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Mechanism of intracellular calcium ($[Ca^{2+}]_i$) inhibition of lipolysis in human adipocytes

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ABSTRACT

We investigated the mechanisms responsible for the anti-lipolytic effect of intracellular Ca^{2+} ($[Ca^{2+}]i$) in human adipocytes. Increasing $[Ca^{2+}]i$ inhibited lipolysis induced by β-adrenergic receptor activation, A1 adenosine receptor inhibition, adenylate cyclase activation, and phosphodiesterase (PDE) inhibition, as well as by a hydrolyzable cAMP analog, but not by a nonhydrolyzable cAMP analog. This finding indicates that the anti-lipolytic effect of $[Ca^{2+}]i$ may be mediated by the activation of adipocyte PDE. Consistent with this theory, $[Ca^{2+}]i$ inhibition of isoproterenol-stimulated lipolysis was reversed completely by the nonselective PDE inhibitor isobutyl methylxanthine and also by the selective PDE 3B inhibitor cilostamide, but not by selective PDE 1 and 4 inhibitors. In addition, phosphatidylinositol-3 kinase inhibition with wortmannin completely prevented insulin's anti-lipolytic effect but only minimally blocked $[Ca^{2+}]i$'s effect, which suggests that $[Ca^{2+}]i$ and insulin may activate PDE 3B via different mechanisms. In contrast, the antilipolytic effect of $[Ca^{2+}]i$ was not affected by inhibitors of calmodulin, Ca^{2+}/calmodulin-dependent kinase, protein phosphatase 2B, and protein kinase C. Finally, $[Ca^{2+}]i$ inhibited significantly isoproterenol-stimulated increases in cAMP levels and hormone-sensitive lipase phosphorylation in human adipocytes. In conclusion, increasing $[Ca^{2+}]i$ exerts an antilipolytic effect mainly by activation of PDE, leading to a decrease in cAMP and HSL phosphorylation and, consequently, inhibition of lipolysis.

Key Words: agouti • cAMP • hormone-sensitive lipase • phosphodiesterase

Previous data from our laboratory demonstrate that agouti protein, a murine obesity gene product, inhibits human adipocyte lipolysis via a Ca^{2+}-dependent mechanism (1), resulting in increased adipocyte lipid storage. In addition, several studies have reported direct anti-lipolytic effects of increasing intracellular calcium ($[Ca^{2+}]i$) under different conditions (2–5). However, the mechanisms responsible for this anti-lipolytic effect of $[Ca^{2+}]i$ are unclear. Although altered interaction between G_i and adenylate cyclase has been reported in one study, others suggest that alteration in adipocyte phosphodiesterase (PDE) activity may be responsible in the anti-lipolytic effect of increasing cellular Ca^{2+} concentrations (3–6).

Adipocyte lipolysis is under acute hormonal regulation (reviewed in 7, 8). Lipolytic hormones, such as catecholamines, act through β-adrenergic receptors, resulting in increases in cAMP, which then activate cAMP-dependent protein kinase A (PKA). The activation of PKA, in turn, phosphorylates and activates hormone-sensitive lipase (HSL), a key enzyme in lipolysis. Insulin, in contrast, acting through the insulin receptor and downstream phosphatidylinositol-3 (PI-3) kinase and possibly other downstream kinases, phosphorylates and activates a PDE kinase, which is now believed to be protein kinase B (PKB) (9). PKB then phosphorylates and activates PDE 3B, a major PDE 3 isoform expressed in adipose tissue, which catalyzes the degradation of cAMP and leads to a decrease in cAMP and PKA activity and inhibition of lipolysis (reviewed in 7, 8).

HSL is regulated primarily by reversible phosphorylation. Phosphorylation of the regulatory site on lipolytic stimulation results in activation of HSL (10–12). Phosphorylation of another serine residue, the basal site, occurs mainly in resting cells and has been shown to exert an anti-lipolytic effect as its phosphorylation prevents the phosphorylation of the regulatory site (13, 14). Recent evidence suggests that additional serine residues phosphorylated during HSL activation are crucial in governing HSL activity (15).

The present study evaluated selectively the effects of $[Ca^{2+}]i$ on each of these steps to investigate the mechanisms responsible for the anti-lipolytic effect of $[Ca^{2+}]i$ in human adipocytes. We report here that depolarizing human adipocytes with 100 mM KCl exerts an anti-lipolytic effect primarily via activation of PDE 3B. This activation results in a decrease in cAMP production, a net decrease in HSL phosphorylation, and, consequently, inhibition of lipolysis.

MATERIALS AND METHODS

Cell culture

We obtained human adipocytes either from Zen-Bio (Research Triangle, NC) or they were differentiated from human adipose tissue stromal vascular cells (Zen-Bio) in our laboratory. To differentiate mature adipocytes, we plated pre-adipocytes at a density of 30,000 cells/cm^2 with pre-adipocyte medium [Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-10 Nutrient Broth (Ham's F-10), 1:1 (v/v) containing 10% fetal calf serum, 15 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and antibiotics]. Cells were then put into differentiation medium [DMEM/Ham's F-10, 1:1 (v/v) containing 10% fetal bovine serum, 15 mM HEPES, 33 μM biotin, 17 μM pantothenate, 100 nM insulin, 1 μM dexamethasone, 0.25 mM isobutyl methylxanthine (IBMX), 1 μM BRL49653, 100 U/ml penicillin, and 100 μg/ml streptomycin]. After three days in differentiation cocktail, cells were changed to and maintained in adipocyte medium [DMEM/Ham's F-10, 1:1 (v/v) containing 3% fetal bovine serum, 15 mM HEPES, 33 μM biotin, 17 μM pantothenate, 100 nM insulin, 1 μM dexamethasone, 100 U/ml penicillin, and 100 μg/ml streptomycin] until fully differentiated. All cells were incubated in nominally serum-free medium [DMEM/Ham's F-10, 1:1 (v/v), 0.2% FBS, 15 mM HEPES, 33 μM biotin, 17 μM pantothenate, 100 U/ml penicillin, and 100 μg/ml streptomycin] overnight before each experiment.

$[Ca^{2+}]i$ measurement

We determined adipocyte $[Ca^{2+}]i$ by using a fura-2 dual wavelength fluorescence imaging system (Intracellular Imaging Inc., Cincinnati, OH). Human pre-adipocytes (Zen-Bio) were plated in 35-mm glass plates (Mat Tek Co., Ashland, MA) and differentiated as described above. Before $[Ca^{2+}]i$ measurement, cells were maintained serum-free overnight and then washed with HEPES balanced salt solution (HBSS, containing components NaCl 138 mM, $CaCl_2$ 1.8 mM, $MgSO_4$ 0.8 mM, NaH_2PO_4 0.9 mM, $NaHCO_3$ 4 mM, glucose 5 mM, glutamine 6 mM, HEPES 20 mM, and bovine serum albumin 1%). Cells were then loaded with 10 μM fura-2 acetoxymethyl ester (fura-2 AM) and incubated at 37˚C in the dark for 2 h. To remove extracellular dye, cells were rinsed with HBSS and kept at room temperature for an additional 1 h for complete hydrolysis of cytoplasmic fura-2 AM. Cells were then mounted on the stage of Nikon TMS-F fluorescence inverted microscope with a Cohu 4915 CCD camera. Fluorescent images were captured alternatively at excitation wavelength of 340 nm and 380 nm with an emission wavelength of 520 nm. Images were analyzed with InCyt Im2 version 4.62 imaging software (Intracellular Imaging Inc.). $[Ca^{2+}]i$ was calculated by using a ratio equation as described previously (16). Each analysis evaluated responses of 6–10 cells. We calibrated images using fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR) to create a calibration curve in solution. Cellular calibration was accomplished by using digitonin (25 μM) and Tris (pH 8.7)/ethylene glyco-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA, 100 mM) to measure maximal and minimal intracellular Ca^{2+} levels (16).

