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A DYNAMIC SYSTEM FOR SUPPRESSION AND RE-EXPRESSION OF INSULIN AND PERVANADATE BIORESPONSES IN RAT ADIPOCYTES

TREATMENT WITH OKADAIC ACID AND STAUROSPORINE

ASSIA SHISHEVA* and YORAM SHECHTER
Department of Hormone Research, The Weizmann Institute of Science, Rehovot 76100, Israel

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Abstract—In previous studies, we demonstrated that while okadaic acid stimulates glucose metabolism, it suppresses the bioresponses of insulin itself in rat adipocytes (Shisheva and Shechter, *Endocrinology* **129**: 2279–2288, 1991). Both stimulation and suppression were attributed to okadaic acid-dependent inhibition of protein phosphatases 1 and 2A. We report here that exposure of adipocytes to staurosporine prior to okadaic acid restored insulin-stimulated actions on glucose metabolism. The effect was half-maximal at staurosporine concentrations as low as 70 nM and was fully expressed (80–87% of the control) at 400–500 nM. Similarly, the insulin-like effect of pervanadate, which was also suppressed by okadaic acid, was restored completely with staurosporine pretreatment. Staurosporine was less effective in restoring cell responses inhibited by high concentrations of okadaic acid, or when added to the cells after okadaic acid. Cell resensitization was unique to staurosporine and could not be produced by various agents that reduce cellular protein kinase A- or protein kinase C-dependent phosphorylation, such as phenylisopropyl adenosine (PIA), K-252a and GF 109203X. Staurosporine (400 nM) partially reversed lipolysis induced by okadaic acid but not that induced by β-adrenergic stimulation. PIA, which antagonized okadaic acid-induced lipolysis to the same extent as staurosporine, was not capable of restoring insulin responses. Further studies aimed at elucidating this reversing effect revealed that staurosporine did not reactivate okadaic acid-inhibited protein phosphatases 1 and 2A in both cellular and cell-free systems. In summary, we report here a unique dynamic system in which insulin and pervanadate bioeffects can be fully suppressed and again re-expressed without reactivation of protein phosphatase 1 or 2A. The precise site for both effects, although still obscure, appears to be downstream from autophosphorylated insulin receptor.

Key words: insulin action; pervanadate; okadaic acid; staurosporine

According to currently accepted concepts, insulin alters cell metabolism initially by binding to its plasma membrane receptor, a step that causes receptor activation and self-phosphorylation. Receptor autophosphorylation, in turn, increases many-fold the intrinsic IR† tyrosine kinase activity [1–3]. The IR-initiated tyrosine-phosphorylating cascade is transformed to a change in the serine/threonine phosphorylation state of key rate-limiting enzymes for glucose, lipid and protein metabolism [4–6]. The intermediate players and events linking tyrosine phosphorylation of IR with serine/threonine-phosphorylated regulatory proteins have been under intensive study in recent years [7–14]. It appears that insulin signal transmission involves tyrosine phosphorylation of a major insulin receptor substrate (IRS-1) [7] and activation of $p21^{ras}$, which occurs predominantly through an increase in nucleotide exchange [8, 9]. $p21^{ras}$ activation seems to be a result of transient interaction between IRS-1, phosphatidylinositol-3′ kinase (PI-3 kinase), an adaptor protein, and the exchange factor [10–13]. $p21^{ras}$ function is required for activation of $p42^{MAP\ kinase}$ (also designated $p42^{erk2}$, extracellular signal regulated kinase), which has emerged as a central point in insulin and growth factor signal transmission [14]. It is not clear at present whether the same signal cascade controls insulin-regulated metabolic effects.

Peroxide(s) of vanadate (pervanadate) is a powerful insulinomimetic agent in rat adipocytes [15–17]. Its mode(s) of action seems to resemble that of the hormone itself since the signal is transmitted via autophosphorylated IR β-subunit [17]. Insulin and pervanadate appear to stimulate tyrosine phosphorylation of similar endogenous substrates [18].

Okadaic acid is a potent cell-permeable inhibitor of serine/threonine-specific protein phosphatases 1 and 2A [19]. By virtue of this feature, okadaic acid activates several insulin-sensitive metabolic pathways

* Corresponding author: Dr. Assia Shisheva, Program in Molecular Medicine, University of Massachusetts Medical Center, 373 Plantation St., Worcester, MA 01605. Tel. (508) 856-6860; FAX (508) 856-4289.

