

ATP-induced calcium mobilization and inositol 1,4,5-trisphosphate formation in H-35 hepatoma cells

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Addition of ATP (but not epinephrine, angiotensin II, vasopressin, or platelet-activating factor) to H-35 hepatoma cells whose cellular lipids have been pre-labelled with [^3H]inositol, causes a rapid increase in [^3H]inositol trisphosphate. In H-35 cells pre-incubated in the presence of $^{45}\text{Ca}^{2+}$, ATP causes a similarly rapid release of $^{45}\text{Ca}^{2+}$. The concentration-effect relationships for inositol trisphosphate formation and Ca^{2+} efflux are similar to those reported previously for differentiated hepatocytes. These results demonstrate that at least one of the Ca^{2+} -mobilizing receptors normally found on hepatocytes is functionally retained in the H-35 hepatoma cell line and thus could provide a useful model for the study of these receptor mechanisms in liver.

(H-35 cell) ATP receptor Ca^{2+} -mobilizing receptor Purinergic receptor Inositol trisphosphate

1. INTRODUCTION

Well characterized immortal tumor cell lines have proven useful experimental tools in a wide variety of areas of cell biology. This is especially true for tumor lines which retain certain differentiated functions of the parent tissue, and can thus be studied under controlled or manipulated environmental conditions. To date, no studies have been reported on the actions of Ca^{2+} -mobilizing receptors in liver tumor (hepatoma) cell lines, presumably because the more conventional receptors (vasopressin, α_1 -adrenergic) have been lost following transformation of these cells. Here we report that one such receptor mechanism, the P_2 -purinergic receptor mechanism, is apparently retained in the H-35 Reuber hepatoma cell line, as evidenced by the ability of micromolar concentrations of ATP to activate inositol trisphosphate (IP_3) formation and to stimulate $^{45}\text{Ca}^{2+}$ efflux. The P_2 receptor mechanism in the H-35 cell line may thus prove to be a useful experimental system for probing the mechanisms of Ca^{2+} -mobilizing receptors in the liver.

2. MATERIALS AND METHODS

2.1. Cell culture

H-35 hepatoma cells were generously provided by M. Czech, University of Massachusetts, and were grown and maintained in T-75 culture flasks in Dulbecco's Minimal Essential Medium (DMEM) containing 10% fetal bovine serum (FBS) with 5% CO_2 in air at 37°C . Prior to experiments cells were transferred to 60×15 mm plastic petri dishes at a concentration of 6×10^5 cells per ml in 3 ml of DMEM containing 10% FBS. After 2 or 3 days the medium was aspirated and replaced with DMEM containing no serum. After 48 h, the medium was removed, and, in inositol phosphate experiments, $20 \mu\text{Ci}$ [^3H]inositol in 2 ml DMEM was added. The [^3H]inositol labeled cells were used in experiments after 40 h of further incubation.

2.2. Determination of [^3H]inositol phosphates and [^3H]inositol lipids

At the end of the 40 h labeling period, the cells were washed twice with sterile Puck's saline and in-

cubated for 1 h in 2 ml serum free DMEM. ATP was added in varying concentrations by aspirating the DMEM and replacing with 1 ml DMEM containing ATP. The reaction was quenched at various times by the addition of 2 ml of 4.5% perchloric acid. The dishes were left on ice for at least 20 min to extract the water soluble inositol phosphates. The perchlorate in the supernatant was precipitated with ice cold KOH and sodium tetraborate buffer (3 ml), vortex-mixed and centrifuged as described [1]. The neutralized supernatant was then applied to a Dowex anion exchange column, and inositol phosphates were eluted with increasing concentrations of ammonium formate as described previously. When isomers of IP_3 were to be determined [^{32}P](1,4,5) IP_3 , prepared from red cell membranes [2], was added along with the perchloric acid. In these experiments, the supernatant was added to 3.3 ml of freon/octylamine (1:1) [3]. The tubes were vortex-mixed vigorously and centrifuged at 2400 rpm for 3 min. The top layer containing the inositol phosphates was removed and applied directly to an HPLC column to determine IP_3 isomer proportions as described [2].

In both inositol phosphate and isomer experiments, total acid insoluble radioactivity (presumably [^3H]phosphoinositides) was removed from the dishes with 2 ml of 1 N NaOH, aided by scraping with a rubber policeman. Inositol phosphate data are expressed as a percentage of the radioactivity in this fraction.

2.3. $^{45}\text{Ca}^{2+}$ efflux from H-35 cells

H-35 cells were cultured to confluent monolayers as described above for determination of inositol phosphates and phosphoinositides. Following incubation in serum free DMEM for 48 h the cells were incubated with $10\ \mu\text{Ci}\ ^{45}\text{CaCl}_2$ in 2 ml of serum free DMEM for 40 h. The medium was replaced 3 times with 2 ml serum free DMEM at 10 min intervals. After 30 min the medium was aspirated and immediately replaced at 30 s intervals, for a total of 10 min. ATP was present in the medium between 5 and 10 min of incubation. The aspirated medium was added to 10 ml of scintillant and counted for radioactivity. At the end of the 10 min incubation, cells were removed from the dish by addition of 2 ml trypsin/EDTA and the total $^{45}\text{Ca}^{2+}$ remaining in the

cells was determined. Apparent first-order rate coefficients for $^{45}\text{Ca}^{2+}$ efflux were calculated as described [4].

