Mechanism of ATP-Induced [Ca\(^{2+}\)]\(_i\) Mobilization in Rat Basilar Smooth Muscle Cells

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**Background and Purpose**—We have previously reported that extracellular ATP activates P\(_{u}\) receptors and increases intracellular free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) by G protein/phospholipase C/inositol 1,4,5-triphosphate pathways in cerebral artery smooth muscle cells. However, the possible contribution of other signaling pathways remains unclear. This study was undertaken to investigate the role of protein tyrosine kinase (PTK) and mitogen-activated protein kinase (MAPK) in mediating ATP-induced Ca\(^{2+}\) mobilization in rat basilar artery smooth muscle cells (RBASMCs).

**Methods**—RBASMCs were freshly isolated, and [Ca\(^{2+}\)]\(_i\), was monitored by fura 2 microfluorimetry. MAPK phosphorylation was studied by the Western blot technique.

**Results**—ATP produced a biphasic [Ca\(^{2+}\)] response, which consists of releasing Ca\(^{2+}\) from internal stores and influx from extracellular space. PTK inhibitors tyrphostin 51 and genistein inhibited [Ca\(^{2+}\)]\(_i\), response to ATP. Tyrphostin A1, an inactive analogue of tyrphostins, failed to reduce the ATP-induced response. MAPK kinase inhibitor PD98059, but not U0126, reduced the ATP-induced [Ca\(^{2+}\)]\(_i\), response. Phosphatidylinositol 3-kinase (PI3-K) tyrosine kinase inhibitor wortmannin, but not janus tyrosine kinase (JAK2) inhibitor AG490, partially inhibited the [Ca\(^{2+}\)]\(_i\), response induced by ATP. In addition, ATP enhanced MAPK phosphorylation in a concentration- and time-dependent manner, and genistein, tyrphostin 51, PD98059, and U0126 inhibited MAPK phosphorylation.

**Conclusions**—Extracellular ATP produced [Ca\(^{2+}\)]\(_i\) elevation and MAPK phosphorylation in RBASMCs, and the effect was regulated by PTK. The role of MAPK in ATP-induced [Ca\(^{2+}\)]\(_i\) elevation is not clear. PI3-K tyrosine kinase and JAK2 tyrosine kinase may not play an important role in the ATP-induced [Ca\(^{2+}\)]\(_i\) response in RBASMCs. (Stroke. 2000;31:1377-1385.)

**Key Words:** calcium ■ protein kinases ■ protein–tyrosine kinase ■ receptors, purinergic P\(_{u}\) ■ rats

A lteration of intracellular free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) concentration plays an important role in the regulation of cerebral vascular smooth muscle tone. Smooth muscle cells contract in response to a rise of [Ca\(^{2+}\)]\(_i\), and relax as the [Ca\(^{2+}\)]\(_i\), decreases. It has been established that extracellular ATP binds with P\(_{u}\) (P\(_{2u}\)) receptors in cerebral vascular smooth muscle cells and generates, through the activation of G protein and phospholipase C (PLC), the inositol 1,4,5-triphosphate (IP\(_3\)) formation, which subsequently releases Ca\(^{2+}\) from IP\(_3\)-sensitive stores.\(^2\)\(^-\)\(^4\) Then ATP activates Ca\(^{2+}\) influx from extracellular space. The effect of ATP may be important in some cerebrovascular disorders\(^5\)\(^-\)\(^7\) such as cerebral vasospasm after subarachnoid hemorrhage. Extracellular ATP may be released from blood clots\(^8\)\(^-\)\(^9\) and contribute to vasospasm in animal models.\(^9\)\(^,\)\(^10\)

However, ATP may possess growth factor effects in smooth muscle cells\(^11\)\(^,\)\(^12\) and produce signal transduction from not only G protein/PLC/IP\(_3\) pathways. Activation of growth factor receptors (tyrosine kinases) has been shown to stimulate the phosphorylation of G proteins, which may then activate a subclass of PLC, leading to an increase in [Ca\(^{2+}\)]\(_i\).
medium containing the following (in mmol/L): NaCl 130, KCl 5, CaCl\(_2\) 0.2, MgCl\(_2\) 1.3, glucose 5, HEPES 10, collagenase (type II, 0.5 g/L), elastase (0.5 g/L), hyaluronidase (type IV-S, 0.5 g/L), and deoxyribonuclease I (0.1 g/L) for 1 hour at room temperature. The rings were triturated gently, plated on glass coverslips, and stored at room temperature for 2 hours for recovery in the buffer solution containing 2 mmol/L CaCl\(_2\).

