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Exogenous ATP and other nucleoside phosphates modulate epidermal growth factor receptors of A-431 epidermoid carcinoma cells

(P2 purinergic receptors/ligand binding/inositol trisphosphate/calcium/receptor phosphorylation)

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ABSTRACT **The binding of epidermal growth factor (EGF) by A-431 human epidermoid carcinoma cells was reduced after exposure of the cells to low concentrations (0.01–1 mM) of ATP and other nucleoside 5′-triphosphates at 37°C, but not at 0°C. This was due to loss of high-affinity EGF binding sites. The modulation was associated with transient increases in inositol phosphate synthesis and intracellular Ca^{2+} and with phosphorylation of the EGF receptor on serine and threonine. There was no evidence for entry of labeled ATP into the cells. ATP appeared to bind to specific cell surface receptors. Such binding was demonstrated directly with the nonmetabolizable ATP analogue adenosine 5′-[β,γ-imido]triphosphate.**

The binding of epidermal growth factor (EGF) to its cell surface receptors is regulated by many different molecules, including EGF itself, other peptide hormones, and tumor-promoting phorbol esters (1, 2). Regulation of EGF binding by all of these agents appears to be mediated through activation of protein kinase C and consequent phosphorylation of the EGF receptor on threonine (3). The phosphorylated EGF receptor has reduced affinity for EGF and a lower tyrosine kinase activity than the unmodified receptor (1).

Extracellular ATP and other nucleoside triphosphates, apparently binding to specific receptors, affect cell metabolism in many ways (4–6). Of interest here are reports that extracellular ATP elevates *myo*-inositol 1,4,5-trisphosphate ($InsP_3$) and Ca^{2+} levels in isolated hepatocytes (7) and $InsP_3$ levels in A-431 epidermoid carcinoma cells (3). These reports suggest that extracellular ATP can regulate EGF receptor expression in intact cells by activating the protein kinase C pathway. Here we show that ATP, as well as other nucleoside triphosphates, does in fact modulate the high-affinity EGF receptor of A-431 cells. The modulation of the EGF receptor is associated with phosphorylation of the receptor on serine and threonine and is preceded by increases in intracellular $InsP_3$ and Ca^{2+}. The process appears to be mediated by the binding of ATP to a specific surface receptor.

MATERIALS AND METHODS

Materials. EGF, purified from mouse submaxillary glands by the method of Savage and Cohen (8), was the generous gift of Akira Komoriya (Meloy Laboratories). EGF was radioiodinated with lactoperoxidase (9) to a specific activity of 300 μCi/μg (1 μCi = 37 kBq). The labeled material, which gave a single band in SDS/11% polyacrylamide gels, was stored at −20°C until used. Polyclonal anti-EGF receptor antibody R1 (10, 11) was kindly provided by Barbara Knowles (Wistar Institute, Philadelphia). Sodium [^{125}I]iodide, [^{32}P]phosphoric acid, [α-^{32}P]ATP, and [γ-^{32}P]ATP were purchased from New England Nuclear. Fixed *Staphylococcus aureus* (Pansorbin) was purchased from Calbiochem. Adenosine 5′-[β,γ-imido]-triphosphate (Ado*PP*[NH]*P*) was obtained from Boehringer Mannheim. [2,8-^{3}H]ATP and [α-^{32}P]Ado*PP*[NH]*P* were obtained from ICN. Specific activity of the latter was 25.0 Ci/mmol and its purity was >98%. All other chemicals were purchased from Sigma. Nucleoside phosphates were >99% pure, according to the supplier's assay.

Cell Culture. A-431 human epidermoid carcinoma cells originated with J. Delarco (National Institutes of Health) and were a gift of Joseph Schlessinger (Rorer Biotechnology Research Laboratories, King of Prussia, PA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Reheis) and 2.4 mg of glucose per ml. Cells were fed every 3–4 days and passed at 1:20 dilution once a week.

