1997

Coupling mechanisms between glucose metabolism and calcium signaling in J774 macrophages.

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COUPLING MECHANISMS BETWEEN GLUCOSE METABOLISM AND CALCIUM SIGNALING IN J774 MACROPHAGES

by

SUDHA DARBHA

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

1997

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSTIY OF ALABAMA AT BIRMINGHAM

Degree Ph.D. Program Cell Biology

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Committee Chair Richard B. Marchase

Title Coupling Mechanisms Between Glucose Metabolism and Calcium Signaling in J774 Macrophages

The primary objective of this dissertation was to investigate the link between glucose metabolism and Ca^{2+} signaling in J774 macrophages. It was found that the ability of these cells to generate Ca^{2+} signals in response to IgG, ATP, and platelet activating factor (PAF) was inhibited following 2-deoxy-D-glucose (2dGlc) treatment or glucose deprivation for 4 min. This inhibition in 2dGlc-treated or glucose-deprived cells is a consequence of compromised intracellular Ca^{2+} stores and decreased influx of Ca^{2+} across the plasma membrane (capacitative Ca^{2+} entry).

In the presence of 2dGlc, a partial release of intracellular Ca^{2+} stores was observed. Since the maintenance of intracellular Ca^{2+} stores involves optimal functioning of the endoplasmic reticulum (ER) Ca^{2+}-ATPase, a requirement for ATP is apparent. However, the mitochondrial inhibitor rotenone decreased cellular ATP levels to the same extent as glucose deprivation but did not cause any release of intracellular Ca^{2+}. This suggests that ATP is not the sole factor involved. Since import of glucose-6-phosphate (Glc-6-P) into the ER has been reported to have an enhancing effect on Ca^{2+} sequestration in liver microsomes, a parallel study between liver microsomes and J774 cells was

performed. The results from this study indicate that although Glc-6-P is imported into J774 microsomes, it does not enhance Ca^{2+} sequestration.

Glucose deprivation or 2dGlc treatment of J774 cells for 4 min also inhibited capacitative entry of Ca^{2+}. Staurosporine, a broad range protein kinase inhibitor, was able to reverse this inhibition. However, the effect of staurosporine was found to be unrelated to inhibition of PKC and PKA, the two serine-threonine kinases implicated in regulation of capacitative Ca^{2+} influx.

Capacitative Ca^{2+} entry was also inhibited in the presence of cytochalasin B iodoacetic acid, glucosamine and mannosamine. In cells treated with 2dGlc, or cytochalasin B, or iodoacetic acid, the decrease in capacitative Ca^{2+} influx was accompanied with lowered cellular ATP. The subsequent addition of staurosporine to these cells restored Ca^{2+} influx to normal. In contrast, the amino sugars inhibited Ca^{2+} influx without an appreciable decrease in cellular ATP. Furthermore, the amino sugar-mediated inhibition could not be reversed by staurosporine.

DEDICATION

I wish to dedicate this dissertation to my grandmother, Smt. M. Ravana, and to my late grandfather, Shri M. Rama Krishnamurty, for the innumerable ways in which they have touched my life with the warmth of their love.

ACKNOWLEDGMENTS

I wish to thank my advisor, Dr. Richard B. Marchase, for all his help. Challenges we faced together have greatly strengthened my belief in scientific reasoning and perseverance. Members of my dissertation committee have been most supportive: and I wish to express my appreciation to each one of them: Drs. Stephen Barnes, Gerald M. Fuller, Thomas H. Howard, and Anne Theibert. I would also like to acknowledge Sherry Crittenden, Diane Moxley, and Deborah Sewell for their administrative assistance.

I wish to thank all of my co-workers including Albert Tousson, Kathy Rostand, Peter Csutora, Peiyan Chen, William Bennett, and, especially, Nancy Veyna-Burke, Denise Auger, and George Thorp. With their friendship and helpful suggestions, Nancy, Denise and George have made my stay in the lab pleasurable and productive.

I would also like to express my appreciation to Balveen who continues to be a source of much strength and support. My parents, my brother, Sekhar, and my sister-in-law, Vardhany, have been very patient and understanding throughout the course of my dissertation work. I wish to thank them for their unwavering support in all the things I do. Finally, a special thanks to Narusu. With every step I take, his affection, encouragement, and friendship grow ever so undauntedly. Having him on my side gives me the strength to do everything in life with zeal and enthusiasm.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
$[Ca^{2+}]i$	Cytoplasmic calcium concentration
Ca^{2+}	Calcium
CIF	Calcium influx factor
DMEM	Dulbecco's modified eagles medium
2dGlc	2-Deoxy-D-glucose
ER	Endoplasmic reticulum
EGTA	Ethylene glycol-bis(b-aminoethyl ether) N, N, N', N'-tetraacetic acid
GFAT	Glutamine-fructose-6-phosphate aminotransferase
Glc	Glucose
Glc-6-P	Glucose-6-phosphate
cGMP	Guanosine 3'5'-cyclic monophosphate
GSD	Glycogen storage disease
GTP	Guanosine triphosphate
GlcNAc	N-acetyl-glucosamine
HBS	HEPES buffered saline
IgG	Immunoglobulin type G
IP_3	Inositol-1,4,5-trisphosphate

LIST OF ABBREVIATIONS (Continued)

MDCK	Madin-Darby canine kidney cells
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
p	Probability
PAF	Platelet Activating Factor
PBS	Phosphate buffered saline
P_i	Inorganic phosphate
PKC	Protein kinase C
PKA	Protein kinase A
Pyr	Pyruvate
SEM, S.E.M.	Standard error of the mean
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
SR	Sarcoplasmic reticulum
Stp	Staurosporine
UDP-Glc	Uridine diphosphoglucose
UTP	Uridine triphosphate

INTRODUCTION

Calcium Signaling

Changes in intracellular Ca^{2+} have been associated with a wide variety of cellular processes ranging from secretion and muscle contraction to gene expression and cell cycle regulation. This ubiquitous role of Ca^{2+} as a second messenger stems from its unique ability to bind tightly and selectively to proteins in a manner that induces large changes in conformation and activity. In contrast to other second messenger molecules which are regulated by chemical modifications, Ca^{2+} levels are regulated intracellularly by compartmentation. Thus, Ca^{2+} levels are altered by transporting Ca^{2+} between its different pools, namely, intracellular endoplasmic reticulum (ER) stores, extracellular media, and the cytoplasm. Mitochondria are also capable of sequestering Ca^{2+}. However, it has been shown that this pool does not participate in acute signaling events (Carafoli, 1982).

On the basis of Ca^{2+} regulatory processes, cells can be broadly classified into two types, excitable and non-excitable. In a typical non-excitable cell (Fig. 1A), Ca^{2+} signals are initiated when Ca^{2+} is released from intracellular, ER pools into the cytoplasm by inositol 1,4,5 trisphosphate (IP_3), which is generated following the activation of tyrosine kinase- or G protein-linked cell surface receptors (Berridge, 1993). These Ca^{2+} signals are maintained by the influx of extracellular Ca^{2+} through the plasma membrane, a process referred to as the capacitative entry of Ca^{2+}. After the signal has transpired, the cytoplasmic Ca^{2+} concentration is restored to a normal submicromolar value of

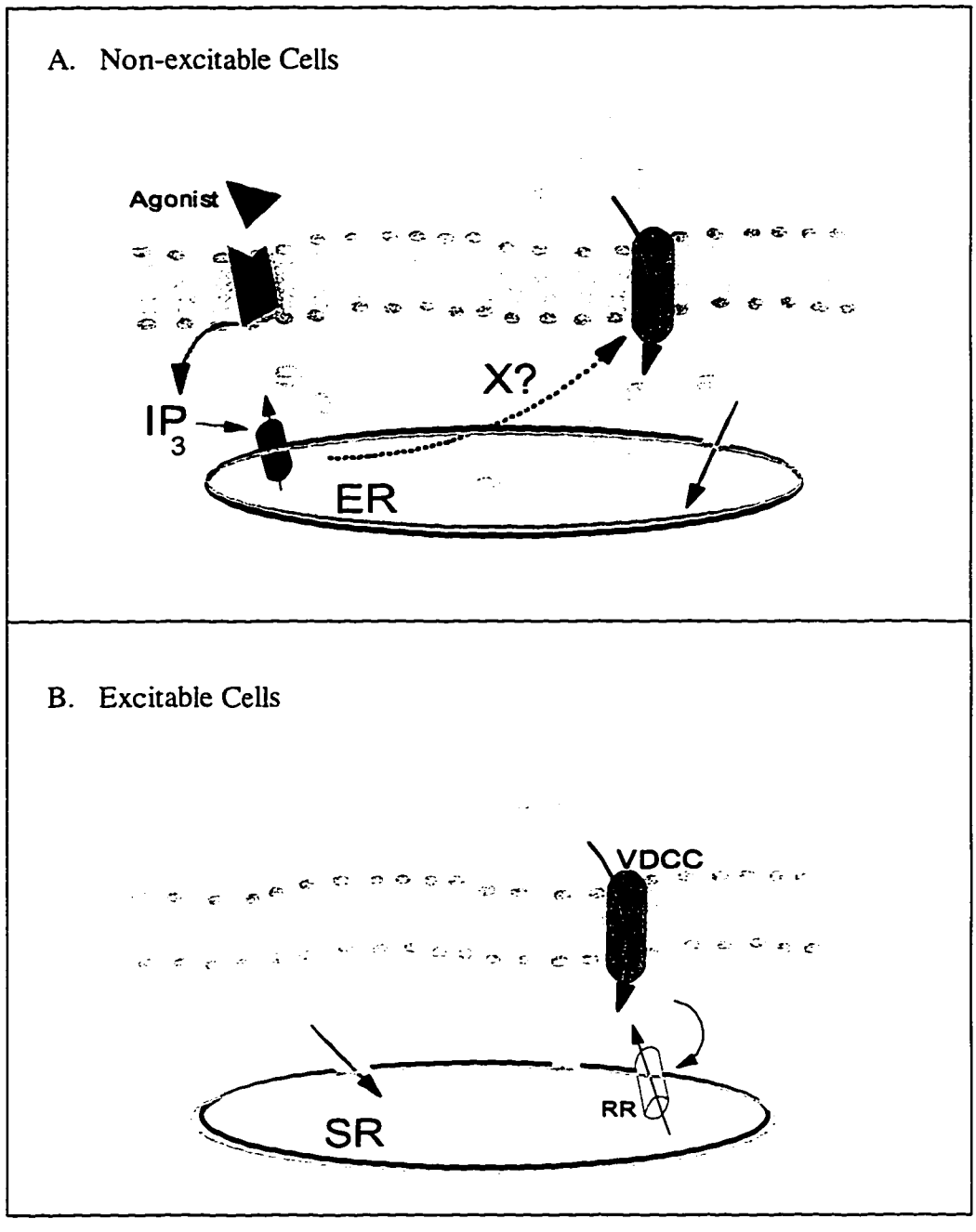

Fig. 1. Calcium signaling in non-excitable and excitable cells. denotes Ca^{2+} ions; X? denotes the, as yet, incompletely understood mechanism for the capacitative influx pathway; RR represents the ryanodine receptor; VDCC stands for voltage dependent Ca^{2+} channel.

approximately 100 nM. This is achieved by the extrusion of Ca^{2+} to the outside with the aid of the plasma membrane Ca^{2+}-ATPase and the Na^{+}-Ca^{2+} exchanger or by sequestration within the ER by the ER Ca^{2+}-ATPase. It should be noted that even in the absence of any stimulus, these ATPases are active. This is due to the fact that maintenance of cytoplasmic Ca^{2+} levels at ~100 nM in the presence of millimolar levels of Ca^{2+} outside the cell and within the ER requires the expenditure of energy.

Excitable cells have the additional capability of initiating a Ca^{2+} signal via voltage-sensitive plasma membrane Ca^{2+} channels. In this case, the Ca^{2+} signal is first initiated by the stimulus-induced influx of extracellular Ca^{2+} (Fig. 1B). The resulting increase in cytoplasmic Ca^{2+} then activates the ryanodine receptor present on the SR/ER, allowing Ca^{2+} to be released from these intracellular Ca^{2+} stores via a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (Clapham, 1995).

Interplay Between Glucose Metabolism and Ca^{2+} Signaling

With the involvement of both ER and plasma membrane Ca^{2+}-ATPases as well as other Ca^{2+} transporting proteins, it is apparent that the precise regulation of intracellular Ca^{2+} concentration is an energy-dependent process. It is, therefore, not surprising that in some cells, alteration of extracellular glucose levels has been found to lead to improper Ca^{2+} regulation. These include smooth muscle cells [Richard Paul, personal communication, 1995], and pancreatic β cells (Roe et al., 1994). In both these cases, a lowering of extracellular glucose is followed by an increase in cytoplasmic Ca^{2+} concentrations. Presumably, this occurred due to the inability of these cells to maintain their intracellular stores of Ca^{2+} in the absence of glycolytically generated ATP. In agreement with these results, an increase in cytoplasmic Ca^{2+} was seen upon the addition

of 2-deoxy-D-glucose (2dGlc) to pulmonary arterial smooth muscle cells (Bright et al., 1995) and rat aortic endothelium (Ziegelstein et al., 1994). However, it is interesting to note that in most of these cases the requirement for glucose could not be overcome by providing the cells with alternate substrates that maintained normal intracellular ATP levels.

A good model system in which to investigate further the link between glucose metabolism and intracellular Ca^{2+} regulation is comprised of the phagocytic cells, macrophages, and neutrophils. Although a direct correlation between extracellular glucose and Ca^{2+} homeostasis has not been established in these cells, the effects of the glucose analogue 2dGlc on phagocytosis has been very well characterized. This analogue of glucose is taken up efficiently by cells and phosphorylated to 2dGlc-6-P but is not metabolized further via the glycolytic pathway. When macrophages or neutrophils were incubated in media containing 2dGlc, the ability of these phagocytes to engulf IgG- or C3b-coated particles was found to be severely compromised (Michl et al., 1976; Boxer et al., 1976). Detailed investigations revealed that the inhibitory effect of 2dGlc could not be explained based on the lowering of cellular ATP levels. Providing alternate substrates such as pyruvate restored ATP levels, but not the phagocytic ability of these cells (Boxer et al., 1976). Compromised protein or glycoprotein synthesis also failed to account for the 2dGlc-mediated inhibition of receptor-mediated phagocytosis (Sung and Silverstein, 1985). Another interesting observation was that 2dGlc seemed to have no effect on the engulfment of non-opsonized particles that are presumably taken up via a non-specific pathway (Michl et al., 1976).

Although the exact role of Ca^{2+} in phagocytosis remains to be elucidated. the occurrence of Ca^{2+} signals during this process has been widely reported (Hishikawa et al., 1991; DiVirgilio et al., 1988). In view of this and the inhibitory effect of 2dGlc on receptor-mediated phagocytosis, we hypothesized that 2dGlc interferes with Ca^{2+} signaling in macrophages. This hypothesis was tested in J774 macrophages, a murine macrophage-like cell line that exhibits predominantly antibody dependent phagocytosis. The results from these investigations are described in the first section.

If the presence of glucose is essential for the normal regulation of cellular Ca^{2+} in these macrophages, the questions that become relevant are as follows: a) which of the Ca^{2+} regulatory processes are inhibited in glucose-deprived or 2dGlc-treated cells? and b) which metabolite of glucose is responsible for these effects? Based on the observations described in the first section, it was possible to isolate two different loci of inhibition in glucose-deprived or 2dGlc-treated cells, the ER Ca^{2+}-ATPase, and the capacitative Ca^{2+} entry pathway. In examining each of these processes, two possibilities were addressed. A specific metabolite of glucose might be involved. Alternatively, metabolism within the cell is compartmentalized such that the Ca^{2+} regulatory processes fueled by glycolytic ATP cannot be driven by ATP that is generated mitochondrially.

Dependence of the ER Ca^{2+}-ATPase on Extracellular Glucose

It has been suggested that glycolytically generated ATP, as opposed to mitochondrially generated ATP, is the preferred substrate for the SR/ER Ca^{2+}-ATPase. This has been proposed by Zhang and Paul (1994) in order to explain their findings regarding the role of glucose during excitation-contraction coupling in porcine carotid arteries. These investigators found that glucose is essential for normal agonist

(norepinephrine) responses and Ca^{2+} handling in these cells. Also, there was a non-specific sensitization to contractile stimuli under glucose-free conditions. They suggested that this was due to a decrease in the ability of SR Ca^{2+}-ATPase to pump Ca^{2+} into the SR efficiently. These effects were not due to any limitation in cellular ATP levels since the presence of β-hydroxybutyrate, an alternate substrate for oxidative metabolism and ATP generation, could not restore the activity to normal. They surmised that this is due to a membrane-associated compartmentation of ATP pools and that the SR Ca^{2+}-ATPase is dependent on the ATP produced locally by membrane-associated glycolytic enzymes (Fig. 2A).

The alternate explanation is based on previous reports indicating that glucose-6-phosphate (Glc-6-P) enhances the ATP dependent uptake of Ca^{2+} into microsomes from liver (Benedetti et al., 1985), kidney (Fulceri et al., 1990) and pancreatic β cells (Wolf et al., 1986). The authors attribute this to the presence of the enzyme glucose-6-phosphatase within the microsomes from these tissue. Glc-6-phosphatase is found most abundantly in liver where it catalyzes the terminal step of the gluconeogenic and glycogenolytic pathways [i.e., the hydrolysis of Glc-6-P to glucose and inorganic phosphate (P_i)] (Burchell and Waddell, 1991). It has been shown that this enzyme is present within the ER with its active site facing the lumen. This entails then the need for translocases that would transport the substrates and products of this enzyme across the ER membrane. Thus, three different transporters have been proposed to exist in order to transport Glc-6-P into and glucose and P_i out of the ER (Fig. 2B). Benedetti et al. suggested that the Glc-6-P mediated enhancement in Ca^{2+} sequestration is due to a non-ionic association of Ca^{2+} with the P_i that is generated following hydrolysis of Glc-6-P.

