ATP- Permeabilizes the Plasma Membrane of Mouse Macrophages to Fluorescent Dyes*

(Received for publication, January 27, 1987)

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Extracellular ATP induces cation fluxes in thioglycolate-elicited mouse peritoneal macrophages and the J774 macrophage cell line apparently due to ligation of a plasma membrane receptor for ATP
tc. We report that ATP permeabilizes the plasma membrane of J774 cells to 6-carboxyfluorescein (376 Da), lucifer yellow (457 Da), and fura-2 (831 Da) but not to trypan blue (961 Da), Evans blue (961 Da), or larger dye conjugates. We employed fluorescence microscopy and quantitative fluorometry to study entry of lucifer yellow into the cytoplasm of J774 cells. Permeabilization to lucifer yellow appears to be mediated by the same ATP- receptor that induces cation fluxes because it was inhibited by divalent cations and low pH, was mediated by the nonhydrolyzable analog adenosine 5'- (β,γ-imido)triphosphate, and because a variant J774 cell line resistant to ATP-induced Rb efflux did not take up lucifer yellow when exposed to ATP. ATP permeabilization was reversed within 5 min by removal of ATP or by addition of divalent cations. ATP also caused a transient increase in lucifer yellow uptake by pinocytosis. These data suggest that ATP ligates a receptor on macrophages which induces the formation of a channel admitting molecules ≤831 daltons into the cytoplasmic matrix and that removal of ATP from the medium causes rapid channel closure.

Investigators have reported various effects of extracellular ATP on different cell types. In some cells, such as murine erythroleukemia cells (7) and Ehrlich ascites tumor cells (8), ATP has been reported to open plasma membrane Ca++, Na+, or Cl- channels. However, in transformed mouse fibroblasts (10) and rat mast cells (3), ATP appears to induce the formation of plasma membrane pores that allow the influx and efflux of larger molecules such as nucleotides. Thus, there is no assurance that all ATP-responsive cells are affected by extracellular ATP in the same fashion or possess similar ATP receptors.

Mouse peritoneal macrophages and the J774 mouse macrophage-like cell line respond to extracellular ATP by membrane depolarization, Na+ influx, K+ efflux, an increase in cytosolic free Ca++, and inhibition of Fc receptor-mediated phagocytosis (11). Cation efflux from J774 cells is not mediated by MgATP2-, which is the prevalent ATP species in physiologic solutions, but rather by ATP4-, which comprises roughly 10% of the ATP present in these solutions, depending on temperature, pH, and divalent cation concentration (12). In the absence of divalent cations, micromolar concentrations of exogenous ATP induce Rb efflux in J774 cells; in the presence of millimolar concentrations of Mg++ or Ca++, millimolar concentrations of ATP are necessary to achieve the same effect. The nonhydrolyzable ATP analog AMP-PNP induces Rb efflux in the absence of divalent cations, confirming that ATP hydrolysis is not required.

The presence of a similar "ATP" receptor has been reported by Gomperts and colleagues in their studies of the rat mast cell (reviewed in Ref. 3). Permeabilization of the rat mast cell plasma membrane is inhibited by divalent cations and acid pH (as is ATP-induced Rb efflux in J774 cells) and is induced only by ATP and a few analogs, but not by other nucleotides. In the rat mast cell, ATP forms plasma membrane channels large enough to allow entry of ethidium bromide and nucleoside triphosphates.

We have investigated the ability of ATP to permeabilize the plasma membrane of J774 cells. We report here that dyes of less than 900 Da entered these cells in the presence of ATP. We used lucifer yellow, which is a fluorescent pH-insensitive dye that has been extensively characterized as a marker for fluid-phase pinocytosis in macrophages (13), as a probe to characterize ATP-mediated permeabilization of these membranes.

*This work was supported by a Clinical Scientist Research Fellowship from the Damon Runyon-Walter Winchell Cancer Fund (to T. H. S.), a generous gift from Samuel Rover, and by United States Public Health Service Grant AI 20516. 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.

