 cells at 0.8 μM inosine vs. 18.6 ± 1% at 25 μM inosine; n = 9 determinations each). In contrast to substrate uptake, true IMPDH activity was not affected by the proliferative rate (Table 1). These findings were observed in each of a variety of β-cell lines (β-HC, βTC, β-TC-tet, and INS-1). In addition, preliminary studies, IMPDH activity was readily detected in each of these six independent preparations of intact islets, averaging 4772 ± 946 cpm/islet. However, in contrast to other cell lines, the uptake of radiolabel into HIT-T15 cells was very low (e.g. 23% of that observed using βHC cells), and a very high percentage (49–50%) of this incorporated inosine was metabolized by IMPDH in HIT-T15 cells, whereas the comparable figure in βHC cells was only 19–20%. Furthermore, in comparison to other cell lines, IMPDH activity reached apparent saturation at very low inosine concentrations (<5 μM) in HIT-T15 cells. These findings are in accord with previous data suggesting impairments of several aspects of purine metabolism in HIT-T15 cells (see Discussion). Therefore, these cells were not studied further.

Major conclusion. Both uptake of label, and activity of IMPDH, can be accurately quantified over a wide range of extracellular inosine (and presumably, intracellular IMP) concentrations, and both variables may be independently modulated.

b. Inhibition of IMPDH by MPA or MZ in intact islets or in hyperplastic or transformed β cells (Fig. 1; Tables 1 and 2)

MPA induced a potent and concentration-dependent inhibition of IMPDH activity in all cell types. The inhibition by MPA was similar when expressed as absolute flux through IMPDH into the medium, or as a percentage of total label incorporated into cells. The IC50 for MPA was in the range of 0.1–0.6 μg/ml (1 μM MPA = 0.32 μg/ml) in all hyperplastic or transformed cell lines, including β-TC13 and β-HC-9 cells (data not shown). However, this figure was ±0.07 μg/ml for HIT-T15 cells. The slower proliferative rates in quiescent β-TC-tet cells (Table 1) was accompanied by a shift to the right in the concentration dependence curves for inhibition by MPA, either at a low or at a high inosine concentration (Fig. 1). Additionally, in these studies a decrease in sensitivity to MPA at 0.8 μM inosine, compared with that at 25 μM inosine, was seen. These latter observations support the unique uncompetitive nature of inhibition by MPA (see Discussion). MZ also inhibited IMPDH in a concentration-dependent fashion with an approximate IC50 of 3 μg/ml in INS-1 or proliferating β-TC-tet cells, whether studied in synchronized or unsynchronized cells (not shown). In intact islets, overnight (16 h) exposure to MPA (25 μg/ml) inhibited IMPDH by 54 ± 4% at 25 μg/ml (n = 5), without changes in insulin or total protein content of the islets. In these preliminary studies, this inhibition seemed to become progressively greater as glucose concentration in the culture medium rose (P < 0.05 by ANOVA; df 6; data not shown), supporting our earlier findings (11) using inhibition of GTP

Results

a. Uptake of substrate and quantitation of IMPDH activity in pancreatic islets and different β-cell lines (Table 1)

The details of the performance and interpretation of this test in β cells has been described by us in detail (29). Cells were synchronized, then treated for 21 h with diluent (DMSO), half-maximal concentrations of MPA (0.3 μg/ml or MZ (20 μg/ml).

i. DNA content

Cells were extracted and assayed by the method of Rymaszewski (40).

Net flux through IMPDH was considered to be the absolute accumulation in the medium of [3H] (following quantitative precipitation of unmetabolized inosine) over a 3-h incubation period (16 h for intact islets), expressed as cpm per well/DNA, after correction for blank values. However, the content of radioactivity released into the medium could be influenced by total cell uptake of label. Therefore, true enzymatic activity of IMPDH is expressed as the ratio between total efflux of tritium and total cell labeling (IMPDH activity, as percent of uptake), where total cell labeling (Uptake) is calculated as the sum of tritium in the medium, which was taken up and/or metabolized, e.g. with the degree of differentiation and/or growth rate (Table 1). Net flux through IMPDH increased with inosine concentration (not shown) in all but one cell line (HIT-T15 cells; see below); it approached or reached saturation at the highest concentrations of inosine tested (25–50 μM). Assessment of IMPDH activity was unaltered by such changes in extracellular concentrations of inosine (e.g. 18.6 ± 2% of cellular uptake of label was metabolized via IMPDH by INS-1 cells at 0.8 μM inosine vs. 18.6 ± 1% at 25 μM inosine; n = 9 determinations each). In contrast to substrate uptake, true IMPDH activity was not affected by the proliferative rate (Table 1). These findings were observed in each of a variety of β-cell lines (β-HC, βTC, β-TC-tet, and INS-1). In addition, preliminary studies, IMPDH activity was readily detected in each of these six independent preparations of intact islets, averaging 4772 ± 946 cpm/islet. However, in contrast to other cell lines, the uptake of radiolabel into HIT-T15 cells was very low (e.g. 23% of that observed using βHC cells), and a very high percentage (49–50%) of this incorporated inosine was metabolized by IMPDH in HIT-T15 cells, whereas the comparable figure in βHC cells was only 19–20%. Furthermore, in comparison to other cell lines, IMPDH activity reached apparent saturation at very low inosine concentrations (<5 μM) in HIT-T15 cells. These findings are in accord with previous data suggesting impairments of several aspects of purine metabolism in HIT-T15 cells (see Discussion). Therefore, these cells were not studied further.