**[Ca\(^{2+}\)]\(_i\) Microfluorimetry**

The method for [Ca\(^{2+}\)]\(_i\) imaging was described previously. The buffer solution for [Ca\(^{2+}\)]\(_i\) measurement was as follows in mmol/L: NaCl 145, CaCl\(_2\) 2, KCl 13, MgCl\(_2\) 1, HEPES 10, and glucose 10. The pH was adjusted to 7.4 with NaOH. Cells were loaded with the fluorescence indicator fura 2-acetoxymethylster (AM) (3 \textmu mol/L) for 30 minutes at room temperature in the extracellular buffer solution. After the cells were loaded, the coverslips were placed in the bottom of a Plexiglas perfusion chamber (volume \textasciitilde 600 \mu L) with 2 openings at each end for perfusion and aspiration. The cells were perfused for 10 minutes before the experiment to allow destereification of the dye. Digital [Ca\(^{2+}\)]\(_i\) imaging was performed by video-microfluorimetry by using a cooled charge-coupled device camera (Princeton Instruments Inc) attached to a Nikon Eclipse microscope (\times 40 Fluor objective; Nikon Inc) and software (Universal Imaging Corp) on a personal computer. A sample illumination was supplied by a 75-W xenon arc lamp, and excitation wavelengths were selected by computer control of a filter wheel. Fluorescence imaging was obtained with alternating excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm through the imaging was performed by using a cooled charge-coupled device camera (Princeton Instruments Inc) attached to a Nikon Eclipse microscope (\times 40 Fluor objective; Nikon Inc) and software (Universal Imaging Corp) on a personal computer. A sample illumination was supplied by a 75-W xenon arc lamp, and excitation wavelengths were selected by computer control of a filter wheel. Fluorescence imaging was obtained with alternating excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm through the microscopic objective. Background fluorescence obtained from a cell-free portion of the same coverslip was subtracted from all recordings before calculation of the 340/380 ratio. EGTA (0.1 mmol/L) was included in Ca\(^{2+}\) free extracellular buffer. The ratio values were converted into [Ca\(^{2+}\)]\(_i\), as previously described. All experiments were performed at room temperature.

**Western Blot Analysis**

Sixty-nine Sprague-Dawley rats, weighing 275 to 300 g each, were used for Western blotting. The basilar arteries of the rats were carefully removed from the brain stem and cleaned of excess connective tissue and blood in a modified Krebs Henseleit bicarbonate solution (Krebs’ solution), which contained the following (in mmol/L): NaCl 120, NaHCO\(_3\) 27, KCl 4.5, CaCl\(_2\) 2.5, MgSO\(_4\) 1, KH\(_2\)PO\(_4\) 1, and dextrose 10. The Krebs’ buffer was bubbled with 95% O\(_2\) and 5% CO\(_2\) air to keep the pH at 7.4. The arteries were homogenized with a supersonic wave (10 seconds 3 times) in an extraction buffer containing the following (in mmol/L): Tris-HCl 50 (pH 7.5), NaCl 100, ethylenediamine tetra-acetic acid 5, phenylmethlysulfonyl fluoride 1, and 100 \mu L of IGEPAL CA-630 for 20 minutes at 4°C. The insoluble materials were removed by centrifugation at 4°C and 13 000 g for 10 minutes. The samples (20 \mu g protein) were separated by SDS-PAGE with the use of 12% acrylamide gel. After electrophoretic transfer of the separated polypeptides to nitrocellulose membranes, the membranes were blocked with the use of 3% nonfat milk in PBS containing 0.1% Tween 20 (Tweens-PBS) for 1 hour. The membranes were then washed with Tween-PBS and incubated at room temperature for 2 hours in a 1:5000 dilution of mouse anti-MAPK antibodies (extracellular signal-regulated kinase [ERK] 1+2 [p\(^{44}\)ERK + p\(^{42}\)ERK], mouse monoclonal antibody). Nitrocellulose membranes were later washed with Tween-PBS and incubated with a 1:5000 dilution of sheep anti-mouse IgG antibody linked with horseradish peroxidase. The enhanced chemiluminescence system was used for visualization of protein bands. The results were quantified by laser densitometry of the films and integrated whole-band analysis. The arteries were treated with genistein (30 \mu mol/L), tyrphostin 51 (30 \mu mol/L), PD98059 (30 \mu mol/L), or U0126 (30 \mu mol/L) for 1 hour at room temperature before treatment with ATP at the indicated concentration or time. After treatment, the arteries were tested by ERK1/2 in-gel kinase assay in the same manner.

**Chemicals**

Fura-2 AM was obtained from Molecular Probes. Genistein, tyrphostin 51, and tyrphostin A1 were obtained from Calbiochem. PD98059, AG490, and wortmannin were obtained from BIOMOL. U0126 was obtained from Promega. Mouse anti-MAPK antibodies were obtained from Zymed Laboratories. Anti-mouse IgG antibody linked with horseradish peroxidase and the enhanced chemiluminescence system were obtained from Amersham Life Science. All other chemicals were purchased from Sigma. The laser densitometer (Quantity One) was acquired from Bio-Rad.