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1The abbreviations used are: AMP-PNP, adenosine 5'- (β,γ-imido)triphosphate; ATPyS, adenosine 5'- O-(3-thio)triphosphate; AMP-PCP, adenosine 5'- (β,γ-methylene)triphosphate; dATP, 2'- deoxyadenosine 5'-triphosphate; DMEM, Dulbecco's modified Eagle's medium; PD, Dulbecco's phosphate-buffered saline without divalent cations; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; PIPES, 1,4-piperazine diethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.
cells. As with ATP-stimulated Rb\textsuperscript{+} efflux, ATP\textsuperscript{+} appeared to be the ATP species that mediated lucifer yellow entry into J774 cells. Permeabilization was rapidly reversed by removal or chelation of extracellular ATP.

**MATERIALS AND METHODS**

**Chemicals**—ATP (special grade), GTP, ITP, and ATP\textsubscript{S} were purchased from Boehringer Mannheim. AMP-PCP and AMP-PNP were purchased from Pharmacia L-P. Biochemicals. dATP and 4100 M, fluorescein isothiocyanate-dextran were bought from Sigma. Lucifer yellow CH, fura-2 pentapotassium salt, and fluorescein-conjugated phalloidin were from Molecular Probes (Eugene, OR), and 6-carboxyfluorescein and Evans blue were from Eastman Kodak (Rochester, NY).

**Cells and Media**—The J774 mouse macrophage cell line was maintained in spinner cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated (56 °C, 30 min) fetal bovine serum. An ATP-resistant variant line of J774 cells, ATPR B2, which was selected for resistance to ATP (12), was grown under identical conditions. For quantitation of dye uptake, cells were plated for 6–18 h in 16-mm tissue culture wells at a density of 5–10 x 10\textsuperscript{4} cells/well. For microscopic studies, cells were plated on No. 1 thickness 12-mm glass coverslips at a density of 1–2 x 10\textsuperscript{5} cells/well. For microscopic studies, cells were plated on No. 1 thickness 12-mm glass coverslips at a density of 1–2 x 10\textsuperscript{5} cells/coverslip. During experiments, cells were bathed in DMEM or buffered salt solution (135 mM NaCl, 5 mM KCl, 10 mM HEPES) as indicated. Cell viability was assessed by trypan blue exclusion (14).

**Microscopy**—To assess the effect of ATP on the uptake of fluorescent dyes, cells plated on coverslips were incubated for 5 min in DMEM or buffered salt solution with or without 5 mM ATP and fluorescent dyes at the indicated concentration: lucifer yellow, 0.5 mg/ml; 6-carboxyfluorescein, 40 mg/ml; fura-2, 10 mg/ml; Evans blue, 1.0 mg/ml; trypan blue, 0.05 mg/ml; fluorescein-phalloidin conjugate, 0.04 mg/ml; or fluorescein-dextran 4100, 5.0 mg/ml. Coverslips were washed 5 times in ice-cold phosphate-buffered saline (PD), mounted on microscope slides, and sealed with a mixture of lanolin, paraffin, and vaseline (mixed 1:1:1). Cells were viewed on a Zeiss Photomicroscope III employing a UV filter set (fura-2) and a fluorescein filter set for fluorescence microscopy. Photographs were taken with 400 ASA Tri-X Pan film, exposed at 1600 ASA, and processed with Diafine film developer.

**Measurement of Fluorescent Dye Uptake**—Cell-associated lucifer yellow and fluorescein-dextran fluorescence was measured as previously described (13). Experiments were performed in 24-well plates; all experiments were performed using triplicate cultures. At the end of each experiment, the 24-well plates were immersed in three beakers of PD, the first containing 0.1 mg/ml bovine serum albumin to remove extracellular dye. 500 ml of 0.05% Triton X-100 was added to each well, and the cells were scraped with rubber policeman. 350 ml of the suspension was added to 750 ml of a solution of 0.05% Triton X-100 and 0.1 mg/ml bovine serum albumin. Fluorescence was measured in a Perkin-Elmer 650-40 fluorescence spectrophotometer. Lucifer yellow fluorescence was measured at an excitation wavelength of 495 nm and an emission wavelength of 540 nm; for fluorescein-dextran uptake was expressed as ng of dye/mg of cell protein.

