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Mechanism of Extracellular ATP-Induced Proliferation of Vascular Smooth Muscle Cells

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SUMMARY

The mitogenic effect of extracellular ATP was examined in cultured rat aortic smooth muscle cells (VSMCs). ATP, 2-methylthio-ATP, and ADP stimulated [^{3}H]thymidine and [^{3}H]leucine incorporation and cell growth. AMP, adenosine, UTP, and P_{2X} agonists showed little of these effects. Reactive blue 2, a P_{2Y} purinoceptor antagonist, was effective in suppressing the mitogenic effect of ATP and 2-methylthio-ATP, indicating that extracellular ATP-induced VSMC proliferation is mediated by P_{2Y} purinoceptors. The P_{2Y} purinoceptor activation was coupled to a pertussis toxin (PTX)-insensitive G protein (G_q) and triggered phosphoinositide hydrolysis with subsequent activation of protein kinase C (PKC), Raf-1, and mitogen-activated protein kinase (MAPK) in VSMCs. In response to ATP, both 42- and 44-kDa MAPKs were activated, and tyrosine was phosphorylated. Western blot analysis using PKC isozyme-specific antibodies indicated that VSMCs express PKC-α, PKC-δ, and PKC-ζ. A complete down-regulation of PKC-α and PKC-δ was seen after 24-hr treatment with 12-*O*-tetradecanoylphorbol-13-acetate. When cells were pretreated with 12-*O*-tetradecanoylphorbol-13-acetate for 24 hr and subsequently challenged with ATP, Raf-1 activation and 42-kDa as well as 44-kDa MAPK tyrosine phosphorylation failed to be induced. These results demonstrate that ATP-induced Raf-1 and MAPK activations involve the activation of PKC-α and PKC-δ. P_{2Y} purinoceptor stimulation with ATP also caused accumulation of c-*fos* and c-*myc* mRNAs. Both Reactive blue 2 and staurosporine significantly blocked this increase by ATP. In conclusion, the mitogenic effect of ATP seemed to be triggered by activation of the G_q protein-coupled P_{2Y} purinoceptor that led to the formation of inositol trisphosphate and activation of PKC. PKC and, in turn, Raf-1 and MAPK were then activated, leading eventually to DNA synthesis and cell proliferation.

Cardiovascular disease remains the main cause of death in the world; atherosclerosis, the principal cause of myocardial and cerebral infarctions, accounts for the major cause of these deaths in the United States and Western Europe (1), probably in Taiwan as well. In the development of advanced lesions of atherosclerosis, abnormal proliferation of VSMCs is known to be a key event. A large number of potent biological mediators are released from blood cells, including growth factors, cytokines, and vasoregulatory molecules, and may participate in the proliferation of VSMCs (2). ATP is stored in the dense bodies of platelets and secreted after activation of platelets (3). In addition to regulating blood vessel contraction and platelet aggregation (4, 5), extracellular ATP has been shown to stimulate cell proliferation, including Swiss 3T3, A431, DDT$_1$MF-2, and renal mesangial cells (6–9). Therefore, ATP may show a long term trophic effect on the growth of VSMCs. In fact, extracellular ATP has been found to play the pivotal role in the control of vascular cell growth and neointima formation after hypertension, renal vascular injury, and atherosclerosis *in vivo* (10, 11).

It has been proposed that the biological effects induced by extracellular adenosine and adenine nucleotides such as ATP and ADP are mediated by specific receptors, termed P_1 and P_2 purinoceptors (12). P_1 purinoceptors preferentially interact with adenosine and AMP. In contrast, P_2 receptors are activated by ATP and ADP, but not by adenosine or AMP, and are further classified into several subtypes, such as P_{2X}, P_{2Y}, P_{2z}, and P_{2u} (13). It is not known which subtype of the P_2 purinoceptor is involved in the growth of VSMCs.

Two distinct intracellular signal pathways that lead to cell proliferation are studied. One is activated by the receptor

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ABBREVIATIONS: VSMC, vascular smooth muscle cell; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; 2MeSATP, 2-methylthio-ATP; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; IP_3, inositol trisphosphate, $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein; PTX, pertussis toxin; PGE_2, prostaglandin E_2; PI, phosphoinositide.

that contains the intrinsic protein tyrosine kinase. This type of receptor is activated by classic growth factors such as PDGF and EGF (14). The other is activated by G protein-coupled receptors such as thrombin, bombesin, and norepinephrine (15–17). Recent studies reveal that the cellular growth mediated by the intracellular signal pathway for the G protein-coupled receptor involves a family of key kinases, MAPKs, which belong to a family of serine/threonine protein kinases (14, 15). MAPKs are activated during proliferation and cell cycle transition triggered by a variety of stimuli (18), thereby indicating that MAPKs are indeed important integrators of receptor-originated signals. Of particular relevance to VSMC hyperplasia in relation to MAPK activity is that certain transcription factors such as c-*fos*, c-*jun*, and c-*myc* have been identified as the substrates of MAPK (19). Thus, MAPKs are thought to be involved in transmitting signals from receptors to the nucleus to regulate gene expression and protein synthesis. The P_2 purinoceptors are G protein-coupled receptors (13), which mediate the many biological effects of ATP. Although the mechanism for ATP-induced blood vessel contraction is well known, the cellular mechanism for ATP-induced VSMC proliferation, including whether the VSMC proliferation induced by P_2 purinoceptor stimulation involves the activation of MAPK, has not been elucidated. Here, we report the results of a series of experiments in an attempt to elucidate the pathway ATP activates for VSMC proliferation. It becomes apparent that extracellular ATP exerts its effect via the G_q protein-coupled, P_{2Y} purinoceptor pathway, including the activation of PKC, Raf-1, and MAPK, which eventually leads to DNA synthesis and cell growth.

Experimental Procedures

Materials. ATP, ADP, and UTP were obtained from Boehringer-Mannheim (Mannheim, Germany). 2MeSATP, α,β-methylene ATP, β,τ-methylene ATP, adenosine, and Reactive blue 2 were purchased from Research Biochemicals (Natick, MA). [τ-^{32}P]ATP (5000 Ci/mmol), [*methyl*-^{3}H]thymidine (25 Ci/mmol), and [^{3}H]leucine (25 Ci/mmol) were obtained from Amersham (Buckinghamshire, UK). All cell culture reagents were obtained from GIBCO BRL (Gaithersburg, MD). Human recombinant MEK (glutathione-*S*-transferase-conjugated) and monoclonal antibodies against mouse ERK1 (against $p44^{mapk}$) and ERK2 (against $p42^{mapk}$), phosphotyrosine (Py20), PKC-α, PKC-β, PKC-τ, PKC-δ, PKC-ϵ, PKC-ζ, and antibody against Raf-1 were obtained from Upstate Biotechnology (Lake Placid, NY). All other chemical reagents were from Sigma Chemical (St. Louis, MO). Drugs were prepared in sterile saline and diluted to a working concentration in DMEM. Unless otherwise noted, the concentrations of the drugs used were 100 μM ATP, 100 μM 2MeSATP, 100 μM Reactive blue 2, 30 nM staurosporine, 50 ng/ml PTX, and 500 nM TPA.