† Abbreviations: IR, insulin receptor; IRS-1, insulin receptor substrate-1, KRB, Krebs–Ringer bicarbonate; BSA, bovine serum albumin; PIA, N^6-phenylisopropyl adenosine; and Me_2SO, dimethyl sulfoxide.

in insulin-responsive cells and tissues, including glucose uptake, glucose oxidation via glycolysis, and glucose incorporation into lipids [20–23]. Paradoxically, while okadaic acid produces insulin-like bioeffects, the cell responses to insulin itself are lost in okadaic acid-pretreated adipocytes, as has been shown recently by several laboratories including our own [21, 22, 24]. The unresponsiveness to insulin is not restricted only to glucose transport, but spreads over to the other insulin bioeffects in adipocytes, including glucose oxidation, its incorporation into lipids, and antilipolysis [22]. The insulin-suppressing effect of okadaic acid cannot be attributed to attenuation of insulin receptor function, since insulin binding, receptor-mediated internalization, receptor autophosphorylation, and its phosphotransferase activity are not altered by okadaic acid treatment [22, 24]. Moreover, the adipocytes are fully refractory to the stimulating actions of vanadate as well [22], which is considered to be a post-receptor insulin mimicker [25, 26]. All these results indicate that okadaic acid inhibits the action of insulin in stimulating glucose and fat metabolism at a step after receptor activation. Since this effect is attributed to the increase of the protein serine/threonine phosphorylation state by virtue of the ability of okadaic acid to inhibit protein phosphatases 1 and 2A [19], in this study we attempted to reinstate insulin effects by inhibiting serine/threonine phosphorylation prior to okadaic acid treatment. Here, we report that staurosporine is capable of restoring the responsiveness to insulin and pervanadate in rat adipocytes despite the persistent inhibition of the protein phosphatases.

MATERIALS AND METHODS

D-[U-^{14}C]-, D[1-^{14}C]-, and D-[6-^{14}C]Glucose were purchased from New England Nuclear (Boston, MA). D-[2-^{3}H]Glucose and [γ-^{32}P]ATP were purchased from Amersham (Buckinghamshire, UK). Collagenase Type 1 (134 U/mg) was obtained from Worthington Biochemicals (Freehold, NJ). Porcine insulin was purchased from the Eli Lilly Co. (Indianapolis, IN). Phosphorylase *b*, phosphorylase kinase, staurosporine, and D,L-isoproterenol–HCl were purchased from the Sigma Chemical Co. (St. Louis, MO). Okadaic acid and K-252a were gifts from Professor Uriel Zor (Department of Hormone Research, The Weizmann Institute of Science, Israel). The inhibitor GF 109203X was donated by Dr. H. Coste (Laboratories Glaxo, France). KRB buffer, pH 7.4, contained 110 mM NaCl, 25 mM $NaHCO_3$, 25 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, and 1.3 mM $MgSO_4$.

Cell preparation and bioassays. Rat adipocytes were prepared from fat pads of male Wistar rats (100–200 g) by collagenase digestion [27]. Cell number was determined by counting the cell suspension in a Burcker chamber. Cell preparation showed more than 95% viability by trypan blue exclusion at least 3 hr after digestion. Cell treatment and bioassays were performed as specified in the figure legends. Glucose oxidation was measured by the conversion of D-[1-^{14}C]glucose to $^{14}CO_2$ [27]. Lipogenesis was performed with D-[U-^{14}C]-, D-[6-^{14}C]- or D-[2-^{3}H]glucose as described elsewhere [28]. Lipolysis was evaluated by measuring glycerol released from adipocytes as described previously [29].

