Feedback Regulation of ATP-induced Ca\(^{2+}\) Signaling in HL-60 Cells Is Mediated by Protein Kinase A- and C-mediated Changes in Capacitative Ca\(^{2+}\) Entry*

(Received for publication, January 2, 1997, and in revised form, June 20, 1997)

Hyosang Lee, Byung-Chang Suh, and Kyong-Tai Kim‡

From the Department of Life Science, Pohang University of Science and Technology, Pohang 790-784, Republic of Korea

Extracellular ATP increases intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) in HL-60 cells. When cells are stimulated with supramaximal concentrations of ATP, although the initial [Ca\(^{2+}\)]\(_i\) increase is similar over a range of 30, 100, and 300 μM ATP, the rate of the return to basal [Ca\(^{2+}\)]\(_i\) level is faster in cells treated with higher concentrations of ATP. This probably results from differences in Ca\(^{2+}\) influx rather than Ca\(^{2+}\) release, since the influx of the unidirectional Ca\(^{2+}\) surrogates Ba\(^{2+}\) and Mn\(^{2+}\) also exhibit similar responses. Furthermore, while 300 μM ATP had an inhibitory effect on the thapsigargin-induced capacitative Ca\(^{2+}\) entry, 30 μM ATP potentiated the response. However, the inhibitory action of 300 μM ATP was blocked by protein kinase C (PKC) inhibitors, such as GF 109203X and chelerythrine, and the potentiating action of 30 μM ATP was blocked by protein kinase A (PKA) inhibitors H89 and Rp-cAMPS. The PKC inhibitors also slowed the decay rate of the Ca\(^{2+}\) response induced by 300 μM ATP, and the PKA inhibitors increased it when induced by 30 μM ATP. In the measurements of PKA and PKC activity, 30 μM ATP activates only PKA, while 300 μM ATP activates both kinases. Taken together, these data suggest that the changes in the ATP-induced Ca\(^{2+}\) response result from differential modulation of ATP-induced capacitative Ca\(^{2+}\) entry by PKC and PKA in HL-60 cells.

Extracellular ATP evokes many physiological effects such as platelet aggregation, neurotransmission, inflammation, and muscle contraction in numerous cell types (1). These various effects of ATP are mediated by plasma membrane P2 purinergic receptors (2). Six subtypes of P2 purinergic receptors, P\(_{2X}\), P\(_{2Y}\), P\(_{2Z}\), P\(_{2U}\), and P\(_{2T}\), were identified in pharmacological and functional studies and supported by cloning data (3). It has been reported that in HL-60 cells extracellular ATP increases the intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) via plasma membrane P\(_{2U}\) and P\(_{2X}\) type receptors (4, 5). We have also shown that extracellular ATP elevates cAMP through a novel type of receptor (6). The P\(_{2U}\) receptor is functionally coupled to phospholipase C (PLC) through pertussis toxin-sensitive and pertussis toxin-insensitive G proteins. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol. The IP\(_3\) produced increases the [Ca\(^{2+}\)]\(_i\) by mobilizing Ca\(^{2+}\) from the intracellular Ca\(^{2+}\) stores. This Ca\(^{2+}\) mobilization activates the plasma membrane Ca\(^{2+}\) influx pathway through Ca\(^{2+}\) release-activated Ca\(^{2+}\) channels (CRAC) and is termed capacitative Ca\(^{2+}\) entry (7, 8). The degree of Ca\(^{2+}\) entry is determined by the filling status of the intracellular Ca\(^{2+}\) store. The P\(_{2X}\) receptor triggers entry of cations; however, it has been shown that the activity is very weak in undifferentiated HL-60 cells. Thus, ATP increases intracellular Ca\(^{2+}\) in HL-60 cells by mobilizing it from the intracellular stores and by influx from the extracellular space. We observed a different rate of decrease in the Ca\(^{2+}\) response, while the peak level remained the same, when HL-60 cells were stimulated with supramaximal concentrations of ATP. There are several mechanisms responsible for Ca\(^{2+}\) removal from the cytosol after the elevation of the [Ca\(^{2+}\)]\(_i\). These mechanisms include sequestering of Ca\(^{2+}\) into intracellular stores, binding to various Ca\(^{2+}\)-binding proteins, and actions by the Ca\(^{2+}\) pump and Na\(^{+}\)/Ca\(^{2+}\) exchanger (9). Among these, the Ca\(^{2+}\) pump, which transports ions across the plasma membrane and into intracellular stores, plays a critical role in reducing the elevated [Ca\(^{2+}\)]\(_i\). The plasma membrane Na\(^{+}\)/Ca\(^{2+}\) exchanger also plays an important role in the control of the intracellular free Ca\(^{2+}\) concentration, exchanging three Na\(^{+}\) for one Ca\(^{2+}\). It appears to have a lower affinity for Ca\(^{2+}\) than the plasma membrane Ca\(^{2+}\) pump and a high capacity for removing increased Ca\(^{2+}\). Thus it operates efficiently when [Ca\(^{2+}\)]\(_i\) is increased beyond 10\(^{-8}\) M. A number of Ca\(^{2+}\)-binding proteins are also involved in buffering the cytosolic Ca\(^{2+}\) concentration. We studied the mechanism by which the different patterns of decrease in Ca\(^{2+}\) occur upon stimulation with supramaximal concentrations of ATP in HL-60 cells. Our results suggest that this difference is not due to the cytosolic Ca\(^{2+}\) removal system, but that it is instead mainly due to changes in capacitative Ca\(^{2+}\) entry by actions of PKA and PKC, which are differentially activated by ATP itself.

