Synergistic Activation of Mitogen-Activated Protein Kinase by Insulin and Adenosine Triphosphate in Liver Cells: Permissive Role of Ca^{2+}

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We have previously demonstrated that insulin and G_q-coupled receptor agonists individually activate mitogen-activated protein kinase (MAPK) in liver cells and both effects involve an influx of extracellular Ca^{2+}. Yet, these agonists have opposing physiological actions on hepatocyte glucose metabolism. We thus investigated the interaction between insulin and the $P2Y_2$ purinergic agonist adenosine triphosphate (ATP) on MAPK in HTC cells, a model hepatocyte cell line, and determined the involvement of cytosolic Ca^{2+}. Insulin and ATP each induced a dose-dependent phosphorylation of p44/42 MAPK that was partially inhibited by EGTA. However, pretreatment with insulin markedly increased the MAPK phosphorylation response to ATP. This potentiation was canceled by chelation of extracellular Ca^{2+} with EGTA. We used patch clamp electrophysiology and fluorescence microscopy to understand the role of intracellular Ca^{2+} in this effect. Insulin and ATP, respectively, induced monophasic and multiphasic changes in membrane potential and intracellular Ca^{2+} as expected. Pretreatment with 10 nmol/L insulin significantly decreased the initial rapid depolarization (inward nonselective cation current [NSCC]), as well as the compounded Ca^{2+} response induced by 100 μmol/L ATP. However, in Ca^{2+}-free conditions, insulin did not modify the Ca^{2+} mobilized from internal pools after stimulation with ATP. Upon Ca^{2+} readmission, internal store depletion by ATP or thapsigargin doubled the rate of capacitative Ca^{2+} influx, whereas insulin increased this influx 1.32-fold. On the other hand, insulin pretreatment counteracted the increased rate of Ca^{2+} influx induced by ATP but not by thapsigargin. In summary, insulin counteracts the membrane potential and Ca^{2+} responses to ATP in HTC cells. However, insulin and ATP effects on MAPK activation are synergistic and Ca^{2+} influx plays a permissive role. Therefore, the opposing metabolic actions of insulin and G_q-coupled receptor agonists involve an interaction in signaling pathways that resides downstream of Ca^{2+} influx.

INSULIN IS THE predominant anabolic hormone that acts on the liver to promote glucose storage and utilization, and to antagonize the action of catabolic hormones, particularly at the level of glucose metabolism. In addition, insulin promotes cation influx into liver cells that is important for its metabolic actions.[1] Recent results from our laboratory have demonstrated that an increase in steady-state intracellular Ca^{2+} caused by insulin couples the tyrosine kinase receptor to mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) activation in isolated rat hepatocyte couplets.[2,3] Both of these signaling components have been found to be physiologically relevant for insulin-mediated stimulation of glycogen and triacylglycerol synthesis in liver cells.[4,5] In addition, we have previously shown that G_q-coupled receptor agonists also stimulate MAPK and that this is partly mediated by Ca^{2+} influx.[6] In contrast, insulin counteracts the Ca^{2+} response induced by G_q-coupled receptor agonists in primary rat hepatocytes,[7,8] as well as the ensuing glycogen phosphorylase activation.[9]

We therefore studied the interactive effect of insulin and adenosine triphosphate (ATP) at the level of MAPK activation We chose ATP as a representative of G_q-coupled receptor agonists because it is a purinergic $P2Y_2$ agonist previously shown to increase cytosolic Ca^{2+} and to activate nonselective cation channels in HTC cells.[10] ATP and other purinergic agonists are also as potent as α_1-adrenergic agonists to induce catabolic actions in both rat and human hepatocytes.[11,12]

We also examined the implication of Ca^{2+} ions in the insulin-ATP interaction by measuring membrane potential and cytosolic Ca^{2+}. Indeed, insulin induces a slow and reversible depolarization of membrane potential in cultured hepatocytes[13] and in HTC cells.[14] Insulin was also shown to antagonize glucagon-induced hyperpolarization in the isolated perfused rat liver and this participates in its inhibition of gluconeogenesis.[15] In contrast, ATP triggers a triphasic membrane potential response in HTC cells, whereby the initial rapid depolarization is due to a nonselective cationic current.[16] The effect of insulin on this purinergic response is unknown.

HTC cells represent a particularly interesting model in which to address such questions. Several observations suggest that HTC cells reflect the behavior of primary hepatocytes. For example, this cell line possesses nonselective cation currents (NSCC) similar to primary rat hepatocytes[17,18] and are more amenable than the latter to patch clamp electrophysiological analysis.[10] In addition, HTC cells express insulin receptors[19] and possess the major components of the insulin receptor signaling cascade.[20] Moreover, the relative abundance of protein kinase C (PKC) isoforms in HTC cells closely resembles that of hepatocytes.[21] Finally, our own work has demonstrated that both insulin and ATP increase cytosolic Ca^{2+} in HTC cells,[14] as is the case in rat hepatocytes.[2,11]

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Thus, the aim of the present work was to study the interaction between insulin and Ca^{2+}-mobilizing agonist ATP in the HTC rat hepatoma cell line. The results show that although insulin counteracts cationic currents and intracellular Ca^{2+} increase induced by ATP, it potentiates the MAPK response to ATP. Influx of Ca^{2+} from the external milieu plays a permissive role in this synergistic action.

MATERIALS AND METHODS

Materials

Phospho-specific p44/42 MAPK (Thr 202/Tyr 204) antibody kit was purchased from Cell Signaling Technology (Beverly, MA) and was chosen because it detects p44 and p42 MAPK (Erk1 and Erk2) only when catalytically activated by phosphorylation at Thr202 and Tyr204.[22,23] Nonphosphospecific antibodies to MAPK (Cell Signaling Technology) were used to ascertain that protein level was not affected by treatment regimens. Bovine insulin, and aprotinin, as well as other chemicals, were purchased from Sigma-Aldrich Canada (Oakville, ON). Fura-2 acetoxymethylester (Fura-2 AM) was purchased from Cedarlane Laboratories (Eugene, OR).

