Effect of Glucocorticoid Receptor Antagonist RU 38486 on Acute Glucocorticoid-Induced Insulin Resistance in Rat Adipocytes

Tatsuo Ishizuka, Toshihiko Nagashima, Kazuo Kajita, Atsushi Miura, Mayumi Yamamoto, Satomi Itaya, Yoshinori Kanoh, Masayoshi Ishizawa, Hiroshi Murase, and Keigo Yasuda

We examined the mechanism of acute glucocorticoid-induced insulin resistance in rat adipocytes using the glucocorticoid receptor antagonist RU 38486. Pretreatment with dexamethasone (DEX) and prednisolone for 60 minutes resulted in 50% inhibition of insulin-induced [^{3}H]2-deoxyglucose (DOG) uptake at 10^{-8} and 10^{-7} mol/L, respectively, in rat adipocytes and 20% and 25% inhibition of insulin-induced [^{3}H]2-DOG uptake, respectively, in soleus muscles. Our previous experiments indicated that DEX and prednisolone alone stimulate protein kinase C (PKC) in rat adipocytes. Accordingly, we examined [^{3}H]DEX binding to PKC from MonoQ column–purified rat brain cytosol. Specific [^{3}H]DEX binding to MonoQ column–purified PKC was observed (*kd*, 56.8 nmol/L; Bmax, 725 fmol/mg protein). Thus, insulin-induced PKC translocation from the cytosol to the membrane was suppressed by pretreatment with 10^{-7} mol/L DEX and 10^{-6} mol/L prednisolone for 60 minutes. During treatment with RU 38486 for 60 minutes, there was no change in the glucocorticoid-induced inhibitory effect on insulin-induced [^{3}H]2-DOG uptake and PKC translocation from the cytosol to the membrane. Moreover, pretreatment with RU 38486 for 120 minutes slightly prevented the DEX-mediated inhibition of insulin-induced glucose uptake. These results suggest that acute glucocorticoid-induced insulin resistance may be mainly mediated through the other non–glucocorticoid receptor pathway.

GLUCOCORTICOID EXCESS causes insulin resistance. However, the mechanisms involved are unknown.[1] It remains unclear whether glucocorticoids affect specific insulin binding,[2] tyrosine kinase activity,[3,4] and/or the glucose transporter.[5] Recently, insulin has been found to stimulate the activity[6] and translocation[7,8] of protein kinase C (PKC) in rat adipocytes. On the other hand, dexamethasone (DEX) stimulates PKC activity without affecting phorbol ester binding activity in a cultured cell line.[9] A dose-response inhibition of insulin-induced [^{3}H]2-deoxyglucose (2-DOG) uptake by DEX and prednisolone in rat adipocytes was observed. In soleus muscle, glucocorticoids also have an inhibitory effect on insulin-induced [^{3}H]2-DOG uptake, as previously reported.[10]

We examined the effect of glucocorticoids and simultaneous addition of the receptor antagonist RU 38486[11] on insulin-induced [^{3}H]2-DOG uptake and translocation of PKC to clarify the mechanism by which glucocorticoid acutely provokes insulin resistance in rat adipocytes and soleus muscles.

MATERIALS AND METHODS

Phosphatidylserine, diolein, histone (type III-S), phenylmethylsulfonyl fluoride (PMSF), leupeptin, bovine serum albumin (BSA), D-glucose, ATP, DEX, and prednisolone were purchased from Sigma Chemical (St Louis, MO). [γ-^{32}P]ATP (3,000 Ci/mmol), [1,2-^{3}H]2-DOG (50 Ci/mmol), L-[1-^{14}C]glucose (47 mCi/mmol), and [6,7-^{3}H]DEX (50 Ci/mmol) were purchased from New England Nuclear (Tokyo, Japan). RU 38486 (17β hydroxy-11β, 4-dimethylaminophenyl-17a-propynyl estra 4,9 diene-3-one) was generously donated by the Roussel-Uclaf Research Centre. All other chemicals were reagent grade or better.