EXPERIMENTAL PROCEDURES

Materials—ATP, UTP, thapsigargin, Triton X-100, Trizma (Tris base), trichloroacetic acid, EGTA, EDTA, sulfipyrazone, MOPS, sodium fluoride, sodium orthovanadate, sodium pyrophosphate, β-glycerophosphate, leupeptin, pepstatin A, aprotinin, phenylmethylsulfonyl fluoride, and IP\(_3\) were purchased from Sigma. Fura-2 pentaacetoxymethyl ester was from Molecular Probes (Eugene, OR), and [\(^{3}H\)]IP\(_3\) and fusic acid; ATP, 8S, 5′-O-(3-thiotriphosphate); Rp-cAMPS, 3′,5′-cyclic monophosphothioate.

* This work was supported by grants from Pohang University of Science and Technology/BRSI Special Fund, the Hallym Academy of Sciences, Hallym University, the Korea Science and Engineering Foundation (KOSEF 95-0401-02), and the Basic Science Research Institute Program (Project BSRF-96-4435) from the Ministry of Education. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Dept. Life Science, POSTECH, San 31, Hyoja dong, Pohang 790-784, Republic of Korea. Tel.: 562-279-2297; Fax: 562-279-2199; E-mail: ttk@vision.postech.ac.kr.

The abbreviations used are: [Ca\(^{2+}\)]\(_i\), intracellular free Ca\(^{2+}\) concentration; CRAC, Ca\(^{2+}\) release-activated Ca\(^{2+}\) channel; fura-2/AM, fura-2 pentaacetoxymethyl ester; IP\(_3\), inositol 1,4,5-trisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; MOPS, 3-(N-morpholino)propanesulfonic acid; ATP, 8S, 5′-O-(3-thiotriphosphate); Rp-cAMPS, 3′,5′-cyclic monophosphothioate.
fraction and used for in vitro PKA activity measurements. All of the following procedures were performed on ice unless stated otherwise. The reaction was initiated by the addition of 10 μl of cell extract to the 30 μl of a test mixture containing 10 μl of Mg2+/ATP mixture containing 75 mM MgCl2, 500 μM ATP, 50 μCi of [γ-32P]ATP (3,000 Ci/μM), and 1 μl of 500 μM flavin. The reaction mixture containing 0.02 μM GF 109203X, 0.9 μM KN62. 10 μM cAMP were added to the reaction mixture with Kemptide for a positive control, and 10 μl of buffer II instead of Kemptide was added to determine the endogenous PKA substrate. All assay components were prepared by using buffer II that contained 20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM MgCl2, 1 mM sodium orthovanadate, and 1 mM dithiothreitol. The reaction mixture was gently vortexed and placed in a 30 °C water bath for 10 min. Then 25 μl of the reaction mixture was transferred to 1 × 3-cm F81 phosphocellulose strips, which were immediately immersed into 0.75% phosphoric acid. The strips were washed three times with 0.75% phosphoric acid and then dehydrated in 95% ethanol, air-dried, and placed into liquid scintillation vials. The radioactivity was quantified in a Beckman LS 8000 liquid scintillation counter.

Assay of PKC Activity—PKC activity was measured by determining the incorporation of 32P from [γ-32P]ATP into histone IIIS as described previously (17, 19), with some modifications. HL-60 cells were harvested and treated with inhibitor mixture containing 10 μM H89 and 1 μM KN62 and then stimulated with 30 or 300 μM ATP and 100 μM PMA. After the stimulation, the cells were washed three times with Locke’s solution and then resuspended in 200 μl of buffer I, which is described in the PKA assay. The cells were sonicated and centrifuged at 100,000 × g for 1 h at 4 °C, and the pellet was saved as the membrane fraction and then was solubilized with the above buffer I containing 1% Nonidet P-40. The reaction was initiated by the addition of 10 μl of solubilized membrane fraction to the 40 μl of reaction mixture containing 10 μl of 500 μM histone IIIS, 10 μl of inhibitor mixture containing 2 μM PKI, PKA inhibitor peptide and 0.9 μM KN62, 10 μl of 500 μM PMA, and 10 μl of the Mg2+/ATP mixture containing 75 mM MgCl2, 500 μM ATP, and 100 μCi of [γ-32P]ATP. All assay components were prepared using buffer II described in the assay of PKA. The reaction mixture was incubated at 30 °C for 10 min, and 25 μl of the reaction mixture was transferred to the F81 phosphocellulose strip. The strips were immersed into the 0.75% phosphoric acid and washed three times for 10 min. After washing, they were rinsed in 95% ethanol, air-dried, and quantified by measuring the radioactivity in a liquid scintillation counter.

