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ed methods for generating them are being developed in many laboratories. However, in many cases, the geometric requirements are not stringent, and technical simplicity and speed are more desirable. Spotted substrates should prove useful in such cases.

We were also able to develop techniques to couple proteins to dots formed from selectively adhesive materials. These modifications allow proteins that are less abundant, pure, or stable to be used in a spotted dish assay, thereby expanding the range of signals that can be tested. Together, these methods should be applicable to assays of a variety of putative cues and the analysis of their effects on diverse cell types.

REFERENCES

1. Arce, V., A. Garces, B. de Bovis, P. Filippi, C. Henderson, B. Pettmann, and O. de-Lapeyriere. 1999. Cardiotrophin-1 requires LIFR β to promote survival of mouse motoneurons purified by a novel technique. *J. Neurosci. Res.* 55:119-126.
2. Bernard, A., E. Delamarche, H. Schmid, B. Michel, H.R. Bosshard, and H. Biebuyck. 1998. Printing patterns of proteins. *Langmuir* 14:2225-2229.
3. Blawas, A.S. and W.M. Reichert. 1998. Protein patterning. *Biomaterials* 19:595-609.
4. Buckley, K. and R.B. Kelly. 1985. Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. *J. Cell Biol.* 100:1284-1294.
5. Burry, R.W. 1980. Formation of apparent presynaptic elements in response to poly-basic compounds. *Brain Res.* 184:85-98.
6. Delamarch, E., A. Bernard, H. Schmid, A. Bietsch, B. Michel, and H. Biebuyck. 1998. Microfluidic networks for chemical patterning of substrates: design and application to bioassays. *J. Am. Chem. Soc.* 120:500-508.
7. Folch, A. and M. Toner. 2000. Microengineering of cellular interactions. *Annu. Rev. Biomed. Eng.* 2:227-256.
8. Johnston, S.A. and D.C. Tang. 1994. Gene gun transfection of animal cells and genetic immunization. *Methods Cell Biol.* 43PtA:353-365.
9. Reznik, G.O., S. Vajda, C.R. Cantor, and T. Sano. 2001. A streptavidin mutant useful for directed immobilization on solid surfaces. *Bioconjug. Chem.* 12:1000-1004.
10. Sanes, J.R. and J.W. Lichtman. 2001. Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat. Rev. Neurosci.* 2:791-805.
11. Scranton, T.W., M. Iwata, and S.S. Carlson. 1993. The SV2 protein of synaptic vesicles is a keratan sulfate proteoglycan. *J. Neurochem.* 61:29-44.
12. Tessier-Lavigne, M. and C.S. Goodman. 1996. The molecular biology of axon guidance. *Science* 274:1123-1133.
13. Walter, J., B. Kern-Veits, J. Huf, B. Stolz, and F. Bonhoeffer. 1987. Recognition of position-specific properties of tectal cell membranes by retinal axons in vitro. *Development* 101:686-696.

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Automated Colorimetric Screen for Apyrase Inhibitors

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ABSTRACT

Apyrases are enzymes that efficiently hydrolyze ATP and ADP and may operate both inside and outside the cell. Although apyrases are important to a variety of cellular mechanisms and uses in industry, there are no available apyrase-specific inhibitors. Colorimetric assays based on the Fiske-Subbarow method for measuring inorganic phosphate are able to detect the release of inorganic phosphate from ATP and other nucleotides. We found that this type of assay could be automated and used to screen for apyrase-inhibiting compounds by assaying

for a reduction in released phosphate in the presence of potential inhibitors. The automation of this assay allowed for the successful screening of a commercially available compound library. Several low molecular weight compounds were identified that, when used at micromolar concentrations, effectively inhibited apyrase activity.

INTRODUCTION

Apyrases are enzymes that hydrolyze nucleoside diphosphates and triphosphates and are dependent on Ca^{2+} or Mg^{2+} for activity (13). The potato apyrase enzyme has a high catalytic activity with the $\text{ATPase } k_{\text{cat}}/K_{\text{M}}$ of approximately $10^8 \text{ M}^{-1} \text{ s}^{-1}$ (8). Apyrase enzymes have been implicated in many cellular functions, including golgi compartment protein glycosylation (6), depletion of parasite host cell ATP levels (15), purine salvage mechanisms (12), viral and cancer mechanisms (4,13), and inhibition of platelet aggregation (1). Besides apyrase function in the cellular mechanisms mentioned above, potato apyrase specifically is an important industrial enzyme used in assays such as luciferase-based microbial contamination tests to clear samples of background ATP (16).