Male Wistar rats weighing 150 to 200 g were fed ad libitum and killed by decapitation. Isolated adipocytes were obtained by collagenase digestion of rat epididymal fat pads[12] in Krebs-Ringer phosphate buffer (pH 7.4) containing 127 mmol/L NaCl, 12.3 mmol/L NaH_2PO_4, 5.1 mmol/L KCl, 1.3 mmol/L $MgSO_4$, 1.4 mmol/L $CaCl_2$, 3% BSA, and 2.5 mmol/L glucose. Adipocytes were washed and preincubated at 37°C in glucose-free Krebs-Ringer-phosphate buffer containing 1% BSA for 30 minutes, and then incubated with or without (control) 10^{-7} mol/L DEX or 10^{-6} mol/L prednisolone (dissolved in <0.01% ethanol) for 60 minutes in combination with or without RU 38486, followed by incubation with 10 nmol/L insulin for 30 minutes. There was no effect of 0.01% ethanol, as used for control, on insulin-induced glucose uptake in rat adipocytes. [^{3}H]2-DOG (0.08 μCi) and unlabeled 2-DOG (0.05 mmol/L) were then added to 300 μL 10% (vol/vol) adipocyte suspension, and uptake of [^{3}H]2-DOG was measured over 1 minute.[13]

In PKC experiments, the reaction was terminated by addition of 20 mmol/L Tris HCl buffer (pH 7.5) containing 0.25 mmol/L sucrose, 1.2 mmol/L EGTA, 0.1 mmol/L PMSF, 20 μg/mL leupeptin, and 20 mmol/L 2-mercaptoethanol (buffer I), washed twice, and homogenized in buffer I. The homogenates were centrifuged for 60 minutes at 105,000 × *g* to obtain cytosol and membrane fractions. The latter was homogenized in buffer I containing 5 mmol/L EGTA, 2 mmol/L EDTA, and 1% Triton X-100. To measure PKC activity of rat adipocytes, the cytosol or solubilized membrane fraction was diluted with 20 mmol/L Tris HCl buffer (pH 7.5) containing 0.5 mmol/L EGTA, 0.5 mmol/L EDTA, and 10 mmol/L 2-mercaptoethanol (buffer II). The samples were then applied to a MonoQ column (0.5 × 0.5 cm; Pharmacia, Uppsala, Sweden) connected to a high-performance liquid chromatography system that had been equilibrated with buffer II as described previously.[14,15] Activation of PKC in rat adipocytes was assayed by changes in subcellular distribution of immunoreactive PKC with the methods described previously.[14,15] Equal amounts of cytosol or membrane-associated fraction were prepared, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and incubated first with polyclonal antiserum raised against synthetic peptide to PKCβ (GIBCO, Grand Island, NY) and second with goat anti rabbit globulin complexed to alkaline phosphatase (Sigma). This immunoblotting method detected a single major immunoreactive band that comigrated on SDS-PAGE and blotted identically with 80-kd (PKCβ) synthetic peptides.

From the Third Department of Internal Medicine, Gifu University School of Medicine, Gifu, Japan.

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Address reprint requests to Tatsuo Ishizuka, MD, Third Department of Internal Medicine, Gifu University School of Medicine, Tsukasamachi 40, Gifu 500, Japan.

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Soleus muscles were excised, and tension was maintained by ligatures attached to the tendon: the two soleus muscles from each rat provided one control and one stimulated sample, as indicated previously.[14] Soleus muscles were incubated at 37°C in Erlenmeyer flasks under 95% O_2/5% CO_2 in 5 mL Krebs-Ringer-bicarbonate buffer (KRBB) containing 119 mmol/L NaCl, 4.8 mmol/L KCl, 1 mmol/L NaH_2PO_4, 1.2 mmol/L $MgSO_4$, 1 mmol/L $CaCl_2$, 24 mmol/L $NaHCO_3$, 12 mmol/L HEPES (pH 7.4), 0.1% BSA, 5 mmol/L glucose, and 2 mmol/L sodium pyruvate, unless otherwise specified.