**Data Analysis**

Data are expressed as mean \pm SEM. Statistical differences between the control and other groups were compared by 1-way ANOVA and then Scheffé’s F procedure if significant variance was found. A value of \(P<0.05\) was considered statistically significant. Applications of agents or changing solutions are indicated in all figures by horizontal lines.

**Results**

The experiments described below were conducted with freshly isolated RBASMCs. As previously described, the [Ca\(^{2+}\)]\(_i\) response of the cells began to "run down" after approximately 30 minutes,\(^17\) and therefore all experiments were done within 30 minutes. To avoid the possible internal Ca\(^{2+}\) store depletion, only 1 agent and 1 concentration were tested in each coverslip. In studies using PTK or MAPK inhibitors, only 1 dose of 1 inhibitor was used in each study to avoid the possible interaction.
Effect of ATP on \([\text{Ca}^{2+}]_i\)

Figure 1A shows that 10 \(\mu\text{mol/L}\) ATP produced a peak \([\text{Ca}^{2+}]_i\) response that was followed by a sustained plateau phase in the presence of extracellular \(\text{Ca}^{2+}\). In the absence of extracellular \(\text{Ca}^{2+}\), 10 \(\mu\text{mol/L}\) ATP produced a transient peak \([\text{Ca}^{2+}]_i\) response without a plateau phase (Figure 1B). The effect of ATP on \([\text{Ca}^{2+}]_i\) was concentration dependent, and 10 \(\mu\text{mol/L}\) ATP produced an almost maximum response. Control indicates the presence of extracellular \(\text{Ca}^{2+}\), and \(\text{Ca}^{2+}\) free indicates the absence of extracellular \(\text{Ca}^{2+}\). A significant difference was found between control and \(\text{Ca}^{2+}\) free. * \(P<0.05\), ** \(P<0.001\). Cell numbers at concentrations of 0.01, 0.1, 1, 10, and 100 \(\mu\text{mol/L}\) of ATP are control 38, 39, 46, 48, and 35 and \(\text{Ca}^{2+}\) free 21, 20, 16, 40, and 38, respectively.

Effect of PTK Inhibitors on \([\text{Ca}^{2+}]_i\)

In this study we tested the effect of PTK inhibitors genistein, tyrphostin 51, and tyrphostin A1, an inactive analogue of tyrphostins, in ATP-induced \([\text{Ca}^{2+}]_i\) elevation. In the presence of extracellular \(\text{Ca}^{2+}\), preincubation with 30 \(\mu\text{mol/L}\) genistein (a selective tyrosine kinase inhibitor competing with an ATP-binding site of tyrosine kinase) for 5 minutes (30 \(\mu\text{mol/L}\), 5 minutes) did not change the resting level of \([\text{Ca}^{2+}]_i\), but instead markedly reduced both peak and plateau \([\text{Ca}^{2+}]_i\) responses to ATP (0.1 to 100 \(\mu\text{mol/L}\)) (Figure 3A and 3B). No further reduction of \([\text{Ca}^{2+}]_i\) response to ATP was achieved when the concentration of genistein increased to 100 \(\mu\text{mol/L}\) (Figure 3A and 3B). Preincubation of tyrphostin 51 (30 \(\mu\text{mol/L}\), 5 minutes), a synthetic PTK inhibitor competing with both ATP and the substrate-binding site of kinase that is structurally different from genistein, showed a similar inhibitory effect on the \([\text{Ca}^{2+}]_i\) response to ATP (Figure 4A and 4B). Again, no further reduction of \([\text{Ca}^{2+}]_i\) response to ATP was achieved when the concentration of tyrphostin 51 was increased to 100 \(\mu\text{mol/L}\). To test the specificity of the PTK inhibitors, tyrphostin A1 was used in the same manner. Tyrphostin A1 (30 \(\mu\text{mol/L}\), 5 minutes) failed to attenuate the \([\text{Ca}^{2+}]_i\) response induced by ATP (Figure 4A and 4B).

Effect of MAPK Kinase Inhibitors on \([\text{Ca}^{2+}]_i\)

In this study we used 2 kinds of MAPK kinase inhibitors: PD98059 and U0126. Figure 5A and 5B shows that pretreatment with PD98059 (30 \(\mu\text{mol/L}\), 5 minutes) attenuated both the peak and plateau responses induced by ATP (0.1 to 100 \(\mu\text{mol/L}\)) in the presence of extracellular \(\text{Ca}^{2+}\). However, U0126 (30 \(\mu\text{mol/L}\), 5 minutes) failed to markedly attenuate the \([\text{Ca}^{2+}]_i\) response (Figure 5A and 5B).