Lipolysis experiments

Differentiated human adipocytes were incubated in serum-free medium overnight before lipolysis experiments. Cells were subjected to the treatments described in each figure legend and table in Krebs-Ringer buffer containing 25 mM HEPES and 1.5% bovine serum albumin (KRH buffer) for 4 h. When we used 100 mM KCl in the treatment, we removed equimolar amounts of NaCl to maintain osmolarity. Lipolysis was determined as glycerol release into the culture medium by an enzymatic fluorometric method (17) by using a microplate fluorometer (Packard Instrument Company, Inc., Downers Grove, IL). Total DNA was measured by CyQUANT cell proliferation assay kit according to manufacturer's instruction (Molecular Probes, Eugene, OR). Cell viability was determined by Trypan blue exclusion examination and medium lactate dehydrogenase (LDH) activity measurement with a LDH-based *in vitro* toxicology assay kit (Sigma, St. Louis, MO).

cAMP measurement

To measure cAMP levels, human adipocytes were incubated in serum-free medium overnight and subjected to isoproterenol (5 nM) stimulation either with or without KCl (100 mM) or insulin (25 nM) in KRH buffer for 10 min. We lysed cells immediately and measured cellular cAMP levels according to manufacturer instruction by using a direct cAMP ^{125}I scintillation proximity assay (SPA) system (Amersham Pharmacia Biotech, Piscataway, NJ). The specific activity of ^{125}I was 2000 Ci/mmol.

^{32}P-labeling of human adipocytes and analysis of adipocyte phosphoproteins by sodium dodecyl sulfate (SDS) –polyacrylamide gel electrophoresis (PAGE)

The analysis of ^{32}P-labeled human adipocyte phosphoprotein was conducted according to Holm et al., Degerman et al., and Castan et al. (8, 18, 19). Human adipocytes were incubated in serum-free medium overnight and prelabeled for 90 min at 37˚C with 0.4–0.8 mCi/ml ^{32}P-orthophosphate (ICN, Costa Mesa, CA) in KRB buffer containing 0.3 mM KH_2PO_4. After labeling, cells were subjected to stimulation by isoproterenol (5 nM) either with or without KCl (100 mM) or insulin (25 nM) for 10 min. The incubations were terminated by the addition of homogenization buffer [50 mM Tris-HCl (pH 7.4), 250 mM sucrose, 1 mM EDTA, 1 mM dithioerythritol, 1 % (v/v) Triton X-100, 10% protease cocktail (Sigma), 1% each of serine phosphatase and tyrosine phosphatase inhibitor cocktail (Sigma)]. Cells were sonicated on ice for 10 s and incubated on ice for 1 h to solubilize all proteins. Insoluble material was removed by centrifugation at 12,500 x g at 4˚C for 10 min. Equal amounts of protein were boiled in Laemmli sample buffer (20) and subject to 10% SDS-PAGE (20). After being stained with Coomassie blue, gels were dried and adipocyte phosphoproteins were visualized by autoradiography.

Immunoprecipitation

Immunoprecipitation of adipocyte phosphoproteins was performed according to Castan et al. (19). Briefly, human adipocytes were prelabeled with 0.4–0.8 mCi/ml ^{32}P-orthophosphate and treated as described above. Adipocyte lysates were then prepared as described above, and equal amounts of lysates were incubated with an anti-HSL antibody (21) at 4˚C overnight on an orbital shaker. Lysates were then incubated with Protein A-Sepharose 4B-CL (Sigma) for 4 h at 4˚C. The immunocomplexe was washed five times with PBS containing 0.1% (w/v) N-laurylsarcosin, boiled in Laemmli sample buffer. The phosphorylated HSL was analyzed as above.

Statistical analysis

All data are expressed as means ± SE. Data were analyzed by using the procedures of SPSS Inc. (Chicago, IL). Difference of lipolysis among treatment groups was compared by using one-way analysis of variance (one-Way ANOVA). A P value < 0.05 is considered significant.

RESULTS

We used KCl as a depolarizing agent to stimulate $[Ca^{2+}]i$ in human adipocytes. We first studied the effect of KCl on human adipocyte $[Ca^{2+}]i$. Figure 1A shows fluorescent imaging of adipocyte $[Ca^{2+}]i$ in pseudocolor, which changes from blue/green in baseline to green/yellow/white on addition of KCl, indicating an increase in $[Ca^{2+}]i$. Quantitation of this response demonstrated a dose-dependent increase in $[Ca^{2+}]i$ in these cells, with 17.86 ± 1.65 nM, 87 ± 5.79 nM, and 158.71 ± 8.55 nM increases over baseline in response to 50, 80, and 100 mM KCl stimulation, respectively (Fig. 1B, $P < 0.05$).

KCl treatment also inhibited 0.1 μM forskolin-stimulated lipolysis in these adipocytes significantly, with the greatest effect at 100 mM (Fig. 1C). During these experiments, cell

viability was determined by both Trypan blue exclusion examination and medium lactate dehydrogenase activity measurement. Cell viability was not different between control and treatment groups (data not shown).

We next tested the ability of 100 mM KCl to inhibit lipolytic responses to the following agonists: isoproterenol, a β-adrenergic receptor agonist; 8-cyclopentyl-1,3-dipropyl xanthine (DPCPX), a selective A1 adenosine receptor antagonist; forskolin, an adenylate cyclase activator; isobutyl methyl xanthine (IBMX), a PDE inhibitor; 8-bromo-cAMP; and dibutyryl cAMP, hydrolyzable and nonhydrolyzable cAMP analogs, respectively, which activate protein kinase A. Figure 2 demonstrates that KCl treatment inhibited all except dibutyryl cAMP induced lipolysis in human adipocytes. 8-bromo-cAMP is susceptible to hydrolysis by PDE, whereas dibutyryl cAMP is resistant to hydrolysis by this enzyme. In addition, insulin, which inhibits adipocyte lipolysis by activation of PDE, also inhibited 8-bromo-cAMP induced lipolysis by 90%; it is, however, unable to inhibit dibutyryl cAMP induced lipolysis (22, 23). Our observation that KCl inhibited 8-bromo cAMP-stimulated lipolysis, although it is unable to inhibit dibutyryl cAMP-induced lipolysis, suggests that KCl may exert its anti-lipolytic effect primarily via activation of PDE, similar to that of insulin. In addition, although KCl completely inhibited isoproterenol and DPCPX and IBMX induced lipolysis, it right-shifted the dose-response curve of forskolin (EC_{50} from 0.17 to 0.60 μM), although the inhibitory effect is still evident even at a maximum dose of forskolin.

To investigate the mechanisms of the anti-lipolytic effect of KCl further, we next studied the ability of different lipolytic agonists to reverse KCl inhibited lipolysis. Figure 3A shows that forskolin, IBMX, and dibutyryl cAMP completely reversed the inhibitory effect of KCl in human adipocytes treated with isoproterenol. These data, combined with data from Figure 2, further suggest that KCl may exert its anti-lipolytic effect via a step before PKA; that is, activation of PDE, and, possibly, inhibition of adenylate cyclase.