3. RESULTS

ATP ($300\ \mu\text{M}$) induced a rapid increase in the rate of $^{45}\text{Ca}^{2+}$ efflux from H-35 cells (fig.1); the response was maximal within 30 s, and had returned to basal levels 3 min after the initial application of ATP, despite the replenishment of medium with fresh ATP every 30 s with this protocol. The transience of this response presumably reflects the finite size of the ATP-sensitive Ca^{2+} pool which would appear to be about 15–20% of exchangeable Ca^{2+} . This value is comparable to the size of the hormone-sensitive pool in hepatocytes [5].

Fig.2 demonstrates that ATP ($100\ \mu\text{M}$) induced a rapid increase in cellular levels of [^3H] IP_3 and [^3H] IP_2 in H-35 cells. Levels of [^3H] IP_3 were maximally increased in 30 s, which could be rapid enough to mediate the $^{45}\text{Ca}^{2+}$ release. The [^3H] IP_3 was separated from other inositol phosphates by the technique of Berridge et al. [6], and thus could

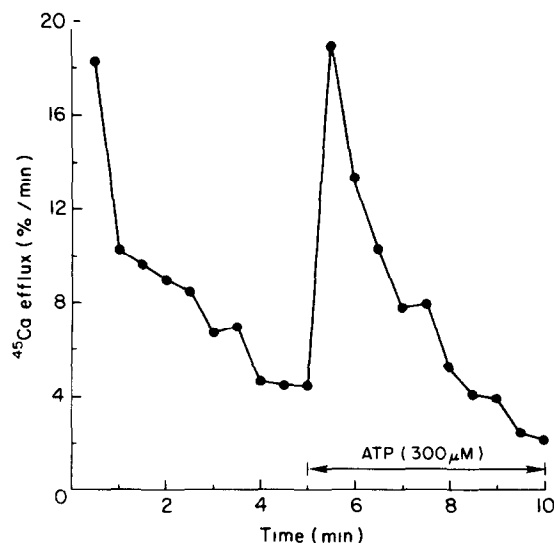


Fig.1. Efflux of $^{45}\text{Ca}^{2+}$ from H-35 hepatoma cells. Cells pre-equilibrated with $^{45}\text{Ca}^{2+}$ for 40 h were incubated sequentially in non-radioactive medium for 30 s intervals for 10 min, as described in section 2. ATP ($300\ \mu\text{M}$) was present from 5 to 10 min. One of three experiments, each similar in result, is shown.

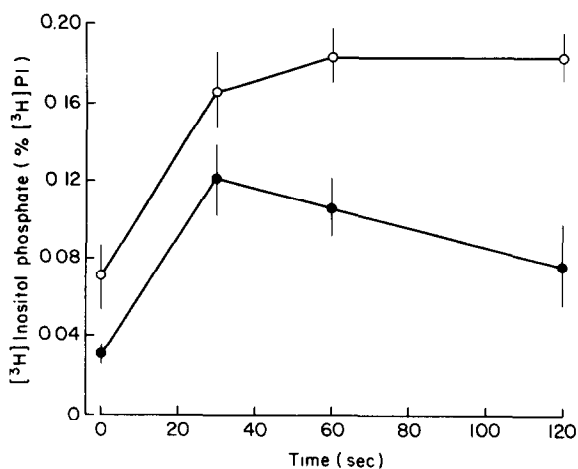


Fig. 2. Time course of increase in [³H]inositol phosphates in H-35 hepatoma cells. Cellular lipids were pre-labeled with [³H]inositol as described in section 2. 100 μ M ATP was added to dishes of cells, which were perchloric acid quenched after 30, 60 or 120 s and analyzed for [³H]IP₃ (●—●) and [³H]IP₂ (○—○) as described [1,6]. The value at $t = 0$ indicates cells prior to stimulation. The data are expressed as % of total acid insoluble radioactivity (primarily [³H]phosphoinositides, [³H]PI). Means \pm SE of three experiments.

contain both or either of the two isomers of IP₃ along with some [³H]IP₄ [7]. Samples taken from unstimulated cells ($n = 2$) and from cells after 30 s stimulation with 300 μ M ATP ($n = 3$) were analyzed by HPLC. There was no detectable [³H]IP₄ in either control or stimulated cells. Control cells contained [³H](1,4,5)IP₃ (0.098, 0.064 %PI) but no detectable [³H](1,3,4)IP₃. On stimulation [³H](1,4,5)IP₃ levels were increased more than 3-fold (0.353 ± 0.063 %PI), and a roughly equivalent amount of [³H](1,3,4)IP₃ was formed (0.290 ± 0.019 %PI).

