Characterization of the Megakaryocyte Secretory Response: Studies of Continuously Monitored Release of Endogenous ATP

By Jonathan L. Miller

Megakaryocytes share a number of structural and chemical properties with their progeny, blood platelets. With the availability of highly purified preparations of megakaryocytes isolated from guinea pig bone marrow, it is now also possible to study functional aspects of these cells. The present work reports the first study of the release of endogenously stored materials in megakaryocytes. Guinea pig megakaryocytes isolated to 75%–90% purity were exposed to thrombin or calcium ionophore (A23187) and the release of ATP was continuously monitored with the luciferin–luciferase reaction. Both maximal extent and initial rate of release were studied. Thrombin-induced release was half-maximal at thrombin concentrations of 0.2–0.5 NIH U/ml. At 4 U/ml thrombin, maximal release was 538 ± 147 nmole ATP/10^9 megakaryocytes. A23187 induced half-maximal responses at concentrations of 7–8 μM. ATP release by ionophore showed a nearly absolute requirement for extracellular calcium, with release by thrombin showing only a partial calcium dependence. Following overnight culture, the response to thrombin was unchanged, whereas ATP release in response to ionophore was consistently increased ($p < 0.01$). By comparison of maximally releasable ATP with total cellular ATP content, the storage pool of ATP in megakaryocytes was determined to comprise only 2%–6% of total megakaryocyte ATP, in contrast to an ATP storage pool of 20%–30% in guinea pig platelets. This difference may reflect further entry of ATP into the storage pool compartment or an enhanced ability of the cell to recognize and respond fully to platelet stimuli as the megakaryocyte reaches full maturity.

BLOOD PLATELETS have been intensively studied as to their secretory as well as aggregatory properties. Platelet secretion of dense granule constituents has been studied as a continuous response over time both by monitoring calcium with the murexide technique[1,2] and ATP with the luciferin–luciferase technique.[3,4] In the precursor cell of the platelet—the bone marrow megakaryocyte—relatively little is known about the platelet type of physiologic responses that are already in place functionally. Substances associated with alpha granules in platelets, including platelet-derived growth factor[5] and platelet factor 4 antigen,[6,7] have recently been demonstrated in megakaryocytes, but the release of these substances from megakaryocytes challenged with platelet stimulatory agents has not been reported.

Fedorko[8] and Schick and Weinstein[9] have demonstrated the capability of megakaryocytes to take up radiolabeled 5-hydroxytryptamine (5-HT), and the subsequent release of this material following stimulation with several platelet agonists has been demonstrated.[8] Studies of megakaryocytes utilizing exogenously added materials are important in terms of establishing the capacity for uptake in these cells. The present studies, however, address and seek to define the pool of endogenous adenosine triphosphate (ATP) available for release, which comprises the "storage pool" of this nucleotide, in megakaryocytes. Kinetics of the continuously monitored release of endogenous ATP are reported, and characteristics of the megakaryococyte secretory response of both fresh and cultured megakaryocytes are presented. Studies of adenosine diphosphate (ADP) release by modifications of the luciferin–luciferase technique are additionally reported.

MATERIALS AND METHODS

Megakaryocytes were isolated from the bone marrow of Hartley strain albino guinea pigs (Wilmington, MA) as previously described.[10,11] In brief, guinea pigs were anesthetized with nembutal (50 mg/kg), the animals sacrificed, and the humeri, femurs, and tibia removed. Marrow was removed from these bones and taken into calcium- and magnesium-free Hanks' balanced salt solution (CMFH) containing 0.38% citrate, 1 m*M* adenosine, and 2 m*M* theophylline. The cells were washed and then layered onto a discontinuous gradient of bovine serum albumin (Fraction V BSA, Armour, Kankakee, IL) for density gradient centrifugation (adjusted by refractive index, with ascending indices, at 20°C, of 1.3694, 1.3662, 1.3630, and 1.3598). The gradients were centrifuged for 30 min at 10,000 *g* (at 4°C), following which all cells remaining above the pellet were pooled, washed in CMFH, and layered on a BSA velocity sedimentation gradient of 1.3430 bottom layer, 2:1 (v/v) of 1.3430:CMFH middle layer, and 1:2 (v/v) of 1.3430:CMFH top layer. Following sedimentation for 30 min at unit gravity (20°C), cells above the second highest interface were discarded and the remaining cells washed in CMFH. Through utilization of a second velocity sedimentation step, yields of 1.4–5.0 × 10^5 megakaryocytes per guinea pig were routinely achieved at purities of 75%–90% of nucleated cells, determined by phase contrast hemocytometer counts of total cells and differential counts of megakaryocytes on Wright-Giemsa-stained cytocentrifuge preparations.[11] No platelets were ever seen in these preparations.