Analysis of Data—Data are summarized as the means ± S.E. EC50 was calculated with the AllFit program (20). We considered differences significant at p < 0.05.

RESULTS

Effect of Extracellular ATP on Cytosolic [Ca2+]i, in HL-60 Cells—In HL-60 cells, ATP increased the [Ca2+]i, in a concentration-dependent manner with maximal and half-maximal effective concentrations (EC50) seen at approximately 10 μM and 85 nM, respectively (Fig. 1A). Fig. 1B illustrates the typical changes in [Ca2+]i, observed in fura-2-loaded HL-60 cells stimulated with maximal concentrations of ATP. Initially, the [Ca2+]i increased rapidly to a peak level and then completely returned to the basal Ca2+ level, even if the stimulant remained present. Notably, the changes in cytosolic Ca2+ exhibited a different desensitization pattern in response to supra-maximal concentrations of ATP as compared with the lower concentrations. Although the peak levels were similar, the rate of return to the basal [Ca2+]i level was faster in cells treated with the higher concentration of ATP. This phenomenon is clearly seen in Fig. 1C. We measured the time from peak response of the Ca2+ signal to the 70% desensitized [Ca2+]i, level as indicated in the inset of Fig. 1C. The data show that the times for return to 30% of the peak level became less in stimulations with increasing concentration of ATP. In other words, the higher the ATP concentration, the faster the return rate. We further analyzed whether changes in the desensitization rate are elicited by Ca2+ release from intracellular stores or Ca2+ influx from the extracellular space, since ATP increases [Ca2+]i, via both pathways.

Effect of Extracellular ATP on Ca2+ Release and IP3 Generation...
concentration-dependent curve of Ca$^{2+}$, maximal IP$_3$ generation was obtained after 15 s. At that time, 300 μM ATP was 62.5% less than when stimulated with 30 μM ATP. Furthermore, the intracellular IP$_3$ level was more sustained in the stimulation with 300 μM as compared with the stimulation with 30 μM ATP, which is enough to maximally mobilize Ca$^{2+}$. Therefore, we conclude that the 300 μM ATP-induced Ca$^{2+}$ release is not less than the 30 μM ATP-induced one and that the difference detected in the Ca$^{2+}$ decay rate is due to changes in the Ca$^{2+}$ influx from the extracellular space.

**Effect of Extracellular ATP on Mn$^{2+}$ Quenching and Ba$^{2+}$ Uptake**—To test whether differences in the falling state of the Ca$^{2+}$ response are due to modulation of the Na$^+$/Ca$^{2+}$ exchanger and Ca$^{2+}$/ATPase activity, we measured Mn$^{2+}$ and Ba$^{2+}$ influx after the addition of ATP. Mn$^{2+}$ and Ba$^{2+}$ are good Ca$^{2+}$ surrogates, since they are not pumped out of the cell, so they can be considered as selective tracers for entry (21, 22). Mn$^{2+}$ uptake was estimated by the quenching of the fura-2 fluorescence when excited at the 360-nm wavelength, which is an isosbestic wavelength and insensitive to variations in Ca$^{2+}$ concentration. Ba$^{2+}$ uptake was estimated by the increase in the fura-2 fluorescence ratio when excited at the 340- and 380-nm wavelength.
ATP shows a concentration-dependent pattern with the higher intensity reflecting Ba\textsuperscript{2+} added to the medium, it caused an increase in the fluorescence similar to the result of Ca\textsuperscript{2+}. Ca\textsuperscript{2+} activity could be the main cause for the rapid desensitization of other metal ions had little or negligible effects (data not shown). The data indicate that the lower concentration of ATP activates the divalent cation influx. This result was also supported by the Ba\textsuperscript{2+} uptake. To measure Ba\textsuperscript{2+} influx, cells were stimulated with ATP in the absence of external Ca\textsuperscript{2+}. When the Ba\textsuperscript{2+} was added to the medium, it caused an increase in the fluorescence intensity reflecting Ba\textsuperscript{2+} uptake. The influx of Ba\textsuperscript{2+} elicited by ATP shows a concentration-dependent pattern with the higher concentrations of ATP triggering less Ba\textsuperscript{2+} uptake. This is similar to the result of Ca\textsuperscript{2+} influx as shown in Fig. 2A. These results suggest that ATP regulates the amount of Ca\textsuperscript{2+} influx, but does not modulate the activity of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger and Ca\textsuperscript{2+}/ATPase. To investigate the involvement of CRAC, we tested the effect of various metal ions on ATP-induced Ca\textsuperscript{2+} signaling, because metal ions are known to block CRAC. Cells were treated with 30 \mu M La\textsuperscript{3+}, Cd\textsuperscript{2+}, Co\textsuperscript{2+}, or Ni\textsuperscript{2+} for 1 min and then stimulated with ATP in Ca\textsuperscript{2+}-containing medium. The difference between the 30 and 300 \mu M ATP-induced Ca\textsuperscript{2+} signals disappeared in the presence of 30 \mu M La\textsuperscript{3+}, whereas the other metal ions had little or negligible effects (data not shown). The data, thus, suggested that changes in CRAC activity could be the main cause for the rapid desensitization of the Ca\textsuperscript{2+} response induced by higher concentrations of ATP.