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findings were, however, confirmed using release at normal or near-normal EC50 for glucose (data not shown); there-
modestly but consistently increased BrdU incorporation. (1.7 or 3.3 mM) glucose, increased BrdU incorporation only inconsistently. High glucose alone (16.7 mM at 0.2% serum) modestly but consistently increased BrdU incorporation. However, the response to both serum and high glucose combined was dramatically synergistic (data not shown); therefore, this combination was chosen for most studies. The effect of glucose (in the presence of 10% serum) required its metabolism and was not merely an osmotic effect, since 3–0-
meethylglucose, mannitol, fructose, and 1-glucose all failed to reproduce the glucose effect (data not shown). The effects of glucose on mitogenesis increased progressively from 1.7 through 16.7 mM glucose, at which point apparent saturation was reached. Overall, the slopes of these glucose concentration-dependence curves were similar at 0.5% FCS compared with that at 10% FCS. The effects of mitogens seemed evident by ~4 h after exposure to stimuli, although these observations were at the limits of assay sensitivity. Provision of guanosine (up to 300 μM), hypoxanthine, or xanthosine (a precursor of GMP), failed to directly alter BrdU incorporation by 21 h (data not shown), in the presence or absence of either low or high concentrations of glucose or serum.

Major conclusions. Glucose or serum (especially in combination) rapidly increases DNA synthesis, although an increase in GNs alone probably does not.

d. Effects of the inhibition of IMPDH on DNA synthesis in β cells (Figs. 2, 3, and 4; and Tables 3 and 4)

MPA led to a concentration-dependent inhibition of BrdU uptake (Table 3). The concentration-dependence curves for inhibition of IMPDH in INS-1 or β-TC-tet cells were almost identical to those for inhibition of BrdU incor-
poration. The concentrations inhibiting IMPDH by 50% were mostly 0.1–0.6 μg/ml, whereas the IC50 for BrdU incorporation was ~0.3 μg/ml (n = 5 complete concentra-
tion-response curves) (Fig. 2). The inhibitory effect of MPA (generally studied at its IC50) was abrogated by inclusion of guanine (100 μM; Fig 2) or 200 μM guanosine (not shown), but not by adenine (Fig. 2). Neither guanine nor adenine had any stimulatory effects on mitogenesis by themselves (see below). MZ (1–100 μg/ml) mimicked the inhibitory responses of MPA, with IC50 of about 7 μg/ml (Fig. 3). The effects of MZ were also prevented by guanine but not by adenine (Fig. 3). BrdU incorporation was also assessed over 45 h, with MPA and BrdU provided only in the final 21 h (Table 4). Under these conditions, the indu-
duction of DNA synthesis in response to high glucose alone, or serum alone (in the presence or absence of ke-
tosacaproic acid), was clearly measurable. Stimulatory effects of either glucose or serum over 45 h were inhibited

| TABLE 2. Effects of MPA (1 μg/ml) on intracellular purines, separated via TLC |
|-----------------------------|-----------------------------|-----------------------------|
| Control | MPA | % change; P |
|-------|-----------------------------|-----------------------------|
| Inosine | 7.14 ± 0.96 | 9.7 ± 1.40 | +36%; P < 0.02 |
| IMP | 4.69 ± 0.95 | 9.68 ± 2.64 | +106%; P < 0.02 |
| GDP | 4.19 ± 0.65 | 3.89 ± 0.41 | −7%; P = ns |
| ATP | 60.86 ± 3.47 | 57.08 ± 3.98 | −6%; P < 0.05 |
| GTP | 6.89 ± 1.50 | 3.07 ± 0.88 | −55%; P < 0.005 |

Values are pooled from six independent experiments (3, INS-1; 2, β-TC-tet(−); 1, intact rat islets), and are comprised of 21–23 determinations for each compound, both in the presence and absence of MPA. Values are expressed as percent of total CPM in individual spots (mean ± SEM). Values for hypoxanthine and ADP are not included due to partial overlap on TLC in some studies.