Despite the interest in apyrase function, there is a lack of useful inhibitors specific for apyrase. Typical inhibition of apyrase activity involves the use of ATP analogues such as ARL 67156 (7,17) or a high concentration of sodium azide (11) or vanadate. However, these inhibitors may be impractical for use in some research or industrial applications and unsuitable as potential therapeutic agents. In fact, literature discussing apyrase is punctuated with comments regarding the lack of apyrase inhibitors (2,14,18).

Colorimetric assays such as the Fiske-Subbarow and malachite green tests for the release of inorganic phosphate are widely used for measuring ATPase activity and other processes involving free phosphate (3,10). Here we discuss an automated, high-throughput screen designed to obtain efficient and effective small-molecule inhibitors of apyrase from a library of compounds. This screen utilizes the Fiske-Subbarow assay and a commercially pre-

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pared apyrase enzyme. The results from this screen indicate that the automated Fiske-Subbarow assay provides for a rapid and reliable method for acquiring apyrase inhibitors. This automated screen could easily be used to identify inhibitors for any ATPase that releases free inorganic phosphate.

MATERIALS AND METHODS

Small-Molecule Library

The small-molecule library DIVER-Set Format F consisting of 9600 compounds was purchased from Chem-Bridge (San Diego, CA, USA). This library was designed to maximize diversity in a library containing a minimum number of compounds. The library was arrayed in 96-well format with 0.1 mg each compound present as a dry film at the bottom of each well. For the present screen, 0.05 mL DMSO were added to each well just before the colorimetric assay, and the Biomek® “mix” function was used repeatedly to dissolve each compound. Once the compounds were dissolved, a 0.01-mL aliquot was transferred to the reaction well that contained the reaction buffer.

Colorimetric Assay

Figure 1A presents a flow chart of this assay. The assay is a variation of the phosphomolybdate complexation assay (5), which forms a blue color in the presence of inorganic phosphate and may be detected visually (Figure 1B). There are four solutions used in the assay: reaction buffer containing ATP, development solutions A and B, and a stop reaction solution C. The reaction buffer consists of 60 mM HEP-ES, 3 mM MgCl₂, 3 mM CaCl₂, and 3 mM ATP adjusted to pH 6.5. ATP was added to the buffer immediately before the assay. Development buffer A consists of a 2% aqueous ammonium molybdate solution. Development buffer B consists of 11% ascorbic acid in 37.5% trichloroacetic acid. Stop reaction buffer C contains 2% trisodium citrate in a 2% acetic acid solution.

Reaction buffer (0.1 mL) was used in each well and was added immediately before the assay. After the addition

of reaction buffer, the 0.01-mL aliquot of DIVERSet compounds was added, at one compound per well. A 0.01-mL (0.1 U) aliquot of ice-cold apyrase enzyme (from potato, grade VI; Sigma, St. Louis, MO, USA) solution (apyrase enzyme reconstituted in reaction buffer minus ATP) was added to each well of the assay plate, and the plates were manually tapped on the work surface to facilitate the mixture of all of the components. Three assay plates were set up during one run of the Biomek assay program. The plates were removed from the work surface and stored in a fume hood at room temperature for 1 h. At the end of this time, the plates were returned to the Biomek work surface for the development assay. Development buffers A and B were mixed in a 1:1.5 ratio immediately before the development phase. The development A:B mixture (0.05 mL) was added to

each well of the assay plate, and each plate was allowed exactly 2 min for the development of the ammonium molybdate-phosphate complex. Control reaction wells will form a blue color within approximately 10 s after the addition of the A:B mixture, and this color darkens over the course of development. After 2 min, 0.05 mL stop reaction solution C was added to each well to avoid the further darkening of each well. Even wells yielding a light blue color continue to darken over time without the addition of solution C. The blue color of control wells corresponded to an A₆₃₀ reading of greater than 0.25. The plates were assayed for the intensity of the blue color immediately after the addition of solution C. Potential positive inhibitors were those in wells giving a lighter blue color than the control, corresponding to an A₆₃₀ reading of approximately 0.15 or less.