In vivo *and* in vitro *measurement of protein phosphatase 1 and 2A activity.* After collagenase digestion and washing with KRB buffer, pH 7.4, containing 0.7% BSA and 0.5 mM glucose, adipocytes (~10^6 cells/0.2 mL) were incubated first in the presence or absence of staurosporine (20 min, 37°) and then in the presence or absence of okadaic acid (20 min, 37°) at concentrations indicated in the figure legends. The cells were then stimulated with insulin (17 nM) or pervanadate (20–50 μM) for an additional 20 min at 37°. Cell suspensions were transferred quickly over silicone oil-containing tubes and spun down for 10 sec to remove the BSA-containing medium and the remaining okadaic acid. Cells were then resuspended in 50 mM HEPES buffer, pH 7.4, and immediately lysed by a freezing/thawing procedure in liquid N_2. Fat was removed by centrifuging (20 sec) cell homogenates through a silicone oil layer [30]. Protein concentrations [31] of the samples were normalized, and the activity of protein phosphatases 1 and 2A was measured in aliquots with [^{32}P]-phosphorylase *a* as a substrate. [^{32}P]-Phosphorylase *a* was prepared by labeling phosphorylase *b* (10 mg) with [γ-^{32}P]ATP for 60 min at 30° in Tris-β-glycerophosphate buffer, pH 8.2, in the presence of phosphorylase kinase, according to the procedure of Cohen *et al.* [32]. The specific activity of the labeled product was between 0.5 and 1×10^6 cpm/nmol. Protein phosphatase activity was measured in an assay mixture (final volume 30 μL, in 50 mM Tris-HCl buffer, pH 7.0, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 5 mM caffeine and 1 mg/mL BSA), containing the adipocytic extracts (~100 ng protein) and [^{32}P]-phosphorylase *a* (~150,000 cpm). Following a 10-min incubation at 30°, the reaction was terminated by adding 10% (w/v) trichloroacetic acid (100 μL). After centrifugation (10 min), the radioactivity of the supernatants (120 μL aliquots) was analyzed with scintillation fluid. $^{32}P_i$ hydrolysis amounted to 15–20% of the radioactivity at the zero time point. These conditions provided linearity of the enzyme assay with respect to the concentration of the enzyme source and time. For the *in vitro* measurements, the cell extracts were incubated with staurosporine (0–200 nM) and/or okadaic acid (0.01 nM to 1 μM) at 22° for 5 min, and then assayed for protein phosphatase activity as described above.

Solutions of staurosporine (up to 100 μM) were prepared in Me_2SO. Lower dilutions were in 40% C_2H_5OH. Okadaic acid was dissolved in 20% Me_2SO up to 250 μM, and the lower dilutions were in water. All control points contained appropriate final concentrations of the solvents. The peroxide(s) of vanadate (pervanadate) was prepared by mixing sodium metavanadate with H_2O_2 in a 1:2 molar ratio for 15 min at 22°, followed by the addition of catalase, as described elsewhere [15]. The concentration of pervanadate generated is denoted by the vanadate concentration. All assays were performed in either duplicate or triplicate. All data are presented as the mean ± SEM (N = 3 or more).

RESULTS

Restoration of cell responsiveness to insulin and pervanadate in okadaic acid-treated adipocytes by staurosporine. To assess whether the adipocytes can restore insulin responses if the phosphorylation of cell protein(s) is decreased prior to phosphatase inhibition by okadaic acid, we took advantage of several cell-permeable, powerful protein kinase inhibitors: staurosporine, K-252a [33], and a novel protein kinase C-specific agent, the bisindolylmaleimide GF 109203X [34]. The choice was based on our previous observation that these agents are weak inhibitors of IR tyrosine kinase [26, 35], and do not influence insulin-regulated actions on glucose metabolism in rat adipocytes at the concentrations used (see below). On the other hand, we have previously identified insulin-dependent actions that are not stimulated by okadaic acid: conversion of [2-^{3}H]- or [6-^{14}C]glucose to lipids, and oxidation of [1-^{14}C]glucose to CO_2 [22]. Thus, we were provided with the opportunity to distinguish clearly the source of the stimulus (okadaic acid or the hormone itself) when the cells were treated simultaneously with the two agents. As shown in Fig. 1A and B, pretreatment of intact rat adipocytes with okadaic acid (30 min, 37°) suppressed insulin-stimulated [2-^{3}H]- and [6-^{14}C]glucose conversion to lipids, in agreement with our previous study [22]. Short exposure of adipocytes to staurosporine (20 min, 37°) prior to okadaic acid treatment reversed cell sensitivity to insulin (Fig. 1). The recovery of insulin-activated [2-^{3}H]glucose conversion to lipids was evident at staurosporine concentrations as low as 20 nM, and the effect was half-maximal at 70 nM (Fig. 1C). At 400–500 nM staurosporine, the response to insulin was restored up to 84 ± 7% of the maximal insulin effect on control cells (Fig. 1A and B). Staurosporine at the concentrations used did not appreciably influence the effects of either insulin or okadaic acid when both agents were applied alone (Fig. 1A and B).

