Formation of ATP from ADP on the external surface of vascular endothelial cells has been attributed to plasma membrane ATP synthase, ectoadenylate kinase (ecto-AK), and/or ectonucleoside diphosphokinase. These enzymes or their catalytic products have been causatively linked to the elaboration of vascular networks and the regulation of capillary function. The amount of ATP generated extracellularly is small, requiring sensitive analytical methods for quantification. Human umbilical vein endothelial cells were used to revisit extracellular ATP synthesis using a reliable tetrazolium reduction assay and multwell plate cultures. Test conditions compatible with AK stability were established. Extracellular AK activity was found to be <1% of the total (intracellular and extracellular), raising the possibility that the external enzyme could have leaked from living cells and/or a few dying cells. To determine whether AK inadvertently leaked from the cells, the activity of another cytoplasmic enzyme, glucose-6-phosphate dehydrogenase (G6PD), was also measured. G6PD is present in the cytoplasm in similar abundance to AK. The activity ratio of G6PD (extracellular/total) was found to be similar to that of AK. Because G6PD in the medium was probably due to leakage, other cytoplasmic macromolecules, including AK, should be released proportionately from the cells. The role of plasma membrane ATP synthase in extracellular ATP formation was examined using Hanks’ balanced salt solution with and without selective inhibitors of AK and ATP synthase activities. With P1-P5-di(adenosine 5’)-pentaphosphate (inhibitor of AK activity), no extracellular ATP synthesis was detected, whereas with oligomycin, piceatannol, and aurovertin (inhibitors of F1F0-ATP synthase and F1-ATPase activities), no inhibition of extracellular ATP synthesis was observed. AK activity alone could account for the observed extracellular ATP synthesis. The possible impact of ADP impurity in the assays is discussed.

Extracellular ATP formation from ADP has been reported in cultures of several lines of human cells, including vascular endothelial cells, dermal keratinocytes, and some lines of tumor cells, and has been attributed to the activities of plasma membrane ATP synthase (PM-ATP synthase) (1–7) and ecto forms of adenylate kinase (AK; ATP-AMP transphosphorylase, myokinase, EC 2.7.4.3) (5, 8–12) and/or nucleoside diphosphokinase (8, 11–14). F1F0-ATP synthase, the principal ATP-forming apparatus of mitochondria and chloroplasts, is a complex, membrane-attached enzyme that generates ATP from ADP and P_i, energized by a transmembrane proton gradient (15). A cogent rationale for PM-ATP synthase in endothelial cells has yet to be developed, even though there are reports that, within the plasma membrane, the protein complex itself may play a regulatory role in angiogenesis, i.e. the process of elaborating the blood capillary network in undervascularized tissues (1, 3, 4). AK, which is widely distributed within the plant and animal kingdoms, is located primarily in the cytoplasm and catalyzes interconversion of ADP and ATP + AMP (see Reaction a in Fig. 1), with K_eq ~ 1. The enzyme is a facilitator of cytoplasmic adenine nucleotide interchange. Plasma membrane-associated AK and certain other phosphate exchange enzymes and glycosidases, including ectonucleoside diphosphokinase and ectonucleotidases, catalyze the interconversion of purine nucleotides, nucleosides, and free purines that can serve as effector substances for P2Y-ligated purinergic pathways on plasma membranes of endothelial cells (11, 12, 16) as well as other types of cells, including dermal (5, 9, 16), neuronal (17–21), etc.

To explore the source of extracellular AK in cultures of endothelial cells, a sensitive and versatile method for evaluating AK activity was devised. It is described and its suitability for this study confirmed at the beginning “Results.” Its use in studies of human umbilical vein endothelial cells (HUVECs) and a line of human embryonic lung fibroblasts (WI38) in culture suggests that enough AK does indeed leak from cells to account for the observed extracellular ATP formation from ADP.

**EXPERIMENTAL PROCEDURES**

**Cells**—Low passage number (3–10 passages) HUVECs from three different sources (American Type Culture Collection 2

---

The abbreviations used are: PM-ATP synthase, plasma membrane ATP synthase; AK, adenylate kinase; HUVECs, human umbilical vein endothelial cells; FBS, fetal bovine serum; G6PD, glucose-6-phosphate dehydrogenase; Ap₅A, P₅-P₅-di(adenosine 5’)-pentaphosphate; INT, p-iodonitrotetrazolium violet; HBSS, Hanks’ balanced salt solution; DPBS, Dulbecco’s phosphate-buffered saline; BSA, bovine serum albumin; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; INT-F, p-iodonitrotetrazolium violet formazan.

---

8 This work was supported by the Higuchi Biosciences Center, Kansas University; the Camille and Henry Dreyfus Foundation (New York); and American Heart Foundation Grant 0151395Z. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed:Dept. of Molecular Biosciences, University of Kansas, 1200 Sunnyside Ave., Lawrence, KS 66045-7534. Tel.: 785-864-3334; Fax: 785-864-5321; E-mail: richter@ku.edu.
(CRL-1730), Cascade Biologics, and GlycoTech Corp.) were used in this study. The American Type Culture Collection (ATCC) was of passage number >10. Most of the experiments were performed using HUVECs from GlycoTech Corp., and the results using them were substantiated with HUVECs from Cascade Biologics and American Type Culture Collection. They were cultivated in HUVEC medium (1:1 Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium supplemented with 100 μg/ml heparin, 15 μg/ml endothelial cell growth supplement, 10% fetal bovine serum (FBS), 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate) in 100-mm tissue culture plates or T-75 tissue culture flasks and, for experimental purposes, in collagen-coated multiwell plates. WI38 cells (ATCC CCL-75) were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium supplemented with the above antibiotics and 10% FBS.

Reagents—AMP (acid form, type V), ADP (disodium salt, equine muscle), ATP (disodium salt, trishydrate), Glu-6-P, glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49; recombinant, Leuconostoc mesenteroides, expressed in Escherichia coli and purified to homogeneity), hexokinase (EC 2.7.1.1; Saccharomyces cerevisiae), a mixture of isozymes as a crystalline suspension in ammonium sulfate, type C-130), AK (rabbit or chicken muscle, essentially salt-free, lyophilized powder), Ap5A, Triton X-100, penicillin G, streptomycin sulfate, phenazine methosulfate, heparin (sodium salt, porcine intestinal mucosa), and phosphate determination reagent (Fiske-SubbaRow) were obtained from Sigma. Other materials were obtained as indicated: β-NADP (sodium salt), U. S. Biochemical Corp. (Cleveland, OH); Tris, Fisher; Bradford reagent, Bio-Rad; p-iodonitrotetrazolium violet (INT), Lancaster Synthesis (Windham, NH); crystalline porcine trypsin, Worthington; basal culture medium (50:50 Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium), Invitrogen; rat tail collagen, BD Biosciences; FBS, Serologicals Corp. (Norcross, GA); Isoton, Coulter Electronics, Inc. (Hialeah, FL); and endothelial cell growth supplement (also called endothelial mitogen), Biomedical Technologies, Inc. (Stoughton, MA). Hanks’ balanced salt solution (HBSS), Dulbecco’s phosphate-buffered saline (DPBS), and DPBSA (DPBS lacking Mg²⁺ and Ca²⁺) were prepared according to Freshney (22).