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Blood for these studies was obtained by cardiac puncture, platelet-rich plasma obtained, and platelets washed as previously described.[11] Both washed platelets and purified megakaryocytes were finally suspended in CMFH containing 3.5% bovine serum albumin (BSA) (Fraction V, Armour). For studies of the effects of calcium upon release, appropriate concentrations of calcium chloride were added as 1/100 volume 2 min prior to the addition of release inducer.

Total protein content of megakaryocytes or platelets was determined by a modification of the Lowry procedure.[12] For these determinations, cells were washed five times in CMFH not containing BSA.

For culture studies, marrow processed by sterile technique was incubated at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium containing 10% (v/v) fetal calf serum (GIBCO, Grand Island, NY), 2.3% (w/v) BSA, 1m*M* L-glutamine, penicillin (250 U/ml), and streptomycin (250 μg/ml) as previously described.[13] Following incubation, cells were washed and resuspended in CMFH containing 3.5% BSA and recounted prior to ATP release studies.

ATP release was performed in a Chronolog Lumi-aggregometer (Haverstown, PA) connected to a dual-channel strip-chart recorder. For megakaryocytes, 0.45 ml of a suspension of megakaryocytes plus 0.05 ml of a 100 mg/ml solution of myokinase-free luciferin–luciferase (Sigma Chemical Co., St. Louis, MO) was stirred at 1200 rpm, 37°C. Release inducers were then added, and ATP release followed continuously. A23187 (Calbiochem-Behring, La Jolla, CA) was dissolved in ethanol and added as 1/100 volume to minimize ethanol exposure. Bovine thrombin (Parke-Davis, Morris Plains, NJ) was stored at −20°C as a 500 U/ml solution in 50% glycerol and diluted just before use. At the end of each measurement, internal ATP standards were added and were used to calculate peak ATP release by the release-inducer. Reaction kinetics with ATP standards were virtually immediate, in contrast to secretory responses by the cells. The recorded responses accordingly reflect the biologic release patterns, rather than the enzymatic assay reaction itself.

In a limited number of experiments the release of ADP from megakaryocytes was also studied, utilizing the phosphoenolpyruvate (PEP)–pyruvate kinase (PK) reaction to convert ADP to ATP.[14] Following the prior measurement of ATP in the presence of luciferin and luciferase, 0.04 ml of a mixture containing 0.5 m*M* PEP (Sigma) and 1000 U/ml PK (Boehringer Mannheim, Indianapolis, IN) was added to the cuvette, resulting in the rapid and complete conversion of ADP to ATP. By reference to subsequently added ADP internal standards, increases in luminescence deriving specifically from the release of ADP by thrombin or ionophore could be calculated. In the absence of added PEP–PK, the conversion of exogenously added ADP to ATP by the cell suspensions themselves was negligible.

Both ATP release and aggregation were monitored simultaneously for platelets. Conditions were as for megakaryocytes, except that 0.05 ml of a 40 mg/ml luciferin–luciferase solution was used.

For analysis of total cellular ATP content, cell suspensions were made 0.3 *N* in perchloric acid, multiply freeze-thawed, extracts neutralized to pH 7.4 with potassium carbonate, and aliquots then assayed by the luciferin–luciferase reaction.