Fig. 1. Inhibition by MPA of IMPDH activity varies with extracellular inosine concentrations. Studies were carried out using proliferating βTC-tet cells [“TET (−)”] or quiescent β-TC-tet cells [“TET (+)”] at low (0.8 μM) or higher (25 μM) inosine concentrations, as indicated in the inset. Data are expressed as x ± SEM from 5 independent experiments, except for n = 3 experiments for β-TC-tet(+) cells at 0.8 μM substrate.

c. Effects of mitogens on DNA synthesis

INS-1 cells were used for most studies of BrdU incorporation, since they are relatively well differentiated, are responsive to several mitogens, and have frequently been used in previous studies of DNA synthesis in β cells (27). The findings were, however, confirmed using β-TC-tet-cells. Both cell lines demonstrated substantial glucose-induced insulin release at normal or near-normal EC50 for glucose (data not shown).

After 21 h of stimulation, high serum (10%) alone at low (1.7 or 3.3 mM) glucose, increased BrdU incorporation only inconsistently. High glucose alone (16.7 mM at 0.2% serum) modestly but consistently increased BrdU incorporation. However, the response to both serum and high glucose combined was dramatically synergistic (data not shown); therefore, this combination was chosen for most studies. The effect of glucose (in the presence of 10% serum) required its metabolism and was not merely an osmotic effect, since 3–0-
meethylglucose, mannitol, fructose, and 1-glucose all failed to reproduce the glucose effect (data not shown). The effects of glucose on mitogenesis increased progressively from 1.7 through 16.7 mM glucose, at which point apparent saturation was reached. Overall, the slopes of these glucose concentration-dependence curves were similar at 0.5% FCS compared with that at 10% FCS. The effects of mitogens seemed evident by ~4 h after exposure to stimuli, although these observations were at the limits of assay sensitivity. Provision of guanosine (up to 300 μM), hypoxanthine, or xanthosine (a precursor of GMP), failed to directly alter BrdU incorporation by 21 h (data not shown), in the presence or absence of either low or high concentrations of glucose or serum.

Major conclusions. Glucose or serum (especially in combination) rapidly increases DNA synthesis, although an increase in GNs alone probably does not.

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d. Effects of the inhibition of IMPDH on DNA synthesis in β cells (Figs. 2, 3, and 4; and Tables 3 and 4)

MPA led to a concentration-dependent inhibition of BrdU uptake (Table 3). The concentration-dependence curves for inhibition of IMPDH in INS-1 or β-TC-tet cells were almost identical to those for inhibition of BrdU incor-
poration. The concentrations inhibiting IMPDH by 50% were mostly 0.1–0.6 μg/ml, whereas the IC50 for BrdU incorporation was ~0.3 μg/ml (n = 5 complete concentra-
tion-response curves) (Fig. 2). The inhibitory effect of MPA (generally studied at its IC50) was abrogated by inclusion of guanine (100 μM; Fig 2) or 200 μM guanosine (not shown), but not by adenine (Fig. 2). Neither guanine nor adenine had any stimulatory effects on mitogenesis by themselves (see below). MZ (1–100 μg/ml) mimicked the inhibitory responses of MPA, with IC50 of about 7 μg/ml (Fig. 3). The effects of MZ were also prevented by guanine but not by adenine (Fig. 3). BrdU incorporation was also assessed over 45 h, with MPA and BrdU provided only in the final 21 h (Table 4). Under these conditions, the indu-
duction of DNA synthesis in response to high glucose alone, or serum alone (in the presence or absence of ke-
tosacaproic acid), was clearly measurable. Stimulatory effects of either glucose or serum over 45 h were inhibited.
by MPA (Table 4) or MZ (data not shown), changes that were prevented by guanosine. The effects on IMPDH activity did not seem to be secondary to growth inhibition because provision of interleukin-1β (2.5–10.0 ng/ml for 21 h) markedly inhibited BrdU incorporation (by 48 ± 6%; n = 6) while inducing only a minor effect on IMPDH activity (215%; n = 6) and interleukin had no effect on uptake (data not shown). In each of three experiments, MPA at its IC50 (0.3 μg/ml) also inhibited (by 46 ± 6%) the increment in total cell content of a second marker of DNA synthesis (proliferating cell nuclear antigen) upon induction by high glucose plus 10% serum (5.32 ± 0.65 to 2.96 ± 0.58 U/ml, df 19; P < 0.02).

**Major conclusions.** Reduction of IMPDH activity impairs DNA synthesis in relation to changes in guanine, but not adenine, nucleotide synthesis.