Effect of Extracellular ATP on Thapsigargin-induced Capacitative Ca\textsuperscript{2+} Entry—In the above experiments, we found that different Ca\textsuperscript{2+} decay rates were caused by changes in Ca\textsuperscript{2+} influx. ATP induces capacitative Ca\textsuperscript{2+} entry through CRAC, which is stimulated by a Ca\textsuperscript{2+} influx factor liberated from the depleted intracellular Ca\textsuperscript{2+} stores by action of IP\textsubscript{3}. To study the regulation of capacitative Ca\textsuperscript{2+} entry, we used thapsigargin, which depletes intracellular Ca\textsuperscript{2+} stores by inhibiting the microsomal Ca\textsuperscript{2+}/ATPase and induces Ca\textsuperscript{2+} influx (23). The differences in Ca\textsuperscript{2+} influx could also be demonstrated when we measured the effect of ATP pretreatment on thapsigargin-induced capacitative Ca\textsuperscript{2+} entry. As shown in Fig. 4, cells were incubated with 100 \mu M thapsigargin (Tg) for 10 min in Ca\textsuperscript{2+}-free medium followed by the addition of 3 mM Ca\textsubscript{Cl}\textsubscript{2}. In solid traces, the treatment with the designated concentrations of ATP was for 1 min prior to the Ca\textsubscript{Cl}\textsubscript{2} addition. The data are representative of seven experiments with similar results.

Effect of PKC and PKA Inhibitors on the ATP Activities in Ca\textsuperscript{2+}/IP\textsubscript{3} System—ATP activates PLC and produces IP\textsubscript{3} and diacylglycerol, which subsequently activates PKC. We have also shown that extracellular ATP triggers elevation of cAMP in HL-60 cells (6). To assess the involvement of PKC and PKA in modulation of capacitative Ca\textsuperscript{2+} entry, we used inhibitors specific for those kinases. GF 109203X and chelerythrine, selective PKC inhibitors, were used to characterize the inhibitory or stimulatory effect of ATP on the capacitative Ca\textsuperscript{2+} entry. Fig. 5A shows what effect pretreatment with protein kinase inhibitors has on the Ca\textsuperscript{2+} transient elicited by thapsigargin. 300 \mu M ATP has a substantial inhibitory effect on thapsigargin-induced capacitative Ca\textsuperscript{2+} entry as seen in Fig. 4. This inhibitory action was antagonized by pretreatment with 1 \mu M GF 109203X, and Ca\textsuperscript{2+} influx was even potentiated in the presence of GF 109203X. Similar effects were obtained when 1 \mu M chelerythrine was used in place of GF 109203X. The results suggest that PKC, when activated by 300 \mu M ATP, inhibits thapsigargin-induced capacitative Ca\textsuperscript{2+} entry.

The involvement of PKA in the 30 \mu M ATP-induced potentiation of capacitative Ca\textsuperscript{2+} entry was investigated by testing the effect of the PKA inhibitors H89 and Rp-cAMPs. Fig. 5B shows the effect that H89 and Rp-cAMPs has on the enhancement of the thapsigargin-induced capacitative Ca\textsuperscript{2+} entry by 30 \mu M.
Feedback Regulation of ATP-induced Ca\(^{2+}\) Signaling

ATP. In cells treated with H89, this potentiation was blocked. The potentiation also disappeared in 20 μM Rp-cAMPS-treated cells. These results suggest the involvement of PKA in the 30 μM ATP-induced enhancement of the capacitative Ca\(^{2+}\) entry.

The effects of protein kinase inhibitors on ATP activity in the desensitization pattern of [Ca\(^{2+}\)]\(_i\) were also investigated. Inhibition of PKA by pretreatment with 2 μM H89 significantly accelerated the decay rate of the 30 μM ATP-induced [Ca\(^{2+}\)]\(_i\), level with little effect on the peak Ca\(^{2+}\) level (Fig. 6A). In contrast, pretreatment with 1 μM GF 109203X slowed the decay rate induced by 300 μM ATP, resulting in a Ca\(^{2+}\) response similar to the 30 μM ATP-evoked response (Fig. 6B). The inhibitory action of 300 μM ATP was slightly enhanced in the presence of PKA inhibitors, while the potentiating effect of 30 μM ATP became even more activated in the presence of PKC inhibitors (data not shown). Thus, the slower decay rate of the 30 μM ATP-induced Ca\(^{2+}\) signal may be the result of the potentiating action of PKA as it increases the capacitative Ca\(^{2+}\) entry induced by ATP, whereas the rapid decay rate in the 300 μM ATP-induced Ca\(^{2+}\) signal might be the result of an inhibitory action by PKC as it blocks the ATP-induced capacitative Ca\(^{2+}\) entry. Taken together, the different desensitization rates of the ATP-induced Ca\(^{2+}\) signals after peak level could be the result of an interplay between inhibition by PKC and activation by PKA of the capacitative Ca\(^{2+}\) entry.