Cell Culture

Rat hepatoma HTC cells were chosen because they express surface membrane channels very similar to those found in primary cultures of rat hepatocytes while offering a greater stability for measurements of membrane currents by the patch clamp method.[10,17] Dr J.Gregory Fitz of the Colorado Health Science Center (Denver, CO) kindly provided the initial HTC cells that are now grown in our laboratory. They were maintained on minimum essential medium (MEM) containing 25 mmol/L HCO_3^-, 2 mmol/L glutamine, 5% fetal bovine serum, and 1% penicillin-streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. All experiments were conducted on cells at in-house passages 4-9. For MAPK experiments, cells were grown to confluence in 100-mm plastic tissue culture dishes and incubated in MEM containing 0.5% serum for 16 to 20 hours prior to experiment. For all other experiments, cells were grown for 24 hours on 22-mm round glass coverslips.

MAPK Phosphorylation Assay

The confluent HTC cells were serum-deprived and then treated with the concentrations of insulin and ATP specified in the figure legends. Cells treated with the vehicle of these hormones were used as controls. To assess the effect of 4 mmol/L EGTA, this agent was administered 15 minutes before and throughout the insulin and/or ATP challenge. Because chelation of extracellular calcium inhibits hepatocyte surface nucleotidase activity, and hence perturbs nucleotide binding to hepatic purinergic receptors,[24] we used the nonhydrolyzable analog of ATP, ATP-γS, for MAPK studies involving EGTA. We verified that this agonist yields comparable calcium and MAPK responses to ATP (data not illustrated). In all experiments, the incubation was stopped by quickly removing the medium and washing the cells twice with ice-cold phosphate-buffered saline (PBS). Petri dishes were then quickly frozen on liquid nitrogen and stored at −80°C pending the enzyme assay. Cells were lysed in 0.4 mL of lysis buffer (25 mmol/L Tris-HCl, pH 7.4, 25 mmol/L NaCl, 1 mmol/L Na ortho-vanadate, 10 mmol/L Na fluoride, 10 mmol/L Na pyrophosphate, 2 mmol/L benzamidine, 0.5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL aprotinin, 1% Triton X-100, and 0.1% sodium dodecyl sulfate [SDS]). Cell lysates were subjected to electrophoresis on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) or nitrocellulose membranes, blotted with phospho-specific p44/42 MAPK (Thr202/Tyr204) antibody (1:2,000) or with non–phospho-specific antibodies (1:1,000), and detected with anti-mouse or anti-rabbit antibody (1:4,000) using an enhanced chemiluminescence kit (New England BioLabs, Beverly, MA). Equal protein loading was routinely ascertained after transfer to PVDF or nitrocellulose membranes by Ponceau Red staining. Band intensities were quantified by scanning densitometry of the developed autoradiograms using the NIH Imager shareware.

Patch Clamp Recording

Membrane currents were measured at room temperature using standard patch clamp recording techniques.[25] Coverslips with adhered HTC cells were placed in a plastic perfusion chamber (100 μL trough) set on the stage of an inverted microscope (Olympus IMT-2, Carsen Medical, Markham, ON) and single cells were selected for study. Cells were continuously superfused (~2 mL/min) with a bath solution (138 mmol/L NaCl, 3.8 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 10 mmol/L HEPES-NaOH, 5 mmol/L D-glucose, 1 mmol/L Na-pyruvate, pH 7.4) at room temperature. An 8-way solenoid valve allowed rapid administration and washout of pharmacological agents. Patch pipettes typically exhibited resistances of 4 to 6 Mohm filled with standard pipette solution (140 mmol/L KCl, 1.2 mmol/L $MgCl_2$, 1 mmol/L EGTA, pH 7.35). All solutions were filtered through 0.22-μm cellulose membranes prior to use.

Whole cell recordings were performed using the perforated patch method described by Horn and Marty.[26] Briefly, the tip of the pipette was capillary filled with the appropriate pipette solution (~3-second immersion) and the rest of the pipette was then backfilled with the same solution containing nystatin (200 μg/mL). After formation of a seal between the pipette and cell membrane, the membrane potential was held at −40 mV until continuity with the cell interior was fully established (20 to 40 minutes), as evidenced from capacitative transients induced by short 20-mV voltage steps given every 5 minutes. Membrane potential was measured in the current clamp (I = 0) mode. After giga-seal formation, electrical signals were amplified and filtered (3 kHz) with a L/M-EPC-7 patch clamp amplifier (Medical Systems Corp, Greenvale, NY), continuously monitored on an oscilloscope and displayed on a chart recorder. Simultaneously, currents were digitized using a 2-channel A/D converter (Instrutech, Elmont, NY) and recorded on standard VHS video magnetic tape. A TL-1 DMA Interface and the PClamp software (v.5.5.1) were used for data acquisition and PClamp v.6.0 was used for data analysis (Axon Instruments, Foster City, CA).

Intracellular Calcium Measurement

HTC cells were loaded with 1.5 μmol/L Fura-2AM for 45 minutes at room temperature in bicarbonate-free MEM medium. Coverslips with adhered cells were transferred into a 100-μL plastic chamber placed on the stage of an inverted microscope coupled to a spectrofluorimeter (Deltascan RF-D4010, Photon Technology International Inc, London, Canada). Cells were perifused at a flow rate of 2 to 3 mL/min with standard buffer (see above) at room temperature. For Ca^{2+}-withdrawal and readmission protocols, we used standard buffer where Ca^{2+} was omitted and 4 mmol/L EGTA was added. We have previously used this protocol to establish the effect of insulin on Ca^{2+} influx in comparison to ATP.[14]Chelation with EGTA was found to effectively abolish Ca^{2+} influx without affecting agonist-induced mobilization from intracellular stores. We have also verified cell viability with the vital dye Trypan blue and found it to be over 90% after 15 to 20 minutes incubation with 4 mmol/L EGTA. Excitation wavelengths were 350 and 380 nm, while fluorescence emission was measured at 505 nm. Intracellular dye calibration was performed in situ by perfusion of 3.5 μmol/L ionomycin containing either 4 mmol/L EGTA (Rmin, 350/380 fluorescence ratio in Ca^{2+}-free solution) or 4 mmol/L $CaCl_2$ (Rmax, 350/380 fluorescence ratio at saturating Ca^{2+}). Once