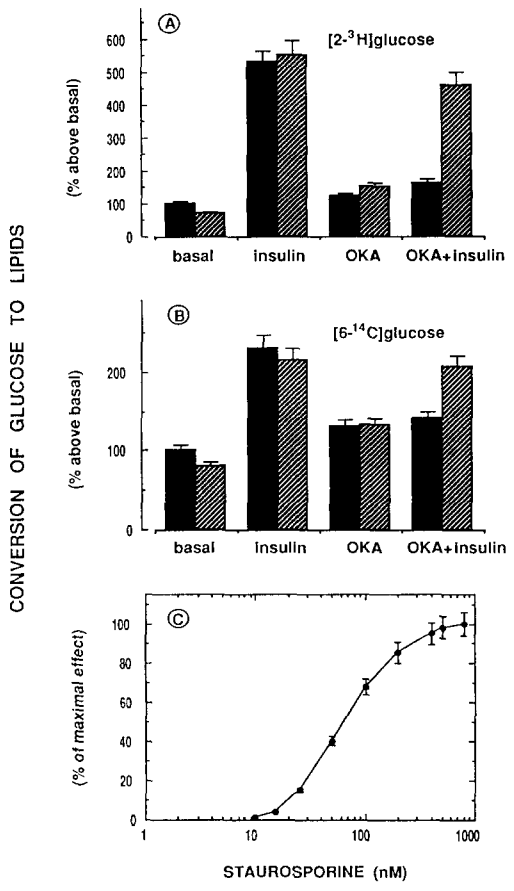

Fig. 1. Re-expression of insulin-stimulated lipogenesis in okadaic acid-treated adipocytes by staurosporine. Adipocytes (2 × 10^4 cells/mL suspended in KRB buffer, containing 0.7% BSA) were incubated in the presence (▨) or absence (■) of 400 nM (A and B) or indicated concentrations of staurosporine (C) for 20 min at 37°. Where indicated, okadaic acid (OKA) was added (300 nM; 30 min; 37°). Cells were then supplemented with either [2-^{3}H]glucose (0.2 mM; 3000 cpm/nmol) (A and C) or [6-^{14}C]glucose (0.2 mM; 1500 cpm/nmol) (B). Lipogenesis was carried out for 1 hr at 37° in the presence or absence of insulin (17 nM). Results are the means ± SEM of three to six different cell preparations. The 100% control values were 0.58 ±0.04 and 2.78 ±0.12 nmol converted [2-^{3}H]-glucose or [6-^{14}C]glucose/10^4 cells/hr, respectively.

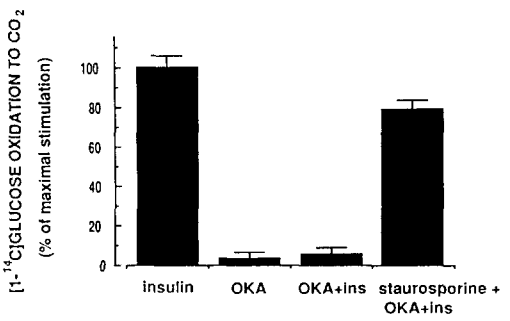

Fig. 2. Re-expression of insulin-stimulated glucose oxidation in okadaic acid-treated adipocytes by staurosporine. Adipocytes (2 × 10^4 cells/ml) were exposed to staurosporine (400 nM, 20 min, 37°) prior to okadaic acid (300 nM, 30 min, 37°). Glucose oxidation ([1-^{14}C]glucose, 1300 cpm/nmol; 0.2 mM glucose) was continued for an additional 1 hr at 37° in the presence or absence of insulin (17 nM), and was estimated by the radioactivity of $^{14}CO_2$ formed. Results are the means ± SEM of three different cell preparations. The basal and insulin-activated levels were 2.52 ± 0.15 and 5.81 ± 0.21 nmol [1-^{14}C]glucose/10^4 cells/hr, respectively.