Xanthine derivatives such as IBMX have been shown to act not only as PDE inhibitors but also as adenosine receptor antagonists (24). The adipocyte A1 adenosine receptor exerts an anti-lipolytic effect by its coupling to Gi, with consequent inhibition of adenylate cyclase (25). In the present study, the selective A1 adenosine receptor antagonist DPCPX alone resulted in a 70% increase in adipocyte lipolysis (Fig. 2B). However, it failed to reverse KCl inhibited lipolysis (Fig. 3A), indicating that the A1 adenosine receptor is not involved in the anti-lipolytic effect of KCl.

We next investigated the involvement of different PDE isoforms in the anti-lipolytic effect of KCl. It has been reported that adipocytes possess several PDE isoform activities (26–28). In the present study, we found that both the PDE 3B inhibitor cilostamide and the PDE 4 inhibitor 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone (rolipram) exhibited comparable lipolytic activities in human adipocytes. Cilostamide (2 μM) caused a 1.7-fold increase in lipolysis (50.19 ± 2.29 vs. 137.17 ± 6.45 nmol/μg DNA, $P < 0.05$), whereas rolipram (50 μM) caused a 1.2-fold increase (42.92 ± 3.43 vs. 92.42 ± 1.94 nmol/μg DNA, $P < 0.05$). Human adipocytes also exhibited less PDE 1 activity, as the PDE 1 inhibitor 8-methoxymethyl IBMX only exerted a 40% increase in lipolysis (40.98 ± 3.74 vs. 57.48 ± 3.42 nmol/μg DNA, $P < 0.05$).

Figure 3B demonstrates that KCl-inhibited lipolysis was reversed completely by the nonselective PDE inhibitor IBMX and by the selective PDE 3B inhibitor cilostamide. However, the selective PDE 1 and 4 inhibitors 8-methoxymethyl IBMX and rolipram failed to reverse the anti-lipolytic effect of KCl. Cilostamide and its derivative OPC 3911 have been shown to inhibit PDE3B activity selectively and to block the anti-lipolytic effect of insulin in adipocytes (26, 27, 29, 30). These data suggest that KCl's anti-lipolytic effects are mediated by the activation of PDE 3B, the same PDE isoform that mediates insulin's anti-lipolytic effect, but not by the PDE 1 or 4 isoforms.

We next compared the anti-lipolytic effect of KCl with that of insulin. Figure 4A shows that increasing insulin concentrations to 10 nM inhibited 0.1 μM forskolin-stimulated lipolysis. However, this activity was inhibited further by the addition of KCl, even at maximum insulin concentrations. In Figure 4B, 100 mM KCl and maximum concentration of insulin (25 nM) exerted an additive anti-lipolytic effect in the presence of increasing concentrations of forskolin. These data indicate that the anti-lipolytic effects of insulin and KCl are additive, suggesting that KCl and insulin may activate PDE 3B via different mechanisms.

Consistent with this, wortmannin, a PI-3 kinase inhibitor, completely reversed the anti-lipolytic effect of insulin. However, it blocked the anti-lipolytic effect of KCl only partially (Fig. 4C). This finding suggests further that KCl and insulin may activate PDE 3B via different mechanisms.

Because calmodulin and Ca^{2+}/calmodulin-dependent protein kinase (Ca^{2+}/CaM protein kinase) are logical targets for KCl action, we then used different calmodulin and Ca^{2+}/CaM protein kinase inhibitors to study whether calmodulin and Ca^{2+}/CaM protein kinase are involved in this effect of KCl. Table 1 demonstrates that the addition of different calmodulin inhibitors trifluoperazine (TFP), N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide (W-7), and calmidazolium, as well as the Ca^{2+}/CaM protein kinase inhibitor KN-62, failed to reverse the anti-lipolytic effect of KCl, even at very high concentrations. Instead, with the exception of calmidazolium, high doses of these compounds exerted independent anti-lipolytic effects comparable with that seen with KCl.

We also investigated the possible involvement of protein kinase C (PKC), protein phosphatase 2B (PP2B, calcinurin), and protein phosphatase 1/2A in the anti-lipolytic effect of KCl. Figure 5 shows that the addition of the PKC and PP 2B inhibitors chelerythrine chloride and FK 506, respectively, failed to affect the anti-lipolytic effect of KCl. However, the PP1/2A inhibitor okadaic acid, at a concentration that inhibits both PP1 and PP2A, abolished the anti-lipolytic effect of KCl completely. These data indicate that a serine/threonin protein phosphatase may be involved in the anti-lipolytic effect of KCl.

We also studied the effect of KCl on adipocyte cAMP level. In Figure 6, 5 nM isoproterenol treatment resulted in a fourfold increase in cAMP level (22.75 ± 2.57 vs. 118.55 ± 4.50 pmol/10^5 cells). However, this increase was blocked by the addition of KCl and insulin to a similar extent.

HSL activity is regulated primarily by reversible phosphorylation. In Figure 7A, 5 nM isoproterenol stimulated a significant increase in HSL phosphorylation in SDS-PAGE separated

adipocyte phosphoproteins. However, this increase was prevented by the addition of either insulin or KCl. This effect was confirmed by immunoprecipitation of adipocyte phosphoproteins with anti-HSL antibody (Fig. 7B).

DISCUSSION

Several studies have reported that increasing $[Ca^{2+}]i$ inhibits adipocyte lipolysis (1–5). However, the mechanism(s) mediating this effect remains unclear. Studies with epidermal growth factor (EGF) shows that the anti-lipolytic effect of EGF, which is mediated through an increase in $[Ca^{2+}]i$, primarily involves the modulation of the interaction between Gi and adenylate cyclase, leading to a reduction in cAMP (5, 6). However, studies of the lipolytic response in hypothyroid rats indicate that thyroid hormone status may modulate adipocyte lipolysis by alterations in cellular Ca^{2+} concentrations that may alter PDE activity (4). In addition, increasing $[Ca^{2+}]i$ by the calcium ionophore A23187 results in stimulation of PDE and a decrease in cAMP levels (3).

In our previous study, we demonstrated that agouti protein, a murine obesity gene product, inhibited adipocyte lipolysis via a postreceptor mechanism, and that this effect is mediated by an increase in $[Ca^{2+}]i$ (1). Further, adipose tissue from obese agouti mutants exhibits reduced lipolytic rate in response to the β-adrenergic receptor agonist epinephrine and the PDE inhibitor theophylline, whereas the response to dibutyryl cAMP, a nonhydrolizable cAMP analog, was normal (31). This finding suggests that increasing $[Ca^{2+}]i$ by agouti may affect the generation or maintenance of cAMP levels, leading to inhibition of lipolysis.