**e. Effects of ketoisocaproic acid (Fig. 4; Tables 3 and 4)**

Ketoisocaproic acid (KIC) also induced a strong mitogenic response in INS-1 cells or β-TC cells, either in the presence or absence of high serum concentration, and after either 21 or 45 h of stimulation (Tables 3 and 4; Fig. 4). In the presence of 10% serum, near-saturation was reached by as low as 500 μM KIC. Another mitochondrial fuel, leucine (10–15 μM), provided in the presence of 10 μM glutamine and 10% serum, reproduced the effects of KIC (data not shown), which were likewise inhibited by MPA or MZ. These effects were prevented by guanine or guanosine but not adenine (Table 4). In these same studies, MPA also markedly reduced, in parallel, the BrdU response to 10% serum plus high glucose (not shown) in a guanine- or guanosine-sensitive fashion. Likewise, MZ inhibited (df 14; P < 0.001) the effect of serum plus glucose in β-TC-tet(−) cells. In β-HC cells, MPA inhibited the effects of glucose plus serum by up to 85% (P < 0.001;
Effects of MPA on stimulation of DNA synthesis (as quantified by BrdU incorporation) in INS-1 cells over a 45-h period: reversibility by guanosine

| Condition | OD ± SEM |
|-----------|----------|
| A         |          |
| 10% serum | 206 ± 42 (8) |
| Serum + MPA 0.3 μg/ml | 129 ± 10 (8) |
| Serum + MPA + guanosine, 200 μM | 207 ± 20 (4) |
| B         |          |
| 10% serum + 5 mM KIC | 338 ± 21 (4) |
| 10% serum + 5 mM KIC + MPA | 164 ± 10 (4) |
| 10% serum + 5 mM KIC + MPA + guanosine | 309 ± 11 (4) |
| C         |          |
| 0.2% serum + 16.7 mM glucose | 495 ± 29 (8) |
| 0.2% serum + 16.7 mM glucose + MPA | 176 ± 18 (8) |
| 0.2% serum + 16.7 mM glucose + MPA + guanosine | 343 ± 38 (4) |
| D         |          |
| 10% serum + 16.7 mM glucose | 501 ± 26 (8) |
| 10% serum + 16.7 mM glucose + MPA | 348 ± 20 (8) |
| 10% serum + 16.7 mM glucose + MPA + guanosine | 569 ± 45 (4) |

* BrdU and MPA were present only during the last 21 h; the agonists were present throughout the entire 45 h.

** Bars: n = number of determinations.

All effects of MPA are significant at the P < 0.003 or greater level (post-hoc analysis following ANOVA). Guanosine restored values to levels not statistically different from control values, except in C, where the reversal was only partial.

**Fig. 5.** MPA (0.3 μg/ml) inhibits BrdU incorporation (an index of DNA synthesis), even in the presence of IGF-1. INS cells were stimulated by 10% serum, 16.7 mM glucose, or both, in the absence or presence of IGF-1 (10 nm, as indicated over the respective pairs of bars; n = 4 determinations for each condition). MPA (open bars) significantly impeded BrdU incorporation in all conditions (P < 0.001, ANOVA followed by Tukey test). The specific agonist(s) are indicated below each pair of bars.

**Fig. 6.** Effects of MPA (0.3 μg/ml) on BrdU incorporation (an index of DNA synthesis) are reversible. INS-1 cells were stimulated by 10% serum alone (3 bars on left) or 10% serum plus 16.7 mM glucose (3 bars on right). Data are expressed as % of untreated control cells (open bars = 100%) in comparison to the continued presence of MPA (solid bars). The latter are compared in turn to results from the withdrawal of MPA during the second period of stimulation, only (stippled bars).

**Major conclusion.** DNA synthesis induced by KIC is dependent on adequate flux through IMPDH.

**f. Relationship to islet cell toxicity (Figs. 5 and 6; Table 5)**

Because both MPA and MZ markedly reduce insulin secretion (10, 11), we considered the possibility that the blockade of the high levels of insulin accumulating in the medium, with its known proliferative effect, might explain the inhibitory effects of MPA on mitogenesis. When bovine insulin (5 μM) was provided during the experimental period, it did augment DNA synthesis somewhat, especially in the presence of high glucose alone. However, under no circumstance was insulin able to circumvent the inhibitory effects of MPA (which were, in contrast, vitiated by guanosine in the same studies; data not shown). Likewise, IGF-1 (10 nm) progressively augmented glucose-, serum-, or glucose plus serum-induced BrdU accumulation (Fig. 5); nevertheless, MPA was still able to inhibit DNA synthesis under every condition (P < 0.001 by one-way ANOVA). Thus, like insulin, IGF-1 was unable to prevent the inhibitory effect of MPA. Assessments of IMPDH activity had been carried out in the presence of added inosine; however, when 25 μM inosine was added, it did not alter BrdU responses to MPA (data not shown).

