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Enhanced Desensitization and Phosphorylation of the β_1-Adrenergic Receptor in Rat Adipocytes by Peroxovanadate

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SUMMARY

Peroxovanadate (PVN) is an insulin-like agent that inhibits the dephosphorylation of the insulin receptor kinase. PVN inhibited the lipolytic action of 0.1 μM isoproterenol by 88%, which is a relatively specific β_1 catecholamine agonist at this concentration, but was largely ineffective against β_3 agonists or forskolin. To determine whether PVN-mediated desensitization of the β_1AR was associated with enhanced phosphorylation, we immunoprecipitated the β_1AR from rat adipocytes that were metabolically labeled with $^{32}PO_4$. Isoproterenol enhanced the net phosphorylation of the β_1AR by 8 ± 2-fold over control. PVN increased the net phosphorylation of the β_1AR by 5 ± 0.5-fold, and together with isoproterenol, they enhanced the phosphorylation of the β_1AR by 2-fold over isoproterenol alone. Phosphoamino acid analysis of the phosphorylated receptor revealed phosphate incorporation into serine that was proportional to the radioactivity incorporated into the immunoprecipitated receptor. PVN inhibited the serine/threonine phosphatase calcineurin, suggesting that inhibition of receptor dephosphorylation may play a role in the actions of PVN. Cyanogen bromide cleavage of the phosphorylated β_1AR generated a phosphoprotein with a molecular mass consistent with carboxyl-terminal phosphorylation. Furthermore, the magnitude of receptor phosphorylation by isoproterenol was 3-fold larger than that due to forskolin, suggesting that the β_1AR is a substrate for the βAR kinase that phosphorylates carboxyl-terminal residues in the β_2AR. Our findings suggest that PVN may be a powerful new tool with which to study the phosphorylation of other G protein-coupled receptors.

The lipolytic effects of catecholamines on fat cells are primarily mediated by βARs. The lipolytic receptors in rat white adipocytes are primarily β_1AR and β_3AR (1–3). The inhibition of lipolysis is mediated primarily via insulin (4, 5). PVN mimics the action of insulin in that it stimulates glucose transport and inhibits the lipolytic action of isoproterenol in rat adipocytes (6–9). Both orthovanadate and PVN inhibit isoproterenol-mediated lipolysis in rat adipocytes, but PVN is more permeable into cells, less toxic, and 100-fold more potent than orthovanadate (6, 7, 9).

The binding of catecholamine agonists to βAR promotes the formation of an agonist receptor/G protein complex that leads to G protein activation of adenylyl cyclase. The cyclase is activated by the receptor signaling pathway or directly by the diterpene forskolin. In adipocytes, the activation of adenylyl cyclase by either paradigm results in the activation of PKA and lipolysis (10).

Signaling by βAR is attenuated via several mechanisms, including uncoupling of the receptor from the effector system as a consequence of its phosphorylation by G protein-coupled receptor kinases and PKA (11, 12). In addition to these acute effects, prolonged incubation of the receptor to the β-agonist promotes agonist-mediated receptor internalization (sequestration) and down-regulation of the number of cell-surface receptors (13, 14). The mechanism of insulin-mediated inhibition of lipolysis is unclear but seems to involve the phosphatidylinositol-3-kinase pathway as the inhibition of phosphatidylinositol-3-kinase by wortmannin blocks the antilipolytic effect as well as the stimulation of glucose transport by insulin (15). PVN has been reported to inhibit the dephosphorylation of the insulin receptor by tyrosine phosphatases (7). The current study was designed to determine the mechanism of the antilipolytic effects of PVN against β-agonists and forskolin. We stimulated lipolysis through several paradigms to assess the similarities and differences between PVN and insulin in inhibition of lipolysis. PVN preferentially inhibited the lipolytic action of β_1 catecholamine receptors and enhanced the net phosphorylation of the β_1AR. The incorporation of ^{32}P into the β_1AR in adipocytes was sufficiently high to perform phosphoamino acid analysis and peptide mapping.

Experimental Procedures

Materials. Bovine serum albumin powder (Bovuminar, lot L59410; containing <0.05 mol fatty acids/mol albumin) was obtained from

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ABBREVIATIONS: β_1AR, β_1-adrenergic receptor; PVN, peroxovanadate; PKA, cAMP-dependent protein kinase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; βARK, β-adrenergic receptor kinase; SDS, sodium dodecyl sulfate; CHW, chinese hamster fibroblast.

Intergen Co. (Purchase, NY), and bacterial collagenase (*Clostridium histolyticum* CLS1, 238 units/ml, No. 4196-SN269) was obtained from Worthington Biochemical Corp. (Freehold, NJ). CL 316,243 [disodium (*R,R*)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethylamino]propyl]-1,3-benzodioxole-2,2-decarboxylate] was a gift from Dr. Thomas Claus (American Cyanamid Co., Pearl River, NY), and CGP 12177 [(−)-4–3–butylamino-2-hydroxypropoxy][5,7]benzimidazole-2-one] was a gift from Ciba-Geigy (Basel, Switzerland).

Sodium orthovanadate was prepared as a 10 mM solution in 25 mM HEPES buffer, pH 7.4. Combinations of vanadate and H_20_2 were prepared by mixing together solutions of vanadate and H_2O_2 at room temperature for 15 min to provide a final concentration of 10 mM vanadate and 1 mM H_2O_2. Excess H_2O_2 was subsequently removed by the addition of 200 μg/ml catalase for 15 min, and the solution was used immediately thereafter. PVN is a mixture of aqueous peroxovanadium complexes, and the concentration added is based on that of vanadate.

Adipocyte preparation. Adipocytes in each experiment were prepared from the epididymal fat pads of three Sprague Dawley rats (200–250 g) fed *ad libitum* (16). The fat pads (4–6 g) were cut into small pieces with scissors and incubated with collagenase (10 mg in 18 ml of buffer divided among four 1-oz. polypropylene bottles) for 60 min in an orbital shaking water bath (Gyratory water bath shaker G76, New Brunswick Scientific). The buffer we used contained 122 mM NaCl, 5 mM KCl, 2.4 mM $NaHCO_3$, 1.4 mM $MgSO_4$, 1.4 mM $CaCl_2$, 1.4 mM Na_2HPO_4, and 25 mM HEPES, plus 4% albumin adjusted to pH 7.4 along with 200 nM adenosine and 2 mM glucose for digestion of the tissue with collagenase. The digest was filtered through nylon mesh with gentle pressure and the adipocytes were washed three times through flotation with albumin-free buffer and then incubated for 10 min in four polypropylene bottles containing 7.5 ml of buffer/bottle without albumin, glucose, or adenosine. The cells were then resuspended to a volume of ~16 ml in buffer containing 4% albumin plus 0.1 unit/ml of adenosine deaminase without added glucose or adenosine. The cell suspension in a volume of 0.2 ml was added to 0.8 ml of the same buffer containing the added agents in 17 × 100 mm polypropylene tubes. We usually incubate 35–75 mg of packed cells (120,000–220,000 adipocytes)/ml medium. The cells were shaken in the orbital shaker bath at 37° for 20 min (100 cycles/min).