Aortic smooth muscle cell isolation and culture. Aortic VSMCs were dissected from freshly harvested Sprague-Dawley rat aortic strips (6–8 weeks old) and plated in 100-mm Petri dishes (20). These VSMCs (explants) were grown in DMEM containing 10% FCS, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 37° in a humidified, 5% CO_2 atmosphere. Explant-derived cells were initially treated with 0.1% trypsin/4 mM EDTA for 3 min at 37°; then, the passage was made conventionally. Cells from passages 5–15 were used for all growth studies. The cells were characterized as smooth muscle cells by morphology and immunostaining with monoclonal antibody specific for smooth muscle α-actin (CGA-7). Cells were subcultured into 24-well plates in a medium containing 10% serum for 24 hr with an initial density of 5×10^4 cells/well. The cells were then placed in the medium without serum for 48 hr to render them quiescent.

Determination of DNA and protein synthesis. DNA and protein synthesis were measured by using [^{3}H]thymidine and [^{3}H]leucine incorporation, respectively (21). Quiescent cells were incubated with P_2 purinoceptor agonists or P_2 agonists plus antagonists in 1 ml of DMEM/FCS-free medium for 20 hr, at which time 1 μCi/ml [^{3}H]thymidine or [^{3}H]leucine was added for pulse labeling. The cells were further incubated for 4 hr and then washed twice with 1 ml of PBS. The cells were treated with 10% trichloroacetic acid to precipitate the acid-insoluble material, from which the DNA was extracted with 0.1 N NaOH. The DNA was collected on a Whatman GF/B filter and washed twice with 5 ml of ice-cold PBS. The filter was then cut and shaken in 3.5 ml of scintillation fluid for 24 hr before counting in a liquid scintillation counter.

Cell counts. VSMCs were seeded at a density of 3×10^4 cells/35-mm dish containing 3 ml of serum-free DMEM supplemented with specified amounts of agonists. The serum-free medium containing agonists was changed replaced with fresh medium every day. On specified days, cells were washed with PBS, harvested with 0.5 ml of trypsin/EDTA, centrifuged, and resuspended in 0.5 ml of DMEM containing 10% FCS. The number of cells was manually counted in a hemocytometer. Triplicate counts were taken for each plate, and quadruplicate plates were used for each determination.

Flow cytometry. To estimate the proportion of cells at various stages in different phases of the cell cycle, cellular DNA content was measured by flow cytometry 24 hr after stimulation of the cells. Cells were trypsinized, centrifuged at $1500 \times g$ for 3 min, washed with PBS, and then cleared of RNA with RNase A (0.1 mg/ml). The DNA was stained with propidium iodide (40 μg/ml) for 30 min at 37°, and 2×10^7 cells were then analyzed for DNA with a FACstar cytofluorometer (Becton Dickinson, Le Pont de Claix, France) by excitation at 488 nm and emission at 585 nm. Flow cytometric determination of DNA content was used as an index of cell proliferation (22).

Assay of IP_3 content. VSMCs were incubated with ATP or Reactive blue 2 plus ATP at 37° for 1 min for IP_3 formation. The reaction was terminated by the addition of 100 μl of ice-cold perchloric acid (20%) followed by a 20-min incubation in an ice bath. The mixture was centrifuged at $2000 \times g$ for 15 min at 4°, the supernatant was collected, and the pH was adjusted to 7.5 with 10 N KOH solution. The $KClO_4$ formed was allowed to precipitate for 30 min at 4° and sedimented at $2000 \times g$ for 15 min at 4°. The amount of IP_3 in the supernatant was determined by radioimmunoassay.

Measurement of $[Ca^{2+}]_i$. VSMCs were cultured on a glass coverslip and incubated in a medium containing 5 μM Fura-2/acetoxymethyl ester, a Ca^{2+} indicator, for 45 min at 37°. The loaded coverslip was then placed in a specially designed holder that positioned the coverslip diagonally in a polymethacrylate cuvette. Each cuvette contained 2.4 ml of Krebs-Henseleit solution that consisted of 117.5 mM NaCl, 5.6 mM KCl, 1.18 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 25.0 mM $NaHCO_3$, 5.5 mM glucose, 25.0 mM HEPES, and 2.5 mM$CaCl_2$. To this cuvette, 100 μl of drug solution containing the appropriate amount of drugs was added to make a total volume of 2.5 ml. The fluorescence was then measured at 37° in a spectrophotometer (CAF-110; Jasco, Tokyo, Japan). The excitation wavelengths were 340 and 380 nm, and the emission wavelength was 510 nm. The ratio of the fluorescence excited at 340 nm (F_{340}) to that at 380 nm (F_{380}) was calculated from the illumination periods and referred as $R_{340/380}$. The ratio was used to estimate the $[Ca^{2+}]_i$ (23).

Immunoblot analysis of PKC isozymes. Confluent VSMCs were washed with PBS and then incubated in DMEM with or without TPA for the indicated time. After the incubation, the cells were washed with ice-cold PBS, scraped, and collected by centrifugation at $1000 \times g$ for 10 min. The collected cells were lysed in ice-cold homogenization buffer that contained 20 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol, 20 mM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM benzamidine. The homogenates were centrifuged at $45,000 \times g$ for 1 hr at 4° to yield the

supernatant (cytosolic extracts) and pellets (membrane fractions). Samples from these two fractions (100 μg of protein) were electrophoresed in 10% SDS-PAGE, and the separated proteins were transferred onto nitrocellulose paper. The nitrocellulose membrane was incubated with specific PKC isozyme monoclonal antibodies (against PKC-α, PKC-β, PKC-τ, PKC-ε, PKC-δ, and PKC-ζ, respectively) in PBST buffer (1:250 dilution) for 4 hr, followed by goat anti-mouse IgG conjugated to alkaline phosphatase (1:2000 dilution) for 60 min. The blots were developed by the addition of alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl-phosphate/Nitro blue tetrazolium (Promega, Madison, WI) in the presence of 0.1 M Tris buffer, pH 9.0, and incubation for 20 min. The immunoreactive bands were visualized and quantified by using a computing densitometer with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Assay of MAPK activity. Quiescent VSMCs were washed with PBS and then treated with ATP or other agents for the indicated time. After washing once with ice-cold PBS, the cells were lysed with ice-cold lysis buffer that consisted of 20 mM Tris·HCl, pH 7.5, 2 mM EDTA, 40 mM sodium pyrophosphate, 50 mM NaCl, 0.1 mM Na_3VO_4, and 0.1% Triton X-100. The cells were then sonicated for 5 sec and centrifuged at 14,000 × *g* for 30 min (model TL-100; Beckman Instruments, Palo Alto, CA). The supernatant was used as the source of MAPK. Aliquots containing an equal amount of protein (2–3 mg) were used for immunoprecipitation with anti-MAPK antibody at 4° for 3 hr. All immune complexes were incubated with protein A/agarose at 4° for 1 hr, and the immune complex bound to protein A/agarose was precipitated by centrifugation. After the immunoprecipitates were washed with 1 ml of the immunoprecipitation buffer, they were treated with 20 μl of sample buffer. For immunoblot analysis of tyrosine residue of MAPK, the immunoprecipitates were electrophoresed in 7.5% SDS-PAGE, transferred onto nitrocellulose membranes, and then incubated with antiphosphotyrosine antibody (Py20) at 25° for 4 hr. After washing, the membranes were incubated with goat anti-mouse IgG conjugated to alkaline phosphatase. The blots were developed by the addition of 5-bromo-4-chloro-3-indolyl-phosphate/Nitro blue tetrazolium in the presence of Tris buffer.

