The Inhibition of Capacitative Calcium Entry Due to ATP Depletion but Not Due to Glucosamine Is Reversed by Staurosporine*

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The capacitative Ca\(^{2+}\) entry pathway in J774 macrophages is rapidly inhibited by the amino sugar glucosamine. This pathway is also inhibited by treatments such as 2-deoxy-D-glucose (2dGlc) or glucose deprivation that inhibit glycolysis and lead to significant decreases in cellular ATP and other trinucleotides. We sought to determine whether glucosamine's effect on capacitative Ca\(^{2+}\) entry was also due to ATP depletion, as has been suggested recently for its link to insulin resistance. In contrast to brief treatments with 2dGlc, there was no significant decrease in ATP following exposure to glucosamine. In addition, the 2dGlc-mediated inhibition of capacitative Ca\(^{2+}\) influx was reversed by staurosporine, a microbial alkaloid that inhibits a broad range of protein kinases. Staurosporine was also able to reverse the inhibition of capacitative Ca\(^{2+}\) entry seen following other treatments that decreased cellular ATP levels, including cytochalasin B and iodoacetic acid. Other inhibitors of protein kinase C, including bisindolylmaleimide, K252a, H-7, and calphostin C, were unable to mimic this effect of staurosporine. However, the inhibition of capacitative Ca\(^{2+}\) influx in the presence of glucosamine was not reversed by staurosporine. These data indicate that the inhibitory action on capacitative Ca\(^{2+}\) entry of glucosamine is distinct from that caused by ATP depletion.

The amino sugar glucosamine has been shown to have a variety of effects on cell and animal physiology. Numerous reports dating from over 40 years ago (1, 2) document that dietary glucosamine is selectively toxic to some experimentally induced tumors in rodents. In addition, Marshall et al. (3) determined that exogenous glucosamine induced insulin resistance in cultured adipocytes in a manner similar to that caused by hyperglycemia but at a 40-fold lower concentration than that required for glucose. They also showed that inhibition of glucose flux through the hexosamine biosynthetic pathway prevented hyperglycemia-induced insulin resistance from developing. These results have been extended to show that insulin resistance develops in animals infused with glucosamine (4, 5) or in cells (6) and animals (7) that overexpress the rate-limiting enzyme in the hexosamine biosynthetic pathway, glutamine:fructose-6-phosphate amidotransferase. Glucosamine treatment has also been shown to elicit the expression of transform-

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1 The abbreviations used are: 2dGlc, 2-deoxy-D-glucose; HBS, Hepes-buffered saline solution; GTP\(_{\gamma}\)S, guanosine 5’-3-O-(thio)triphosphate; Tg, thapsigargin; stp, staurosporine.
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EXPERIMENTAL PROCEDURES

**Cell Culture and Media—**J774 cells (American Type Culture Collection) were cultured at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) and 1% penicillin/streptomycin. Hepes-buffered saline solution (HBS) comprised 125 mM NaCl, 5 mM KCl, 1 mM MgSO\(_4\), 1 mM KH\(_2\)PO\(_4\), 10 mM NaHCO\(_3\), 1 mM CaCl\(_2\), 20 mM Hepes, pH 7.3.

**Measurement of Cytoplasmic Free \( \text{Ca}^{2+} \) ([Ca\(^{2+}\)]\(_i\)) with Fura-2 AM—**J774 cells were washed in serum-free Dulbecco's modified Eagle's medium and resuspended in Dulbecco's modified Eagle's medium containing 1 mg/ml bovine serum albumin and 2.5 mM probenecid. Probenecid was added to decrease the leakage of Fura-2 (42). After a 30-min incubation at 37 °C, the cells were centrifuged, washed, and resuspended in HBS. Fluorescence measurements were performed in a fluorescence spectrophotometer (Photon Technologies Inc.) with the cells incubated at 37 °C, the cells were centrifuged, washed, and resuspended in HBS. Fluorescence intensities were measured with excitation wavelengths of 340 and 380 nm.