Cell Culture Protocol—Cells were grown to near confluence in the indicated medium in 100-mm tissue culture plates or T-75 tissue culture flasks, with nutrient replenishment 1 day prior to use. The culture vessels used for experiments were coated with type 1 rat tail collagen according to the manufacturer’s instructions. The inoculum for an experiment was prepared by incubating the cell layer at 37 °C in trypsin/EDTA solution (0.01% crystalline porcine trypsin and 0.1% EDTA in DPBSA) until the cells had detached from the dish, terminating the tryptic action by adding a small volume of medium containing 5% FBS. The cell suspension was centrifuged briefly at low speed (300 rpm) in a clinical centrifuge, and the cells were suspended in the appropriate medium. The cell density was determined by electronic particle counting in Isoton on a CASY 1 Model TT counter and sizer (Scha¨rfe System GmbH, Reutlingen, Germany). The cells were diluted in medium to the desired density, seeded in culture vessels, and incubated in a humidified CO₂ incubator (5% CO₂ and 95% air) at 37.5 °C.

Assay Mixture and Protocol—The principles and protocol for preparing the secondary assay mixture (see Table 1) have been described by Haslam et al. (23, 24). The crystalline suspension of G6PD was diluted 1:8 in 60 mM Tris (pH 7.8), dispensed in small volumes in sealed vials, and stored at −20 °C. To test for AK activity, the cells were seeded in collagen-coated 6- or 12-well plates at densities needed to attain the desired confluence (usually total) during the growth period (1–4 days); 2.5 × 10⁵ cells/well in a 6-well plate reached confluence overnight. The growth medium was then removed; the plates were washed as indicated; the primary assay mixture (see Table 1) was added; and the plates were incubated, usually for 30 min at 37 °C. The supernatant liquid was then transferred to a microcentrifuge tube containing 20 μl of 9.75 mM Ap₅A, an inhibitor of AK activity (25), at 0 °C; centrifuged at 3000 × g for 1 min; and transferred to a clean tube on ice. The liquid was dispensed at 0.15 ml/well in a 96-well plate using a multichannel pipetter, and 0.05 ml of secondary assay mixture (see Table 1) was added, avoiding bubble formation. The plate contents were mixed for 30 s on a plate shaker or Vortex mixer (fast enough to stir the contents without causing spillage or foaming) and then incubated at 37 °C. At the specified times, the plates were again vortexed, and the A₄₉₀ values were determined on a multwell plate reader (AutoReader II, Ortho-Clinical Diagnostics, Inc., Ranir, NJ).

When cellular G6PD activity was being tested, the primary assay mixture was modified. ADP, hexokinase, and bovine serum albumin (BSA) were omitted, and 0.125 mM NADP was added where indicated to stabilize G6PD; also where indicated, DPBS, DPBSA, or another medium was substituted for HBSS. In the secondary assay mixture, G6PD was omitted; 4.2 mM Glu-6-P was added; and the NADP concentration was adjusted to compensate for the amount added to the primary assay mixture. All subsequent steps were identical to those in the AK assay protocol.

F₇ₐ-ATPase Assay—Soluble mitochondrial F₇ₐ-ATPase was prepared from beef heart according to the method of Knowles and Penefsky (26). The ATPase activity was determined according to Taussky and Shorr (27), measuring inorganic phosphate formed from ATP/µg of F₇ₐ-ATPase protein, determined by the method of Bradford (28). To determine F₇ₐ-ATPase activity and its inhibition by test substances, the enzyme solution (5 µg/ml) was incubated with Ap₅A or a putative inhibitor for 5 min at 37 °C in 25 mM Tricine (pH 8.0). Then, 0.5 ml of the assay mixture (25 mM Tricine (pH 8.0), 25 μM Na₂SO₄, 4 mM ATP, and 2 mM MgCl₂) was added, and the mixture was incubated for 2 min. The reaction was stopped with 1 ml of 0.5 M trichloroacetic acid and chilled to 0 °C. One ml of Fiske-SubbaRow color reagent was added, and the system was held at 0 °C for another 5 min and then warmed to room temperature. Absorbances at 740 nm were determined on a spectrophotometer.

Cell Proliferation Studies—To determine the effects of potential inhibitors of ATP synthase on proliferation, the cells were seeded at 1 × 10⁵ cells/well in 0.5 ml of medium containing the test substance in 12-well plates. At specified times
thereafter, the cells were trypsinized, suspended in Isoton, counted, and sized on a CASY 1 Model TT counter.

RESULTS

Multiwell Colorimetric Assay for AK—AK activities were determined using a 96-well plate tetrazolium reduction assay (AK/INT assay) (Fig. 1). The methodology was based in part on similar assays: the lactate dehydrogenase/INT (23) and G6PD/INT (24) assays for determining viable cell numbers and the ATP/INT assay for measuring ATP concentrations in solution.3 The reactions of the AK/INT assay were carried out in two steps. In Step 1, samples with unknown AK activity were incubated at 37 °C for a specified time in the primary assay mixture (Table 1). AK catalyzed the conversion of ADP to ATP plus AMP, and in turn, the ATP phosphorylated glucose to Glu-6-P. The AK-catalyzed reaction was halted at selected times by adding Ap5A and chilling to 0 °C. When the AK activity of cells was being determined, the solutions were centrifuged after Step 1 for 1 min at 3000 g to remove cellular debris. In Step 2, 0.15-ml aliquots of the mixtures from Step 1 were transferred to wells of a 96-well plate; 0.05-ml portions of the secondary assay mixture were added to each well; and the plate was incubated for at least 60 min at 37 °C, long enough for stoichiometric conversion of INT to INT formazan (INT-F) via the Glu-6-P produced in Step 1 (Fig. 1).