In this study, we used KCl to stimulate $[Ca^{2+}]i$ by depolarization, which exerted a potent anti-lipolytic effect. KCl treatment inhibited lipolysis induced by isoproterenol, a β-adrenergic receptor agonist; forskolin, an adenylate cyclase activator; IBMX, a PDE inhibitor; and 8-bromo cAMP, a hydrolyzable cAMP analog, which activates protein kinase A. However, lipolysis induced by dibutyryl cAMP, a nonhydrolyzable cAMP analog, was not affected by KCl, suggesting that KCl inhibition of adipocyte lipolysis may be mediated primarily by the activation of PDE. In addition, KCl inhibition of isoproterenol-induced lipolysis was reversed completely by IBMX and dibutyryl cAMP, further confirming that the anti-lipolytic effect of KCl is mediated primarily by the activation of adipocyte PDE and at a step before protein kinase A, as the addition of dibutyryl cAMP bypasses PDE. Consistent with this finding was our demonstrating that KCl treatment prevented isoproterenol stimulated cAMP production in adipocytes significantly, leading to a reduction in adipocyte cAMP level. In addition, this results in a net decrease in HSL phosphorylation and, consequently, inhibition of lipolysis. This finding is consistent with both *in vitro* and *in vivo* data demonstrating that agouti protein inhibits adipocyte lipolysis via an increase in $[Ca^{2+}]i$ (1). Obese agouti mutant mice express agouti protein in adipose tissue, resulting in an increase in $[Ca^{2+}]i$ (32, 33). This increase in $[Ca^{2+}]i$ results in reduced adipose tissue lipolysis in response to β-adrenergic stimulation and PDE inhibition, whereas the response to dibutyryl cAMP remains normal (31), which suggests a defect in the maintenance of cAMP levels in these animals.

In this study, we showed that KCl inhibition of isoproterenol-stimulated lipolysis was also reversed by forskolin. This finding may simply be due to the high concentration of forskolin used in this study, which may overwhelm the maximum capacity of adipocyte phosphodiesterase.

However, a possible involvement of adenylate cyclase in the anti-lipolytic effect of KCl cannot be ruled out, as the alteration in the interaction between G_s/G_i and adenylate cyclase has been implicated in the anti-lipolytic effect of EGF, which is also mediated by an increase in $[Ca^{2+}]i$ (5, 6). In addition, type V and VI adenylate cyclase have been shown to be specifically inhibited by submicromolar concentrations of Ca^{2+} (34). Further studies are needed to explore these possibilities.

More than 10 PDE isoforms have been discovered and characterized (35, 36). Several studies have shown that adipocytes possess several PDE isoform activities (26–28). In 3T3-L1 adipocytes and rat epididymal fat cells, a low-Km, cGMP-inhibitable PDE isoform (identified as PDE 3B in 37), the PDE isoform that mediates the anti-lipolytic activity of insulin (30), accounts for the major particulated PDE activity (26, 27). A cAMP-specific PDE isoform (identified as PDE 4 in 37) is the major cytosolic PDE isoform (26, 27). In addition, the adipocyte cytosolic fraction also contains distinct Ca^{2+}/calmodulin- (26) and cGMP-stimulated PDE activity, albeit to a lesser extent (26, 27), which represents PDE 1 and 2, respectively, according to the nomenclature proposed in ref 37. Human adipose tissue also contains more than one PDE activity (28).

In this study, we found that human adipocytes contain comparable PDE 3B and PDE 4 activity, and less PDE 1 activity, as the selective PDE 3B, 4 and 1 inhibitors cilostamide, rolipram, and 8-methoxymethyl IBMX exert 1.7-, 1.2-fold, and 40% increase in lipolysis, respectively. However, KCl inhibition of isoproterenol-stimulated lipolysis was reversed only by the selective PDE 3B inhibitor cilostamide, which suggests that the anti-lipolytic effect of KCl is mediated by the activation of PDE 3B, the same PDE isoform mediating the anti-lipolytic effect of insulin. However, we showed that the anti-lipolytic effects of KCl and insulin are additive, suggesting that KCl and insulin may activate PDE 3B via different mechanisms. Consistent with this finding, PI-3 kinase inhibition by wortmannin, which has been shown to be critical in insulin-induced activation of PDE 3B (38), completely abolished the anti-lipolytic effect of insulin. However, it blocked the effect of KCl only partially. These data further indicate a different regulatory effect of insulin and KCl on PDE 3B.

Insulin-mediated activation of PDE 3B has been shown to be dependent on PI-3 kinase (38). The generation of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate by PI-3 kinase leads to the recruitment and phosphorylation of the serine-threonine kinase protein kinase B (PKB) by 3'-phosphoinositide-dependent kinase-1 (PDK1, reviewed in 39). PKB then phosphorylates and activates PDE 3B (9, 18, 39), leading to a reduction in cAMP and inhibition of lipolysis. However, it is not clear which pathway leads to the activation of PDE 3B by KCl. In addition, as new members of the PDE family have been identified recently (36), it is possible that KCl may activate new PDE isoforms (other than PDE 3B), which are not yet well characterized.

Calmodulin and Ca^{2+}/calmodulin-dependent protein kinases are logical targets for $[Ca^{2+}]i$. However, to our surprise, we found that they are not involved in the anti-lipolytic effect of KCl. Nor did we find other Ca^{2+}-dependent pathways, protein kinase C (PKC), and phosphatase 2B (PP2B, calcinurin) to be involved in the anti-lipolytic activity of KCl. However, the addition of okadaic acid, at a concentration that inhibits both phosphatase 1 and 2A (PP1 and 2A),

completely abolished the effect of KCl. This finding may suggest a possible involvement of a serine/threonine protein phosphatase in the anti-lipolytic activity of KCl. Further studies are needed to elucidate which phosphatase is involved in the effect of KCl and the precise role of the phosphatase in this process.

In conclusion, we demonstrated that KCl increases $[Ca^{2+}]i$ via depolarization, which exhibits potent anti-lipolytic effects in human adipocytes. This effect is mediated primarily by activation of adipocyte PDE and a reduction in cAMP levels, leading to a decrease in HSL phosphorylation and, consequently, inhibition of lipolysis.

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Table 1

Effects of calmodulin and Ca^{2+}/calmodulin dependent protein kinase inhibitors on KCl-inhibited lipolysis

Treatment	Glycerol (nmol/μg DNA)
Control	12.10 ± 0.78
Isoproterenol 5 nM	21.28 ± 1.36 *
Isoproterenol 5 nM + KCl 100 mM	13.01 ± 0.50
+ TFP 10 μM	13.25 ± 0.64
+ TFP 50 μM	10.34 ± 0.67
+ TFP 100 μM	9.90 ± 0.78
+ W-7 10 μM	11.54 ± 0.66
+ W-7 50 μM	10.94 ± 0.48
+ W-7 100 μM	7.27 ± 0.79
+ Calmidazolium 10 nM	13.46 ± 0.85
+ Calmidazolium 50 nM	13.11 ± 0.39
+ Calmidazolium 100 nM	13.43 ± 0.54
+ KN-62 1 μM	13.54 ± 0.40
+ KN-62 10 μM	9.43 ± 0.44
+ KN-62 50 μM	8.10 ± 0.28