Assay of lipolysis and glucose oxidation. Rat fat cells were incubated under the conditions given in the table and legends to the figures. At the end of the incubation, 0.1 ml of 1 N HCl was added to each tube, the tubes were heated in a boiling water bath for 1 min and then cooled, and the extracts were neutralized by the addition of 0.1 ml of 1 N NaOH. A 50-μl aliquot of the medium was removed for analysis of glycerol according to the one-step enzymatic fluorimetric procedure of Boobis and Maughan (17).

For studies on glucose oxidation, we incubated adipocytes in the presence of a trace amount (0.2 μM) of D-[1-^{14}C]glucose and measured the release of [^{14}C]CO_2 by collecting the carbon dioxide on filter papers in hanging wells containing NaOH suspended from serum stoppers that sealed the incubation tubes. Glucose transport is rate limiting for glucose oxidation at low concentrations of glucose, and this assay has the added advantage of detecting effects of inhibitory agents on glucose oxidation. Glucose oxidation is expressed as percent conversion of the added [1-^{14}C]glucose to carbon dioxide, and ~500,000 dpm was present in each tube.

Immunoprecipitation of the β_1AR. The procedure for immunoprecipitation of the β_1AR was based on that of Kiely *et al.* (18). Freshly dissociated rat fat cells were washed twice with phosphate-free HEPES buffer containing 4% albumin and resuspended at a cell volume-to-buffer ratio of 1:4. Cell suspension (3×10^7 cells containing 1 mCi of $^{32}PO_4$ in 5 ml of buffer) were incubated for 2 hr at 37° to equilibrate the [^{32}P]ATP pool in these cells. The cells were washed twice with warm phosphate-free HEPES buffer and then incubated for 20 min at 37°. The cells were allowed to float for 2 min, and the medium was aspirated and replaced with 1 ml of ice-cold lysis buffer containing 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.6 mM dithiothreitol, 20 mM Tris·HCl, pH 7.4, 50 mM NaF, 40 mM sodium pyrophosphate, 50 mM potassium phosphate, 10 mM sodium molybdate, 2 mM orthovanadate, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 0.1 mg/ml benzamidine, and 0.1 mg/ml bacitracin. The cells were boiled for 5 min, and the homogenate of fat and solubilized protein was transferred to two screw-top Eppendorf tubes and centrifuged at 14,000 × g_{av} for 5 min at 4°. The aqueous phase between the floating fat cake and the insoluble precipitate was carefully aspirated and placed in another Eppendorf tube containing 4 μl of 0.5 M iodoacetamide/ml solution. The homogenate was incubated on ice for 1 hr (in the dark) to alkylate the reduced proteins. The particulate material was separated by a 10-min spin in the microfuge.

Immunoprecipitation of the β_1AR used an antiserum prepared against a peptide corresponding to amino acids 396–408 in the carboxyl-terminal domain of the rat β_1AR (19). Equal amounts of homogenates were incubated with 200 μl of nonimmune protein A agarose (equilibrated in RIPA buffer composed of 20 mM Tris·HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% SDS) for 2 hr in a rotating platform. The resin was spun down, and the solution was transferred to a new tube containing 40 μl of a slurry containing the β_1AR antibody/protein A-agarose complex and then incubated at 4° overnight on a rotating platform. The resin was washed four times with 1 ml of ice-cold RIPA buffer and once more with the same buffer without detergents. Then, 100 μl of Laemmli sample buffer containing 20 mM dithiothreitol was added, and the slurry was boiled for 5 min to release the IgG/βAR complex from the resin. The proteins were separated through electrophoresis in 10% acrylamide gels containing 0.1% SDS, dried, and exposed to autoradiography. The relative amounts of ^{32}P incorporated into the βAR band was determined through densitometric scanning of the X-ray film with a densitometer.

Phosphoamino acid analysis of immunoprecipitated β_1AR. After electrophoresis on 10% acrylamide gels, the proteins were transferred electrophoretically to polyvinylidene difluoride nylon membranes. The transfer buffer contained 100 μM orthovanadate to prevent the dephosphorylation of the ^{32}P-labeled amino acids. The membranes were autoradiographed, and the bands corresponding to phosphorylated receptor protein were excised. The blot was acid-hydrolyzed with 6 N HCl for 2 hr at 100°, and the ^{32}P-labeled phosphoamino acids were purified through chromatography on Dowex 50W-X2 (H^+) 100–200 mesh (20). The phosphoamino acids were then separated through thin layer electrophoresis (50 V/cm, 60 min) with glass plates precoated with cellulose acetate (20 × 20 cm). Radioactive amino acids were visualized with the use of autoradiography and identified through comparison with standards stained with 0.2% ninhydrin in acetone.

Effect of PVN on calcineurin. Bovine brain calcineurin was assayed for protein phosphatase activity by measuring the breakdown of *p*-nitrophenyl phosphate at 410 nm with a recording spectrophotometer as described by Martin and Graves (21). The assays were done in 25 mM 3-(*N*-morpholino)propanesulfonic acid buffer, pH 7.0, in the presence of 1 mM $MnCl_2$, 10 μg/ml calmodulin, and 10 μg/ml calcineurin.

Peptide mapping of immunoprecipitated β_1ARs. After electrophoresis on 10% acrylamide gels, the proteins were transferred electrophoretically to nitrocellulose membranes in the presence of 100 μM orthovanadate. The membranes were autoradiographed, and the bands corresponding to the phosphorylated receptor were cut out. The membrane was placed in an Eppendorf tube and submerged in 0.5 ml of 70% (v/v) formic acid containing 100 mg/ml cyanogen bromide (Sigma) for 1.5 hr at room temperature (22). At the end of the digestion, the sample was centrifuged, and the supernatant was collected and dried in a Speed-Vac concentrator. The dried peptides were dissolved in sample buffer and subjected to electrophoresis on a 10% acrylamide gel in tricine cathode buffer. To determine the molecular mass of the phosphorylated peptides, a set of seven polypeptides ranging from 16,950 to 2,510 da (Sigma MW-SDS-17S kit) were electrophoresed next to the proteolized sample. At the end of the run,

the gel was fixed, stained with Coomassie Brilliant G, and destained with 10% acetic acid. The gel was dried and exposed to an X-ray film with one intensifying screen for 7 days.

