# Palmitic acid stimulates glucose incorporation in the adipocyte by a mechanism likely involving intracellular calcium

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**Abstract** The effect of palmitic acid on basal and insulin-stimulated incorporation of glucose into rat adipocytes was studied. Palmitic acid (2.40 mM) stimulated basal as well as insulin-stimulated glucose incorporation in rat adipocytes three- and twofold, respectively. Similar degrees of stimulation of basal glucose oxidation by palmitate were also observed. The ability of palmitic acid to stimulate glucose uptake was additive with respect to the stimulation induced by insulin and was proportional to the palmitic acid concentration between 0.15 mM and 2.40 mM. Stimulation of glucose incorporation by palmitic acid was inhibited by preincubating the cells with quin2-AM, which accumulates intracellularly yielding the trapped chelator form, quin2, which binds intracellular $Ca^{2+}$. The concentration of quin2-AM required for half-maximal inhibition of palmitic acid stimulated glucose incorporation was 3.8 ± 1.2 μM (mean ± SEM). The inhibition of palmitic acid-stimulated glucose incorporation by quin2-AM (10 μM) was overcome by incubating cells with the $Ca^{2+}$ ionophore, A23187, in the presence of extracellular $Ca^{2+}$ (2.6 mM). Chelation of extracellular $Ca^{2+}$ with EGTA did not significantly affect the magnitude of palmitic acid-stimulated glucose incorporation. Dantrolene (12.5–100 μM) failed to affect basal or palmitic acid-stimulated glucose incorporation. These findings suggest that palmitic acid stimulates incorporation of glucose in the adipocyte by a mechanism dependent upon intracellular but not extracellular $Ca^{2+}$. — **Thode, J., H. A. Pershadsingh, J. H. Ladenson, R. Hardy, and J. M. McDonald.** Palmitic acid stimulates glucose incorporation in the adipocyte by a mechanism likely involving intracellular calcium. *J. Lipid Res.* 1989. **30:** 1299–1305.

**Supplementary key words** calcium • fatty acid • glucose metabolism • insulin • A23187

It is well documented that insulin and free fatty acid (FFA) turnover are interrelated (1,2). Under physiological conditions insulin is known to inhibit catecholamine-stimulated lipolysis in adipocytes (1–3). FFA flux is greatly increased in pancreatectomized, alloxan-diabetic, or diazoxide-treated animals (4–6). Not only does insulin regulate FFA turnover by depressing FFA release, but plasma FFA also regulates insulin secretion (7–9). It has also been shown that FFA stimulates glucose transport (10) as well as glucose incorporation (11) into isolated adipocytes.

There is now evidence that the saturated FFA, palmitic acid, increases $^{45}Ca^{2+}$ uptake in rat calvaria (12). Other investigators, however, found that unsaturated but not saturated FFA had a similar effect on $^{45}Ca^{2+}$ uptake in bone discs (13), i.e., an increase in $^{45}Ca^{2+}$ uptake. Although it has been suggested that $Ca^{2+}$ complexed with palmitic acid possesses biological activity (12), these speculations are based on $^{45}Ca$ uptake measurements by cells and interpretation of such experimental data can be difficult and easily misinterpreted (14).

The mechanism leading to increased glucose incorporation into fat cells by palmitic acid is unknown and has not been extensively studied, whereas the action of insulin on glucose transport and incorporation has been investigated extensively (2,15). Although the mechanism whereby insulin stimulates glucose transport in adipocytes remains controversial, there is compelling evidence implicating $Ca^{2+}$ as an important component (16).

We demonstrate that the free fatty acid palmitic acid (saturated $C_{16}$) stimulates glucose incorporation in isolated rat adipocytes in an additive manner to the effects of insulin. We further demonstrate that maneuvers designed to chelate intracellular calcium prevent palmitic

Abbreviations: HEPES, n-2-hydroxy-ethyl piperazine-N'-2-ethanesulfonic acid; Dantrolene, 1-[5-(*p*-nitrophenyl)-furfurylidine]amino]hydantion sodium hydrate; quin2, (2-[2-bis-[carboxymethyl]amino-5-methyl-phenoxy)-methyl]-6-methoxy-8-bis-[carboxymethyl]aminoquinoline; quin2-AM, (2-[2-bis-[carboxymethyl]amino-5-methyl-phenoxy)methyl]-6-methoxy-8-bis-[carboxymethyl]aminoquinolinetrakis-[acetoxymethyl] ester); FFA, free fatty acids; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid; BSA, bovine serum albumin.