*$P < 0.05$ vs. control

Fig. 1

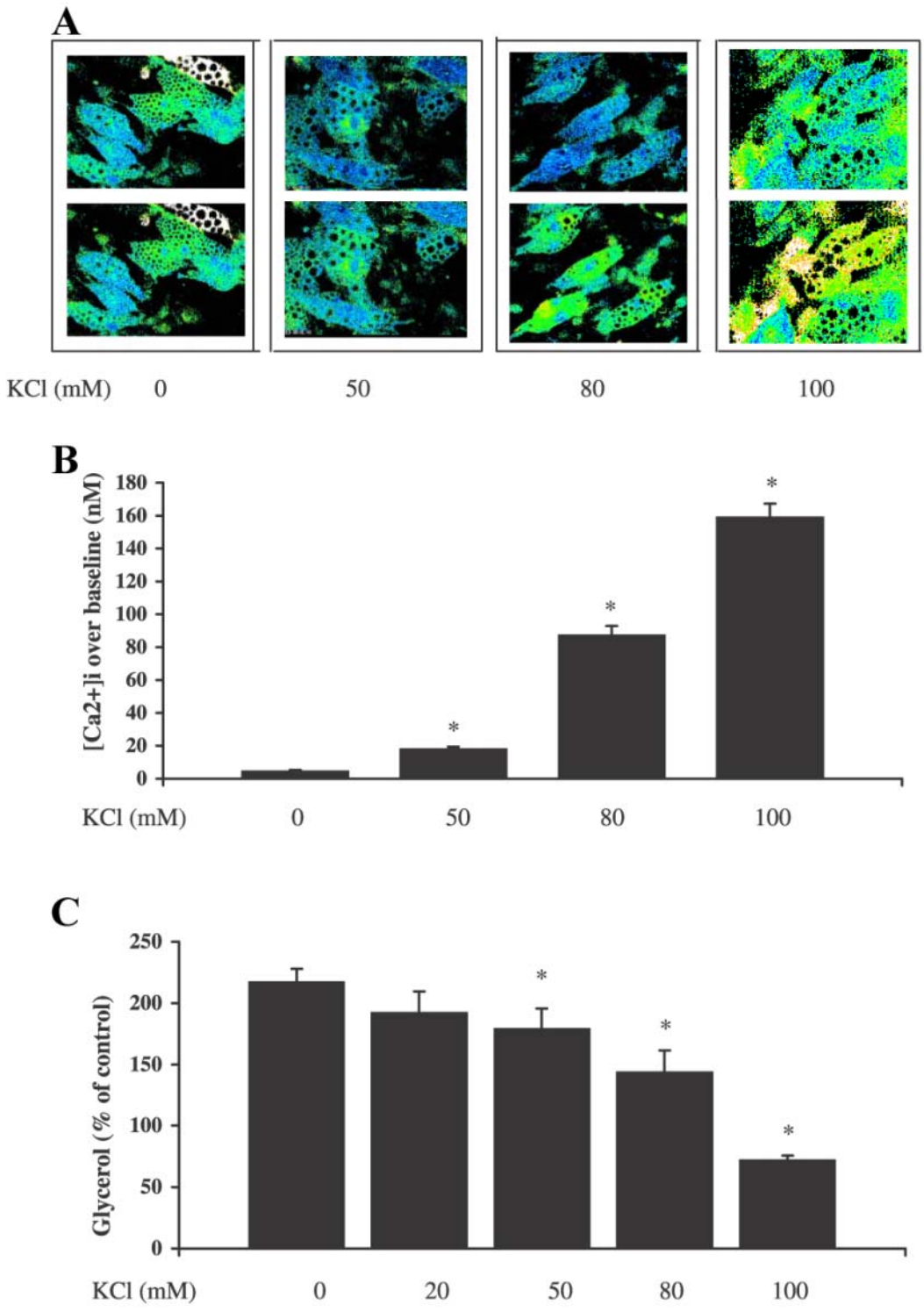

Figure 1. Effect of KCl on human adipocyte $[Ca^{2+}]i$ (A and B) and forskolin-stimulated lipolysis (C). A) Human pre-adipocytes were plated and differentiated into mature adipocytes. Adipocytes were loaded with fura-2/AM and $[Ca^{2+}]i$ was measured by using a fura-2 dual wavelength imaging system. The upper and lower panels represent fluorescent image of adipocyte $[Ca^{2+}]i$ before and after the addition of vehicle (H_2O), 50, 80, and 100 mM KCl, respectively.
B) Quantification of KCl-stimulated increase in $[Ca^{2+}]i$. *$P < 0.05$ versus control. **C)** Human adipocytes were treated with 0.1 μM forskolin and the indicated concentrations of KCl for 4 h. Glycerol release was measured as nmol/μg DNA and expressed as percentage of control. Data are expressed as mean ± SE for six experiments. *$P < 0.05$ vs. no KCl treatment.

Fig. 2

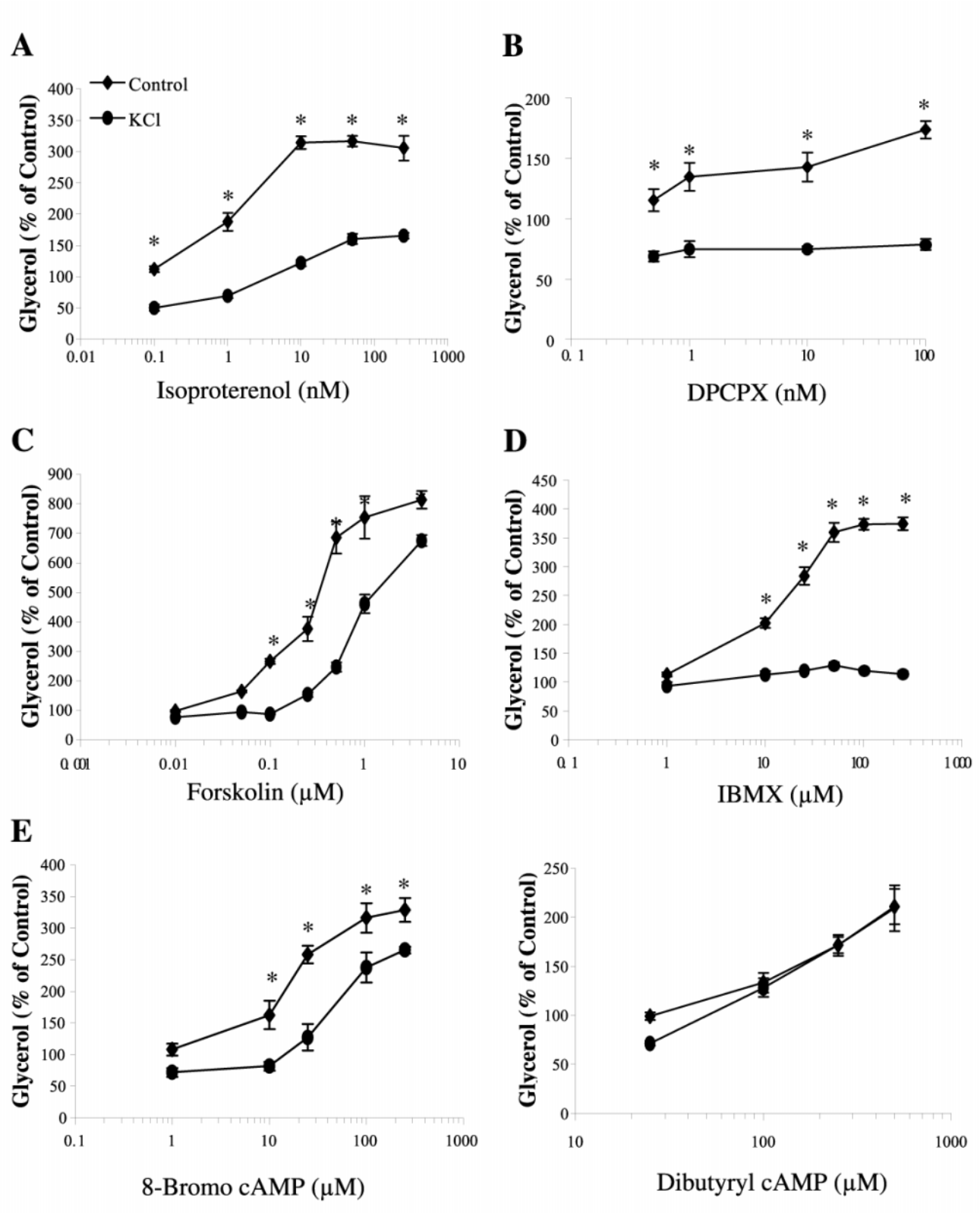

Figure 2. Effect of 100 mM KCl on lipolysis in human adipocytes stimulated by various agonists. Human adipocytes were treated with KCl and isoproterenol **(A)**, DPCPX **(B)**, forskolin **(C)**, IBMX **(D)**, 8-bromo-cAMP **(E)** and dibutyryl cAMP (**F**) for 4 h. Glycerol release was measured as nmol/μg DNA and expressed as percentage of control. Data are expressed as mean ± SE for six experiments. * $P < 0.05$ versus KCl treatment. DPCPX: 8-cyclopentyl-1,3-dipropyl xanthine. IBMX: isobutyl methyl xanthine.