Results

We compared the effects of insulin and PVN on glucose oxidation and lipolysis stimulated by 0.1 μM isoproterenol or 3.3 nM CL 316,243, which activate β_1AR and β_3AR, respectively (23, 24). Insulin inhibited the activation of lipolysis by isoproterenol and CL 316,243 by 42% and 61%, respectively (Table 1). PVN inhibited isoproterenol-stimulated lipolysis by 50% but was largely ineffective against CL 316,243-stimulated lipolysis. Another facet of the metabolic effects of insulin on adipocytes was the stimulation of glucose oxidation. Insulin and PVN stimulated glucose oxidation by 2.5–3-fold, and this stimulation was unaffected by isoproterenol or CL 316,243 (Table 1). These data suggest that PVN inhibited lipolysis due to catecholamines acting on β_1AR, whereas insulin inhibited the lipolytic activation by β_1AR and β_3AR. The assumption that the action of 0.1 μM isoproterenol is limited to β_1AR is based on the following information. Granneman (23) reported that the activation of adenylyl cyclase activity by 100 nM isoproterenol in rat adipocyte membranes was completely blocked by a β_1-selective catecholamine antagonist. Hollenga *et al.* (25) found that the lipolytic stimulation due to 100 nM isoproterenol was blocked by 100 nM CGP 20712A, a β_1 catecholamine antagonist. We found that 10 μM concentration of the β_1AR antagonist atenolol blocked 53 ± 8% (mean ± standard error of five experiments) of the increase of lipolysis due to 10 nM isoproterenol and 60 ± 7% of the increase in cAMP due to 100 nM isoproterenol (data not shown). These data and others given below provide ample evidence that activation of lipolysis by 0.1 μM isoproterenol was primarily caused by the activation of β_1AR in rat fat cells.

The influence of low and high concentrations of PVN on isoproterenol-stimulated lipolysis revealed that high concentrations of PVN attenuated the increase in lipolysis that was generated by 10 nM isoproterenol (Fig. 1). The lipolysis generated by 100 nM isoproterenol was maximal, and high concentrations of PVN inhibited by 50% the increase in lipolysis that was produced by 100 nM isoproterenol, which is in agreement with the data in Table 1. However, the antilipolytic

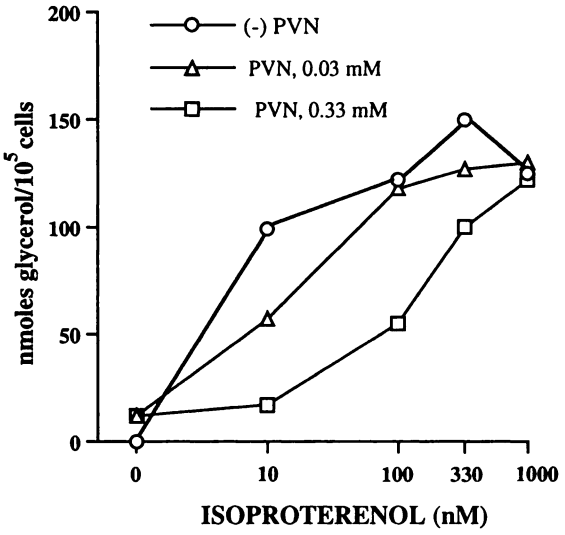

Fig. 1. Effect of PVN on isoproterenol-stimulated lipolysis. Rat adipocytes were incubated in albumin-free buffer for 10 min. The buffer was removed, the cells were suspended in 4% albumin, and then 185,000 cells/ml were incubated for 20 min in the presence of the indicated concentrations of isoproterenol either without, with 0.033 mM PVN, or with 0.33 mM PVN. The data are the mean of two paired experiments.

action of PVN against β_1AR activation by isoproterenol was reversed with micromolar concentrations of isoproterenol (Fig. 1). High concentrations of isoproterenol may coactivate β_3AR-mediated lipolysis that seems to be insensitive to inhibition by PVN (Table 1). To better understand this phenomenon, the effects of another selective β_3-agonist and forskolin on PVN and insulin actions in adipocytes were investigated (Table 2). In these experiments, we used CGP-12177, which at low nanomolar concentrations antagonizes β_1AR and β_2AR and at higher concentrations activates β_3AR (26). To ensure that the CGP-12177 response is devoid of a β_1AR component, we included 10 μM of the β_1-selective antagonist atenolol. The activation of lipolysis by isoproterenol, CGP-12177, and forskolin was reduced by insulin. PVN reduced the activation of lipolysis by isoproterenol by 81% but had no effect on CGP-12177- or forskolin-mediated lipolysis (Table 2).

The finding that PVN inhibited lipolysis that was mediated by β_1AR but not β_3AR suggested that PVN selectively desen-

TABLE 1

PVN, unlike insulin, preferentially inhibits the stimulation of lipolysis by isoproterenol but not by CL 316,243

Rat adipocytes were resuspended in 4% albumin (170,000/ml) and incubated for 20 min in the presence of H_2O_2 plus catalase (control) and the indicated additions. Values are the mean ± standard error of five paired replications.

Addition	[1-^{14}C]Glucose oxidation				
	Control	+Insulin (10 nM)		+PVN (0.1 mM)	
	% oxidation	*% oxidation*	*% of control*	*% oxidation*	*% of control*
None	1.8 ± 0.5	5.3 ± 0.3	294[a]	5.2 ± 1.0	289[a]
Isoproterenol (0.1 μM)	1.7 ± 0.3	4.4 ± 0.6	259[a]	5.3 ± 1.3	312[a]
CL 316,243 (3.3 nM)	2.0 ± 0.8	5.4 ± 1.0	275[a]	5.8 ± 1.5	290[a]
	Lipolysis				
	nmol		*% of control*	*nmol*	*% of control*
None	20 ± 5	11 ± 4	58	20 ± 6	100
Isoproterenol (0.1 μM)	160 ± 35	93 ± 28	58	80 ± 20	50[a]
CL 316,243 (3.3 nM)	140 ± 35	55 ± 23	39[a]	113 ± 32	81

[a] $p < 0.05$.

TABLE 2

PVN inhibits the stimulation of lipolysis by isoproterenol but not that by CGP 12177 or forskolin

Rat adipocytes were resuspended in 4% albumin (170,000/ml) incubated for 20 min in the presence of H_2O_2 plus catalase and the indicated additions. Values are the mean ± standard error of four paired replications, and the effects of insulin or peroxovanadate are given as percent of control values.