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acid from stimulating glucose incorporation, whereas removal of extracellular $Ca^{2+}$ was ineffective.

## EXPERIMENTAL PROCEDURES

### Cell preparation

Adipocytes were isolated from the epididymal fat pads of male Sprague-Dawley rats (120–150 g) by the collagenase digestion method of Rodbell (17). Cells were washed 3 times and resuspended in a $Ca^{2+}$-Krebs-Ringer phosphate (KRP) buffer supplemented with pyruvate. The buffers contained 1.4 mM $CaCl_2$, 128 mM NaCl, 5.2 mM KCl, 1.4 mM $MgSO_4$, 10 mM Na pyruvate, 30 g/l BSA, and 10 mM Na phosphate, pH 7.4, at 37°C.

### Standard and palmitic acid buffer preparations

The standard buffer contained 4.5 g/dl human albumin (fatty acid free, dialyzed for 24 h against albumin-free buffer), 2.6 mM $CaCl_2$, 130 mM NaCl, 5 mM glucose, 4 mM KCl, 3 mM $Na_2HPO_4$, 1.4 mM $MgSO_4$, 5 mM HEPES, pH 7.4, at 37°C. The palmitate buffer was prepared by adding palmitic acid (99% pure, Sigma) to the standard buffer to saturating concentrations by the hexane-Celite method of Spector and Hoak (18). Unless otherwise indicated, the final concentration of palmitic acid was 2.40 mM as determined by an enzymatic colorimetric method (NEFAC, Wako Pure Chemical Industries, Osaka, Japan).

### Glucose incorporation assays

Glucose incorporation in adipocytes was assayed by a filtration method. Aliquots (200 $\mu$l) of a 2.5 $\times$ $10^6$ cells/ml adipocyte suspension ($\approx$500,000 cells/tube) were added to assay tubes containing standard buffer (295 $\mu$l) without and with various concentrations of palmitic acid, $CaCl_2$, EGTA, and other reagents as indicated. Whenever present, insulin concentration was 1.25 nM. After preincubation for 15 min at 37°C, measurement of uptake was initiated by addition of 25 $\mu$l[$^{14}$C-U]-D-glucose (0.24 $\mu$Ci) and terminated after a 15-min incubation by addition of ice-cold 2 mM $HgCl_2$ KRP buffer. The cells were filtered on Whatman GF/C filters and counted by liquid scintillation spectrometry (19).

Where quin2-AM and A23187 were used they were added at the beginning of the 15-min preincubation from stock solutions containing DMSO. The final concentration of DMSO never exceeded 1%. Controls contained identical concentrations of DMSO. Na pyruvate (10 mM) was included in buffers used for these experiments to protect against possible toxic effects of quin2-AM. This was necessary since hydrolysis of quin2-AM within cells produces toxic byproducts such as formaldehyde and causes depletion of cellular ATP (20). Pyruvate protects against these toxic effects by preventing depletion of intracellular ATP in adipocytes (16) and was therefore included in all buffer solutions. Dantrolene was added from stock solutions containing glycerol. Controls contained identical concentrations of glycerol, never exceeding 1%. In a few experiments, the oxidation of glucose was measured by the procedure of Gliemann (21). For these experiments, approximately 250,000 cells were pre-incubated for 15 min in dialyzed, fatty acid-free buffer or palmitate buffer prior to measurement of glucose oxidation over a 45-min period. The data are expessed as nmol of glucose oxidized/mg of cell protein per 45 min.

The assay for measuring glyceride-fatty acid and glyceride-glycerol was performed according to the procedure of May (22). Briefly, cells were incubated for 1 h, followed by an overnight lipid extraction using chloroform–methanol 1:1. The organic phase was separated and washed twice with KCl(7.4 mg/ml)–methanol 1:1. The chloroform was taken to dryness and the residue was saponified for 2 h at 60°C with 2 ml of saturated ethanolic KOH. The samples were cooled, acidified, and the fatty acids were extracted three times with heptane. Both the heptane and remaining ethanolic solution were taken to dryness and counted after the addition of Aquasol.