When metabolic viability (MTS test), cell number and percentage of viable cells (cell counting plus trypan blue exclusion), and DNA content were assessed in INS-1 cells at the concentrations of MPA or MZ generally used (Table 5), only minor and inconsistent changes were seen, and these were considerably less than the decrements in BrdU incorporation (Table 5). The number of unattached INS-1 cells in the medium, and their viability, were also unaltered (not shown). In a second well-differentiated cell line (β-HC6), similar results were seen. Additionally, the effects of MPA on BrdU uptake were totally reversible using serum alone as stimulus (to
TABLE 6. Summary of effects of mitogens on uptake of hypoxanthine or inosine (the precursors of IMP), and on IMPDH enzymatic activity

| ANOVA for effects of serum and/or glucose | Significant effect of glucose or IMPDH activity | P value |
|------------------------------------------|-----------------------------------------------|---------|
| **A. Hypoxanthine label**                |                                               |         |
| i. Uptake                                |                                               | P = 0.011 |
| ii. IMPDH activity                       |                                               | P = 0.005 |
| **B. Inosine label**                     |                                               | P = 0.0066 |
| i. Uptake                                |                                               |         |
| ii. IMPDH activity                       |                                               |         |

*Data from INS-1 and β-TC-tet cells are expressed as % of control values. Significance levels were obtained using post hoc Fisher’s test following ANOVA.

**TABLE 5. Effects of MPA (0.3 μg/ml) on various indices of cell viability: discordance with Rates of DNA Synthesis**

| Control | MPA | \(\bar{x} \pm SE\) |
|---------|-----|---------------------|
| BrdU incorporation | 3.06 ± 0.11 | 2.78 ± 0.14 | \(n = 27, P = 0.011\) |
| DNA content (μg/well) | 3.85 ± 0.43 | 3.29 ± 0.38 | \(n = 10, P = 0.0005\) |
| Cell number (×10^6) | 94.8 ± 0.6 | 91.9 ± 0.9 | \(n = 10, P = 0.02\) |
| Metabolic viability (O.D., arbitrary units) | 0.193 ± 0.003 | 0.181 ± 0.003 | \(n = 10, P = 0.034\) |

*Data in "B" are from two independent experiments using synchronized INS-1 cells.

**Major conclusions.** β-cell mitogens increase substrate uptake and/or IMPDH enzymatic activity rapidly but persistently.

**Effects of mitogens on IMPDH expression or protein (Fig. 7)**

Using semiquantitative RT-PCR, it was first established that INS-1 cells (Fig. 7), β-TC-tet cells, or rat islets (not shown) expressed IMPDH 2 and, with lower abundance, IMPDH 1. In addition, expression of IMPDH 1 and IMPDH 2 was readily observed by Northern blot analysis in INS-1, β-HC, or β-TC cells with transcripts at \(~2.6\) kb (type 1 IMPDH) or \(~1.7\) kb (type 2 IMPDH) (data not shown). IMPDH protein was also detected in β-TC-tet cells; INS-1 cells could not be studied because rat proteins are not recognized by the antibody used. To assure that we could detect changes in gene expression or cellular content of IMPDH protein, we first determined the responses to GTP depletion and excess, as have others (23, 42). When MPA (1 μg/ml) was applied to INS-1 cells for 21 h and then washed out, gene expression of IMPDH 2 increased to 204% of diluent control \(1.87 \pm 0.44\) to 3.44 \pm 0.50; ratios of IMPDH 2 to housekeeping gene; \(n = 6\) pairs; \(P = 0.0003\). A lower concentration of MPA (0.3 μg/ml) had a similar, though smaller, effect, whereas MZ also increased IMPDH 2 mRNA to 153% at 12 h and 296% of control by 24 h. MPA treatment also increased IMPDH mass to 133 \pm 14% of control \(n = 6\); \(P < 0.004\). In contrast, expression (mRNA) of the constitutive isoform (IMPDH 1) did not change (not shown). IMPDH activity also had increased from initial values from 26.3 \pm 8\ (diluent) to 32.6 \pm 8\% (df 6; \(P < 0.02\)) after wash-out of MPA.¹

Conversely, increases in GNs, as induced by application of guanosine, led to a profound inhibition of IMPDH activity. For example, provision of 300 μM guanosine inhibited IMPDH ac-

¹ Note that this is a substantial underestimation, since MPA is not easily washed from cells (Ref. 22); furthermore, such estimates include both the inducible and constitutive isoforms of IMPDH, of which only the former can be physiologically modulated.
FIG. 7. IMPH 2 is expressed in INS-1 cells, detected using RT-PCR with TaqMan technology. The x-axis depicts the cycle at which the fluorescent signal (y-axis) rises above baseline. The relationship between the gene of interest (here, IMPDH) and a housekeeping gene (here, GAPDH) is a semiquantitative measure of relative abundance. Each point represents the mean of three replicate determinations.