Figure 2. Summary of effects of ATP on \([\text{Ca}^{2+}]_i\). Both peak (A) and plateau (B) \([\text{Ca}^{2+}]_i\) responses were concentration dependent in the presence or absence of extracellular \(\text{Ca}^{2+}\), and 10 \(\mu\text{mol/L}\) ATP produced an almost maximum response. Control indicates the presence of extracellular \(\text{Ca}^{2+}\), and \(\text{Ca}^{2+}\) free indicates the absence of extracellular \(\text{Ca}^{2+}\). A significant difference was found between control and \(\text{Ca}^{2+}\) free. * \(P<0.05\), ** \(P<0.001\). Cell numbers at concentrations of 0.01, 0.1, 1, 10, and 100 \(\mu\text{mol/L}\) of ATP are control 38, 39, 46, 48, and 35 and \(\text{Ca}^{2+}\) free 21, 20, 16, 40, and 38, respectively.

Figure 3. Effect of genistein on \([\text{Ca}^{2+}]_i\) induced by ATP. The peak (A) and plateau (B) \([\text{Ca}^{2+}]_i\) responses to ATP (0.1 to 100 \(\mu\text{mol/L}\)) are shown. Genistein (30, 100 \(\mu\text{mol/L}\), preincubated with cells for 5 minutes, significantly reduced the \([\text{Ca}^{2+}]_i\) elevation induced by ATP, which was applied with genistein. A significant difference was found between control and genistein-treated cells. * \(P<0.05\), ** \(P<0.001\). Increased peak indicates the net maximum \([\text{Ca}^{2+}]_i\) response (=Peak – Resting Level). Increased plateau indicates the net \([\text{Ca}^{2+}]_i\) values (=Plateau – Resting Level). Cell numbers at concentrations of 0.1, 1, 10, and 100 \(\mu\text{mol/L}\) of ATP are control 39, 46, 48, and 35; genistein 100 \(\mu\text{mol/L}\) 22, 21, 24, and 18; and genistein 30 \(\mu\text{mol/L}\) 18, 15, 25, and 18, respectively. There was no significant difference between genistein 100 \(\mu\text{mol/L}\) and genistein 30 \(\mu\text{mol/L}\).
Effect of PTK and MAPK Inhibitors in the Absence of Extracellular [Ca\(^{2+}\)]

Since the peak [Ca\(^{2+}\)] response was a mixture of a release from intracellular stores and an influx from extracellular space (see Figure 2A), the effect of PTK or MAPK inhibitors against Ca\(^{2+}\) released from intracellular stores could not be adequately studied in the presence of extracellular Ca\(^{2+}\). Thus, we tested the effect of PTK and MAPK inhibitors in the absence of extracellular Ca\(^{2+}\) (Figure 6). When the cells were preincubated with 30 μmol/L genistein for 5 minutes, genistein attenuated the peak [Ca\(^{2+}\)] response at only 1 μmol/L of ATP. At the higher concentrations of ATP (10 and 100 μmol/L), genistein failed to attenuate the [Ca\(^{2+}\)] elevation. In contrast, tyrphostin 51 and PD98059 (30 μmol/L, 5 minutes each) attenuated peak [Ca\(^{2+}\)] response induced by every concentration of ATP. U0126 was not used since it failed to reduce ATP-induced [Ca\(^{2+}\)] elevation. These results indicated that tyrphostin 51 and PD98059, but not genistein, inhibited Ca\(^{2+}\) release from intracellular stores on RBASMCs. The inconsistency of the effect of genistein in the absence and presence of external Ca\(^{2+}\) may be due to its additional effect on the initial Ca\(^{2+}\) entry that might interfere with a Ca\(^{2+}\)-induced Ca\(^{2+}\)-release pathway in RBASMCs. For example, genistein is more effective in reducing the effect of hemolysate at low but not high concentrations in the absence of external Ca\(^{2+}\) in RBSMCs.

Effect of Phosphatidylinositol 3-Kinase Inhibitor on [Ca\(^{2+}\)\(_{i}\)]

We used a phosphatidylinositol 3-kinase (PI3-K) inhibitor, wortmannin, to determine that the ATP-induced [Ca\(^{2+}\)] mobilization is regulated by PI3-K. Wortmannin is also a well-known myosin light-chain kinase inhibitor, but this effect appeared at a higher concentration (ID\(_{50}\) 200 μmol/L). At the lower concentration, it can inhibit the PI3-K without effect on myosin light-chain kinase.

Pretreatment of wortmannin (30 μmol/L, 5 minutes) attenuated both peak and plateau responses at lower concentrations of ATP (0.1 and 1 μmol/L). However, at the higher concentrations of ATP (10 and 100 μmol/L), wortmannin had no effect on [Ca\(^{2+}\)] elevation (Figure 7A and 7B).