Incubation with Nucleotides and EGF Binding Assay. Two days before an experiment, cells were seeded in wells of a 24-well culture plate to reach about 10^6 cells per well on the day of the experiment. For assay, each well was washed twice with 0.5 ml of phosphate-buffered saline (PBS: 0.14 M NaCl/0.02 M phosphate buffer/0.01 M KCl, pH 7.0) containing 0.1% bovine serum albumin (BSA). Washed cells were first incubated in serum-free DMEM supplemented with 0.1% BSA and 2.4 mg of glucose per ml, with added nucleoside phosphates as appropriate. Unless otherwise stated, the final pH of the mixture was 7.5. After this incubation at 0°C or at 37°C, cells were chilled and washed twice with 0.5 ml of PBS/0.1% BSA. Mixtures of 0.1–0.2 nM ^{125}I-labeled EGF and 0.1–40 nM unlabeled EGF were added to washed cells in 250 μl of binding buffer (140 mM NaCl/5 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/20 mM Hepes/0.1% BSA, final pH 7.0) and the plates were incubated on ice for 4 hr, by which time specific binding reached a plateau. Nonspecific binding was always <5% of the total binding. After incubation, cells were washed three times with cold PBS/0.1% BSA and then lysed in 0.1 M NaOH/1% SDS/2% Na_2CO_3 (250 μl per well). Two hundred microliters of the lysate was taken for ^{125}I determination with a Beckman γ counter. All experiments were done in duplicate and mean values were computed after normalization for number of cells per well, determined by counting cells trypsinized from duplicate wells.

The affinities (K_a) and the number of EGF binding sites were calculated from binding data by using the LIGAND curve-fitting program of Munson and Rodbard (12).

^{32}P-Labeling of Cells and Immunoprecipitation of EGF Receptor. Cells cultured as for binding experiments were

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Abbreviations: EGF, epidermal growth factor; $InsP_3$, inositol 1,4,5-trisphosphate; Ado*PP*[NH]*P*, adenosine 5′-[β,γ-imido]triphosphate; TPA, "12-tetradecanoylphorbol 13-acetate" (phorbol 12-myristate 13-acetate); PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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washed three times with 1 ml of 130 mM NaCl/20 mM Hepes/0.1% BSA, pH 7.2, and then cultured in 0.5 ml of phosphate-free DMEM containing 0.1% BSA and 0.2 mCi of carrier-free [^{32}P]phosphoric acid per ml. In some experiments the medium also contained either ATP, EGF, or phorbol 12-myristate 13-acetate ("12-tetradecanoylphorbol 13-acetate," TPA). After incubation for 30 min at 37°C, the cells were chilled on ice water, washed with cold PBS/0.1% BSA, and lysed according to procedures used previously in this laboratory (13, 14).

Aliquots of different lysates, with equal radioactivities, were incubated with 10% *S. aureus* (Pansorbin, Calbiochem) for 1 hr at 4°C. The cleared supernatant was incubated for 2 hr at 4°C with anti-EGF receptor antibody, prebound to *S. aureus*. The immunocomplexes were washed six times with 150 mM NaCl/10 mM Tris·HCl/0.5% Nonidet P-40/0.5% sodium deoxycholate/0.05% SDS, pH 8.0. Details of the solubilization of the washed precipitate and its analysis in 5–15% gradient polyacrylamide gel have been given (10, 14).

EGF receptor bands located by autoradiography were cut from the dried gel and each piece was dissolved by heating at 65°C overnight with 100 μl of 30% H_2O_2 in a tightly capped vial. Protosol (1 ml, NEN) was added to the liquefied gel and mixed with 5 ml of a toluene-based scintillation mixture. Radioactivity was then determined by liquid scintillation counting.