The kinase assay was performed using ^{32}P phosphorylation of MBP as a measurement of MAPK activity as described by Morinelli *et al.* (24). The reaction mixture (containing 5 mM β-glycerophosphate, 20 mM HEPES, 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.02% Triton X-100, 0.1 mM Na_3VO_4, 0.05 mM ATP, 5 μCi [τ-^{32}P]ATP, and 1 mg/ml MBP) was incubated with 20 μl of the protein sample for 20 min at 25°. The reactions were terminated after 20 min at 30° by spotting 15 μl of the reaction mixture onto phosphocellulose paper (1 × 2 cm, Whatman p81). The paper was washed five times with phosphoric acid and dried under a heat lamp. The amount of ^{32}P incorporated into MBP was determined in a scintillation counter.

In some experiments, MAPK activity "in gel" was measured by the phosphorylation of MBP. The phosphorylated MBP was separated in 10% SDS-PAGE, and the extent of MBP phosphorylation was determined by autoradiography.

Assay of Raf-1 activity. Raf-1 activity was assayed by using a recombinant human MEK fused to glutathione-*S*-transferase as a substrate. Cell lysates were immunoprecipitated with 5 μg of the mouse anti-Raf-1 antibody and protein A/agarose. After washing with immunoprecipitation buffer as above, the immunoprecipitates were incubated with kinase buffer containing 100 μg of recombinant MEK and 5 μCi [τ-^{32}]ATP at 25° for 5 min. The reaction was then stopped, and the mixture was assayed for Raf-1 activity on p81 paper or autoradiographically after separation in 10% SDS-PAGE as described above.

RNA isolation and Northern blot analysis. Total RNA was extracted essentially as described by Chomczynski and Sacchi (25), fractionated with 1% agarose/3% formaldehyde gel electrophoresis, and transferred to a nylon filter by capillary action. The Northern blot procedure was as follows. The sample was UV cross-linked, prehybridized in hybridization buffer (50% formaldehyde, 10× standard saline/phosphate/EDTA buffer (1× = .15 M NaCl, 0.001 mM NaH_2PO_4, 0.001 mM EDTA, pH 7.4), 0.1% SDS, 5× Denhardt's solution, and 90 μg/ml salmon sperm DNA) at 42° for 3 hr, and hybridized in the same buffer with ^{32}P-labeled mouse c-*fos* and c-*myc* cDNA probes at 42° for 16 hr. After hybridization, the blots were washed twice in 500 ml of 2× standard saline citrate (1 × =0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and 0.1% SDS at room temperature. The blots were partially dried and exposed to X-ray film (Kodak X-OMAT film) at −70° with an intensifying screen.

Statistical analysis. The pA_2 value for antagonist was calculated according to the following: −log ([antagonist]/[dose ratio − 1]) (27). One-way analysis of variance was used for multiple-group comparisons, and the Student's *t* test was used for other comparisons. $p < 0.05$ was considered statistically significant.

Results

Effects of extracellular adenosine and adenine nucleotides on [^{3}H]thymidine and [^{3}H]leucine incorporation. Cell proliferation must be preceded by DNA and protein synthesis; therefore, the capability of extracellular adenosine and adenine nucleotides to stimulate the incorporation of [^{3}H]thymidine into DNA and [^{3}H]leucine into protein was first determined using the method described in Experimental Procedures. As seen in Fig. 1, both ATP and ADP produced a concentration-dependent increase in [^{3}H]thymidine and [^{3}H]leucine incorporation into serum-deprived, quiescent VSMCs, respectively. The concentrations of ATP that produced a 50% increase (EC_{50}) in [^{3}H]thymidine and [^{3}H]leucine incorporation were 3.0 ± 0.2 and 3.8 ± 0.4 μM, respectively. The EC_{50} values for ADP-induced [^{3}H]thymidine and [^{3}H]leucine incorporation were 5.6 ± 0.8 and 6.5 ± 0.9 μM, respectively. ATP and ADP (100 μM) stimulated [^{3}H]thymidine incorporation to ~500 ± 20% and ~450 ± 27%, respectively, over basal levels. ATP was more potent than AMP and adenosine (100 μM) in stimulating the incorporation (58 ± 18% for AMP and 64 ± 12% for adenosine) (Fig. 1, a and b). Furthermore, the P_1 purinoceptor agonists (*R*)-N^6-phenylisopropyladenosine and 5′-*N*-ethylcarboxamidoadenosine were not found to stimulate [^{3}H]thymidine and [^{3}H]leucine incorporation (data not shown). Adenosine-5′-[β,τ-imido]triphosphate, an ATP analogue that is resistant to hydrolysis, is as effective as ATP in producing the incorporation (528 ± 38%). During the incubation of VSMCs with 100 μM ATP for 24 hr, the amount of extracellular ATP decreased 14 ± 5%, with a generation of its hydrolyzed metabolites, mainly ADP. Further hydrolysis of ADP to AMP and adenosine also occurred to a lesser extent (5 ± 3%) after 24 hr of incubation.

Effects of P_2 purinoceptor agonists and an antagonist on DNA synthesis. To determine whether the mitogenic effect of extracellular ATP was mediated by the activation of P_2 purinoceptors, various P_2 purinoceptor subtype agonists and an antagonist were tested for their ability to stimulate [^{3}H]thymidine incorporation. As shown in Fig. 1a, 2MeSATP (0.1–100 μM), which binds preferentially to P_{2Y} receptors (26), induced an increase in [^{3}H]thymidine incorporation similar to that obtained with ATP. In contrast, P_{2X} agonists α,β-methylene ATP and β,τ-methylene ATP (26) as well as P_{2u} agonist UTP, at concentrations of ≥100 μM, failed to mimic the ATP response (35 ± 15% for α,β-methylene ATP, 45 ± 15% for β,τ-methylene ATP, and 80 ± 17% for UTP). The effect of the putative P_{2Y} purinoceptor antagonist Reactive blue 2 on ATP-induced increase in [^{3}H]thymidine