**RESULTS**

**ATP Permeabilizes J774 Cells to Fluorescent Dyes**—J774 cells adherent to glass coverslips were incubated in DMEM or buffered salt solution containing 0.5 mg/ml lucifer yellow at 37 °C for 5 min, washed at room temperature, and examined by fluorescence microscopy. Little lucifer yellow fluorescence was seen, and this fluorescence was found within peripheral cytoplasmic vacuoles characteristic of pinosomes (13). When cells were exposed to 5 mM ATP in addition to lucifer yellow for 5 min, washed, and observed, they demonstrated diffuse lucifer yellow staining of the cytoplasmic and nuclear matrices (Fig. 1).

Using this microscopic assay, we found that ATP also permeabilized the plasma membrane of J774 cells to 6-carboxyfluorescein and pentacarboxyflurcarboxyflurcboxyfluorescein and pentacarboxylate fura-2, dyes that are membrane impermeant under most conditions. J774 cells remained impermeable to trypan blue, Evans blue, a fluorescein-phalloidin conjugate, and a 4100-Da fluorescein-dextran conjugate. Therefore, ATP permeabilizes the J774 plasma membrane to molecules of up to 831 Da (fura-2), but less than 961 Da (trypan blue).

**Lucifer Yellow Uptake during ATP Permeabilization**—We measured the time course of lucifer yellow entry into ATP-permeabilized J774 cells in medium containing 100 μg/ml lucifer yellow. Lucifer yellow uptake in the absence of ATP comprised at most 2% of the uptake seen in the presence of ATP (Fig. 2) and is due to pinocytosis of lucifer yellow (13). Lucifer yellow entry in the presence of ATP was rapid and reached a plateau of 700 ng of lucifer yellow/mg of cell protein at 30 min. Assuming the volume of J774 cells is 5.5 μl/mg cell protein (11), uptake of 700 ng of lucifer yellow/mg of cell protein reflects an intracellular lucifer yellow concentration of 130 μg/ml, or roughly equal to the extracellular lucifer yellow concentration.

ATP did not promote lucifer yellow entry below 18 °C;
lucifer yellow entry increased dramatically between 18 and 37 °C (Fig. 3). In contrast, in the absence of ATP uptake of lucifer yellow occurred by pinocytosis and as expected increased linearly with temperature from 4 to 37 °C (13, 16).

ATP-induced Lucifer Yellow Uptake in J774 Cells Is Maximal at Alkaline pH—In the presence of divalent cations and at neutral pH, ATP exists in a number of ionic forms, principally MgATP\(^{2+}\), CaATP\(^{2+}\), HATP\(^{3+}\), and ATP\(^{4-}\). ATP-induced cation fluxes in J774 cells are mediated by ATP\(^{4-}\) rather than the more prevalent MgATP\(^{2+}\) or any of the protonated species of ATP present (12). To determine whether ATP\(^{4-}\) mediates entry of lucifer yellow into J774 cells we first examined the effect of pH on lucifer yellow uptake. ATP-induced lucifer yellow entry into J774 cells occurred maximally at pH 8.0-8.5; lucifer yellow entry was barely detectable below pH 6.5 (Fig. 4). Since the reaction HATP\(^{3+}\) → H\(^+\) + ATP\(^{4-}\) has a pK of 6.95, this result is consistent with our hypothesis that ATP\(^{4-}\) is the ligand mediating lucifer yellow uptake.

Divalent Cations Inhibit ATP-induced Permeabilization—Because of the high affinities of ATP\(^{4-}\) for Mg\(^{2+}\) and Ca\(^{2+}\), the amount of ATP\(^{4-}\) available to promote cation fluxes in J774 cells is largely dependent on the divalent cation concentration of the medium; divalent cations have an inhibitory effect on ATP-induced Rb\(^{+}\) efflux at any given ATP concentration (12). To determine whether lucifer yellow uptake is similarly inhibited by divalent cations, we measured intracellular accumulation of lucifer yellow in the presence of 5 mM ATP and varying concentrations of Mg\(^{2+}\) or Ca\(^{2+}\) (Table I).