Determination of Phospho Amino Acids in EGF Receptor. While various ligands were added, A-431 cells were ^{32}P-labeled as described in the previous section except that the radioactivity added was increased to 1 mCi per well. The EGF receptor was immunoprecipitated and gel-purified as described above. After excision of the receptor band from the dried gel, the ^{32}P-labeled receptor was hydrolyzed and recovered from the gel by incubation with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (50 μg/ml in 1.5 ml of 50 mM ammonium carbonate, pH 8.0) for 6 hr at 37°C as described by Reynolds *et al.* (15). The residual gel was extracted again with 1 ml of H_2O, which was combined with the first extract. The combined extracts were centrifuged and the supernatant was filtered (Millex-GV$_4$, 0.22 μm), lyophilized, dissolved in H_2O, and relyophilized. Tryptic peptides were then hydrolyzed in 6.0 M HCl at 105°C for 3 hr and HCl was removed by lyophilization. The radioactivity of [^{32}P]phosphate incorporated into total protein (trichloroacetic acid-insoluble fraction) before immunoprecipitation was determined as described above, and the amounts of samples applied to a cellulose TLC plate were normalized so that they represented equal amounts of radioactivity from trichloroacetic acid-insoluble proteins. Two-dimensional thin-layer electrophoresis was carried out, first at pH 1.9 and then at pH 3.5, as described by Cooper *et al.* (16). Unlabeled standards were also run; their locations were detected by ninhydrin and acid molybdate staining (17).

Measurement of Intracellular Ca^{2+} with Indo-1 Calcium Indicator. Special dishes were prepared for measuring the intracellular Ca^{2+} of cultured cells. A hole of 18-mm diameter was made at the center of 35-mm Falcon dishes and closed with a round coverglass of 22-mm diameter. A-431 cells (10^5 cells per ml; 2 ml) were inoculated into the dishes and cultured for 2 days in DMEM containing 10% fetal bovine serum. The medium was removed and cells were incubated at 37°C for 15 min with fresh culture medium that contained 20 μM indo-1 pentakis(acetoxymethyl) ester. The excess dye solution was rinsed off and cells were covered with 1 ml of PBS/0.1% BSA or, in some experiments, with PBS/0.1% BSA/0.5 mM EDTA. They were then analyzed immediately on the ACAS 470 work station (Meridian Instruments, Okemos, MI) (18) at room temperature. Intracellular indo-1 was viewed by scanning a laser beam (355-nm light) over single cells in steps of 2.5 μm and detecting fluorescence emission at both 400 and 485 nm. Scans were taken at 10-sec intervals and images of 20–25 scans were stored in the ACAS computer. Intracellular Ca^{2+} concentrations were computed from the two fluorescence images (400 and 485 nm) of each cell before and after addition of ATP, according to the procedure of Grynkiewicz *et al.* (19), by using the ACAS software.

Measurement of Inositol Mono-, Bis-, and Trisphosphates. Inositol phosphate formation by A-431 cells before and after addition of ATP to extracellular fluid was measured according to Downes and Wusteman (20).

Binding of an ATP Analogue to A-431 Cells. A-431 cells (3×10^5) were inoculated in each well of a 24-well plate and cultured for 2 days. They were washed twice with binding buffer (PBS/0.1% BSA/6 mM glucose) and preincubated in the same buffer at 37°C for 30 min. The preincubated cells were then washed twice with cold binding buffer and incubated with 0.5 ml of binding buffer containing 1 μCi of [α-^{32}P]Ado*PP*[NH]*P* and various concentrations of nonradioactive Ado*PP*[NH]*P* in duplicate at 0°C for 2.5 hr; during that time the binding reached equilibrium. The cells in each well were then quickly washed with binding buffer at 0°C and lysed in 1 mM Tris/0.1% SDS. The solubilized radioactivity was measured in a liquid scintillation counter. Nonspecific binding was estimated in the presence of 1 mM unlabeled Ado*PP*[NH]*P*.