Addition	Lipolysis				
	Control	+Insulin (10 nM)		+PVN (0.1 mM)	
	nmol		*% of control*	*nmol*	*% of control*
Basal	30 ± 20	10 ± 10	32	15 ± 20	50
Isoproterenol (0.1 μM)	285 ± 40	180 ± 30	63[a]	34 ± 20	12[a]
CGP 12177 (1 μM) +atenolol (10 μM)	200 ± 50	42 ± 14	21[a]	204 ± 60	102
Forskolin (10 μM)	185 ± 55	61 ± 22	33[a]	220 ± 75	120

[a] $p < 0.05$ based on paired comparisons.

sitized the β_1AR in adipocytes. Desensitization of βAR seems to be mediated by the phosphorylation of the agonist-occupied receptor by βARK or PKA (11, 12, 27). In this case, PVN may enhance the phosphorylation of the agonist-occupied β_1AR, which contains in the carboxyl terminus and the third cytoplasmic loop several serine/threonine residues in a favorable context for phosphorylation, but not the β_3AR, which lacks these sites (28, 29). Therefore, we tested these alternatives by determining whether β-agonists increase the phosphorylation of the β_1AR in intact adipocytes and whether this phosphorylation could be enhanced by PVN. The ATP pools in adipocytes were labeled with [^{32}P]orthophosphate; then, adipocytes were incubated with 0.1 μM isoproterenol in the absence or presence of 0.1 mM PVN for 20 min. The β_1ARs were immunoprecipitated from unfractionated adipocytes by a β_1-selective anti-peptide antibody (19). The data in Fig. 2 demonstrate that the major phosphorylated species that was immunoprecipitated was a 68-kDa peptide equal in size to the β_1ARs in adipocytes (30). PVN increased the phosphorylation of the 68-kDa peptide by 5 ± 0.5-fold over H_2O_2 plus catalase (three experiments). Isoproterenol increased the phosphorylation of the 68-kDa peptide by 8 ± 2-fold over control (three experiments). The phosphorylation of the β_1AR was markedly enhanced after exposure to isoproterenol plus PVN by 2-fold over isoproterenol alone. These data indicate that the β_1AR is phosphorylated as a consequence of β_1-agonist activation and that receptor phosphorylation is markedly enhanced by PVN.

We next determined the time course for isoproterenol-mediated phosphorylation of the β_1AR in adipocytes. In these experiments, adipocytes were exposed to 0.1 mM PVN in the absence or presence of 0.1 μM isoproterenol for 0, 5, 10, and 20 min (Fig. 3A). Half-maximal stimulation of the β_1AR occurred within 5 min, and phosphorylation was maximal by 10 min. To determine the relationship between the concentration of isoproterenol and the magnitude of β_1AR phosphorylation, metabolically labeled rat fat cells were exposed to 10, 100, and 1000 nM isoproterenol in the presence of 0.1 mM PVN for 20 min (Fig. 3B). The phosphorylation of the β_1AR by 10 nM isoproterenol was minimal. This is expected as phosphorylation is a consequence of substantial receptor occupancy and at this concentration the β_1AR is not maximally occupied (31). At 100 nM isoproterenol, maximal β_1AR phosphorylation was attained. The data in Fig. 3 indicate that the kinetics of the β_1AR phosphorylation *in vivo* in rat adipocytes are comparable to the kinetics of phosphorylation of epitope-tagged human β_1AR in HEK 293 cells (32).

Isoproterenol and forskolin phosphorylate βAR primarily via βARK and PKA, respectively (12, 27, 33). The next sets of experiments were designed to compare the magnitude of β_1AR phosphorylation by isoproterenol and forskolin and their target amino acids. Rat fat cells were metabolically labeled with 1 mCi of $^{32}PO_4$-O_4 for 2 hr and then exposed to 0.1 μM isoproterenol, 0.1 mM PVN, and isoproterenol plus PVN for 20 min (Fig. 4A). In addition, rat fat cells were exposed to 50 μM forskolin and PVN for 20 min. The intensities of the phosphorylated 68-kDa bands in response to isoproterenol and PVN were equivalent. The intensity of the 68-kDa band increased by 10-fold when isoproterenol and PVN were added simultaneously. Forskolin and PVN together increased band intensity by 3-fold over PVN alone. Phosphoamino acid analysis of the 68-kDa bands revealed that serine was the phosphorylated amino acid after β_1-agonist or forskolin exposure (Fig. 4B). The relative intensities of the phosphoserine bands were 1 and 1.3 for PVN or isoproterenol alone. The addition of isoproterenol or forskolin to PVN increased serine phosphorylation by 16- and 3-fold, respectively. Therefore, the fold increase in β_1AR phosphorylation was proportional in magnitude to that of serine phosphorylation.

The synergy between PVN and isoproterenol in phosphorylating the β_1AR may be caused by either enhanced phosphorylation or diminished dephosphorylation. Protection of the phosphorylated state of the receptor is more likely because this is the predominant mechanism by which PVN enhances the actions of insulin. In this regard, PVN acts as a tyrosine phosphatase inhibitor (7). Because serine phosphorylation of the β_1AR was enhanced by PVN, we determined whether PVN can inhibit serine/threonine phosphatases (Fig. 5). Calcineurin is a calcium-dependent type 2B protein phosphatase that displays serine/threonine protein phosphatase activity. The inhibition of calcineurin *in vitro* by PVN occurred at an IC_{50} of 0.17 μM and was essentially complete at 3.3 μM (Fig. 5). These concentrations of PVN are 1000-fold lower than those used on intact adipocytes because they were performed *in vitro* with purified enzymes, whereas the action of PVN on adipocytes requires entry into the cell and is associated with binding to other proteins.

The rat β_1AR has several potential phosphorylation sites for βARK and PKA. The consensus sequences for phosphorylation by βARK are unknown but involve the cluster of serine/threonine residues in the distal portion of the cytoplasmic tail of the β_2AR (27, 33). In analogy to the β_2AR, the rat β_1AR has 13 serine/threonine residues in its carboxyl-terminal tail that could be potential sites for phosphorylation by βARK (28). The sequence Lys-Arg-Arg-Pro-Ser^{301} in the