**Inhibitors—**2dGlc was added to decrease the leakage of Fura-2 (42). After a 30-min incubation at 37 °C, the cells were centrifuged, washed, and resuspended in HBS. Fluorescence measurements were performed in a fluorescence spectrophotometer (Photon Technologies Inc.) with the cells suspended in a cuvette in a temperature-controlled chamber (37 °C) equipped with a magnetic stirrer. The fluorescence intensity was measured at 340 nm with excitation wavelengths of 340 and 380 nm. [Ca\(^{2+}\)] was calculated as described by Grynkiewicz et al. (43):  
\[
[	ext{Ca}^{2+}] = \frac{K_P \times (R - R_{\text{min}})/(R_{\text{max}} - R)}{S_{R_{\text{max}}} / S_{R_{\text{min}}}},
\]
where \(K_P\) is the Fura-2 dissociation constant for Ca\(^{2+}\) (224 nm), \(R\) is the ratio of the intensities at 340 nm and 380 nm, \(R_{\text{min}}\) and \(R_{\text{max}}\) are the \(R\) values at 0 and saturating levels of Ca\(^{2+}\), respectively. \(S_{R_{\text{max}}} / S_{R_{\text{min}}}\) is the ratio of the intensities at 380 nm excitation under \(R_{\text{max}}\) and \(R_{\text{min}}\) conditions. In presentations in which traces overlap, base-line values ranged between 70 and 120 nm with no significant differences being seen among the various conditions.

**Entry Assay—**J774 cells were washed in serum-free Dulbecco's modified Eagle's medium and resuspended in Dulbecco's modified Eagle's medium containing 1 mg/ml bovine serum albumin and 2.5 mM probenecid. Probenecid was added to decrease the leakage of Fura-2 (42). After a 30-min incubation at 37 °C, the cells were centrifuged, washed, and resuspended in HBS. Fluorescence measurements were performed in a fluorescence spectrophotometer (Photon Technologies Inc.) with the cells suspended in a cuvette in a temperature-controlled chamber (37 °C) equipped with a magnetic stirrer. The fluorescence intensity was measured at 340 nm with excitation wavelengths of 340 and 380 nm. [Ca\(^{2+}\)] was calculated as described by Grynkiewicz et al. (43):  
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where \(K_P\) is the Fura-2 dissociation constant for Ca\(^{2+}\) (224 nm), \(R\) is the ratio of the intensities at 340 nm and 380 nm, \(R_{\text{min}}\) and \(R_{\text{max}}\) are the \(R\) values at 0 and saturating levels of Ca\(^{2+}\), respectively. \(S_{R_{\text{max}}} / S_{R_{\text{min}}}\) is the ratio of the intensities at 380 nm excitation under \(R_{\text{max}}\) and \(R_{\text{min}}\) conditions. In presentations in which traces overlap, base-line values ranged between 70 and 120 nm with no significant differences being seen among the various conditions.

**Assay for Intracellular ATP Levels—**Cellular ATP levels were determined using an ATP assay kit (Calbiochem) based on firefly luciferase-catalyzed oxidation of d-luciferin. The emitted light was quantitated by luminometry. Cells (10⁶/ml) were incubated in HBS containing the indicated nutrients or inhibitors for 4 min at 37 °C. The cells were then centrifuged and the extracts were neutralized with 5 mM K₂CO₃. A 10-µl aliquot of the extract was added to 400 µl of HBS buffer, and the reaction was initiated by addition of the luciferase.

**RESULTS**

**Staurosporine Reverses the 2dGlc-mediated Inhibition of Capacitative \( \text{Ca}^{2+} \) Influx—**The addition of thapsigargin to Fura-2-loaded J774 cells leads to a sustained elevation of [Ca\(^{2+}\)]. This irreversible inhibitor of the endoplasmic reticulum Ca\(^{2+}\)-ATPase causes depletion of intracellular Ca\(^{2+}\) stores and thereby activates the capacitative influx of Ca\(^{2+}\) (15). The elevated plateau value of [Ca\(^{2+}\)], i.e. −100 nm higher than the starting base line in J774 macrophages under our standard assay conditions (Fig. 1A), reflects the new balance between Ca\(^{2+}\)-extruding mechanisms active at the plasma membrane and the ongoing capacitative influx of Ca\(^{2+}\).