RESULTS

Modulation of EGF Binding by ATP and Other Nucleotides. As reported by others (21–23), A-431 cells bind EGF at both high- and low-affinity sites. This is evident in the Scatchard plot of EGF binding (Fig. 1*A*). The calculated K_a is $\approx 5 \times 10^9$ M^{-1} for the high-affinity site and $3–5 \times 10^7$ M^{-1} for the low-affinity site. There are 20,000–30,000 high-affinity sites, and ≈10 times as many low-affinity sites, per cell. Incubation of cells with 1 mM ATP at 0°C for 30 min before binding EGF had no effect on the number or quality of binding sites (Fig. 1*A*). However, incubation with the same concentration of ATP at 37°C reduced the number of high-affinity sites to about one-third or less of controls (Fig. 1*B*).

EGF binding at 37°C was affected by ATP concentrations as low as 0.01 mM (Fig. 2*A*). Concentrations of ATP as high as 10 mM had no effect on EGF binding if cells were incubated on ice, instead of at 37°C (Fig. 2*A*). Some effect of 1 mM ATP was detectable after 5 min at 37°C, but the maximum effect required 15–30 min (Fig. 2*B*). ATP was

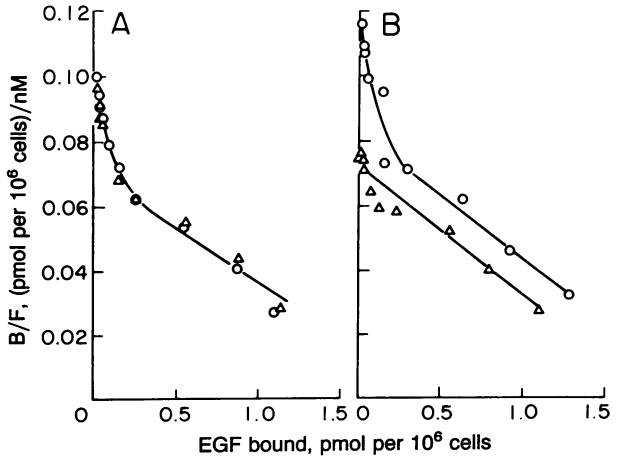

FIG. 1. Effects of incubation temperature on ATP-induced modulation of EGF receptors. Cells were incubated with 1 mM ATP (△) or with buffer (○) for 2.5 hr at 0°C (*A*) or for 30 min at 37°C (*B*) before measurement of EGF binding. B/F, bound/free.

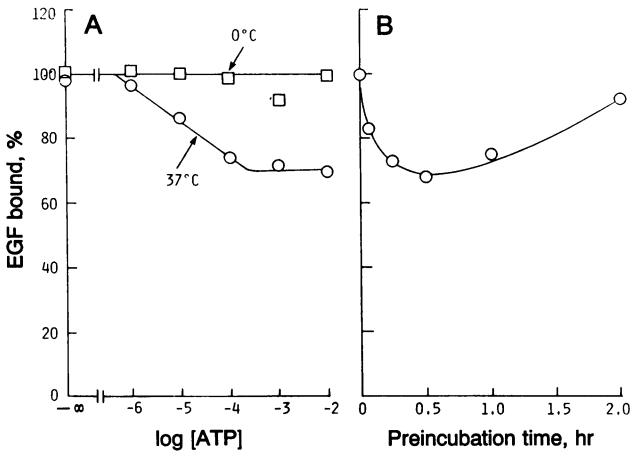

FIG. 2. Effects of ATP concentration and incubation time on subsequent binding of EGF to its receptors on A-431 cells. (*A*) Inhibition of EGF binding as a function of ATP concentration in incubation at 0°C (□) or 37°C (○). (*B*) Effect of time of preincubation with 1 mM ATP on subsequent binding of EGF. Binding was assayed with a low concentration (0.2 nM) of ^{125}I-labeled EGF in order to detect the changes of high-affinity binding sites.

effective in reducing EGF binding when cells were incubated over a pH range from 6 to 8.5 (data not shown).

