Extracellular purine nucleotides elicit a diverse range of biological responses through binding to specific cell surface receptors. The ionotropic P2X subclass of purinoreceptors respond to ATP by stimulation of calcium ion permeability; however, it is unknown how P2X purinoreceptor activation is linked to intracellular signaling pathways. We report that stimulation of PC12 cells with ATP results in the activation of the mitogen-activated protein (MAP) kinases ERK1 and ERK2 and was wholly dependent upon extracellular calcium ions. Treatment of the cells with adenosine, AMP, ADP, UTP, or α,β-methylene ATP was without effect; however, MAP kinase activation was abolished by pretreatment with suramin and reactive blue 2. The calcium-activated tyrosine kinase, Pyk2, acts as an upstream regulator of the MAP kinases and became tyrosine phosphorylated following treatment of the cells with ATP. We have ruled out the involvement of depolarization-mediated calcium influx because specific blockers of voltage-gated calcium channels did not affect MAP kinase activation. These data provide direct evidence that calcium influx through P2X2 receptors results in the activation of the mitogen-activated protein (MAP) kinases ERK1 and ERK2 and was wholly dependent upon extracellular calcium ions. Treatment of the cells with adenosine, AMP, ADP, UTP, or α,β-methylene ATP was without effect; however, MAP kinase activation was abolished by pretreatment with suramin and reactive blue 2. The calcium-activated tyrosine kinase, Pyk2, acts as an upstream regulator of the MAP kinases and became tyrosine phosphorylated following treatment of the cells with ATP. We have ruled out the involvement of depolarization-mediated calcium influx because specific blockers of voltage-gated calcium channels did not affect MAP kinase activation. These data provide direct evidence that calcium influx through P2X2 receptors results in the activation of the MAP kinase cascade. Finally, we demonstrate that a different line of PC12 cells respond to ATP through P2Y2 purinoreceptors, providing an explanation for the conflicting findings of purine nucleotide responsiveness in PC12 cells.

Adenine nucleotides act through specific cell surface receptors to provoke a variety of biological responses (1, 2). In the nervous system, extracellular ATP has been postulated to serve as a classical neurotransmitter and to also act as a co-transmitter through its coordinate packaging and release with norepinephrine and acetylcholine (3). The analysis of these roles of ATP has been complicated by its catabolism into other biologically active species and the unexpected diversity of cell surface purinoreceptors. Purinoreceptors have been classified into two primary classes, the P1 receptors are responsive to adenosine, whereas the P2 class receptors respond to a variety of purine nucleotides, including ATP. Presently, twelve P2 subtypes have been identified and assigned to two mechanistically distinct subclasses of purinoreceptors (1). The metabolotropic purinoreceptors of the P2Y subclass (formerly P2u, P2t, and P2y) initiate their biological actions by G-protein-dependent activation of phospholipase C and subsequent elevation of intracellular calcium levels through liberation of calcium from internal stores (4). The P2X purinoreceptors comprise a distinct subclass of receptors that are ligand-gated calcium channels functionally related to glutamate and nicotinic acetylcholine receptors. The ionotropic P2X receptors regulate intracellular calcium levels through the ligand-stimulated increase in calcium permeability; thus their actions are dependent upon extracellular calcium ions. Although the ability of the P2X receptors to evoke a rise in intracellular calcium levels is well documented, it is entirely unclear how these receptors are linked to intracellular signaling pathways subsuming their biological actions. In PC12 cells, activation of P2X receptors has been shown to be responsible for stimulation of neurotransmitter release (5–9). A nervous system-specific P2X receptor subtype, P2X2, was initially cloned from PC12 cells and exhibits a distinctive pharmacological profile (10) and has been implicated in mediating many of the biological actions of ATP. Michel et al. (11) have recently demonstrated that the P2X2 receptor appears to be principally responsible for the ATP-stimulated calcium influx in PC12 cells, extending and confirming a number of earlier reports of the involvement of the P2X subclass in mediating the action of ATP.

PC12 cells have also been reported to respond to purinergic agonist through other classes of purinoreceptors, giving rise to an extensive and controversial literature (1, 4). Recently, Solt-off and colleagues (12) have demonstrated that a line of PC12 cells respond to purinergic ligands through P2Y receptors. A direct comparison of these two PC12 cell lines has revealed that they exhibit distinctly different responses to ATP mediated through either P2X2 or P2Y receptors, demonstrating diversity in purinoreceptor sensitivity within PC12 cell lines.