For [1,2-^{3}H]2-DOG uptake experiments, soleus muscles were first preincubated for 30 minutes in KRBB, incubated for 60 minutes in glucose-free KRBB with or without (controls) DEX or prednisolone in combination with or without RU 38486 simultaneously, and then incubated with 100 nmol/L insulin for 30 minutes. [^{3}H]2-DOG (1 μCi) and unlabeled 2-DOG (0.1 mmol/L) were then added, and incubation was continued for 10 minutes. After incubation, tissues were removed, rapidly rinsed in isotope-free medium, blotted, weighed, homogenized in 0.5% trichloroacetic acid, and counted simultaneously for ^{14}C and ^{3}H. Corrections for [^{3}H]2-DOG in tissue samples unrelated to specific transport were determined by measurement of radioactivity of L-[1-^{14}C] glucose. Uptake of [^{3}H]2-DOG in soleus muscles was linear with respect to time and was inhibited by cytochalasin B.

For [^{3}H] DEX-specific binding to purified PKC from rat brain, MonoQ column–purified PKC was obtained using the HPLC system as described previously,[14,15] and then collected samples were fivefold concentrated in buffer I containing 5 mmol/L EGTA, 2 mmol/L EDTA, and 5% glycerol. MonoQ column–purified PKC (5 to 10 μg protein), 50 nmol/L [^{3}H]DEX, 1,000-fold DEX, and 100-fold RU 38486 were incubated for 24 hours at 4°C with and without 0.5 mmol/L Ca^{2+}, and separated by addition of charcoal dextran. Radioactivity in the supernatant was measured by a liquid scintillation counter. For [^{3}H]DEX, specific binding to free adipocytes was performed as previously described.[16] Briefly, free adipocytes (80 μL), 50 nmol/L [^{3}H]DEX, and 1,000-fold unlabeled DEX in the presence or absence of 100-fold RU 38486 were incubated for 60 minutes at 37°C, separated by passing through Whatman filter paper, and rinsed several times with an excess of 0.9% saline. Radioactivity in the report was counted by a liquid scintillation counter.

RESULTS

Effects of Glucocorticoids and the Receptor Antagonist RU 38486 on Insulin-Induced [^{3}H]2-DOG Uptake

Pretreatment with DEX (10^{-7} mol/L) and prednisolone (10^{-6} mol/L) for 60 minutes resulted in 43% and 50% decreases of insulin-induced [^{3}H]2-DOG uptake in rat adipocytes. (We selected the concentration of DEX and prednisolone according to a dose-response experiment.[10]) Simultaneous addition of RU 38486 (10^{-4} mol/L) to glucocorticoids resulted in no definite change of glucocorticoid-mediated inhibition of insulin-induced [^{3}H]2-DOG uptake in rat adipocytes (Fig 1). Neither DEX nor prednisolone alone stimulated [^{3}H]2-DOG uptake. Preincubation with DEX for 2 hours resulted in a 60% inhibition of insulin-induced [^{3}H]2-DOG uptake without RU 38486 and a 45% inhibition with RU 38486. There is no difference between simultaneous and prior incubations with RU 38486 for 60 minutes in glucocorticoid-mediated insulin resistance. These results suggest that pretreatment with DEX for 60 minutes causes inhibition of insulin-induced [^{3}H]2-DOG uptake, mainly due to the non–glucocorticoid receptor-mediated mechanism. Pretreatment with a higher concentration of RU 38486 ($>10^{-3}$ mol/L) alone provoked a decrease of insulin-induced [^{3}H]2-DOG uptake in rat adipocytes (data not shown).