The nonmetabolizable ATP analogue Ado*PP*[NH]*P* was 1–2 orders of magnitude less effective than ATP in reducing EGF binding at high-affinity sites (data not shown). Incubation of cells with nucleoside phosphates other than ATP had some effect on subsequent EGF binding. At 1 mM, ADP was less effective than ATP; AMP had no detectable effect. One millimolar UTP or GTP reduced EGF binding, but to a smaller extent than in cells incubated with ATP. Incubation with CTP had no effect on subsequent EGF binding. 5′-Di- and 5′-monophosphates of guanosine, uridine and cytidine had little or no effect on subsequent EGF binding (data not shown).

Cells were incubated with either [γ-^{32}P]ATP or [α-^{32}P]ATP to determine the fate of added ATP. No radioactivity was incorporated into cells incubated with 0.1 mM [α-^{32}P]ATP, whereas about 0.5% of the radioactivity was associated with cells after incubation with [γ-^{32}P]ATP at 37°C for 30 min. We found that 33% of added [γ-^{32}P]ATP had been hydrolyzed into ADP and inorganic phosphate under these conditions. Hence the cell-associated radioactivity was likely due to entry of free [^{32}P]phosphate; indeed, 95% of the cell-associated radioactivity was trichloroacetic acid-soluble. Thus, no more than 0.02% of radioactive phosphate from the exogenous [γ-^{32}P]ATP added to A-431 cultures was incorporated into any cellular macromolecules. Even this overestimates the entry of intact ATP, since cells were sampled after 30 min of incubation. These facts suggest that the main effect of added ATP is extracellular.

Inositol Phosphate Metabolism After Addition of ATP. The synthesis of Ins*P*$_3$ and inositol bisphosphate increased significantly within 15–30 sec of addition of 1 mM ATP. These increases were followed by an increase in inositol monophosphate 1–2 min after addition of ATP (Fig. 3). The effects of extracellular ATP on the EGF receptor are similar to those of TPA or diacylglycerols. The latter, which are formed upon breakdown of phosphatidylinositol bisphosphate, also stimulate the phosphorylation of the EGF receptor. Besides diacylglycerols, inositol phospholipid metabolism yields Ins*P*$_3$.

Ca^{2+} Mobilization in A-431 Cells by Extracellular ATP. Ins*P*$_3$ is believed to mobilize intracellular Ca^{2+}. Accordingly, we used the Ca^{2+} probe indo-1 to look for effects of extracellular ATP on intracellular Ca^{2+} (Fig. 4). ATP at 0.01–1 mM

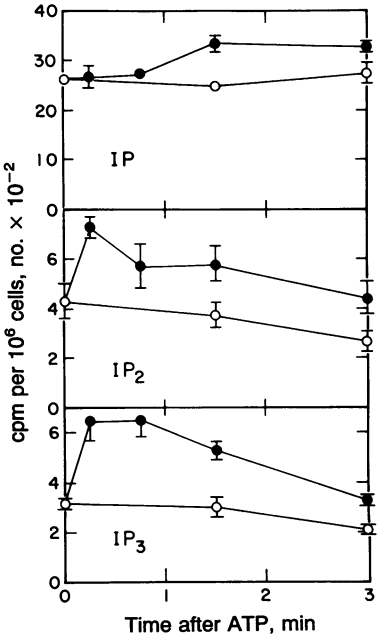

FIG. 3. Time course of synthesis of inositol mono-, bis-, and trisphosphates (IP, IP$_2$, and IP$_3$) in A-431 cells incubated without (○) or with (●) 1 mM ATP.

rapidly increased the intracellular Ca^{2+} in most cells. Maximum values were reached as early as 30 sec after addition of ATP. Free Ca^{2+} then decreased, reaching its original level within 3 min. Single cells differed considerably in their response to extracellular ATP, but responses were evoked in some cells by ATP concentrations as low as 0.01 mM. The increases observed in Ca^{2+} were due to mobilization of intracellular Ca^{2+}, since the same results were obtained whether or not 0.5 mM EDTA was present in the medium (data not shown).