Figure 1. Apyrase inhibitor screen. (A) Flow chart of colorimetric assay for apyrase inhibitors. An automated screen was designed to identify apyrase inhibitors. The screen utilized a phosphomolybdate complexation that resulted in a dark blue color when phosphate was liberated from ATP by the apyrase enzyme. The inhibition of the apyrase enzyme should result in less phosphate released from ATP and therefore less blue color in the reaction well. (B) Apyrase normally liberates inorganic phosphate from ATP, resulting in a dark blue color in the reaction well (left) after complexation with molybdate. Control wells without enzyme are clear after the development step (center). A light blue color (right) indicates less free phosphate and a potential apyrase inhibitor.

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Robotics

The Biomek 2000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA, USA) was used for all liquid-handling steps of both the enzyme inhibition and development assays. The BioWorks™ software version 2.0 was used to create programs to carry out the enzyme inhibition and development assays. The configuration of the work surface of both assays is shown in Figure 2. Figure 2A shows the enzyme inhibition setup used to transfer the compounds from mother plates to assay plates and to add the reaction mixture. Figure 2B shows the setup of the development step of the assay. Before the addition of the apyrase enzyme to the assay, it was kept on ice; otherwise, the assays were carried out at room temperature.

Kinetic Assays and Specificity Tests

The relative level of apyrase inhibition by inhibitory compounds from the screen was quantified by performing a colorimetric assay similar to that described above. In a 100- μ L reaction, 0.1 U apyrase enzyme was added to reaction buffer containing concentrations of ATP ranging from 1 to 2.25 mM per reaction over a 30-s time course. The resulting absorbance was then compared to an inorganic phosphate standard. The phosphate standard was established by the addition of known quantities of sodium phosphate solution to wells in 10-nmol increments. The development and stop reactions were performed as described above. The absorbance of each well was determined spectrophotometrically using a Dynatech MR700 (Dynex Technologies, Chantilly, VA, USA) at a wavelength of 630 nm. A substrate velocity series of apyrase enzyme using 1–2.25 mM ATP was used to calculate K_m and V_{max} . Lineweaver-Burk plots (primary plots) were used to determine the type of inhibition. Secondary plots of change in slope of the primary plots versus inhibitor concentration and $1/V_{app}$ versus inhibitor concentration were used to obtain K_i and K_i' values, respectively.

For specificity tests, white potato acid phosphatase (type VII; Sigma) activity was measured using the reaction

buffer at pH 4.8, and the reaction duration was 30 min at 37°C; bovine intestinal mucosa alkaline phosphatase (type VII; Sigma) activity was measured at pH 9.8, and the reaction duration was 30 min at 37°C. Inhibition to luciferase was measured by comparing the RLU of an assay well containing 0.1 mL reaction buffer, pH 7.0, with ATP (500 μ M) and 0.1 mL luciferin/luciferase (Promega, Madison, WI, USA) in the presence of 10 μ g inhibitor compound, with those wells receiving buffer and luciferin/luciferase, but no compound. All measurements were taken using a luminometer (Dynex Technologies).

RESULTS AND DISCUSSION

A small-molecule library of 9600 diverse compounds arrayed in a 96-well format was screened for compounds, which inhibited the apyrase enzyme. The screen involved dissolving each compound and adding an aliquot of a 0.01-mL sample to a reaction well, a buffered solution containing ATP, and the apyrase enzyme to each well. Under normal conditions, apyrase liberates phosphate from ATP and ADP, and the free inorganic phosphate reacts with ammonium molybdate, forming a blue color with an A_{630} reading of approximately 0.25. The absorbance is an in-

indicator of the amount of phosphate liberated during the reaction. The concentration of ATP used in the screen (3 mM) was saturating, such that only strong inhibitors of the apyrase enzyme would come through as positive. A positive result would be one that resulted in a lower absorbance than the control reactions. Similar screens for apyrase or other ATPase inhibitors could also be performed using methods that measure the remaining ATP in the reaction, such as the luciferase/luciferin system (9). We chose the phosphomolybdate complexation assay for released inorganic phosphate for its simplicity and extremely low cost, in view of the high throughput required for this screen.