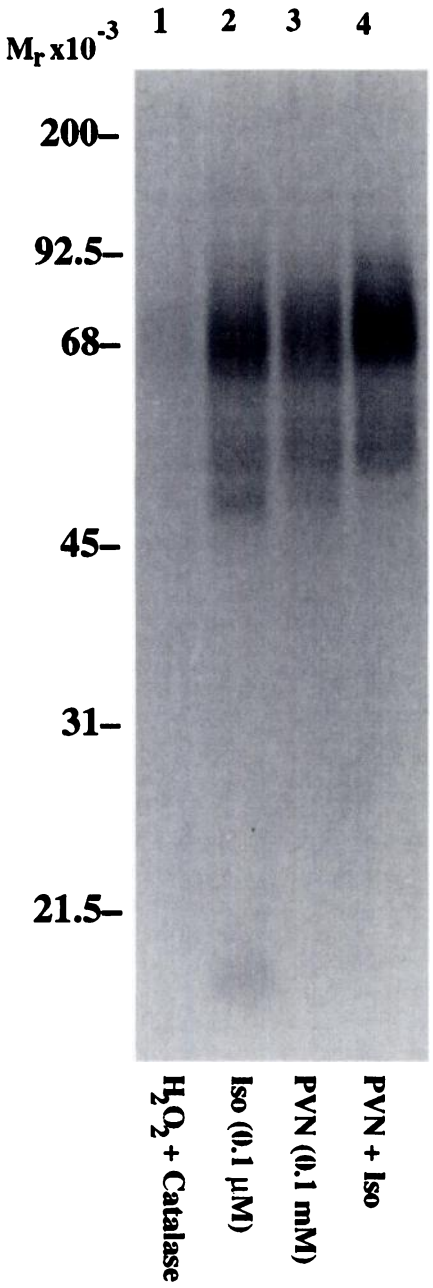

Fig. 2. Effects of PVN and isoproterenol (*Iso*) on phosphorylation of the β_1AR of adipocytes. Adipocytes from one rat were incubated for 2 hr in 5 ml of phosphate-free buffer containing 4% albumin and 1 mCi of $^{32}PO_4$-O_4 for each condition. At the end of this incubation, the buffer was removed, and the cells were resuspended in 4% albumin and then incubated for 20 min in 5 ml of buffer with H_2O_2 plus catalase (*lane 1*), 0.1 μM isoproterenol (*lane 2*), 0.1 mM PVN (*lane 3*), or isoproterenol plus PVN (*lane 4*). The β_1AR was immunoprecipitated with a β_1AR-specific antiserum bound to protein A-agarose. Agarose beads containing the immune complexes were boiled and subjected to electrophoresis on 10% acrylamide gels. The gels were dried and exposed to X-ray film for 3 days with one intensifying screen. The intensity of the bands was compared through the use of a PDI laser densitometer. The relative intensity of the 68-kDa band was 1-, 8 ± 2-, 5.6 ± 0.5-, and 15.4 ± 2.5-fold, respectively, for *lanes 1–4*. The experiment was replicated three times with similar results.

third cytoplasmic loop of the β_1AR matches the consensus sequence for phosphorylation by PKA (12). To determine whether the phosphorylation of the β_1AR by β-agonists occurred at the carboxyl terminus, the third cytoplasmic loop, or both, we used peptide mapping methodology to localize the phosphoserines. For the β_1AR, it is possible to discriminate through cyanogen bromide cleavage between phosphoserines in the carboxyl-terminal region and those in the third cytoplasmic loop. Cyanogen bromide cleavage of the methionines in 68-kDa β_1AR peptide generates two phosphopeptides, a 78-amino acid phosphopeptide extending from 239 to 317 encompassing all of the third cytoplasmic loop and a 146-amino acid phosphopeptide extending from 318 to 464 that contains the entire carboxyl-terminal domain (Fig. 6A). β_1ARs were immunoprecipitated from rat fat cells that were exposed to isoproterenol and PVN, electrophoresed on 10% acrylamide gels, and transferred electrophoretically to nitrocellulose. After autoradiography, the 68-kDa phosphopeptide was excised, proteolized with cyanogen bromide, and then subjected to electrophoresis on a Tris-tricine system. As shown in Fig. 6B, complete cleavage was achieved of the 68-kDa protein through cyanogen bromide. Cyanogen bromide cleavage generated a phosphopeptide that migrated with a molecular mass of ~14 kDa, which is consistent with the expected mobility of the carboxyl-terminal peptide. These data support the notion that the major kinase involved in phosphorylating the rat β_1AR is βARK.

Another kinase that is involved in phosphorylating this class of receptors is PKA. The data in Fig. 4 indicate that forskolin, which bypasses the receptor and activates all isoforms, adenylyl cyclase phosphorylated the β_1AR to ~30% of the level achieved with isoproterenol. To determine the contribution of the βARK versus the PKA pathway to β_1AR phosphorylation, adipocytes were metabolically labeled with 0.1 mCi of $^{32}PO_4$ to enhance the sensitivity of the phosphorylation assay (Fig. 7). In this experiment, PVN-mediated phosphorylation was minimal. PVN with 0.1 μM isoproterenol or forskolin increased the relative intensity of the 68-kDa peptide by 14- and 4-fold, respectively. Thus, the magnitude of forskolin-mediated phosphorylation was 30% of that of isoproterenol, which is consistent with the data in Fig. 4A. The combination of isoproterenol and forskolin in the presence of PVN resulted in 2.5 ± 0.5-fold greater phosphorylation than with just isoproterenol. Therefore, there is synergism between forskolin- and isoproterenol-mediated phosphorylation of the β_1AR in rat adipocytes.

Discussion

PVN exerts insulin-like effects on rat and human adipocytes (6–9). Therefore, we compared the effects of insulin and PVN on the stimulation of glucose oxidation and inhibition of lipolysis. Our results confirm that insulin stimulation of glucose oxidation is mimicked by PVN. However, the effects of PVN and insulin on inhibition of lipolysis by forskolin, β_1-agonists, and β_3-agonists were most telling concerning the difference between the actions of insulin and PVN. Insulin inhibited forskolin-, β_1-agonist-, and β_3-agonist-mediated lipolysis, whereas the antilipolytic action of PVN was restricted to β_1-catecholamine stimulation. Therefore, different mechanisms underlie the antilipolytic actions of insulin and PVN. Further evidence that PVN is not a true insulin-like agent comes from the studies of Fantus *et al.* (7), which showed that PVN does not activate the insulin receptor kinase *in vitro*. Instead, PVN inhibited tyrosine dephosphorylation of the labeled receptor by a crude preparation of phos-