Statistical data are presented as mean ± SE for *n* experiments unless otherwise specified. Differences in ATP release and cell content in fresh and cultured cells were analyzed by the 2-tailed, paired t test. Half-maximal effective doses of agonists were usually directly apparent from the experimental data. Additionally, half-maximal doses and maximal ATP release following thrombin stimulation were calculated from double-reciprocal plots of data with a Hewlett-Packard (Corvallis, OR) Model 85 desk-top computer. Proportionate weighting was used in fitting the least-squares regression line to the experimental points.[15]

RESULTS

Secretory responses of isolated megakaryocytes could be continuously monitored in terms of ATP released following the addition of an appropriate stimulus (Fig. 1). Utilizing the chart speed indicated, addition of ATP standards to the megakaryocyte suspension resulted in virtually instantaneous peaks of luciferin–luciferase-mediated luminescence responses. Peak heights following stimulation of the cells were always well within the linear range of peaks from

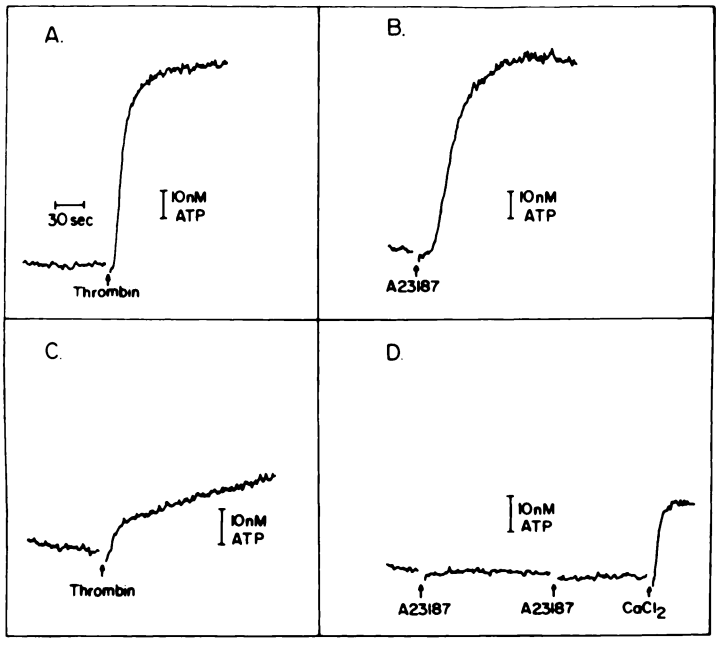

Fig. 1. Release of ATP induced by thrombin and A23187 from guinea pig megakaryocytes. Stirred cuvettes containing 0.45 ml of 50,000 megakaryocytes/ml in 3.5% BSA-CMFH and 0.05 ml of 100 mg/ml luciferin–luciferase solution. Thrombin (4 U/ml) or A23187 (25 μ*M*) added at times indicated. (A, B) $CaCl_2$ (2.5 m*M*) added 2 min prior to release inducer. (C, D) No $CaCl_2$ added prior to inducing agents. In D, 2.5 m*M* $CaCl_2$ is shown added following the second addition of A23187.

additions of ATP standards. The rates and maximal extents of light production thus faithfully reflected the biologic response patterns of the megakaryocytes (or platelets), rather than the enzymatic assay reaction itself.

Release induced by thrombin showed partial dependence on extracellular calcium, in terms of both total extent (Table 1) and, even more strongly, maximal rate of release (Fig. 1A and C). In contrast to thrombin, A23187 showed a virtually complete dependence on the presence of extracellular calcium in order to elicit release. In six of seven experiments, no ATP was released from megakaryocytes by A23187 in the absence of added external calcium, whereas in the presence of physiologic calcium concentrations, strong release occurred (Table 1 and Fig. 1B and D). Guinea pig platelets challenged with A23187 also showed a strong dependence on the level of external calcium, although typically not as absolute a requirement as seen with the megakaryocytes (Table 1). Thrombin-induced release in platelets was relatively less sensitive to external calcium.

Thrombin (4 U/ml) induced the release of 340 ± 110 and A23187 (25 μM) the release of 615 ± 183 nmole ADP/10^9 megakaryocytes ($n = 4$). Calcium was maintained at 2.5 mM and BSA at 3.5% for these experiments.