Calcium plays well defined and critical roles in the regulation of a diverse range of intracellular events. Elevation of intracellular calcium levels provokes the activation of a number of protein serine/threonine kinases and signaling cascades (13, 14). The recent discovery of a calcium-stimulated tyrosine kinase, Pyk2, has provided a mechanistic linkage between calcium and well described signaling systems dependent upon protein tyrosine phosphorylation, most prominently, the MAP kinase cascade (15).

The MAP kinases ERK1 and ERK2 are components of a
critical signaling pathway linking membrane receptors to cytoplasmic and nuclear effectors (16). Signaling through the MAP kinase cascade is initiated through activation of p21ras that results in the serial phosphorylation and activation of Raf family members, MAP/ERK kinase and the MAP kinases. Once activated, the MAP kinases phosphorylate cytoplasmic effectors and are translocated to the nucleus where they act on transcription factors and mediate changes in gene expression.

The goal of the present study was to establish the linkage of the ionotonic calcium influx through P2X2 purinoreceptors to activation of intracellular signaling cascades and the stimulation of the MAP kinases.

**EXPERIMENTAL PROCEDURES**

**Materials**

Nerve growth factor (NGF) was purchased from Austral Biological (San Ramon, CA). All other reagents were from Sigma unless otherwise specified. Reactive blue 2 was purchased from Polysciences (Warrington, PA). Antibodies to the EGF receptor, ERKs, and Pyk2 were from Santa Cruz (Santa Cruz, CA). The anti-phospho-phosphotyrosine antibody, 4G10, was from Upstate Biotechnology Inc. (Lake Placid, NY). The anti-phospho-MAP kinase antibody was purchased from New England Biolabs (Beverly, MA).

**Methods**

Cell Culture—The PC12 cells used in this study were obtained from Dr. Eric Shooter (Stanford University) and have been maintained in this laboratory since 1988 under conditions similar to those described originally by Greene and Tischler (17). This line of PC12 cells was cultured in DMEM containing 10% horse serum and 5% bovine serum albumin in an atmosphere of 10% CO2. Cells were serum-starved for 16 h prior to harvest in DMEM containing 0.5% fetal bovine serum. A second line of PC12 cells was obtained from Dr. Stephen Soltoff (Harvard University) and cultured in DMEM containing 5% horse serum and 5% fetal bovine serum in an atmosphere of 5% CO2. The cells were harvested by trituration in phosphate-buffered saline (PBS), pH 7.4, containing 0.1% bovine serum albumin and 0.1% dextrose. The cells were collected by centrifugation, resuspended in PBS (2 × 10^6 cells/ml), and treated as indicated. Following treatment, cells were collected by centrifugation and resuspended in TEV buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 100 mM NaCl, 1 mM sodium orthovanadate) containing 20 mM p-nitrophenyl phosphosphate, 1 mg/ml aprotinin, and 1 mg/ml bacitracin. Lysis was performed by sonication. Lysates were centrifuged at 13,000 rpm for 15 min at 4 °C. Protein concentration was determined by the method of Bradford (18) using BSA as standard.

**Phospho-MAP Kinase and Phosphotyrosine Detection**—The anti-phospho-MAP kinase antibody specifically detects only the activated, dually phosphorylated forms of the MAP kinases ERK1 and ERK2. Aliquots of the cellular lysates (50 μg of protein) were resolved using 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked overnight at 4 °C in Tris-buffered saline containing 0.1% Tween 20 (TBS-Tween) and 5% BSA. Membranes were then incubated at 25 °C for 1 h in the presence of anti-phospho-MAP kinase antibody (1:1000 dilution into TBS-Tween) and then washed three times in TBS-Tween for 15 min each. The membranes were then incubated in horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 h at 25 °C (1:4000 dilution into 5% milk in TBS-Tween). The membranes were then washed three times in TBS-Tween for 15 min, and the MAP kinases were visualized by chemiluminescence. Tyrosine phosphoproteins were detected by Western blot analysis using the anti-phosphotyrosine antibody, 4G10 (Upstate Biotechnology Inc.). Aliquots of cellular lysates (50 μg of protein) were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes, blocked in 5% BSA, and probed with the indicated antibody.