**Effect of Extracellular UTP on Cytosolic [Ca\(^{2+}\)]\(_i\) in HL-60 Cells**—Since it has been shown that P2X receptors were present and coupled to PLC in HL-60 cells, we treated the cells with UTP and measured the return rate of the [Ca\(^{2+}\)]\(_i\), level. Fig. 7A illustrates the times for return to 30% of the peak level in response to supramaximal concentrations of UTP: the higher the UTP concentration, the faster the return rate. However, although the phenomenon was similar to that of ATP, the rate of return to the basal [Ca\(^{2+}\)]\(_i\) level was not as remarkable compared with that induced by ATP in Fig. 1C. We further analyzed whether changes in the desensitization rate involve PKC and PKA in the modulation of the UTP-induced capacitative Ca\(^{2+}\) entry using kinase inhibitors. Fig. 7B shows that pretreatment with GF 109203X slowed the decay rate induced by 300 μM UTP. However, inhibition of PKA by pretreatment with H89 had no effect on the return rate of the 30 μM UTP-induced [Ca\(^{2+}\)]\(_i\) level (data not shown). The difference in the desensitization pattern between ATP and UTP might result from different activations of effector enzymes, such as PLC and adenylyl cyclase. In HL-60 cells, UTP has no effect on cAMP production (6). Therefore, the results suggest that the effect of PKA increasing the capacitative Ca\(^{2+}\) entry is not involved in UTP-treated cells and that PKC alone acts in the desensitization of the UTP-induced Ca\(^{2+}\) response.
Effects of ATP on cAMP Generation, IP$_3$ Production, and Protein Kinase Activity—To assess agonist concentration-dependent differential activation of PKC and PKA, we measured the production of cAMP and IP$_3$. Fig. 8 shows the production of cAMP and IP$_3$ induced by various concentrations of ATP. The maximal increase of cAMP was obtained with 300 μM ATP. The EC$_{50}$ value was 19.2 μM. Particularly, for 30 and 300 μM ATP, respectively, the cAMP levels reached 137.2 ± 9.3 and 190.2 ± 7.7 over the basal cAMP level of 25.3 ± 4.2. The amounts of IP$_3$ caused by 30 and 300 μM ATP were 27.7 ± 5.7 and 67.0 ± 5.5 pmol/mg of protein, respectively, while the basal IP$_3$ level was 18.0 ± 3.1. There was only a slight increase in the IP$_3$ level in the response to 30 μM ATP, whereas the cAMP level was already significantly increased at that concentration. On the other hand, during stimulation with 300 μM ATP, cAMP was produced maximally, and IP$_3$ was also dramatically increased over the basal IP$_3$ level, suggesting that both PKA and PKC might be highly activated. We directly measured the activities of the protein kinases induced by different concentrations of ATP. Fig. 9A shows that stimulation with 30 μM ATP induced strong activation of PKA similar to 300 μM ATP. However, stimulation with 30 μM ATP produced a relatively weak activation of PKC (Fig. 9B), indicating that PKA was more significantly activated than PKC during the 30 μM ATP stimulation. However, PKC seems to have a dominant effect during the highly activated state of PKA and PKC as occurs with the 300 μM ATP treatment, because inhibition of the capacitative Ca$^{2+}$ entry was only exhibited during the stimulation with 300 μM ATP.

To investigate the interrelation between PKA and PKC, thapsigargin-induced capacitative Ca$^{2+}$ entry was measured in cells treated simultaneously for 1 min with 100 nM PMA and 30 μM ATP. As shown in Table I, PMA by itself has an inhibitory effect on the thapsigargin-induced capacitative Ca$^{2+}$ entry. Moreover, the 30 μM ATP-induced potentiation of the capacitative Ca$^{2+}$ entry also disappeared when cells were simultaneously treated with PMA. Thus it seems likely that strong activation of PKC has a dominant effect on the capacitative Ca$^{2+}$ entry, even during a state of strongly activated PKA. Similarly, the dominant effect of PKC might cause the rapid decline of the 300 μM ATP-induced Ca$^{2+}$ response.

DISCUSSION

The present study demonstrates that ATP stimulation with maximal concentrations of ATP causes different decay rates for the Ca$^{2+}$ signal after obtaining similar peak levels if supramaximal concentrations of ATP are used. We suggest that this is a result of homologous feedback regulation of PKC and PKA activation. ATP increases intracellular Ca$^{2+}$ by the release of Ca$^{2+}$ from the intracellular stores and by influx from the extracellular space. Most of the Ca$^{2+}$ increase was caused by capacitative Ca$^{2+}$ entry activated by the store depletion. It has also been reported that ATP activates nonselective cation channels permeable for Ca$^{2+}$ and Na$^+$ in dibutyryl cAMP-differentiated HL-60 cells (24). Recently, Buell et al. (5) demonstrated the presence of P$_{2X_1}$ in HL-60 cells. The current through the P$_{2X_1}$ was barely detected in undifferentiated HL-60 cells. However, the current was markedly increased in differentiated cells. We cannot exclude the involvement of this nonselective cation channel in the homologous desensitization of the ATP-induced Ca$^{2+}$ response; however, its contribution would be small, since we used undifferentiated cells.