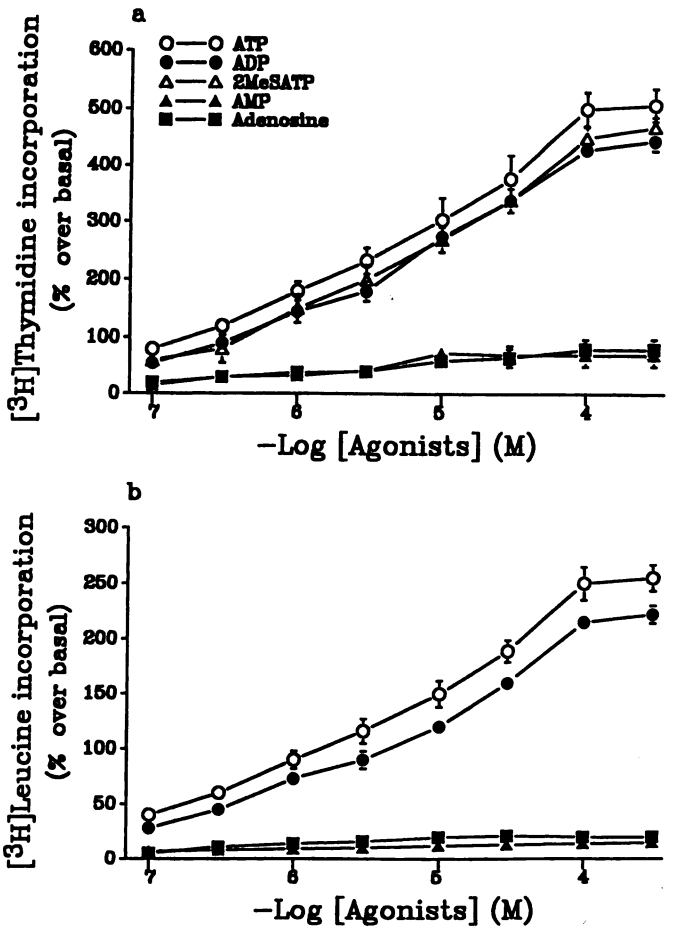

Fig. 1. Effects of extracellular adenosine and adenine nucleotides on DNA (a) and protein (b) synthesis in VSMCs. [^{3}H]Thymidine and [^{3}H]leucine incorporations into DNA and protein respectively were determined, as described in Experimental Procedures, in synchronized cells stimulated by ATP, ADP, AMP, adenosine, and 2MeSATP in serum-free medium for 24 hr. Cells were labeled during the final 4 hr of incubation. The basal amount of [^{3}H]thymidine and [^{3}H]leucine incorporation was 0.3 ± 0.02 dpm × 10^4 and 0.9 ± 0.1 dpm × 10^4, respectively. Data are mean ± standard error of five independent experiments, each tested in quadruplicate.

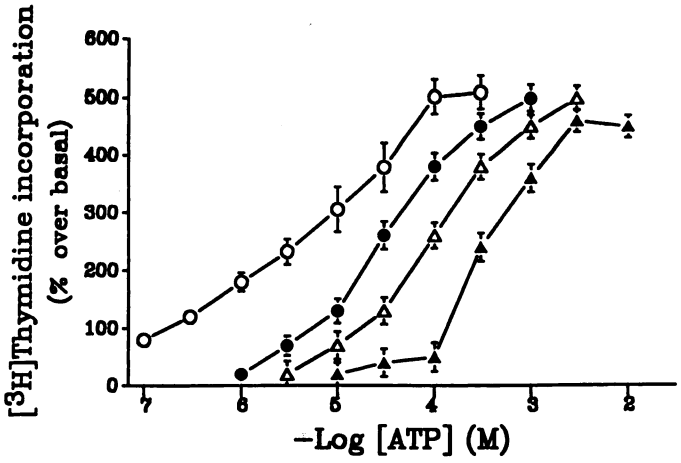

Fig. 2. Effect of Reactive blue 2 on ATP-stimulated [^{3}H]thymidine incorporation. Quiescent cells were stimulated with various concentrations of ATP in the presence of 0.1% dimethylsulfoxide as a solvent control (○) or in the presence of three increasing levels of Reactive blue 2 (10 μM, ●; 30 μM, △; 100 μM, ▲) for 24 hr. Cells were labeled during the final 4 hr of incubation. The basal amount of [^{3}H]thymidine incorporation was 0.4 ± 0.05 dpm × 10^4. Data are mean ± standard error of five independent experiments, each in quadruplicate.

TABLE 1

Effect of extracellular adenine nucleotides on the number of VSMCs

Synchronized VSMCs were incubated with 0.9% saline (resting), 10% FCS, and 100 μM ATP, ADP, AMP, and adenosine in serum-free medium. On the indicated day, the cells were washed with PBS, trypsinized, and counted with a hemocytometer. Data are mean ± standard error of four independent experiments, each performed in quadruplicate.

Treatment	Cell numbers	
	Day 1	Day 3
	-fold	
Resting	1.00 ± 0.00	1.16 ± 0.14
FCS	1.78 ± 0.04[a]	4.11 ± 0.22[a]
ATP	1.49 ± 0.07[a]	3.55 ± 0.16[a]
ADP	1.32 ± 0.06[b]	3.20 ± 0.10[a]
AMP	1.09 ± 0.11	1.14 ± 0.12
Adenosine	1.00 ± 0.09	1.16 ± 0.15

[a] $p < 0.001$, [b] $p < 0.05$ compared with the respective resting value.

incorporation is shown in Fig. 2. Reactive blue 2 at concentrations of ≤10 μM produced a parallel shift to the right of the concentration-response curve of ATP; there was no depression in the maximum response to ATP, even with the highest dose of Reactive blue 2 (100 μM) tested. When the data were presented as a Schild plot, pA_2 and pA_{10} values of 5.8 (5.6–6.0 for 95% confidence limits) and 4.9 (4.7–5.1) with a slope of 1.05 were obtained (Fig. 2). These results indicated that Reactive blue 2 behaved as a competitive antagonist of the P_{2Y}-purinoceptor in VSMCs.

Effect of ATP on cell number. To determine whether the [^{3}H]thymidine and [^{3}H]leucine incorporation correlated cell proliferation, we checked whether ATP and other purinoceptor agonists induced an increase in cell number. The VSMCs were grown in serum-free medium in the absence (resting) or presence of ATP, ADP, AMP, or adenosine, and the number of cells was counted. As shown in Table 1, both ATP and ADP indeed increased the number of cells. In contrast, treatment with AMP or adenosine did not significantly increase the cell number. The magnitude of the mitogenic effect of ATP was comparable to that of FCS (10%). The mitogenic effect was also assessed by flow cytometry. As shown in Fig. 3a, in unstimulated quiescent VSMCs, 97% of the cells were in the growth-arrested (G_0/G_1) phase of the cell cycle, whereas after 24-hr stimulation with ATP, 30% of the VSMCs entered the S phase of the cell cycle (Fig. 3b). Reactive blue 2 nearly completely abolished this response by ATP (Fig. 3c). These observations indicated that the increase in [^{3}H]thymidine incorporation and the mitogenic effect were the manifestation of the P_{2Y} purinoceptor activation evoked by ATP.