**Immunoprecipitation of Pyk2 and EGF Receptor**—PC12 cells were collected and resuspended in PBS or calcium-free PBS containing 1 mM EGTA and then stimulated for 2 min with 100 μM ATP or 20 μg/ml EGF or stimulated for 5 min with 75 mM KCl. The cells were lysed in RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate) containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. The lysates were cleared by centrifugation, and the cleared lysates were preincubated with protein A-agarose beads for 30 min on ice. Aliquots contain-
stimulated with 100 μM ATP for the indicated periods of time. The cells were collected and lysed, and aliquots (50 μg of protein) were subjected to Western blot analysis using the phospho-specific ERK antibody to determine levels of ERK activation. Blots were stripped and reprobed with an anti-ERK antibody to confirm uniform loading.

FIG. 2. Time course of ATP-stimulated ERK activation in PC12 Cells. PC12 cells were deprived of serum for 16 h and were then stimulated with 100 μM ATP for the indicated periods of time. The cells were collected and lysed, and aliquots (50 μg of protein) were subjected to Western blot analysis using the phospho-specific ERK antibody to determine levels of ERK activation. Blots were stripped and reprobed with an anti-ERK antibody to confirm uniform loading.

FIG. 3. Removal of extracellular calcium blocks ATP-mediated activation of the MAP kinases. PC12 cells were serum starved for 16 h prior to stimulation with ATP, UTP, ADP, AMP, adenosine, or NGF (50 ng/ml) for 5 min at 37 °C. The nucleotides were all used at a concentration of 100 μM. B, PC12 cells were stimulated with ATP (100 μM), α,β-methylene (bMeth, ATP (100 μM), or NGF (50 ng/ml) for 5 min. The effect of the antagonists suramin (Sur, 300 μM) and reactive blue 2 (RB, 300 μM) were evaluated by preincubation for 30 min prior to stimulation of the cells with ATP (100 μM) for 5 min. Cont., control. Aliquots of the cellular lysates were subject to Western blot analysis using a phospho-specific ERK antibody to detect MAP kinase activation. The Western blots were reprobed using an anti-ERK antibody to control for protein loading.

FIG. 4. Effects of purinergic agonists and antagonists. A, PC12 cells were serum starved for 16 h prior to stimulation with ATP, UTP, ADP, AMP, adenosine, or NGF (50 ng/ml) for 5 min at 37 °C. The nucleotides were all used at a concentration of 100 μM. B, PC12 cells were stimulated with ATP (100 μM), α,β-methylene (bMeth, ATP (100 μM), or NGF (50 ng/ml) for 5 min. The effect of the antagonists suramin (Sur, 300 μM) and reactive blue 2 (RB, 300 μM) were evaluated by preincubation for 30 min prior to stimulation of the cells with ATP (100 μM) for 5 min. Cont., control. Aliquots of the cellular lysates were subject to Western blot analysis using a phospho-specific ERK antibody to detect MAP kinase activation. The Western blots were reprobed using an anti-ERK antibody to control for protein loading.

the MAP kinases was because of the action of ionotrophic P2X receptors. Moreover, treatment of the cells with dantrolene, which blocks the release of intracellular calcium by acting as an antagonist to the inositol triphosphate receptors, had no effect on the activation ATP-stimulated activation of the MAP kinases (data not shown). This finding verifies that extracellular calcium and not calcium from internal stores was responsible for the activation of the ERKs. One possible consequence of elevated calcium levels is the activation of cPKC isoforms; however, down-regulation of PKC by chronic phorbol ester treatment of the cells had no effect upon the activation of the MAP kinases by extracellular ATP, suggesting that MAP kinase activation occurs independently of PKC action (data not shown).

Ligand-specific Activation of the MAP Kinases ERK1 and ERK2 in PC12 Cells—To investigate both the specificity of the response and the identity of the receptors involved in the activation of the MAP kinase cascade, a battery of purinoceptor ligands were tested for their ability to activate the MAP kinases in PC12 cells (Fig. 4). Each P2 receptor subtype is defined by its relative response to different purinergic ligands (1, 4). UTP has been demonstrated to be a selective agonist of the P2Y2 and P2Y4 subclasses of purinergic receptors but showed no ability to activate the MAP kinases in our PC12 cells at a concentration of 100 μM or greater (Figs. 4 and 8). Because the metabolic breakdown products of ATP stimulate other purinoceptor subtypes, we tested their possible participation in the response. However, ADP, AMP, and adenosine were without effect. These data rule out the involvement of P1 and metabolotropic P2Y receptors.