### Materials

Dantrolene and porcine insulin were generous gifts from Norwich Eaton Pharmaceuticals, Inc. (Norwich, NY) and Lilly Research Laboratories (Indianapolis, IN), respectively. A23187, quin2-AM, fatty acid-free human albumin, palmitic acid, and Celite were from Sigma (St. Louis, MO). [$^{14}$C]-D-glucose was obtained from ICN Biochemicals (Cleveland, OH).

## RESULTS

### Effect of palmitic acid and insulin on D-glucose incorporation

Palmitic acid (2.40 mM) increased both basal and insulin-stimulated glucose incorporation into rat adipocytes by approximately threefold (4.2 to 13.0 pmol/500,000 cells per 30 min) and twofold (16.1 to 28.9 pmol/500,000 cells per 30 min), respectively (**Fig. 1**). Insulin, at a physiologically saturating concentration of 1.25 nM, stimulated D-glucose incorporation in both control and palmitic acid buffer approximately fourfold (4.2 to 16.9 pmol/500,000 cells per 30 min) and twofold (13.0 to 28.9 pmol/500,000 cells per 30 min), respectively. The stimulatory effects of palmitic acid and insulin on D-glucose incorporation were roughly additive (Fig. 1). To identify whether the increase in glucose incorporation could be due to a decrease in glucose oxidation

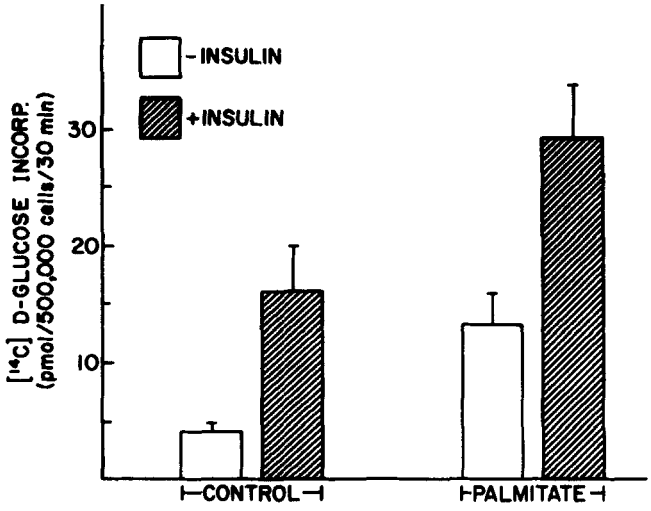


**Fig. 1.** Effect of insulin and palmitic acid on D-glucose incorporation. Adipocytes were preincubated with standard buffer (control) or palmitate buffer with (hatched bars) or without (open bars) 1.25 nM insulin. The data are means ± SEM from 11 separate experiments.

and thus a trapping of glucose, we assessed glucose oxidation at two palmitate concentrations (1.9 mM and 2.5 mM). We observed a twofold increase (169 ± 15 to 408 ± 41 nmol/ mg per 45 min) at 2.5 mM palmitate and a fourfold increase (141 ± 6 to 667 ± 19 nmol/mg per 45 min) at 1.9 mM palmitate. These increases were similar to those observed for glucose incorporation.

### Effect of extracellular calcium on palmitic acid-stimulated glucose incorporation

Extracellular calcium had no significant effect on the ability of palmitic acid to enhance glucose incorporation (**Fig. 2**). For these experiments, the calcium chelator EGTA (0.1 mM) was present in all assays and $CaCl_2$ was added to give the total calcium concentrations shown. At zero extracellular calcium (EGTA only), palmitic acid stimulated the D-glucose uptake approximately twofold.

### Effect of various palmitic acid concentrations on glucose incorporation with or without extracellular calcium

As shown in **Fig. 3**, D-glucose incorporation was directly proportional to the palmitic acid concentration within the range investigated (0.15–2.40 mM). Over this range of palmitate, there was no indication of saturation of the stimulatory effect. In these experiments, the presence of extracellular calcium at the physiological concentration of 2.6 mM caused a small increase in the glucose incorporation of similar magnitude at all palmitic acid concentrations.