If megakaryocytes were not maintained in protein-containing media following the final velocity sedimentation step in the isolation process, poor secretory responses were consistently observed. In the absence of added albumin but with 2.5 mM $CaCl_2$ present, 4 U/ml thrombin was found to induce the release of only 50 nmole ATP/10^9 megakaryocytes, and in the absence of both added albumin or added calcium, thrombin was unable to induce the release of any ATP. All subsequent studies were accordingly performed in buffer containing 3.5% BSA.

With calcium maintained at 2.5 mM, the effect of thrombin concentration on maximal extent of megakaryocyte ATP release was studied (Fig. 2). Release of

Table 1. Calcium Dependence of Stimulated ATP Release*

	Thrombin	A23187
Megakaryocytes		
No added $CaCl_2$	180 ± 109 (8)	11 ± 11 (7)
+ 2.5 mM $CaCl_2$	538 ± 147 (13)	725 ± 154 (13)
Platelets		
No added $CaCl_2$	14.5 ± 2.7 (4)	1.4 ± 0.9 (4)
+ 2.5 mM $CaCl_2$	17.6 ± 1.8 (9)	13.0 ± 3.0 (6)

*Megakaryocytes or platelets isolated as described in the text were suspended in CMFH containing 3.5% BSA and challenged with thrombin (4 U/ml) or A23187 (25 μM) in the presence or absence of added calcium (see Fig. 1 for details of procedure). Results are expressed as nmole ATP released/10^9 cells. Mean ± SE for (n) experiments.

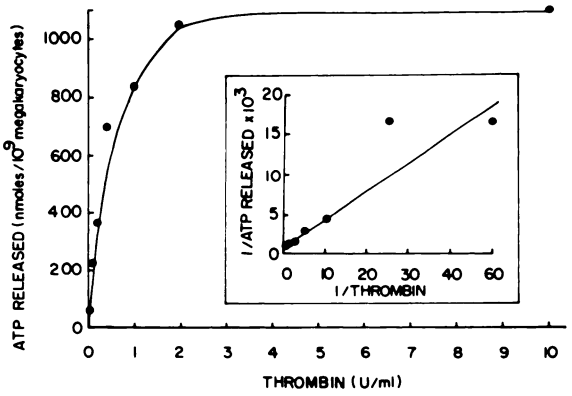

Fig. 2. Effect of thrombin concentration on ATP release from megakaryocytes. Conditions as described in Fig. 1, with 2.5 mM $CaCl_2$ added 2 min prior to thrombin. Inset: Double-reciprocal plot of same data fitted by least-squares analysis ($r = 0.92$).

ATP showed a hyperbolic response pattern to increasing concentrations of thrombin. When replotted on double-reciprocal coordinates (Fig. 2, inset), these data were fit by a least-squares regression line having a correlation coefficient (r) of 0.92. From the double-reciprocal plot, the thrombin concentration for half-maximal ATP release was 0.45 NIH U/ml thrombin, and the maximal ATP release in this experiment was 1260 nmole ATP/10^9 megakaryocytes. Half-maximal release in megakaryocytes typically occurred between 0.2 and 0.5 U/ml thrombin, whereas guinea pig platelets appeared somewhat more sensitive to thrombin, with half-maximal responses typically occurring at 0.08–0.10 U/ml.

Megakaryocyte response to the calcium ionophore A23187 was also strongly concentration-dependent. Both maximal extent and initial rate of ATP release showed a half-maximal response at 7–8 μM A23187 (Fig. 3). Responses of guinea pig platelets under the same conditions showed a similar dependency on ionophore concentration.