Similarly, short exposure of intact adipocytes to staurosporine (400 nM; 20 min; 37°) prior to okadaic acid reinstated the ability of insulin to stimulate [1-^{14}C]glucose oxidation, which also had been blunted by treatment with okadaic acid alone (Fig. 2).

It is noteworthy that if the agents were added vice versa (staurosporine after okadaic acid), the reversal of the insulin effect amounted to only 34% of maximal insulin stimulation (Table 1). This fact shows that the staurosporine-dependent event, supposedly inhibition of protein phosphorylation, should precede the inhibition of protein phosphatases in order to reverse cell sensitivity to insulin. Also, staurosporine (400 nM) was much less effective in

Table 1. Effects of different conditions and protein kinase C inhibitors in regenerating insulin activation of lipogenesis

Conditions	[2-^{3}H]Glucose conversion to lipids (% of maximal insulin stimulation)
Insulin	100
Okadaic acid (300 nM)	15 ± 2.1
Staurosporine + okadaic acid (300 nM)*	84 ± 7.0
Okadaic acid (300 nM) + staurosporine†	34 ± 2.3
Okadaic acid (1 μM)	10 ± 0.5
Staurosporine + okadaic acid (1 μM)*	35 ± 2.5
K-252a (1 μM) + okadaic acid (300 nM)	8 ± 0.5
GF 109203X (1 μM) + okadaic acid (300 nM)	10 ± 0.8

Lipogenesis was carried out for 1 hr at 37° in the presence of insulin (17 nM) in all samples. The basal and insulin-activated levels of lipogenesis were 0.58 ± 0.04 and 2.09 ± 0.12 nmol/10^4 cells/hr, respectively. Values are means ± SEM of four separate experiments.

* Adipocytes were pretreated first with staurosporine (400 nM; 20 min; 37°) and then with the indicated concentrations of okadaic acid (20 min; 37°).

† The addition of the agents is in reversed order.

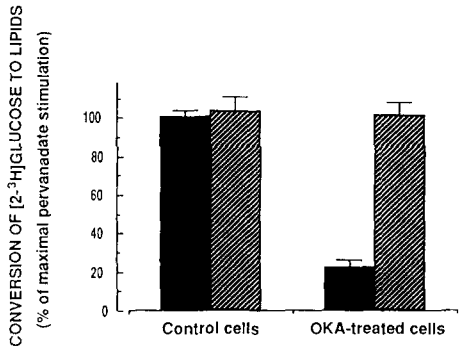

Fig. 3. Re-expression of pervanadate-stimulated lipogenesis in okadaic acid-treated adipocytes by staurosporine. Adipocytes (2 × 10^4 cells/mL) were incubated in the presence (▨) or absence (■) of staurosporine (400 nM, 20 min, 37°) and then with/without okadaic acid (300 nM, 30 min, 37°). Lipogenesis was carried out in the presence of pervanadate (20 μM) for 1 hr at 37°. Results are the means ± SEM of three different cell preparations. The basal and pervanadate-activated levels were 0.58 ± 0.04 and 2.60 ± 0.14 nmol/10^4 cells/hr of [2-^{3}H]glucose, respectively.

reversing insulin-dependent lipogenesis suppressed by high concentrations of okadaic acid (1 μM). The effect was restored to only 42% of the level found in cells treated with 300 nM okadaic acid (Table 1).

K-252a (1 μM), an analog of staurosporine, and the bisindolylmaleimide GF 109203X (1 μM) were ineffective in restoring insulin-stimulated [2-^{3}H]-glucose conversion to lipids in okadaic acid-treated cells (Table 1). At the concentrations used, both agents did not appreciably influence either insulin- or okadaic acid-dependent lipogenesis (not shown).

As with insulin, staurosporine (400 nM) did not inhibit the insulin-like effect of pervanadate in stimulating lipogenesis, while okadaic acid (300 nM) suppressed it almost completely (Fig. 3). To assess whether staurosporine is capable of reversing cell responsiveness to pervanadate as well, the intact adipocytes were exposed to staurosporine (20 min, 37°) prior to okadaic acid (30 min, 37°). As shown in Fig. 3, pervanadate-stimulated [2-^{3}H]glucose conversion to lipids was fully restored.