As we reported previously (30), a 4-min pretreatment with 2dGlc inhibits the capacitative Ca\(^{2+}\) influx pathway in these cells (Fig. 1E). We speculated that local changes in ATP levels caused by 2dGlc could lead to a selective decrease in the activity of a protein kinase that was necessary for a sustained capacitative Ca\(^{2+}\) entry, thus altering the equilibrium between antagonistic kinases controlling this response. Staurosporine is a microbial alkaloid that was initially described as an inhibitor of protein kinase C but has since been shown to be a broad range inhibitor of protein kinase activity (34). Staurosporine augments capacitative Ca\(^{2+}\) influx in rat parotid acinar (36) and mesangial cells (38) and modulates Ca\(^{2+}\) responses in Jurkat T lymphocytes (39). Also, in Xenopus oocytes the \(t_\text{50}\) of inhibition of capacitative Ca\(^{2+}\) entry by GTP\(^-\)S was found to be increased by staurosporine (37). Most recently, a staurosporine-sensitive kinase was shown to be critical to the Ca\(^{2+}\)-dependent down-regulation of capacitative Ca\(^{2+}\) entry in human submandibular gland cells (35). The effect of staurosporine on capacitative Ca\(^{2+}\) influx in J774 macrophages was therefore tested both with and without 2dGlc pretreatment.

We found that a 5-min incubation with staurosporine, either prior to or after 2dGlc addition, resulted in approximately an 80% reversal of the inhibition seen in the presence of 2dGlc alone (Fig. 1, C and D). As can be seen by comparing Fig. 1, A with E, the initial peak height following the addition of thapsigargin, in addition to the capacitative plateau, is lower in the presence of 2dGlc. This is due not only to the inhibition of capacitative Ca\(^{2+}\) influx by 2dGlc, but to the ability of 2dGlc to partially deplete intracellular, thapsigargin-sensitive Ca\(^{2+}\) stores (30). Upon the addition of staurosporine, the 2dGlc-mediated inhibition of capacitative Ca\(^{2+}\) influx is relieved, although the release of Ca\(^{2+}\) from intracellular stores is not reversed (Fig. 1 and data not shown). Unlike the finding in rat parotid acinar cells (44), staurosporine did not affect the capacitative Ca\(^{2+}\) entry pathway in untreated J774 cells (Fig. 1B), even at concentrations of up to 150 nM. This suggests that the effect of staurosporine is not due to an inhibition of the plasma membrane Ca\(^{2+}\)-ATPase or the opening of a different Ca\(^{2+}\) channel, but rather to a reversal of the inhibition of capacitative Ca\(^{2+}\) entry seen with 2dGlc.

The inhibition of capacitative Ca\(^{2+}\) influx by 2dGlc can also be demonstrated by its addition after the pathway has been activated. In Fig. 2 Fura-2-loaded cells were treated with thapsigargin, and once a plateau value for [Ca\(^{2+}\)], was established, 2dGlc was added. Within ~1 min of its addition [Ca\(^{2+}\)] decreased. The subsequent addition of 40 nM staurosporine caused [Ca\(^{2+}\)] to return to the initial, higher plateau value. Following the recovery of Ca\(^{2+}\) influx by staurosporine, treatment with SKF 96365, an inhibitor of Ca\(^{2+}\) influx via the capacitative entry pathway (45), caused [Ca\(^{2+}\)], to return to base line. This, like the finding that staurosporine itself caused
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Effects of Staurosporine on Other Inhibitory Treatments That Deplete ATP—In order to determine whether the effect of staurosporine is limited to reversing only 2dGlc-mediated inhibition of capacitative Ca\(^{2+}\) entry, we tried alternate ways of inhibiting glycolysis. The addition of iodoacetic acid also caused a decrease in Ca\(^{2+}\) influx that was reversed by the subsequent addition of staurosporine (Fig. 3A). Addition of cytochalasin B, an inhibitor of glucose transport (46), also caused a decrease in Ca\(^{2+}\) influx similar to that seen upon the addition of 2dGlc (Fig. 3B). The subsequent addition of staurosporine restored the influx to normal. Dihydrocytochalasin B, which has a similar effect to cytochalasin B on the cytoskeleton but does not interfere with glucose transport (47), had no effect on capacitative Ca\(^{2+}\) influx (data not shown). This is in agreement with our previous results indicating that glucose deprivation inhibits the influx of Ca\(^{2+}\) via the capacitative entry pathway (30). Thus, it appears that staurosporine is able to reverse compromised capacitative Ca\(^{2+}\) entry that is accompanied by and presumably due to a decrease in cellular ATP levels.