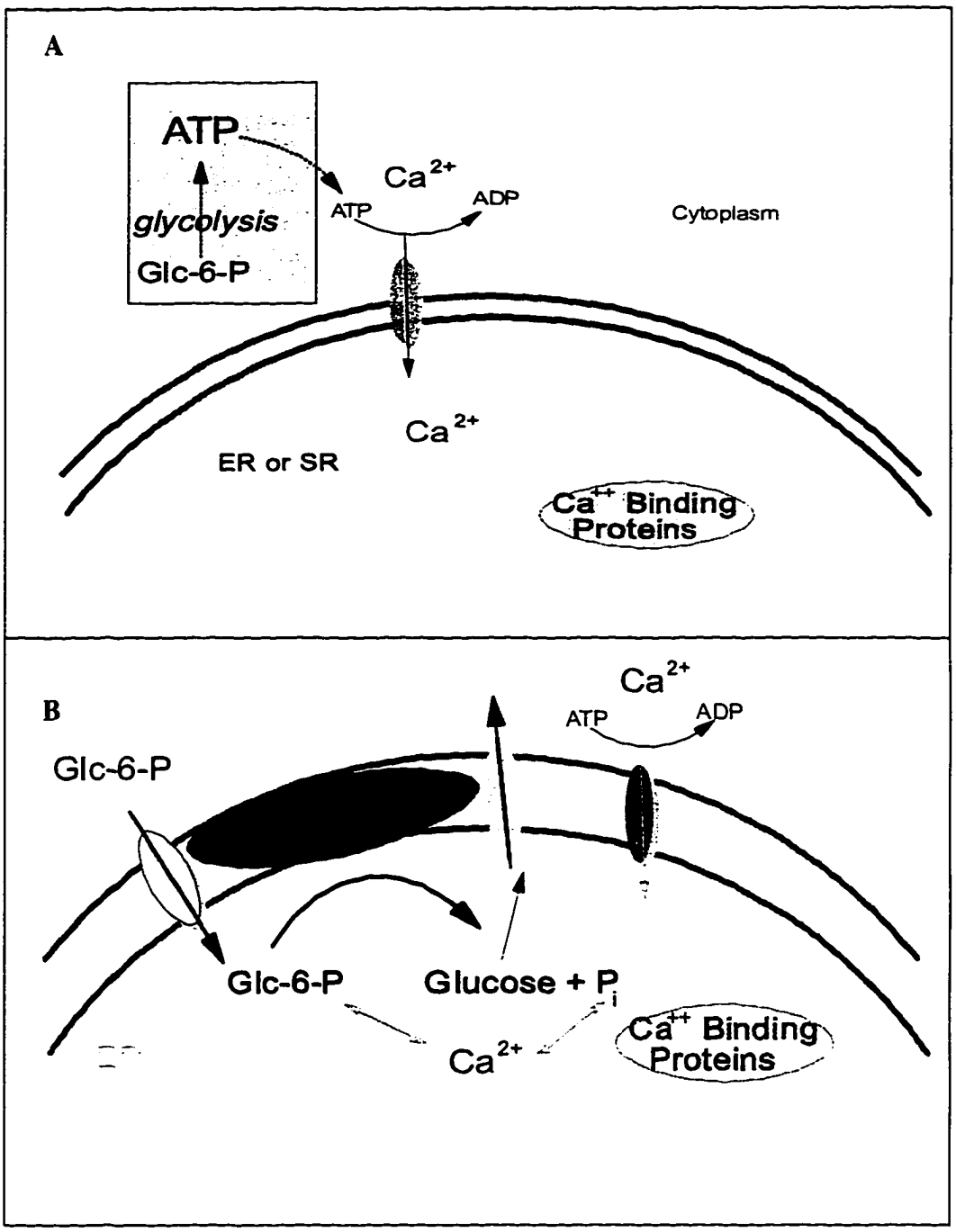

Fig. 2. Models for Glc-6-P mediated enhancement of Ca^{2+} sequestration in the ER. (A) Compartmentation hypothesis and (B) Glucose-6-phosphatase hypothesis.

Alternatively, results from our laboratory (Veyna-Burke, 1996) suggest that Glc-6-P can, itself, bind to Ca^{2+} within the lumen, thereby enhancing the sequestration of Ca^{2+}. In either case, the common step involved is the uptake of Glc-6-P into the ER.

The assumption that Glc-6-P import is a key factor in intracellular Ca^{2+} regulation is supported by some of the recent findings regarding patients suffering from glycogen storage disease type I (GSD I). GSD I is an inherited metabolic disorder that is characterized by a defect in the liver glucose-6-phosphatase. In general, patients suffering from GSD I are unable to break glycogen down, and part of their symptomology includes an enlarged liver and severe hypoglycemia (Burchell and Waddell, 1991). In GSD Ia, a subtype of GSD I, the phosphatase itself is defective, whereas in GSD Ib the function of Glc-6-P transporter is compromised. Surprisingly, the phagocytic cells of GSD Ia patients have been noted to be normal, whereas those from GSD Ib patients are defective (Gitzelmann and Bosshard, 1993). Part of the defect seems to lie in the ability of these phagocytes to mobilize Ca^{2+} normally (Kilpatrick et al., 1990; Korchak et al., 1993). It seems then that Glc-6-P import into the ER may be a determinant for the proper sequestration of Ca^{2+}. Although the presence of Glc-6-phosphatase has been demonstrated using staining methods in the macrophage ER, its physiological role or its biochemical activity has not been studied. Therefore, Glc-6-P import as well as the rate of hydrolysis of Glc-6-P was characterized using permeabilized J774 cells and J774 microsomes. Subsequently, the effect of Glc-6-P on Ca^{2+} uptake into the ER vesicles was examined. The results from these studies are discussed in the second section.

Regulation of Capacitative Ca^{2+} Influx by Glucose

As mentioned earlier, in macrophages and other non-excitable cells, Ca^{2+} influx across the plasma membrane is triggered following a release of Ca^{2+} from intracellular, IP_3-sensitive stores. The formation of IP_3 is not a prerequisite for this influx to be initiated. This is best demonstrated by the use of thapsigargin, a specific inhibitor of the ER-Ca^{2+} ATPase (Kwan et al., 1990). The inhibition of this Ca^{2+} pump is followed by a rapid leakage of Ca^{2+} from intracellular stores and a subsequent activation of the capacitative Ca^{2+} entry pathway (Fig. 3). Since the entry of Ca^{2+} appears to be regulated by the state of filling of intracellular stores, it has been referred to as the capacitative influx of Ca^{2+} (Putney, 1986). Recently, the plasma membrane channel that allows for such an influx of Ca^{2+} has been shown to be homologous to the drosophila trp/trpl proteins (Philipp et al., 1996) and coexpression of these Drosophila proteins reconstitutes capacitative influx in Xenopus oocytes (Gillo et al., 1996).

As described in the first section, the capacitative entry pathway in J774 macrophages is inhibited within 4 min of 2dGlc treatment or glucose deprivation. In agreement with these results, Lien et al. (1995) have shown that in MDCK cells the capacitative influx of Ca^{2+} can be enhanced by the addition of extracellular glucose. Efforts to elucidate the role of glucose in this pathway is further complicated by the fact that the precise mechanism of activation of capacitative Ca^{2+} influx is, as yet, unknown.

One of the models for capacitative Ca^{2+} influx suggests the presence of a diffusible messenger termed calcium influx factor (CIF) (Randriamampita and Tsien, 1993). CIF is thought to be released upon depletion of intracellular stores and diffused to the plasma membrane where it activates the capacitative entry channel (Fig. 3A). Preliminary efforts

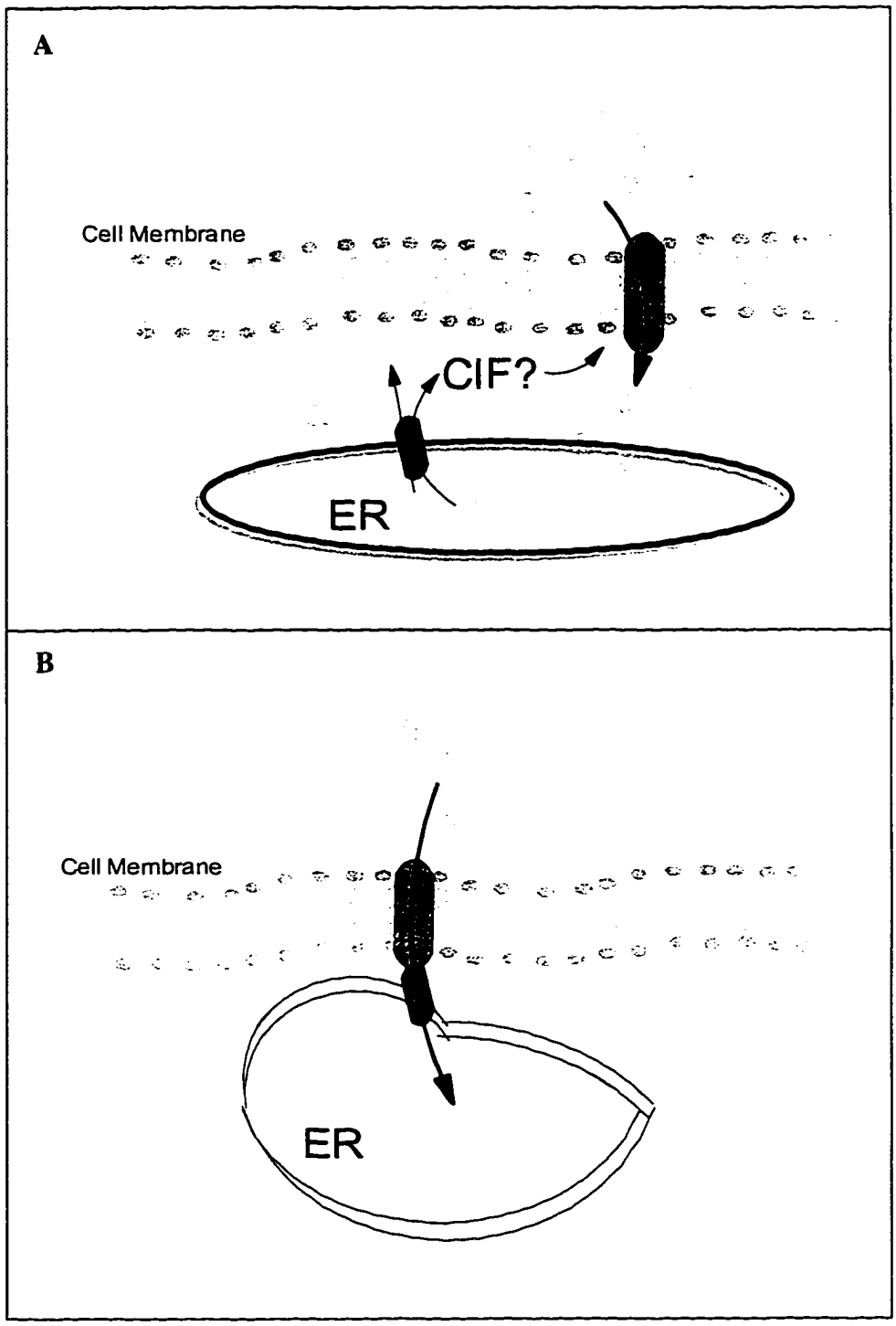

Fig. 3. Models for the mechanism of activation of capacitative Ca^{2+} entry. (A) Model based on the existence of a diffusible calcium influx factor, CIF. (B) Conformational coupling model involving the association of the IP_3 receptor with the plasma membrane.

to isolate and characterize CIF from crude extracts of Jurkat cells has indicated that this factor has a molecular weight of less than 500 daltons, contains a phosphate group that is essential for its activity, and has vicinal hydroxyls. It has been suggested that while some of the CIF is preformed and stored within intracellular compartments, de novo synthesis might also be involved (Randriamampita and Tsien, 1993).

Several second messengers and protein kinases have also been implicated in regulating the activation state of the capacitative influx pathway (Berridge, 1995) including cGMP (Bischof et al., 1995), tyrosine kinases (Sargeant et al., 1993), PKA (Chen et al., 1993), and PKC (Petersen and Berridge, 1994). Heterotrimeric G proteins (Jaconi et al., 1993) as well as small molecular weight G proteins (Fasolato et al., 1993) have been proposed to play regulatory roles in the capacitative entry pathway. Feedback regulation by increases in $[Ca^{2+}]_i$ has also been observed (Louzao et al., 1996). While it is possible that some of these kinases or phosphatases could be involved in the regulation of the putative second messenger (CIF), an alternate model has also been proposed. It has been suggested that the mechanism whereby the emptied ER stores can lead to activation of the capacitative Ca^{2+} channel involves a conformational change in the ER or its proteins upon the release of Ca^{2+} (Fig. 3B). This change in conformation may result in the juxtaposition of the IP_3 receptor with the plasma membrane thereby activating Ca^{2+} influx (Irvine, 1990; Putney, 1986). The kinases/phosphatases implicated in the regulation of Ca^{2+} influx could be affecting this conformational change in the ER.

In relevance to our observations regarding the inhibitory effects of 2dGlc on the capacitative Ca^{2+} entry, it has been reported that this process is very sensitive to cellular ATP levels (Gamberucci et al., 1994; Marriott and Mason, 1995). However, it is worth

noting that in J774 cells, although ATP levels following incubation with the mitochondrial inhibitor, rotenone, were similar to those seen under glucose-free conditions, rotenone had little effect on the capacitative influx of Ca^{2+}. It appears, therefore, that in these cells the capacitative influx of Ca^{2+} is exclusively dependent on the presence of glycolytic ATP.

Alternatively, some other metabolite of glucose or a cellular metabolite that is dependent on the presence of glycolytically generated ATP may be involved. The possibility that ATP itself is not the key molecule is indicated by observations in RBL-2H3 cells. Parekh and Penner (1995) have shown that Ca^{2+} influx, via the capacitative Ca^{2+} channel, was not inhibited in the absence of ATP. Furthermore, microinjection of 2 mM ATP in these cells led to an inactivation of this Ca^{2+} influx pathway. They suggest that this decrease in Ca^{2+} influx involves the activation of a protein kinase.

In an attempt to further investigate the role of glucose in capacitative Ca^{2+} entry, two lines of investigation were pursued. In the first, attempts were made to either mimic or reverse the 2dGlc block on capacitative influx using inhibitors/activators of protein kinases that have already been implicated in the regulation of this pathway. The assumption here was that 2dGlc is inhibiting capacitative influx at a specific signal transduction event. As a result of these investigations, it was found that staurosporine, a broad range serine-threonine kinase inhibitor, was able to reverse the 2dGlc-mediated inhibition of capacitative Ca^{2+} entry. This effect was further investigated and characterized as described in the third section.

The second approach was based on the use of alternate monosaccharides that might exert the same inhibitory effects as 2dGlc. If a metabolite other than glycolytic ATP is involved, then its identification may be made possible by studying the metabolism of

these alternate sugars. To this end, the effects of the amino sugars, glucosamine and mannosamine, were tested. The metabolism of glucosamine is depicted in Figure 4. Glucosamine is a naturally occurring amino monosaccharide. Biosynthesis of glucosamine-6-phosphate from fructose-6-phosphate is catalyzed by the enzyme glutamine-fructose-6-phosphate aminotransferase (GFAT). Glucosamine that is internalized from extracellular media is phosphorylated by hexokinase and, therefore, bypasses the GFAT step. Unlike 2dGlc, glucosamine does not inhibit glycolysis. However, prolonged exposure to glucosamine can lead to an accumulation of UDP-N-acetylglucosamine accompanied by depletion of both ATP and UTP pools (Plagemann and Erbe, 1973; Hawkins et al., 1997).

Addition of glucosamine to J774 macrophages led to decreased ability of these cells to activate capacitative Ca^{2+} influx as described in Chapter 3. In relevance to this, recently it has been reported that microinjection of CDP-Glc or UDP-Glc into Xenopus oocytes leads to a release of intracellular Ca^{2+} as well as activation of Ca^{2+} influx. Furthermore, in their efforts to isolate CIF, Kim et al. (1996) detected the presence of pyrimidine nucleotide-sugar derivatives in partially purified extracts from Jurkat lymphocytes. Thus, the interference with pyrimidine nucleotide-sugars by glucosamine might account for its inhibitory effects on capacitative Ca^{2+} entry.

Statement of the Problem

As discussed thus far, the primary aim of this dissertation was to investigate the role of glucose metabolism in the regulation of intracellular Ca^{2+} in J774 macrophages. In summary, the specific questions that were addressed are as follows: 1) which of the Ca^{2+} regulatory processes are altered in 2dGlc-treated or glucose-deprived J774 macrophages?

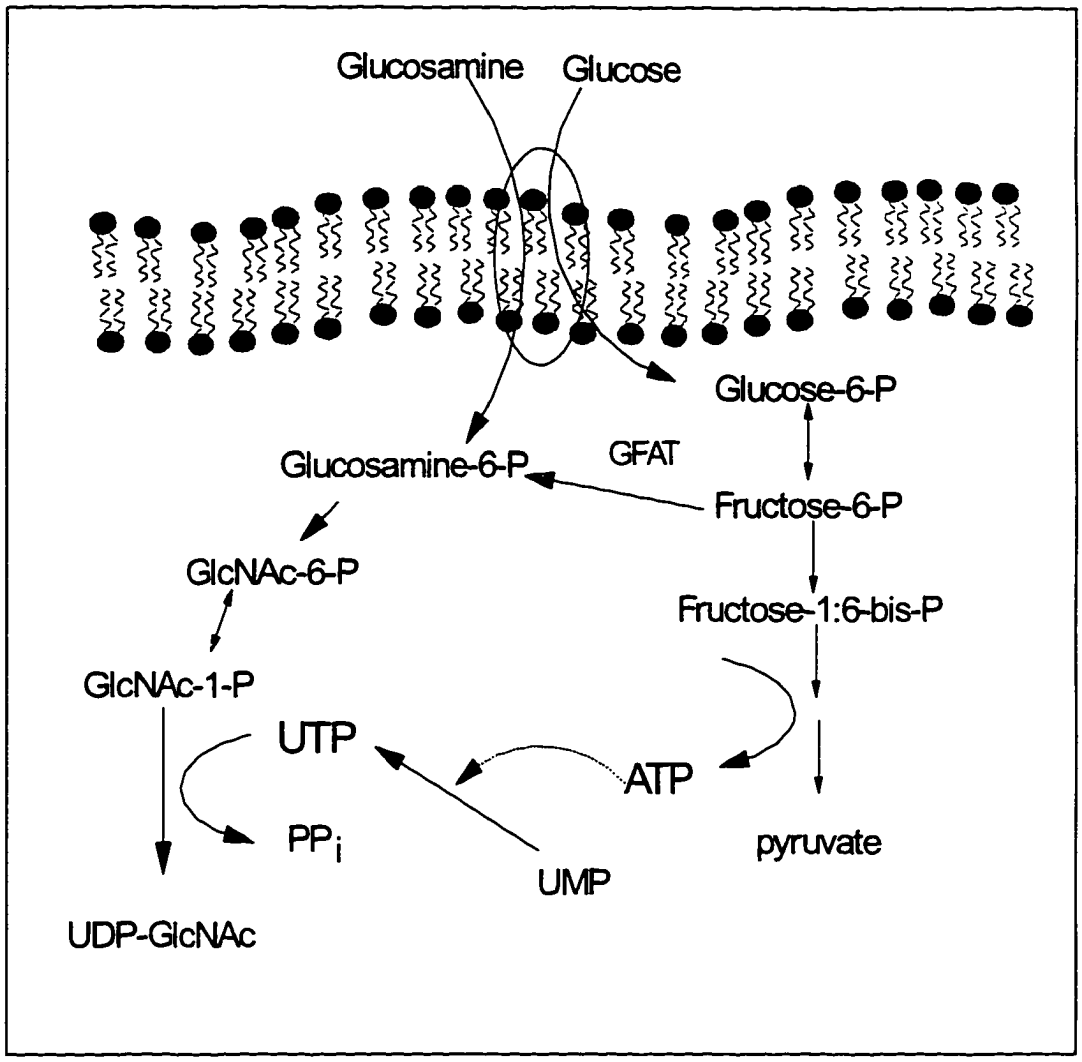

Fig. 4. Metaboiism of glucosamine. The main metabolic pathways involved in the utilization of extracellular glucosamine as well as the biosynthesis of glucosamine are shown.

2) Is Glc-6-P directly involved in the normal functioning of the ER-Ca^{2+} ATPase? and 3) Does the dependence of capacitative Ca^{2+} entry on extracellular glucose reflect a specific need for ATP in this process?