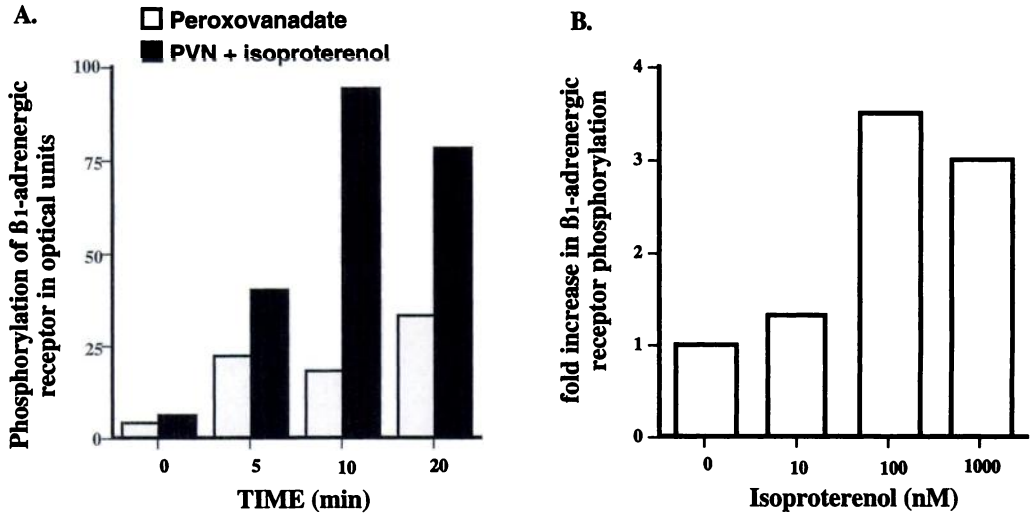

Fig. 3. Time course and concentration dependence of isoproterenol-mediated phosphorylation of the β_1AR in adipocytes. A, Rat adipocytes were incubated for 2 hr in 5 ml of phosphate-free buffer containing 4% albumin and 1 mCi of $^{32}PO_4$-O_4 for each condition. At the end of this incubation, the buffer was removed, and the cells were resuspended in 4% albumin and then incubated for 5, 10, and 20 min in 5 ml of buffer with 0.1 mM PVN plus 100 nM isoproterenol. The β_1AR was immunoprecipitated, and the immune complexes were resolved through electrophoresis on 10% acrylamide gels. B, Metabolically labeled rat fat cells were exposed to 10, 100, and 1000 nM isoproterenol for 20 min. The β_1AR was immunoprecipitated, and the immune complexes were resolved through electrophoresis on 10% acrylamide gels. The gels were dried and exposed to X-ray film for 3 days with one intensifying screen. The intensity of the bands was compared through the use of a laser densitometer. The experiment was replicated three times with similar results.

photyrosine phosphatase. Therefore, we tested whether this analogy can adequately explain why the inhibition by PVN was restricted to β_1 catecholamines. We reasoned that PVN was inhibiting β_1 catecholamine-mediated lipolysis by augmenting agonist-promoted desensitization of β_1AR but not β_3AR in adipocytes. Desensitization of G protein-coupled receptors is associated with their phosphorylation (27, 33). The phosphorylation of the β_2AR by PKA and βARK is intimately involved in its desensitization, whereas the β_3AR is resistant to short term agonist-mediated desensitization and is not phosphorylated in response to agonist (27, 29, 33). No data are available concerning the phosphorylation of the β_1AR in adipocytes or directly linking desensitization of this physiologically relevant response to receptor phosphorylation.

Radionucleotide labeling of the ATP pools and immunoprecipitation of the adipocyte β_1AR revealed several insights concerning the phosphorylation of this βAR subtype. First, the time course of β_1AR phosphorylation indicated that this phenomenon occurred rapidly, as is the case for β_2AR phosphorylation (33). Second, the β_1AR was exquisitely sensitive to phosphorylation by isoproterenol, and nanomolar concentrations of the β-agonist generated marked increase in its phosphorylation. The phosphorylation of transiently transfected epitope-tagged β_1AR in CHW cells by 10 μM isoproterenol resulted in ^{32}P incorporation similar to that achieved with 0.1 μM isoproterenol in rat fat cells (32). Therefore, *in vivo* phosphorylation of these receptors may occur at physiologically attainable concentrations of catecholamines. Third, PVN increased the extent of β_1AR phosphorylation by isoproterenol and forskolin several-fold over either agent alone and revealed that these agents enhanced serine phosphorylation in the β_1AR. Therefore, PVN seems to be a general inhibitor of receptor-associated serine as well as tyrosine phosphatases, which markedly improves the extent of phosphorylation of receptor tyrosine kinases and G protein-coupled receptors.

The mechanism of PVN-mediated increase in β_1AR phosphorylation is unknown. Posner *et al.* (34) found that endosomal rat liver serine/threonine phosphatases were unaffected by peroxovanadium compounds under conditions in which a 10 μM concentration of the compound inhibited insulin receptor dephosphorylation. As a control, a 10 μM concentration of okadaic acid inhibited the serine/threonine phosphatase activity by 100%. Swarup *et al.* (35) reported that orthovanadate was a potent inhibitor of *p*-nitrophenyl phosphate and phosphoserine histone hydrolysis as well as of phosphoserine histone breakdown by lectin-purified phosphatase activity solubilized from membranes. In contrast, the breakdown of phosphoserine histone by the phosphatase activity present in the soluble fraction of TCRC-2 cells was unaffected by vanadate (35). Our data with calcineurin indicate that the breakdown of *p*-nitrophenyl phosphate by this enzyme is also inhibited by PVN (Fig. 5). The available data suggest that membrane-bound protein phosphatases are inhibited by PVN and that at least one of these enzymes can degrade phosphoserine as well as phosphotyrosine residues of proteins. Pitcher *et al.* (36) reported that a phosphatase related to protein phosphatase type 2A and associated with particulate fractions in bovine brain dephosphorylated the β_2AR. In this study, we show that calcineurin, a serine phosphatase related to protein phosphatases type 2B, can be inhibited by PVN *in vitro* at a concentration that is 1000-fold lower than that added to intact adipocytes. Pitcher *et al.* (36) found negligible receptor phosphatase activity in the presence of Ca^{2+}/calmodulin, indicating that protein phosphatase 2B may not play a role in dephosphorylating the receptor. Therefore, the target for PVN action on β_1AR awaits further characterization.

The use of PVN provided several new key pieces of information concerning the mechanism of phosphorylation of the β_1AR *in vivo*. Earlier studies on β_1AR desensitization in human nueroblastoma SK-N-MC cells proposed that the