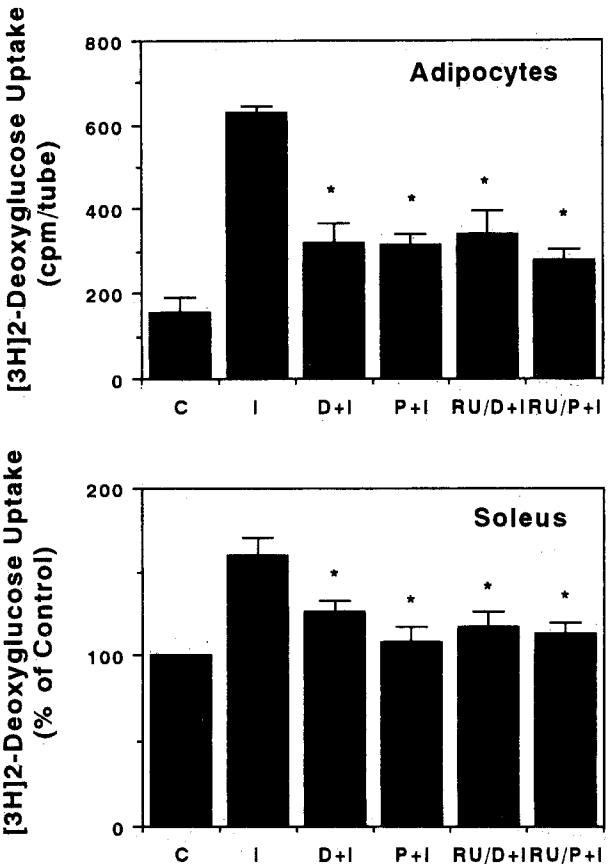


Fig 1. Insulin-stimulated [^{3}H]2-DOG uptake after pretreatment with DEX (D + I), prednisolone (P + I), RU 38486 (RU) plus DEX (RU/D + I), and RU plus prednisolone (RU/P + I) and the control value (C) in rat adipocytes (upper panel) and soleus muscles (lower panel). Rat adipocytes and soleus muscles were preincubated with 10^{-7} mol/L DEX alone, 10^{-6} mol/L prednisolone alone, 10^{-6} mol/L DEX plus 10^{-4} mol/L RU, or 10^{-6} mol/L prednisolone plus 10^{-4} mol/L RU for 60 minutes and then stimulated with 10 and 100 nmol/L insulin, respectively. Insulin stimulated [^{3}H]2-DOG uptake was measured. Values are the mean ± SEM of 3 to 5 separate experiments. *$P < .01$ *v* insulin-stimulated value in adipocytes; *$P < .05$ *v* insulin-stimulated value in soleus muscle.

In soleus muscle, 10^{-7} mol/L DEX and 10^{-6} mol/L prednisolone pretreatment for 60 minutes provoked 21% and 32% decreases in insulin-induced [^{3}H]2-DOG uptake, respectively. Simultaneous addition of RU 38486 (10^{-4} mol/L) with glucocorticoids did not release insulin-induced [^{3}H]2-DOG uptake from the inhibitory effect of glucocorticoids (Fig 1).

Effect of RU 38486 on [^{3}H]DEX Binding to Rat Brain, Adipocyte, Soleus Muscle Cytosolic Protein, and Free Adipocytes

[^{3}H]DEX binding to rat brain cytosol (450 μg protein) was displaced by unlabeled DEX, and was completely blocked by addition of various concentrations of RU 38486 (Fig 2A).