Other nucleoside phosphates had effects on intracellular Ca^{2+} that paralleled their effects in down-regulating EGF receptors. GTP, UTP, ADP, and Ado*PP*[NH]*P* all significantly increased intracellular Ca^{2+}, while CTP and AMP had no effect (Table 1). Readdition of ATP had no further effect on Ca^{2+} in cells previously exposed to ATP or to Ado*PP*-[NH]*P*.

External ATP Increases Phosphorylation of EGF Receptor. McCaffrey *et al.* (22) reported that incubation of A-431 cells with phorbol ester extensively phosphorylates the EGF binding sites at serine and threonine residues, resulting in signif-

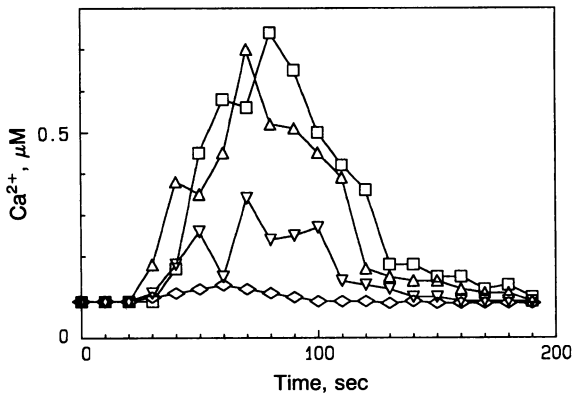

FIG. 4. Intracellular Ca^{2+} of four A-431 cells exposed to ATP. Cells were loaded with indo-1 as described in the text and transferred to 1.0 ml of fresh PBS/0.1% BSA. After measurement of baseline levels of Ca^{2+} (first three points), ATP was added to a final concentration of 0.1 mM.

Table 1. Intracellular Ca^{2+} in A-431 cells exposed to Ado*PP*[NH]*P* and various nucleoside phosphates

Compound(s)*	Number of cells examined	Intracellular Ca^{2+}, % of control†, (mean ± SE)	P‡
Exp. 1			
ATP	10	148 ± 7.4	0.01
ATP + ATP§	10	87 ± 4.4	NS
Ado*PP*[NH]*P*	10	244 ± 10.4	0.001
Ado*PP*[NH]*P* + ATP¶	8	106 ± 4.5	NS
Exp. 2			
ATP	10	151 ± 14.1	0.05
ADP	10	199 ± 20.6	0.001
AMP	9	104 ± 4.1	NS
Exp. 3			
ATP	9	252 ± 14.8	0.001
GTP	7	217 ± 14.4	0.001
UTP	11	240 ± 8.9	0.001
CTP	6	115 ± 9.2	NS

*Concentration, 0.1 mM, except for Ado*PP*[NH]*P* and AMP, which were at 1 mM.
†Control values for experiments 1, 2, and 3 were 0.112 ± 0.047 μM (n = 40), 0.135 ± 0.0021 μM (n = 30), and 0.190 ± 0.0806 μM (n = 38), respectively. Comparisons of control with treated cells were made using control subgroups of 8–11 cells each.
‡Probability of the increase observed; NS, not significant.
§Cells were treated with 0.1 mM ATP, washed with PBS/0.1% BSA, and then exposed to 0.1 mM ATP.
¶Cells were treated first with 1 mM Ado*PP*[NH]*P*, washed with PBS/0.1% BSA, and then exposed to 0.1 mM ATP.

icant loss of high-affinity EGF binding sites from the cell surface. To see whether external ATP also changed receptor phosphorylation, we immunoprecipitated EGF receptor with a molecular weight of 170,000–180,000 from the lysate of ^{32}P-labeled cells. As reported previously (22), TPA and EGF itself stimulated the phosphorylation of EGF receptor (2.6- and 2.9-fold, respectively; Table 2). ATP also stimulated the phosphorylation; EGF receptor phosphorylation increased