REGULATION OF INTRACELLULAR CALCIUM IS CLOSELY LINKED TO GLUCOSE METABOLISM IN J774 MACROPHAGES

by

S. DARBHA AND R. B. MARCHASE

Cell Calcium 20(4): 361-371

SUMMARY

The effects of 2-deoxy-D-glucose (2dGlc) and glucose deprivation were investigated in the J774 murine macrophage-like cell line. 2dGlc addition or glucose deprivation for 4 min led to an inhibition in the transient increase in cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) that otherwise occurs in response to three different agonists: IgG, ATP, and platelet activating factor. This inhibition was preceded by a partial release of Ca^{2+} from intracellular, thapsigargin-sensitive stores. In contrast, the transition from 5 to 30 mM glucose caused a decrease in $[Ca^{2+}]_i$ and a corresponding increase in thapsigargin-sensitive sequestered Ca^{2+}. The effects of an alternate glycolytic inhibitor, NaF, and a mitochondrial inhibitor, rotenone, were also tested. These inhibitors caused neither a release of Ca^{2+} from intracellular stores nor an inhibition in any of the agonist responses. The capacitative influx of extracellular Ca^{2+} following depletion of intracellular stores was also found to be selectively inhibited by the prior addition of 2dGlc or with glucose deprivation. In addition, when an elevated plateau of $[Ca^{2+}]_i$ was established by the irreversible depletion of intracellular Ca^{2+} stores, the addition of 2dGlc caused a decrease in the on-going capacitative entry of Ca^{2+}.

INTRODUCTION

The glucose analogue 2-deoxy-D-glucose (2dGlc) has been shown to inhibit receptor-mediated phagocytosis in both macrophages (Michl et al., 1976) and neutrophils (Boxer et al., 1976). In addition, T cell-mediated cytolysis (MacDonald and Cerottini, 1979) and antibody-dependent, eosinophil-mediated lysis of schistosomula (David et al., 1977) are also inhibited by 2dGlc. Since 2dGlc is efficiently internalized by glucose transporters and converted to 2dGlc-6-P by hexokinase (Sung and Silverstein, 1985) but

not metabolized to ATP, its inhibitory effects might initially be attributed to ATP depletion. However, closer examinations demonstrated that the inhibitory effects were not due to limitations in total cellular ATP levels (Michl et al., 1976; Boxer et al., 1976; MacDonald and Cerottini, 1979) nor to 2dGlc's inhibitory effect on glycoprotein synthesis (MacDonald and Cerottini, 1979; Sung and Silverstein, 1985).

Glucose itself has been shown to be necessary for several cellular responses that are mediated by the release of Ca^{2+} from intracellular stores. These include processes as diverse as smooth muscle contractility following exposure to norepinephrine or pressor hormones (Adams and Dillon, 1989; Zhang and Paul, 1994) sperm capacitation (Fraser and Herod, 1990), and neural transmission both *in vivo* (Lewis et al., 1974) and *in vitro* (Macaluso et al., 1992; Cox and Bachelard, 1982). The dependence of these processes on glucose was seen despite the use of alternate metabolic substrates that maintained total cellular ATP at normal levels. In addition, a direct correlation between the concentration of extracellular glucose and cytoplasmic Ca^{2+} levels in the absence of external stimuli has been demonstrated in pancreatic β cells (Roe et al., 1994), cultured kidney cells (Lien et al., 1995), and aortic smooth muscle [R. Paul, personal communication, 1995]. In these cells, a transition from low to high concentrations of glucose was accompanied by a decrease in the cytoplasmic free calcium concentration ($[Ca^{2+}]_i$). In contrast, the addition of 2dGlc has been shown to increase $[Ca^{2+}]_i$ in pulmonary arterial smooth muscle cells (Bright et al., 1995) and rat aortic endothelium (Ziegelstein et al., 1994).

Several of these authors (Zhang and Paul, 1994; Lien et al., 1995) have attributed their observations to a pool of ATP, generated by glycolysis, that cannot be replaced by mitochondrially-generated ATP. In particular, it has been proposed that this pool is

necessary to power the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases (SERCAs). These SERCAs are required to maintain the high levels of Ca^{2+} sequestered within the SR/ER, which in turn are necessary for agonist-mediated Ca^{2+} signaling. In the absence of glucose, this pool of ATP is suggested to be insufficient to maintain these Ca^{2+} stores, while an increase in extracellular glucose is proposed to result in a more efficient SERCA-mediated sequestration of intracellular Ca^{2+}.

Here, we report investigations of the effects of 2dGlc on the intracellular regulation of Ca^{2+} in J774 cells, a murine macrophage-like cell line that exhibits predominantly antibody-dependent phagocytosis (Hibbs et al., 1988). In these macrophages, activation of cell-surface Fc receptors causes a transient increase in cytoplasmic Ca^{2+} levels (DiVirgilio et al., 1988). As in most non-excitable cells, this Ca^{2+} signal, initiated by release of Ca^{2+} from intracellular stores, is followed by an entry of extracellular Ca^{2+} into the cell. This process, referred to as the capacitative influx of Ca^{2+} (Putney and Bird, 1993), reinforces the initial Ca^{2+} signal and also serves to refill the intracellular stores.

The data presented here indicate that a portion of the intracellular Ca^{2+} stores in J774 macrophages is dependent on the presence of extracellular glucose. We also show that the capacitative entry pathway in these cells is inhibited in the absence of extracellular glucose or the presence of 2dGlc. In addition, 2dGlc treatment or glucose deprivation causes a marked inhibition in the ability of these cells to generate Ca^{2+} signals in response to three different agonists: IgG, ATP, and platelet activating factor (PAF).

MATERIALS AND METHODS

Cell Culture. J774 cells (American Type Culture Collection) were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco-BRL, Gaithersburg, MD,USA) and 1% penicillin/streptomycin.

Buffers and Stimulants. HEPES based saline solution (HBS): 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 10 mM $NaHCO_3$, 5 mM pyruvate, 20 mM HEPES, pH 7.3. HBS-Ca^{2+}: HBS containing 1 mM $CaCl_2$. HBS-EGTA: HBS with 1 mM EGTA. Aggregated IgG was prepared by incubating human IgG (Sigma, St. Louis, MO, USA) at 63°C for 30 min. Ultra-centrifugation was performed 145,000 g for 1 h at 4°C. The resulting pellet was homogenized and resuspended in phosphate buffered saline (PBS), pH 8.0. 250 μg/ml of the IgG were used to stimulate the J774 cells. Mg-ATP (Sigma, St. Louis, MO, USA) was used at a final concentration of 10 μM. PAF (Sigma, St. Louis, MO, USA) was added to a final concentration of 10 nM (Prpic et al., 1988).

Measurement of Cytoplasmic Calcium With Fura-2/AM. J774 cells were washed in serum-free DMEM and resuspended in DMEM containing 1 mg/ml BSA and 2.5 mM probenecid. Probenecid was added to decrease the leakage of Fura-2 (DiVirgilio et al., 1988). In nearly all cases, parallel experiments were done in the absence of probenecid and comparable results were obtained. Fura-2/AM (Molecular Probes, Eugene, OR, USA) was added to a final concentration of 2 μM. 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 (Spex Industries 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 500 nm with excitation

wavelengths of 340 and 380 nm. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. (1985):

$$[Ca^{2+}]_i = K_d ¥ (R-R_{min})/(R_{max}-R) \times S_{f2}/S_{b2} \quad \text{Eq1}$$

where K_d is the Fura-2 dissociation constant for Ca^{2+} (224 nM), R is the ratio of the intensities at 340 nm and 380 nm, and R_{min} and R_{max} are the R values at 0 and saturating levels of Ca^{2+}, respectively. S_{f2}/S_{b2} is the ratio of the intensities at 380 nm excitation under R_{min} and R_{max} conditions.

Assays for Intracellular ATP Levels. Cellular ATP levels were determined using an ATP assay kit (Calbiochem, San Diego, CA, USA) based on firefly luciferase-catalyzed oxidation of d-luciferin. The emitted light was quantitated by luminometry (Analytical Luminescence Laboratory). Cells (10^5/ml) were incubated in HBS-Ca^{2+} containing the indicated nutrients or inhibitors for 4 min at 37°C. A 10 µl aliquot was then added to the kit's releasing agent, and the reaction was initiated by addition of the enzyme. ATP calibrations containing the sugars and inhibitors were assessed, and none interfered with the assay.

RESULTS

2dGlc or Glucose Deprivation Inhibits Agonist-Induced Increases in $[Ca^{2+}]_i$. Free cytoplasmic Ca^{2+} levels ($[Ca^{2+}]_i$) in J774 cells were monitored using the ratiometric Ca^{2+} indicator Fura-2 (Grynkiewicz et al., 1985). As reported previously, stimulating the F_c receptor with aggregated IgG (DiVirgilio et al., 1988) resulted in a transient increase in $[Ca^{2+}]_i$ (Fig. 1A), and subsequent stimulation of a purinergic receptor with 10 µM ATP (Greenberg et al., 1988) elicited an additional Ca^{2+} response. We then tested the ability of

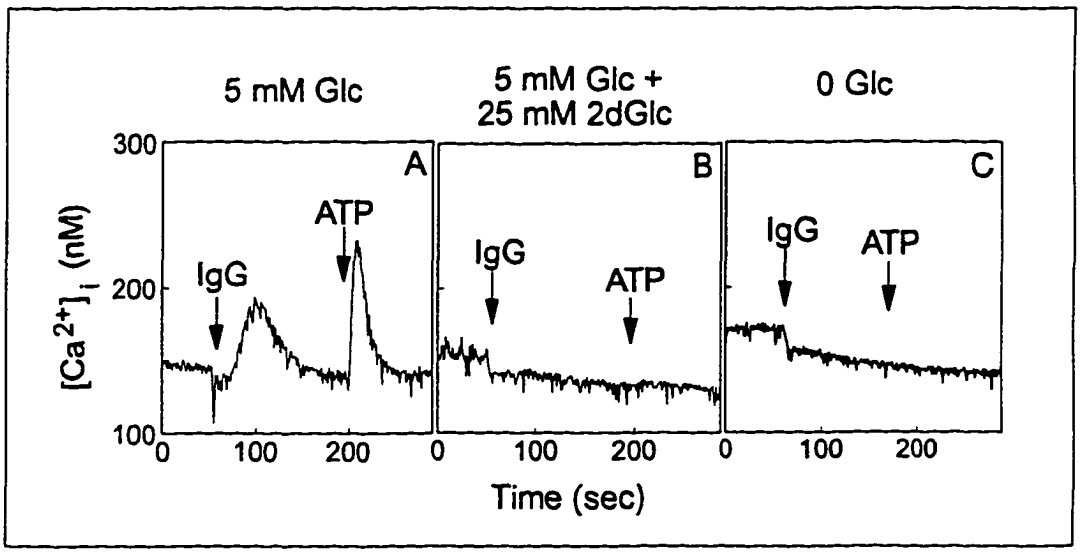

Fig. 1. Responses in cytoplasmic free Ca^{2+} to aggregated IgG and ATP in control, 2dGlc-treated, or glucose-deprived cells. J774 cells were loaded with 2 μM Fura-2/AM, washed, resuspended in HBS-Ca^{2+}, and subjected to a 3 min pre-incubation in HBS-Ca^{2+} containing (A) 5 mM Glc, (B) 5 mM Glc, 5 mM pyruvate and 25 mM 2dGlc and (C) 5 mM pyruvate. The cells were then stimulated sequentially with 250 μg/ml aggregated IgG and 10 μM ATP. Fluorescence intensity was measured at an emission wavelength of 500 nm with alternating excitation wavelengths of 340 and 380 nm. $[Ca^{2+}]_i$ was calculated as described in Materials and Methods. The results shown above are representative of three similar experiments (Glc=glucose).

these cells to respond to the same stimulants following a 4 min incubation in a buffer containing 25 mM 2dGlc in addition to 5 mM glucose and 5 mM pyruvate. As shown in Figure 1B, this brief 2dGlc treatment resulted in a complete failure of these cells to exhibit $[Ca^{2+}]_i$ signals in response to both IgG and ATP. The effect of incubating J774 cells for 4 min in a glucose-free buffer containing 5 mM pyruvate was also tested (Fig. 1C). This too led to a complete inhibition in the agonist-induced Ca^{2+} responses.

We then looked for changes in $[Ca^{2+}]_i$ levels that may be occurring in 2dGlc-treated cells prior to the addition of agonists. We found that the addition of 2dGlc itself resulted in an increase in $[Ca^{2+}]_i$. The subsequent effect on three different agonists was then tested. As shown in Figure 2, 2dGlc inhibited the response to IgG (100% inhibition in all cases), ATP (67.4 $\pm$ 5.8 % inhibition) and PAF (53.6 $\pm$ 8.9% inhibition). The degree of inhibition thus varied among agonists, with the response to IgG being the most sensitive. In each case, the 2dGlc-mediated inhibition was preceded by an increase in $[Ca^{2+}]_i$. In the examples shown in Figure 2, agonists were added approximately 3 min after exposure to 2dGlc. With longer intervals, $[Ca^{2+}]_i$ returned nearly to baseline, but the degree of inhibition was at least as great as that depicted (Fig. 1B and data not shown).

PAF has been reported to activate macrophages in a variety of different ways, including an enhancement in phagocytic index (Ichinose et al., 1994). In neutrophils, an increased expression of $F_{c\gamma 2}R$ has been observed within 5 min of incubation with PAF (Fukuchi et al., 1991). Therefore, the effects of sequential additions of PAF and IgG on $[Ca^{2+}]_i$ responses were examined. Under control conditions the response to IgG when added after PAF (Fig.3A) was comparable to that seen when IgG was added alone

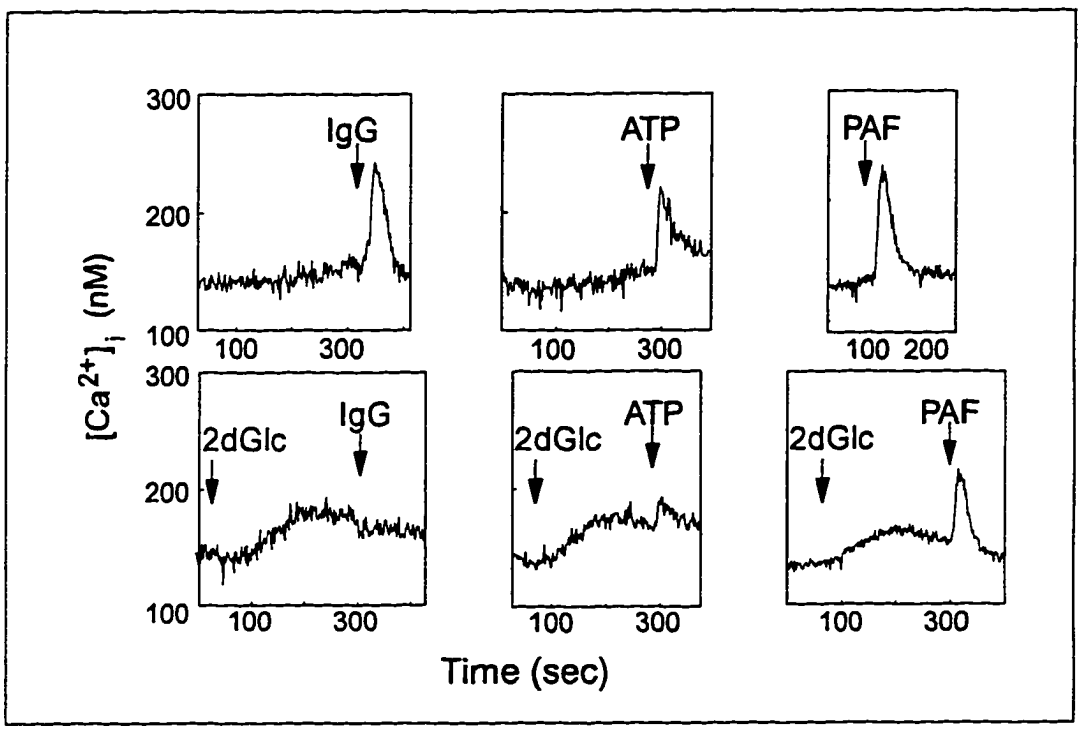

Fig. 2. 2dGlc-mediated inhibition of Ca^{2+} signals is preceded by an increase in $[Ca^{2+}]_i$. Fura-2/AM loaded J774 cells were suspended in HBS-Ca^{2+} containing 5 mM Glc and 5 mM pyruvate. The responses to IgG, ATP, and PAF are shown. The upper panels are control responses. In the lower panels, 25 mM 2dGlc was added as indicated, followed by the corresponding agonist. Here, unlike the data shown in Figure 1, the ATP response in the presence of 2dGlc is not completely abolished. This is due to the differences in the times the cells were suspended in 2dGlc prior to addition of ATP. The tracings shown are representative of at least three experiments.

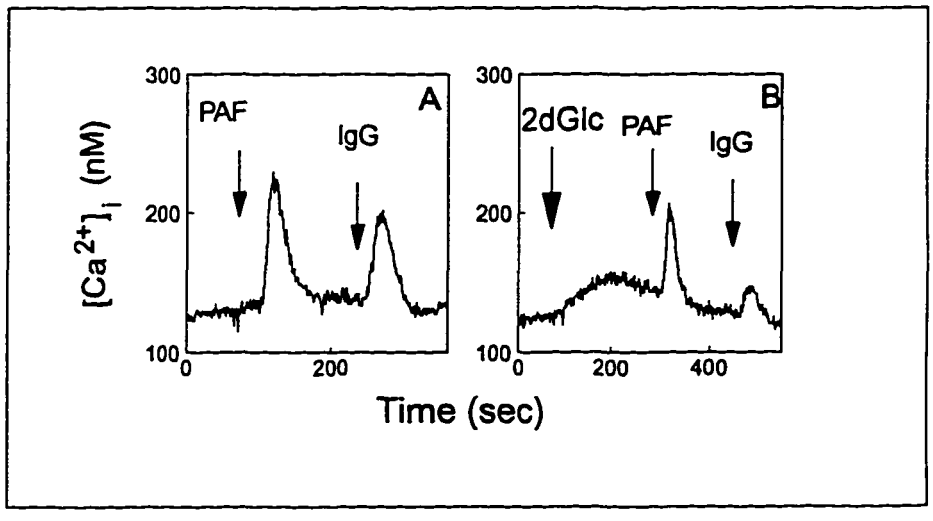

Fig. 3. PAF partially reverses the inhibition of IgG-induced Ca^{2+} signal in 2dGlc-treated cells. Fura-2/AM loaded cells were suspended in HBS-Ca^{2+} containing 5 mM Glc and 5 mM pyruvate. The sequential additions of PAF and IgG are shown. (A) control, (B) 25 mM 2dGlc was added as indicated. The data shown are representative of three replicate experiments.

Fig. 2A). However, in 2dGlc-treated cells, the addition of PAF prior to IgG partially restored the IgG response (Fig. 3B compared to Fig. 2). Thus, not only was PAF more resistant to 2dGlc's inhibitory effect, but it also enhanced the response to IgG following exposure to the metabolic inhibitor. Further studies are required to test which, if any, of the reported effects of PAF is related to its influence on the generation of IgG-induced Ca^{2+} signals.

The effects of other metabolic inhibitors and glucose removal on agonist response were then examined. Unlike the addition of 2dGlc, the addition of a mitochondrial inhibitor, rotenone, or an alternate glycolytic inhibitor, NaF, did not lead to an increase in $[Ca^{2+}]_i$. Also, these inhibitors failed to cause an inhibition in the responses to IgG and PAF (Fig. 4). The effect of glucose deprivation, however, closely paralleled the inhibitory effect of 2dGlc, as was expected from the result shown in Figure 1. Also, the baseline $[Ca^{2+}]_i$ in glucose-free media was slightly elevated. This indicates that removal of glucose causes an increase in $[Ca^{2+}]_i$, which is similar to the effect seen upon addition of 2dGlc. It is also apparent that a partial response to PAF can be observed even though the response to IgG has been completely inhibited (Fig. 4D,E).