### Effect of quin2-AM loading on palmitic acid-stimulated glucose incorporation

Quin2-AM enters the cell and is cleaved to form the trapped calcium chelator quin2. It is therefore valuable as a probe to explore the possible dependence of the palmitic acid effect on intracellular calcium. The stimulatory effect of palmitic acid on glucose incorporation by adipocytes decreased with increasing concentrations of quin2-AM (**Fig. 4**). The concentration of quin2-AM required for half-maximal inhibition ($IC_{50}$) of palmitic acid stimulated glucose incorporation was (mean ± SEM) 3.8 ± 1.2 μM (n = 4) and the maximum inhibition ($I_{max}$) at a saturating quin2-AM concentration was 89 ± 3% (n = 4) as calculated from a Dixon plot of the data (not shown) (23). Note in the insert, the small (≈4 pmol) decrease in basal glucose incorporation observed at the lowest concentration of quin2-AM. Although small, this effect was consistently observed.

We also analyzed the effect of quin2-AM (30 μM) on a specific aspect of glucose incorporation, namely the incorporation of D-[U-$^{14}$C]glucose into glyceride-fatty acids and glyceride-glycerol in the presence of 2 mM palmitate. In two separate experiments, quin2-AM inhibited the incorporation of D-[U-$^{14}$C]glucose into glyceride-fatty acids and glyceride-glycerol by an average of 54% and 48%, respectively.

### Restoration of palmitic acid-stimulated glucose incorporation with A23187 and $Ca^{2+}$

If the effect of quin2-AM was due to chelation of intracellular calcium, then restoration of intracellular calcium

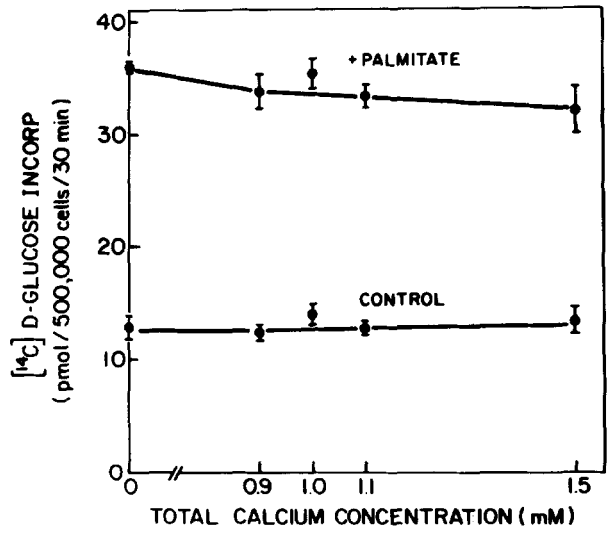


**Fig. 2.** Effect of extracellular calcium on palmitic acid-stimulated glucose incorporation. Adipocytes were preincubated with various concentrations of calcium. The calcium chelator EGTA (0.1 mM) was uniformly present throughout. Data are means ± SEM of triplicate determinations from a representative experiment.

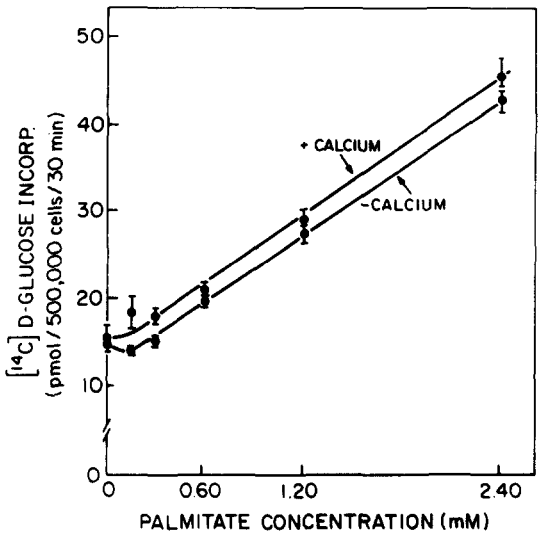


**Fig. 3.** Effect of various palmitic acid concentrations on glucose incorporation with or without extracellular calcium. Adipocytes were preincubated with various concentrations of palmitic acid with or without calcium as indicated. The data are means ± SEM of triplicate determinations from a representative experiment.

should also restore the ability of palmitic acid to enhance glucose incorporation in quin2-AM-loaded cells. This was assessed by first incubating adipocytes with quin2-AM at a submaximal concentration (10 μM) for inhibition of palmitic acid-stimulated glucose incorporation. Intracellular calcium was restored by incubating these cells with 2.6 mM $CaCl_2$ and various concentrations of the $Ca^{2+}$ ionophore, A23187. D-Glucose incorporation was monitored in the presence and absence of palmitic acid (2.40 mM).