An excess of glucose can overcome the 2dGlc-mediated inhibition in capacitative Ca\(^{2+}\) influx. As shown in Fig. 3C, an inhibition of capacitative Ca\(^{2+}\) entry can also be achieved by the addition of 5 mM 2dGlc to cells suspended in medium containing 1 mM glucose. Upon the addition of excess glucose (10 mM), the inhibition is reversed. As expected, the reversal in this case is accompanied by an increase in cellular ATP levels (data not shown).

Inhibition of Capacitative Ca\(^{2+}\) Entry by Glucosamine and Mannosamine—We next sought to determine the effects of a 4-min incubation with monosaccharides other than 2dGlc on the activation of capacitative Ca\(^{2+}\) influx by thapsigargin. As shown in Fig. 4, galactose, mannose, N-acetylglucosamine, and 3-O-methylglucose were without effect. However, glucosamine and mannosamine caused nearly complete inhibition in the ongoing elevation of Ca\(^{2+}\), indicating an absence of capacitative Ca\(^{2+}\) influx.

The inhibition in capacitative Ca\(^{2+}\) entry by these amino sugars can also be demonstrated in a Ca\(^{2+}\)-add-back experiment. Fura-2-loaded J774 cells were suspended in a nominally Ca\(^{2+}\)-free buffer. Glucosamine was added 5 min prior to the addition of thapsigargin. Following release from intracellular stores, Ca\(^{2+}\) was added to the medium so that the entry of Ca\(^{2+}\) via the capacitative Ca\(^{2+}\) pathway could be observed in isolation (Fig. 5). Glucosamine caused little change in the release of intracellular Ca\(^{2+}\) from stores by thapsigargin. However, the capacitative entry of Ca\(^{2+}\) was clearly inhibited.

We asked if staurosporine could reverse the inhibition of the capacitative Ca\(^{2+}\) influx seen in the presence of the amino sugars. As shown in Fig. 6, staurosporine was unable to reverse the inhibition in capacitative Ca\(^{2+}\) influx that is brought about by the action of glucosamine or mannosamine.

Intracellular ATP Levels Are Not Altered by Glucosamine or Staurosporine—Since glucosamine’s link to insulin resistance has been attributed to ATP depletion (11), we asked if, like 2Glc, short term glucosamine treatments sufficient to inhibit capacitative Ca\(^{2+}\) entry would lead to drops in cellular ATP levels. A 4-min treatment in the presence of the amino sugars did not decrease ATP levels to an extent that was statistically significant. This is in contrast to the much more substantial decreases obtained with 2dGlc (Fig. 7).

The effect of staurosporine on cellular ATP levels was also investigated. Staurosporine had no effect on ATP levels in the presence or absence of 2dGlc. Thus, staurosporine is not revers-
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**FIG. 5.** Inhibition of capacitative Ca$^{2+}$ entry by glucosamine in a Ca$^{2+}$ add-back protocol. Glucosamine (25 mM) was added 5 min prior to the addition of Tg in the nominal absence of extracellular Ca$^{2+}$. At the time indicated 1.5 mM extracellular Ca$^{2+}$ was added. Trace shown is representative of three replicate experiments.

**FIG. 6.** Effect of staurosporine on capacitative Ca$^{2+}$ influx in the presence of amino sugars. Fura-2-loaded cells were suspended in HBS containing 5 mM glucose and 5 mM pyruvate. Thapsigargin was added as indicated. The cells were pretreated in the following manner: 4 min in 25 mM glucosamine (A), 4 min in 25 mM glucosamine followed by 4 min in 40 mM stp (B), 4 min in 25 mM mannosamine (C), 4 min in mannosamine followed by 4 min in stp (D), and no addition (E). All conditions were assessed at least three times with comparable results.

**FIG. 7.** Intracellular ATP measurements. ATP content was assayed as described under “Experimental Procedures.” The cells were incubated in HBS containing 5 mM glucose and 5 mM pyruvate along with the indicated components. The concentrations used for the various additives are as follows. Glucosamine (GlcN) and mannosamine (ManN), 25 mM; stp, 40 mM; 2dGlc, 25 mM; iodosacetic acid (IAA), 1 mM; cytochalasin B (cytB), 10 mM. The data obtained under test conditions were compared with control using paired t tests. Means are from four determinations. **, $p > 0.05$; ***, $p < 0.001$.