Suitability of the AK/INT Assay for Measuring AK Activity—In a model study of AK activity using the AK/INT assay, three different concentrations of rabbit muscle AK (27, 53, and 107 milliunits/ml) were used in 3.5 ml of primary assay mixture in the wells of a 6-well plate. AK activity was terminated at selected times by transferring 1.5-ml aliquots of the mixture to tubes containing Ap5A (final Ap5A concentration of 130 μM)

TABLE 1

Assay mixtures for Steps 1 and 2 of the AK/INT assay

| Primary assay mixture in HBSS | Secondary assay mixture in water |
|-----------------------------|---------------------------------|
| ADP, 200 μM                | Tris, 0.333 M (pH 7.8)           |
| BSA, 20 μg/ml              | NADP, 0.5 mM                    |
| HK, 8.45 milliunits/ml     | INT, 1.25 mM                    |
|                            | Triton X-100, 0.2% (w/v)        |
|                            | PMS, 0.02 mM                    |
|                            | G6PD, 141 milliunits/ml         |

FIGURE 1. Reaction scheme for the AK/INT assay. The asterisks indicate the reagents included in the primary (Step 1) and secondary (Step 2) assay mixtures. The Amax for INT-F was 492 nm. G: glucose; G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; PMS, phenazine methosulfate; AK (rate-limiting); HK, hexokinase; G6PD, G6PD.

FIGURE 2. AK/INT assay: model study using purified rabbit muscle AK. A, effect of AK activity on the primary reaction. Three different concentrations of rabbit muscle AK were used in 1.5 ml of primary assay mixture at 37 °C (Fig. 1, Step 1; and Table 1): 27 (■), 53 (○), and 106 (▲) milliunits/ml. The AK reaction was halted as indicated by adding 20 μl of 9.75 mM Ap5A and chilling. Then, 0.15-ml aliquots of the supernatant solutions were dispensed in a 96-well plate; 0.05-ml portions of the secondary assay mixture were added to each well; and the plate was incubated for at least 60 min at 37 °C, long enough for stoichiometric conversion of INT to INT formazan (INT-F) via the Glu-6-P produced in Step 1 (Fig. 1).

B, INT reduction: function of AK activity. A492 data at 10 min of primary incubation from A are plotted as a function of AK activity at 0, 27, 53, and 106 milliunits/ml. The AK reaction was stopped as indicated by adding 20 μl of 9.75 mM Ap5A and chilling. Then, 0.15-ml aliquots of the supernatant solutions were dispensed in a 96-well plate, and 0.05 ml of secondary assay mixture was added. The plate was incubated for 90 min at 37 °C with periodic A492 determinations. Each data point indicates the means ± S.D. (n = 8). B, INT reduction: function of AK activity. A492 data at 10 min of primary incubation from A are plotted as a function of AK activity at 0, 27, 53, and 106 milliunits/ml. Data points are the means ± S.D. (n = 8). The line was drawn by linear regression analysis. C, kinetics of INT-F formation from ATP in the AK/INT assay. ATP was used at 200 μM, and both ADP and AK were left out of the AK/INT assay mixture. Other conditions were the same as those used for the standard AK assay. By 60 min of secondary incubation, the reaction was complete (net A492 = 1.53).
Relative Abundance of AK Inside and Outside HUVECs—Intracellular and extracellular AK activities of HUVECs were determined as follows. Cells were seeded at two different densities (2.5 \times 10^5 cells/well for external AK activity and 5 \times 10^4 cells/well for total AK activity) in 6-well plates with 3 ml of HUVEC medium and incubated for 24 h. The cultures were

on ice. Then, 0.15-ml aliquots were transferred to wells of a 96-well plate, and the secondary assay mixture was added. The 96-well plate was incubated at 37 °C with periodic measurements. At 27 milliunits/ml AK, the rate of formation of ATP from ADP was constant for at least 30 min (Fig. 2A). At higher AK activities, the rate of ATP formation in Step 1 departed from linearity after 10 min of incubation because of ADP depletion. During the first 10 min, however, the rate of ATP formation from ADP was proportional to the activity of AK (Fig. 2B). ATP reference standards (200 \mu M ATP in the primary assay mixture instead of ADP and hexokinase) eliminated Reaction a in Fig. 1 and were used in this and all AK/INT assays. INT reduction in Step 2 was complete by 60 min (net \Delta A_{492} = 1.53 at 60 min; 1 absorbance unit at 492 nm = 20 nmol of INT-F/well in a 96-well plate) (Fig. 2C).

Stability of Rabbit Muscle AK in HBSS—AK was unstable at 37 °C in dilute aqueous solution (half-time of \sim 8 min) (Fig. 3A), but could be stabilized, at least in part, by BSA. The concentration of BSA needed for half-maximal stabilization (EC_{50}) of rabbit muscle AK was \sim 1 \mu g/ml (Fig. 3B). Whether other substances in the AK/INT assay stabilize the enzyme was explored. Commercial rabbit muscle AK was incubated for 30 min at 37 °C with AMP, ADP, ATP, Mg^{2+}, Triton X-100, or BSA, and its activity was determined by the AK/INT assay. Of these reagents, only BSA prevented rapid and complete loss of AK activity (Fig. 3C).

Stability of Rabbit Muscle AK in HBSS in the Presence of Living Cells—To determine the stability of extracellular AK in the presence of living cells, HUVECs were seeded at 2.5 \times 10^5 cells/well in a 12-well plate containing 3 ml of HUVEC medium. Additional wells had medium only; the plates were incubated for 24 h at 37 °C. The medium was removed, and the wells were washed zero to three times, 3 ml/wash, with HBSS. Then, 1 ml of a solution of rabbit muscle AK (prepared freshly on ice at 156 milliunits/ml in HBSS) with or without 20 \mu g/ml BSA was added to designated wells, and the plates were incubated at either 37 or 0 °C for 30 min. The supernatant solutions were transferred to tubes on ice and centrifuged, and 0.15-ml portions of the supernatants were transferred to the wells of a 96-well plate on ice. Then, 0.05 ml of modified secondary assay mixture was added, and the plate was incubated at 37 °C. The modified secondary assay mixture contained 600 \mu M ADP and 25.35 milliunits/ml hexokinase, so the concentrations of these components were equal to those used in the standard primary incubation. The final concentrations of all reagents were the same as those used in the AK/INT assay. The absorbances at 492 nm were determined at 60 min (Table 2). At 37 °C without BSA and cells, AK was completely inactivated by 30 min of preincubation (Table 2, row 3). Even at 0 °C, much of the activity was lost (row 1); but with 20 \mu g/ml BSA, the activity was maximal (row 2). At 37 °C in the presence of BSA but without a cell monolayer, a substantial portion of the activity was lost (row 4). At 37 °C in the presence of cells that had been washed zero to three times with HBSS, AK was stabilized (rows 5–8), apparently unaffected by the HBSS washings.