Increasing concentrations of Ca\(^{2+}\) or Mg\(^{2+}\) from 1 to 5 mM caused decreased lucifer yellow entry, and the inhibitory effect of the two cations was additive at intermediate divalent cation concentrations. Mg\(^{2+}\) was more inhibitory than Ca\(^{2+}\) at the same concentration, consistent with the greater affinity of ATP\(^{4-}\) for Mg\(^{2+}\) than for Ca\(^{2+}\).

The inhibitory effect of Mg\(^{2+}\) also was assessed by determining the concentration of ATP needed to promote half-maximal lucifer yellow uptake in buffered salt solution over 5 min at 37 °C with different concentrations of Mg\(^{2+}\); in the absence of divalent cations, 0.5 mM ATP was required, and in the presence of 1 mM Mg\(^{2+}\), 1 mM ATP was necessary.

The ability of Mg\(^{2+}\) to inhibit ATP-induced lucifer yellow influx was confirmed by fluorescence microscopy. J774 cells incubated at 37 °C in DMEM containing lucifer yellow, 5 mM ATP, 5.2 mM Mg\(^{2+}\), and 1.8 mM Ca\(^{2+}\) for up to 30 min displayed lucifer yellow only in cytoplasmic vesicles, consistent with pinocytic uptake of this dye. When 5 mM EDTA was added subsequently for 5 min and the cells were washed in PBS, lucifer yellow was seen to diffusely stain the cytoplasm.

A Nonhydrolyzable ATP Analog Induces Lucifer Yellow Uptake—Permeabilization of the J774 plasma membrane to lucifer yellow was not induced by ITP, GTP, dATP, adenosine, or AMP (Table II). ATP-PNP was nearly as effective as ATP. Of the nonhydrolyzable ATP analogs, AMP-PNP pro-
moted lucifer yellow influx, while AMP-PCP did not. In contrast to its lack of effect on Rb⁺ efflux, ADP induced a small increase in lucifer yellow uptake (12).

J774 cells also were examined by fluorescence microscopy after incubation in DMEM containing 5 mM EDTA, lucifer yellow, and 5 mM of the above nucleotides and ATP analogs. Cells exposed to ATP⁺-S, AMP-PNP, and ADP displayed diffuse fluorescence, confirming that the increased intracellular lucifer yellow detected spectrophotometrically represents membrane permeabilization. In the presence of the other compounds, lucifer yellow was present only in pinocytic vesicles.

ATP Permeabilizes Mouse Peritoneal Macrophages—Lucifer yellow entered the cytoplasm of thioglycolate-elicited mouse peritoneal macrophages treated with 5 mM ATP in DMEM for 5 min at 37 °C. Thus, the capacity of ATP to promote lucifer yellow entry through the plasma membrane is not restricted to continuous cell lines or to transformed cells.

ATP Does Not Promote Uptake of Lucifer Yellow in an ATP-resistant J774 Cell Line—Variant J774 cell lines were selected previously for resistance to extracellular ATP by repeatedly exposing J774 cells to 10 mM concentrations of this nucleotide and subsequent cloning of the resistant cells (12). One of these cell lines, clone ATPR B2, did not efflux "Rb⁺" in the presence of 10 mM ATP in divalent cation-free medium; thus this clone was at least 200-fold less sensitive to extracellular ATP than the parent J774 cell line. Similarly, ATPR B2 cells did not take up lucifer yellow when they were treated with 5 mM ATP in DMEM for 5 min at 37 °C. This result confirms the specificity of the ATP effect and suggests the importance of specific plasma membrane structures in mediating these effects.