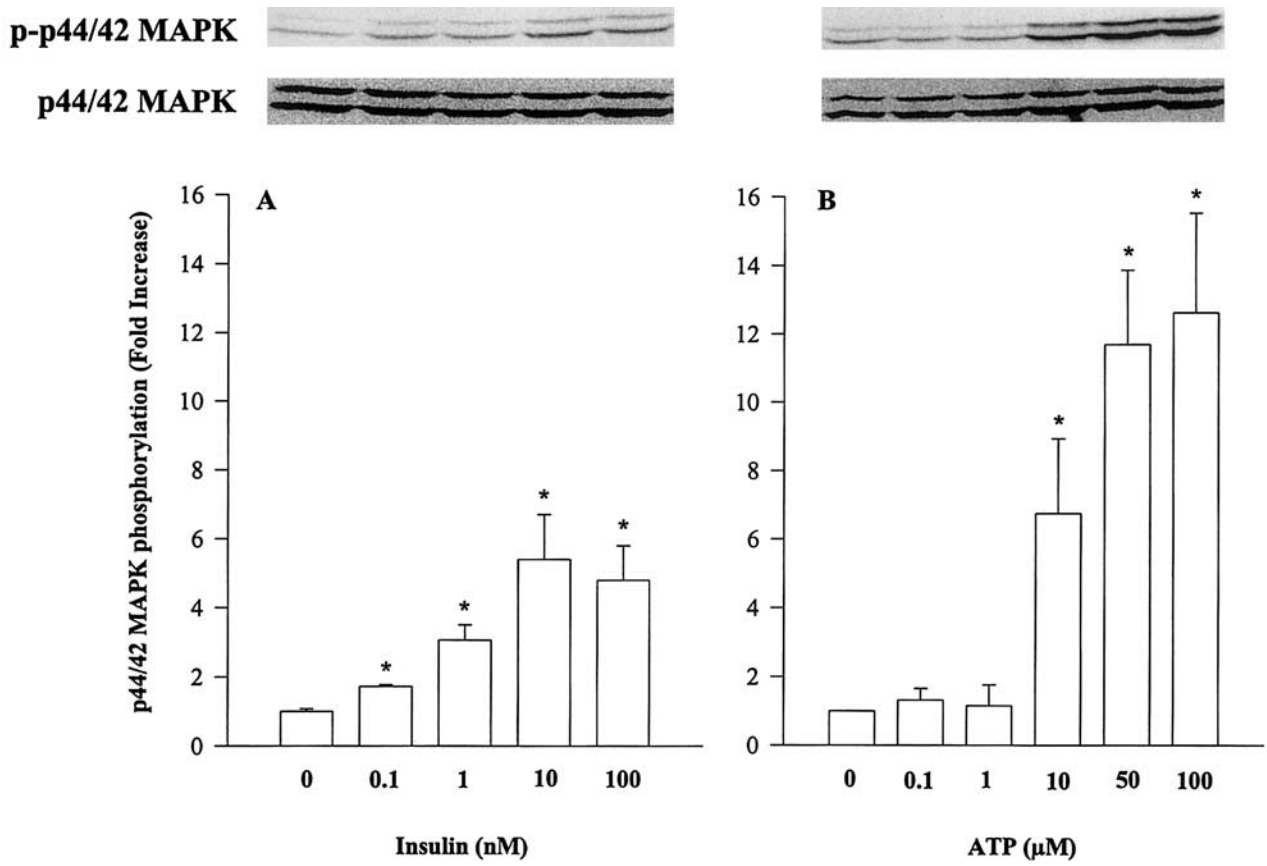

Fig 1. Insulin and ATP induce concentration-dependent p44/42 MAPK phosphorylation. HTC cells were incubated in MEM containing 0.5% serum for 16-20 h and treated with the indicated concentrations of insulin (A) or ATP (B) for 5 min. Cells were lyzed and cell lysates were processed as described in the Methods for immunoblotting with anti-phospho-p44/42 MAPK or anti-p44/42 MAPK antibody to determine the phosphorylation state and the protein level of p44/42 MAPK, respectively. Representative immunoblots of each of these are presented for insulin (A) or ATP (B) dose-responses. The phosphorylation of p44/42 MAPK bands were quantified by densitometry using NIH imager and expressed as fold-increase over baseline signal density. Results are expressed as the mean ± SEM of 3 (A) or 4 (B) different experimental series. *Significantly different from control ($P < .001$).

corrected for autofluorescence, the fluorescence ratios (350/380) were transformed into Ca^{2+} concentrations using the OSCAR software supplied by Photon Technology International Inc.

Statistical Analysis

Significant differences between group means were evaluated by analysis of variance (ANOVA) or by paired or unpaired Students' *t* test as appropriate using the SigmaStat software (Jandel Scientific, San Rafael, CA). Differences in the frequency of appearance of the rapid depolarization induced by ATP was evaluated by the nonparametric chi-square test.

RESULTS

Insulin and ATP Induce MAPK Phosphorylation in HTC Cells

HTC cells were treated with increasing concentrations of insulin or ATP for 5 minutes. The cells were then lysed and analyzed for phosphorylated p44/42 MAPK as described in the Methods. As shown in Fig 1, insulin and ATP led to a dose-dependent increase in p44/42 MAPK phosphorylation. A detectable increase in p44/42 MAPK phosphorylation was induced by as little as 0.1 nmol/L insulin and the maximum response occurred at concentration of 10 nmol/L (Fig 1A), a dose-response relationship consistent with several other insulin-dependent actions in cultured rat hepatocytes.[3,27,28] In comparison, the Gq-coupled $P2Y_2$ receptor agonist ATP was found to induce a maximal phosphorylation of p44/42 MAPK that was twice as important as that induced by 10 nmol/L insulin (Fig 1B). Additional time-course experiments also confirmed that insulin and ATP induced a typical bi-phasic pattern of MAPK response, with an inital peak at 5 minutes and a later one between 60 and 120 minutes (data not illustrated).