**FIG. 8.** Effects of other inhibitors of protein kinase C on capacitative Ca$^{2+}$ entry. Fura-2-loaded cells were suspended in HBS containing 5 mM glucose and 5 mM pyruvate. Tg was added as indicated, and after a plateau value was reached, 25 mM 2dGlc was added. This was followed by the addition of 1 mM calphostin C (A), 500 nM H-7 (B), 1 mM K252a (C), and 200 mM bisindolylmaleimide (D). All kinase inhibitors were tested at least three times with comparable results.

...mimic the effect of staurosporine (Fig. 8). A dose response with staurosporine (Fig. 9) also revealed that its effect is not likely to be due to protein kinase C inhibition, since the IC$_{50}$ for protein kinase C inhibition has been reported to be ~5 mM (34), whereas a concentration of ~18 mM is half-maximal for the reversal of the inhibition of capacitative Ca$^{2+}$ influx in our experiments.

Other experiments were performed to determine whether activators of protein kinase C might cause a decrease in capacitative Ca$^{2+}$ entry similar to that seen with 2dGlc. Phorbol myristate acetate, an activator of protein kinase C, did not have any effects on the capacitative influx pathway in these cells in the presence or absence of 2dGlc. Phorbol myristate acetate has been shown previously to inhibit the Ca$^{2+}$ response to IgG in these cells (42). The finding that in our experiments phorbol myristate acetate was again able to suppress the response to IgG excludes the possibility that under the conditions/concentrations used the phorbol ester was ineffective in activating protein kinase C in our cells (data not shown).

**DISCUSSION**

We had shown previously an inhibition of capacitative Ca$^{2+}$ influx within 4 min of 2dGlc treatment or glucose deprivation in J774 macrophages (30). Here, we found that staurosporine, a microbial alkaloid that inhibits a broad range of protein kinases, is able to reverse the 2dGlc-mediated inhibition of the capacitative Ca$^{2+}$ influx pathway. The decreases in influx following other means of inhibiting glycolysis were also reversed by staurosporine. However, the identity of staurosporine’s target remains unclear, as protein kinase C does not appear to be the object of its action.

We also found that the amino sugars glucosamine and mannosamine inhibited capacitative Ca$^{2+}$ influx. The inhibitory action of these amino sugars appears to be distinct from that of 2dGlc. This conclusion is based in part on our observation that in the presence of either of these sugars there was no significant decrease in ATP levels. In addition, all treatments investigated led to an inhibition of the capacitative Ca$^{2+}$ pathway that was accompanied by a decrease in cellular ATP were reversed by the addition of staurosporine. In contrast, the inhibitory effects of glucosamine and mannosamine were not reversed by this kinase inhibitor.

The mechanism by which staurosporine restores the block in capacitative Ca$^{2+}$ entry caused by depletion of ATP is still undetermined. It is likely that protein kinases play regulatory
and possibly counter-balancing roles in controlling the magnitude of capacitative Ca\(^{2+}\) influx. It is possible that an activating kinase is preferentially inhibited when ATP levels fall, allowing an inhibitory kinase with a lower \(K_m\) for ATP to dominate. This could be responsible for the inhibition seen with 2dGlc and other glycolytic inhibitors. In such a model, staurosporine is proposed to selectively inhibit the proposed inhibitory kinase so as to allow the system to re-achieve a balance permissive for capacitative Ca\(^{2+}\) influx. A related possibility is that a staurosporine-sensitive inhibitory kinase with a lower \(K_m\) for ATP is kept in check by phosphorylation. When ATP falls, it becomes dephosphorylated and in turn phosphorylates and thereby inhibits an element important for capacitative Ca\(^{2+}\) entry.

In evaluating such models it should be noted that an activating kinase does not appear to be essential for the initiation of capacitative Ca\(^{2+}\) entry, at least in patch clamp experiments. When such experiments are carried out with no ATP in the pipette solution capacitative Ca\(^{2+}\) entry activates within minutes of whole cell break-in (33). The relationship between this mechanism of activation and the necessity for ATP in whole cell experiments observed both by us (30) and, for instance, Gamb caricci et al. (28) is still unclear. It should also be noted that a decrease in GTP cannot be experimentally dissociated from a decrease in ATP (33). Thus, the inhibitory effects observed with 2dGlc could be due to decreases in a trinucleotide other than ATP.