Fig. 3 summarizes concentration-effect data for ATP-induced ⁴⁵Ca²⁺ efflux. The values were calculated as the net increase in rate coefficient in the first 30 s interval after ATP addition. A substantial (about 2-fold) increase in efflux was detected with 3 μ M ATP; maximal and half-maximal responses were obtained with 100 and 10 μ M concentrations, respectively.

Concentration-effect data were also obtained for inositol phosphate generation, again using the value obtained at 30 s after addition of ATP

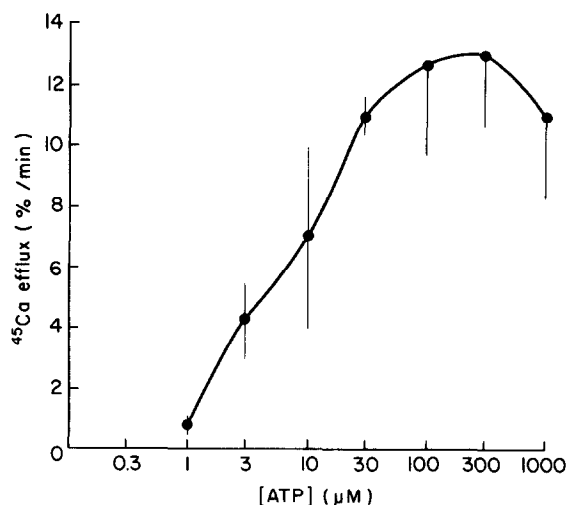


Fig. 3. Concentration-effect relationship for ATP-induced ⁴⁵Ca²⁺ efflux. The protocol was as for fig. 1. The basal efflux just prior to ATP addition was subtracted from the maximal increase obtained at 30 s after ATP addition. Means \pm SE of three experiments.

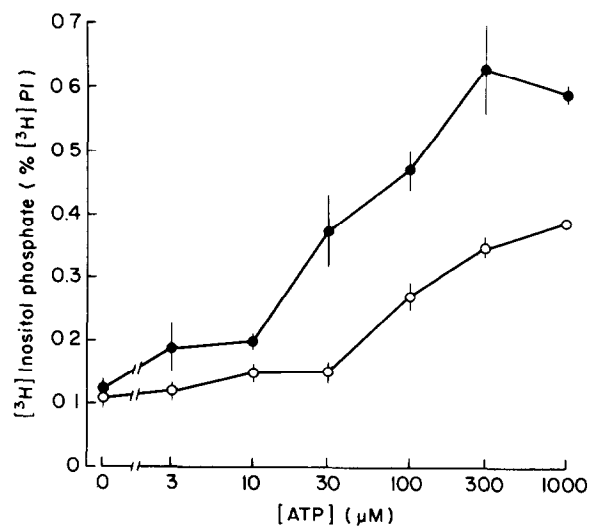


Fig. 4. Concentration-effect relationship for ATP-induced formation of [³H]IP₃ (●—●) and [³H]IP₂ (○—○). The protocol was as for fig. 2, except that the perchloric acid was added 30 s after the indicated concentration of ATP. Means \pm SE of four experiments.

(fig. 4). Somewhat higher concentrations of ATP were required for generation of [³H]IP₃, with a half-maximal increase occurring at about 30 μ M, and a maximal increase at 300 μ M.

4. DISCUSSION

The first report of agonist effects of exogenous ATP on the liver was by Jenkinson and Koller [8] who found that ATP hyperpolarized cells of guinea-pig liver slices in a manner similar to α -adrenoceptor agonists. Subsequent studies in a number of laboratories demonstrated that ATP produces biochemical effects in the liver which are mechanistically indistinguishable from the more extensively investigated α -adrenoceptor or peptide receptor effects [9–11]. Since the putative receptor mediating these effects prefers ATP to adenosine, it would be characterized as a P_2 -type purinergic receptor [12].

In preliminary studies on the effects of various agonists on the formation of [3 H]IP $_3$, ATP produced a significant increase in the level of this putative mediator; negative results were obtained with phenylephrine (10 μ M), angiotensin II (1 μ M), vasopressin (1 μ M), and platelet activating factor (10 nM). The reason why, as a result of transformation, all but one of the Ca $^{2+}$ and phosphoinositide-linked receptors normally present on hepatocytes are no longer expressed is not known. However, the ATP mechanism appears to be functionally intact. ATP caused a rapid increase in cellular [3 H]IP $_3$, and the concentration-effect relationship (fig.4) was similar to that reported for rat hepatocytes by Charest et al. [11]. Also in agreement with Charest et al. [11], the curve for Ca $^{2+}$ -mobilization (fig.3) appears to lie somewhat to the left (lower ATP concentrations) of that for IP $_3$ production, a finding consistent with the idea that IP $_3$ formation may precede Ca $^{2+}$ mobilization in the stimulus-response coupling pathway in the H-35 cells, as previously suggested for the hepatocyte [13,14]. Thus, the P_2 -type ATP receptor mechanism in the H-35 hepatoma cell line may be a useful model system for understanding the properties and regulation of Ca $^{2+}$ and phosphoinositide-linked receptors in the liver.

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