Megakaryocyte ATP released by the highest con-

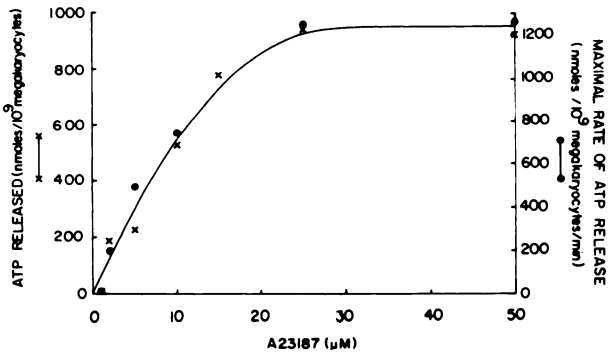

Fig. 3. Effect of ionophore A23187 concentration on both total amount (X) and maximal rate (●) of ATP released from megakaryocytes.

centrations employed of either thrombin (Fig. 2) or A23187 (Fig. 3) constituted only about 2%–6% of the total cellular ATP content (15.9 ± 2.0 nmole/10^6 megakaryocytes, n = 6). In contrast, the releasable pool of ATP in guinea pig platelets comprised 20%–30% of total cellular ATP (63 ± 2.4 nmole/10^9 platelets, n = 4).

Total megakaryocyte protein content was 1.1 ± 0.4 mg protein/10^6 cells (n = 5), and total platelet protein content measured 2.1 ± 0.04 mg protein/10^9 cells (n = 5). Megakaryocytes and platelets were accordingly quite similar in terms of nmole ATP/mg protein (14 for megakaryocytes, 30 for platelets). Isolated megakaryocytes maintained in culture for 24 hr showed no significant differences in their responsiveness to thrombin in comparison with freshly isolated cells ($p > 0.5$) (Fig. 4, left panel). Responsiveness to A23187 during this period, in contrast, was consistently increased ($p < 0.01$) (Fig. 4, right panel). Megakaryocytes continued to show a virtually complete dependence on external calcium for the support of ionophore-induced secretion. Total cellular ATP content of pairs of fresh and cultured megakaryocytes during this same period showed no significant differences ($p > 0.5$).

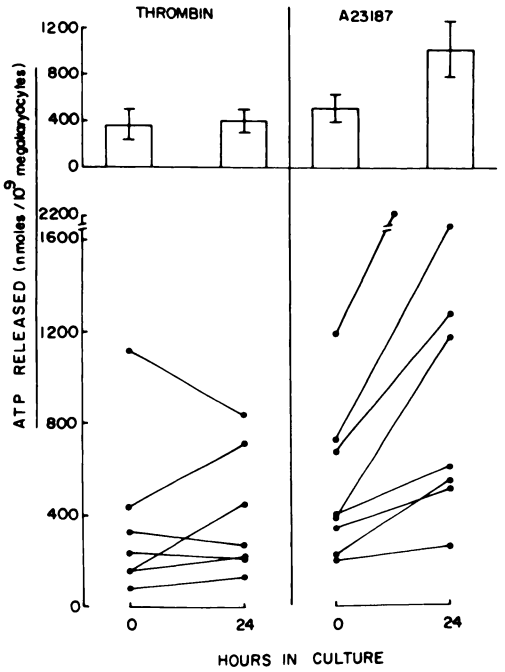

Fig. 4. Response of megakaryocytes to thrombin (4 U/ml) and A23187 (25 μM) prior to and following 24 hr in culture. Calcium (2.5 mM) added just prior to release-inducing agent. Individual experiments shown as connected data points below, with mean ± SE for all experiments shown above.

DISCUSSION

The present studies demonstrate that guinea pig megakaryocytes freshly isolated from bone marrow already contain a pool of adenine nucleotides available for release upon exposure of the megakaryocytes to appropriate platelet stimuli. Thus, 400–700 nmole ATP and only slightly less ADP (300–500 nmole) are released per 10^9 cells by thrombin. The calcium ionophore A23187 is capable of releasing approximately 600–900 nmole ATP and 400–800 nmole ADP/10^9 cells. Whereas the storage pool of ATP comprises 20%–30% of total cellular ATP in guinea pig platelets, the proportion of megakaryocyte ATP releasable by thrombin stimulation is lower, comprising only 2%–6% of the total cellular ATP content. In terms of total cellular content, one megakaryocyte contains about 200–300 times as many nanomoles of ATP as does one platelet. Yet the number of molecules of ATP maximally releasable per megakaryocyte is only about 34 times as great as that per platelet when thrombin is the release inducer, or about 63 times as great when A23187 is the inducing agent (Table 1). These findings accordingly suggest that the megakaryocytes have a proportionately smaller releasable pool of ATP than do their progeny. When cellular ATP/protein contents are compared between megakaryocytes and platelets, in contrast, the values are actually quite close (approximately 14 nmoles ATP/mg protein for megakaryocytes and 30 for platelets). Additionally, the cellular estimates of megakaryocyte ATP content in the present work, utilizing the luciferin–luciferase reaction, are close to those reported by Levine and Webster[16] using gradient anionic exchange HPLC and u.v. detection.