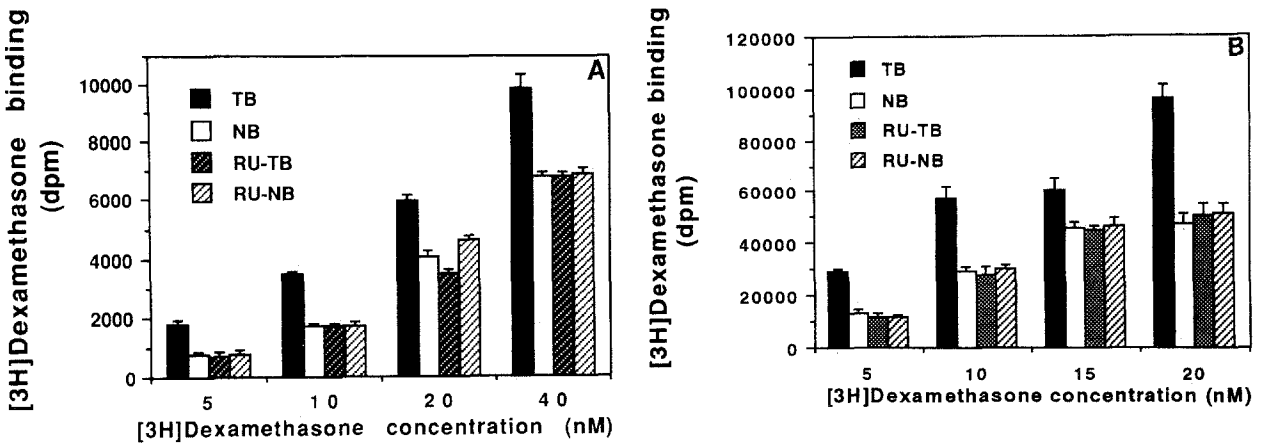


Fig 2. Effect of RU 38486 on [^{3}H]DEX specific binding to rat brain cytosol (A) and free adipocytes (B). Rat brain cytosol (450 μg) or packed free adipocytes (80 μL), [^{3}H]DEX, and 100-fold DEX were incubated for 24 hours at 4°C or 1 hour at 37°C in the presence or absence of 100-fold excess RU 38486. Binding activity was measured. Total binding, nonspecific binding, total binding in the presence of RU 38486, and nonspecific binding in the presence of RU 38486 are abbreviated as TB, NB, RU-TB, and RU-NB. Values are the mean ± SEM of 3 separate experiments.

However, [^{3}H]DEX binding to rat adipocyte and soleus muscle cytosol (500 to 1,000 μg protein) was not displaced by unlabeled DEX, and was not changed by addition of RU 38486 (data not shown). Therefore, as previously indicated in [^{3}H]DEX binding to free adipocytes,[16] [^{3}H]DEX binding to free adipocytes displaced by unlabeled DEX and completely blocked by addition of a 100-fold excess of RU 38486 (Fig 2B).

[^{3}H]DEX Binding to MonoQ Column–Purified PKC From Rat Brain

In the presence of 0.5 mmol/L Ca^{2+} [^{3}H]DEX binding to partially purified PKC was displaced by unlabeled DEX, and was not changed by addition of RU38486 (Fig 3). However, in the absence of 0.5 mmol/L Ca^{2+}, specific [^{3}H]DEX binding was not observed. [^{3}H]DEX specific binding to PKC was saturated by the high concentration of [^{3}H]DEX (Fig 4A), and Scatchard analysis of [^{3}H]DEX binding showed a *kd* of 56. 8 nmol/L and maximum binding of 725 fmol/mg protein (Fig 4B).

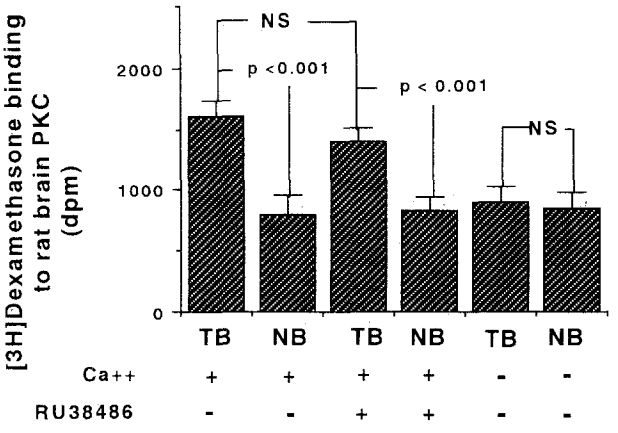


Fig 3. Effect of Ca^{2+} and RU 38486 on [^{3}H]DEX specific binding to MonoQ column–purified PKC. One milligram protein was applied onto MonoQ column connected to a HPLC system. Partially purified PKC (specific activity, 2,000 to 3,000 pmol/min/mg protein), [^{3}H]DEX, and 1,000-fold DEX were incubated for 24 hours at 4°C in the presence or absence of 0.5 mmol/L Ca^{2+} and/or 100-fold excess RU 38486. TB, total binding; NB, nonspecific binding. Values are the mean ± SEM of 5 separate experiments.