Mechanism of mitogenic effect of ATP on VSMCs. We attempted to determine the mechanism responsible for the mitogenic effect of ATP on VSMCs by performing the following experiments. Cells were preincubated for 24 hr with PTX (50 ng/ml; an inhibitor of G_i and G_0 protein-coupled process) or for 30 min with staurosporine (30 nM; a PKC inhibitor) or indomethacin (10 μM; a cyclo-oxygenase inhibitor), and we then added ATP and measured the [^{3}H]thymidine incorpora-

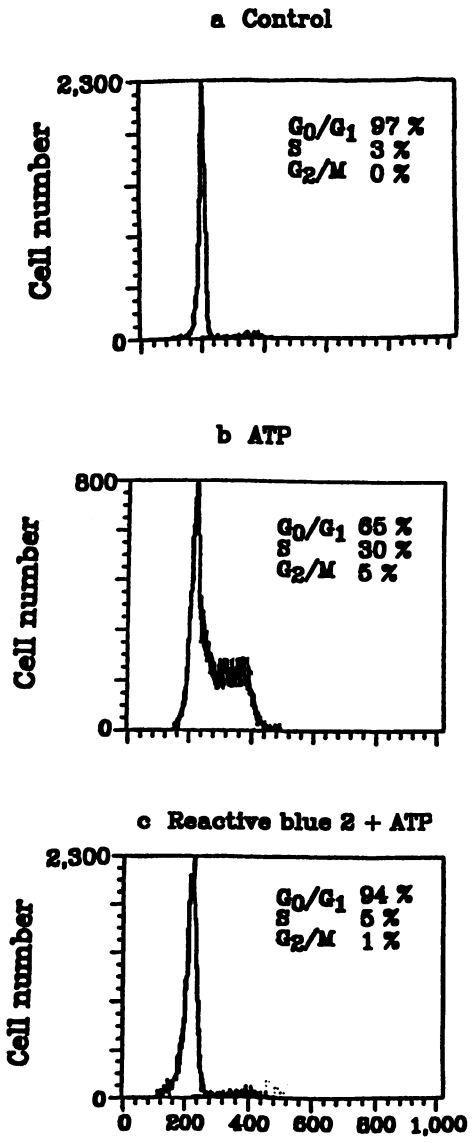

Fig. 3. Effect of ATP on the transition of VSMCs from the G_0/G_1 phase into the S phase of the cell cycle and its inhibition by Reactive blue 2. The VSMCs, kept quiescent for 48 hr (a), after 24-hr incubation with ATP (100 μM) alone (b), or after 24-hr incubation with ATP and Reactive blue 2 (100 μM) (c), were examined with flow cytometry. There were initially 2×10^7 cells/ml. The percentage of the cells at each phase stage of the cell cycle is shown.

tion. As shown in Fig. 4, staurosporine caused an almost 100% inhibition of the ATP-induced [^{3}H]thymidine incorporation, which at concentration (30 nM) almost completely blocked the activity of PKC. Pretreatment of VSMCs with 50 ng/ml PTX for 24 hr entirely blocked (96 ± 3%) the PTX-induced [^{32}P]ADP ribosylation of the PTX-sensitive α subunit of the G protein. Transducin (100 ng) was used as a positive control. Neither PTX nor indomethacin affected the ATP-stimulated [^{3}H]thymidine incorporation. It should be noted that none of these inhibitors were cytotoxic to the cells at the concentrations used (data not shown). ATP did not affect the formation of cAMP and PGE_2 (data not shown). Taken together, these data indicate that PTX-insensitive G protein

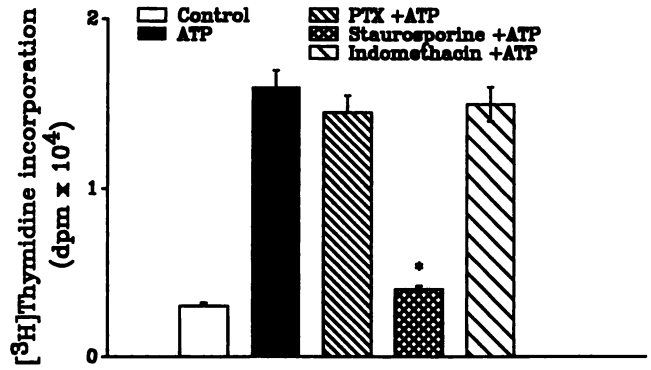

Fig. 4. Effects of specific inhibitors on ATP-stimulated [^{3}H]thymidine incorporation. [^{3}H]Thymidine incorporation into VSMCs was measured 24 hr after the addition of ATP (100 μM). Before the addition, the cells were pretreated, respectively, with 10 μl of saline (control), 50 ng/ml PTX, 30 nM staurosporine, and 10 μM indomethacin for 30 min (except PTX was for 24 hr). *Control bar*, [^{3}H]thymidine incorporation in unstimulated quiescent cultures. Data are mean ± standard error of five independent experiments, each in quadruplicate. *, $p < 0.001$ compared with the ATP-treated cells.

(G_q) and PKC were involved in the ATP mitogenic signal system, which was effected via the activation of the P_{2Y} purinoceptor(s).

Transductional pathway linked to P_{2Y} purinoceptor-mediated stimulation of cell proliferation. It seems that the P_{2Y} purinoceptor in the signal pathway was involved in the [^{3}H]thymidine incorporation; this was examined next. The hydrolysis of PI seemed to be the early signal transduction pathway in the control of cell proliferation (7, 8). In VSMCs, ATP rapidly (≤1 min) increased IP_3 formation to 1.5 ± 0.2-fold after stimulation (Fig. 5a). A rapid rise was observed in $[Ca^{2+}]_i$ from ~100 to 300 nM at 30 sec after the addition of ATP. $[Ca^{2+}]_i$ declined after the peak and reached a stable $[Ca^{2+}]_i$ value of ~220 nM within 6 min (Fig. 5b). Preincubation of cells with Reactive blue 2 for 30 min caused an almost 100% inhibition of the ATP-induced IP_3 formation and the intracellular Ca^{2+} mobilization, whereas preincubation with PTX for 24 hr did not show such a blocking effect (Fig. 5, a and b). PTX alone had no effect on basal $[Ca^{2+}]_i$.

MAPKs, another group of components in the signal transduction pathway, have been shown to be activated during the transition of entering the S phase from the G_0/G_1 phase of the cell cycle (18). Therefore, we checked whether ATP activated MAPK. As shown in Fig. 6a, ATP induced a biphasic activation of MAPK in VSMCs: a rapid phase (a 5-fold increase over basal level) appeared at 5 min and after 4 hr, and a late and sustained phase with an elevated (2-fold) activity could still be seen. ATP activated MAPK in a concentration-dependent manner with an EC_{50} of 4.3 ± 0.4 μM. This was similar to [^{3}H]thymidine and [^{3}H]leucine incorporation. Reactive blue 2 (100 μM) completely inhibited the MAPK activation induced by ATP, whereas little such inhibitory effect was observed in PDGF-, angiotensin II-, or norepinephrine-treated cells.

Two isoforms of MAPK have been identified: one with a molecular mass of 42 kDa and one with a molecular mass of 44 kDa. Also, the activation of MAPK requires phosphorylation at both tyrosine and threonine residues (28, 29). Therefore, we next investigated whether the ATP-induced activation of MAPK was accompanied by tyrosine phosphorylation. Cell lysates were immunoprecipitated with anti-mouse