We also tested the effect of these metabolic inhibitors on cellular ATP levels. As shown in Table 1, 2dGlc treatment caused a more pronounced decrease in cellular ATP levels than glucose deprivation, rotenone, or NaF. The effects of NaF were most variable, perhaps due to the pleiotropic nature of its action (Tojyo et al., 1991). However, the decrease in cellular ATP levels upon treatments with 2dGlc, rotenone, or upon glucose deprivation do not appear to reflect the effects these have on generation of Ca^{2+} signals. Rotenone caused drops in total cellular ATP levels that were comparable to those seen in

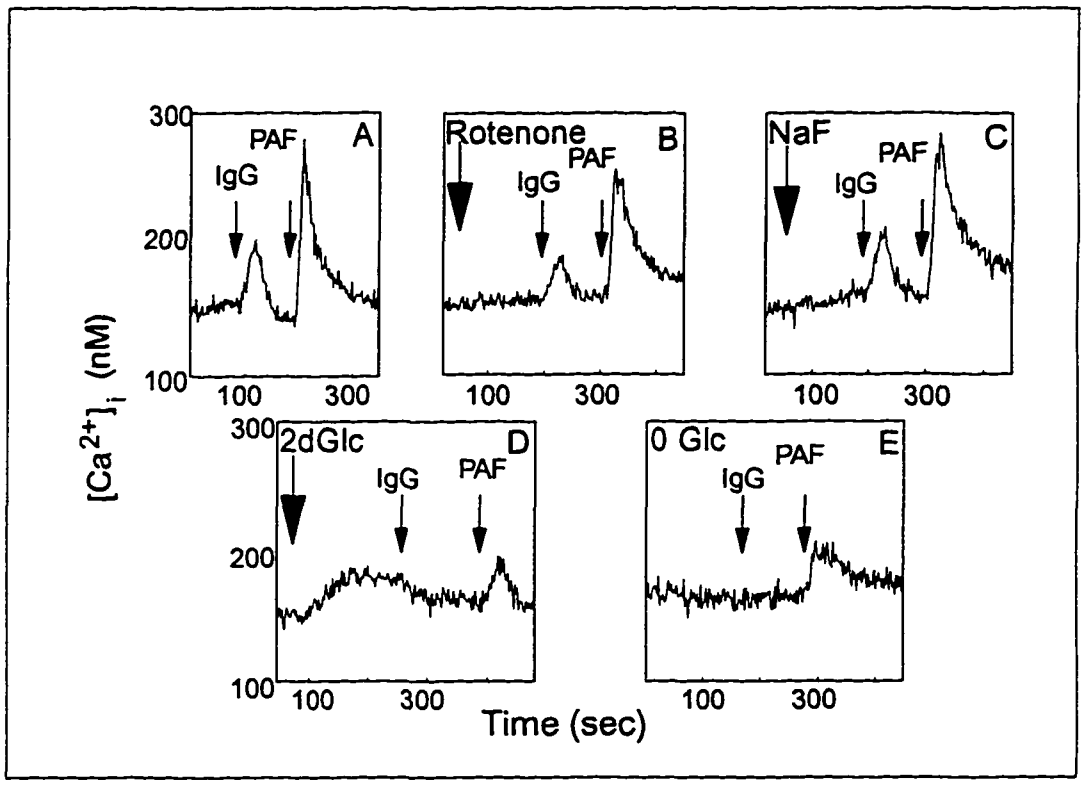

Fig. 4. Agonist-induced Ca^{2+} signals in the presence of various metabolic inhibitors. Fura-2/AM loaded cells were suspended in HBS-Ca^{2+} containing 5 mM Glc and 5 mM pyruvate {except in (E)}. Responses to IgG and PAF are shown following the addition of: (A) control, no addition, (B) 4 μM rotenone, (C) 1 mM NaF, (D) 25 mM 2dGlc. In (E), cells were suspended in Glc-free HBS-Ca^{2+} containing 5 mM pyruvate just prior to starting the experiment.

Table 1. ATP Content of J774 Cells 4 Min After Addition of Nutrients/Inhibitors

NUTRIENT/INHIBITOR	ATP CONTENT (% +/- s.e.m.)
5 mM Glc + 5 mM Pyr	100
5 mM Glc	98.1 +/- 5.6*
5 mM Pyr	81.4 +/- 3.9**
25 mM 2dGlc + 5 mM Glc + 5 mM Pyr	61.4 +/- 3.3**
1 mM NaF + 5 mM Glc + 5 mM Pyr	84.4 +/- 8.0**
4 μM Rot + 5 mM Glc + 5 mM Pyr	78.5 +/- 4.0**
30 mM Glc + 5 mM Pyr	93.8 +/- 4.1*

[Glc: glucose, Pyr: pyruvate, Rot: rotenone]

Cellular ATP levels were determined using an ATP assay kit (Calbiochem) based on firefly luciferase-catalyzed oxidation of d-luciferin as described in Materials and Methods. ATP calibrations containing the sugars and inhibitors were assessed, and none interfered with the assay. 100% ATP level corresponds to 486 +/- 78.6 pmols/10^5 cells. SEM= Standard Error of the Mean. The data obtained under test conditions were compared to control (5 mM Glc + 5 mM Pyr) using the paired *t* test. * Corresponds to $p > 0.1$. ** These results were significantly different from control, $p < 0.02$.

the absence of glucose. Yet, the effect of rotenone on the agonist responses was not as profound as that seen with glucose removal. Also, 2dGlc treatment and glucose deprivation had very different effects on ATP levels, but their inhibitory effects on intracellular Ca^{2+} responses were comparable. Thus, the inhibition in agonist responses upon 2dGlc treatment or glucose deprivation is likely to be unrelated to depletion of total cellular ATP levels.

2dGlc Results in a Partial Release of Ca^{2+} From the ER While the Addition of Glucose Enhances Ca^{2+} Sequestration. The experiments shown in Figures 1-4 were performed in the presence of extracellular Ca^{2+}. To investigate more closely the source of the 2dGlc-mediated increase in $[Ca^{2+}]_i$, Fura-2 loaded cells were suspended in a Ca^{2+}-free buffer. In these experiments thapsigargin, a selective inhibitor of the SERCAs (Kwan et al., 1990), was used as a measure of total ER Ca^{2+} pools. The addition of thapsigargin to these cells allows detection of a rapid and complete leakage of Ca^{2+} from the ER store that is normally balanced by the action of the SERCAs. This release can be monitored as an increase in $[Ca^{2+}]_i$ as shown in Figure 5A. The addition of 2dGlc after thapsigargin did not result in any change in $[Ca^{2+}]_i$. In Figure 5B, 2dGlc was added prior to thapsigargin. In this case, addition of 2dGlc caused an increase in $[Ca^{2+}]_i$ (peak increase of 21.1 ± 1.4 nM) indicating that it is releasing Ca^{2+} from intracellular stores. Further, a diminished response in $[Ca^{2+}]_i$ to thapsigargin (integrated area = 57.1 ± 6.1% of control; compare Fig. 5B to Fig. 5A) was seen when the inhibitor was added after 2dGlc. Taken together, these results demonstrate that 2dGlc releases Ca^{2+} from an intracellular, thapsigargin-sensitive store. When incubation times with 2dGlc were extended, no additional decreases in thapsigargin-sensitive stores was observed (data not shown).

These results do not appear to be due to the osmotic effects of 2dGlc addition since the removal of glucose also caused an increase in $[Ca^{2+}]_i$ (Fig. 4E). However, two experiments were carried out to further substantiate this. In the first, the concentration of2dGlc was decreased to 5 mM. This concentration of 2dGlc was sufficient to evoke a release of Ca^{2+} from thapsigargin-sensitive stores when the ambient glucose concentration was reduced to 1 mM (data not shown). This suggests that the ratio between glucose and

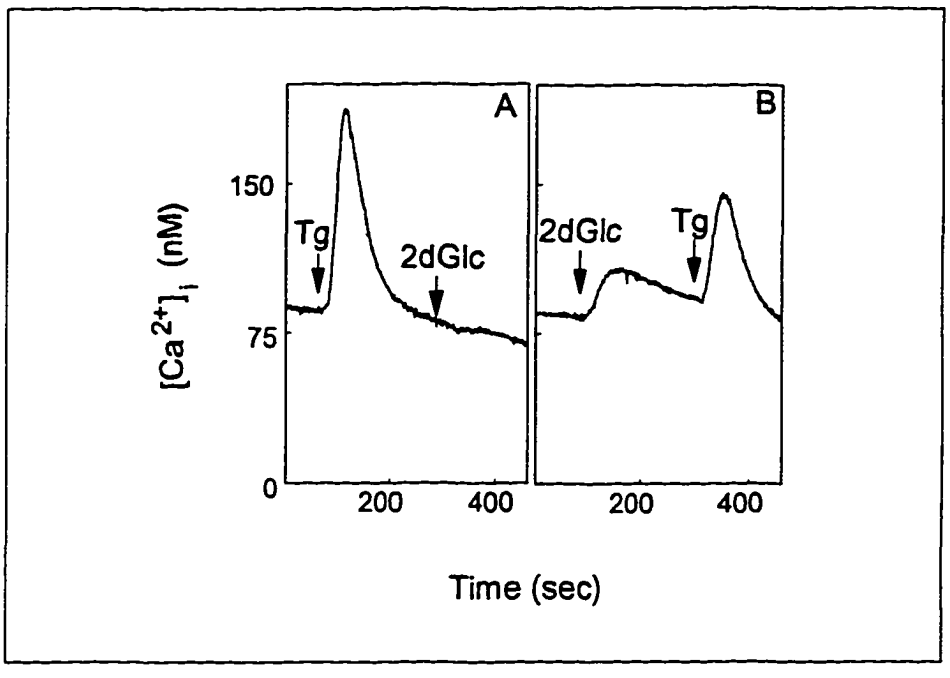

Fig. 5. Effects of 2dGlc on the thapsigargin-sensitive intracellular Ca^{2+} pool. Fura-2/AM loaded J774 cells were resuspended in HBS-EGTA containing 5 mM Glc and 5 mM pyruvate. 2dGlc (25 mM) was added either (A) after the depletion of Ca^{2+} pools by 200 nM thapsigargin (Tg) or (B) prior to the addition of thapsigargin. The baseline Ca^{2+} levels are lower than those seen in Fig. 1 due to the presence of extracellular EGTA. Data shown are representative of at least four similar experiments.

2dGlc concentrations is critical, as was seen for the 2dGlc-mediated inhibition of phagocytosis in both macrophages (Michl et al., 1976) and neutrophils (Boxer et al., 1976). As a second control that addresses both the osmotic effects of 2dGlc addition and the possibility of short-term ATP depletion due to sugar phosphorylation by hexokinase, 25 mM glucose was added to cells initially in Ca^{2+}-free media containing 5 mM glucose (Fig. 6). In contrast to the results with 2dGlc, a decrease in $[Ca^{2+}]_i$ (24.7 ± 1.9 nM decrease from initial baseline) accompanied by a subsequent increase in the apparent size of the thapsigargin-releasable Ca^{2+} pool (44.3 ± 8.4 nM increase in peak height), was observed. This suggests that the added glucose facilitated an increased sequestration of cytoplasmic Ca^{2+} into the ER. The result was most striking in J774 cells in which the Ca^{2+} pools were partially depleted, but in no case was an increase in $[Ca^{2+}]_i$, such as that which accompanies 2dGlc addition, observed. The shift from lower to higher glucose concentration causes an augmentation of intracellular Ca^{2+} stores without any apparent increase in cellular ATP levels (Table 1). A similar decrease in $[Ca^{2+}]_i$ in response to glucose due to enhanced sequestration by the ER has been reported in pancreatic β cells prior to the influx of Ca^{2+} through the plasma membrane that is characteristic of these cells (Roe et al., 1994), as well as in MDCK cells (Lien et al., 1995) and aortic smooth muscle cells [R. Paul, personal communication, 1995].

2dGlc Inhibits Agonist-Induced Ca^{2+} Release From the ER Without Completely Depleting the Intracellular Stores. Figures 1-4 demonstrate that 2dGlc inhibits the agonist-mediated Ca^{2+} responses in the presence of extracellular Ca^{2+}. As noted in the Introduction, the agonist-mediated increase in $[Ca^{2+}]_i$ shown in these figures has two components, the release of Ca^{2+} from intracellular stores and the capacitative influx of

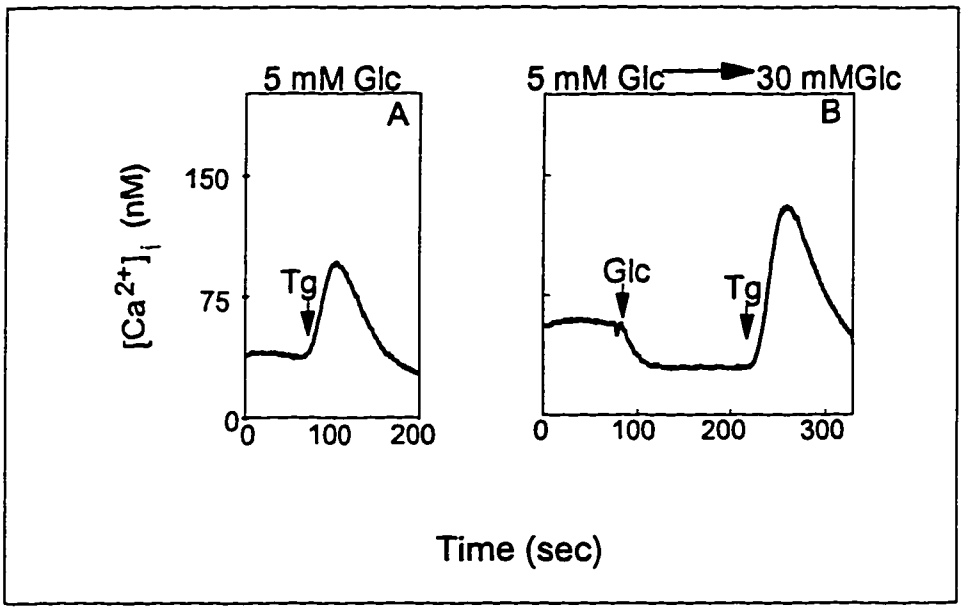

Fig. 6. Effect of Glc on the thapsigargin-sensitive intracellular Ca^{2+} pool. Fura-2/AM loaded J774 cells were suspended in HBS-EGTA containing 5 mM Glc and 5 mM pyruvate. (A) Thapsigargin (Tg) was added to deplete the intracellular stores of Ca^{2+}; (B) 25 mM Glc was added prior to the addition of thapsigargin. This experiment is one of three similar experiments.

extracellular Ca^{2+} into the cell. The latter phase, however, can be eliminated simply by removing extracellular Ca^{2+}. The next set of experiments was designed to specifically examine the effect of 2dGlc on the ability of the agonists to mobilize intracellular Ca^{2+} stores. Figure 7A shows the sequential addition of IgG, PAF, and thapsigargin in the absence of extracellular Ca^{2+}. The effect of 2dGlc on these responses in Ca^{2+}-free media is shown in Figure 7B. The addition of 2dGlc caused, as expected, a release of Ca^{2+} from intracellular stores. This was followed by an inhibition in agonist-induced Ca^{2+} signals (100% inhibition of IgG signal in all cases and 41.3 $\pm$ 8.7% inhibition of PAF signal) but only a modest attenuation of the thapsigargin-induced release from the ER. It is thus apparent that the cells failed to respond normally to IgG and PAF despite the fact that a substantial amount of Ca^{2+} remained in the thapsigargin pool following the partial release by 2dGlc.

2dGlc or Glucose Deprivation Also Inhibits Capacitative Ca^{2+} Entry. We next addressed the effects of 2dGlc on the secondary phase of Ca^{2+} signaling, the capacitative influx of Ca^{2+}. IgG, PAF, and thapsigargin were now added sequentially to cells suspended in a Ca^{2+}- containing buffer (Fig. 7C). Compared to the Ca^{2+}-free condition shown in Figure 7A, the peak responses were greater, and the final plateau of $[Ca^{2+}]_i$ following the addition of thapsigargin was elevated (~100 nM higher than the starting baseline). The plateau value of Ca^{2+} presumably represents the balance between the Ca^{2+}-extruding mechanisms active at the plasma membrane and the capacitative influx. It persists for at least 10 min with little attenuation (data not shown). The increase in peak response as well as the elevated plateau value of $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} is attributed to the sustained activation of the capacitative influx pathway. In the

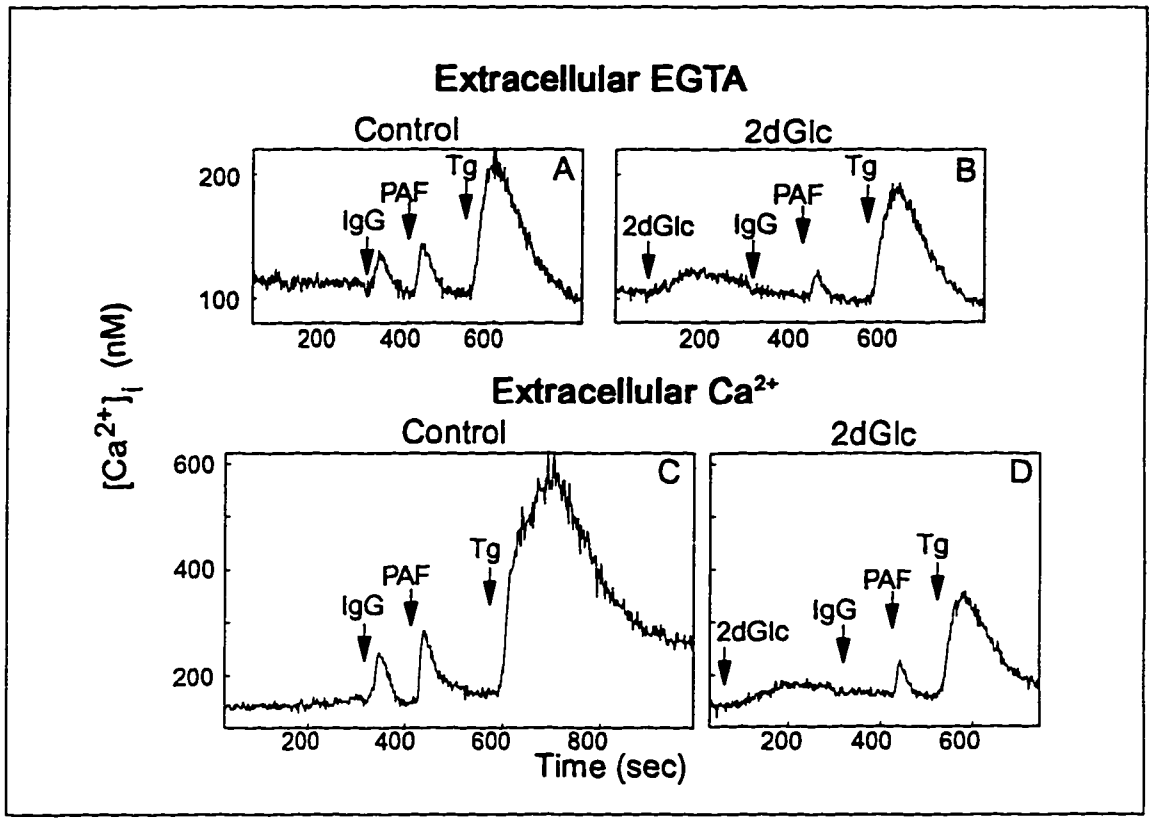

Fig. 7. 2dGlc inhibits agonist responses even though most of the thapsigargin-sensitive Ca^{2+} pool is still intact. (A) Fura-2/AM loaded cells were suspended in HBS-EGTA buffer containing 5 mM Glc and 5 mM pyruvate. The sequential addition of IgG, PAF, and thapsigargin (Tg) is shown. (B) Cells were suspended in HBS-EGTA containing 5 mM Glc and 5 mM pyruvate. 25 mM 2dGlc was added prior to the addition of IgG, PAF, and thapsigargin. (C) IgG, PAF, and thapsigargin were added to Fura-2/AM loaded cells suspended in HBS-Ca^{2+} containing 5 mM Glc and 5 mM pyruvate. (D) Fura-2/AM loaded cells were suspended in HBS-Ca^{2+} containing 5 mM Glc and 5 mM pyruvate. 25 mM 2dGlc was added prior to the addition of IgG, PAF, and thapsigargin.

experiment shown in Figure 7D, IgG, PAF, and thapsigargin were added to 2dGlc-treated cells in Ca^{2+}-containing buffer. In this case, not only does 2dGlc cause a partial release of Ca^{2+} from intracellular stores, it also appears to completely negate the capacitative influx component of the responses.