Effect of Ca^{2+} Chelation on Insulin- and ATP-Induced MAPK Phosphorylation.

As in several other cell types, Ca^{2+} ions have been reported to be an important component of both tyrosine kinase and G_q-coupled receptor stimulation of p44/42 MAPK activity in primary rat hepatocytes.[2,6] We thus examined the role of extracellular Ca^{2+} in insulin- and ATP-γS–induced p44/42 MAPK phosphorylation in HTC cells. For this purpose, we

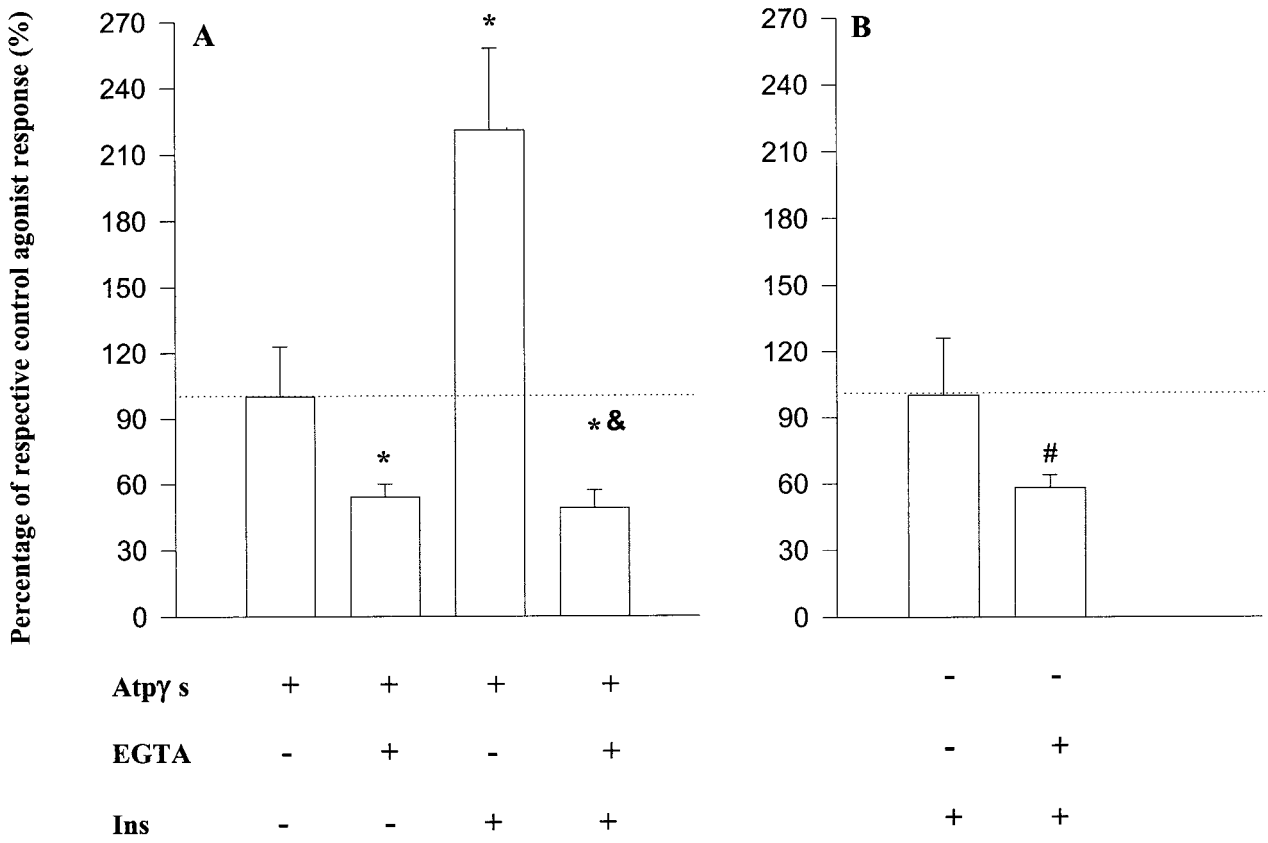

Fig 2. Effect of EGTA on Insulin and ATP-γS–induced p44/42 MAPK phosphorylation. HTC cells were preincubated in MEM medium containing 4 mmol/L EGTA for 15 min before 10 nmol/L insulin and 100 μmol/L ATP-γS were added for 5 min. Cells were analyzed for p44/42 MAPK phosphorylation as described in the legend of Fig 1. Stimulations of MAPK phosphorylation were normalized to responses (100%) obtained with 100 μmol/L ATP-γS alone (A) or with 10 nmol/L insulin alone (B). It must be noted, as shown in Fig 1, that responses to 10 nmol/L insulin were half as intense in absolute terms (fold-increase over unstimulated controls) as those to ATP. Hence, histogram bars of the right panel would lie approximately at the 50% and 25% levels in the left panel, respectively. Results are expressed as the mean ± SEM of 4 to 14 different experimental series. Significantly different from ATP-γS (*$P < .05$) or insulin (#$P < .05$); significantly different from ATP-γS + insulin (&$P < .05$).

preincubated the cells in the presence of 4 mmol/L EGTA for 15 minutes before addition of ATP or insulin. As previously demonstrated, this protocol allows the maintenance of a proper mobilization of internal Ca^{2+} from internal pools by G_q-coupled receptor agonists and does not affect insulin binding to its receptor in rat hepatocytes[2] or in HTC cells.[14] As shown in Fig 2, EGTA pretreatment diminished both ATP-γS– (Fig 2A) and insulin-induced (Fig 2B) p44/42 MAPK phosphorylation, significantly and to the same extent (percent of respective control). This confirms that an influx of Ca^{2+} is required for the full expression of both insulin- and ATP-γS–mediated responses in HTC cells. Control experiments ascertained that pretreatment of cells with EGTA for 15 minutes did not significantly alter basal MAPK phosphorylation (results not shown).