Inhibition of okadaic acid-induced lipolysis by staurosporine. Okadaic acid stimulates basal lipolysis in rat adipocytes [20, 22, 36], an effect which may result from an increased phosphorylation of hormone-sensitive lipase [20]. Insulin is ineffective in reversing this lipolysis [22]. However, staurosporine pretreatment (400 nM; 30 min; 37°) of intact adipocytes inhibited okadaic acid-dependent lipolysis by 50.6 ± 5% (Table 2). Thus, staurosporine, but not insulin [22], can largely reduce lipolysis in okadaic acid-treated cells, a fact suggesting that this agent can induce protein phosphatase 2A-independent dephosphorylation. The antilipolytic action of staurosporine was not due to protein kinase A inhibition; the agent did not reverse either isoproterenol- nor cholera toxin-induced lipolysis even at their submaximal concentrations. In this case, staurosporine slightly increased lipolysis (by 30%) as it increased the basal level as well (Table 2).

Lack of restoration of cell responses to insulin following treatment with PIA. Okadaic acid lipolytic action in rat adipocytes can be also reversed by a nonmetabolizable analog of adenosine, PIA [20]. In our hands, the antilipolytic effect of PIA was also observed. At 10 nM, PIA antagonized okadaic acid-induced lipolysis to the same extent as staurosporine

Table 2. Effect of staurosporine on lipolysis induced by okadaic acid and β-adrenergic agents

Additions	Glycerol released (% of okadaic acid stimulation)
Basal	0
Staurosporine	15
Okadaic acid (300 nM)	100
+ staurosporine	50
Isoproterenol (50 nM)	240
+ staurosporine	320
Isoproterenol (5 nM)	60
+ staurosporine	78
Cholera toxin (140 ng/mL)	510
+ staurosporine	530

Adipocytes were exposed to staurosporine (400 nM; 20 min; 37°) prior to the addition of the indicated agents. Lipolysis was evaluated over a period of 1 hr or of 2.5 hr for cholera toxin. The basal- and okadaic acid-induced lipolysis values were 10.3 ± 1.2 and 58.2 ± 3.2 nmol glycerol/2 × 10^5 cells/hr, respectively (means ± SEM, N = 5).

Table 3. Lack of re-expression of insulin bioeffects following treatment with PIA

Conditions	Glycerol released (nmol/2 × 10^5 cells/hr)	[2-^{3}H]Glucose conversion to lipids (nmol/10^4 cells/hr)
Control cells		
Basal	10.3 ± 1.2	0.43 ± 0.04
Insulin	9.8 ± 0.5	2.28 ± 0.15
Okadaic acid	58.2 ± 3.2	0.53 ± 0.02
Okadaic acid + insulin (17 nM)	59.5 ± 4.1	0.71 ± 0.06
PIA-treated cells		
Basal	8.3 ± 0.5	0.41 ± 0.04
Insulin	9.3 ± 0.7	2.20 ± 0.20
Okadaic acid	32.1 ± 1.4	0.50 ± 0.04
Okadaic acid + insulin (17 nM)	33.2 ± 2.1	0.64 ± 0.05

Adipocytes (2 × 10^4 cells/mL for lipogenesis, and 4 × 10^5 cells/mL for lipolysis, suspended in KRB buffer containing 0.7% and 1% BSA, respectively) were incubated in the presence or absence of PIA (10 nM) for 10 min at 37°. Where indicated, okadaic acid (300 nM) was added and the cells were incubated for an additional 40 min at 37°. Lipogenesis and lipolysis were carried out for 1 hr at 37°. Lipolysis was evaluated by the amount of glycerol released. Values are means ± SEM of four separate experiments.

(50%, Table 3). However, addition of PIA (10 nM) did not restore the ability of insulin to stimulate lipogenesis in okadaic acid-treated cells, as opposed to staurosporine (Table 3). Thus, cell resensitization is unique to staurosporine and could not be produced by another antilipolytic agent.