Effect of RU 38486 on Glucocorticoid-Induced PKC Translocation in Rat Adipocytes

Glucocorticoids alone apparently caused the redistribution of PKC from cytosol to membrane in rat adipocytes. During treatment with 10^{-7} mol/L DEX and 10^{-6} mol/L prednisolone, cytosolic PKC activity was decreased to 55% and 58% for 60 minutes and membrane-associated PKC activity was increased to 175% and 257% for 60 minutes, respectively. Cytosolic PKC stores were decreased during treatment with glucocorticoids for 60 minutes (Fig 5).

Simultaneous addition of RU 38486 (10^{-4} mol/L) to glucocorticoids resulted in no definite change of glucocorticoid-induced PKC translocation in rat adipocytes.

Effects of Glucocorticoids and/or RU 38486 on Insulin-Induced PKCβ Translocation in Rat Adipocytes

Immunoblot analysis of PKCβ immunoreactivity is shown in Fig 6. DEX (10^{-7} mol/L) and prednisolone (10^{-6} mol/L) pretreatment for 60 minutes suppressed insulin-induced redistribution of PKC from cytosol to membrane for 10 minutes, respectively. Simultaneous addition of RU 38486 (10^{-4} mol/L) to glucocorticoids did not release insulin-induced PKCβ translocation for 10 minutes from the inhibitory effect of glucocorticoids.

DISCUSSION

It has been reported that acute and chronic glucocorticoid treatment causes insulin resistance.[1,2] However, the mechanism by which glucocorticoids induce insulin resistance remains unclear. We have examined whether the acute glucocorticoid effect on insulin action is mediated by the glucocorticoid

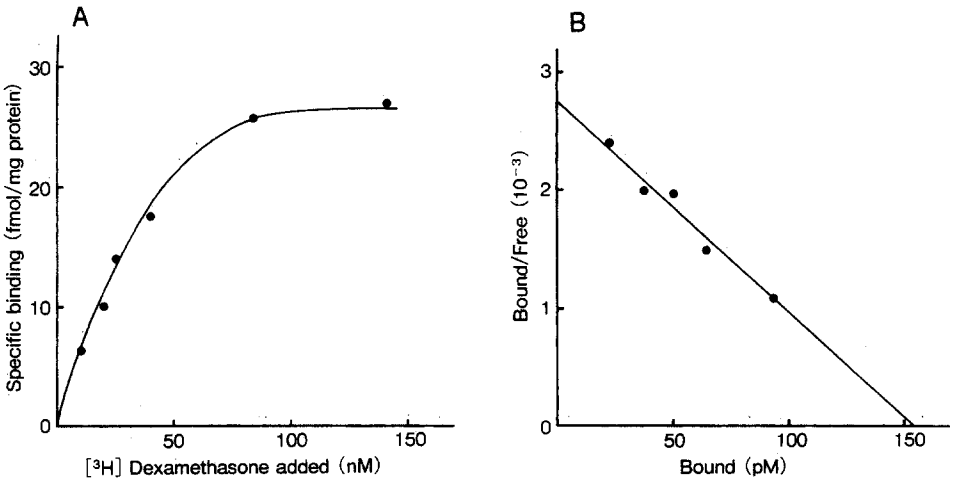


Fig 4. [³H]DEX specific binding to MonoQ column–purified PKC (A) and its Scatchard analysis (B). Values are the mean of 5 separate experiments.