Upon completion of the preliminary screen, many compounds showed some inhibition of apyrase activity; however, a re-screen by hand revealed that most of the compounds identified in the first round were false positives. The high frequency of false positives may be due to the program of the Biomek at the point in which the apyrase enzyme is aliquoted to the reaction well. It was noted that, because of the small volume of enzyme solution pipetted to the reaction well, the solution would occasionally remain at the end of the tip or the side of the reaction well after the completion of the tip-touch function. Hence, the enzyme would never reach the reaction well,

Figure 2. Workstation configuration of the Biomek robot used for the automated screen. The Setup configuration (A) allowed for the transfer of compounds from library plates to reaction wells in assay plates containing buffer, ATP, and apyrase enzyme. After a 1-h reaction period, the plates were aligned in the Development configuration (B) to start and stop the development reaction and to visualize the blue color and the results of each test.

Table 1. Apyrase Inhibitor Structures and Specificity

	Class I NGXT 1913	Class II NGXT 199	Class III NGXT195

Apyrase	65%	27%	21%
Alkaline Phosphatase	25%	0%	0%
Acid Phosphatase	0%	27%	22%
Luciferase	8%	32%	67%

and the result would appear as complete inhibition. This problem could easily be corrected in future screens by dispensing the enzyme solution within the volume of the reaction solution and possibly by using the mix or blowout functions of the Biomek.

After the secondary round of screening, 17 positives were identified. Preliminary pharmacophore analysis indicates that several distinct classes of compounds were isolated. Table 1 shows the structures of the representative compounds of three classes, a sul-

fonimide (class I), a N-naphthylbenzoic (class II), and a naphthylacetyl hydrazone (class III). The strength of the inhibition of the 17 inhibitors ranged from 10% to 65% inhibition, as measured by absorbance of the blue color in the reaction well. In addition to the strength of inhibition, these 17 compounds were tested for their specificity to the apyrase enzyme. Other enzymes tested included both acid and alkaline phosphatases and luciferase. Table 1 shows the results of these specificity tests.

Representative compounds from the three classes were chosen for kinetic analysis, and the K_i or K_i' and type of inhibition were determined for each. For these assays, we used the commercially prepared apyrase enzyme used in the screen. Fifty- and 200- μ M concentrations of apyrase-inhibitor compounds NGXT195, NGXT 199, and NGXT 1913 were used to test for the type of inhibition of the compound.

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Figure 3. Kinetics of inhibition of apyrase inhibitors. Lineweaver-Burk plots of compounds representative of the three classes of apyrase inhibitors reveal that they act by different inhibition mechanisms. The K_i or K_i' of each compound is listed. ■ represents 0 μM inhibitor compound, ● represents 50 μM inhibitor compound, and ▲ represents 200 μM inhibitor compound.

Lineweaver-Burk plots (Figure 3) were used to determine the type of inhibition. The K_i or K_i' value for each compound is listed.

The inhibitors obtained in the screen described here may be used to elucidate the function and involvement of apyrase enzymes in cellular processes. Since the inhibitors possess a range of activity and specificity to apyrase and other ATPases, they may be used to ascertain which ATPases are functioning at a particular time point in the cell cycle or during development.

The ease of use of small molecule chemical inhibitors make them an attractive experimental option for studying enzyme function when compared to techniques such as antisense and genetic knockouts. For most academic laboratories, screening for small molecules specific to their particular application would not be considered a realistic option. However, academically priced chemical libraries and the availability of robotic equipment may allow for increasing numbers of investigators to obtain enzyme inhibitors "tailor-made" to their area of research. The screen described here could be modified to screen for any enzymatic function involving the release of inorganic phosphate. This could include screening for inhibitors specific to other ATPases or phosphatases. Such screening by investigators in diverse areas of research would undoubtedly yield compounds with both academic and commercial applications.