tivity to $11 \pm 5\%$ of control ($n = 6$ experiments; $P < 0.002$). This inhibition followed a log[guanosine]-linear [IMPDH] concentration-dependent response. In contrast, provision of control nucleosides (adenosine or xanthosine) did not mimic these effects of guanosine. Effects of guanosine on IMPDH activity were accompanied by reductions in IMPDH mass. By 12 h, 200 $\mu$M guanosine reduced IMPDH protein (using semiquantitative analysis) to $35 \pm 9\%$ of control ($n = 7; P = 0.005$). At 21 h, IMPDH mass was reduced by $51\% \pm 6\%$ ($n = 6; P = 0.008$). The control nucleosides, adenosine, caused much more modest declines, which were significantly less ($P = 0.005$) than those induced by guanosine. In addition, in each of two time course studies (4 h through 36 h), 200 $\mu$M guanosine reduced IMPDH 2 mRNA, reaching decrements of $-45\%$ at 12 h, $-46\%$ at 24 h, and $-41\%$ at 36 h, comparing guanosine to no nucleoside. In eight additional experiments in which the proper comparison was made (guanosine vs. adenosine), guanosine still reduced IMPDH 2 gene expression (difference $= 30 \pm 11\%; n = 8$ pairs; $P = 0.02$).

Once it had been demonstrated that we were able to detect changes in IMPDH activity, expression, and protein, the responses to physiologic mitogens were assessed. Neither glucose nor serum (alone or in combination) detectably increased IMPDH 2 mRNA at 21 h. IMPDH protein was not increased by glucose or serum, alone or in combination (high glucose alone $= 115 \pm 9\%$ of control; $n = 5; P = ns$; high glucose plus high serum $= 92 \pm 6\%$ of control; $n = 8; P = ns$; serum alone $= 74 \pm 8\%$ of control; $n = 8; P = 0.014$).

**Major conclusions** (Table 7). IMPDH expression and activity are detectably modulated by classical product feedback; that is, they are decreased by elevations in GN and increased by GN depletion. However, the effects of glucose on IMPDH activity cannot be explained by changes in gene expression, at least at early time points. These data suggest that IMPDH 2 is regulated by at least one more, unidentified mechanism other than changes in gene expression, probably at the enzyme level (see Table 7 and Discussion).

**Table 7. Summary of changes observed in IMPDH expression and activity**

| Condition | Enzyme Activity | mRNA (RT-PCR) | Protein mass (immunoblotting) |
|-----------|-----------------|---------------|------------------------------|
| 1. ↓ GTP (MPA or MZ) | ↓ | ↓ | ↓ |
| 2. ↑ GTP (guanosine) | ↓ | ↓ | ↓ |
| 3. Glucose in low serum | ↑ | → | → |
| 4. Glucose in high serum | ↑ | → | → |

"$\rightarrow$ no change.
↑ or ↓ small change.
↑ or ↓ moderate change.
↑ ↓ ↓ dramatic change.
See text for details.

**Discussion**

Although reduction of total GTP content rapidly impairs DNA synthesis and later induces apoptosis in tumor β cells or in neonatal rat islet cells (12–14), steady-state levels of GTP alone may not provide an adequate index of the availability of specific pools of GTP directed toward DNA replication. These GTP pools are small, compartmentalized, and turn over rapidly (43), and thus their assessment is problematic. Although IMPDH inhibitors reduce cellular GTP content in β cells by up to 80%, much of the remaining GTP is probably sequestered in larger, inert pools (44) that tend to mask dynamic changes in smaller pools. For these reasons, it has been suggested (28) that assessments of the flux through the cytosolic biosynthetic cascade for guanine nucleotides (GNs) might provide a valuable index. Therefore, after optimizing an assay capable of reliably detecting IMPDH activity in intact β cells, we sought to provide evidence supporting or refuting the formulation that IMPDH is (at least) a permissive factor in β-cell replication, as assessed by DNA synthesis. Synthesis of DNA during the S phase of the cell cycle was mostly assessed by an ELISA for BrdU. BrdU is a thymidine analog that is incorporated into newly synthesized DNA during the S phase of the cell cycle of actively proliferating cells. Use of BrdU has been shown in other cells to have greater sensitivity, reproducibility, and stimulation index than the more traditional assays of thymidine incorporation (45), while obviating the need for radioisotopes.