Relative Abundance of AK Inside and Outside HUVECs—Intracellular and extracellular AK activities of HUVECs were determined as follows. Cells were seeded at two different densities (2.5 \times 10^5 cells/well for external AK activity and 5 \times 10^4 cells/well for total AK activity) in 6-well plates with 3 ml of HUVEC medium and incubated for 24 h. The cultures were...
Extracellular ATP Synthesis

**TABLE 2**

Stabilization of rabbit muscle AK by BSA and by HUVECs

A 12-well plate (with or without $2.5 \times 10^5$ HUVECs/well) was incubated with HUVEC medium for 1 day, and the wells were then washed zero to three times with HBSS. Rabbit muscle AK (156 milliunits/ml) in HBSS with and without 20 µg/ml BSA was added as indicated, and the plate was incubated at the specified temperature for 30 min. The supernatant solutions were washed once with 3 ml of HBSS with and without 20 µg/ml BSA. The supernatant solutions were chilled, centrifuged, and dispensed at 0.15 ml/well in a 96-well plate. Then, 0.05 ml of secondary assay mixture supplemented with 600 µM ADP and 25 milliunits/ml hexokinase, was added. The plate was incubated at 37 °C, and the $A_{492}$ values were determined at 60 min.

| Well no. | Temp. during preincubation | Cells |
|---------|--------------------------|-------|
|         | ℃                        |       |
| 1       | 0                        | –     |
| 2       | 0                        | –     |
| 3       | 37                       | –     |
| 4       | 37                       | –     |
| 5       | 37                       | 1     |
| 6       | 37                       | 1     |
| 7       | 37                       | +     |
| 8       | 37                       | +     |

| BSA (200 µg/ml) | $A_{492}$ at 60 min of secondary incubation (± S.D.) |
|-----------------|---------------------------------------------------|
|                 |                                                   |

**TABLE 3**

Intra- and extracellular AK activities in HUVEC cultures

HUVECs were seeded at $2.5 \times 10^5$ (wells 1–3) and $5 \times 10^4$ (wells 4 and 5) cells/well, grown for 1 day, and washed with HBSS with and without BSA. Then, 1 ml of primary assay mixture with and without BSA was added. In wells 4 and 5, the primary assay mixture was supplemented with 0.05% Triton X-100. The plate was incubated at 37 °C for 30 min, and the supernatants were collected in chilled tubes containing Ap5A, centrifuged, and dispensed in the wells of a 96-well plate. Then, 0.05 ml of secondary assay mixture was added; the plate was incubated for 55 min; and $A_{492}$ values were determined. The ratio of extracellular to total AK activity is ~0.006. Values in parentheses are S.D. values ($n = 5$).

| Well no. | BSA in wash | BSA in primary incubation | Triton X-100 in primary incubation | $A_{492}$ at 55 min of secondary incubation (nM/µl) | ATP formed from ADP (nM/h/1×10^6 cells) |
|----------|-------------|--------------------------|-----------------------------------|---------------------------------------------------|-------------------------------------|
| 1        | –           | –                        | –                                 | 0.030 (0.008)                                      | 40.0 (10.7)                         |
| 2        | +           | –                        | –                                 | 0.031 (0.004)                                      | 41.3 (5.3)                          |
| 3        | +           | +                        | –                                 | 0.026 (0.009)                                      | 34.7 (12.0)                         |
| 4        | –           | –                        | +                                 | 0.968 (0.027)                                      | 6454 (180)                          |
| 5        | +           | +                        | +                                 | 0.977 (0.037)                                      | 6514 (247)                          |

washed once with 3 ml of HBSS with and without 20 µg/ml BSA. To the denser cultures was added 1 ml of primary assay mixture with and without BSA, and to the less dense cultures was added 1 ml of primary assay mixture containing 0.05% Triton X-100, which lysed the cells. The plates were incubated for 30 min at 37 °C, and the supernatant solutions were collected in centrifuge tubes containing Ap5A on ice. The tubes were centrifuged, and the supernatants were transferred to the wells of a 96-well plate at 0.15 ml/well. The secondary assay mixture was added; the plates were incubated for 55 min; and the absorbances at 492 nm were obtained (Table 3). BSA in the primary incubation made little difference to the AK activity of either the supernatant medium or the medium plus cells. Extracellular AK was found to be a very small fraction (~0.6%) of the whole cultures.

*Extracellular and Intracellular G6PD Activities*—To determine whether the abundance of AK in the HUVEC medium was unusually large, a comparison was made with that of another major cytoplasmic enzyme, G6PD. G6PD can be stabilized in aqueous solution by the addition of NADP, one of its substrates, and assayed using a modified secondary assay mixture via Reactions c and d (Fig. 1). G6PD was omitted from the modified mixture; the only G6PD present was cell-derived, and in this system, it was rate-limiting. The substrate Glu-6-P was added at 4 mM to the modified mixture, and the NADP concentration was adjusted to compensate for that added to stabilize the enzyme. HUVECs were seeded at $2.5 \times 10^5$ cells/well in 2 ml of medium in a 6-well plate and incubated overnight. The cultures were washed with HBSS and incubated for 30 min with 1.5 ml of HBSS, DPBS, or DPBSA supplemented with 0.125 mM NADP; one well was incubated with HBSS without NADP. The incubation solutions were chilled, centrifuged, and dispensed at 0.15 ml/well in a 96-well plate. The modified secondary assay mixture was added and the plate incubated. To the fifth well of the 6-well plate was added 1.5 ml of fresh medium plus 0.5 ml of secondary assay mixture, modified as described above but with NADP at the standard concentration. The contents of this well were mixed by pipetting up and down, distributed at 0.2 ml/well in the 96-well plate, and incubated at 37 °C. The $A_{492}$ values were determined periodically. The sixth well of the 6-well plate was used to obtain a cell count.

The rate of INT reduction with the whole culture extract (fifth well) was much greater than with the supernatant medium (first four wells), and an $A_{492}$ value was obtained within the first 10 min of incubation. The kinetics of the assays of the supernatant medium are shown in Fig. 4. The results indicate that, in HBSS and DPBS, the cells released similar amounts of G6PD during the primary incubation. In DPBSA, the release was somewhat greater. The G6PD activities/h/1×10^6 cells, determined by linear regression analysis, are shown in Table 4.

**AK Activities in Human Endothelial and Non-endothelial Cells**—If, as has been suggested, extracellular AK activity is associated mainly with cells of the vascular endothelium (3, 11), the same function would not be expected to occur with cells of most other tissues, including human fibroblasts. Therefore, the extracellular AK activity of cultures of human fibroblasts (WI38) was determined and compared with that of HUVECs. The two cell lines were seeded at $5 \times 10^4$ cells/well in a 6-well plate to determine total activity and $2.5 \times 10^6$ cells/well to determine extracellular activity. The cultures were incubated for 24 h, washed once with HBSS, and overlaid with 1 ml of...
primary assay mixture with or without 0.05% Triton X-100. The wells containing Triton X-100 (for total AK activity) were incubated for 20 min at 37 °C, and those without Triton X-100 (for extracellular activity) were incubated for 60 min, at which times AK activity was halted with Ap5A and chilling. The supernatant solutions were centrifuged, and 0.15 ml/well in a 96-well plate, and assayed by the G6PD/INT assay at 37 °C (23). The A₄₉₂ values were plotted as a function of time of incubation.