Fig. 3

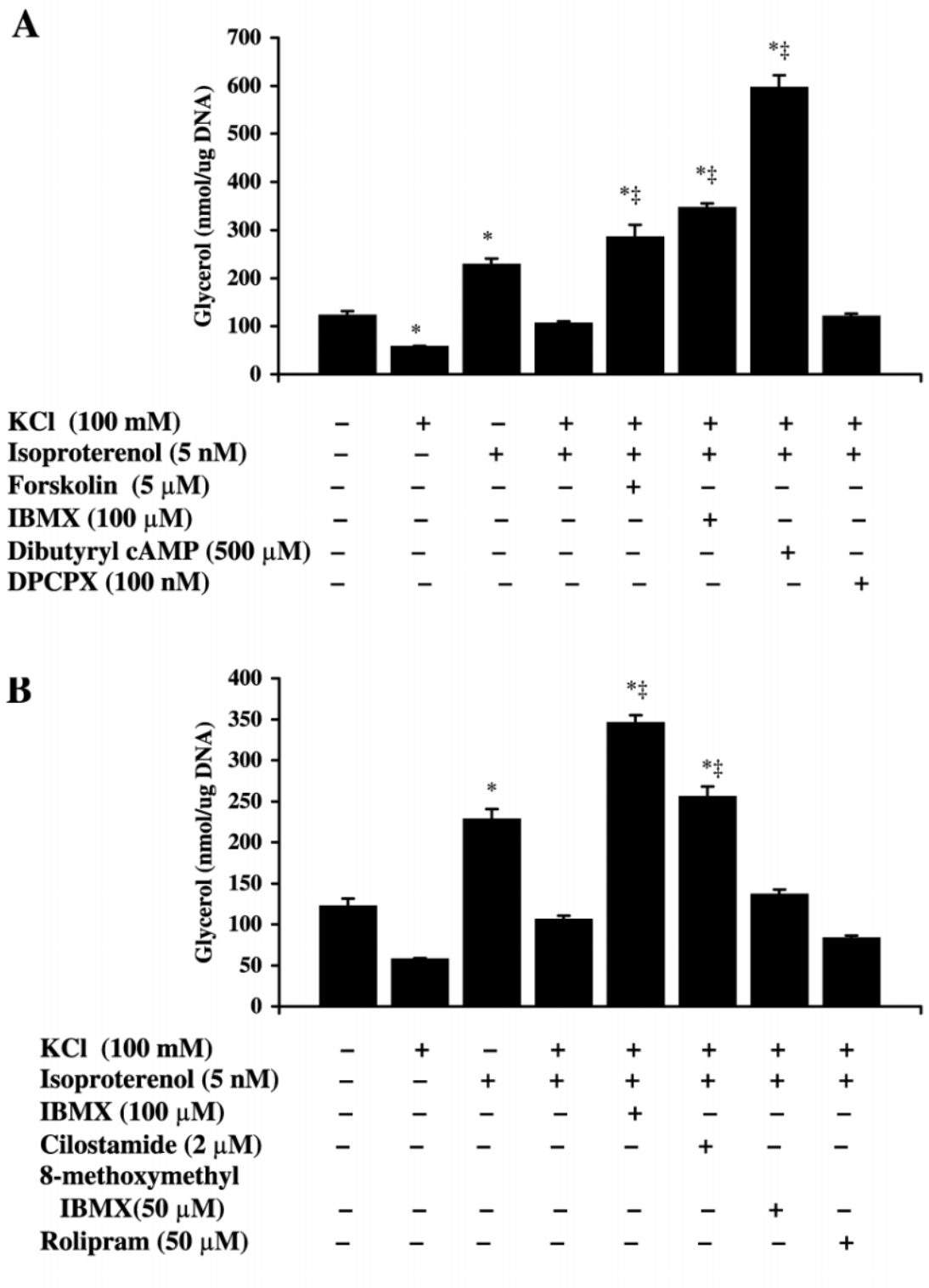

Figure 3. Effects of different agonists on the reversal of KCl inhibition of isoproterenol-stimulated lipolysis. Human adipocytes were treated with isoproterenol, KCl, and the indicated agonists for 4 h. Data are expressed as mean ± SE for six experiments. * $P < 0.05$ vs. control. ‡ $P < 0.05$ versus KCl + Isoproterenol. DPCPX: 8-cyclopentyl-1,3-dipropyl xanthine. IBMX: isobutyl methyl xanthine.