Lack of staurosporine effect in reactivating protein phosphatases 1 and 2A. Since okadaic acid inhibits protein phosphatases 1 and 2A [19], we examined whether the enzymes were reactivated by staurosporine treatment, in both cell-free and cellular systems. In cell-free experiments, staurosporine (5–800 nM) had no effect on the activities of okadaic acid-inhibited protein phosphatases 1 and 2A; an IC_{50} value of ~4 nM okadaic acid was determined with or without staurosporine (Fig. 4A). Similarly, in intact adipocytes, staurosporine (400 nM) did not affect the basal activity of either phosphatase, nor did it reactivate either enzyme in okadaic acid-treated cells (Fig. 4B). To avoid the masking effect of the intracellular presence of okadaic acid, in separate experiments the inhibitor (0.1 nM to 1 μM) was included directly in the phosphatase assay. No measurable differences in the phosphatase activity between the extracts of staurosporine-treated and -untreated cells were observed (not shown).

DISCUSSION

Recently several laboratories, including our own, have observed that treatment of rat adipocytes with okadaic acid, a powerful, cell-permeable inhibitor of serine/threonine-specific protein phosphatases 1 and 2A [19], blunts insulin-regulated actions on glucose and lipid metabolism [20–22, 24]. The main aim of this study was to find agents and conditions capable of resensitizing the cells to the actions of the hormone. We hypothesized that okadaic acid-induced desensitization to insulin is related to an increase in the phosphorylation state of a crucial putative protein(s) as a direct or indirect result of protein phosphatase 1 and/or 2A inhibition. Therefore, if initially the phosphorylation of this putative protein(s) does not occur, the cell may remain sensitive to insulin despite the persistent inhibition of both phosphatases. On the basis of this hypothesis, our search was directed toward several serine/threonine kinase inhibitors. A necessary prerequisite was that these agents must be indifferent to the metabolic effects of the hormone itself.

Among the several agents studied here, staurosporine (400–500 nM) was the one able to reinstate almost completely insulin responses in okadaic acid-treated adipocytes. Similarly, the effect of pervanadate in stimulating lipogenesis, which was also blocked by okadaic acid, was fully restored by staurosporine, despite the continuous presence of the protein serine/threonine phosphatase inhibitor.

We initially considered and ruled out several adverse cell effects, not related to our general hypothesis: (i) staurosporine did not perturb the permeation of okadaic acid into cell interiors; okadaic acid stimulated lipogenesis with overlapping concentration–response curves in staurosporine-treated and -untreated adipocytes (not shown); (ii) staurosporine did not increase IR β-subunit autophosphorylation in insulin- or pervanadate-treated cells (not shown); and (iii) staurosporine did not interfere with the inhibiting effect of okadaic acid on protein phosphatase 1 and 2A activities; no staurosporine-dependent reactivation of the protein phosphatases was detected in either cellular or cell-free systems (Fig. 4). This result supported our assumption that the re-expression of insulin effects by staurosporine does not result from direct counteraction to the phosphatase inhibition.

Staurosporine is a powerful inhibitor of protein kinase C and protein kinase A [33]. However, several lines of evidence show that cell resensitization is not related to protein kinase C and/or protein kinase A inhibition: (i) the protein kinase C-specific inhibitor GF 109203X [34], as well as K-252a, an