**TABLE 4**

Total and extracellular G6PD activities in HUVECs

| Well no. | Culture fraction | Assay medium       | Glu-6-P oxidized (μmol/h/10⁵ cells) |
|----------|------------------|--------------------|-------------------------------------|
| 1        | Extracellular medium | HBSS + NADP      | 36.2 (2.4)                           |
| 2        | Extracellular medium | HBSS              | 4.1 (1.8)                            |
| 3        | Extracellular medium | DPBS + NADP       | 55.8 (3.3)                           |
| 4        | Extracellular medium | DPBS              | 39.6 (4.0)                           |
| 5        | Total (cells + medium) | HUVEC          | 3972 (70)                            |

**FIGURE 4.** G6PD released from HUVECs into HBSS, DPBS, and DPBSA. HUVECs were seeded at 2.5 × 10⁵ cells/well in 2 ml of medium in a 6-well plate and incubated overnight. Cultures were washed with HBSS and incubated 30 min with 1.5 ml of NADP-supplemented HBSS (●), DPBS (○), or with unsupplemented HBSS (▲). Supernatant solutions were centrifuged, dispensed at 0.15 ml/well in a 96-well plate, and assayed by the G6PD/INT assay at 37 °C (23). The A₄₉₂ values are plotted as a function of time of incubation.

**TABLE 5**

Total and external AK activities in HUVECs and WI38 cells

| Cell type | μmol/h/10⁵ cells | External/total |
|-----------|-----------------|----------------|
| HUVEC     | 11.4 (0.5)      | 0.14 (0.08)    | 1.20 |
| WI38      | 5.90 (0.16)     | 0.20 (0.12)    | 3.38 |

When the reaction had gone to completion, the positive control (200 μM ATP but no cells) produced a net A₄₉₂ of 1.228 ± 0.040 (Fig. 5B); with ADP as substrate, the corresponding net A₄₉₂ was only 0.009 ± 0.003 (Fig. 5B and inset), indicating that 0.75% of the ADP was actually ATP. Absorbances for the solutions that had been incubated with HUVECs are shown in Fig. 5A. They are, of course, much lower than those for the ATP control (Fig. 5B). Without Ap5A, a significant amount of ATP was formed from ADP; with Ap5A, essentially none was formed (Fig. 5A, difference between ○ and ▲).

**Effects of ATP Synthase Inhibitors and Ap5A on Purified F₁-ATPase—**To determine whether Ap5A inhibits ATP synthase, a situation that could render it useless as a diagnostic agent for determining extracellular AK inhibition, Ap5A and well known inhibitors of the synthase were incubated with ATP plus isolated bovine heart mitochondrial F₁-ATPase, and P₁ formation was monitored. At 10 μM, two of the authentic F₁-ATPase inhibitors, piceatannol (30–33) and aurovertin B (34), strongly inhibited F₁-ATPase activity (Table 6). Oligomycin, an inhibitor of F₁,F₀-ATP synthase, would not be expected to act on the F₁-ATPase preparation because it does not contain the F₀ subunits; it was inactive in this assay (Table 6). Ap5A at concentrations up to 100 μM, a concentration close to that used for AK inhibition, did not inhibit F₁-ATPase.

**Effects of Piceatannol and Oligomycin on HUVEC Proliferation—**Piceatannol and oligomycin were tested for their effects on HUVEC proliferation. The cells were seeded in 24-well plates at 1 × 10⁴ cells/well in 0.5 ml of HUVEC medium supplemented with 0, 5, 10, or 20 μM inhibitor, and cell numbers were monitored for 3 days. Oligomycin at 5 and 10 μM inhibited cell proliferation, and 20 μM resulted in massive cell loss. Piceatannol was much less inhibitory, registering only 80% of the control cell number at day 3. The cell size was essentially unchanged over the course of the study (no inhibitor, 6.47 ± 0.36 pl; 10 μM piceatannol, 6.25 ± 0.19 pl; 10 μM oligomycin, 5.99 ± 0.21 pl; n = 4 in each case). Ap5A up to 200 μM had no effect on either cell number or size.

**Effect of Oligomycin and Piceatannol on Extracellular ATP Formation—**Piceatannol and oligomycin (up to 20 μM) were tested for their effects on ATP formation from ADP by HUVECs.
Cells were seeded at 1.5 × 10^6 cells/well in 10 ml of HUVEC medium in 100-mm tissue culture dishes and incubated for 24 h. The medium was removed, and the cell layers were washed once with 10 ml of HBSS. Three ml of primary assay mixture with and without 200 μM ADP and 130 μM Ap5A was added, and the plate was incubated for 30 min at 37 °C. The supernatants were centrifuged and dispensed at 0.15 ml/well in a 96-well plate. The secondary assay mixture was added, and the plate was incubated at 37 °C with periodic A_{492} readings. ADP without Ap5A, ○; ADP with Ap5A, ▼; without ADP and without Ap5A, ▲; without ADP and with Ap5A, ●; 6-well plate without cells but with medium, ▲; 6-well plate without cells but with medium and with primary assay mixture, ○. The results are consistent with the concept that the extracellular ATP was formed from ADP exclusively via AK.

**DISCUSSION**

Extracellular synthesis of ATP from ADP by cells of or related to the vascular endothelium, epidermis, or some tumor cells has been attributed to proton gradient-driven F, F0-ATP synthase activity of the plasma membrane (1–6), ecto-AK (5, 8–12, 17–19), and/or ectonucleoside diphosphokinase (8, 11–14). These enzymes, either by their presence in or proximity to the plasma membrane or by their catalytic functions, have been implicated in tissue plasia and/or differentiated functions of the constituent cells of the vascular or dermal epithelia (1, 3–5, 9–12). The amounts of the enzymes needed to catalyze extracellular ATP formation at the observed rates are very small indeed, and the formative basis of this ATP might be otherwise explained. The experiments performed in this study were intended to resolve some of the uncertainties about the enzymes responsible for extracellular ATP synthesis.