We next examined the effects of glucose deprivation and the addition of NaF or rotenone on capacitative entry. Figure 8A shows the response of the J774 macrophages to thapsigargin in the presence of extracellular Ca^{2+}. As explained previously, the capacitative entry of Ca^{2+} not only bolsters the initial increase in $[Ca^{2+}]_i$ following the addition of thapsigargin, but also results in the establishment of an elevated plateau of $[Ca^{2+}]_i$. The effects of rotenone and NaF are shown in Figures 8B,C. Neither of these caused a substantial inhibition in the peak response to thapsigargin (83.3 $\pm$ 3.5% and 94.7 $\pm$ 5.6% of control, respectively). In contrast, both 2dGlc treatment and glucose deprivation led to a strong inhibitory effect on the initial thapsigargin -induced increase in$[Ca^{2+}]_i$ (38.7 $\pm$ 9.8% and 32.1 $\pm$ 12.9% of control, respectively). The elevations over baseline of the final $[Ca^{2+}]_i$ plateaus were also lower in the presence of 2dGlc (22.4 $\pm$ 6.9% of control) and in the absence of glucose (20.5 $\pm$ 15.2% of control). This level of inhibition in the plateau value of $[Ca^{2+}]_i$ was not seen with rotenone (60.9 $\pm$ 15.6% of control) or NaF (91.7 $\pm$ 4.8% of control).

The thapsigargin-induced release of Ca^{2+} from intracellular stores and the influx of extracellular Ca^{2+} can be visualized as two separate events when the experiment is initiated in the absence of extracellular Ca^{2+}. The addition of thapsigargin causes a release of Ca^{2+} from intracellular ER stores; but because of the absence of extracellular Ca^{2+} the capacitative influx of Ca^{2+} is not a factor. However, when Ca^{2+} is added back to the

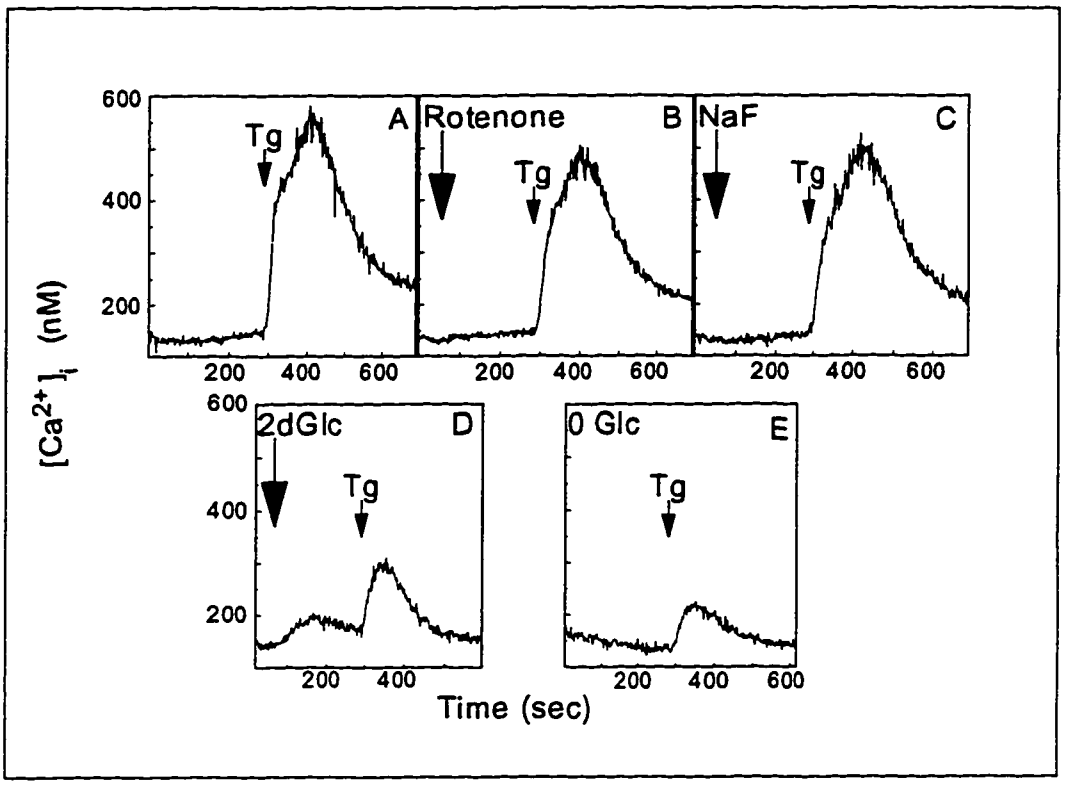

Fig. 8. Thapsigargin-induced capacitative entry in the presence of various metabolic inhibitors. Fura-2/AM loaded J774 cells were suspended in HBS-Ca^{2+} containing 5 mM Glc and 5 mM pyruvate. Prior to the addition of thapsigargin the cells were treated with: (A) control, no addition, (B) 4 μM rotenone , (C) 1 mM NaF, or (D) 25 mM 2dGlc. In (E) the cells were suspended in Glc-free HBS-Ca^{2+} containing 5 mM pyruvate immediately before the start of the experiment. The tracings are representative of three similar experiments.

extracellular media, it rapidly enters the cell through the already activated capacitative influx pathway. Such an experiment is shown in Figure 9A,B. The addition of 2dGlc clearly inhibits the capacitative influx of Ca^{2+} (Fig. 9B). In Figure 9C, the plateau value of $[Ca^{2+}]_i$ was first established following the addition of thapsigargin and the restoration of Ca^{2+} into the media. 2dGlc was then added. This caused a rapid decrease in the plateau, indicating that 2dGlc inhibits the on-going capacitative entry of Ca^{2+}.

DISCUSSION

The data presented here show that in J774 macrophages the addition of 2dGlc or the removal of glucose leads to an inhibition of $[Ca^{2+}]_i$ responses to IgG, ATP, and PAF. Though an inhibition was seen with all the agonists, there was clearly an increased sensitivity in the IgG response to 2dGlc treatment and glucose deprivation.

As noted in the Introduction, 2dGlc has been shown to inhibit F_c receptor-mediated phagocytosis (Michl et al., 1976; Boxer et al., 1976) when added at the same ratios (5 2dGlc: 1 glucose) as we have used here. These investigators also reported that the effects of 2dGlc were not due to its effects on cellular ATP levels. NaF, an alternate glycolytic inhibitor, did not have any effect on agonist-mediated $[Ca^{2+}]_i$ responses, although its effects on cellular ATP were more variable than those due to 2dGlc. The lack of effect of NaF on $[Ca^{2+}]_i$ correlates well with the observations by Piccolo and co-workers that 30 min treatments with NaF did not cause any inhibition in the levels of phagocytosis by human (D'Onofrio et al., 1977) and mouse macrophages (Cifarelli et al., 1979). However, further investigations are necessary in relating our findings to the effects that 2dGlc and NaF have on F_c receptor-mediated phagocytosis since the precise role of

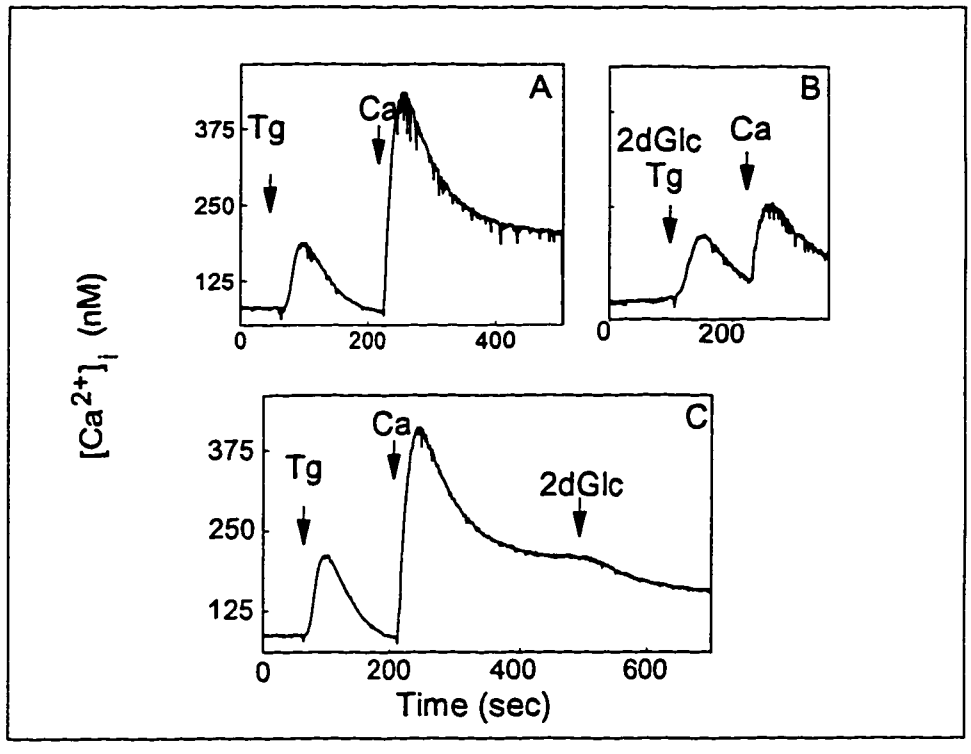

Fig. 9. Effects of 2dGlc on the capacitative entry of Ca^{2+} in a Ca^{2+} add-back protocol. Fura-2/AM loaded J774 cells were suspended in HBS-EGTA buffer containing 5 mM Glc and 5 mM pyruvate. (A) Thapsigargin (Tg) was added, followed by Ca^{2+}, while $[Ca^{2+}]_i$ was monitored; (B) 25 mM 2dGlc was added along with thapsigargin at the time indicated. (C) 25 mM 2dGlc was added after Ca^{2+}. Results shown are representative of three similar experiments.

Ca^{2+} in the phagocytic process remains to be elucidated (Hishikawa et al., 1991). (DiVirgilio et al., 1988).

The inhibition of agonist-induced Ca^{2+} responses in the presence of 2dGlc appears to be related to our findings that (i) 2dGlc releases Ca^{2+} from intracellular thapsigargin-sensitive Ca^{2+} stores, while addition of glucose causes an enhancement in this pool; and (ii) the capacitative influx of Ca^{2+} is inhibited under the experimental conditions of 2dGlc treatment or glucose deprivation. Each of these effects and their relation to agonist-mediated Ca^{2+} responses are discussed later.

Partial Release of ER Ca^{2+}. The release of Ca^{2+} from intracellular stores following the addition of 2dGlc raises two questions: (i) what is the mechanism whereby this release occurs? and (ii) how is this release relevant to the agonist-induced Ca^{2+} responses? Several investigators have reported effects of glucose (Lien et al., 1995; Roe et al., 1994; Zhang and Paul, 1994) or 2dGlc (Bright et al., 1995; Ziegelstein et al., 1994) on intracellular Ca^{2+} in other cell types. Some have suggested the presence of an ATP pool generated by glycolysis that is small relative to total cellular ATP but serves specific cellular functions. For instance, in the case of smooth muscle, Zhang and Paul (1994) concluded that the absence of glucose led to higher than normal $[Ca^{2+}]_i$ due to ineffective Ca^{2+} sequestration by the SERCAs. This was found despite the presence of an oxidative metabolic substrate that maintained overall ATP at normal levels. Based on this hypothesis our data would then imply that in the presence of 2dGlc or the absence of glucose, the J774 SERCAs are inhibited due to a lack of glycolytically generated ATP.

A second possibility is that an intermediate glycolytic metabolite is important to SERCA activity. Dukes et al. (1994) have suggested that glycolytically produced NADH

is the metabolite necessary for the normal functioning of ATP-dependent K^+ channels in pancreatic β cells. They proposed that this NADH was subsequently converted to ATP in the mitochondria since addition of rotenone to these cells inhibited the glucose-induced oscillations in $[Ca^{2+}]_i$. In contrast to these observations, we found that, in J774 macrophages, rotenone had no significant effect on intracellular Ca^{2+} regulation.

Both of these hypotheses are based on the assumption that the release of ER Ca^{2+} we see here in the presence of 2dGlc is due to depletion of a specialized pool of cellular ATP. The assumption that a lack of the specialized ATP pool inhibits SERCA activity would explain the initial, 2dGlc-mediated release of Ca^{2+} from intracellular stores. It does not, however, explain why, in the continued presence of 2dGlc, $[Ca^{2+}]_i$ does not stay elevated until nearly all of the thapsigargin-sensitive pool of Ca^{2+} has been emptied. We have observed that $[Ca^{2+}]_i$ values return to baseline within a relatively short (~100 s) period following the release of ER Ca^{2+} by 2dGlc (see Fig. 7). The return of $[Ca^{2+}]_i$ to a baseline indicates a decrease in the rate of leakage of ER Ca^{2+} relative to its rate of removal from the cytoplasm. In fact, nearly 60% of the thapsigargin-sensitive pool is intact following 2dGlc addition. This result suggests that SERCA activity in these cells is not dependent solely on glycolytically generated ATP. It is plausible that only a portion of the ATP required for an optimal SERCA activity is derived from a glycolytically generated ATP pool, whereas the remainder is derived from oxidative metabolism. In this case it would be expected that, following 2dGlc treatment and inhibition of glycolysis, a new steady state of SERCA activity would be attained. However, this would be inconsistent with our results indicating that rotenone has no effect on SERCA activity.

These inconsistencies may be explained by considering the possibility that 2dGlc causes the partial release of ER Ca^{2+} independent of its effects on cellular ATP levels. Roe et al. (1994) have suggested that a metabolite downstream of glyceraldehyde-3-phosphate but upstream of pyruvate was essential for the initial decrease in $[Ca^{2+}]_i$ seen with increased levels of extracellular glucose. Alternatively, Benedetti and co-workers have demonstrated that the glycolytic metabolite glucose-6-phosphate (Glc-6-P) directly enhances the ATP-dependent sequestration of Ca^{2+} in permeabilized hepatocytes (Benedetti et al., 1987), as well as in microsomes from both liver (Benedetti et al., 1985) and kidney (Fulceri et al., 1990). In liver, Glc-6-P is efficiently imported into the ER as a required step in glycogenolysis. It is then cleaved to glucose and P_i by an intra-organellar Glc-6-phosphatase (Burchell and Waddell, 1991), and the P_i produced is thought to enhance intralumenal Ca^{2+} sequestration (Benedetti et al., 1985). The effects of P_i presumably reflect the capacity of multivalent anions to allow a higher level of efficiency of SERCA activity (Korge and Campbell, 1994; Yu and Inesi, 1995). It is of interest to note that 2dGlc-6-P is not efficiently imported into the ER in liver [our unpublished observations], and that it competitively inhibits the import of Glc-6-P (Arion et al., 1972). It is possible that a similar, Glc-6-P dependent pathway for Ca^{2+} sequestration also exists in macrophages, since the presence of Glc-6-phosphatase system in these cells has been previously demonstrated (Nichols and Setzer, 1981). The hypothesis, then, would be that a decrease in Glc-6-P levels due to the addition of 2dGlc or glucose deprivation would lead to the release of the 'Glc-6-P-sensitive' portion of the ER Ca^{2+} stores. It is also of interest to note that in the subtype of glycogen storage disease (GSD Ib) that is characterized by a defect in the Glc-6-P import mechanism into the ER, not only is there

an inability of the liver to break glycogen down but also a loss of function of the phagocytic cells (Gitzelmann and Bosshard, 1993). Furthermore, the phagocytic cells from these patients exhibit a compromised capacity to mobilize Ca^{2+} from intracellular stores (Kilpatrick et al., 1990; Korchak et al. 1993).

Regardless of the mechanism involved, 2dGlc-mediated partial release of ER Ca^{2+} leads to a significant inhibition of $[Ca^{2+}]_i$ signals. The inhibition occurs despite the fact that most of the thapsigargin-sensitive Ca^{2+} pools remain intact following 2dGlc treatment. It is as yet not clear whether in non-excitable cells there are two pools within the thapsigargin-sensitive Ca^{2+} stores, one sensitive and the other insensitive to IP_3. If this is the case, then it is conceivable that 2dGlc may be causing a more pronounced depletion of the IP_3-sensitive pools. In light of recent reports on effects of luminal Ca^{2+} concentrations on IP_3 sensitivity, another possibility is that a decrease in luminal Ca^{2+} concentration, brought about by 2dGlc, may result in the inhibition of agonist responses (Nunn and Taylor, 1991; Tanimura and Turner, 1996). Further studies would be necessary to elucidate both the mechanism of the 2dGlc-mediated release of Ca^{2+} from intracellular stores and its relevance to agonist-induced Ca^{2+} signals.

Capacitative Influx. Little is known about the capacitative influx pathway, and several different mechanisms have been proposed to explain this phenomenon. Some of the molecules that have been suggested to regulate this pathway include an unknown, low molecular weight, diffusible messenger molecule (Randriamampita and Tsien, 1993; Parekh et al., 1993), a small GTP binding protein (Bird and Putney, 1993), cGMP and nitric oxide (Bischof et al., 1995).

Recent reports (Gamberucci et al., 1994; Marriott and Mason, 1995) have indicated that a decrease in cellular ATP content leads to a reduction in capacitative influx. Gamberucci et al. (1994) showed an inhibition in Ca^{2+} influx in the presence of rotenone although the magnitude of this effect appeared to vary among the cell types that were examined. Our observations in J774 cells are that mitochondrial inhibition by rotenone does not cause the significant change in capacitative influx seen in the presence of 2dGlc or upon glucose removal. It is possible that J774 macrophages rely exclusively on glycolytic ATP for optimal activation of the capacitative entry pathway or that some other glucose metabolite is involved.