Preincubation with quin2-AM and 0.001 μM A23187 decreased palmitic acid-stimulated glucose incorporation by 51.4% (**Fig. 5**). Addition of increasing concentrations of A23187 restored the ability of palmitic acid to stimulate D-glucose incorporation with complete restoration being achieved at 10 μM A23187 (111% of control) (Fig. 5). Although A23187 concentrations of 0.001, 0.01, and 0.1 μM significantly inhibited basal glucose incorporation, concentrations of 1.0 and 10 μM had no effect on basal activity (Fig. 5).

### Effect of dantrolene on palmitic acid-stimulated glucose incorporation

Dantrolene, a putative inhibitor of $Ca^{2+}$ release from the endoplasmic reticulum, was used to determine whether $Ca^{2+}$ release from the endoplasmic reticulum plays a role in the mechanism whereby palmitic acid stimulates glucose incorporation. Although 12.5 μM dantrolene caused a small (30%) enhancement of stimulation of glucose incorporation by palmitic acid, concentrations from 12.5 to 100 μM did not affect either palmitic acid-stimulated or basal glucose incorporation (**Fig. 6**).

## DISCUSSION

The effect of insulin on glucose transport and metabolism, and on FFA metabolism, in particular, in the adipocyte and the mechanism involved in the ability of insulin to regulate these functional parameters have been the subjects of intense investigation (15,16,21,24–29). In general, there is an overall reciprocal relationship between the oxidation of glucose and fatty acid substrates. With increased

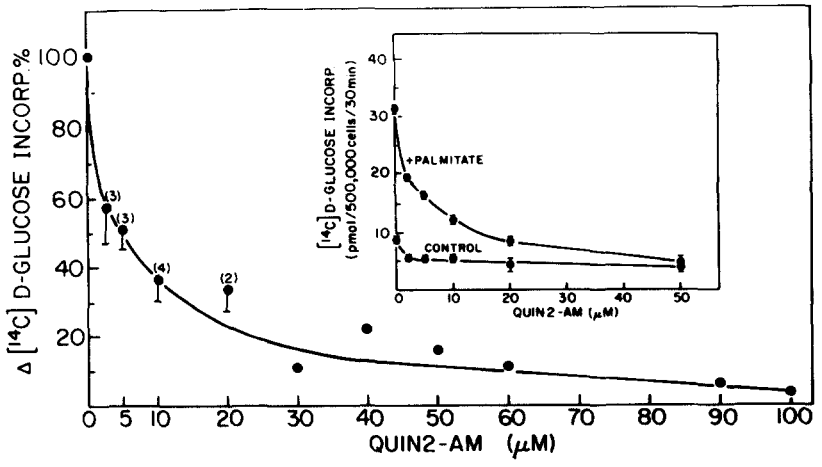


**Fig. 4.** Effect of quin2-AM loading on palmitic acid-stimulated D-glucose incorporation. Adipocytes were preincubated with various concentrations of quin2-AM with and without palmitic acid as indicated. Results are expressed as percentage of palmitic acid-stimulated D-glucose incorporation as a function of Quin2 AM concentration. Data points are means ± SEM from one to four separate experiments as indicated ($IC_{50}$ = 3.8 ± 1.2 μM). The insert shows the effect of Quin2-AM loading on palmitic acid-stimulated D-glucose incorporation expressed as pmol glucose incorporation from a representative experiment. Data are mean ± SEM of triplicates.

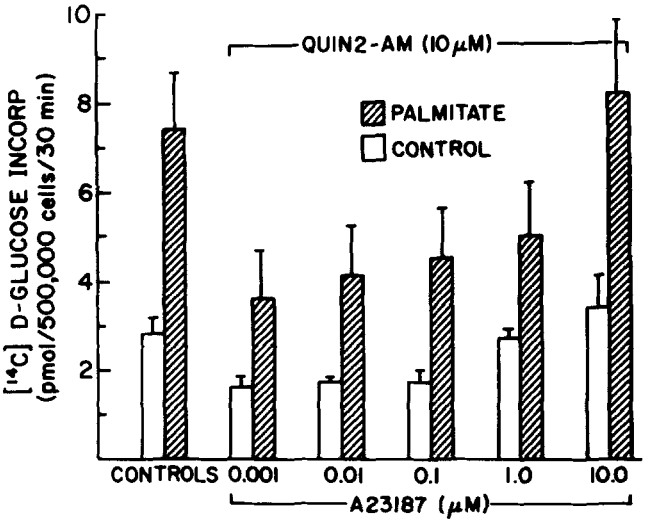


**Fig. 5.** Restoration of palmitic acid-stimulated glucose incorporation with A23187 and calcium. Adipocytes were preincubated in standard buffer (open bars) and palmitic acid buffer (hatched bars) with and without 10 μM quin2-AM as indicated on the graph. A23187 was added to the incubation medium at the concentrations indicated and D-glucose was assayed as described in Materials and Methods. The data are means ± SEM of three separate experiments.