**Analytical Methods**—To monitor the synthesis of small amounts of ATP from ADP, a simple, reliable, and sensitive multwell plate assay, the AK/INT assay, was designed. It is a modification of the ATP/INT assay of Colvert et al. (3) for measuring ATP and of the G6PD/INT assay (24) for determining the number of viable cells.
animal cells in culture. The AK/INT assay (outlined in Fig. 1) has two steps, each involving two reactions: Step 1, Reactions a and b; and Step 2, Reactions c and d. By intent, AK activity is rate-limiting. In Reaction a, AK enables the formation of ATP and AMP from ADP, the $K_{eq}$ of which is $\sim 1$. By coupling it to Reaction b, ADP plus glucose is stoichiometrically converted to Glu-6-P and AMP. Reaction a can be terminated as needed by the addition of Ap$_5$A (25). Because the ADP generated by Reaction b is reused in Reaction a, the Glu-6-P produced in Step 1 is the molar equivalent of the added ADP that is consumed in Reaction a, i.e. $2 \text{ ADP} + 2 \text{ glucose} \rightarrow \text{2 AMP} + 2 \text{ Glu-6-P}$. In Step 2, Tris (pH 7.8), G6PD, NADP, phenazine methosulfate, INT, and Triton X-100 are added, and the mixture is incubated until Reactions c and d have gone to completion ($\sim 60$ min under the conditions used here). The ATP/INT assay involves only Reactions b–d (Fig. 1). Fig. 2C shows the kinetics of the AK/INT assay for 30 nmol of ATP/well in the 96-well plate format. As little as 1 nmol of ATP provided a net $A_{492}$ reading of 0.04 on the plate reader (total volume of liquid in the well, 200 $\mu l$).

**Validation of the AK/INT Assay**—The AK/INT assay was validated with purified rabbit muscle AK (Fig. 2, A and B). At low AK activity, in this case, 27 milliunits/ml, the net $A_{492}$ was a direct function of incubation time (Fig. 2A). At 27, 53, and 106 milliunits/ml, the net $A_{492}$ values were proportional to the AK activity for the first 10 min (Fig. 2B) but not at later times (Fig. 2A). Departure from proportionality at the later times was due to depletion of ADP during the assay. When the reaction had gone to completion, 30 nmol of ADP/well (i.e. 0.15 ml of 200 $\mu M$ ADP) produced an $A_{492}$ of 1.2–1.5 (Fig. 2C).

**AK Stability in Aqueous Solution**—Because AK is unstable in aqueous solution, the conditions used to measure its activity in culture fluids had to be validated. Purified rabbit muscle AK was used for this purpose. (Purified chicken muscle AK behaved identically.) In water at 37 °C, rabbit muscle AK had a half-time of $\sim 8$ min (Fig. 3A). However, in the presence of 1 $\mu g/ml$ BSA, 50% of the enzymatic activity remained at 30 min (Fig. 3B). At 0 °C, such loss of activity was greatly reduced (Table 2, compare rows 1 and 3). Several other solutes used or produced in the AK/INT assay (AMP, ADP, ATP, Triton X-100, and Mg$^{2+}$) had little or no protective effect on the enzyme at 37 °C (Fig. 3C). In the presence of a cell monolayer, even after three washings with HBSS, there was essentially no loss of AK activity (Table 2, rows 5–8). The beneficial effect of cells, even washed three times, was greater than that of 200 $\mu g/ml$ BSA (Table 2, compare rows 4 and 8). Something in the culture environment, either residual cell-adherent FBS or the plasma membrane of the cells themselves, must stabilize the enzyme.

**Relative Abundance of AK Inside and Outside HUVECs**—AK could access the medium of HUVEC cultures either by programmed transfer to or through the plasma membrane (ecto-AK) or simply by leakage due to compromised integrity of the plasma membrane or death of a small fraction of the cell population. If the passage of AK into the medium were due to a defective plasma membrane, the enzymatic activity would probably be small but proportional to the intracellular AK concentration. Extracellular and total AK activities were measured and are reported in terms of nanomoles of ATP produced from ADP by way of AK/h/1 $\times 10^6$ cells (Table 3). The total AK activity was very large compared with the extracellular activity and, for practical purposes, considered to be equal to the intracellular activity. To measure extracellular AK activity, it was necessary to use substantially more cells per culture than when measuring total AK activity. Although BSA was included in the preliminary wash and/or the primary incubation mixture in some of the cultures, there was no apparent advantage to having it there (Table 3). For total activity, Triton X-100 was included in the primary assay mixture to lyse the cells. It did not adversely affect the enzymatic activity. For the secondary incubation, the concentration of Triton X-100 in the mixture was adjusted so that assay conditions for all samples were the same.

The total AK activity of the HUVEC cultures was calculated to be $\sim 6500$ nmol of ATP formed from ADP/h/1 $\times 10^6$ cells, whereas the extracellular AK activity was only 40 nmol, $\sim 0.6\%$ of the total. The very small external activity was consistent with either low ecto-AK activity or slow AK leakage from the cytoplasm, for whatever reason. Differential trypan blue staining counts indicated the existence of 1–5% stained (dead) cells, values too large and inconsistent to confirm the observed extracellular AK activity (data not shown). Trypan blue staining is obviously less sensitive than extracellular/intracellular enzyme ratios for monitoring possible cell incontinence.

**Relative Abundance of G6PD Inside and Outside HUVECs**—An approach to determining whether extracellular AK had leaked from the cytoplasm was to measure the extracellular activity of another, relatively abundant cytoplasmic enzyme, G6PD. It, too, is unstable in aqueous solution but is stabilized by NADP. G6PD activity was measured with the same sensitivity as that for AK activity using the reactions in Step 2 of the AK/INT assay (Fig. 1). If extracellular AK activity were due to “ecto-AK” (i.e. AK actively transported to or through the plasma membrane), other enzymes of the cytoplasm would likely not experience the same proportionate translocation. On the other hand, if it were due to cell leakage or death, other cytoplasmic enzymes should be present externally in similar relative abundance.

In HUVEC cultures, G6PD accessed the extracellular medium in small but measurable amounts (Fig. 4). More was released during 30 min of incubation at 37 °C in DPBSA than in either DPBS or HBSS. This greater loss of G6PD from the cells in DPBSA than in DPBS or HBSS could be due to the absence of divalent cations in DPBSA. Without NADP in the primary assay mixture, no extracellular G6PD was detected (Fig. 4), con-

**TABLE 6**

| Inhibitor   | P formed from ATP | mol/mg protein/min (mean) | % of control |
|-------------|------------------|--------------------------|-------------|
| Ap$_5$A (10 $\mu M$) | 25.46 (21.04)     | 121                      |
| Ap$_5$A (100 $\mu M$) | 25.00 (21.04)     | 119                      |
| Oligomycin A (10 $\mu M$) | 26.84 (26.18)     | 102                      |
| Piceatannol (10 $\mu M$) | 9.28 (30.56)      | 30.4                     |
| Aurovertin (10 $\mu M$)  | 9.48 (27.86)      | 34.0                     |
sistent with the stabilizing effect of NADP on the enzyme. In HBSS, the proportion of extracellular to total G6PD was similar to that for AK, 0.9 versus 0.6%, respectively (Tables 3 and 4). Extracellular G6PD is most likely from cell leakage or death. Under such circumstances, similar loss of other cytoplasmic macromolecules, including AK, would be expected. The findings reveal that there are losses of cytoplasmic macromolecules from cells into their bathing medium, at least into the “physiological saline” solutions used here and in studies of extracellular and plasma membrane enzymes (1, 3, 4, 11, 12, 17–20).