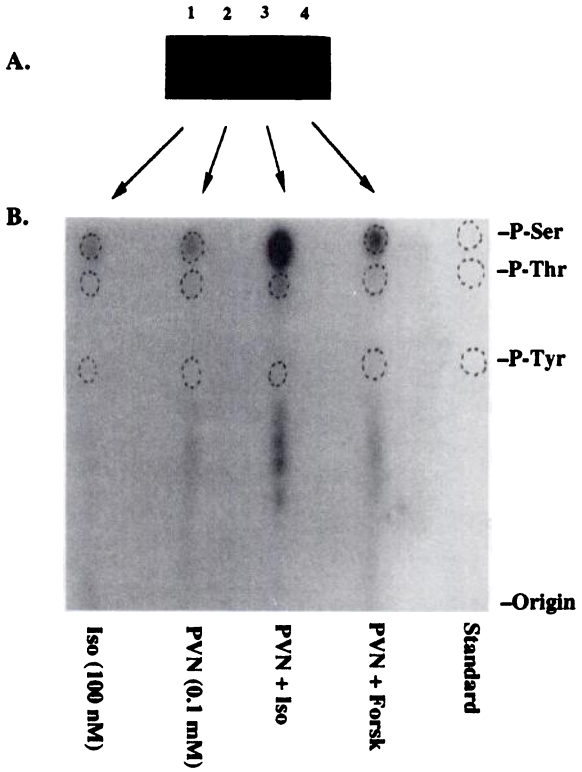

Fig. 4. Phosphoamino acid analysis of phosphorylated β_1ARs. A, Rat adipocytes were labeled with 1 mCi of $^{32}PO_4$-O_4 for 2 hr and then exposed to 0.1 μM isoproterenol (*Iso*) (*lane 1*), 0.1 mM PVN (*lane 2*), PVN plus isoproterenol (*lane 3*), or PVN plus 50 μM forskolin (*Forsk*) (*lane 4*) for 20 min at 37°. The immunoprecipitated β_1AR was subjected to electrophoresis on 10% acrylamide gels and then transferred electrophoretically to polyvinylidene difluoride nylon membranes. The blots were exposed to X-ray film for 3 days with two intensifying screens. The relative intensities of the 68-kDa bands were 1-, 1.3-, 10-, and 3-fold, respectively, for *lanes 1–4*. B, The 68-kDa bands were excised, and the proteins were hydrolyzed with 6 N HCl for 2 hr at 100°. The ^{32}P-labeled phosphoamino acids were separated through thin layer electrophoresis. The migration of phosphoserine (*P-Ser*), phosphothreonine (*P-Thr*), and phosphotyrosine (*P-Tyr*) was monitored using standard phosphoamino acids, and their positions were localized by staining with ninhydrin. The plates were exposed to X-ray film for 8 days with one intensifying screen. The relative intensities of the phosphoserine bands were 1-, 1.5-, 16-, and 3-fold, respectively, for *lanes 1–4*. The experiment was replicated three times with similar results.

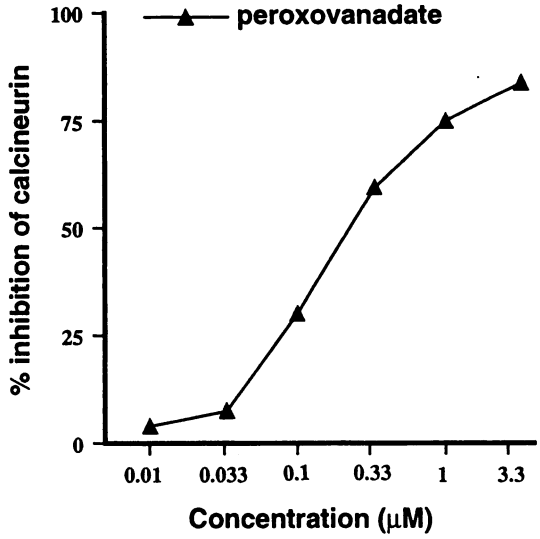

Fig. 5. Inhibition of calcineurin activity by PVN. Calcineurin purified from bovine brain (10 μg/ml) and calmodulin (10 μg/ml) were suspended in 3-(*N*-morpholino)propanesulfonic acid, pH 7, buffer. The inhibition of *p*-nitrophenyl phosphate hydrolysis by PVN was measured over a 5-min incubation. Peroxovanadate inhibition of calcineurin occurred with an IC_{50} of 0.16 μM. Data are the average of four experiments.

β_1AR is desensitized by PKA to a larger extent than βARK (37). The desensitization of transiently transfected β_1AR in CHW cells, however, resembled that of the β_2AR, which is a prominent target for phosphorylation by βARK. Moreover, the desensitization of the β_1AR in rat adipocytes is due mainly to agonist occupation of the β_1AR (i.e., homologous) as β_3AR activation did not desensitize the β_1AR, discounting a significant role for PKA in this process (23). Finally, the desensitization of the β_1AR can be intensified by coexpression of βARK and β-arrestin, which is thought to bind the phosphorylated receptor and uncouple it from G_s (38). Our data reveal that isoproterenol increased the phosphorylation of the β_1AR by ~15-fold. The extent of β_2AR phosphorylation in lipid vesicles composed of purified receptor, heterotrimeric G protein, and βARK was ~7 mol Pi/mol receptor (39). In intact cells, however, the increase in βAR phosphorylation corresponded to ~2–3 mol Pi/mol receptor (32). The combined effect of PVN and β-agonist on β_1AR phosphorylation indicates that the capacity to phosphorylate the receptor in intact adipocytes is high and may be comparable to the extent of *in vitro* phosphorylation. The serines in the carboxyl terminus of the β_2AR have been implicated in β-agonist-mediated phosphorylation because numerically they were abundant in that region and carboxyl-terminal truncation corresponded to loss of βARK-mediated phosphorylation. The use of PVN provided direct evidence that carboxyl-terminal serines in the β_1AR were phosphorylated by β-agonists. Furthermore, PVN desensitized lipolysis in response to β_1 catecholamines, indicating a direct link between physiological desensitization and biochemical modification of carboxyl-terminal serines by phosphorylation. In agreement with the data of Freedman *et al.* (32), it appears that β_1AR phosphorylation is very similar to β_2AR phosphorylation.

The extent of phosphorylation of the β_1AR in adipocytes by forskolin, which maximally increased cAMP levels and PKA activities, was less than that of isoproterenol when both agents were incubated separately in the presence of PVN. In general, the phosphorylation on PKA sites is observed at low nanomolar concentrations of isoproterenol, whereas micromolar concentrations of isoproterenol are required for phosphorylation by βARK (27, 33). The adipocyte provided an excellent model for testing these alternatives because this cell approximates the *in vivo* situation and the receptor under study is the wild-type β_1AR in its native environment. The extent of phosphorylation of the β_1AR by an intermediate concentration of isoproterenol (100 nM) in the presence of PVN was 3-fold greater than forskolin, indicating that βARK was activated by nanomolar concentrations of isoproterenol rather than by the micromolar concentrations that were required in immortalized cells. The exquisite sensitivity of the β_1AR to phosphorylation reinforces the importance of cellular setting in imparting signaling sensitivity and specificity. The model for the phosphorylation of the βAR activation by βARK assumes that the activation of the βAR by agonist catalyzes the activation and dissociation of $G_{s\alpha}$/GTP from βγ.