availability of carbohydrate, glucose furnishes the major body fuels. In response to stimulation by insulin, lipolysis is minimal, lipogenesis is maximized, and tissue triglycerides accumulate. Conversely, when the supply of carbohydrates is limited as during starvation, there is an increased release of FFA mobilized from triglycerides (30). Insulin antagonizes the influence of catabolic hormones on FFA release from triglycerides (31, 32).

Most studies of glucose are actually studies of glucose transport and are performed at low glucose concentrations (33). However, as stated by Gliemann and Rees (34), this seems paradoxical from a physiological point of view considering that the plasma glucose concentration varies between 4 and 8 mM. In our study, the adipocytes were incubated in buffer containing glucose at a concentration of 2.8 mM, close to normal physiological concentration and sufficient to ensure measurement of glucose metabolism rather than transport. Saggerson (11) and Gliemann, Rees, and Foley (33) have shown that, in rat adipocytes under basal conditions in which glucose is at or near physiological levels, glucose is incorporated predominantly into glycerol and fatty acids. Insulin or palmitic acid increased the incorporation of glucose predominantly into glycerol with palmitic acid stimulation (11). Our observation that palmitic acid, like insulin, stimulates glucose incorporation into adipocytes is similar to the observations of Saggerson (11). The effect is similar to that described by several groups for catecholamines on glucose transport (19,35–37) and suggests that the effect of catecholamines may be secondary to FFA release. Palmitic acid also caused a two- to fourfold increase in the oxidation of glucose, indicating that the increases in glucose incorporation we observed were not due to an inhibition of glucose oxidation.

There have been discrepancies concerning the types of fatty acids that influence glucose transport. Some investigators have reported that unsaturated fatty acids are more effective than saturated fatty acids (38–40) and even that saturated fatty acids have no effect (39,40). Others have found that saturated fatty acids stimulate glucose transport (10,11). Likewise, the magnitude of the reported effects of fatty acids on glucose transport have been variable. We found the magnitude of the effect of palmitic acid on glucose incorporation similar to the effect of insulin under the same experimental conditions (threefold and fourfold, respectively). These observations are in contrast

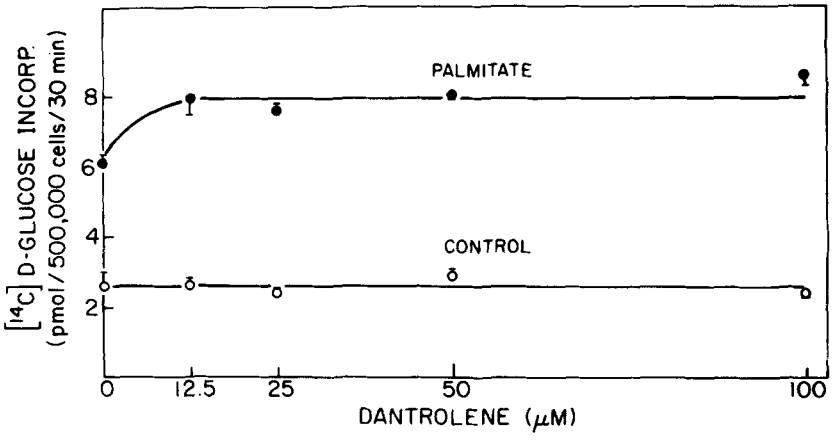


**Fig. 6.** Effect of Dantrolene on palmitic acid-stimulated glucose incorporation. Adipocytes were preincubated with (●) or without (○) palmitic acid with various concentrations of Dantrolene. D-Glucose incorporation was assayed as described in Materials and Methods. The data represents mean ± SEM of triplicate determinations from a representative experiment.