Previously, staurosporine had been shown in a variety of cell types to augment capacitative Ca\(^{2+}\) influx (36–39). In several of these experiments staurosporine's effect targeted a kinase other than protein kinase C, since other inhibitors of this enzyme were, as seen here, ineffective. The most parsimonious explanation for the data presented here is that staurosporine's effect is on a protein kinase that is capable of inactivating capacitative Ca\(^{2+}\) entry and that is relatively resistant to the initial decreases in intracellular ATP brought about by glycolytic inhibitors.

The assertion that glucosamine can affect biological processes via a mechanism independent of ATP depletion contrasts with arguments recently put forth by Hresko et al. (11). They reported that glucosamine treatment of 3T3-L1 adipocytes dramatically decreased cellular ATP and that subsequently this prevented normal levels of insulin-stimulated protein phosphorylation from occurring. Furthermore, they suggested that this was the mechanism responsible for the insulin resistance caused by glucosamine in these cells. They went on to suggest that many of the other biological effects of glucosamine were likely to be due to ATP depletion. However, in the data presented here and in a related study by Bounelis et al.\(^2\) we did not observe significant decreases in ATP in response to short term glucosamine treatments. The difference between our findings and those reported by Hresko et al. (11) would appear to be due to the provision of alternate fuels from which ATP can be generated. In our experiments 5 mM glucose and 5 mM pyruvate were present along with glucosamine. In the experiments reported by Hresko et al. (11) the cells were starved for glucose for several hours prior to the addition of glucosamine. This no doubt exacerbated ATP depletion and would appear to be a significant procedural difference relative to the experiments reported here. Under conditions in which glucose is present along with glucosamine, the data presented here and elsewhere\(^3\) suggest that a non-ATP-dependent inhibition of capacitative Ca\(^{2+}\) entry must be considered as a mechanism by which glucosamine affects cell physiology. In addition, a recent publication by Kim et al. (5) showed following glucosamine treatment no inhibition of insulin-stimulated phosphorylation of the insulin receptor or IRS-1. The authors concluded that the inhibition caused by glucosamine was at a step distal to these early phosphorylation events.

In a related study by Bounelis et al.,\(^2\) we have determined that short term glucosamine inhibits capacitative Ca\(^{2+}\) entry in Jurkat T lymphocytes, RBL-2 cells, and BHK-21 cells. In those experiments, the influx and metabolism of glucosamine was followed utilizing \(^3\)H-glucosamine. We observed an increase in intracellular levels of glucosamine, glucosamine-6-P, and UDP-GlcNAc. The most likely candidate for mediating the effect of extracellular glucosamine on capacitative Ca\(^{2+}\) entry is the initial intracellular metabolite of glucosamine, glucosamine 6-phosphate. Whole-cell patch clamp experiments in RBL-2 cells determined that glucosamine 6-phosphate, but not other intracellular metabolites of glucosamine, inhibited the trans-plasma membrane current \(I_{\text{CRAC}}\), the Ca\(^{2+}\) release activated Ca\(^{2+}\) current regulated by Ca\(^{2+}\) store depletion and responsible for capacitative Ca\(^{2+}\) entry in those cells. We propose that this metabolite is also responsible for the inhibition seen here in J774 cells.

Increased flux through the glucosamine pathway that could lead to the accumulation of intracellular glucosamine metabolites has been reported to be a response to hyperglycemia (49). In addition, such increases may occur in response to dietary glucosamine, which is currently being widely used as an alternative treatment for osteoarthritis (12). An inhibition in capacitative Ca\(^{2+}\) entry due to excessive hexosamine biosynthesis could affect a number of physiological processes, including several important to the capacity to combat infections and the onset of inflammation. For instance, increases in [Ca\(^{2+}\)] have been shown to accompany phagocytosis in macrophages (42) and to be necessary for phagosome/lysosome fusion in neutrophils (50). However, phagosome/lysosome fusion in macrophages appears to be a Ca\(^{2+}\)-independent event (51).