Fig. 4

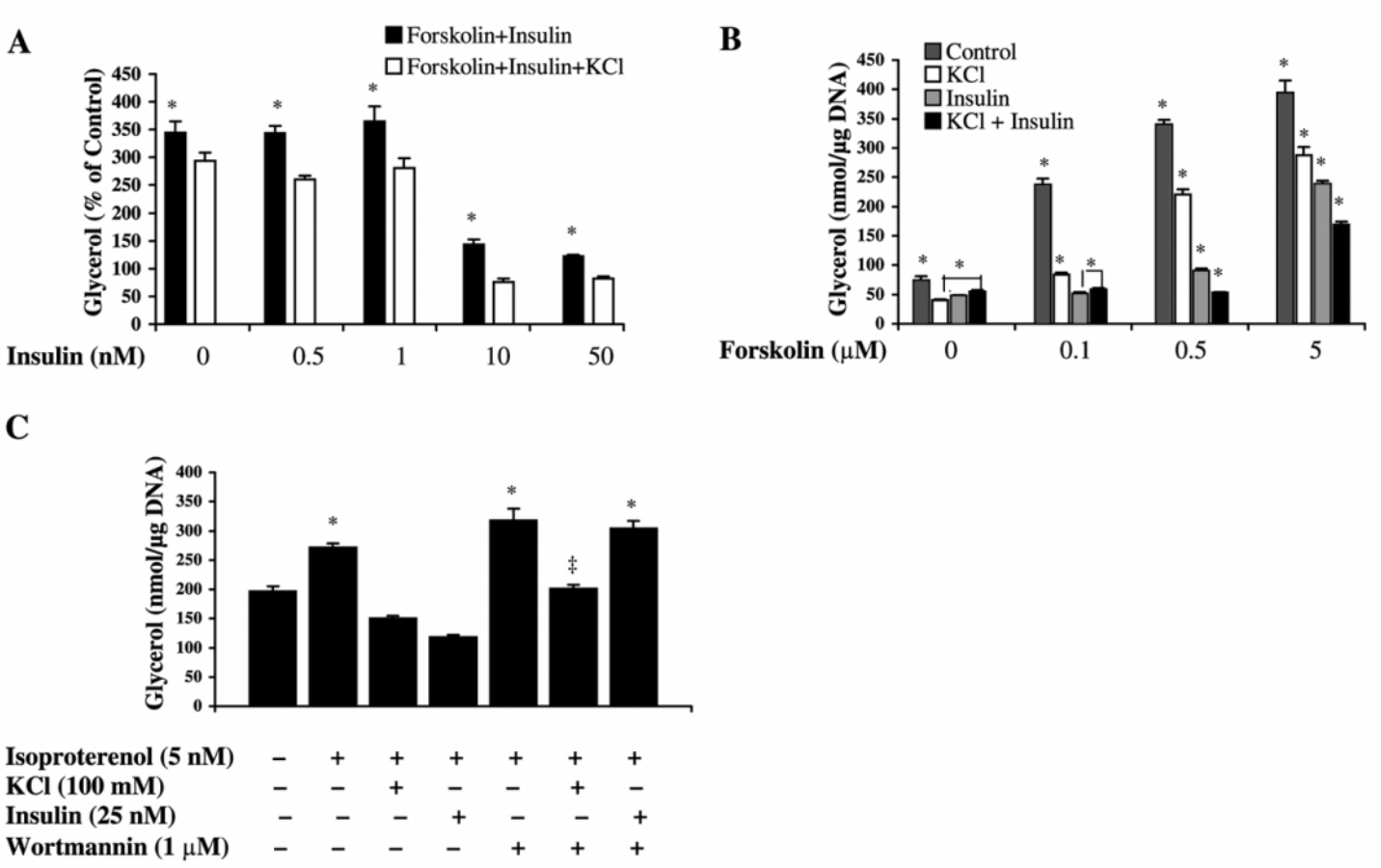

Figure 4. The anti-lipolytic effect of KCl and insulin are additive. A) Human adipocytes were treated with 0.1 µM forskolin, 100 mM KCl, and different concentrations of insulin for 4 h. Glycerol release was measured as nmol/µg DNA and expressed as percentage of control. Data are expressed as mean ± SE for six experiments. * $P < 0.05$ versus KCl treatment. **B)** Human adipocytes were treated with 25 nM insulin, 100 mM KCl, and different concentrations of forskolin for 4 h. Data are expressed as mean ± SE for six experiments. * $P < 0.05$ within each cluster. **C)** Human adipocytes were treated with isoproterenol in the presence of either KCl or insulin and either with or without wortmannin for 4 h. Data are expressed as mean ± SE for six experiments. * $P < 0.05$ versus control. ‡ $P < 0.05$ vs. Isoproterenol + Wortmannin.

Fig. 5

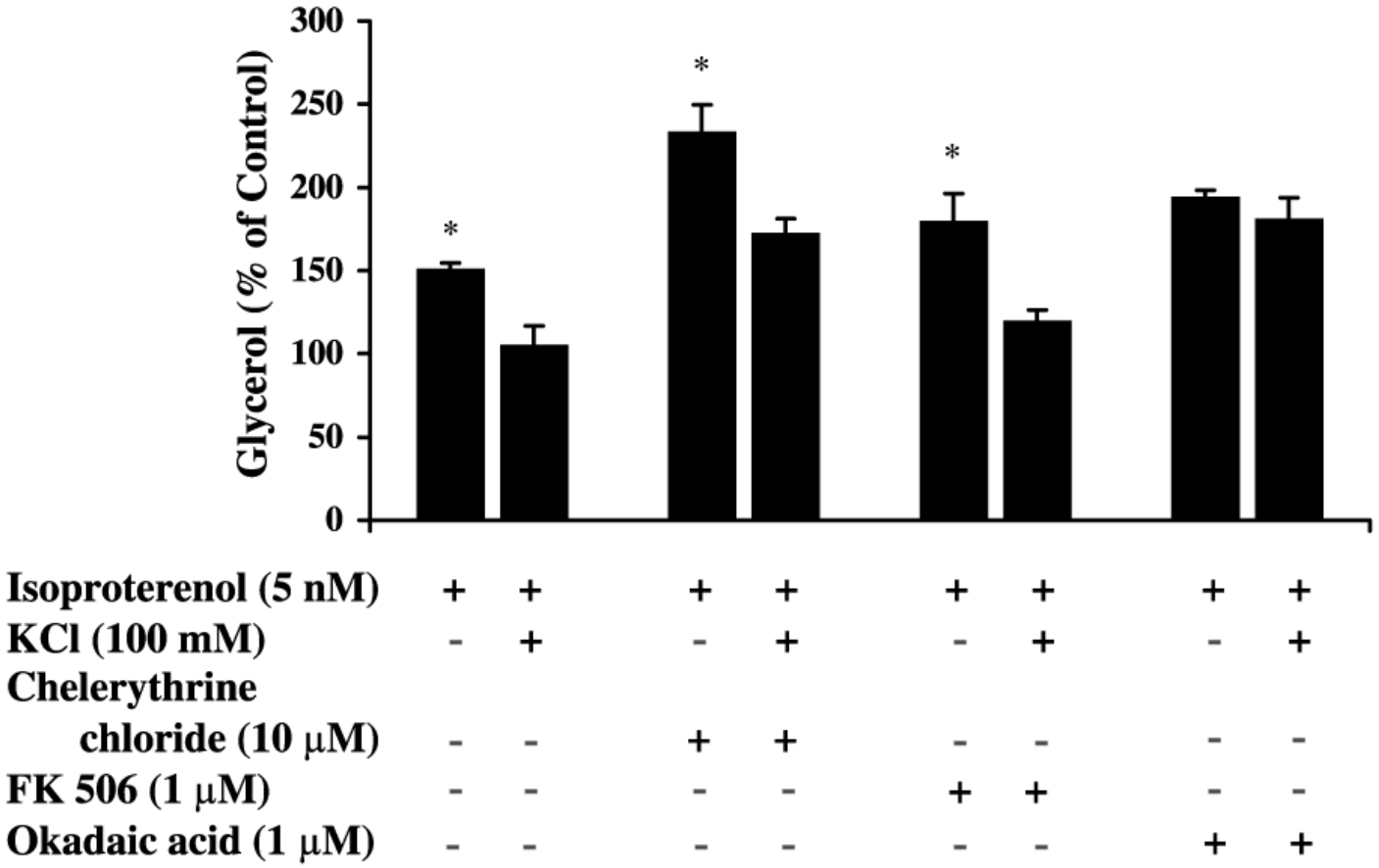

Figure 5. Effects of protein kinase C (PKC), protein phosphatase 2B (PP2B), and protein phosphastase (PP1/2A) inhibitors on the anti-lipolytic effect of KCl. Human adipocytes were treated with isoproterenol and KCl in the presence of PKC, PP2B or PP1/2A inhibitor chelerythrine chloride, FK 506, or okadaic acid for 4 h. Glycerol release was measured as nmol/μg. DNA and expressed as percentage of control. Data are expressed as mean ± SE for six experiments. * $P < 0.05$ vs. KCl treatment.