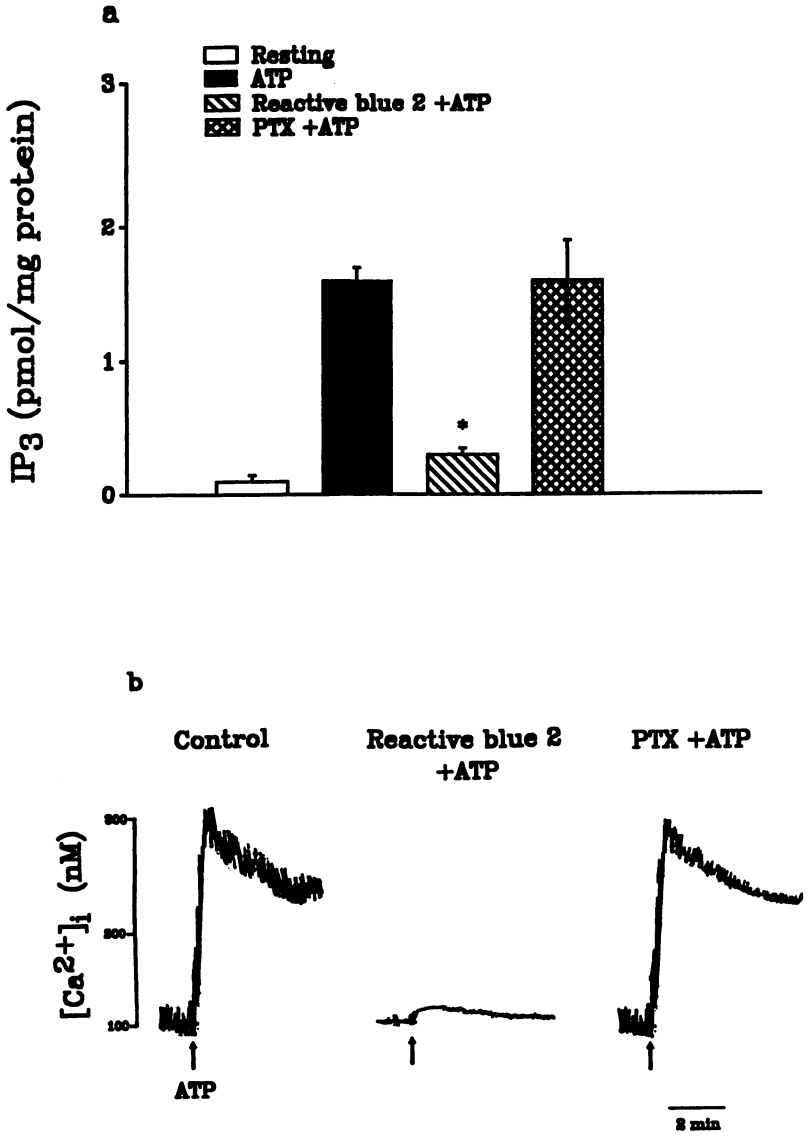

Fig. 5. Effect of ATP induced increase in IP_3 formation and $[Ca^{2+}]_i$ of VSMCs and its inhibition by Reactive blue 2. The cells were treated with 10 μl of saline (control), Reactive blue 2 (100 μM, 30-min pretreatment), or PTX (50 ng/ml, 24-hr pretreatment), followed by incubation with 100 μM ATP for 1 min for IP_3 measurement (a) or for 6 min for $[Ca^{2+}]_i$ measurement (b). The IP_3 formation and changes in $[Ca^{2+}]_i$ were determined as described in Experimental Procedures. Data are mean ± standard error of four independent experiments, each in triplicate. *, $p < 0.001$ compared with ATP-treated cells.

MAPK antibody; then, Western blotting with antiphosphotyrosine antibody (Py20) was carried out. As shown in Fig. 6b, tyrosine phosphorylation in both 42- and 44-kDa MAPKs was observed at 2 min after stimulation with ATP, reaching a maximum at 5 min and then markedly decreasing at 60 min, which is consistent with the above result. Because ATP has been shown to increase IP_3 and evoke diacylglycerol generation from the hydrolysis of PI, resulting in intracellular Ca^{2+} mobilization and PKC activation, the role of PKC in the ATP-induced MAPK activation was examined. The activation of MAPK by ATP was almost completely inhibited by staurosporine but not by W7, a Ca^{2+}/calmodulin kinase inhibitor (data not shown). To determine which PKC isoform is involved in the ATP-induced MAPK activation, the expression of PKC isoforms in VSMCs was characterized by Western blot analysis. Using isoform-specific antibodies, PKC-α, PKC-δ, and PKC-ζ were found to be expressed in VSMCs. A 1-hr exposure of cells to 500 nM TPA induced 90 ± 4%, 95 ± 3%, and 5 ± 3% translocation of PKC-α, -δ, and -ζ, respectively, from the cytosol to the membrane fraction (seven experiments) (Fig. 7a). However, after a 24-hr treatment with TPA, a significant decline in the total amount of PKC-α and PKC-δ immunoreactivity in the membrane and cytosol was seen (Fig. 7a). After 24 hr of TPA treatment, both PKC-α and PKC-δ were almost completely down-regulated (8 ± 2% and 5 ± 3%, respectively; seven experiments). In contrast, the expression of PKC-ζ in both cytosol and membrane was not altered by TPA treatment. When cells were pretreated with TPA for 24 hr and subsequently challenged with ATP, 42- and 44-kDa MAPK tyrosine phosphorylation failed to be induced (8 ± 4% and 6 ± 3% over basal levels, respectively; seven experiments) (Fig. 7b). These results demonstrate that ATP-induced MAPK activation involves the activation of PKC-α and PKC-δ.

We also assessed the upstream region of MEK, the Raf-1 activity. By immunoprecipitating Raf-1 from cell lysates, we observed a rapid increase (66 ± 5% over basal level) in the Raf-1 activity on ATP stimulation (Fig. 8). Again, when cells were pretreated with TPA for 24 hr or with Reactive blue 2 for 30 min and subsequently challenged with ATP, Raf-1 activation failed to be induced (3 ± 2% and 5 ± 3%, respectively) (Fig. 8).

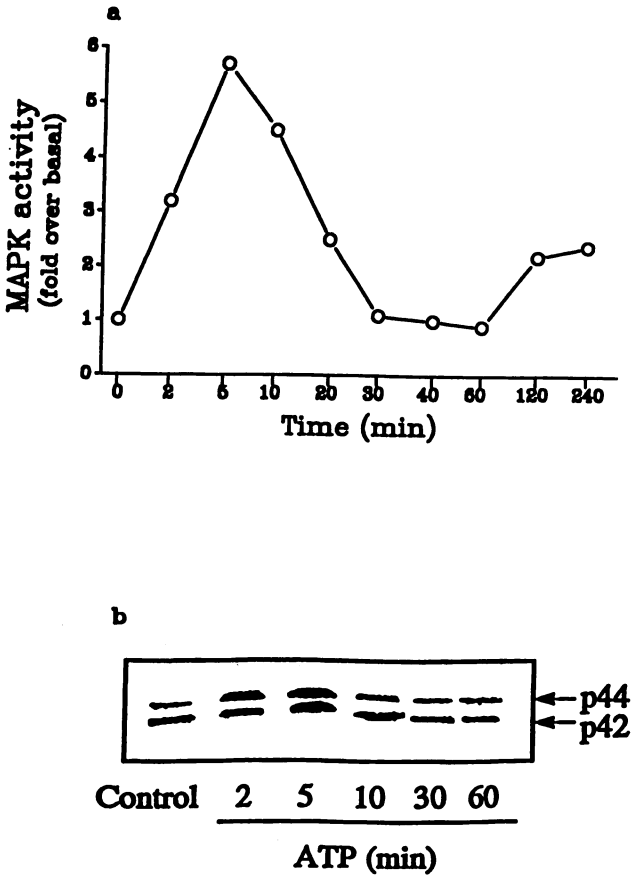

Fig. 6. Kinetics of the activation of MAPK (a) or of tyrosine phosphorylation of 42 kDa and 44 kDa MAPK (b) in VSMCs stimulated by ATP. Quiescent VSMCs were stimulated with ATP (100 μM) for the indicated time periods. Immunoprecipitation of cell lysates with anti-mouse MAPK antibody, MAPK activity, and Western blot analysis with antiphosphotyrosine antibody (Py20) were performed as described in Experimental Procedures. *Arrows*, two isoforms of MAPK in VSMCs, 42 and 44 kDa.