Figure 4. Effect of tyrphostin 51 and tyrphostin A1 on [Ca\(^{2+}\)]\(_{i}\) induced by ATP. The peak (A) and plateau (B) [Ca\(^{2+}\)] responses to ATP (0.1 to 100 μmol/L) are shown. Tyrphostin 51 (30, 100 μmol/L, preincubated with cells for 5 minutes, significantly reduced the [Ca\(^{2+}\)] elevation induced by ATP, which was applied with tyrphostin 51. In contrast, tyrphostin A1 (30 μmol/L), an inactive analogue of tyrphostins, failed to attenuated the [Ca\(^{2+}\)] response induced by ATP (0.1 to 100 μmol/L). A significant difference was found between control and tyrphostin 51–treated cells, and there was no significant difference between control and tyrphostin A1–treated cells. *P<0.05, **P<0.001. Increased peak indicates the net maximum [Ca\(^{2+}\)] response (=Peak–Resting Level). Increased plateau indicates the net [Ca\(^{2+}\)] values (=Plateau–Resting Level). Cell numbers at concentrations of 0.1, 1, 10, and 100 μmol/L of ATP are control 39, 46, 48, and 35; tyrphostin 51 100 μmol/L 26, 26, 32, and 35; and tyrphostin A1 30 μmol/L 22, 30, 18, and 24, respectively. There was no significant difference between tyrphostin 51 100 μmol/L and tyrphostin 51 30 μmol/L.

Figure 5. Effect of MAP kinase inhibitors PD98059 and U0126 on [Ca\(^{2+}\)]\(_{i}\) induced by ATP. The peak (A) and plateau (B) [Ca\(^{2+}\)] responses to ATP (0.1 to 100 μmol/L) are shown. PD98059 (30 μmol/L), preincubated with cells for 5 minutes, significantly reduced the [Ca\(^{2+}\)] elevation induced by ATP, which was applied with PD98059. U0126 (30 μmol/L) attenuated only the peak [Ca\(^{2+}\)] elevation induced by ATP (1 μmol/L). A significant difference was found between control and PD98059- and U0126–treated cells. *P<0.05, **P<0.001. Increased peak indicates the net maximum [Ca\(^{2+}\)] response (=Peak–Resting Level). Increased plateau indicates the net [Ca\(^{2+}\)] values (=Plateau–Resting Level). Cell numbers at concentrations of 0.1, 1, 10, and 100 μmol/L of ATP are control 39, 46, 48, and 35; PD98059 30 μmol/L 34, 23, 21, and 19; and U0126 30 μmol/L 18, 24, 22, and 24, respectively.
Effect of Janus Tyrosine Kinase Inhibitor on 
[Ca^{2+}]

Preincubation of janus tyrosine kinase (JAK2) JAK2 inhibitor AG490 (10 μmol/L, 5 minutes) failed to attenuate a [Ca^{2+}] response induced by ATP (Figure 7A and 7B).

Effect of ATP on MAPK Phosphorylation

We tested the effect of ATP on MAPK immunoprecipitation and the effect of PTK and MAPK inhibitors on ATP-enhanced MAPK immunoprecipitation using Western blot analysis.

Figure 8A shows that ATP enhanced ERK1/2 immunoprecipitation in a dose-dependent manner. The top of the figure shows that MAPK immunoprecipitation was enhanced by an increase of ATP concentration after 10 minutes of incubation. The dose-dependent curve represents the summary of 3 separate studies. The effect of 10 and 100 μmol/L ATP is significantly enhanced from control. Figure 8B demonstrates that ATP enhanced ERK1/2 immunoprecipitation in a time-dependent manner. The top of the figure shows that MAPK immunoprecipitation was enhanced at 3 minutes and peaked at 10 minutes by 10 μmol/L ATP. There was a slight increase of MAPK immunoprecipitation at 1 minute but without statistical significance. The time-dependent curve represents the summary of 3 separate studies. Figure 8C shows that ATP (10 μmol/L, 10 minutes) enhanced MAPK immunoprecipitation and PTK inhibitors genistein and tyrphostin 51 and that MAPK kinase inhibitors PD98059 and U0126 attenuated the effect of ATP to a similar degree.

Discussion

In the present study the results can be summarized as follows. First, ATP produced [Ca^{2+}], peak and plateau responses in freshly isolated RBASMCs. The peak response mainly reflects a Ca^{2+} release from intracellular stores, and the plateau response reflects Ca^{2+} entry from extracellular space. Second, tyrosine kinase inhibitors genistein and tyrphostin 51 attenuated the effect of ATP on [Ca^{2+}]. The inhibitory effect of genistein primarily affected Ca^{2+} entry from extracellular space; however, tyrphostin 51 affected both the release from intracellular stores and the entry from extracellular space. Third, the MAPK kinase inhibitor PD98059 attenuated both the peak and plateau [Ca^{2+}], elevations induced by ATP; however, another MAPK kinase inhibitor, U0126, failed to markedly attenuate the [Ca^{2+}] response. Fourth, the PI3-K inhibitor wortmannin slightly attenuated the [Ca^{2+}] elevation induced by a lower concentration of ATP. The JAK2 inhibitor AG490 had no effect on ATP. Finally, ATP enhanced MAPK (ERK1/2) immunoprecipitation, which was attenuated by PTK inhibitors and MAPK kinase inhibitors.