Fig. 6

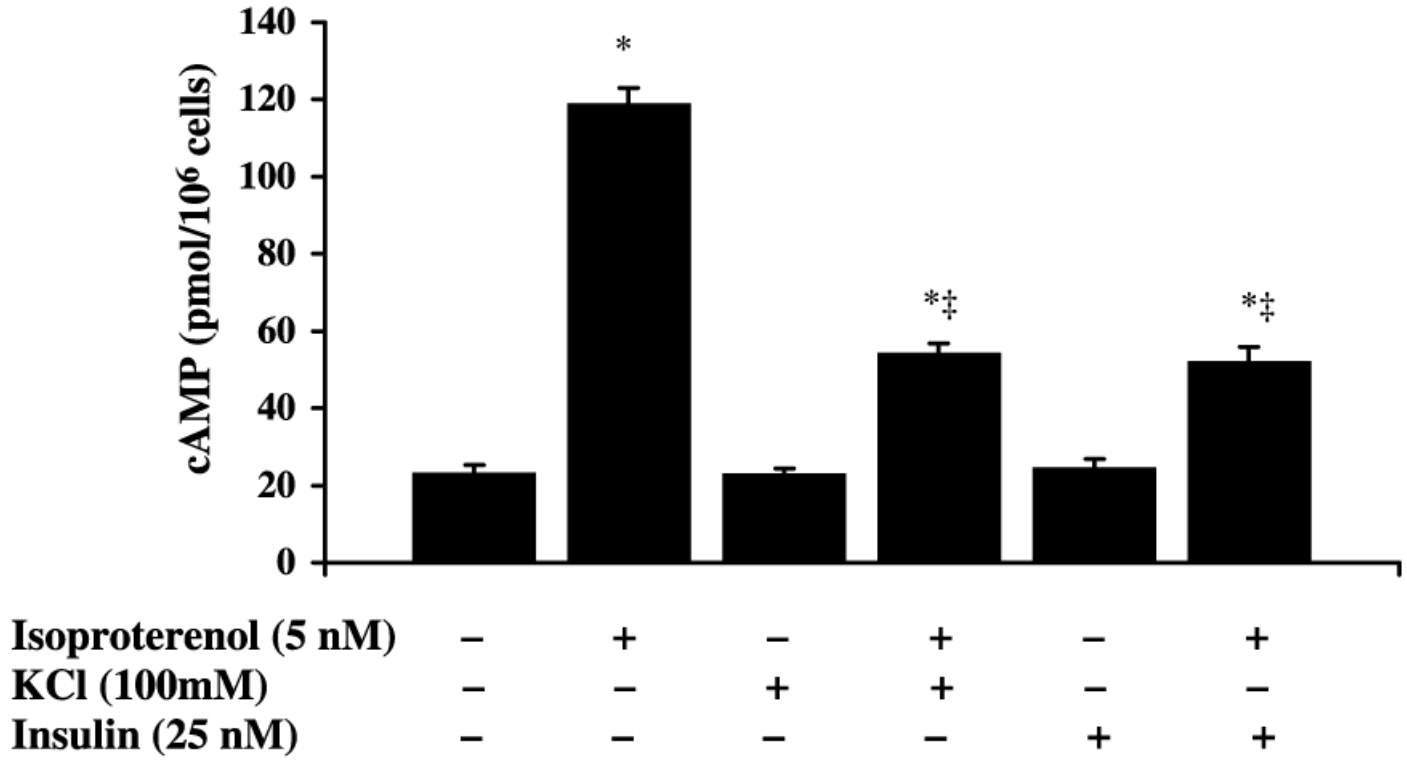

Figure 6. Effects of KCl and insulin on human adipocyte cAMP levels. Human adipocytes were treated with isoproterenol, KCl and insulin alone or isoproterenol in the presence of either KCl or insulin for 10 min. Cells were immediately lysed, and cAMP content was measured by using a cAMP ^{125}I-scintillation proximity assay (SPA) system. * $P < 0.05$ vs. control. ‡ $P < 0.05$ vs. Isoproterenol treatment.

Fig. 7

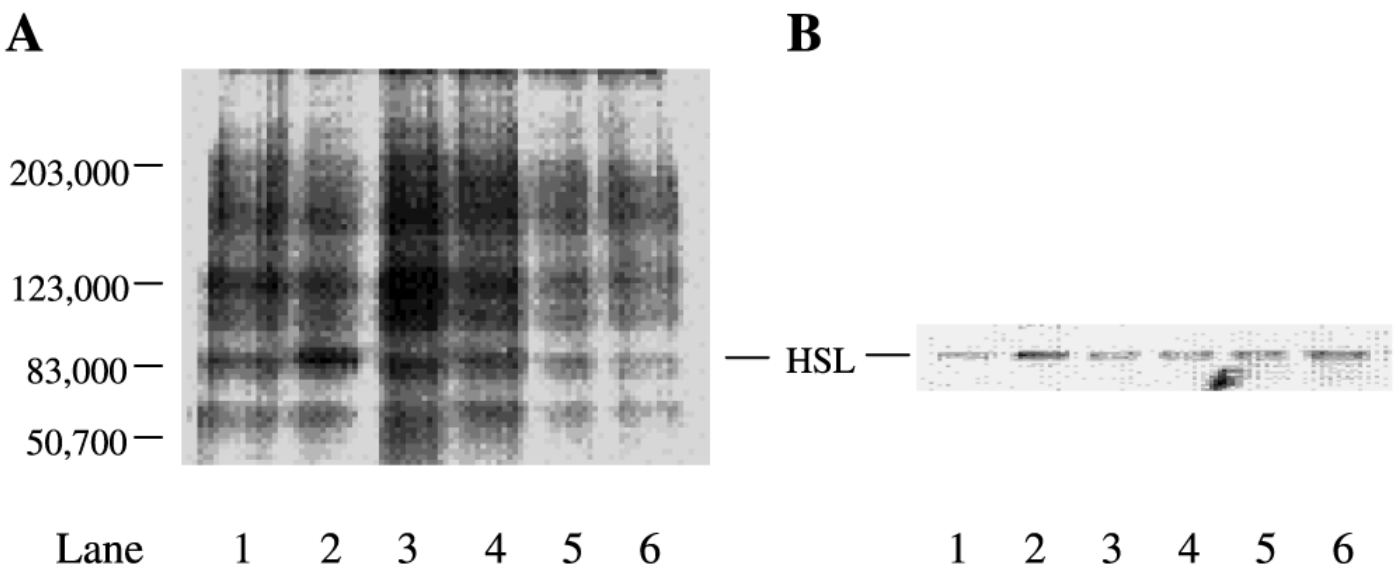

Figure 7. Effects of KCl and insulin on HSL phosphorylation. Human adipocytes were prelabeled with ^{32}P-orthophosphate in KRH buffer with 0.3 mM KH_2PO_4 for 90 min. Cells were then treated with isoproterenol, KCl, and insulin alone or isoproterenol in the presence of either KCl or insulin for 10 min. Cells were lysed in homogenization buffer. Equal amounts of adipocyte lysates were either directly subjected to 10% SDS-PAGE or incubated with anti-HSL antibody. The immunoprecipitates were washed and subjected to 10% SDS-PAGE. Gels were stained with Coomassie blue, dried, and visualized by autoradiography. **A)** Adipocyte phosphoproteins separated by SDS-PAGE. Lane 1: control. Lane 2: isoproterenol. Lane 3: insulin. Lane 4: insulin + isoproterenol. Lane 5: KCl. Lane 6: KCl + isoproterenol. **B)** Immunoprecipitation of adipocyte phosphoproteins with anti-HSL antibody. Lane 1: control. Lane 2: isoproterenol. Lane 3: KCl. Lane 4: KCl + isoproterenol. Lane 5: insulin. Lane 6: insulin + Isoproterenol.