receptor. Now, we indicate that RU 38486 did not prevent the glucocorticoid-modulated inhibitory effect on insulin-stimulated [³H]2-DOG uptake and PKC translocation in rat adipocytes and soleus muscles. RU 38486 is the first steroid capable of antagonizing the acute and chronic effects of glucocorticoids in vitro and in vivo in different animal target tissues without exhibiting any agonist activity even at large doses.[11] We used 20 nmol/L to 100 μmol/L RU 38486, which fully inhibited binding of glucocorticoid to its receptor. Accordingly, preincubation with glucocorticoids plus RU 38486 must block receptor-mediated glucocorticoid effects. Interestingly, glucocorticoid still inhibited insulin-induced [³H]2-DOG uptake treated by RU 38486 (Fig 1). Moreover, the effect of RU 38486 on glucocorticoid-induced activation of PKC activity did not occur in rat adipocytes (Fig 5). These results indicate that glucocorticoids directly activate PKC enzyme activity, due to the binding of glucocorticoids to the regulatory unit of PKC (Figs 3 and 4). In addition to these results, we must explain the difference in the stimulatory effect of glucose uptake between glucocorticoid and phorbol ester. This difference may be due to the translocation of glucose transporter by glucocorticoid and phorbol ester. Previous study indicated that phorbol ester stimulates glucose transporter translocation from low-density microsome to plasma membrane.[17] On the other hand, glucocorticoids per se suppressed the translocation of glucose transporter to the cell surface, as indicated by Garvey et al.[5] Figure 6 also shows that the inhibitory effect of glucocorticoids on insulin-induced translocation of PKCβ remains unchanged by addition of RU 38486. Previous investigators indicated that inhibition of glucose transport by DEX requires synthesis of RNA during the first 45 to 60 minutes after steroid addition using cycloheximide and actinomycin D.[18] Moreover, they indicated that DEX-induced inhibition of basal and insulin-stimulated glucose uptake was prevented by actinomycin D in rat adipocytes.[19] On the other hand, recently, insulin-induced PKCβ mRNA alternative splicing is very fast, within 5 minutes, and unexpectedly, insulin-induced PKC isoform mRNA changes should be fast.[20] DEX-mediated PKC mRNA changes may also be fast. Therefore, actinomycin D blocks protein and RNA synthesis and subsequently prevents insulin- or DEX-mediated action. In our experiments, DEX-induced inhibition of insulin-induced glucose uptake was slightly prevented by RU 38486 (15% recovery of insulin-induced glucose uptake) when incubated with DEX for 2 hours. Thus, pretreatment with DEX for 60 minutes may cause inhibition of insulin-induced glucose uptake, mainly due to the non–glucocorticoid receptor-mediated pathway.

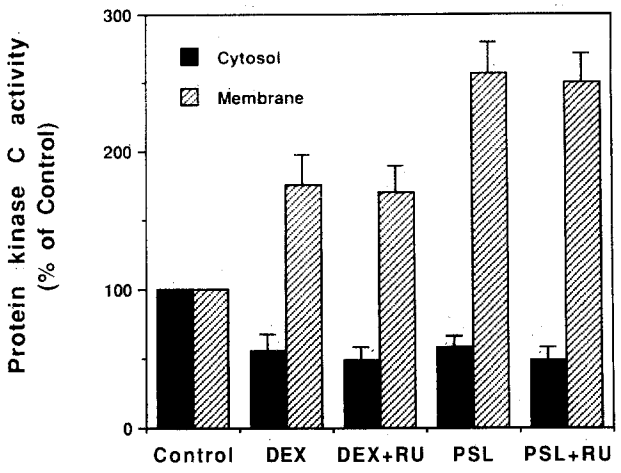
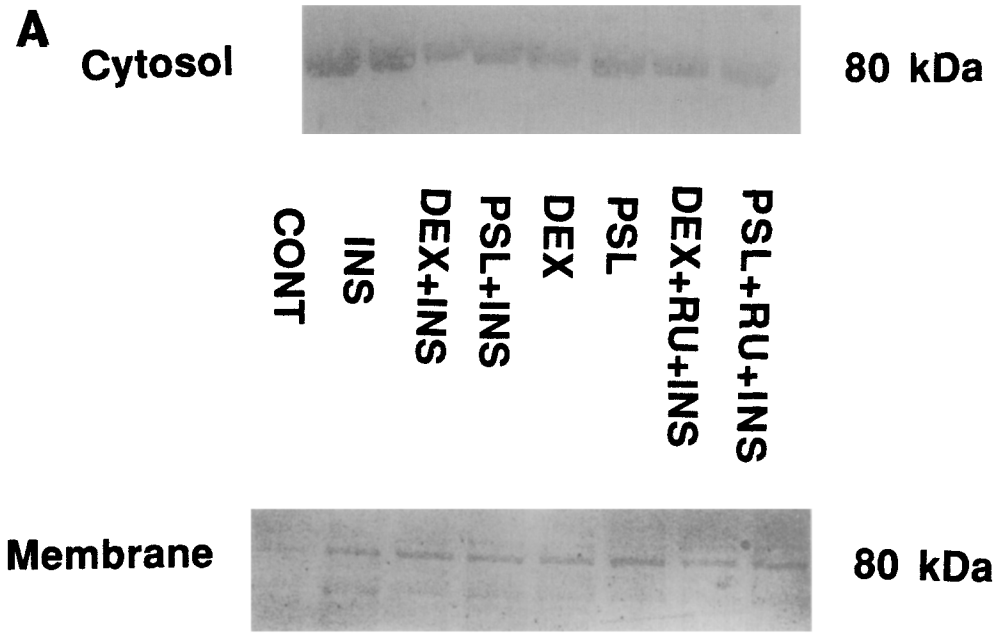