Since the purified megakaryocyte cell suspensions employed in these studies are heterogeneous with respect to cellular maturity,[10] it is possible that the relatively low proportion of releasable ATP reflects the presence of megakaryocytes that have not yet reached full functional maturity. As megakaryocytes mature, more ATP may enter the storage pool compartment and hence become available for release. Alternatively, ATP may enter a storage pool compartment relatively early in megakaryocyte maturation, but the ability of the cell to recognize and respond fully to platelet stimuli may be a relatively late event in maturation. Cytochemical studies[17,18] have suggested that nucleotide-containing organelles are in fact present in megakaryocytes, whereas granule storage of 5-HT does not normally occur to an appreciable extent in megakaryocytes in situ. Even immature megakaryocytes, however, appear capable of accumulating ^{3}H-5-HT in comparable amounts as do mature megakaryocytes.[9] Whether or not the more immature megakaryocytes can actually be induced to undergo release by thrombin or ionophore is not presently known.

In the course of the present studies it was found that maintenance of isolated megakaryocytes in protein-containing media prolonged their responsiveness to thrombin and to ionophore, as well as supporting a greater absolute amount of secretion (see Results). The relatively high concentrations of ionophore required for maximal secretion in these studies is most likely due to the high protein content of the 3.5% BSA-CMFH utilized. It is apparent, however, that at a concentration of 25 μM, A23187 is only at the beginning of the plateau region of the concentration–response curves (Fig. 3). Moreover, the failure of even 50 μM A23187 to produce more than about 6% of total ATP release argues strongly against a lytic effect in these studies.

Guinea pig megakaryocytes showed a striking dependence on the presence of extracellular calcium for ionophore-induced release (Fig. 1 and Table 1). Thrombin, in contrast, showed a significant, but distinctly more relative, dependence on external calcium. This pattern was generally similar to that seen with blood platelets from the same animals (Table 1). It would appear from these studies that in both guinea pig platelets and megakaryocytes, A23187 is not able to promote secretion via the release of calcium from an intracellular site, as is believed to occur during A23187-induced secretion in human platelets.[19] Rather, an inward flux of calcium into the cell is likely required for the ionophore to induce secretion. Thrombin would also appear to utilize external calcium for a maximal secretory response, yet is able to evoke at least a partial secretory response by an additional mechanism. A similar variation in calcium requirement by thrombin and ionophore to support secretion and protein phosphorylation has been reported in rabbit platelets.[20–22]

Following overnight maintenance culture of megakaryocyte suspensions, thrombin was able to elicit secretory responses equivalent to those seen in freshly isolated megakaryocytes (Fig. 4). This finding is important, as it indicates that the immediate effects as well as recovery from inhibitory drugs may now be studied in a relatively constant experimental system. Leven and Nachmias[23,24] have also reported the ability of guinea pig megakaryocytes to manifest a spreading response to ADP following overnight culture, but a comparison with freshly isolated cells was not presented.

An intriguing finding in the present studies is the consistently increased release of ATP induced by ionophore following overnight culture (Fig. 4). Since a comparable increase was not seen with thrombin, it appears more likely that this increase reflects a change in the ionophore–calcium activation mechanism, rather than in the actual amount of ATP available for release. As megakaryocytes cultured overnight maintain their strong dependency on external calcium for ionophore-induced release, acquisition of an intracellular calcium pool susceptible to activation by ionophore would not appear a likely explanation for this finding. Further studies are in progress to examine additional aspects of ionophore-induced cell activation in short-term megakaryocyte cultures.

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