Our data indicate that the differences in the Ca$^{2+}$ signal were caused not by Ca$^{2+}$ release but by influx. However, it is possible that the different rates of desensitization could also be the result of a differential activity of the cytosolic Ca$^{2+}$-removing system. There are two major pathways by which to decrease the [Ca$^{2+}$]. One is the pumping out of Ca$^{2+}$ from the cell exterior by Na$^+$/Ca$^{2+}$ exchanger and/or by plasma...
Feedback Regulation of ATP-induced Ca\(^{2+}\) Signaling

It has been reported that the capacitative Ca\(^{2+}\) entry is blocked by metal ions with an efficiency order of La\(^{3+}\) > Zn\(^{2+}\) > Cd\(^{2+}\) > Be\(^{2+}\) = Co\(^{2+}\) > Mn\(^{2+}\) > Ni\(^{2+}\) > Sr\(^{2+}\) > Ba\(^{2+}\) (7). We found the same desensitization pattern between stimulations with 30 and 300 \(\mu M\) ATP in the presence of La\(^{3+}\). This is consistent with the notion that the different desensitization rates of the ATP-induced Ca\(^{2+}\) signals resulted from different feedback regulation of the capacitative Ca\(^{2+}\) entry evoked by ATP itself.

Fig. 9. Effects of ATP on protein kinase activity. A, PKA activity induced by different concentrations of ATP. PKA activity was determined after stimulation of cells with 30 or 300 \(\mu M\) ATP for 3 min in Locke’s solution as described under “Experimental Procedures.” 10 \(\mu M\) cAMP was added to the reaction mixture with Kemptide to determine positive control. The experiments were performed three times in triplicate, and the values are the means ± S.E. *p < 0.01, compared with the control. B, PKC activity in the membrane fraction induced by different concentrations of ATP. Cells were treated with 30 and 300 \(\mu M\) ATP or 100 \(\mu M\) PMA for 3 min in Locke’s solution, and membrane fraction was prepared to measure the PKC activity as described under “Experimental Procedures.” The experiments were carried out twice in triplicate and the values are the means ± S.E. **p < 0.01, compared with the control.

Table I

| Treatment          | Net increase of [Ca\(^{2+}\)]\(_r\) | Percentage of control |
|--------------------|---------------------------------|-----------------------|
| Control            | 804 ± 28                        | 100                   |
| ATP                | 1229 ± 533                      | 153                   |
| PMA                | 628 ± 22                        | 78                    |
| ATP + PMA          | 658 ± 34                        | 82                    |

\(^a\)p < 0.01, compared with the control.

Net membrane Ca\(^{2+}\)/ATPase. The other is the pumping of Ca\(^{2+}\) into the intracellular stores by Ca\(^{2+}\)/ATPase. The Na\(^+/Ca\(^{2+}\) exchanged may not be directly involved in the phenomena of the present study, because stimulation of cells with ATP in Na\(^+\)-free medium or in the presence of the Na\(^+/Ca\(^{2+}\) exchanger blocker benzamil did not affect the desensitization pattern of the Ca\(^{2+}\) responses elicited by supramaximal concentrations of ATP (data not shown). It has been reported that PKC stimulates Ca\(^{2+}\) efflux by activation of plasma membrane Ca\(^{2+}\)/ATPase in neutrophils (25). It seems unlikely that the activation of the Ca\(^{2+}\) efflux was involved in the fast return to basal level at higher concentrations of ATP, because unidirectional Ca\(^{2+}\) surrogates, Mn\(^{2+}\) and Ba\(^{2+}\), showed a similar pattern as Ca\(^{2+}\).

Using ATP as an agonist, we had to consider whether a particular cellular response was caused by the activation of a P2 purinergic receptor per se. ATP is rapidly hydrolyzed to adenosine by extracellular ATPase and nucleotidase (26). But adenosine itself is a potent signaling substance. To assess this potential problem, we tested the hydrolysis-resistant ATP analog, adenosine ATP\(_{\gamma}\)S, and obtained the same results as in the stimulations with ATP (data not shown). For example, ATP\(_{\gamma}\)S also showed the differences in the Ca\(^{2+}\) decay rate and the biphasic effect on the thapsigargin-induced capacitative Ca\(^{2+}\) entry. These results indicate that the changes in the decay rate of elevated [Ca\(^{2+}\)]\(_i\), were due to ATP and not its metabolite.

In the experiments with UTP, the rate of return to the basal Ca\(^{2+}\) level increased as the UTP concentration was raised, but the decay rate was not as prominent as with ATP. Recently, we (6) showed that ATP, but not UTP, elevates the cAMP level in HL-60 cells. A similar effect of ATP was found in human lymphocytes and neutrophils (27–29). It has been reported that the capacitative Ca\(^{2+}\) entry is blocked by metal ions with an efficiency order of La\(^{3+}\) > Zn\(^{2+}\) > Cd\(^{2+}\) > Be\(^{2+}\) = Co\(^{2+}\) > Mn\(^{2+}\) > Ni\(^{2+}\) > Sr\(^{2+}\) > Ba\(^{2+}\) (7). We found the same desensitization pattern between stimulations with 30 and 300 \(\mu M\) ATP in the presence of La\(^{3+}\). This is consistent with the notion that the different desensitization rates of the ATP-induced Ca\(^{2+}\) signals resulted from different feedback regulation of the capacitative Ca\(^{2+}\) entry evoked by ATP itself.