PTK and MAPK in ATP-Induced [Ca^{2+}], Changes

It is well known that PTK and MAPK play important roles in cell growth, cell proliferation, and smooth muscle contraction.\(^\text{5,11,13,21}\) In particular, the ERK pathway is well documented for mitogenic response, and it is phosphorylated by mitogen-ac-
tivated protein/ERK kinase. It has been established that ATP activates ERK1/2 in cardiac myocytes, coronary artery smooth muscle cells, and renal mesangial cells. ATP or UTP binds with P_{2\alpha} receptors and stimulates smooth muscle cell proliferation through MAPK cascade.

In this study genistein and tyrphostin 51 reduced the effect of ATP on [Ca^{2+}]_{i} to a similar extent in the presence of extracellular Ca^{2+}. Genistein also reduced the effect of UTP on [Ca^{2+}]_{i} in RBASMCs (preliminary data). However, in the absence of external Ca^{2+}, tyrphostin 51 produced a more pronounced inhibitory effect than that of genistein, indicating that tyrphostin 51 may be stronger in the inhibition of Ca^{2+} release from internal stores. The differential effects between genistein and tyrphostin 51 might be due to their action against different tyrosine kinases or different isotypes.

MAPK kinases inhibitor PD98059, but not U0126, attenuated a [Ca^{2+}]_{i} elevation induced by ATP. PD98059 reduced [Ca^{2+}]_{i} elevation not only in the presence of external Ca^{2+} but also in the absence of external Ca^{2+}, indicating that PD98059 reduced both Ca^{2+} release from internal stores and Ca^{2+} entry. MAPK should be downstream of a tyrosine kinases cascade activated by G protein–coupled receptors, and thus the mechanism of inhibitory effect on [Ca^{2+}]_{i} by MAPK kinase inhibitors was not clear. There are several pieces of evidence that support a possible role of MAPK in ATP-induced [Ca^{2+}]_{i} elevation in RBASMCs. First, MAPK, especially ERK, is Ca^{2+} dependent and could retroactively modulate [Ca^{2+}]_{i}. Second, a result similar to that of our results was published that showed that PD98059 attenuated the [Ca^{2+}]_{i} elevation induced by a G protein–coupled receptor agonist angiotensin II in rat mesenteric vascular smooth muscle cells. In another study, PD98059 completely inhibited the peak and plateau [Ca^{2+}]_{i}, responses induced by the leukemia inhibitory factor in cardiomycocytes. Third, PD98059 reduced the peak [Ca^{2+}]_{i} elevation by ATP and leukemia inhibitory factor, indicating that PD98059 might inhibit a Ca^{2+} release from internal stores.

However, there are several pieces of evidence against a possible role of MAPK in the regulation of [Ca^{2+}]_{i} elevation induced by ATP. First, MAPK kinase inhibitor U0126 was reported to be more potent in the inhibition of MAPK kinase than PD98059. U0126 produced a greater reduction than PD98059 of endothelin-1–induced contraction of rabbit basilar artery. However, U0126 failed to markedly inhibit the effect of ATP in this study. Second, a recent study suggested that PD98059 is nonspecific and that it can inhibit Ca^{2+} current via the L-type Ca^{2+} channel in rat middle cerebral artery. Indeed, in our preliminary studies both PD98059 and U0126 reduced KCl-induced contraction in rabbit basilar artery. Third, even though ATP enhanced MAPK immunoprecipitation in RBASMCs, the time courses of the effect of ATP on the [Ca^{2+}]_{i} elevation and MAPK immunoprecipitation are different. ATP (10 \mu mol/L) induced a [Ca^{2+}]_{i} elevation within several seconds to 1 minute, but at the same concentration, ATP induced a significant enhancement of MAPK immunoprecipitation after 3 to 10 minutes (Figure 8B). Even though there was a slight increase of MAPK at 1 minute (we are not sure if this slight increase of MAPK might lead to a [Ca^{2+}]_{i} elevation), the result was not statistically significant. Fourth, the effect of ATP on MAPK (Figure 8C) and the inhibitory actions of genistein, tyrphostin 51, PD98059, and U0126 might confirm that ATP enhanced MAPK immunoprecipitation through tyrosine kinase but might not offer any information as to whether MAPK regulates ATP-induced [Ca^{2+}]_{i} elevation. On the contrary, U0126 abolished the effect of ATP on MAPK immunoprecipitation but failed to reduce ATP-induced [Ca^{2+}]_{i} elevation, indicating that MAPK might not contribute to the regulation of [Ca^{2+}]_{i} by ATP.

Even though it is uncertain whether MAPK is involved in ATP-induced [Ca^{2+}]_{i} elevation, this study confirmed that ATP enhanced MAPK immunoprecipitation in RBASMCs. We have shown that ATP enhanced the ERK1/2 immunoprecipitation in a time- and dose-dependent manner and that this effect of ATP was abolished by MAPK kinase inhibitors PD98059 and U0126. Since PTK is upstream of MAPK, it is not a surprise that PTK inhibitors genistein and tyrphostin 51 attenuated the ERK1/2 immunoprecipitation induced...
by ATP. These results are consistent with those of several previous publications that extracellular ATP induced the ERK1/2 phosphorylation in cardiac myocytes, coronary artery smooth muscle cells, and renal mesangial cells.