Fig 5. Glucocorticoid-induced redistribution of PKC in rat adipocytes. Rat adipocytes (25% packed cells) were incubated with 10^{-7} mol/L DEX, 10^{-6} mol/L prednisolone (PSL), 10^{-7} mol/L DEX plus 10^{-4} mol/L RU 38486, or 10^{-6} mol/L prednisolone plus 10^{-4} mol/L RU 38486 for 60 minutes. At each indicated time, adipocytes were washed, homogenized, and centrifuged in buffer I. Cytosolic and membrane-associated PKC activity were measured. Values are the mean of 5 separate experiments.

We conclude that, first, glucocorticoids directly activate (translocate) PKC in rat adipocytes; second, preincubation with glucocorticoids decreases cytosolic PKC activity, which causes a decrease of insulin-induced [³H]2-DOG uptake and translocation of PKC; and third, [³H]DEX binding to PKC was found in the presence of RU 38486 and Ca^{2+} in vitro. Finally, results from examination of the glucocorticoid receptor antagonist RU 38486 may show evidence of a direct action of glucocorticoids on PKC per se mainly, but not glucocorticoid receptor–mediated action in acute glucocorticoid-induced insulin resistance.



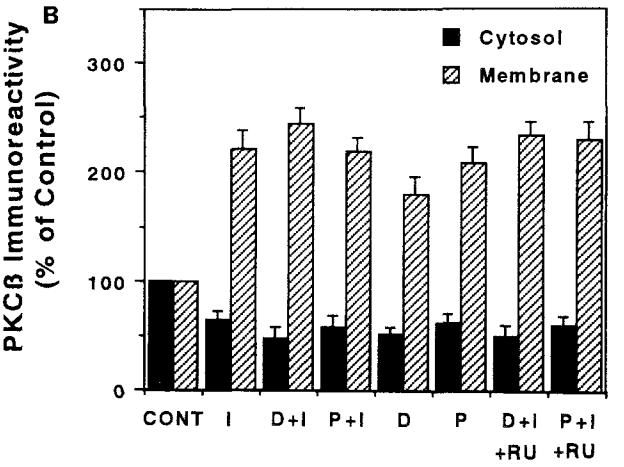


Fig 6. PKCβ immunoreactivity treated by various conditions in rat adipocytes. Adipocytes were stimulated by 10 nmol/L insulin for 10 minutes after pretreatment with 10^{-9} mol/L DEX (DEX + INS), 10^{-8} mol/L prednisolone (PSL + INS), 10^{-9} mol/L DEX plus 10^{-4} mol/L RU 38486 (DEX + RU + INS), or 10^{-8} mol/L prednisolone plus 10^{-4} mol/L RU 38486 (PSL + RU + INS) for 60 minutes. Adipocytes were stimulated by 10^{-9} mol/L DEX alone (DEX) or 10^{-8} mol/L prednisolone alone (PSL) for 60 minutes or 10 nmol/L insulin alone (INS) for 10 minutes. Control sample (CONT) was incubated in KRP buffer for 70 minutes. Each sample was equally incubated for 70 minutes. Cytosolic and membrane-associated proteins (30 μg) were applied to SDS-PAGE for transblotting and immunostaining of PKCβ. This immunoblot (A) is a representative experiment of 3 separate experiments. Laser densitometric analysis is also shown (B).

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