Until now, little is known about the signaling between the intracellular Ca\(^{2+}\) store and the plasma membrane CRAC. It has been proposed that the signal is mediated via Ca\(^{2+}\)-entry factors, which include calcium influx factor (27, 28), heterotrimeric G protein (29), and small G protein (30), or is mediated via direct interaction between the IP\(_3\) receptor and the plasma membrane Ca\(^{2+}\) channel (31). Recently, there were some reports describing the cloning and functional expression of a mammalian homologue to the Drosophila eye-specific trp gene (32–34). It was identified as a Ca\(^{2+}\)-permeable cation channel that is activated by calcium store depletion. Although it was not clearly shown that TRP is the I\(_{\text{CRAC}}\) protein, there is a possibility that TRP should be classified as one of the CRAC family (35). The molecular regulatory mechanism of signaling between the Ca\(^{2+}\) store depletion and CRAC is controversial and complicated. There is some evidence that protein phosphorylation is involved in the regulation of capacitative Ca\(^{2+}\) entry. In Xenopus oocytes and lymphocytes, protein phosphatase inhibitor potentiates Ca\(^{2+}\) influx (36). Tyrosine kinase inhibitor blocks the bradykinin- and thapsigargin-induced Ca\(^{2+}\) influx in lymphocytes and in human foreskin fibroblast cells (37, 38). Protein kinase C-dependent phosphorylation plays a key role in the modulation of the capacitative Ca\(^{2+}\) entry, too. In the insulin-secreting cell line RINm5F, PKC activates capacitative Ca\(^{2+}\) entry (39). On the contrary, PKC stimulation has been shown to inhibit capacitative Ca\(^{2+}\) entry in thyroid cells.
In human neutrophils, formyl-methionyl-leucyl-phenylalanine and PMA inhibited capacitative Ca\(^{2+}\) entry, which was mediated by PKC. Capacitative Ca\(^{2+}\) entry caused by Drosophila photoreceptor activation is inhibited by PKC as well. Here, we suggest that 300 \(\mu\)M ATP preferentially inhibits capacitative Ca\(^{2+}\) entry by PKC activation. However, little is known about the involvement of PKA in the capacitative Ca\(^{2+}\) entry. It has been reported that activation of PKA had a biphasic effect on Ca\(^{2+}\) entry-evoked currents in thapsigargin-treated Xenopus oocytes. Application of dibutyryl cAMP at 1–10 \(\mu\)M inhibited the current, whereas at 1–10 \(\mu\)M potentiated the current. We show here that 30 \(\mu\)M ATP preferentially activates capacitative Ca\(^{2+}\) entry by relatively strong PKA activation rather than PKC activation. This activation is not the result of the further emptying the intracellular Ca\(^{2+}\) stores because Ca\(^{2+}\) stores may be fully depleted after thapsigargin treatment for 10 min, and no Ca\(^{2+}\) increase was detectable upon subsequent ATP treatment. We also tested the effect of prostaglandin E\(_2\) on the thapsigargin-induced capacitative Ca\(^{2+}\) entry. Prostaglandin E\(_2\) activates adenyl cyclase and increases intracellular cAMP concentration, but there were no detectable changes in the Ca\(^{2+}\) signal and IP\(_3\) generation in HL-60 cells. Prostaglandin E\(_2\) also potentiates the thapsigargin-induced capacitative Ca\(^{2+}\) entry as shown in the stimulation with 30 \(\mu\)M ATP. This result also suggests that PKA activates the capacitative Ca\(^{2+}\) entry in HL-60 cells.

In many cell types, functional effects elicited by extracellular ATP are related to a Ca\(^{2+}\) increase. Therefore, the Ca\(^{2+}\) increase must be tightly regulated to maintain cellular homeostasis and to exert physiological effects. The fine regulation of the ATP-induced Ca\(^{2+}\) signal could be achieved in a feedback mode with PKC and PKA, which are differentially activated according to the extent of stimulation caused by different ATP concentrations.

Acknowledgments—We are grateful to Dr. J. S. Chun, H. D. Chae, and M. J. Park for valuable discussion of the assays of protein kinase concentrations. We also thank G. Hoschek and H. M. Kim for editing this manuscript.