Intact cells were required because glucose, a major mitogen of β cells, must be metabolized intracellularly to exert its effects. Cultured cells were used in most of the current studies because primary β cells from adult islets replicate extremely slowly, and detection of decrements from basal level of $-0.2\%$–$0.4\%$ of dividing cells (2, 46) would not be logistically possible. However, the cell lines studied retained mitogenic responsiveness to all physiologic agonists (as well as robust glucose-induced insulin release). It was critical to include studies of several dissimilar cell lines so that any findings could not be readily ascribed to any peculiarity of a single cell line. Although the cell lines were derived via very different approaches, they responded similarly. For example, β-TC13 cells, the β-TC-tet line, and β-HC cells, are all derived from transgenic mice expressing the SV40 Tag under the control of the insulin promoter. The former two are insulinoma cells, whereas β-HC cells (and quiescent β-TC-tet cells) are hyperplastic β cells and are more differentiated. In further contrast, INS-1 cells are derived from radiation-induced
rat β-cell tumors, whereas HIT-T15 cells are clonal, being derived from Syrian hamster islets transformed by SV40. Growth rates also differ between cell types. Thus, the observations reported herein are not restricted in terms of clonality, transformation, degree of differentiation, growth rate, species of derivation, or expression of SV40 Tag, and therefore the conclusions are likely to be physiologically relevant. Nonetheless, additional studies involving proliferation of normal β cells are desirable. However, we did observe, in these and earlier studies (25), that the HIT-T15 cells, although demonstrating similar qualitative findings, displayed some quantitative abnormalities in nucleobase uptake and/or incorporation of purine bases into cellular pools. Therefore, HIT-T15 cells should be used only with extreme caution in future studies as a model of purine cellular pools. Therefore, HIT-T15 cells should be used only with extreme caution in future studies as a model of purine physiology in β cells.

The assay was also validated, in part, by measurements of intracellular purines. In addition to the expected decline of GNs induced by MPA, a large percentage rise in IMP levels was seen, as observed in other cells (47–50). IMP can accumulate via four mechanisms: 1) accumulation proximal to the block in IMPDH; 2) increases in availability of the phosphoribosylpyrophosphate (PRPP) required for IMP synthesis, due in turn to the loss of feedback by GNs (51–53), which inhibits PRPP synthetase; 3) the GTP dependence of conversion of IMP to AMP at the adenylosuccinate synthetase step; and 4) inhibition by GNs of the retroconversion of AMP back to IMP by adenosine deaminase (54). Thus, measurements of the concomitant rise in intracellular IMP and its derivatives may be a particularly useful index of IMPDH inhibition in addition to decrements in GNs. In our studies, GTP fell by only 55% at an MPA concentration that inhibited IMPDH activity by ~80–86%. Similarly, in the studies of Balzarini and De Clercq (28), little or no decline in GTP was detected after a 5-h exposure to MPA, by which time IMPDH had been inhibited by >30–50%.

b. Effects of mitogens and inhibitory effects of MPA or MZ

Marked inhibition of IMPDH in intact islets and in all β-cell lines tested was achieved by MPA or MZ, which are highly selective inhibitors of IMPDH in β cells and elsewhere (12, 15, 18, 26, 53). The unique nature of the uncompetitive inhibition by MPA of IMPDH (55) was confirmed in the current studies, again validating the assay. Uncompetitive inhibitors become increasingly potent as substrate concentrations rise toward the V_{max}. Indeed, the inhibitory effect of MPA was greater at the higher isonine concentration (see Fig. 1) (55). Even when the effects of MPA were expressed in relationship to the total cellular uptake of label, potent inhibition was seen. Although the mass of intracellular IMP derived via the uptake of isonine precursor cannot be rigorously assessed by these studies, it is interesting to note that approximations of half-maximal IMPDH activity for most of the cell lines occurred at ~4–12 μM isonine. Taking into account the small amounts of preformed IMP in cells, this figure is reasonably close to the published K_m for IMP for IMPDH in mammalian cells (mean ~20 μM; Refs. 55–57). Taken together, these data suggest that this assay should be useful to study IMPDH activity in many β-cell lines over a wide range of endogenously formed intracellular IMP concentrations.

It is of interest that the inhibition induced by MPA seemed to be greater in faster-growing cells. Because precursor uptake was greater in these cells, they would indeed be predicted to be more sensitive to MPA, as indicated above. However, in addition, the inducible (type 2) isoform of IMPDH is the one implicated in previous studies of the role of IMPDH in cell cycle progression, mitogenesis, and cell survival (15, 20, 21, 58, 59). Isoform 2 of IMPDH is more sensitive to inhibition by MPA than is the constitutive isoform (60); this fact might also contribute to the greater sensitivity to MPA in rapidly proliferating cells. In all cell lines, however, either MPA or MZ induced a potent inhibition of BrdU incorporation at concentrations virtually identical to those inhibiting IMPDH. PCNA content [a measure of cell proliferation maximum in the late G_{1} and S phases (Refs. 41, 61)] was also inhibited by MPA. In addition, rigorous and extensive testing excluded nonspecific toxic effects as the cause of these effects. Thus, there can be little doubt that restriction of IMPDH activity severely hampers mitogenesis of β cells.