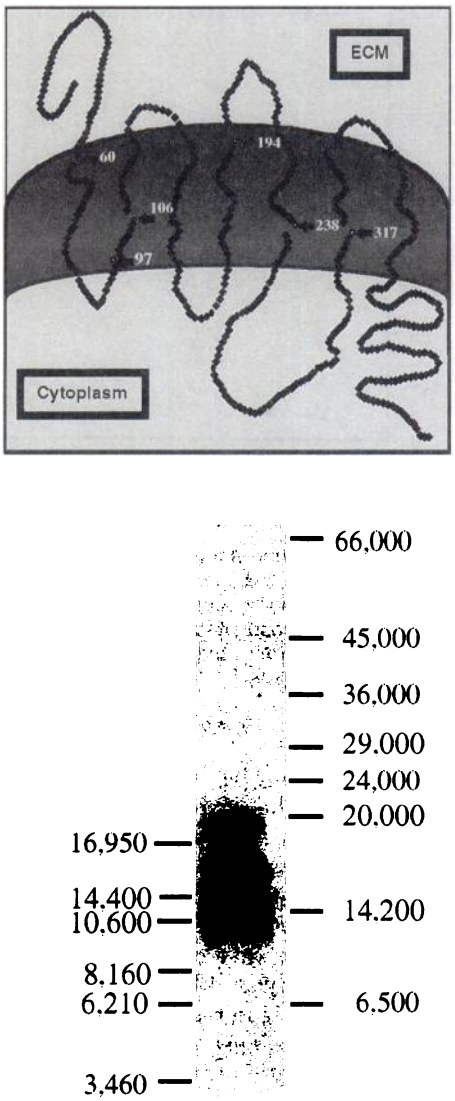

Fig. 6. Cyanogen bromide digestion of the phosphorylated β_1AR. A, Localization of cyanogen bromide-cleaved methionine residues within the intermembranous and extramembranous domains of the rat β_1AR. B, Rat adipocytes metabolically labeled with 1 mCi of $^{32}PO_4$-O_4 were exposed to 0.1 mM PVN plus 100 nM isoproterenol for 20 min at 37°. The β_1AR was immunoprecipitated, subjected to electrophoresis on 10% acrylamide gels, and transferred electrophoretically to nitrocellulose in the presence of 0.1 mM orthovanadate. The blot was exposed to X-ray film, and the band corresponding to the phosphorylated β_1AR was excised. The nitrocellulose (3 × 10 mm) was submerged in 0.5 ml of 70% (v/v) formic acid containing 100 mg/ml cyanogen bromide for 1.5 hr at room temperature. The dried peptides and the molecular mass standards were dissolved in tricine sample buffer and subjected to electrophoresis on a 10% acrylamide gel in tricine cathode buffer. The gel was dried and exposed to an X-ray film with one intensifying screen for 8 days.

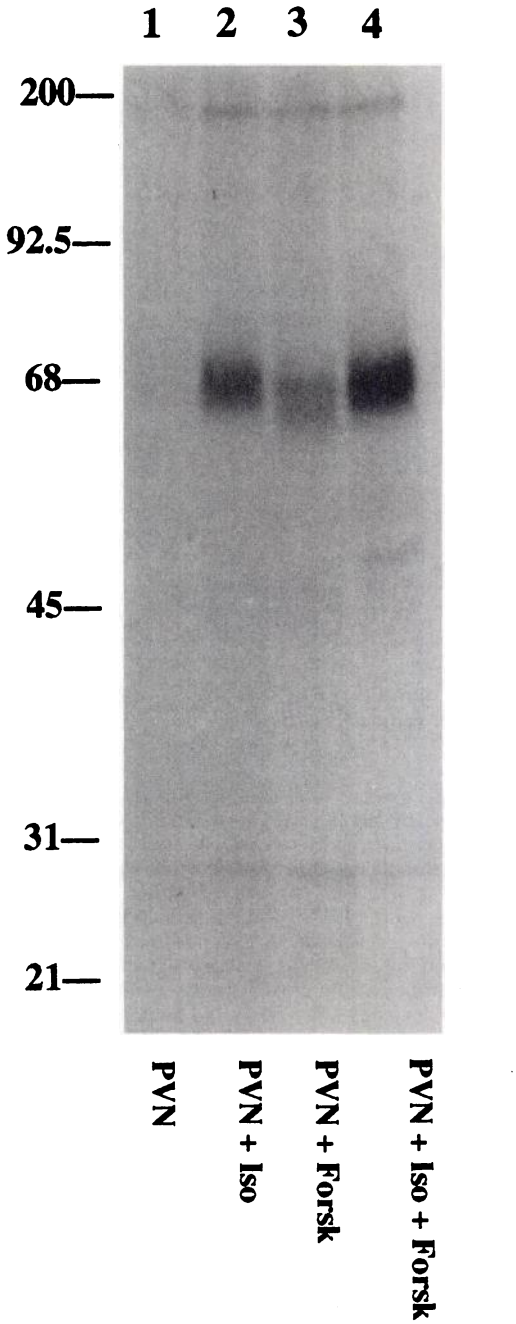

Fig. 7. Effect of PVN on isoproterenol (*Iso*)- and forskolin (*Forsk*)-mediated phosphorylation of the β_1AR in adipocytes. Rat adipocytes were incubated for 2 hr in 5 ml of phosphate-free buffer containing 4% albumin and 0.1 mCi of $^{32}PO_4$-O_4 for each condition. At the end of this incubation, the buffer was removed, and the cells were resuspended in 4% albumin and then incubated for 20 min in 5 ml of buffer with 0.1 mM PVN (*lane 1*), PVN with 0.1 μM isoproterenol (*lane 2*), PVN with 50 μM forskolin (*lane 3*), or PVN plus isoproterenol and forskolin (*lane 4*). The β_1AR was immunoprecipitated, and the immune complexes were resolved through electrophoresis on 10% acrylamide gels. The gels were dried and exposed to X-ray film for 6 days with one intensifying screen. The intensity of the bands was compared through the use of a laser densitometer. The relative intensity of the 68-kDa band was 1-, 14 ± 3-, 7.6 ± 2-, and 31 ± 5-fold for *lanes 1–4*. The experiment was replicated three times with similar results.

Subsequently, βARK binds to βγ and phosphorylates the G protein-coupled receptor responsible for exposing βγ (40). Coincubation of β-agonist with forskolin increased the magnitude of phosphorylation over that attained with either agent alone or their additive effects. This suggests that phosphorylation by this combination was enhanced either by recruiting additional serine/threonine residues or by increasing the extent of phosphorylation of residues by PKA through the removal of stearic hindrance and/or charge effects in the inactive receptor, which reduce the efficiency of its phosphorylation by PKA. The latter possibility may play a greater role in PKA-mediated phosphorylation of β_1AR than of β_2AR. The potential site for phosphorylation by PKA in the third cyto-

plasmic loop of the β_1AR has a sequence of Lys-Arg-Arg-Pro-Ser[301]. This domain is situated adjacent to a cluster of prolines that provide substantial rigidity and stability to the third cytoplasmic loop of the β_1AR. Therefore, a conformational change in the β_1AR structure after β-agonist binding may expose this domain and facilitate the phosphorylation of Ser[301] by PKA.

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