The P2X receptors comprise the ionotrophic subclass of purinoceptors, of which seven subtypes have been identified (1). We observed a highly selective effect of ATP on the activation of the MAP kinases and an insensitivity of the response to α,β-methylene ATP. This latter finding is of some importance because it also clearly discriminates between the response of P2X1 and P2X2 receptor subtypes, both of which have been reported to be expressed on PC12 cells (19). P2X1 receptors are activated by α,β-methylene ATP, whereas P2X2 receptors are not. These data indicate that ATP-mediated activation of the MAP kinases was because of the action of P2X2 receptors. Moreover, the EC50 for ATP of the P2X1 receptor is approximately 1 mM (1), which differs from the EC50 of P2X2 receptors, consistent with the dose response observed here (Fig. 1). The effect of ATP on P2X2 purinoceptors is selectively antagonized by suramin and reactive blue 2 (8, 9, 11, 20). Pretreatment of PC12 cells with either of these agents at concentrations that block ATP-driven calcium influx (11) for 30 min prior to stimulation with ATP abolished the ability of ATP to stimulate the MAP kinases (Fig. 4). The sensitivity of the response to these antagonists is consistent with the identification of a P2X2 receptor linked to activation of the MAP kinases and rules out the involvement of P2X4 and P2X6 purinoceptors. P2X3 and P2X5 receptors are not known to be expressed in PC12 cells. Reverse transcription-polymerase chain reaction

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We directly compared the PC12 cell line main-
in signaling pathway (Fig. 7).

**Clonal Differences in PC12 Cell Response to Purinergic Stimulation**—Recently, Soltoff and colleagues (12) reported that PC12 cells utilized P2Y receptors in their response to ATP stimulation. We directly compared the PC12 cell line main-
in this study was found not to express the P2X7 (P2Z) purinergic receptor. 2

**L-type Calcium Channels Are Not Linked to MAP Kinase Activation**—ATP has been reported to result in the depolarization of PC12 and other cells (6). One consequence of membrane depolarization is to open voltage-sensitive calcium channels. We tested whether the ATP-stimulated activation of the MAP kinases was because of movement of calcium ions through these channels. PC12 cells were treated with the selective L-type calcium channel blockers, diltiazem, nifedipine, and verapamil. Preincubation of the cells with each of these drugs for 30 min at a concentration of 100 μM was followed by ATP stimulation for 5 min. All of the channel blockers applied failed to inhibit the activation of ERK1 and ERK2 by ATP, demonstrating that L-type channels are not involved in this response (Fig. 5).

**ATP Treatment Induces the Tyrosine Phosphorylation of Pyk2**—Exposure of PC12 cells to ATP resulted in the induction of the tyrosine phosphorylation of Pyk2 (Fig. 6), which is correlated with its enzymatic activation (15). Treatment of the cells with KCl also stimulated Pyk2 tyrosine phosphorylation because of depolarization of the cells (15). EGFP treatment did not change Pyk2 tyrosine phosphorylation levels. The ability of ATP or KCl to stimulate Pyk2 phosphorylation was abolished when the incubations were performed in the absence of calcium in the medium. ATP stimulated the rapid and evanescent activation of PYK2, reaching maximal levels within 2 min and returning to basal levels within 5 min (Fig. 6). The kinetics of PYK2 activation was similar to that observed with the MAP kinases.

The EGF receptor has been shown to become tyrosine phosphorylated in response to elevation of intracellular calcium levels following depolarization (21) or ligand-stimulated Gαq and Gq11/12 activation (22) and acts as a surrogate signaling intermediate mediating the activation of the MAP kinase cascade. We tested whether ATP treatment of PC12 cells would enhance EGF receptor tyrosine phosphorylation. We were unable to detect any change in phosphotyrosine levels in response to either ATP or KCl. These data suggest that the ATP-stimulated activation of the MAP kinase cascade does not employ the EGF receptor as an intermediate in the activation of this signaling pathway (Fig. 7).

**Clonal Differences in PC12 Cell Response to Purinergic Stimulation**—Recently, Soltoff and colleagues (12) reported that PC12 cells utilized P2Y receptors in their response to ATP stimulation. We directly compared the PC12 cell line main-
tained in this laboratory with those provided by Dr. Soltoff. The PC12 cell line used in this study were uniform, round, and refractile when viewed by phase microscopy, similar to the original clonal PC12 line described by Greene and Tischler (17). In contrast, the PC12 cell line obtained from Dr. Soltoff was exhibiting a pleiomorphic appearance and were phase gray (Fig. 8A).