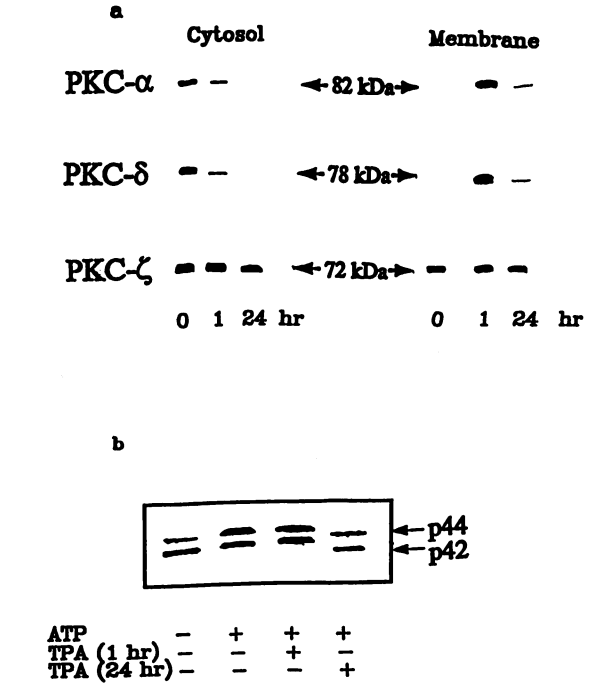

Fig. 7. Translocation and down-regulation of PKC isoforms in VSMCs in response to TPA (a). Effect of 24-hr pretreatment of TPA on the ATP-induced tyrosine phosphorylation of 42- and 44-kDa MAPK (b). Cells were treated with 0.1% dimethylsulfoxide or 500 nM TPA for 1 or 24 hr, and then cytosolic and membrane fractions were prepared. The proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose paper, and immunodetected with PKC-α-, PKC-δ-, or PKC-ζ-specific antibodies, as described in Experimental Procedures. For ATP-induced tyrosine phosphorylation of 42- and 44-kDa MAPK, cells were pretreated with 500 nM TPA for 24 hr and then challenged with 100 μM ATP. Immunoprecipitation of cell lysates with anti-mouse MAPK antibody and then Western blot analysis with Py20 were performed as described in Experimental Procedures.

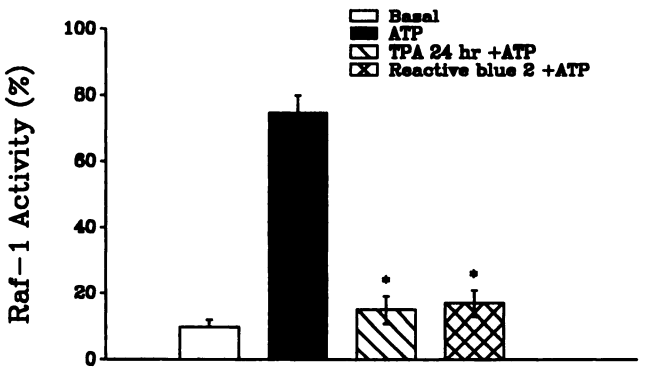

Fig. 8. Activation of Raf-1 kinase in VSMCs treated with ATP and its inhibition by TPA (24-hr pretreatment) or Reactive blue 2. Quiescent cells were incubated with 500 nM TPA for 24 hr or 100 μM Reactive blue 2 for 30 min and then stimulated with ATP (100 μM) for 5 min. Cell lysis, immunoprecipitation of Raf-1, and assay of Raf-1 kinase activity in the immunocomplexes were carried out as described in Experimental Procedures. Data are mean ± standard error of five independent experiments. *, $p < 0.001$ significantly different compared with ATP alone.

Effect of ATP on production of mRNA of transcription factors. As mentioned earlier, certain transcription factors (e.g., c-*fos* and c-*myc*) have been identified as substrates for MAPK (15, 28). Therefore, the production of the mRNAs of these factors was checked. As shown in Fig. 9, ATP caused a significantly increase in c-*fos* and c-*myc* mRNAs. Both Reactive blue 2 and staurosporine completely blocked this increase by ATP, whereas PTX did not.

Discussion

ATP stimulates [^{3}H]thymidine incorporation and growth of rat aortic smooth muscle cells, which is consistent with the observations of others (6, 8, 30). The ATP concentration that induces the mitogenic effect ranges from 0.1 to 100 μM. Such concentrations might be generated *in vivo*, in particular, under conditions such as platelet aggregation (3) and vascular cell release (12), two processes probably occurring during transluminal angioplasty. Under these conditions, locally released ATP, in synergy with other factors such as PDGF and EGF, could play an important role in initiating the VSMC proliferation characteristic of late postangioplasty restenosis (31).

AMP, adenosine, and P_1 agonists fail to show the mitogenic effect. Therefore, ATP seems to show its mitogenic effect by activating various P_2 purinoceptor subtypes. There are at least four subtypes: P_{2X}, P_{2Y}, P_{2z}, and P_{2u} (13). As shown above, Reactive blue 2, which preferentially inhibits P_{2Y} re-

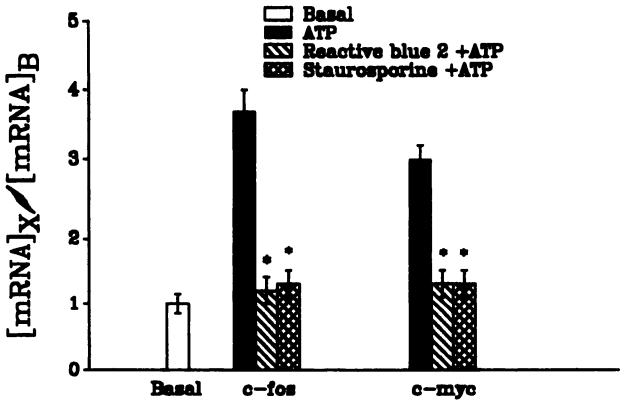

Fig. 9. Accumulation of c-*fos* and c-*myc* mRNAs in VSMCs treated with ATP and its inhibition by Reactive blue 2 and staurosporine. Cells were incubated with Reactive blue 2 or staurosporine for 30 min and then were stimulated with ATP (100 μM) for 30 min (c-*fos*) or 4 hr (c-*myc*). The c-*fos* and c-*myc* mRNAs were obtained by Northern blotting. The ordinate is the ratio of the mRNAs accumulated after stimulation by the drugs ($[mRNA]_X$) to the basal concentration of mRNA ($[mRNA]_B$). Data are mean ± standard error of four independent experiments. *, $p < 0.001$ compared with the respective control.