Table 2. EGF, TPA, and ATP stimulation of EGF receptor phosphorylation

Treatment	^{32}P cpm		Relative value
	In immunoprecipitate*	Normalized for cell number	
None	248 (31)	218	1
TPA (100 ng/ml)	594 (37)	564	2.6
EGF (150 nM)	667 (34)	637	2.9
ATP (1 mM)	392 (17)	362	1.7
ATP (0.1 mM)	265	235	1.1

*Radioactivity in EGF receptor band obtained by SDS/polyacrylamide gel electrophoresis of immunoprecipitate. Values in parentheses are for bands obtained with nonimmune serum; these values were not subtracted.

about 70% in 1 mM ATP but <10% in 0.1 mM ATP (Table 2) or 1 mM AMP (data not shown). Thus, ATP at concentrations that reduce the number of high-affinity EGF binding sites also stimulates phosphorylation of EGF receptor.

Phospho amino acids were isolated from immunoprecipitated EGF receptor. Receptor from EGF-treated cells was phosphorylated on serine, threonine, and tyrosine. Receptor from TPA-treated cells was phosphorylated largely on serine, and receptor from ATP-treated cells was phosphorylated on both serine and threonine (Fig. 5).

Binding of ATP and Ado*PP*[NH]*P* to A-431 Cells. The results suggest that A-431 cells bear binding sites for ATP and other nucleoside phosphates. We attempted to demonstrate ATP binding sites directly under conditions that inhibited cellular ectonucleases. Binding of 0.1 μM [^3H]ATP in the presence of 0.5 mM EDTA (to inhibit ectonucleotidases) and of 0.5 mM adenosine (to block P1 purine nucleoside receptors) approached saturation after about 4 hr at 20°C. The plateau in binding did not appear to be due to an equilibrium between uptake and degradation of the ligand, since no [^3H]adenosine was detected in the incubation supernatants (data not shown).