The response of the two cell lines to purinergic ligands and NGF was compared (Fig. 8B). The PC12 cell line maintained in this laboratory exhibited a robust activation of the MAP kinases in response to NGF and also responded to ATP, albeit less robustly. This cell line did not respond to UTP, as described above (Fig. 4). The PC12 cell line used in the study by Soltoff et al. (12) responded to treatment of ATP and UTP by activation of the MAP kinases, a response consistent with their conclusion that this response was mediated by P2Y receptors.
pleiomorphic appearance, and refractive. A PC12 cell line obtained from Dr. S. Soltoff exhibited

line used in this study, and maintained in this laboratory, top panel, A

These results demonstrate that clonal variation accounts for

of purinoreceptors. These receptors are ubiquitously expressed

of biological actions reflects the involvement of multiple classes

ATP can stimulate the rapid depolarization of cells as well as

biological effects that are mediated by purinoreceptors (2, 3).

of purinergic agonists. A, phase micrographs of the PC12 cell line used

in this study and maintained in this laboratory (top panel) are round

and refractive. A PC12 cell line obtained from Dr. S. Soltoff exhibited

pleomorphic appearance (bottom panel). B, the two PC12 cell lines, the

line used in this study (left panels), and the line obtained from Dr. S.

Soltoff (right panels) were deprived of serum for 16 h and then treated

with 100 mM ATP, UTP for 2 min or 50 ng/ml NGF for 5 min. Aliquots

of these lysates were subjected to Western blot analysis using the

phospho-specific ERK antibody to determine levels of ERK activation

(top panels). Blots were stripped and reprobed with an anti-ERK anti-

body to confirm uniform loading (bottom panels). Cont., control.

Significantly, the response of the latter cells to NGF was of

approximately the same magnitude as that produced by ATP.

These results demonstrate that clonal variation accounts for

the differences seen in these two studies.

DISCUSSION

Extracellular ATP and its catabolites have a diverse range of

biological effects that are mediated by purinoreceptors (2, 3).

ATP can stimulate the rapid depolarization of cells as well as

mediate long-lived changes in cellular metabolism. This range

of biological actions reflects the involvement of multiple classes

of purinoreceptors. These receptors are ubiquitously expressed

and comprise several distinct subclasses. There has been sub-

stantial confusion over the identity and actions of the puri-

nceptors, owing largely to the lack of specific agonists or an-

tagonists. Molecular analysis of purinoreceptors and their

effectors has now established that ATP acts principally

through ionotrophic P2X and metabotropic P2Y receptor sub-

classes. The linkage of the P2Y receptors to intracellular sig-

naling events occurs through G-protein-coupled pathways,

most notably those activating phospholipase C (4). Interest-

ingly, both P2X and P2Y receptor subclasses stimulate an

increase in intracellular calcium levels but accomplish this

through mechanistically distinct pathways. It is of particular

interest that the biological effects elicited by the two receptor

subtypes are also distinct and provide direct evidence that the

cell must possess sophisticated mechanisms to selectivity de-

ploy calcium (and its effectors) to drive specific intracellular

signaling systems (1, 2, 4, 23). One of the major unresolved

issues surrounding the action of P2X receptors is how they are

linked to intracellular signaling systems that subserve their

specific biological effects. The present study provides direct

evidence that calcium flux through the P2X2 receptors results

in the activation of the MAP kinases.