The release of Ca^{2+} from intracellular stores induced by 2dGlc might by itself be expected to activate the capacitative influx pathway. However, the inhibitory effect of 2dGlc on capacitative influx seen after treatments with agonists or thapsigargin appears to take precedence as no influx due to 2dGlc-mediated depletion is seen (data not shown).

In summary, in the presence of extracellular Ca^{2+}, 2dGlc treatment or glucose deprivation of J774 cells has two distinct effects. First, it causes a partial release of Ca^{2+} from intracellular stores. Second, the capacitative influx of Ca^{2+}, which is activated by depletion of Ca^{2+} from the ER, is significantly attenuated. In terms of our model these effects may relate to agonist-mediated Ca^{2+} signals in the following way: 2dGlc treatment or glucose deprivation releases a portion of the ER Ca^{2+} pool which then leads to an attenuation of IP_3-mediated responses. At the same time, the ability of these macrophages to refill this pool of Ca^{2+} is also inhibited. The net effect is the observed block in the generation of Ca^{2+} signals in response to IgG, ATP, and PAF.

ACKNOWLEDGMENTS

We thank C. Roger White for his valuable help in use of the SPEX spectrofluorometer. This work was supported by NIH AR 207614 and the Fifty 50 Foods Research Program of the Juvenile Diabetes Foundation International. We thank Sherry Crittenden for her expertise with editorial assistance.

REFERENCES

Adams, G.R., and Dillon, P.F. (1989) Glucose dependence of sequential norepinephrine contractions of vascular smooth muscle. Blood Vessels, *26*: 77-83.

Arion, W.J., Wallin, B.K., Carlson, P.W., and Lange, A.J. (1972) The specificity of glucose 6-phosphatase of intact liver microsomes. J. Biol. Chem., *247*: 2558-2565.

Benedetti, A., Fulceri, R., and Comporti, M. (1985) Calcium sequestration activity in rat liver microsomes. Evidence for a cooperation of calcium transport with glucose-6-phosphatase. Biochim. Biophys. Acta., *816*:267-277.

Benedetti, A., Fulceri, R., Romani, A., and Comporti, M. (1987) Stimulatory effect of glucose 6-phosphate on the non-mitochondrial Ca^{2+} uptake in permeabilized hepatocytes and Ca^{2+} release by inositol trisphosphate. Biochim. Biophys. Acta, *928*: 282-286.

Bird, G.S.J., and Putney, J.W., Jr. (1993) Inhibition of thapsigargin-induced calcium entry by microinjected guanine nucleotide analogues. J. Biol. Chem., *268*: 21486-21488.

Bischof, G., Brenman, J., Bredt, D.S., and Machen, T.E. (1995) Possible regulation of capacitative Ca^{2+} entry into colonic epithelial cells by NO and cGMP. Cell Calcium, *17*: 250-262.

Boxer, L.A., Baehner, R.L., and Davis, J. (1976) The effect of 2-deoxyglucose on guinea pig polymorphonuclear leukocyte phagocytosis. Cell. Physiol, *91*: 89-102.

Bright, R.T., Salvaterra, C.G., Rubin, L.J., and Yuan, X.-J. (1995) Inhibition of glycolysis by 2-DG increases $[Ca^{2+}]_i$ in pulmonary arterial smooth muscle cells. Am. J. Physiol, *269*: L203-L208.

Burchell, A., and Waddell, I.D. (1991) The molecular basis of the hepatic microsomal glucose-6-phosphatase system. Biochim. Biophys. Acta, *1092*:129-137.

Cifarelli, A., Pepe, G., Paradisi, F., and Piccolo, D. (1979) The influence of some metabolic inhibitors on phagocytic activity of mouse macrophages in vitro. Res. Exp. Med. (Berl.), *174*:197-204.

Cox, D.W.G., and Bachelard, H.S. (1982) Attenuation of evoked field potentials from dentate granule cells by low glucose, pyruvate + malate, and sodium fluoride. Brain Res., *239*:527-536.

David, J.R., Butterworth, A.E., Remold, H.G., David, P.H., Houba, V., and Sturrock, R.F. (1977) Antibody-dependent, eosinophil-mediated damage to ^{51}Cr-labeled schistosomula of *Schistosoma Mansoni*:Effect of metabolic inhibitors and other agents which alter cell function. J. Immunol., *118*:2221-2229.

DiVirgilio, F., Meyer, B.S., Greenberg, S., and Silverstein, S.C. (1988) F_c receptor-mediated phagocytosis occurs in macrophages at exceedingly low cytosolic Ca^{2+} levels. J. Cell Biol., *106*:657-666.

D'Onofrio C., Paradisi F., and Piccolo D. (1977) The influence of some metabolic inhibitors on in vitro phagocytozing macrophages. Med. Microbiol. Immunol., *163*:195-207.

Dukes, I.D., McIntyre, M.S., Mertz, R.J., Philipson, L.H., Roe, M.W., Spencer, B., and Worley, I.J.F. (1994) Dependence on NADH produced during glycolysis for β-cell glucose signaling. J. Biol. Chem., *269*:10979-10982.

Fraser, L.R., and Herod, J.E. (1990) Expression of capacitation-dependent changes in chlortetracycline fluorescence patterns in mouse spermatozoa requires a suitable glycolysable substrate. J. Reprod. Fert., *88*: 611-621.

Fukuchi, Y., Sato, M., Yamashita, T., and Koyama, J. (1991) Low ligand binding activities of two distinct types of Fcψ receptor on guinea-pig peripheral blood polymorphonuclear leukocytes are differentially improved by proteolysis or platelet activating factor. Mol. Immunol, *29*:583-591.

Fulceri, R., Romani, A., Pompella, A., and Benedetti, A. (1990) Glucose 6-phosphate stimulation of MgATP-dependent Ca^{2+} uptake by rat kidney microsomes. Biochim. Biophys. Acta, *1022*:129-133.

Gamberucci, A., Innocenti, B., Fulceri, R., Banhegyi, G., Guinti, R., Pozzan, T., and Benedetti, A. (1994) Modulation of Ca^{2+} influx dependent on store depletion by intracellular adenine-guanine nucleotide levels. J. Biol. Chem., *269*:23597-23602.

Gitzelmann, R., and Bosshard, N.U. (1993) Defective neutrophil and monocyte functions in glycogen storage disease type Ib: a literature review. Eur. J. Pediatr., *152*:S33-S38.

Greenberg, S., DiVirgilio, F., Steinberg, T.H., and Silverstein, S.C. (1988) Extracellular nucleotides mediate Ca^{2+} fluxes in J774 macrophages by two distinct mechanisms. J. Biol. Chem., *263*:10337-10343.

Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem., *260*:3440-3450.

Hibbs, M.L., Classon, B.J., Walker, I.D., McKenzie, I.F., and Hogarth, P.M. (1988) The structure of the murine Fc receptor for IgG. Assignment of intrachain disulfide bonds, identification of N-linked glycosylation sites, and evidence for a fourth form of Fc receptor. J. Immunol., *140*:544-550.

Hishikawa, T., Cheung, J.Y., Yelamarty, R.V., and Knutson, D.W. (1991) Calcium transients during Fc receptor-mediated and nonspecific phagocytosis by murine peritoneal macrophages. J. Cell Biol., *115*:59-66.

Ichinose, M., Hara, N., Sawada, M., and Maeno, T. (1994) A flow cytometric assay reveals an enhancement of phagocytosis by platelet activating factor in murine peritoneal macrophages. Cellular Immun., *156*:508-518.

Kilpatrick, L., Garty, B.Z., Lundquist, K.F., Hunter, K., Stanley, C.A., Baker, L., Douglas, S.D., and Korchak, H.M. (1990) Impaired metabolic function and signaling defects in phagocytic cells in glycogen storage disease type 1b. J. Clin. Invest., *86*:196-202.

Korchak, H.M., Garty, B.Z., Stanley, C.A., Baker, L., Douglas, S.D., and Kilpatrick, L. (1993) Impairment of calcium mobilization in phagocytic cells in glycogen storage disease type 1b. Eur. J. Pediatr., *152*:S39-S43.

Korge, P., and Campbell, K.B. (1994) Local ATP regeneration is important for sarcoplasmic reticulum Ca^{2+} pump function. Am. J. Physiol., *267*:C357-C366.

Kwan, C.Y., Takemura, H., Obie, J.F., Thastrup, O., and Putney, J.W, Jr. (1990) Effects of MeCh, thapsigargin, and La^{3+} on plasmalemmal and intracellular Ca^{2+} transport in lacrimal acinar cells. Amer. J. Physiol.:Cell Physiol., *258*:C1006-C1015.

Lewis, L.D., Ljunggren, B., Ratcheson, R.A., and Siesjo, B.K. (1974) Cerebral energy state in insulin-induced hypoglycemia, related to blood glucose and EEG. J. Neurochem., *23*:673-679.

Lien, Y.-H.H., Wang, X., Gillies, R.J., and Martinex-Zaguilan, R. (1995) Modulation of intracellular Ca^{2+} by glucose in MDCK cells: role of endoplasmic reticulum Ca^{2+}-ATPase. Am. J. Physiol., *268*: F671-F679.

Macaluso, C., Onoe, S., and Niemeyer, G. (1992) Changes in glucose level affect rod function more than cone function in the isolated, perfused cat eye. Invest. Ophthalmol. Vis. Sci., *33*:2798-2808.

MacDonald, H.R., and Cerottini, J.C. (1979) Inhibition of T cell-mediated cytolysis by 2-deoxy-D-glucose:dissociation of the inhibitory effect from glycoprotein synthesis. Eur. J. Immunol., *9*:466-470.

Marriott, I., and Mason, M.J. (1995) ATP depletion inhibits capacitative Ca^{2+} entry in rat thymic lymphocytes. Am. J. Physiol., *269*:C766-774.

Michl, J., Ohlbaum, D.J., and Silverstein, S.C. (1976) 2-Deoxyglucose selectively inhibits Fc and complement receptor-mediated phagocytosis in mouse peritoneal macrophages. J. Exp. Med., *144*:1465-1483.

Nichols, B.A., and Setzer, P.Y. (1981) Cytochemical localization of glucose-6-phosphatase in rabbit mononuclear phagocytes during differentiation. J. Histochem. and Cytochem., *29*:317-320.

Nunn, D.L., and Taylor, C.W. (1991) Luminal Ca^{2+} increases the sensitivity of Ca^{2+} stores to inositol 1,4,5-trisphosphate. Mol. Pharmacol., *41*:115-119.

Parekh, A.B., Terlau, H., and Stuhmer, A. (1993) Depletion of $InsP_3$ stores activates a Ca^{2+} and K^+ current by means of a phosphatase and a diffusible messenger. Nature, *364*:814-818.

Prpic, V., Uhing, R.J., Weiel, J.E., Jakooi, L., Gawdi, G., Herman, B., and Adams, D.O. (1988) Biochemical and functional responses stimulated by platelet-activating factor in murine peritoneal macrophages. J. Cell Bio., *107*:363-372.

Putney, J.W.,Jr., and Bird, G.S.J. (1993) The signal for capacitative calcium entry. Cell, *75*:199-201.

Randriamampita, C., and Tsien, R.Y. (1993) Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. Nature, *364*, 809-813.

Roe, M.W., Mertz, R.J., Lancaster, M.E., Worley, J.F., III, and Dukes, I.D. (1994) Thapsigargin inhibits the glucose-induced decrease of intracellular Ca^{2+} in mouse islets of Langerhans. Am. J. Physiol., *266*:E852-E862.

Sung, S.S., and Silverstein, S.C. (1985) Role of 2-deoxy-D-glucose in the inhibition of phagocytosis by mouse peritoneal macrophage. Biochim. Biophys. Acta, *845*:204-215.

Tanimura, A., and Turner, R.J. (1996) Calcium release in HSY cells conforms to a steady-state mechanism involving regulation of the inositol 1,4,5-trisphosphate receptor Ca^{2+} channel by luminal [Ca^{2+}]. J. Cell Bio., *132*:607-616.

Tojyo, Y., Tanimura, A., Matsui, S., Matsumoto, Y., Sugiya, H., and Furuyama, S. (1991) NaF-induced amylase release from rat parotid cells is mediated by PI breakdown leading to Ca^{2+} mobilization. Am. J. Physiol., *260*:C194-C200.

Yu, X., and Inesi, G. (1995) Variable stoichiometric efficiency of Ca^{2+} and Sr^{2+} transport by the sarcoplasmic reticulum ATPase. J. Biol. Chem., *279*:4361-4367.

Zhang, C., and Paul, R.J. (1994) Excitation-contraction coupling and relaxation in porcine carotid arteries are specifically dependent on glucose. Am. J. Physiol., *36*:1996-2004.

Ziegelstein, R.C., Cheng, L., Aversano, T., Ouyang, P., Lakatta, E.G., and Silverman, H.S. (1994) Increase in rat aortic endothelial free calcium mediated by metabolically sensitive calcium release from endoplasmic reticulum. Cardiovasc. Res., *28*:1433-1439.

INTERACTION BETWEEN GLUCOSE-6-PHOSPHATASE AND Ca^{2+}-ATPASE SYSTEMS: A COMPARISON BETWEEN RAT LIVER AND J774 CELLS

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In preparation for Journal of Cellular Physiology

Format adapted for dissertation

ABSTRACT

A partial loss of intracellular Ca^{2+} stores in 2-deoxy-D-glucose (2dGlc)-treated and glucose-deprived J774 macrophages was previously noted (Darbha and Marchase, 1996). In this report, it is noted that upon 2dGlc treatment or glucose deprivation of these cells, glucose-6-phosphate (Glc-6-P) levels decreased to approximately 17% and 10% of control values, respectively. It has previously been shown that Glc-6-P enhances Mg-ATP dependent sequestration of Ca^{2+} in microsomal preparations from both liver (Benedetti et al., 1985) and kidney (Fulceri et al., 1990). Here, the possibility that a similar pathway for Ca^{2+} sequestration exists in J774 macrophages was investigated. Glc-6-P uptake into J774 microsomes was determined using light scattering techniques and [^{14}C]Glc-6-P import. The concentration of Glc-6-P inside microsomes reached values that were comparable to that present in the assay buffer. However, Ca^{2+} uptake assays performed with permeabilized J774 cells, as well as with microsomes from these cells, revealed no detectable enhancement in Ca^{2+} sequestration in the presence of Glc-6-P.

INTRODUCTION

It has previously been demonstrated that Glc-6-P enhances the ATP-dependent sequestration of Ca^{2+} in microsomes from liver (Benedetti et al., 1985) and kidney (Fulceri et al., 1990). In liver, Glc-6-P is efficiently imported into the endoplasmic reticulum (ER) as a required step in glycogenolysis. It is then cleaved to glucose and P_i by an intra-organeller Glc-6-phosphatase, and the P_i produced is thought to enhance intralumenal Ca^{2+} sequestration (Burchell and Waddell, 1991). The effect of P_i presumably reflects the capacity of multivalent anions to allow a higher level of efficiency of Ca^{2+}-ATPase activity (Inesi and de Meis, 1989). Other investigators from our laboratory suggest a different

mechanism for Glc-6-P-mediated enhancement in Ca^{2+} sequestration (Veyna-Burke, 1996). Their data indicate that the enhancement in Ca^{2+} sequestration by Glc-6-P is due to a direct association between Glc-6-P and Ca^{2+} within the microsomes.

As previously reported, in J774 macrophages 2-deoxy-D-glucose (2dGlc) treatment or glucose deprivation resulted in a disruption of Ca^{2+} regulation (Darbha and Marchase, 1996). 2dGlc is converted to 2dGlc-6-P by hexokinase (Sung and Silverstein, 1985) but is not metabolized further by glycolytic enzymes. Addition of 2dGlc to J774 cells caused a partial release of intracellular Ca^{2+} stores. A similar release of intracellular stores was seen when the cells were incubated in glucose-free media (Darbha and Marchase, 1996). It is apparent that in 2dGlc-treated and glucose-deprived cells, Glc-6-P levels would be affected. In this report, the possibility that in J774 cells Glc-6-P enhances Ca^{2+} sequestration is addressed. The existence of such a pathway might provide a simple explanation for the release of intracellular stores in glucose-deprived cells.

An investigation into Glc-6-P mediated Ca^{2+} sequestration within the ER in J774 macrophages was also prompted by observations made in GSD Ib (glycogen storage disease type Ib) patients. In these patients, there is an inability of the liver to break glycogen down due to an impairment of Glc-6-P transport into the lumen of the ER. Along with the clinical symptoms of hepatomegaly that is common to all subtypes of GSD, type Ib patients also suffer from neutropenia and defects in phagocytic functions (Gitzelmann and Bosshard, 1993). Furthermore, it was shown that neutrophils from GSD Ib patients were compromised in their ability to generate Ca^{2+} signals (Kilpatrick et al., 1990; Korchak et al., 1993). The link between defective Glc-6-P import in the liver and the dysfunction of phagocytic cells in GSD Ib patients remains unclear. The existence of a Glc-6-P import

pathway in the macrophage ER has not been previously described. This issue is, therefore, addressed here.

MATERIALS AND METHODS

Cell Culture. J774 cells (American Type Culture Collection) were cultured at 37° C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco-BRL, Gaitersburgh, MD, USA) and 1% penicillin/streptomycin.

Preparation of J774 Microsomes. J774 cells were suspended at 10^8 cells/ml in homogenization buffer [0.34 M sucrose, 10 mM Tris-HCl, 1mM EDTA, 0.5 mM DTT, 1 mM PMSF, 1 μM pepstatin, 1mM benzamidine, pH 7.2]. All of the following steps were performed on ice. The cells were homogenized in a pyrex, hand-held homogenizer, and the homogenate was centrifued at 2700 g for 15 min. The supernatant was diluted to 0.25 M sucrose using homogenization buffer without sucrose and centrifuged at 80,000 g for 30 min. The pellet was washed once in the appropriate assay buffer.

Preparation of Liver Microsomes. Liver microsomes were isolated as previously described (Benedetti et al., 1985). Briefly, a liver homogenate in 0.154 M KCl and 3 mM EDTA at pH 7.4 was centrifuged at 2700 g for 10 min. The resulting supernatant was centrifuged at 80,000 g for 30 min. Liver from a male Sprague-Dawley rat (approximately 200 g) was minced and homogenized in a glass homogenizer with a motor driven teflon pestle in 0.154 M KCl and 3mM EDTA, pH 7.4. The homogenate was centrifuged at 2700 g for 15 min. The supernatant was centrifuged at 80,000 g and resuspended in the appropriate assay buffer.

Light Scattering. Light scattering assays were performed as previously described (Fulceri et al., 1992). Microsomes were suspended at 50 mg/ml in a buffer containing 100

mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 20 mM Mops, pH 7.2. Prior to the start of the assay, the microsomes were diluted 1:600 in 5 mM K-Pipes buffer, pH 7.0. 3 ml of the diluted microsomal suspension in a cuvette was placed in a temperature-controlled (25^0C) fluorimeter (Photon Technology Inc., S. Brunswick, NJ, USA) chamber equipped with a stirrer. Osmotically induced changes in microsomal vesicle size were initiated by the addition of 0.3 ml stock solutions of different solutes. The light scattering intensity was monitored at 400 nm at right angles to the incoming beam.

The rationale for these experiments is the following. The increase in osmolarity causes the microsomal vesicles to shrink as water leaves the vesicles. This causes increased light scattering. If the solute that has been added is permeable to the vesicles, both the solute and water will enter the vesicles and the microsomes will return to their original size. Thus, the initial increase in scattered light will be followed by a decrease. However, if the solute added is impermeable, then the microsomes will remain in their shrunken state, and there will be a sustained increase in the intensity of scattered light.