Interaction Between Insulin and ATP on MAPK Phosphorylation

We next verified the effect of insulin on ATP-γS–induced MAPK phosphorylation. We used a protocol where 10 nmol/L insulin was administered to HTC cells 1 minute prior to and throughout the subsequent 5-minute administrations of ATP-γS 100 μmol/L. Surprisingly, such coadministration of insulin markedly increased the response to ATP-γS (Fig 2A). In fact, this increase represented more than twice the control ATP-γS response (set to 100% in Fig 2A). Additional time-course experiments ascertained that insulin did not interfere with the kinetics of MAPK activation induced by ATP-γS (results not illustrated). Finally, when insulin and ATP-γS were coadministered in HTC cells that had been pretreated for 15 minutes with EGTA, MAPK phosphorylation dropped to a level similar to experiments with ATP-γS and EGTA but without insulin. Thus, the potentiation by insulin of ATP-induced MAPK phosphorylation appears to be entirely dependent on the presence of extracellular Ca^{2+}.

Interaction Between Insulin and ATP on NSCC

We next performed whole-cell patch clamp recordings to study the effects of insulin on membrane potential changes induced by the Ca^{2+}-mobilizing agonist, ATP. In the current clamp mode, 100 μmol/L ATP induced a typical triphasic response of membrane potential (V_m) as previously described in these cells by Fitz and Sostman.[16] As shown by these

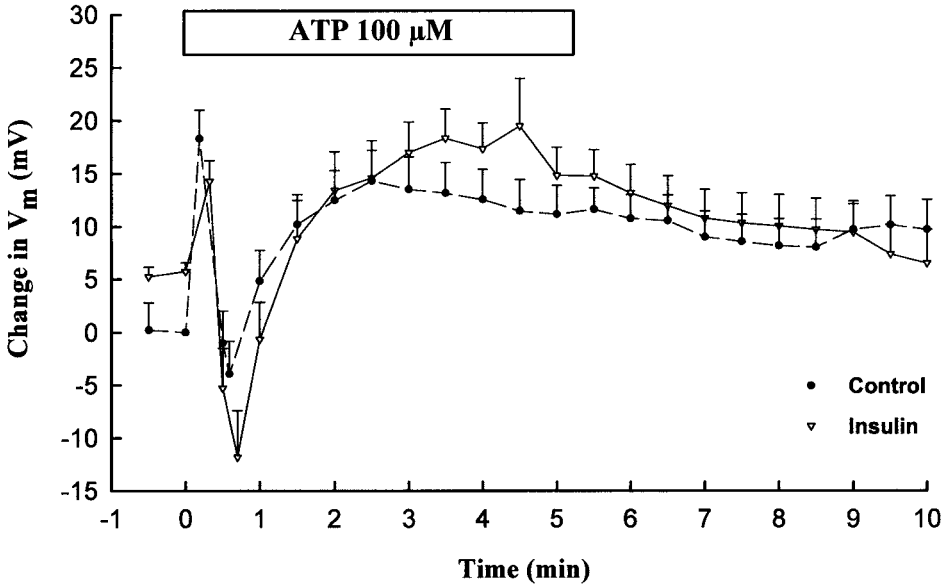

Fig 3. Effect of 10 nmol/L insulin on membrane potential changes induced by ATP in HTC cells. In control experiments (•), membrane potential changes were measured in HTC cells that were perfused with 100 μmol/L ATP for a period of 5 min followed by 5 min of washout. In experiments with insulin (▽), cells were treated with the peptide for 5 min prior to and during the subsequent 5 min challenge with ATP. Data represent the average of 6 separate experiments with an initial membrane potential (V_m) of −41.8 ± 5.0 mV for insulin-pretreated cells and of 24 individual cells, which had an initial V_m of −46.3 ± 2.6 mV for controls.

investigators, the initial rapid depolarization represents cation influx through nonselective channels[16] and was observed in 84% (26/31) of cases. When insulin (10 nmol/L) was present 5 minutes prior to and throughout the subsequent 5-minute administrations of ATP, the initial rapid depolarization was observed in only 50% (8/16) of cases ($P < .05$ by χ^2 test). Figure 3 illustrates the membrane potential changes (relative to steady-state V_m) obtained with ATP, in the presence or absence of insulin, for experiments where an initial depolarization was observed. With ATP alone, the rapid initial depolarization averaged 18 ± 3 mV (n = 26), while the subsequent repolarization peaked at −4 ± 3 mV (n = 31). As previously reported, insulin 10 nmol/L decreases baseline Vm in HTC cells[14] and the curve thus starts at a depolarized value. In the continued presence of insulin, the ATP-induced rapid depolarization, expressed in relation to the V_m observed immediately before ATP addition, decreased from18 ± 3 mV (n = 26) to 12 ± 2 mV (n = 8, $P < .05$). Insulin pretreatment also caused the peak repolarization to reach a lower level (−12 ± 4 mV, n = 16). However, the extent of this repolarization (expressed in relation to the peak initial depolarization) was unchanged. Similarly, the presence of insulin did not affect the slow depolarization phase of the triphasic membrane potential response to ATP. Thus, insulin specifically antagonizes ATP-induced membrane depolarization due to an inward non-selective cationic current component.