PC12 cells have been extensively used as a model system to

investigate both growth factor and purinoreceptor action. ATP

has been demonstrated to depolarize PC12 cells (6), raise

intracellular calcium levels (7, 11, 24), and elevate phosphoinos-

tide levels (25). The principal biological effect of ATP is its action

as a secretagogue, causing the release of catecholamines (5–7,

8, 9). However, the cloning and functional characterization of

purinoreceptor subtypes has provided insight into the biologi-

cal actions of these molecules (1). Considerable controversy

exists over the involvement of the various purinoreceptors in

the response of PC12 cells to purine agonists. This study dem-

onstrates that much of the confusion over the action of puri-

nergic ligands can be accounted for by the use of PC12 cell lines

expressing different classes of purinergic receptors. The recent

report of Soltoff and colleagues that PC12 cells responded to

ATP by activation of the MAP kinases through P2Y receptors

lead us to directly compare the cell line used in that study with

those maintained in this laboratory. It is significant that we

found dramatic differences in both the morphology of the two

cell lines and in their sensitivity to purinergic ligands, reflect-

ive of their expression of different purinoreceptor classes. The

two populations of PC12 cells were maintained under different

culture conditions that may have presented different selection

pressures on the cells and may account for the loss of the

original morphological phenotype described by Greene and

Tischler (17). The results presented here are an illustration of

the inherent problem with the use of this cell type, and they

provide a clear demonstration of the phenotypic instability of

PC12 cells.

We have shown that exposure of PC12 cells to ATP results in

the rapid and transient activation of the MAP kinases. The data

strongly suggest that this is because of activation of the

P2X2 receptor. We have ruled out the involvement of P1 puri-

nergic receptors on the basis of the absence of an effect of

adenosine or AMP, which act as agonists at these receptors.

The ATP-mediated activation of the MAP kinases in PC12 cells

is not because of metabotropic P2Y purinoreceptors as evi-

denced by the insensitivity of this response to UTP, ADP, and

the absolute dependence of the effect on extracellular calcium.

The metabotropic, G-protein-linked P2Y receptors have been

shown to mediate the activation of the MAP kinases in cortical

astrocytes (23, 26, 27) and another line of PC12 cells (12).

Michel et al. (11) have convincingly argued that P2Z receptors

are not responsible for ATP-stimulated calcium permeability in

PC12 cells. Further, we have demonstrated that this receptor

subclass is not expressed in our PC12 cells.

The ionotrophic P2X subclass of receptors comprise a struc-

turally related family of ligand-gated ion channels with two

transmembrane domains that are likely to assemble in homo-

meric and heteromeric oligomers, forming a calcium-specific

channel (1). The ATP-stimulated calcium influx (11) and MAP

kinase activation in PC12 cells is a consequence of the activa-
tion of the P2X2 subtype. The ATP EC$_{50}$ of approximately 25 μM was observed for both MAP kinase activation and calcium influx (11), distinguishing the P2X2 from the P2X1 receptor, because the latter exhibits a much lower sensitivity (EC$_{50}$ = 1 μM) to ATP. A distinctive feature of the P2X2 subtype is its insensitivity to α,β-methylene ATP (1, 11). α,β-Methylene ATP was unable to activate the MAP kinases; similarly, ATP-stimulated calcium influx was not inhibited by this agent (11). These data suggest that neither the P2X1 nor the P2X3 subtype is activated, because both are sensitive to the action of this ligand. This finding has been confirmed using an electrophysiological approach (28). Moreover, we found that the ATP stimulation of the MAP kinases was blocked by suramin and reactive blue 2. These findings are consistent with the observation that ATP-stimulated calcium influx (7, 11, 25) and dopamine release (20) were inhibited by these agents. P2X4 and P2X6 subtypes are not sensitive to purinergic inhibitor, suramin (1). This observation provided further support for the identification of the P2X2 subtype being responsible for both MAP kinase activation and calcium influx (11) in PC12 cells.

Calcium ions are able to provoke a diverse range of intracellular events. There is ample evidence that the cell possesses sophisticated mechanisms to compartmentalize and selectively deploy calcium and that its effectors elicit specific cellular effects. The biological effects of changes in intracellular calcium concentration are dependent upon the mechanisms through which calcium levels are elevated (14), as evidenced by the ability of activation of L-type calcium channels to promote cellular survival, whereas ligand-gated calcium influx through N-methyl-D-aspartic acid receptors is linked to excitotoxic cell death. It is presently not understood how these specific biological effects of calcium are achieved. Depolarization of neurons results in elevation of intracellular calcium levels through the opening of voltage-gated calcium channels (14). Extracellular ATP elicits the depolarization of PC12 cells (20). We have provided data indicating that agents that block L-type calcium channels have no effect on the ability of ATP to activate the MAP kinases, indicating that the calcium permeability is mediated principally by the P2X receptor. There is presently no evidence indicating the opening of voltage-sensitive calcium channels subsequent to ATP-mediated depolarization (7, 11, 25). Paradoxically, K$^+$-stimulated depolarization of these cells results in opening of the voltage-sensitive calcium channels and activation of the MAP kinases (Fig. 6 and Refs. 21 and 29).