There are other environmental characteristics that can affect plasma membrane permeability, including, for example, HEPES buffer (35). Both AK and G6PD were detected in the clarified medium, indicating that they are in solution rather than anchored to the plasma membrane.

Cell-type Specificity of Extracellular ATP Synthesis—PM-ATP synthase has been reported in vascular endothelial and epidermal cells (3–5, 9). Extracellular ATP was indeed produced from ADP by HUVECs, a normal human endothelial cell line, but also by WI38 cells, a human fibroblastoid cell line, in this case, at 0.14 and 0.20 μmol of ATP/h/1 × 10⁶ cells, respectively (Table 5). The ratio of external to total AK activity was even greater for WI38 cells than for HUVECs. In the case of the total AK activity, ATP synthesis could not have been due to PM-ATP synthase because the plasma and mitochondrial membranes were permeabilized with Triton X-100. Both external and total ATP syntheses can be attributed to AK activity. This outcome was confirmed in part by experiments showing equivalence between ADP consumed and INT-F formed (data not shown). Equivalence requires recycling of ADP as indicated in Step 1 (Fig. 1).

Substrate Purity—ATP is a common contaminant of commercial ADP. Several ADP lots were analyzed for contamination and found to contain from <0.5 to ~5% ATP. Unless its presence is recognized, the contaminant could be interpreted as a product of ATP synthase or other ATP-synthesizing enzymatic activity. In this study, such contamination was routinely monitored: only those ADP lots having <1% ATP were used, and the results were corrected for the contribution of contaminating ATP. Some reports of PM-ATP synthase activities may not have taken this pitfall into consideration. For example, in the studies of Arakaki et al. (1) using HUVECs, the kinetics of ATP formation revealed that accretion of extracellular ATP ceased within 30 s. Contaminating ATP could be some or all of the ATP reported as product. The results, given as nanomoles of ATP/mg of protein (specific concentration), did not reveal how much of the added ADP was actually converted to ATP. The denominator, milligrams of protein (presumably in the supernatant medium, HEPES-buffered saline), would be very small, particularly after such brief exposure to cells, making the calculated specific concentration large. Alternatively, the brevity of the reaction period could be due to ADP exhaustion, but this seems unlikely because a large amount (200 μM) was used. Yet another is enzyme inactivation, likewise unlikely in so short a time period.

Our determinations of extracellular ATP at early time periods are similar to those of Arakaki et al. (1) but revealed that the amount of ATP found in the extracellular fluid was equivalent to that contaminating the ADP. Furthermore, as the concentration of the ADP was increased or decreased, so did the amount of detected ATP, always in proportion to the contaminant. Arakaki et al. also indicated that the specific concentration of extracellular ATP was large compared with that of intracellular ATP. This outcome, although counterintuitive, could be explained if, in the case of intracellular ATP, the denominator of the calculation were protein content of the cell layer, which would be large compared with that of the medium (assuming that the specific concentration of ATP in the medium was based on the protein content of that liquid). A further complication is that the exogenous ADP with its attendant ATP (1–10 nmol/ml in our experience), being excluded from the cytoplasm, would not add to the internal ATP pool as it does to the external pool.

Role of PM-ATP Synthase in the Formation of Extracellular ATP—Extracellular ATP formation by intact HUVECs was examined in the presence and absence of a number of specific inhibitors, including Ap₅A, oligomycin, and piceatannol. Ap₅A selectively and strongly inhibits AK (25); oligomycin inhibits F₁Fₒ-ATP synthase (36); and piceatannol inhibits F₁-ATPase (30, 31).

The IC₅₀ values of Ap₅A for purified rabbit and chicken muscle AK, as well as for the HUVEC and WI38 enzymes, both intracellular and extracellular, were almost identical, ~1 μM (data not shown). At 130 μM Ap₅A, AK activity was completely repressed, consistent with the likelihood that this enzyme is responsible for most, if not all, of the ATP generated in the medium (Fig. 5A). In the assay, the net A₄₉₂ values were corrected for the contribution of ATP to both ADP and Ap₅A (0.90 and 0.53%, respectively). It should be noted that Ap₅A up to 200 μM did not inhibit beef heart mitochondrial F₁-ATP synthase. Moreover, up to 20 μM oligomycin did not inhibit HUVEC extracellular ATP synthesis. Under these conditions, it is probably safe to say that PM-ATP synthase either was not present in the plasma membrane or, if present, was not active.

Piceatannol inhibition of ATP synthase is not as diagnostic as oligomycin inhibition because it has additional inhibitory targets, including several protein-tyrosine kinases (30, 31) and, in our experience, small but measurable effects on adenylate kinase and hexokinase. It is a strong inhibitor of F₁-ATPase. Not surprisingly, it does inhibit HUVEC proliferation but not as strongly as oligomycin. Possibly, piceatannol does not traverse the plasma membrane as readily as oligomycin.

PM-ATP Synthase and Angiostatin—PM-ATP synthase is reported to be an angiostatin receptor on the plasma membrane of endothelial cells. Angiostatin is a kringle domain-containing polypeptide derived from either plasmin or plasminogen (3, 4, 29, 37). An interaction between angiostatin and ATP synthase has been suggested to down-regulate proliferation and migration of vascular endothelial cells, with possible important normal and anti-oncological outcomes (3, 4). Evidence for the presence of ATP synthase in the plasma membrane of HUVECs was addressed in several ways by Moser et al. (3), including immunological fluorescence labeling of the plasma membrane using polyclonal IgG raised against the α-subunit of F₁-ATP synthase, followed by either fluorescence microscopy or flow cytometry. Labeling was done so as to preclude access of the
antibody to the cytoplasm, which is abundantly endowed with F1F0-ATP synthase and which, if labeled, could compromise interpretation of the results. However, the fluorescence images were less than definitive and could be interpreted as showing cytoplasmic labeling. This alternative reading of the fluorograms is supported by the corresponding transmission light photomicrograph of the same cells: they appeared to be permeabilized, i.e. only two-dimensional. The flow cytometric studies are also in need of critical reassessment. Labeling profiles of the cells similar to those in the study would result if the cells were inadvertently permeabilized during staining. The staining protocol involved centrifuging the living cells at high speed (microcentrifuging) to separate them from the primary antibody. Such treatment challenges the integrity of the plasma membrane by compression, with possible consequent exposure of mitochondrial ATP synthase to the antibody. The evidence for PM-ATP synthase needs verification.