**PI3-K and JAK2 Tyrosine Kinases in ATP-Induced [Ca\(^{2+}\)]\(_i\) Changes**

Since PTK inhibitors abolished the enhancement of MAPK and reduced the [Ca\(^{2+}\)]\(_i\) elevation by ATP, PTK might be involved in ATP-induced signaling. Thus, we have studied 2 other tyrosine kinases, PI3-K and JAK2, and examined their inhibitors in ATP-induced [Ca\(^{2+}\)]\(_i\), elevation.

PI3-K is an important component of the signal transduction systems activated by the tyrosine kinase receptor. Scharenberg and Kinet suggested that PI3-K is involved in the regulatory processes that produce IP\(_3\) accumulation and then control Ca\(^{2+}\) influx via Ca\(^{2+}\)-induced Ca\(^{2+}\) release. In addition, the PI3-K inhibitor wortmannin has been reported to inhibit Ca\(^{2+}\) entry in porcine aortic endothelial cells, human neutrophils, and human platelets. It was reported that PI3-K also reduced angiotensin AT\(_1\) receptor–stimulated and G\(_{\beta\gamma}\) complexes–stimulated L-type Ca\(^{2+}\) channel current in venous myocyte. Recently, Wilden et al showed that ATP stimulated coronary artery smooth muscle cell proliferation, which is required for independent activation of both the ERK and PI3-K signaling pathways.

In this study the PI3-K inhibitor wortmannin slightly attenuated the peak and plateau [Ca\(^{2+}\)]\(_i\), elevations induced by a lower concentration of ATP (0.1 and 1 \(\mu\)mol/L) in RBASMCs. Wortmannin failed to reduce [Ca\(^{2+}\)]\(_i\) elevation induced by a higher concentration of ATP. Our results indicate that PI3-K might not play an important role in ATP-induced [Ca\(^{2+}\)]\(_i\), elevation. The reason for the discrepancy between our results and those of others, as mentioned above, might be due to differences in tissue preparation, species, agonists, or cerebral arterial smooth muscle cells that were used in this study. A similar discrepancy was also observed in our previous studies; wortmannin abolished the contractile effect of endothelin-1 but not hemolyset in rabbit basilar arteries.

Cytosolic kinases JAK2 and MAPK, which mediate vascular smooth muscle proliferation, are an important mitogenic signaling cascade. It was reported that this pathway responded to growth factor and G protein–coupled receptors. AG-490 is a specific inhibitor of the JAK2 tyrosine kinase, and it prevents JAK/signal transducer and activators of transcription and the Ras/Raf-1/MAPK cascade. In our study AG-490 failed to reduce ATP-induced [Ca\(^{2+}\)]\(_i\), mobilization, indicating that the JAK2 pathway might not be involved. This study, however, does not rule out the possible role of JAK2 in other agonist-induced contraction since AG-490 abolished the contraction induced by endothelin-1 in rabbit basilar artery.

We previously showed that the P\(_{2x}\) (P\(_{2y}\)) receptor was more predominant than either P\(_x\) or P\(_y\) in freshly isolated RBASMCs. ATP or UTP released Ca\(^{2+}\) from internal stores, and the effect was reduced by pertussis toxin, by the PLC inhibitor 2-nitro-4-carboxyphenyl N,N-diphenylcarbamate (NCDC), and by the Ca\(^{2+}\) pump inhibitor thapsigargin. The calcium entry induced by ATP or UTP was partially attenuated either by the receptor-operated Ca\(^{2+}\) channel blocker SK&F96365 or by the voltage-dependent Ca\(^{2+}\) channel blocker verapamil. The P\(_x\) receptor antagonist suramin reduced the effect of ATP or UTP in RBASMCs. In this study we demonstrated that ATP enhanced MAPK immunoprecipitation in RBASMCs. The effect of ATP might be mediated by PTK (upstream regulator) since PTK inhibitors abolished the action of ATP on MAPK. The effect of ATP on [Ca\(^{2+}\)]\(_i\), was mediated partially by PTK. Even though the MAPK inhibitor PD98059 inhibited the [Ca\(^{2+}\)]\(_i\), mobilization induced by ATP, the role of MAPK in ATP-induced [Ca\(^{2+}\)]\(_i\), elevation remains unclear because of the discrepancy between the effect of PD98059 and U0126 and the different time course between [Ca\(^{2+}\)]\(_i\), mobilization and MAPK immunoprecipitation. PI3-K and JAK2 tyrosine kinases might have a limited role, if any, in the effect of ATP on [Ca\(^{2+}\)]\(_i\), mobilization in RBASMCs.