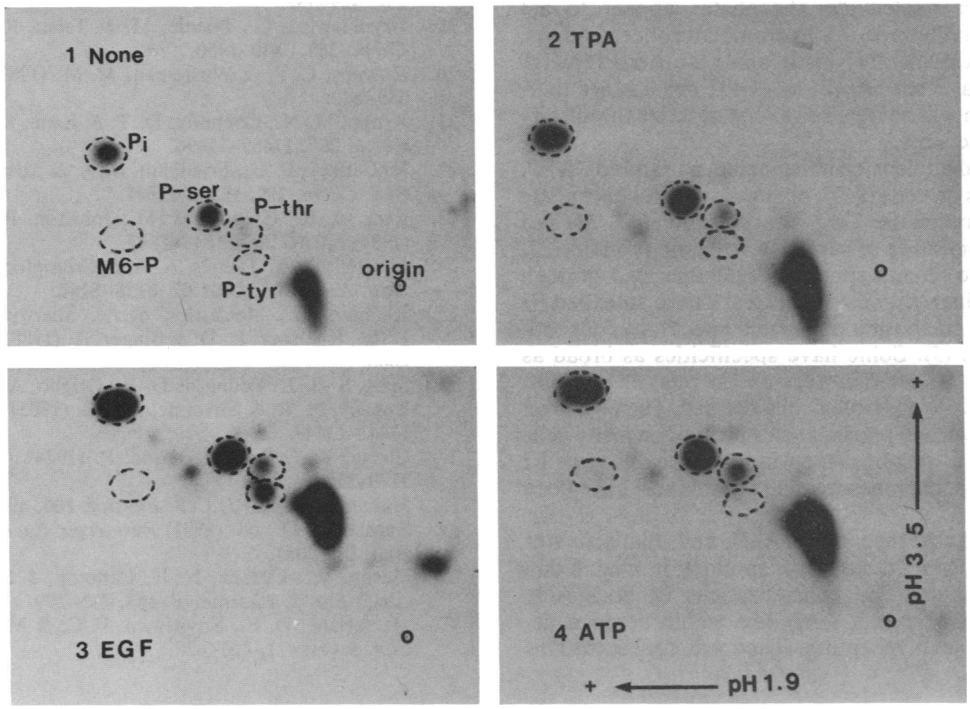

FIG. 5. Phospho amino acids of isolated EGF receptors. Receptors were purified and hydrolyzed for phospho amino acid analysis. Culture conditions: *1*, no additions to the medium; *2*, TPA (100 ng/ml); *3*, EGF (150 nM); *4*, ATP (1 mM). Positions of standards are indicated: P_i, inorganic phosphate; P-ser, phosphoserine; P-thr, phosphothreonine; P-tyr, phosphotyrosine; M6-P, mannose 6-phosphate.

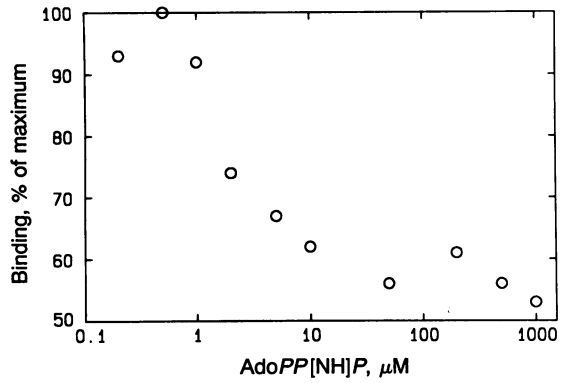

FIG. 6. Displacement of α-[^{32}P]Ado*PP*[NH]*P* from surface receptors by unlabeled Ado*PP*[NH]*P*.

We confirmed this result in medium without EDTA by using the nonhydrolyzable ATP analogue Ado*PP*[NH]*P*. Binding of α-[^{32}P]Ado*PP*[NH]*P* at 0°C was saturable, with a maximum reached within 2.5 hr (data not shown), and was blocked by 1 mM unlabeled Ado*PP*[NH]*P* (Fig. 6).

DISCUSSION

Incubation of A-431 cells with ATP and other nucleoside phosphates reduced the number of high-affinity binding sites for EGF. Receptor modulation by ATP is associated with increased receptor phosphorylation on serine and threonine. The rapid metabolic changes in A-431 cells after binding of nucleoside phosphates, increases in inositol bis- and trisphosphates and mobilization of cell Ca^{2+}, indicate that the effect of ATP on EGF receptors is probably mediated through hydrolysis of inositol lipids and subsequent activation of protein kinase C. This pathway for regulation is known to be activated after binding of EGF to its receptor (1, 2) and similar mechanisms appear to be effective in down-regulating antigen receptors (24) and CD4 antigens (25) of T cells.

ATP and other nucleoside phosphates appear to act through specific receptors. At micromolar concentrations, both ATP and its nonhydrolyzable analogue Ado*PP*[NH]*P* bound saturably and specifically to A-431 cells. More thorough investigation will be required to characterize the affinity and number of the sites.

Nucleosides and nucleoside phosphates, particularly ATP, have many effects on cells (3, 5), changing inositol phosphate levels (5) and intracellular Ca^{2+} concentrations (7, 26) and inducing phosphorylation of surface membrane proteins (27, 28). Most of these effects appear to be moderated through nucleoside phosphate-specific receptors. These so-called P2 receptors vary in nucleoside phosphate specificity, depending upon cell type (5). Some have specificities as broad as those shown here (some examples are in refs. 29 and 30). These specificities are physiologically defined. The high level of ectonucleotidases and phosphatases present on many cells makes it difficult to demonstrate binding of agonists to P2 receptors, though some measurements of this sort have been made (31).

We have defined a P2 receptor on A-431 cells that activates metabolism of phosphatidylinositol, resulting in modulation of the EGF receptor. The concentrations of nucleoside phosphates effective in this assay are within a range described for other such receptors. They are concentrations that may be achieved by release of nucleoside phosphates from viable cells (6). Hence nucleoside phosphates are potentially physiological modulators of growth factor receptor activity *in vivo*.

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