The MAP kinases ERK1 and ERK2 are responsible for propagation of mitogenic signals in response to growth factor stimulation, resulting in changes in cellular morphology, metabolism, and gene expression. In PC12 cells, the ERKs have been demonstrated to be activated by growth factor stimulation with NGF and EGF. The receptors for these factors are themselves receptor tyrosine kinases that undergo autophosphorylation, with the resulting phosphorysosine residues catalyzing the formation of a signaling complex leading to the activation of p21$^{ras}$, p21$^{ras}$ is an essential element of this cascade and when activated directly stimulates the phosphorylization and activation of B-Raf, MAP/ERK kinase, and the MAP kinases. A central finding of this study is that extracellular ATP, acting over a P2X purinergic receptor, leads to the phosphorylation and activation of the ERKs. We demonstrate that the mechanism of ERK activation is distinct from that induced by NGF, both by its time course and by its dependence upon extracellular calcium. We were unable to detect any effect of ATP on the activation of other MAP kinase superfamily members, including the stress-activated protein kinases (also termed c-Jun N-terminal kinases or JNKs) or p38 MAP kinase pathways (data not shown).

The linkage of changes in calcium ion levels to the activation of the MAP kinases is illustrative of the complexity of calcium-based signaling events. Presently, several mechanistically distinct signaling processes have been associated with activation of p21$^{ras}$ and the MAP kinases. The K$^+$-stimulated depolarization of PC12 cells or N-methyl-D-aspartic acid treatment of cortical neurons results in the activation of the MAP kinases as a consequence of calcium influx through L-type channels and the N-methyl-D-aspartic acid receptor, respectively (13, 14). The elevation of intracellular calcium levels results in the activation of p21$^{ras}$ and the subsequent serial activation of protein kinases comprising the MAP kinase cascade. The recent discovery of a calcium-activated protein-tyrosine kinase, Pyk2, has raised the possibility that it too may be a critical intermediate in the activation of p21$^{ras}$ and the MAP kinases. Lev and colleagues (15) have demonstrated that Pyk2 activation can be stimulated by elevation of intracellular calcium levels. The activated Pyk2 forms complexes with the adapter proteins Shc and Grb2 and the p21$^{ras}$ nucleotide exchange factor, Sos, leading to MAP kinase activation. The observation of ATP-stimulated, calcium-dependent phosphorylation of Pyk2 strongly suggests the involvement of this enzyme in the P2X2 receptor-driven MAP kinase activation.

In PC12 cells, plasma membrane depolarization has been reported to stimulate the tyrosine phosphorylation of the EGF receptor, which has been postulated to act as a surrogate whose phosphorytrosine residues serve to catalyze the assembly of a signaling complex mediating the activation of p21$^{ras}$ (21). This is an unusual use of a growth factor receptor in a calcium-initiated signaling pathway. We were unable to confirm this observation and failed to observe any change in EGF receptor tyrosine phosphorylation in response to depolarization, despite a robust stimulation of Pyk2 phosphorylation. Moreover, we found that extracellular ATP did not alter the phosphorytosine content of the EGF receptor, indicating that the purinoreceptor-mediated calcium influx does not utilize the EGF receptor as a signaling intermediate leading to MAP kinase activation. In some cell types, the activation of heterotrimeric G-protein-coupled receptors also results in the activation of the MAP kinases as a consequence of increased intracellular calcium liberated from internal stores (22). The exact nature of the involvement of tyrosine kinases in this scheme is not presently clear (30), and the specific signaling molecules employed are likely to be cell type-specific.

The mechanisms subserving the biological effects of the ionotropic P2X receptors have not been extensively investigated, and the present study documents that purinoreceptors in this class employ well characterized signal transduction pathways to mediate their biological effects. A critical unresolved issue is how calcium ion influx is coupled to the upstream elements regulating the activation of the MAP kinases. The recent identification of novel calcium-regulated signaling intermediates has opened new avenues for investigation of these events.

Acknowledgments—We thank Dr. George Dubyak for assistance, advice, and encouragement, for comments on the manuscript, and for performing reverse transcription-polymerase chain reaction analysis of P2X7 receptor expression. We thank Dr. Stephen Soltoff for generously providing us with the line of PC12 cells. We also thank Maria Bamberger for assistance.

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