ATP-induced Permeabilization of J774 Cells Is Reversed by Removal of ATP from the Medium—To determine whether the J774 cell plasma membrane remained permeable to lucifer yellow after the removal of ATP from the medium, we incubated J774 cells in buffered salt solution containing 5 mM ATP for 5 min, washed the cells, and added medium containing lucifer yellow to the cells for successive 5-min intervals after removal of ATP. Cells incubated with lucifer yellow for the 5-min interval immediately following removal of ATP from the medium exhibited substantial uptake of lucifer yellow, reflecting continued membrane permeability (Fig. 5). During the next 5-min interval much less lucifer yellow entered the cells, and this amount continued to diminish over subsequent 5-min intervals.

To determine whether the small amount of lucifer yellow entering cells subsequent to the 0–5-min interval appeared within the cytoplasmic matrix, we examined cells treated as in the above experiment by fluorescence microscopy. Diffuse cytosolic fluorescence was present in cells incubated with lucifer yellow during the 0–5-min interval. In cells incubated with lucifer yellow during subsequent 5-min intervals, lucifer yellow fluorescence appeared only in cytoplasmic vesicles. Thus, membrane permeability persisted for up to 5 min after ATP was removed from the cells; however, at all times thereafter lucifer yellow was unable to cross the plasma membrane.

ATP-induced permeabilization was also reversed by the addition of sufficient Mg²⁺ to chelate all extracellular ATP. In these experiments, J774 cells incubated in DMEM containing 0.5 mg/ml lucifer yellow and 5 mM ATP for 5 min accumulated 976 ng of lucifer yellow/mg of cell protein, and cells incubated with lucifer yellow and ATP for 10 min accumulated 1761 ng of lucifer yellow/mg of cell protein. In contrast, cells incubated for 10 min in medium containing lucifer yellow and ATP to which 5 mM MgSO₄ was added after the first 5 min contained only 726 ng of lucifer yellow/mg of cell protein. The addition of MgSO₄ after 5 min prevented further accumulation of lucifer yellow. Because these cells accumulated less lucifer yellow than cells exposed to ATP and lucifer yellow for 5 min, it is possible that lucifer yellow may have effluxed from the cells after permeabilization was reversed.

ATP Causes a Transient Increase in Pinocytic Uptake of Lucifer Yellow in J774 Cells—To determine whether the increase in lucifer yellow uptake detected in the 5–10 min interval after removal of ATP from the medium was due to an increase in pinocytic uptake rather than a persistent increase in plasma membrane permeability, we assessed the uptake of 4100-Da fluorescein-dextran during successive 5-min intervals after exposure to ATP. During a 5-min incubation with fluorescein-dextran and ATP, J774 cells did not accumulate more fluorescein-dextran than cells incubated without ATP (data not shown), confirming our microscopic observation that 4100-Da fluorescein-dextran does not enter ATP-permeabilized cells. However, over the two subsequent 5-min intervals the amount of fluorescein-dextran internalized by cells increased and thereafter gradually decreased (Fig. 5). Fluorescence microscopy of parallel samples showed only vesicular uptake of fluorescein-dextran. Since fluorescein-dextran does not cross the plasma membrane after ATP permeabilization, this transient increase in fluorescein-dextran uptake indicates that during cell recovery from ATP treatment there is an increase in pinocytic uptake of the dye. These results support the microscopic observation that the J774 plasma membrane is no longer permeable to lucifer yellow after 5 min. These findings are consistent with those of Cohn and Parks (17), who found that adenine nucleotides increase the number of pinocytic vesicles in mouse peritoneal macrophages.

**DISCUSSION**

These studies demonstrate that extracellular ATP increases the permeability of the J774 plasma membrane not only to...
small cations such as Na\textsuperscript{+}, K\textsuperscript{+}, and Rb\textsuperscript{+}, but also to molecules as large as 831 Da. Membrane permeability to fluorescent dyes was induced by ATP\textsuperscript{4-} rather than MgATP\textsuperscript{2-}, as was ATP-mediated \textsuperscript{86}Rb\textsuperscript{+} efflux (12). Both dye uptake and cation fluxes were caused by ATP, AMP-PNP, and ATP\textsubscript{2-}S, but not by nucleoside triphosphates other than ATP. These data suggest that monovalent cation fluxes and dye permeability are mediated by the same membrane receptor for ATP\textsuperscript{4-} and that this ATP\textsuperscript{4-} receptor induces the formation or opening of a nonelective transmembrane channel with a relatively large pore size. All the dyes tested, both those that were permeant and those that were impermeant, are anionic (sulfated or carboxylated) molecules; it is, therefore, unlikely that the apparent size specificity of the pore induced by extracellular ATP\textsuperscript{4-} is due to differences in charge.