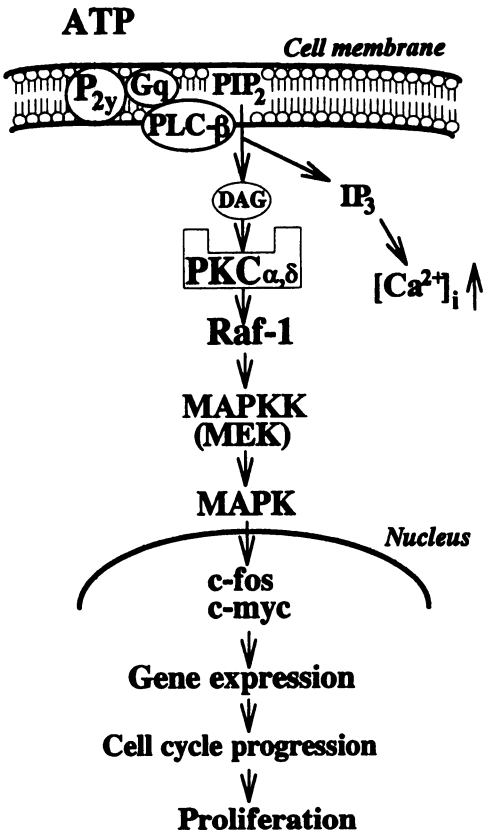

Fig. 10. Schematic representation of extracellular ATP-induced proliferation of VSMC.

ceptor (32), completely blocked the mitogenic effect of ATP and its signal transduction system (i.e., the IP_3 formation, $[Ca^{2+}]_i$ increase, MAPK activation, and accumulation of the mRNA of transcription factors). In contrast, P_{2X} agonists (α,β-methylene ATP and β,γ-methylene ATP) and P_{2u} agonist (UTP) did not show the stimulatory effect. Furthermore, 2MeSATP, P_{2Y} agonist, was to ATP in increasing [^{3}H]thymidine incorporation. Thus, ATP seems to induce VSMC proliferation via the activation of P_{2Y} purinoceptor(s). However, Malam-Souley *et al.* (33) found that both P_{2u} and P_{2Y} receptors are expressed in aortic VSMCs from Wistar rats. The reason for this discrepancy is unknown; it may be due to strain differences in the animals used (i.e., Wistar versus Sprague-Dawley rats).

The activation of the P_{2Y} receptor is linked to the stimulation of PI hydrolysis, which produces two second messengers, diacylglycerol and IP_3 (13). The former is known to activate PKC and the latter to release Ca^{2+} from the intracellular storages. The activation of PKC and increase of $[Ca^{2+}]_i$ seem to account for most of the early proliferative events (14). Such a pathway is apparently activated by the growth-promoting agents coupled to G protein, such as angiotensin II (34), endothelin (35), and norepinephrine (17). We showed that 100 μM ATP caused the increase in IP_3 formation and $[Ca^{2+}]_i$. Thus, the PI hydrolysis is presumably stimulated by ATP and may be essential for the mitogenic effect on VSMCs. In general, P_{2Y} purinoceptors are found to be coupled to G proteins in most cell types. ATP, however, did not affect the cAMP formation, thus excluding the possibility that the P_{2Y} receptor is linked to G_S or G_i. This is reinforced by the observation that the ATP-induced VSMC proliferation was unaffected by the inhibition of G_i with PTX pretreatment. However, it was abolished by staurosporine at concentrations that inhibit PKC. Taken together, it seems that PTX-insensitive G protein (G_q instead of G_i) and PKC are involved in the ATP-stimulated mitogenic signals via the activation of P_{2Y} purinoceptor(s).

The effect of ATP facilitates the transition of the cells from the G_0/G_1 phase to the S phase of the cell cycle as shown in the result of flow cytometry. Recently, a novel group of serine/threonine kinases, MAPKs, have been shown to be activated during the transition of entering the S phase from the G_0/G_1 phase of the cell cycle (18). Furthermore, MAPKs are activated by various growth factors [e.g., PDGF (36), EGF (14), thrombin (14), angiotensin II (34), and vasopressin (37)]. A recent study shows that the late and sustained MAPK activation is an obligatory event for growth factor-induced cell cycle progression (39). We have also shown that stimulation with 100 μM ATP resulted in a biphasic activation of MAPK. This result is consistent with the finding of Huwiler and Pfeilschifter (9), who showed that MAPKs are activated by ATP in rat renal mesangial cells. Furthermore, this activation is accompanied by tyrosine phosphorylation, which is a part of the established mechanism for the activation of MAPK (38). As far as we know, this is the first demonstration that ATP induces tyrosine phosphorylation that signifies the activation of MAPK in rat VSMCs. The P_2 purinoceptor is associated with G protein (13), and on the basis of its predicted sequence, it lacks an intracellular tyrosine kinase domain (13). Thus, the stimulation of the MAPK activity by ATP is probably indirect. Vasopressin, angiotensin II, thrombin, and phorbol ester stimulate PKC in VSMCs and induce activation of Raf-1 (40), which in turn activate MAPKs (37) in these cells. Thus, we postulate that the activation of MAPK occurs downstream of PKC in the signal transduction pathway responsive to ATP in VSMCs. This is supported by the observation that the activation of MAPK was sensitive to staurosporine, a PKC inhibitor, but not to W7, a Ca^{2+}/calmodulin kinase inhibitor. Furthermore, the down-regulation

of PKC-α and PKC-δ with pretreatment of the cells with TPA suppressed the activation of Raf-1 and MAPK induced by ATP.

Recent studies indicate that extracellular signals cause cell proliferation by modulating transcription factor activity in the nucleus via protein phosphorylation cascades (41), and certain transcription factors have been shown to be substrates for MAPK (15, 29). In the current study, we show that ATP activates MAPK and produces c-*fos* and c-*myc* mRNAs in VSMCs. Both Reactive blue 2 and staurosporine completely blocked such increase. Therefore, it seems that the activation of MAPK by the signal from the P_{2Y} purinoceptor on the cell surface led to phosphorylation of the transcription factors, which, in turn, led to the expression of genes for DNA synthesis and cell proliferation.

Recently, Graves *et al.* (42) demonstrated that PDGF induces a rapid formation of cAMP through a mechanism that includes MAPK-mediated activation of cytosolic phospholipase A_2, release of arachidonic acid, synthesis of PGE_2, and the subsequent activation of adenylyl cyclase in human arterial smooth muscle cells. In our study, ATP did not stimulate arachidonic acid release, PGE_2, or cAMP synthesis, although ATP increased MAPK activity. Therefore, activation of MAPK alone is insufficient for stimulation of PGE_2 and cAMP synthesis through cytosolic phospholipase A_2 in rat VSMCs.

In summary, the mechanism of VSMC proliferation induced by ATP may be as shown in Fig. 10. ATP binds to a P_{2Y} purinoceptors/G_q complex and stimulates the hydrolysis of PI, producing IP_3, which causes the rise of $[Ca^{2+}]_i$ and diacylglycerol, which activate PKC. The activation of PKC in turn activates MAPK through the Raf/MEK pathway, causing phosphorylation of transcription factors, which then induces gene expression, leading to DNA synthesis, which results in growth of the cell. Future studies will be directed toward analyzing whether other cytoplasmic kinases are activated through the stimulation of P_2 purinoceptor(s).

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