Interaction Between Insulin and ATP on Cytosolic Ca^{2+} Homeostasis

We next investigated the effects of insulin on ATP-induced Ca^{2+} responses. As shown in Fig 4, two 2-minute pulses of 100 μmol/L ATP were administered to HTC cells at an interval of 5 minutes. In such conditions, the second ATP response (evaluated as the area under the curve [AUC]) amounted to 79% ± 6% of the first one (n = 26, $P < .05$, Fig 4A). In a second set of experiments, 1 minute prior to the second ATP pulse, cells were perifused with 10 nmol/L insulin, which was also present throughout the subsequent ATP administration. Insulin itself triggered a small rise in cytosolic Ca^{2+} as previously observed[14] and evidenced in Fig 4B by the small shoulder preceding the second ATP-induced response. However, such coadministration of insulin (10 nmol/L) decreased the second ATP compound Ca^{2+} response to 56% ± 3% of the first one (n = 42, Fig 4B, $P < .05$) and this was significantly smaller than in controls without insulin ($P < .001$). Thus, in HTC cells, insulin counteracts Ca^{2+} mobilization by ATP in agreement with the previously described inhibitory effects of insulin on responses to Ca^{2+}-mobilizing agonists in rat hepatocytes.[7]

Finally, we used a protocol of Ca^{2+} withdrawal and readmission[29] to study the interactive effect of insulin with the Ca^{2+} influx triggered by internal store depletion. Administration of 100 μmol/L ATP in Ca^{2+}-free conditions (1 minute prior to and throughout the subsequent Ca^{2+} readmission) induced a sharp transient rise in cytosolic Ca^{2+} as expected from mobilization of internal pools (not illustrated) and doubled the initial rate of Ca^{2+} influx upon external Ca^{2+} readmission (Table 1). When introduced 1 minute prior to ATP in Ca^{2+}-free conditions, insulin (10 nmol/L) did not affect the mobilization of internal stores by the purinergic agonist as evaluated by the area under the Ca^{2+}-versus-time curve (3.2 ± 0.9 μmol/L *v* control value of 4.4 ± 0.7 μmol/L, difference not significant [NS]). However, in the continued presence of insu-

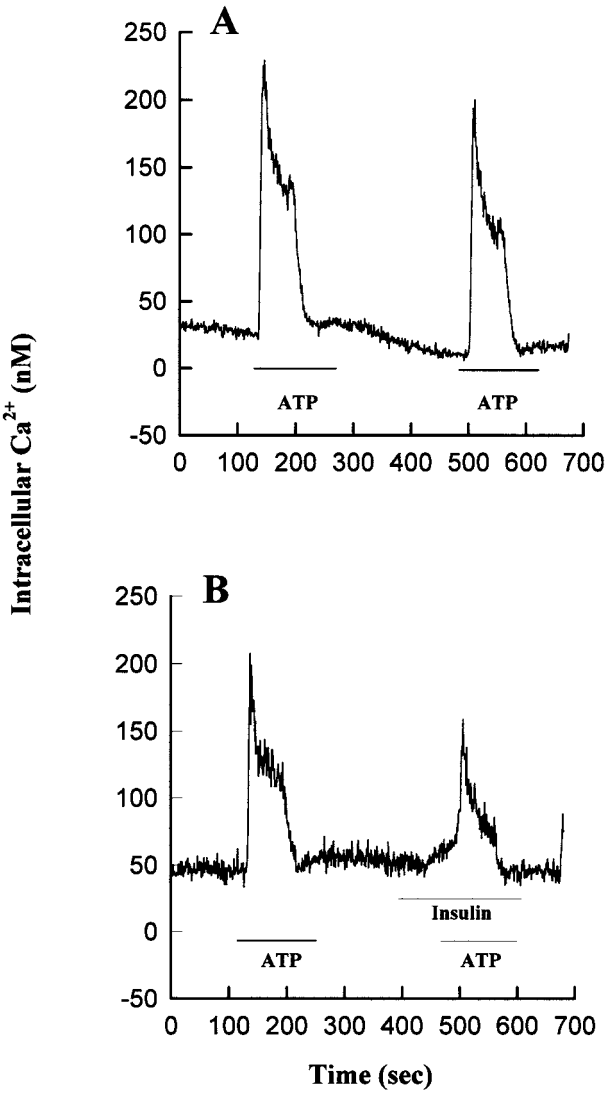

Fig 4. Effect of insulin on ATP-induced Ca^{2+} response in HTC cells. (A) Representative trace of the cytosolic Ca^{2+} response to two 2-min pulses of 100 μmol/L ATP administered at an interval of 5 min. The compound response to the second ATP pulse amounted to 79% ± 6% of the first as measured by the ratio of the respective areas under the cytosolic Ca^{2+}-*v*-time curves (AUC2/AUC1, n = 26). (B) In this representative trace, 10 nmol/L insulin was introduced 1 min prior to and during the second 2-min challenge with ATP. Insulin significantly decreased the second ATP response to 56% ± 3% of the first one (AUC2/AUC1, n = 42).

lin, ATP failed to increase the slope of the Ca^{2+} influx upon readmission of extracellular Ca^{2+} (Table 1). Similar experiments were performed with thapsigargin 0.5 μmol/L to deplete intracellular Ca^{2+} stores without the intervention of second messengers. Coadministration of 10 nmol/L insulin did not have a significant effect on either the AUC of the thapsigargin response in Ca^{2+}-free conditions (data not illustrated) or on the initial slope of Ca^{2+} influx upon readmission of external Ca^{2+} (Table 1). Thus, insulin counteracts the Ca^{2+} influx induced by ATP but not by thapsigargin. This indicates that insulin may interfere with the $P2Y_2$ purinergic receptor signaling mechanisms, or enhance feedback inhibition of Ca^{2+} influx, rather than modulate the communication between the emptying of internal Ca^{2+} pools and the capacitative Ca^{2+} influx.

DISCUSSION

Our laboratory has previously observed that insulin can trigger Ca^{2+} influx into isolated rat hepatocyte couplets and that this effect was important for the full expression of the signaling pathway to MAPK activation.[2] Parallel studies with G_q-coupled receptor agonists demonstrated clearly that these agents also stimulate MAPK activity in primary rat hepatocytes and that Ca^{2+} influx was equally important in mediating this effect.[6] We therefore wondered how insulin would affect the response of hepatocyte MAPK to G_q-coupled receptor agonists, and chose ATP as a representative of the latter.