REFERENCES

1. Beukers, M.W., I.M. Pirovano, A. van Weert, C.J. Kerkhof, A.P. IJzerman, and W. Soudijn. 1993. Characterization of ecto-ATPase on human blood cells. A physiological role in platelet aggregation? *Biochem. Pharmacol.* 46:1959-1966.
2. Bodas, E., J. Aleu, G. Pujol, M. Martín-Satúe, J. Marsal, and C. Solsona. 2000. ATP crossing the cell plasma membrane generates an ionic current in *Xenopus* oocytes. *J. Biol. Chem.* 275:20268-20273.
3. Durward, E. and W.J. Harris. 1998. Colorimetric method for detecting amplified nucleic acids. *BioTechniques* 25:608-614.
4. Dzhandzhugazyan, K.N., A.F. Kirkin, P. Thor Straten, and J. Zeuthen. 1998. Ecto-ATP diphosphohydrolase/CD39 is overexpressed in differentiated human melanomas. *FEBS Lett.* 430:227-230.
5. Fiske, C.H. and Y. Subbarow. 1925. The colorimetric determination of phosphorous. *J. Biol. Chem.* 66:375-400.
6. Gao, X., V. Kaigorodov, and Y. Jigami. 1999. *YND1*, a homologue of *GDA1*, encodes a membrane-bound apyrase required for golgi N- and O-glycosylation in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 274:21450-21456.
7. Gendron, F.P., E. Halbfinger, B. Fischer, M. Duval, P. D'Orleans-Juste, and A.R. Beaudoin. 2000. Novel inhibitors of nucleoside triphosphate diphosphohydrolases: chemical synthesis and biochemical and pharmacological characterizations. *J. Med. Chem.* 43:2239-2247.
8. Handa, M. and G. Guidotti. 1996. Purification and cloning of a soluble ATP-diphosphohydrolase (apyrase) from potato tubers (*Solanum tuberosum*). *Biochem. Biophys. Res. Commun.* 218:916-923.
9. Karamohamed, S. and G. Guidotti. 2001. Bioluminometric method for real-time detection of ATPase activity. *BioTechniques* 31:420-425.
10. Kirchgesser, M. and N. Dahlmann. 1990. A colorimetric assay for the determination of acid nucleoside triphosphatase activity. *J. Clin. Chem. Clin. Biochem.* 28:407-411.
11. Knowles, A.F. and A.K. Nagy. 1999. Inhibition of an ecto-ATP-diphosphohydrolase by azide. *Eur. J. Biochem.* 26:349-357.
12. Ngo, H.M., E.O. Ngo, D.J. Bzik, and K. Joiner. 2000. *Toxoplasma gondii*: are host cell adenosine nucleotides a direct source for purine salvage? *Exp. Parasitol.* 95:148-153.
13. Plesner, L. 1995. Ecto-ATPases: identities and functions. *Int. Rev. Cytol.* 158:141-214.
14. Sakakibara, T., S. Murakami, N. Hattori, M. Nakajima, and K. Imai. 1997. Enzymatic treatment to eliminate the extracellular ATP for improving the detectability of bacterial intracellular ATP. *Anal. Biochem.* 250:157-161.
15. Silverman, J.A., H. Qi, A. Riehl, C. Beckers, V. Nakaar, and K. Joiner. 1998. Induced activation of the *Toxoplasma gondii* nucleoside triphosphate hydrolase leads to depletion of host cell ATP levels and rapid exit of intracellular parasites from infected cells. *J. Biol. Chem.* 273:12352-12359.
16. Stanley, P.E., B.J. McCarthy, and R. Smither. 1989. ATP Luminescence: Rapid Methods in Microbiology. Blackwell Scientific Publications, Boston, MA.
17. Westfall, T.D., C. Kennedy, and P. Sneddon. 1996. Enhancement of sympathetic purinergic neurotransmission in the guinea-pig isolated vas deferens by the novel ecto-ATPase inhibitor ARL 67156. *Br. J. Pharmacol.* 117:867-872.
18. Ziganshin, A.U., C.H.V. Hoyle, and G. Burnstock. 1994. Ecto-enzymes and metabolism of extracellular ATP. *Drug Dev. Res.* 32:134-146.

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