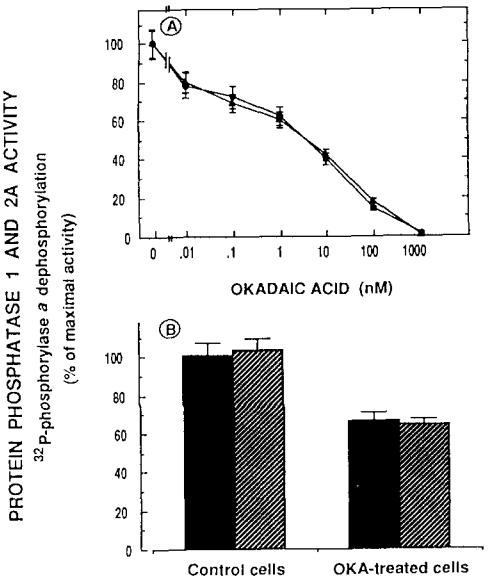

Fig. 4. Lack of staurosporine effect in reactivating okadaic acid-inhibited protein phosphatases 1 and 2A in cell-free (A) and cellular (B) systems. (A) Adipocytic extracts (~100 ng protein) were incubated in the presence or absence of staurosporine (200 nM) for 5 min at 22°. Okadaic acid was then added at the indicated concentrations. Protein phosphatase activity was measured (10 min; 30°) with [^{32}P]phosphorylase *a* as a substrate (~7 μM; specific activity 0.5 to 1×10^6 cpm/nmol) in an assay mixture containing 50 mM Tris–HCl buffer (pH 7.0), 0.1 mM EGTA, 0.1% β-mercaptoethanol, 5 mM caffeine and 1 mg/mL BSA. After precipitation of the proteins, the amount of $^{32}P_i$ released was analyzed in a β spectrometer. The 100% control values were 20,150 ± 850 cpm (basal) and 20,650 ± 980 cpm (staurosporine). (B) Intact adipocytes (10^6 cells/0.2 mL) were incubated in the presence (▨) or absence (■) of staurosporine (400 nM) for 20 min at 37° and/or okadaic acid (300 nM) for an additional 20 min at 37°, as described under Materials and Methods. Protein phosphatase activity of lipid-free cell homogenates was then measured as described above. Results are the means ± SEM of three different cell preparations. The 100% control value was 12,850 ± 580 cpm.

analog of staurosporine [33], did not restore insulin bioeffects in okadaic acid-treated cells (Table 1); (ii) PIA, a nonhydrolyzable agonist of adenosine, which causes inhibition of adenylate cyclase and reduces protein kinase A activity [37], did not restore insulin responses (Table 3); and (iii) staurosporine did not antagonize β-adrenergic-stimulated lipolysis (Table 2). Finally, cell resensitization was not a direct result of the antilipolytic effect of staurosporine on okadaic acid-induced lipolysis, since PIA inhibited okadaic acid-evoked lipolysis, yet the insulin responses were not reinstated (Table 3). It might be hypothesized, therefore, that a staurosporine-sensitive kinase (other than protein kinase C or protein kinase A) counteracts the serine/threonine protein phosphatase inhibition. The site of this counteraction is presently unknown. The fact that okadaic acid abrogates a variety of insulin bioresponses in adipocytes, starting with glucose influx, suggests that a crucial dephosphorylating event(s) is required at an early step in the transduction of the insulin signal. An increase of serine phosphorylation of IR brings about inhibition of its autophosphorylation [38, 39], but okadaic acid does not suppress receptor β-subunit autophosphorylation [21]. This result, together with our previous observation that okadaic acid does not alter IR tyrosine kinase activity in adipocytes [22], indicates that the crucial serine/threonine sensitive step is after receptor activation. It has been observed recently that okadaic acid causes a marked suppression of IRS-1 tyrosine phosphorylation in 3T3-L1 adipocytes [40] and rat fat cells (Shisheva A and Shechter Y, unpublished data). This inhibition has been attributed to the increased serine/threonine phosphorylation state of the activated insulin receptor and/or IRS-1, thereby reducing the ability of the receptor to recognize and phosphorylate IRS-1 [40]. In fact, in resting cells IRS-1 appears to be highly phosphorylated on serine residues [41, 42]. Therefore, the regulation of IRS-1 may be combined by serine/threonine phosphorylation, which could also influence its interaction with downstream effectors. However, okadaic acid, via increasing serine/threonine phosphorylation, may influence other early steps along the chain of events initiated by activated insulin receptor. Examples in this respect might be PI-3 kinase and $p42^{erk2}$; activation of both is inhibited markedly by an increase of serine/threonine phosphorylation [43, 44]. Further studies will reveal the exact locus of okadaic acid-induced blockade on insulin and pervanadate bioeffects and staurosporine counteraction.

In summary, the results presented herein show that while okadaic acid causes cell desensitization to insulin and pervanadate responses, staurosporine restores them without reactivation of protein phosphatase 1 or 2A. This dynamic cell system "off/on" may be a useful model for further studies of the signal-transducing mechanism(s) of insulin action, in general, and the molecular mechanism(s) related to insulin resistance, in particular.

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