ATP is an interesting and physiologically relevant G_q-coupled receptor agonist in the liver. Indeed, extracellular nucleotides such as uridine triphosphate (UTP) and ATP act on liver cells mainly via purinergic $P2Y_2$ receptors[10,11,30] and trigger IP_3 formation, intracellular Ca^{2+} mobilization, and activation of glycogen phosphorylase in both rat[11] and human[12] hepatocytes. In fact, the catabolic effects of ATP are comparable to those of α_1-adrenergic agonists in hepatocytes of both species.[11,12] More recently, ATP has also been found to play a significant role in the autocrine control of cell volume and secretory functions of liver cells.[31] Finally, extracellular ATP activates several ion channels in liver cells, including stretch-activated and nonselective cation channels,[10,32] as discussed further below.

As observed in primary hepatocytes, we found that both insulin and ATP induce a dose-dependent activation of MAPK in HTC cells, and that this effect was partly dependent on the presence of extracellular Ca^{2+}. However, the results of the present study clearly demonstrate that insulin amplifies the activation of MAPK induced by ATP. Indeed, the level of ATP-induced phosphorylation of MAPK was more than dou-

Table 1. Direct and Interactive Effects of Insulin, ATP, and Thapsigargin on the Initial Ca^{2+} Influx Rate Observed Upon Readmission of External Ca^{2+} After a Period of Ca^{2+}-Free Perfusion

	Control	Insulin (10 nmol/L)
Control	1.0 (n = 36)	1.32 ± 0.32 (n = 22)*
ATP (100 μmol/L)	1.99 ± 0.54 (n = 16)*	1.05 ± 0.38 (n = 16)
Thapsigargin (0.5 μmol/L)	1.92 ± 0.29 (n = 10)*	1.76 ± 0.32 (n = 10)*

NOTE. Ratios reported represent the initial rate of Ca^{2+} influx (nmol/L/s, measured over the initial 15 seconds of the cytosolic Ca^{2+}-*v*-time curve upon readmission of external Ca^{2+} after a 15-minute period of Ca^{2+}-free perfusion) observed for individual experiments divided by the average value observed in daily controls (control set to equal 1.00). Direct effects of insulin (10 nmol/L) or ATP (100 μmol/L) were observed when added 2 minute prior to external Ca^{2+} readmission. Thapsigargin (Tg 0.5 μmol/L) was present for 5 minutes before the return to normal Ca^{2+} bathing conditions. Interactive effects of insulin on Ca^{2+} influx triggered by internal store depletion was studied by introducing insulin 1 minute prior to and throughout the subsequent ATP or thapsigargin administration.

*Significantly different from control value by paired *t* test: $P < .01$.

bled by prior insulin administration. This was surprising in view of the physiological antagonistic action of insulin on catabolic effects of G_q-coupled receptor agonists, which is also reflected at the level of membane events and signal transduction pathways. Indeed, insulin counteracts glucagon-induced membrane hyperpolarization in the isolated perfused rat liver. This effect is important to mediate the inhibition by insulin of the gluconeogenesis triggered by glucagon.[15] Insulin also interferes with the cytosolic Ca^{2+} response to G_q-coupled receptor agonists,[7,8] and with the ensuing activation of glycogen phosphorylase.[9] We therefore examined in greater detail the interaction between insulin and ATP on cytosolic Ca^{2+}.

Indeed, our previous studies demonstrated that both the Ca^{2+} influx and MAPK activity stimulated by insulin were sensitive to Ca^{2+}-channel blockers nickel and gadolinium, but not to verapamil. Interestingly, this pharmacological profile is similar to that found for so-called capacitative Ca^{2+} influx pathways induced in hepatocytes by mobilization of internal Ca^{2+} stores with G_q-coupled receptor agonists or with thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase.[33] The molecular entities responsible for this capacitative Ca^{2+} influx in liver parenchymal cells are still unknown. However, nonselective cation channels (NSCC) described by Lidofsky et al on rat hepatocytes and HTC cells are activated in response to G_q-coupled agonists[17] and are sensitive to nickel and NSCC-inhibitor SKF96365 but not to verapamil.[18] These properties make these channels likely candidates for store depletion-induced Ca^{2+} influx pathways.[33] ATP-induced Ca^{2+} influx was also shown to proceed through stretch-activated NSCC channels sensitive to gadolinium and cell swelling.[32,34] In view of their similar charateristics and participation in ATP-activated $Ca^{2\pm}$ influx, these 2 NSCC channels could represent the same entity. However, in the absence of more detailed molecular information (no such channel has yet been cloned from the liver), this question remains open. Insulin also induces Ca^{2+} influx and inward NSCC currents in HTC cells, with a pharmacological sensitivity profile similar to NSCC.[14] Recently, Kilic et al confirmed that insulin increases plasma membrane conductance in HTC cells and further showed that it causes the recruitment of channel-containing vesicles in these cells,[35] implicating the necessity of an intact microtubule network for insulin effects on membrane channels. Overall, current data thus suggests that insulin activates NSCC and other channels in liver cells.

In the present study, our results show that insulin decreases the frequency of appearance, as well as the amplitude, of the ATP-induced rapid initial depolarization of HTC cell membrane potential. This initial part of the triphasic response of membrane potential to $P2Y_2$ purinergic agonists was clearly shown by Fitz and Sostman to represent NSCC activation.[16] Hence, our data suggest that insulin may inhibit or interfere with NSCC activation by G_q-coupled purinergic agonists. Insulin also significantly diminished the compounded cytosolic Ca^{2+} response triggered by purinergic stimulation. Insulin thus antagonized the response to a G_q-coupled receptor agonist in HTC cells, as expected from its physiological role and as previously observed in isolated rat hepatocytes.[7,9] Again, however, insulin itself increases intracellular Ca^{2+} and depolarizes membrane potential in HTC cells as mentioned above.[14] We thus set out to determine at what level insulin was acting on the Ca^{2+} response to ATP.