Mitogens can rapidly increase the apparent uptake and phosphoribosylation of some nucleosides (62–64), an event that correlates with the onset of S phase (62). This increase in uptake is, in fact, largely due to intracellular retention of precursor when the availability of PRPP is increased (63, 64). This finding is particularly relevant to β cells because glucose augments cellular content of PRPP (48, 65). This formulation is supported by the fact that another mitogenic fuel that is not a hexose (KIC) augmented IMPDH but not substrate uptake. Whatever the mechanism, an increase in net isonine or hypoxanthine uptake might mimic an increase in true IMPDH enzyme activity by providing increased substrate (IMP) availability. While glucose did increase label uptake, it also increased true IMPDH enzymatic activity. Thus, in β cells, mitogens increase net flux through IMPDH by two mechanisms: 1) substrate uptake; and 2) direct modulation of enzyme activity. The mechanism(s) by which glucose or KIC increase true IMPDH activity with little or no change in gene expression, are unclear. At the least, glucose’s effect clearly requires its metabolism since four other unmetabolized, or poorly metabolized, sugars failed to mimic it. The regulation of both IMPDH activity and expression in general is very poorly understood. However, the possible mechanisms might include carboxyl methylatation (or possibly other posttranslational modifications) at critical cysteine residues in the nucleotide binding site (66); end-product feedback mechanisms (56); and modifications of the K_m for IMP or NAD (55). Additional studies will be required to address this question. It is of interest that, in contrast, spontaneous rates of proliferation of the cell lines seemed correlated in general with substrate uptake but not IMPDH activity. Thus, it seems likely that the mechanisms of growth stimulation differ between acute activation of mitogenesis and chronically elevated rates of cell division. This distinction has been largely overlooked in the extant literature.
c. Possible mechanisms, and Summary

The effects of MPA could not be attributed to secondary decrements in ATP or ATP/ADP ratio, which declined little or not at all (this study and Ref. 10); furthermore, adenine or adenosine (which maintain normal ATP levels; Ref. 10) did not restore cell proliferation. Parenthetically, the content of cellular adenine nucleotides is probably maintained, at least in part, by the large rise in IMP, which can be directly converted to AMP.

In contrast, the inhibition of proliferation by MPA or MZ was closely related to inhibition of GNs. There is substantial evidence that the effects of GNs on mitogenesis may be exerted at several levels. Some are direct, including conversion to their deoxy derivatives, direct incorporation into nucleotides, or support for GTP-dependent, RNA-primed DNA synthesis (67). However, other, indirect effects are likely present; these involve modulation of several key regulators of the cell cycle. We reported that MPA reduced activity of the cyclin D1-cyclin-dependent kinase 4(Cdk4) cascade in neonatal rat islet cells (14); this observation is of particular relevance in view of recent reports that targeted disruption (unpublished data) that GTP depletion induces p21CIP1/WAF1, a potent cell cycle inhibitor acting on several cyclin-dependent kinases, as well as on DNA polymerase δ. Sherley et al. have suggested that the effects of p53, another potent cell cycle inhibitor, may act via changes in the expression and activity of IMPDH, since transfection into cells of IMPDH reversed the inhibition of mitogenesis induced by p53 (24, 70, 71). Finally, glucose does rapidly increase GTP/GDP ratio in rat islets (36) and simultaneously activates several MPA-inhibitable small GTP-binding proteins involved in cell cycle progression (14, 15, 72). Evaluation of each of these possible mechanisms will require additional studies.

In summary, adequate activity of IMPDH, acting via the production of GNs, appears to be required for cell cycle progression and DNA synthesis in β cells—that is, it is a competence factor, as defined by Thompson (73). This modulation of IMPDH during mitogenesis does not appear to be a secondary epiphenomenon, since commensurate changes in IMPDH did not appear under several conditions (such as provision of serum or interleukin-1β), wherein rates of mitogenesis were altered. However, it also seems unlikely that an increase in IMPDH activity can, per se, initiate DNA synthesis, as indicated by the lack of induction of DNA synthesis by precursors of GTP. Thus, increases in IMPDH activity might potentiate mitogenesis induced by physiologic fuels, or sustain it over time as a secondary effect. Such regulation of IMPDH may be germane to the differentiation, growth, and survival of β cells (including those used for purposes of transplantation) as well as, possibly, other endocrine cells. Given that the concentrations of MPA that inhibit β-cell proliferation and induce apoptosis (12) are well within the range of concentrations of MPA when used clinically as an immunosuppressive agent during transplantation (74), these observations may also have clinical relevance.

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