Preparation of [^{14}C] Glc-6-P. [^{14}C]Glc-6-P was prepared from [^{14}C] glucose [NEN-Dupont, Boston, MA, USA; uniformly labeled, 318 mCi/m mol] and ATP [Sigma, St. Louis, MO, USA]. The hexokinase reaction was performed for 16 h in 50 mM Tris pH 7.6 containing 500 μCi ^{14}C glucose, 5 mM $MgCl_2$, 50 mM ATP, and 1 unit hexokinase. Glc-6-P was separated from glucose by descending paper chromatography using Whatman No.1 paper. The solvent used was 2 volumes 1 M ammonium acetate plus 5 volumes 95% ethanol.

[^{14}C]Glc-6-P uptake. Glc-6-P uptake assay was adapted from a previously described method for uptake of nucleotide sugars into microsomes (Perez and Hirschberg, 1987).

Microsomes from J774 cells were prepared, as described previously, and resuspended in 50 mM Tris Cacodylate buffer, pH 6.5. The uptake was initiated by the addition of 1mM Glc-6-P and 4.5 μCi of [^{14}C] Glc-6-P to 100 μg of microsomes. The total volume was 1 ml. Following a 5 min incubation, 2 ml of ice cold incubation buffer was added, and the microsomes were separated by centrifugation at 100,000 g at 4^{0}C for 25 min. The radioactivity associated with supernatant was determined (input counts). The surface of the microsomal pellet was washed three times with ice cold water, and the pellet was then sonicated in 500 μl ice cold water and extracted with 8% $HClO_4$. The precipitate was removed by centrifugation at 30,000 g at 4^{0}C for 15 min. The supernatant was counted, and the fraction uptake determined.

Glc-6-P hydrolysis. Glc-6-P hydrolysis rate was assessed by measuring the production of glucose using a glucose dehydrogenase-based assay. Following a 15 min incubation of the microsomes in 50 mM Tris Cacodylate buffer containing 1 mM Glc-6-P, ice cold buffer was added, and the microsomes were centrifuged at 80,000 g. The supernatant was assayed for the amount of glucose by adding an aliquot of it to a sodium phosphate-based assay buffer containing 50 μM NADP, 1.042 unit mutarotase and 1 unit/ml glucose dehydrogenase. The production of NADPH was monitored fluorimetrically (Photon Technology Inc., S. Brunswick, NJ, USA) using an excitation wavelength of 340 nm and emission wavelength set at 460 nm.

$^{45}Ca^{2+}$ uptake assay. $^{45}Ca^{2+}$ uptake assays were performed as reported (Benedetti et al., 1985). The assay buffer was 100 mM KCl, 5 mM NaCl, 5 mM sodium azide, 5 mM $MgCl_2$, 1 mM ATP, 5 mM phosphocreatine, 5U/ml phosphocreatine kinase, 20 μM $CaCl_2$, and 0.1 μCi/ml $^{45}CaCl_2$ (NEN-Dupont, Boston, MA, USA), pH7.0. Microsomes were used

at 100 μg protein/ml. Glc-6-P and 2dGlc-6-P were added, as noted. Following the incubation, the microsomes were filtered through prewetted cellulose nitrate filters (pore size 0.2 μm). The filters were washed with assay buffer without $^{45}CaCl_2$ and placed in 5 ml of scintillation fluid. Radioactivity was monitored using a Packard Tri-Carb 4000 scintillation counter.

Fura-2 based Ca^{2+} uptake assays. Fluorescence measurements were performed in a fluorescence spectrophotometer (Photon Technology Inc., S. Brunswick, NJ, USA) with microsomes or permeabilized cells suspended in a cuvette in a temperature-controlled chamber (37^0C) equipped with a magnetic stirrer. The fluorescence intensity was measured at 500 nm with excitation wavelengths of 340 and 380 nm. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al (1985): $[Ca^{2+}]_i = K_d \times (R-R_{min})/(R_{max}-R) \times S_{f2}/S_{b2}$, where K_d is the dissociation constant for Ca^{2+} (224 nM), R is the ratio of the intensities at 340 nm and 380 nm, and R_{min} and R_{max} are the R values at 0 and saturating levels of Ca^{2+}, respectively. S_{f2}/S_{b2} is the ratio of the intensities at 380 nm excitation under R_{min} and R_{max} conditions.

RESULTS

Import of Glc-6-P and 2dGlc-6-P into liver microsomes. The use of light scattering assays to assess the permeability of microsomes to various solutes, including Glc-6-P, has previously been demonstrated (Fulceri et al., 1992; Forsyth et al., 1993). As explained under experimental procedures, in this assay osmotically induced changes in the size of microsomes are followed by monitoring the intensity of the scattered light at right angles to the incidence beam. KCl and sucrose were used as controls for permeant and impermeant solutes respectively. As shown in Figure 1 when KCl was added to liver

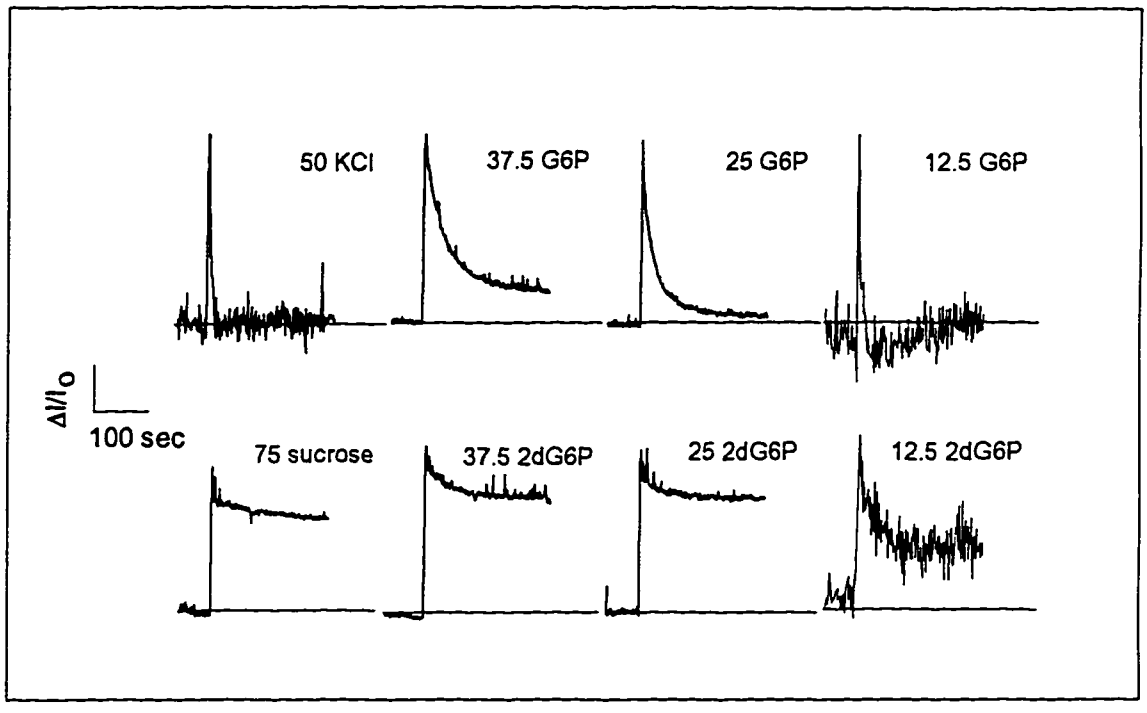

Fig. 1. Permeability of liver microsomes to Glc-6-P and 2dGlc-6-P. Light scatter measurements were performed as described under experimental procedures. Microsomes were suspended in 3 ml of a low osmolarity buffer (5 mM K-Pipes). A 0.3 ml aliquot of the concentrated solution of the different solutes was added. The resulting final concentration in mM of the respective solutes is indicated. Io=initial intensity of scattered light and ΔI=final intensity-initial intensity.

microsomes, the intensity of scattered light at first increased then was followed by a rapid return to the initial lower intensity. The addition of sucrose, however, yielded a sustained increase in the intensity of scattered light. In agreement with previously published results, Glc-6-P was readily permeable to liver microsomes at all concentrations used. We next tested the permeability of these microsomes to 2dGlc-6-P. The effect of 2dGlc-6-P was in contrast to Glc-6-P. At all concentrations, addition of 2dGlc-6-P was followed by a sustained increase in the intensity of scattered light. This indicates that the liver microsomes are relatively impermeable to 2dGlc-6-P. Our results are in agreement with the previously published data by Arion et al. (1972). These investigators reported that although in disrupted microsomes 2dGlc-6-P could be hydrolyzed at rates similar to that of Glc-6-P, in intact microsomes it is a very poor substrate for Glc-6-phosphatase. They surmised that this is due to the inability of 2dGlc-6-P to be efficiently transported into the microsomes.

Effect of Glc-6-P and 2dGlc-6-P on $^{45}Ca^{2+}$ Uptake in Liver Microsomes. The import of Glc-6-P into the ER is accompanied by its hydrolysis into glucose and P_i by the Glc-6-phosphatase enzyme present within the lumen of the ER. It has been proposed that there is a cooperation between the Glc-6-phosphatase and the Ca^{2+}-ATPase systems. The P_i produced upon the hydrolysis of Glc-6-P by the phosphatase has been proposed to enhance the ATP dependent uptake of Ca^{2+}. The non-ionic association of Ca^{2+} with P_i within the lumen would reduce the concentration of free Ca^{2+} within the lumen. This would relieve the inhibition of Ca^{2+}-ATPase activity that normally occurs when free Ca^{2+} concentration within the lumen increases. Owing to the lack of permeability of 2dGlc-6-P to liver microsomes, it is reasonable to expect that 2dGlc-6-P would be unable to mimic the effect of Glc-6-P in enhancing the uptake of Ca^{2+}. As shown in Figure 2, while Glc-6-P

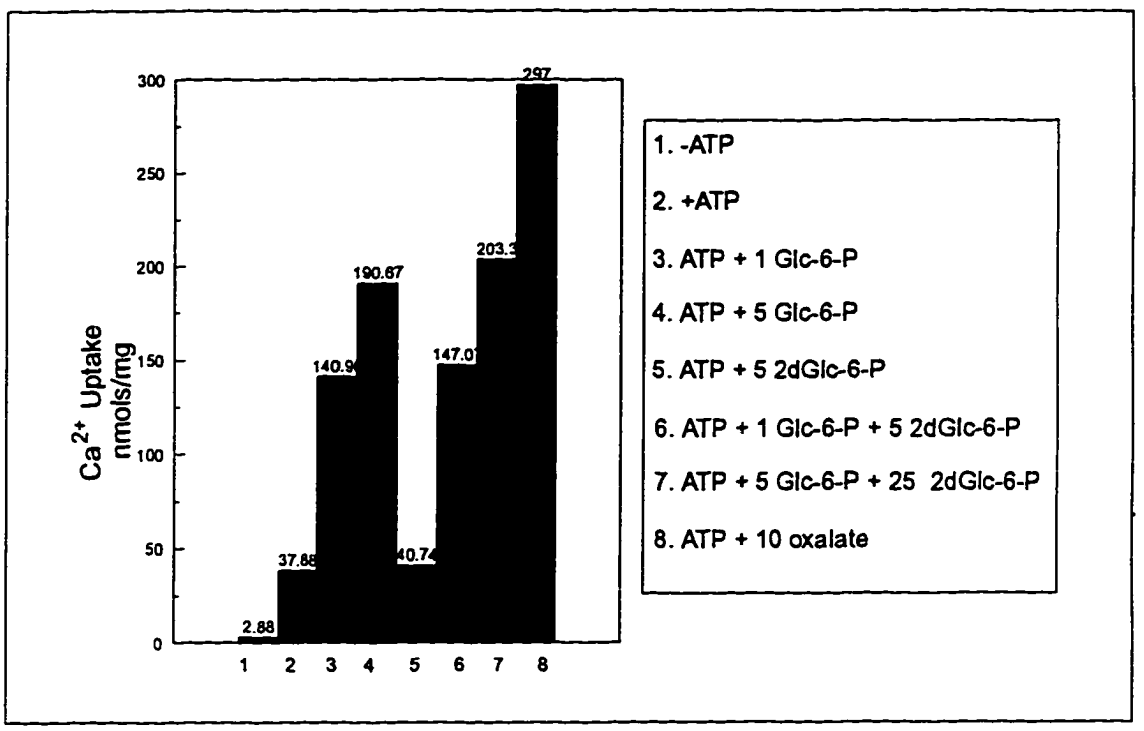

Fig. 2. $^{45}Ca^{2+}$ uptake in liver microsomes. $^{45}Ca^{2+}$ uptake assays were performed as described under experimental procedures. Incubation was performed for 30 min in an intracellular-like buffer containing mM amounts of the components indicated. ATP, when present, was added to a final concentration of 1 mM along with an ATP regenerating system.

caused an enhancement of Ca^{2+} uptake to levels similar to those previously reported by Benedetti et al (1985), 2dGlc-6-P had no effect. Also, 2dGlc-6-P was unable to compete with Glc-6-P when used at a 5-fold excess of Glc-6-P. Previous reports (Arion et al., 1972) indicate that in intact microsomes, where transport is the limiting factor, 2dGlc-6-P is a weak inhibitor of Glc-6-P hydrolysis (K_i 15 mM). Our results are consistent with this. We did not investigate the effects of further increasing the ratio between Glc-6-P and 2dGlc-6-P.

Glc-6-P levels in intact J774 cells. In intact J774 macrophages, we have shown that the removal of glucose or the addition of 2dGlc causes a partial depletion of ER Ca^{2+} stores. Based on the results shown previously and those published elsewhere (Benedetti et al., 1985), it is apparent that in liver, Glc-6-P can enhance the uptake of Ca^{2+} into the ER. If in J774 cells Glc-6-P has a similar effect on Ca^{2+} sequestration, it might provide for a simple explanation for our observation in intact, glucose-deprived cells. Therefore, Glc-6-P levels in intact cells were measured following 2dGlc treatment or glucose deprivation for 4 min. As shown in Figure 3, under these conditions there was a considerable drop in cellular Glc-6-P levels.

Hydrolysis of Glc-6-P in J774 Microsomes. Although the presence of Glc-6-phosphatase system in neutrophils and macrophages has been demonstrated with staining methods, it has not been biochemically characterized in these cells. We, therefore, measured hydrolysis of Glc-6-P in J774 microsomes. Using glucose dehydrogenase based assays, the hydrolysis rate was found to be 2.08+/-0.6 nmol/min/mg. This rate of hydrolysis of Glc-6-P is about 50-fold lower than in liver (reported value of approximately 100 nmol/min/mg).

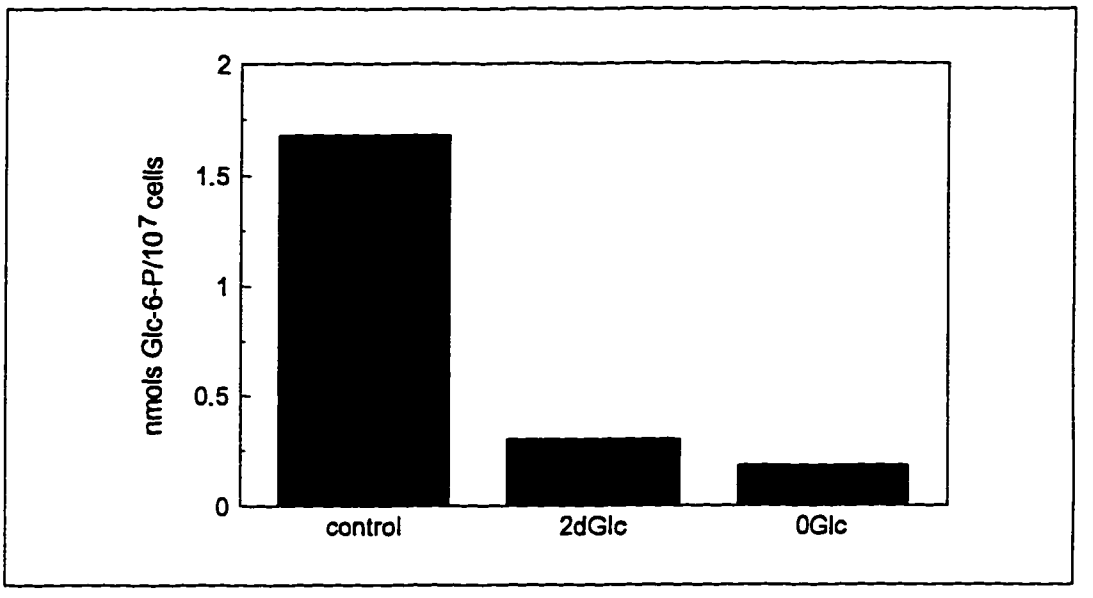

Fig. 3. Glc-6-P measurements in intact J774 cells. J774 cells were incubated for 4 min in HEPES-based saline buffer containing the following. 5 mM glucose and 5 mM pyruvate [control]; 5 mM glucose, 5 mM pyruvate, and 25 mM 2dGlc [2dGlc]; or 5 mM pyruvate [0Glc]. The cells were centrifuged and extracted with 1M $HClO_4$ at 4^0C. The extracts were neutralized with 5 M K_2CO_3, and the Glc-6-P content was assayed fluorimetrically using a Glc-6-P dehydrogenase-based assay as previously described (Lowry and Passonneau, 1972).

Benedetti et al. have suggested that in liver, the enhanced uptake of Ca^{2+} in the presence of Glc-6-P is due to a non-ionic association of Ca^{2+} with the Pi that is produced following the hydrolysis of Glc-6-P to glucose and Pi. As noted earlier, an alternate explanation has been proposed by others from our laboratory (Veyna-Burke, 1996). These investigators have suggested that the enhancement of Ca^{2+} uptake by Glc-6-P can be explained based on an association between Glc-6-P and Ca^{2+} within the lumen. Thus, in J774 microsomes, a similar situation might exist. In this case, hydrolysis of Glc-6-P is not the limiting factor.

^{14}C Glc-6-P Uptake in J774 Microsomes. In order to test this alternate hypothesis in J774 macrophages, we measured the uptake of [^{14}C]Glc-6-P into J774 microsomes. As shown in Figure 4, the concentration of Glc-6-P inside these microsomes was approximately the same as its concentration outside. Under similar conditions, the uptake of Glc-6-P in liver microsomes is comparable to that observed here. Phlorizin has been previously shown to competitively inhibit the uptake of Glc-6-P into liver microsomes (Arion et al., 1980). As shown in Figure 4, the inclusion of 1 mM phlorizin in the assay buffer led to a decrease in the amount of Glc-6-P accumulated within the J774 microsomes. This reflects the specificity of Glc-6-P uptake.