Our results indicate that the modulation of the ATP-induced Ca^{2+} response by insulin was due to an antagonism of Ca^{2+} influx rather than an effect on Ca^{2+} mobilization from internal stores. Indeed, in Ca^{2+}-free bathing conditions, purinergic agonist stimulation yields only Ca^{2+} mobilization from internal stores. This was not affected by the presence of insulin as measured by AUC analysis of the Ca^{2+} response. On the other hand, the initial rate of Ca^{2+} influx observed upon subsequent readmission of external Ca^{2+} after ATP stimulation (representing capacitative Ca^{2+} entry) was strongly inhibited by the concurrent administration of insulin. As mentioned, this Ca^{2+} entry presumably occurs through NSCC pathways. Our results on membrane potential and cytosolic Ca^{2+} thus concur to show that insulin inhibits ATP-induced Ca^{2+} influx.

To gain further insight on the underlying mechanisms, experiments were performed with thapsigargin to deplete internal Ca^{2+} stores directly without interfering with G_q-coupled signaling components. Thapsigargin, like ATP, induced a doubling of the rate of Ca^{2+} influx after the Ca^{2+} withdrawal and readmission protocol. This confirms that the depletion of internal Ca^{2+} stores triggers capacitative Ca^{2+} influx in HTC cells, as seen in hepatocytes.[36] As was the case for ATP, insulin did not have an effect on the extent of thaspsigargin-induced Ca^{2+} mobilization from internal stores. However, unlike results with ATP, insulin pretreatment did not have a significant effect on the Ca^{2+} influx caused by store depletion with thapsigargin. This implicates that insulin is not interfering with the putative signal(s) that couples physical depletion of internal Ca^{2+} stores with capacitative Ca^{2+} influx.[37] On the other hand, our study clearly shows that insulin antagonizes ATP-induced Ca^{2+} influx.

Several mechanisms could be involved in this effect. First, insulin could modulate negatively the number of purinergic receptors present on the hepatocyte membrane. If this was true, then we would have expected insulin to diminish the mobilization of Ca^{2+} from internal stores induced by ATP. Our AUC analysis in Ca^{2+}-free conditions clearly demonstrates that insulin is devoid of such an effect. Consequently, we can also shed reasonable doubt on the possibility that insulin is interfering with other components of purinergic receptor signaling leading to Ca^{2+} mobilization from internal stores. This could include altered phospholipase C activity, enhanced IP_3 degradation or diminished IP_3 receptor density or responsiveness.

An interesting alternative hypothesis would be that insulin is somehow enhancing ATP-induced PKC stimulation. Indeed, Fitz et al have clearly shown that NSCC channels were strongly inhibited by conventional (Ca^{2+}- and diacylglycerol-dependent) isoforms of PKC.[10] They also suggested that this may represent a form of feedback inhibition to terminate agonist-induced Ca^{2+} responses. Hence, insulin may amplify the stimulation of conventional PKC by ATP. However, this effect cannot be mediated through Ca^{2+} ions since insulin diminishes the compounded Ca^{2+} response to ATP.

On the other hand, several nonconventional (Ca^{2+}-independent, but diacyglycerol-dependent) and atypical (Ca^{2+}- and diacyglycerol-independent) isoforms of PKC are known to be implicated in the action of insulin on rat hepatocytes.[38,39] This

brings forth the interesting possibility that stimulation of such PKC isoforms by insulin may strengthen the negative feedback action of conventional PKCs activated by G_q-coupled receptor agonists or act synergistically wih the latter. This would explain the antagonism, by insulin, of both membrane potential and Ca^{2+} responses to ATP observed herein. It is conceivable that insulin- and ATP-activated PKC isoforms may act on similar or different sites on NSCC, but such detailed determinations will only be made possible by the still-awaited cloning and molecular characterization of these NSCC.

This hypothesis also offers an explanation for the synergistic effect of insulin on ATP-induced MAPK stimulation observed in the present study. Indeed, for both G_q-coupled receptor agonists and insulin, MAPK activation may implicate PKC-dependent pathways in hepatocytes,[6] as in other cells.[40] It is therefore possible that convergent stimulation of MAPK, through different PKC isoforms induced by insulin and ATP, explains the synergy of the 2 hormones at that level in HTC cells. Moreover, our results with EGTA to chelate external Ca^{2+} support this hypothesis. Indeed, conventional PKC activation by G_q-coupled receptor agonists in hepatocytes requires Ca^{2+} as does MAPK activation.[6] Similarly, MAPK activation by insulin is also dependent on Ca^{2+} influx into rat hepatocytes,[2] as confirmed herein for HTC cells. Finally, Ca^{2+} influx impacts very early in insulin-mediated signal transduction, namely just distal of IRS-1 tyrosine phosphorylation, as shown recently by our laboratory in rat hepatocytes.[3] Therefore, insulin-induced nonconventional and atypical PKC activation is also expected to lie downstream of Ca^{2+} influx. Indeed, when insulin and ATP were coadministered in cells where external Ca^{2+} was chelated, the resultant MAPK activation resembled that seen in EGTA-treated cells stimulated with ATP alone. Hence, Ca^{2+} ions play a permissive role in the synergistic effect of insulin on ATP-induced MAPK activation.

Physiologically, our results confirm the antagonism by insulin of responses to Ca^{2+}-mobilizing hormones. On the other hand, the synergistic action of insulin and ATP on MAPK may be relevant in the context of the comitogenic actions of tyrosine kinase– and G_q-linked receptor agonists in liver cells.[41] Indeed, MAPK is implicated in proliferative responses in liver cells as in other cell types.

In conclusion, insulin counteracts the membrane potential and Ca^{2+} responses to ATP in HTC cells, as expected from the physiological antagonistic interaction between these anabolic and catabolic hormones in liver cells. At the level of MAPK activation, however, insulin and ATP effects are synergistic and this requires extracellular Ca^{2+} influx into liver cells.

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