In conclusion, ATP was produced from ADP in the extracellular media of HUVECs and WI38 cells in culture. AK catalyzed the process. The presence of AK in the media appears to be due to cell leakage or death rather than programmed export from the cells. Extracellular ATP synthesis from ADP by PM-ATP synthase was not detected. Interpretation of these results and the results of others awaits detailed reassessment of the experiments.

REFERENCES

1. Arakaki, N., Nagao, T., Niki, R., Toyofuku, A., Tanaka, H., Kuramoto, Y., Emoto, Y., Shibata, H., Magota, S., and Higuti, T. (2003) Mol. Cancer Res. 1, 931–939
2. Das, B., Mondragon, O. H., Sadeghian, M., Hatcher, V. B., and Norin, A. J. (1994) J. Exp. Med. 180, 273–281
3. Moser, T. L., Stack, M. S., Asplin, I., Enghild, J. J., Hojrup, P., Everitt, L., Hubchak, S., Schnaper, H. W., and Pizzo, S. V. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2811–2816
4. Moser, T. L., Kenan, D. J., Ashley, T. A., Roy, J. A., Goodman, M. D., Misra, U. K., Cheek, D. J., and Pizzo, S. V. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6656–6661
5. Burrell, H. E., Wlodarski, B., Foster, B. J., Buckley, K. A., Sharpe, G. R., Quayle, J. M., Simpson, A. W. M., and Gallagher, J. A. (2005) J. Biol. Chem. 280, 29667–29676
6. Martinez, L. O., Jacquet, S., Esteve, J. P., Rolland, C., Cabezon, C., Champagne, E., Pineau, T., Georgeaud, V., Walker, J. E., Teree, F., Collet, X., Perret, B., and Barbaras, R. (2003) Nature 421, 75–79
7. Chi, S. L., and Pizzo, V. (2006) Cancer Res. 66, 875–882
8. Donaldson, S. H., Picher, M., and Boucher, R. C. (2002) Am. J. Respir. Cell Mol. Biol. 26, 209–215
9. Dixon, C. J., Hall, J. F., and Boarder, M. R. (2003) Br. J. Pharmacol. 138, 272–278
10. Picher, M., and Boucher, R. C. (2003) J. Biol. Chem. 278, 11256–11264
11. Yegutkin, G. G., Henttinen, T., and Jalkanen, S. (2001) FASEB J. 15, 251–260
12. Yegutkin, G. G., Henttinen, T., Samburski, S. S., Spychala, J., and Jalkanen, S. (2002) Biochem. J. 367, 121–128
13. Lazarowski, E. R., Boucher, R. C., and Harden, T. K. (2000) J. Biol. Chem. 275, 31061–31068
14. Buxton, I. L., Kaiser, R. A., Oxhorn, B. C., and Cheek, D. J. (2001) Am. J. Physiol. 281, H1657–H1666
15. Boyer, P. D. (2000) Biochim. Biophys. Acta 1458, 252–262
16. Dixon, C. J., Bowler, W. B., Littlewood-Evans, A., Dillon, J. P., Bilge, G., Sharpe, G. R., and Gallagher, J. A. (1999) Br. J. Pharmacol. 127, 1680–1686
17. Chahwala, S. B., and Cantley, L. C. (1984) J. Biol. Chem. 259, 13717–13722
18. Rozengurt, E., Heppel, L. A., and Friedman, I. (1977) J. Biol. Chem. 252, 4584–4590
19. Carrasco, A. J., Dzeja, P. P., Alekseev, A. E., Pucar, D., Dingman, L. V., Abraham, M. R., Hodgson, D., Bienengraeber, M., Puceat, M., Janssen, E., Wieringa, B., and Terzic, A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7623–7628
20. Buckley, K. A., Golding, S. L., Rice, J. M., Dillon, J. P., and Gallagher, J. A. (2003) FASEB J. 17, 1401–1410
21. Teuscher, L., and Weidlich, V. (1985) Biomed. Biochem. Acta 44, 493–495
22. Freshney, R. I. (1983) Culture of Animal Cells, Alan R. Liss, Inc., New York
23. Haslam, G., Wyatt, D., and Kitos, P. A. (2000) Cytotechnology 32, 63–75
24. Haslam, G., Richter, M. L., Wyatt, D., Ye, Q.-Z., and Kitos, P. A. (2005) Anal. Biochem. 336, 187–195
25. Sinev, M. A., Sineva, E. V., Ittah, V., and Ilaas, E. (1996) Biochemistry 35, 6425–6437
26. Knowles, J., and Penefsky, H. (1972) J. Biol. Chem. 247, 6617–6623
27. Taussky, H. H., and Shorr, E. (1953) J. Biol. Chem. 202, 675–685
28. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
29. Folkman, J. (2000) J. Natl. Cancer Inst. 92, 94–95
30. Zheng, J., and Ramirez, V. D. (1999) Biochem. Biophys. Res. Commun. 261, 499–503
31. Zheng, J., and Ramirez, V. D. (2000) Br. J. Pharmacol. 130, 1115–1123
32. Scow, C. J., Chue, S. C., and Wong, W. S. (2002) Eur. J. Pharmacol. 443, 189–196
33. Oliver, J. M., Burg, D. L., Wilson, B. S., McLaughlin, J. L., and Geahlen, R. L. (1994) J. Biol. Chem. 269, 29697–29703
34. van Raaij, M. J., Abrahams, J. P., Leslie, A. G. W., and Walker, J. E. (1996) Biochem. Biophys. Acta 130, 6913–6917
35. Douglas, G. C., Swanson, J. A., and Kern, D. F. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6425–6437
36. Bohem, M., and Penefsky, H. (1972) J. Biol. Chem. 247, 6617–6623
37. Taussky, H. H., and Shorr, E. (1953) J. Biol. Chem. 202, 675–685
38. Knowles, J., and Penefsky, H. (1972) J. Biol. Chem. 247, 6617–6623
39. Taussky, H. H., and Shorr, E. (1953) J. Biol. Chem. 202, 675–685
40. Knowles, J., and Penefsky, H. (1972) J. Biol. Chem. 247, 6617–6623
41. Taussky, H. H., and Shorr, E. (1953) J. Biol. Chem. 202, 675–685
42. Knowles, J., and Penefsky, H. (1972) J. Biol. Chem. 247, 6617–6623
43. Taussky, H. H., and Shorr, E. (1953) J. Biol. Chem. 202, 675–685
44. Knowles, J., and Penefsky, H. (1972) J. Biol. Chem. 247, 6617–6623
45. Taussky, H. H., and Shorr, E. (1953) J. Biol. Chem. 202, 675–685