REFERENCES

1. Gordon, J. L. (1986) Biochem. J. 233, 309–319
2. Dubayk, G. A., Cowen, D. S., and Meuller, L. M. (1988) J. Biol. Chem. 263, 18108–18117
3. Fredholm, B. B., Abbracchio, M. P., Burnstock, G., Daly, J. W., Harden, T. K., Jacobson, K. A., Leff, P., and Williams, M. (1994) Pharmacol. Rev. 46, 143–156
4. Dubyk, G., and El-moataassim, C. (1993) Am. J. Physiol. 265, C577–C606
5. Buell, G., Michel, A. D., Lewis, C., Collo, G., Humphrey, P. F. A., and Surprent, A. (1996) Blood 87, 2659–2664
6. Choi, S. Y., and Kim, K. T. (1997) Biochem. Pharmacol. 53, 429–432
7. Berridge, M. J. (1995) Biochem. J. 312, 1–11
8. Putney, J. W., and Bird, G. St. J. (1995) Cell 75, 199–201
9. Carafoli, E. (1987) Annu. Rev. Biochem. 56, 395–433
10. Suh, B. C., and Kim, K. T. (1994) Biochem. Pharmacol. 47, 1262–1266
11. Di Virgilio, F., Fasolato, C., and Steinberg, T. H. (1988) Biochem. J. 256, 959–963
12. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
13. Sage, S. O., Merritt, J. E., Hallam, T. J., and Rink, T. J. (1989) Biochem. J. 258, 923–926
14. Suh, B. C., and Kim, K. T. (1995) J. Neurochem. 64, 2500–2508
15. Challis, R. A., Chilvers, E. R., Willecocks, A. L., and Nahorski, S. R. (1990) Biochem. J. 265, 421–427
16. Suh, B. C., Lee, C. O., and Kim, K. T. (1995) J. Neurochem. 64, 1071–1079
17. Rokoski, R., Jr. (1986) Methods Enzymol. 99, 3–6
18. Davies, W. A., Berghorn, K. A., Albrecht, E. D., and Pepe, G. J. (1993) Endocrinology 132, 2491–2497
19. Noland, T. A., Jr., and Dimino, M. J. (1986) Biochim. Biophys. Acta 853, 555–565
20. De Lean, A., Munson, P. J., and Rodbard, D. (1978) Am. J. Physiol. 235, E97–E102
21. Suh, B.-C., Song, S.-K., Kim, Y.-K., and Kim, K.-T. (1996) J. Biol. Chem. 271, 32753–32759
22. Schilling, W. P., Rajan, L., and Stohl-Jager, E. (1989) J. Biol. Chem. 264, 12838–12848
23. Takemura, H., Hughes, A. R., Thastrup, O., and Putney, J. W. Jr. (1989) J. Biol. Chem. 264, 12266–12271
24. Krautwurst, D., Seifert, R., Hescheler, J., and Schultz, G. (1992) Biochem. J. 288, 1025–1035
25. Lagast, H., Pozzan, T., Waldregel, F. A., and Lew, P. D. (1984) J. Clin. Invest. 73, 878–883
26. Zimmermann, H. (1992) Biochem. J. 285, 345–365
27. Randriamampita, C., and Tsien, R. Y. (1993) Nature 364, 809–814
28. Parikh, A. B., Terlau, H., and Stuhmer, W. (1995) Nature 374, 814–818
29. Jaconi, M. E. E., Lew, D. P., Monod, A., and Krause, K.-H. (1995) J. Biol. Chem. 268, 26075–26078
30. Bird, G. St. J., and Putney, J. W. Jr. (1993) J. Biol. Chem. 268, 21486–21488
31. Irvine, R. F. (1990) FEBS Lett. 263, 5–9
32. Zit, C., Zobel, A., Obukhov, A. G., Harteneck, C., Luckoff, A., and Schultz, G. (1998) Neuron 20, 357–364
33. Randriamampita, C., and Tsien, R. Y. (1995) J. Biol. Chem. 270, 29–32
34. Lee, K.-M., Toscas, K., and Villereal, M. L. (1994) J. Biol. Chem. 268, 9945–9948
35. Tepel, M., Kuhnapefl, S., Thielmeier, G., Teupe, C., Schlottmann, R., and Zidek, W. (1994) J. Biol. Chem. 269, 26239–26242
36. Szabo, J., Goecke, B. (1994) FEBS Lett. 339, 307–311
37. Tanaka, M., and Schultz, G. (1996) Annu. Rev. Biochem. 65, 651–659
38. Zite, C., Zobel, A., Obukhov, A. G., Harteneck, C., Kalkbrenner, F., Luckoff, A., and Schultz, G. (1998) Neuron 20, 1189–1196
39. Clapham, D. E. (1996) Neuron 16, 1069–1072
40. Randriamampita, C., and Tsien, R. Y. (1995) J. Biol. Chem. 270, 29–32
41. Lee, K.-M., Toscas, K., and Villereal, M. L. (1994) J. Biol. Chem. 268, 9945–9948
42. Tepel, M., Kuhnapefl, S., Theilmeier, G., Teupe, C., Schlottmann, R., and Zidek, W. (1994) J. Biol. Chem. 269, 26239–26242
43. Bode, H.-P., Goke, B. (1994) FEBS Lett. 339, 307–311
44. Tanaka, M., and Schultz, G. (1996) Annu. Rev. Biochem. 65, 651–659
45. Hardie, R. C., Peretz, A., Suss-Toby, E., Rom-Glas, A., Bishop, S. A., Selinger, Z., and Minke, B. (1993) Nature 363, 634–637
46. Peterson, C., and Berridge, M. J. (1998) Biochem. J. 307, 663–668

\(^{2}\) H. Lee, B.-C. Suh, and K.-T. Kim, unpublished data.