These results agree in most respects with the investigations of Gomperts and colleagues on the effects of ATP on the permeability of the rat mast cell (3, 9). ATP-induced permeability of rat mast cells also depends on the concentration of ATP\textsuperscript{4-} and, therefore, also is maximal in alkaline medium and inhibited by divalent cations. Furthermore, molecules such as nucleotides can enter mast cells in the presence of ATP. The transformed 3T6 fibroblast cell line similarly can be permeabilized by ATP\textsubscript{2-}S and AMP-PNP, but also by AMP-PCP (18).

The inability of ATP to induce plasma membrane permeabilization in the ATPR B2 clone provides further evidence that permeabilization to dyes and cation fluxes is mediated by the same receptor. In addition, our ability to select ATP-resistant clones is strong evidence that ATP-induced permeabilization results from ligation of a specific membrane molecule and is not a nonspecific effect of ATP on the plasma membrane.

Two observations can be made considering the large size and apparent lack of ion specificity of the ATP-induced channel. First, it is possible that ATP-mediated membrane permeabilization is not the physiologically relevant event induced by exogenous ATP. The amount of ATP necessary to raise intracellular free [Ca\textsuperscript{2+}] is considerably less than the quantities required for the induction of ion fluxes (11). ATP-induced increases in cytosolic [Ca\textsuperscript{2+}] may be the physiologic response induced by extracellular ATP, and permeabilization may reflect the cellular response to supraphysiologic concentrations of ATP that have little physiologic relevance.

Second, because the ATP-induced pore is large enough to permit the efflux of many cytosolic molecules, it is possible that the primary function of the ATP-induced channel is to allow certain intracellular molecules to flow into a confined extracellular space, such as might be formed by two cells adherent to one another, as occurs when macrophages form a syncytium around a foreign body or an infectious agent, or to allow such molecules to flow from one cell into another permeabilized cell. In this latter instance, the ATP\textsuperscript{4-} receptor might be analogous to the gap junction, which forms pores of about the same size as the ATP-induced channel (19). Macrophages form intimate contacts with a variety of cell types, such as lymphocytes, platelets, and endothelial cells. This mechanism of intercellular signaling might allow macrophages to influence or be affected by such cells and may, therefore, be important in regulation of processes such as inflammation, immunity, or atherogenesis.

Although the membrane receptors and channels responsible for ATP-induced permeabilization have not been identified and the physiologic relevance of these events is unclear, there is considerable evidence that J774 cells and thioglycolate-elicited mouse peritoneal macrophages possess specific cell surface receptors that recognize ATP\textsuperscript{4-} and that activation of these receptors does not require ATP hydrolysis. At present it is unclear whether these receptors are ligand-operated channels or whether these receptors are signaling devices that activate other membrane molecules, leading to pore formation. In addition, although it appears that dye entry and cation fluxes are mediated by the same ATP\textsuperscript{4-} receptor, it is not clear whether these events are mediated by the same channels.

In any event, ATP\textsuperscript{4-}-induced permeabilization may have considerable usefulness as a method of introducing small molecules into macrophages: it has already been effectively employed in this manner in the rat mast cell (20). In addition, the ability to introduce a variety of small molecules into a continuous cell line may allow the identification and selection of cell lines that express mutations in cytoplasmic metabolism or intracellular transport of those molecules. Recent studies of cytoplasmic degradation of proteins (21), intracellular processing of viral antigens (22), and the mechanism of multiple drug resistance in cancer cells (23) have highlighted the importance of transport of polypeptides and other small molecules through and from the cytoplasm; these and other processes may be amenable to analysis by this technique.

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