However, an issue that remains to be clarified is whether the Ca\(^{2+}\) pathways induced by ATP are separately or jointly regulated by G protein–coupled receptor activation or/and the activation of PTK or MAPK. As mentioned above, ATP binds with P\(_{2x}\), receptors and, through G protein/PLC pathways, releases Ca\(^{2+}\) from internal stores in RBASMCs. In this study ATP releases Ca\(^{2+}\) by activation of PTK (and MAPK?) in the same cells. Even though we do not have solid evidence regarding whether these 2 pathways are interrelated, there are several reasons for speculation. First, it is known that there are cross-links between signals of G protein–coupled and growth factor receptors, and many G protein–coupled receptor agonists such as angiotensin II, 5-hydroxytryptamine, and endothelin produce Ca\(^{2+}\) elevation and contraction more or less by activation of tyrosine kinase and MAPK. Second, ATP may possess growth factor effects in smooth muscle cells, and the growth factor–like effect of ATP may be mediated by P\(_{2x}\) receptors. Third, inhibitors of PTK or MAPK reduce contractions induced by angiotensin II, 5-hydroxytryptamine, endothelin, or several other agents and reduce ATP-induced Ca\(^{2+}\) elevation in this study. Thus, there is a possibility that ATP-induced Ca\(^{2+}\) elevation in RBASMCs is jointly regulated by G protein/PLC/IP, and PTK/MAPK pathways, and these 2 pathways may be interrelated. Nevertheless, more experiments are needed to draw such a conclusion.

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**References**

Aoki et al studied the mechanisms involved in ATP-induced calcium elevations in freshly isolated rat basilar artery smooth muscle cells. Extracellular ATP acts on cell-surface receptors and causes a peak increase in intracellular calcium, followed by a sustained plateau. The peak is due to a combination of intracellular calcium release and extracellular calcium influx, and the plateau is due predominantly to calcium influx. The authors use pharmacological inhibitors of protein tyrosine kinases and mitogen-activated protein kinase (MAPK) to assess the contribution of these pathways to the calcium response. Unfortunately, the results are not entirely consistent. When extracellular calcium is present, both phases
are reduced by inhibitors of protein tyrosine kinases, genistein and tyrphostin 51, whereas only tyrphostin 51 reduces the peak in the absence of extracellular calcium. Inhibitors of MAPK, PD98059 and U0126, also had differing effects in that only PD98059 inhibited calcium increases. All 4 drugs seemed to attenuate the ability of ATP to induce MAPK immunoprecipitation. Thus, there is no correlation between the effect of the drugs on calcium response and on MAPK phosphorylation. Wortmannin, an inhibitor of phosphatidylinositol 3-kinase, attenuated the peak and plateau calcium responses to ATP. Finally, an inhibitor of Janus tyrosine kinase, AG-490, had no effect on the calcium response to ATP. The disparate effects of the pharmacological inhibitors may be due to several factors, including nonspecific effects and differential inhibition of different kinases, as mentioned by the authors. The operation of the various intracellular kinases are only incompletely understood, and further delineation of the effects of ATP must await the discovery and/or development of more specific drugs and/or the use of other experimental techniques. At this point, however, the conclusions of the authors are reasonable as stated in the abstract.

Extracellular ATP has several effects on smooth muscle. It contracts arterial smooth muscle by acting directly on purinoceptors but may relax arteries by an endothelium-dependent mechanism. ATP also causes smooth muscle proliferation by activation of the MAPK pathway. The authors suggest that ATP may be important in the cerebral vasospasm that occurs after subarachnoid hemorrhage. This is based on studies in animal models which showed that ATP could contract cerebral arteries of monkeys in vivo,1 femoral arteries of rats in vivo,2 and dog cerebral arteries in vitro.3 On the other hand, we found that ATP concentrations in the subarachnoid space of monkeys and humans after subarachnoid hemorrhage are too low to cause significant vasospasm.4,5 Thus, while ATP may contribute to the early phases of vasospasm, it is unlikely to be of any importance to the prolonged, clinically important phase of vasospasm. Furthermore, the argument that processes developing early after the hemorrhage start an unstoppable process that will lead to vasospasm regardless of the continuing presence of blood clot also is erroneous.5 Phenotypic change in the smooth muscle cells that may be associated with proliferation of the cells and with intimal hyperplasia may be epiphemomena. Burnstock6,7 suggested that purine nucleotides, including ATP, may be important in numerous other conditions, including migraine headache and various other painful diseases. The work of Aoki et al may be important from this point of view as well. One theory of migraine is that there is an initial vasoconstriction, followed by vasodilation. Burnstock6 suggested that both phases may be mediated by ATP, with constriction due to direct action of ATP on smooth muscle cells and dilation due to an endothelium-mediated effect. It is not clear whether this theory is correct or not, but it is clear that, in addition to critical intracellular functions, extracellular ATP and other purine and pyrimidine nucleotides seem to be involved in critical physiological and possibly pathological processes.

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