with the glucose transport experiments by Joost and Steinfelder (10) showing considerably smaller effects of palmitic acid than insulin on glucose transport. We believe this difference is explained by the difference in the metabolic parameter being monitored (glucose incorporation vs. glucose transport) and the fact that the highest concentration of palmitic acid in their study was 0.8 mM, compared with 2.40 mM in ours. We find the effect of palmitic acid on glucose incorporation to be proportional to the palmitic acid concentration with no indication of saturation even at 2.4 mM. Some of the discrepancies could be due to the method by which the fatty acids are added to the medium. The studies in which the fatty acids were added by procedures that removed or eliminated solvents (10,11), including the studies reported here, found an influence of saturated fatty acids. Further work will be required to clarify these methodologic differences.

Because FFA cause increased $^{45}Ca$ fluxes in rat calvaria (12,13), and since $Ca^{2+}$ appears to have a fundamental role in the stimulatory effect of insulin on glucose metabolism (15), we explored the possibility that $Ca^{2+}$ also was essential for the stimulation of glucose incorporation by palmitic acid. If the presence of palmitic acid leads to an elevation in intracellular $Ca^{2+}$, this elevation might be effectively eliminated in adipocytes loaded with quin2-AM. This is what we observed with quin2-AM giving an $IC_{50}$ of 3.8 $\mu$M which is 7 times lower than the $IC_{50}$ (26 $\mu$M) for quin2-AM inhibition of insulin-stimulated glucose transport and 3 times lower ($IC_{50}$ = 11 $\mu$M) for the corresponding effect of quin2-AM on glucose oxidation (16). Furthermore, the inhibitory effect of quin2-AM preincubation was fully restored by supplementing the buffer with the $Ca^{2+}$ ionophore A23187 and 2.6 mM $Ca^{2+}$, further suggesting a $Ca^{2+}$-dependent mechanism.

It is possible that quin-2 could be acting independently of calcium to inhibit palmitic acid-mediated glucose incorporation. This is difficult to directly assess; however, the reversibility of the effect by A23187 would argue against such an effect.

It is unlikely that the effect of palmitic acid is mediated through rapid influx of extracellular calcium since removal of extracellular calcium by chelation of EGTA failed to prevent the action of palmitic acid. It is also unlikely that calcium release from the endoplasmic reticulum is involved since dantrolene exerted no inhibitory effect on the ability of palmitic acid to stimulate glucose incorporation over the concentration range tested. Dantrolene has been reported to inhibit release of stored calcium from sarcoplasmic reticulum of skeletal muscle (41,42) and the endoplasmic reticulum of pancreatic islet cells (43).

Palmitic acid may increase intracellular calcium via inhibition of the $Ca^{2+}$ pumps in the plasma membrane as proposed for insulin (16,24) or by release from mitochondria. Alternatively, $Ca^{2+}$ may be permissive, being required along with palmitic acid, perhaps as the $Ca^{2+}$-palmitic acid complex, to increase glucose incorporation. In such a case a net increase in $Ca^{2+}$ in the cytoplasm need not be postulated. Elucidation of the exact mechanism will require further study.

Although our data indicate that quin2-AM inhibits the incorporation of D-[U-$^{14}C$]glucose into glyceride-fatty acids and glyceride-glycerol in the presence of palmitate, this does not necessarily indicate that the calcium-dependent enzymes involved in glyceride synthesis are being affected by quin2-AM. The rate-limiting calcium-dependent step could still be glucose transport. If quin 2-AM inhibits glucose transport then less glucose would be available for the metabolic enzymes, resulting in decreased incorporation of glucose into glyceride-fatty acids and glyceride-glycerol.

The stimulatory effect of palmitic acid on glucose incorporation may represent a regulatory mechanism in adipose tissue and provide an insulin-independent supply of glycerol phosphate required for the excess fatty acids released during stimulation of lipolysis (2,10). The stimulatory effect of palmitic acid on glucose incorporation may also play an important physiological role in diabetic ketoacidosis where levels of FFA as high as 2.3 mM have been reported (44). The FFA may therefore represent an insulin-independent regulatory mechanism for glucose incorporation, and play an important survival role to sustain intermediary glucose metabolism in the absence of endogenous insulin during ketoacidosis. The effects of palmitic acid on glucose metabolism in the other major insulin target tissues (liver and skeletal muscle) must be explored to test the importance of FFA in regulating glucose metabolism in the intact animal.

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