In addition, capacitative Ca\(^{2+}\) entry is important to certain aspects of gene regulation. The best described examples of this involve transcription mediated by members of the nuclear factor of activated T cells family. These transcription factors require capacitative Ca\(^{2+}\) entry in order to provide for the ongoing activation of calcineurin and the subsequent sustained nuclear localization of nuclear factor of activated T cells family

FIG. 9. Dose-dependent effect of staurosporine on capacitative Ca\(^{2+}\) influx. Tg was added to Fura-2-loaded cells suspended in HBS containing 5 mM glucose and 5 mM pyruvate, and a stable plateau of [Ca\(^{2+}\)] was achieved. At each of the staurosporine concentrations used, the new plateau value of Ca\(^{2+}\) was then measured following the addition of nothing further or stp alone (A); the latter is depicted as a percent of the former; 25 mM 2dGlc or 25 mM 2dGlc and then 4 nM staurosporine (B); 25 mM 2dGlc or 40 nM stp and then 2dGlc (C). In B and C the percent reversal of the 2dGlc-mediated inhibition seen as a result of staurosporine treatment is depicted. Each determination is the mean of three independent experiments.

2 P. Bounelis, Z. Su, E. A. Johnson, H. McFerrin, W. Bennett, J. E. Blalock, and R. B. Marchase, submitted for publication.
members (40). This transcription factor family is critical to production of a number of cytokines that greatly influence both the selection of peripheral T cell populations and the function of other cells more directly involved in combating infections. For instance, granulocyte-macrophage colony-stimulating factor is dependent upon a nuclear factor of activated T cells family member for its transcriptional control (40). Such alterations in cytokine environment could be expected to have effects on host defense against a variety of infectious agents.

Because glucosamine metabolites increase during hyperglycemia (49), the inhibition of capacitative Ca$^{2+}$ entry seen here may be relevant to the inability of diabetic individuals to effectively combat infections. For instance, Rayfield et al. (52) found a striking correlation between the prevalence of infection and mean plasma glucose levels in diabetic outpatients. Consistent with these data is a more recent report by Zerr et al. (48) in which infection rates increased with blood glucose in patients recovering from chest surgery. Those individuals with the highest blood glucose levels suffered from rates of infection more than 15 times those seen in a control population. Interestingly, implementation of a glucose control protocol led to a 40% drop in infection rate among the diabetic population.

Last, glucosamine is currently being widely used as an alternative treatment for osteoarthritis (12). The data presented here suggest that, rather than acting to enhance glycosaminoglycan synthesis, a change in cytokine profiles and subsequent decreases in inflammatory immune responses could underlie the putative effectiveness of such treatments.

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REFERENCES
1. Quastel, J. H., and Cantero, A. (1953) Nature 171, 252–254
2. Friedman, S. J., and Skehan, P. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1172–1176
3. Marshall, S., Bacote, V., and Traxinger, R. R. (1991) J. Biol. Chem. 266, 4769–4712
4. Rossetti, L., Hawkins, M., Chen, W., Gindi, J., and Barrilai, N. (1995) J. Clin. Invest. 96, 132–140
5. Kim, Y.-B., Zhu, J.-S., Zierath, J. R., Shen, H.-Q., Baron, A. D., and Kahn, B. B. (1999) Diabetes 48, 310–320
6. Crook, E. D., Daniels, M. C., Smith, T. M., and McClain, D. A. (1993) Diabetes 42, 1289–1296
7. Hebert, L. F., Jr., Daniels, M. C., Zhou, J., Crook, E. D., Turner, R. L., Simmons, S. T., Neidigh, J. L., Zhu, J. S., Baron, A. D., and McClain, D. A. (1996) J. Clin. Invest. 98, 930–936
8. Daniels, M. C., Kansal, P., Smith, T. M., Paterson, A. J., Kudlow, J. E., and McClain, D. A. (1993) Mol. Endocrinol. 7, 1041–1048
9. Kolm-Litty, V., Sauer, U., Nerlich, A., Lehmann, R., and Schleicher, E. D. (1998) J. Clin. Invest. 101, 160–169
10. Wang, J., Lin, R., Hawkins, M., Barrilai, N., and Rossetti, L. (1998) Nature 393, 684–688
11. Hresko, R. C., Heimberg, H., Chi, M.-Y., and Mueckler, M. (1999) J. Biol. Chem. 274, 20658–20668
12. Theodosakis, J., Adderly, B., and Fox, B. (1997) The Arthritis Cure, St. Mar-