Glc-6-P Dependent Uptake of Ca^{2+} in Permeabilized J774 Cells. Having ascertained that a pathway for Glc-6-P import exists in J774 cells, we next tested the effect of Glc-6-P on Ca^{2+} uptake in permeabilized J774 cells. Ca^{2+} levels in the buffer(outside the ER) were measured with Fura-2. Thus, an uptake of Ca^{2+} is monitored as a decrease in fluorescence of the dye. The addition of ATP resulted in a rapid decrease of extra-organeller [Ca^{2+}]. Thapsigargin, an inhibitor of the ER Ca^{2+} ATPase, was then added. The

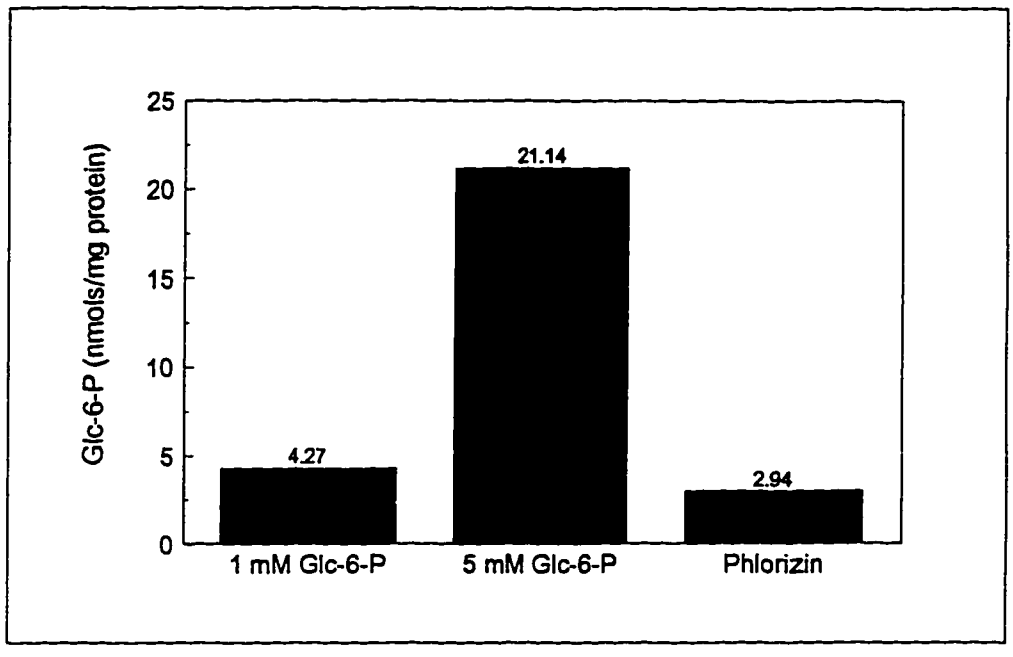

Fig. 4. [^{14}C] Glc-6-P uptake in J774 microsomes. Glc-6-P uptake was measured as described under experimental procedures in the presence of 1 mM Glc-6-P, 5 mM Glc-6-P, or 1 mM Glc-6-P + 2 mM phlorizin. The estimated concentration of Glc-6-P inside the microsomes under these conditions (assuming a volume of 4μl/mg of microsomal protein) is 1.06 mM, 5.29 mM, and 0.74 mM, respectively.

release of Ca^{2+} into the buffer following the addition of thapsigargin was used as a measure of the Ca^{2+} sequestered specifically within the ER. As shown in Figure 5, under control conditions, most of the decrease upon addition of 1 mM ATP is accounted for by uptake into the thapsigargin-releasable pool. An estimate of the total Ca^{2+} released by thapsigargin in an intact cell (Fig. 5, legend) shows that the level of Ca^{2+} uptake in the permeabilized system is comparable to that in an intact cell. However, under these conditions, no Glc-6-P dependent enhancement of Ca^{2+} uptake or release was apparent.

Glc-6-P Dependent Uptake of $^{45}Ca^{2+}$ in J774 Microsomes. In the experiments shown in Figure 2 and those reported previously (Benedetti et al., 1985) for liver microsomes, the uptake of Ca^{2+} was carried out at a higher extra-organeller Ca^{2+} concentration. In order to compare the data obtained with J774 microsomes with those in liver, $^{45}Ca^{2+}$ uptake experiments were carried out with J774 microsomes. Furthermore, in $^{45}Ca^{2+}$ experiments, the buffering effects of Ca^{2+}-binding dyes are avoided. The Ca^{2+} concentrations and other conditions used in these experiments were similar to those used for liver microsomes. The results from these investigations are shown in Figure 6. Two points are worth noting. First, Glc-6-P was unable to enhance ATP-dependent Ca^{2+} uptake, at all concentrations used. Second, even in the presence of oxalate, the amount of Ca^{2+} that could be trapped within these microsomes was much less compared to that in liver.

Assuming a volume of 4µl/mg protein, the Ca^{2+} concentration with ATP is nearly 1 mM while with the addition of oxalate, it increases to approximately 3.5 mM. From Figure 2, it is apparent that in liver microsomes the ATP-dependent uptake is around 7 mM while the maximal uptake obtained with oxalate reaches to nearly 75 mM. The half-maximal binding of Glc-6-P with Ca^{2+} has been reported to occur at ~ 7 mM (Luttrell, 1993). Thus,

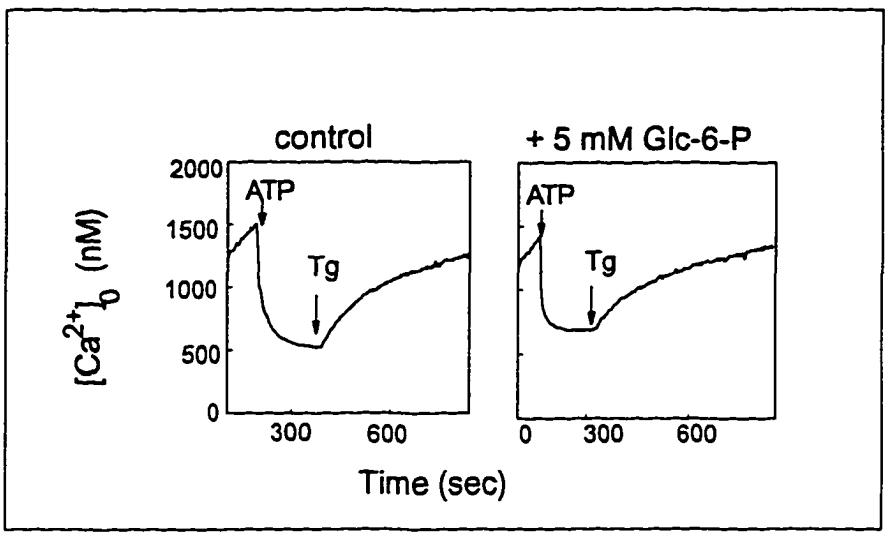

Fig. 5. Fura-2 based assay for the uptake of Ca^{2+} in permeabilized J774 cells.
J774 cells were suspended at 5 x 10^6 cells/ml. Permeabilization was carried out in an intracellular-like buffer by the addition of saponin (50 μg/ml). After 5 min, the cells were washed and resuspended in the assay buffer containing ATP regenerating system and 1 μM Fura-2, pentapotassium salt. Where indicated, 5 mM Glc-6-P was also present in the buffer. The uptake of Ca^{2+} was initiated by the addition of 1 mM ATP as shown. Thapsigargin (Tg, 200 nM) was added in order to estimate the amount of Ca^{2+} taken up into the ER. The 500 nM increase in extra-organeller Ca^{2+} corresponds to a Ca^{2+} release of 500 pmol/ml. The amount of Ca^{2+} released by a single cell would be about 10^{-7} nmol.

Estimation of thapsigargin-releasable Ca^{2+} in intact J774 cells was performed as follows. The area under the curve following the addition of thapsigargin to intact cells (calculated using the trapezoidal rule) was 189 μM. Assuming the diameter of a single cell to be 15 μm, the intracellular volume is 1.68 x 10^{-9} ml. Thus, in a single cell the amount of Ca^{2+} released by thapsigargin is ~ 3 x 10^{-7} nmol.

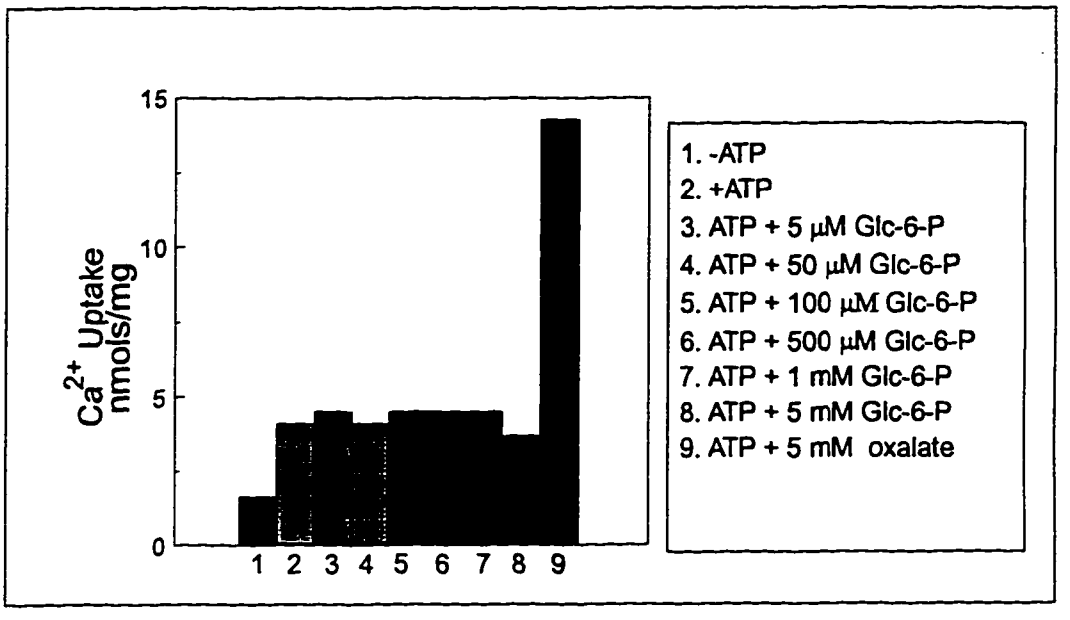

Fig. 6. $^{45}Ca^{2+}$ uptake in J774 microsomes. $^{45}Ca^{2+}$ uptake in J774 microsomes was assayed as described, for liver microsomes. Incubation was performed in an intracellular-like buffer with the indicated components.

it is reasonable to assume that the inability of Glc-6-P to enhance Ca^{2+} uptake in J774 microsomes is due to the lower levels of luminal Ca^{2+}.

Specificity of the Glc-6-P Importer in J774 Microsomes. From the results described thus far in permeabilized J774 cells and J774 microsomes, it is apparent that Glc-6-P is not able to mediate an enhancement in Ca^{2+} uptake into the ER in these cells. However, it appears that macrophages are capable of importing Glc-6-P into the ER. Furthermore, the results shown in Figure 4 support the idea that this import is mediated via a specific importer. Other investigators from our laboratory have observed that Glc-6-P importer in liver is distinct from that in brain and cardiac tissues. In microsomes prepared from brain or cardiac tissue, the microsomes are permeable to both 2dGlc-6-P and Glc-6-P in contrast to liver where only Glc-6-P can be imported (Fig. 1). As noted earlier, in GSD Ib patients the Glc-6-P import pathway in liver is compromised. This defect in liver is accompanied by a dysfunction in neutrophils. It was, therefore, of interest to see if the Glc-6-P import pathway in J774 microsomes is comparable to liver microsomes rather than cardiac or brain microsomes. Using light scatter experiments the permeability of J774 microsomes to 2dGlc-6-P was assessed. As shown in Figure 7, the permeability of 2dGlc-6-P was similar to that of Glc-6-P.

DISCUSSION

In this report, the Glc-6-phosphatase system present in liver microsomes is compared to that in J774 cells. The results presented here indicate the following differences between the two. The rate of hydrolysis of Glc-6-P in J774 microsomes is at least 50-fold lower than the values reported in liver. Consistent with this, in light scatter assays the uptake of Glc-6-P appears to be much slower in J774 microsomes. 2dGlc-6-P is relatively impermeant in liver

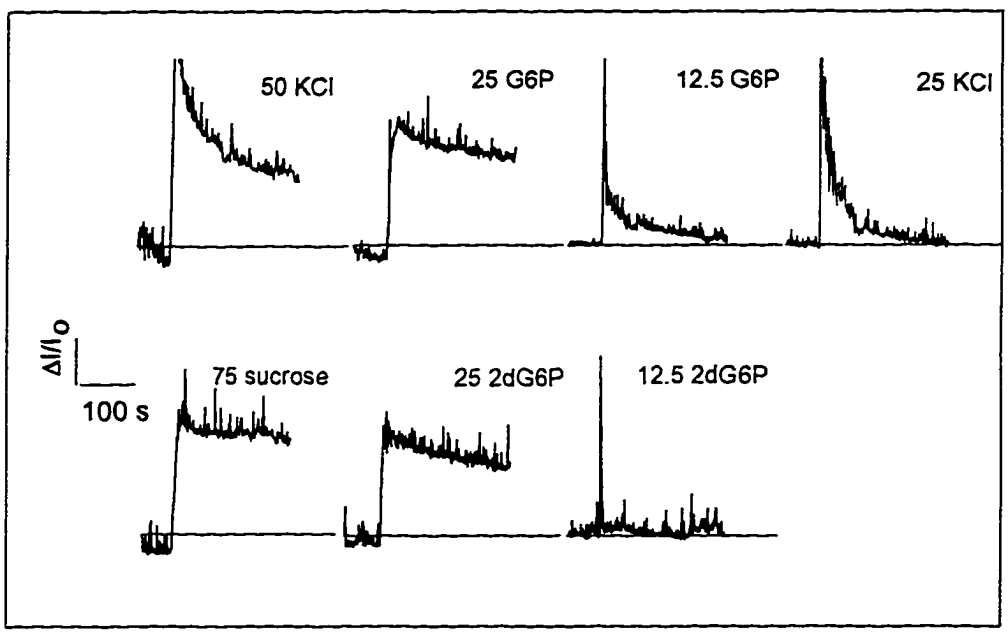

Fig. 7. Permeability of J774 microsomes to Glc-6-P and 2dGlc-6-P. Light scatter assays were performed as described for liver microsomes. [Fig. 1, legend]

whereas in J774 microsomes its uptake is similar to Glc-6-P. However, the Glc-6-P import pathway in J774 microsomes appears to be similar to the one in liver, in that phlorizin was able to compete with the import of Glc-6-P. Also, the concentration of Glc-6-P accumulated within J774 microsomes is nearly the same as its concentration outside. Under similar conditions (where there is no uptake of Ca^{2+} into the microsomes) such an observation has been made in liver microsomes as well.

With regard to a cooperation between the Glc-6-phosphatase and the Ca^{2+} ATPase systems, J774 microsomes are in marked contrast to liver microsomes. In the case of liver a 5- to 6-fold enhancement in ATP-dependent Ca^{2+} uptake is observed in the presence of Glc-6-P. No such Glc-6-P-mediated enhancement in Ca^{2+} sequestration was observed in J774 microsomes. As noted earlier, it appears that the Ca^{2+} concentration in J774 microsomes is much lower and may not be able to support an association between Glc-6-P and Ca^{2+}. It is also of interest to note that in brain and in liver, the Glc-6-P concentration inside the microsomes is different in the presence of ATP (and Ca^{2+}) than in its absence. In the absence of ATP and Ca^{2+}, Glc-6-P concentrations reach the same levels as that present outside (Veyna-Burke, 1996). However, when the Ca^{2+}-ATPase is active then accumulation of Ca^{2+} is accompanied with an increased accumulation of Glc-6-P. In J774 cells, Glc-6-P did not cause an enhancement in Ca^{2+} sequestration. Conversely, there was no enhancement in Glc-6-P accumulation in the presence of Ca^{2+} and ATP (data not shown).

Due to the observation that the Glc-6-P import pathway has no effect on Ca^{2+} sequestration in J774 cells, the questions that were addressed in the Introduction remain unanswered. In the case of GSD Ib, it is possible that human macrophages and neutrophils express a Glc-6-P import pathway that is similar to that found in liver. Thus the hypothesis

that a defective Glc-6-P importer in these phagocytic cells is causing their dysfunction in GSD Ib patients might still be valid. Alternatively, as suggested previously, the defect in Glc-6-P importer in liver might be accompanied with a different defect in the phagocytic cells. It has been observed that transport of glucose into the cell is inefficient in neutrophils from GSD Ib (Bashan et al., 1993).

In J774 cells, 2dGlc releases only a portion of the intracellular Ca^{2+} stores (Darbha and Marchase, 1996). Our hypothesis that decreased Glc-6-P levels directly cause the release is not valid. There are two alternate explanations for the observed release of ER Ca^{2+} stores in glucose-deprived cells. First, a component in addition to ATP might be required to maintain this 'glucose-sensitive' pool. For instance, cAMP and cGMP have been shown to alter Ca^{2+} uptake into the ER (Brune and Ullrich, 1992). It is possible that in glucose-deprived or 2dGlc-treated J774 cells, there is a down-regulation of a second messenger. This would lead to a decreased activity of the Ca^{2+}-ATPase and hence the partial release of Ca^{2+} from intracellular stores.

The second explanation is based on the presence of heterogeneous Ca^{2+} stores. It has been previously suggested that the ER Ca^{2+}-ATPase is specifically dependent on glycolytic ATP. The ER Ca^{2+}-ATPase and glycolysis within the cell are thought to be compartmentalized so as to be in close association with each other. In J774 cells, 2dGlc releases only a portion of the ER Ca^{2+} store. This suggests that only a portion of the ER stores is dependent on glycolysis, and the rest is fueled by mitochondrial ATP. Inconsistent with this assumption is our observation that rotenone has no effect on intracellular Ca^{2+} stores. It is possible that it is not the compartmentation of glycolysis that determines the efficient maintenance of intracellular Ca^{2+} stores, but rather the location of the ER stores of

Ca^{2+}. Thus, in the presence of rotenone, mitochondrial ATP generation is decreased, but the cells may be able to compensate for this loss by increased glycolysis. The sites of glycolytic ATP production might be sufficiently distributed so as to be able to maintain all of the intracellular Ca^{2+} stores. However, when glucose is removed, ATP that is generated mitochondrially might not be as accessible to some portions of the ER stores. These stores of Ca^{2+} would, therefore, be lost. Based on this suggestion it would follow that Ca^{2+} stores within the cell are heterogeneous. This has indeed been reported recently (Golovina and Blaustein, 1997).

REFERENCES

Arion, W.J., Lange, A.J., and Walls, H.E. (1980) Microsomal membrane integrity and the interactions of phlorizin with the glucose-6-phosphatase system. J. Biol. Chem., *255*:10387-10395.

Arion, W.J., Wallin, B.K., Carlson, P.W., and Lange, A.J. (1972) The specificity of glucose 6-phosphatase of intact liver microsomes. J. Biol. Chem., *247*:2558-2565.

Bashan, N., Potahnik, R., Peist, A., Peleg, N., Moran, A., and Moses, S.W. (1993) Deficient glucose phosphorylation as a possible denominator and its relation to abnormal leucocyte function, in glycogen storage disease 1b patients. Eur. J. Pediatrics, *152*:S44-S48.

Benedetti, A., Fulceri, R., and Comporti, M. (1985) Calcium sequestration activity in rat liver microsomes. Evidence for a cooperation of calcium transport with glucose-6-phosphatase. Biochim. Biophys. Acta., *816*:267-277.

Brune, B., and Ullrich, V. (1992) Cyclic nucleotides and intracellular-calcium homeostasis in human platelets. Eur. J. Biochem., *207*:607-613.

Burchell, A., and Waddell, I.D. (1991) The molecular basis of the hepatic microsomal glucose-6-phosphatase system. Biochim. Biophys. Acta, *1092*:129-137.

Darbha, S., and Marchase, R.B. (1996) Regulation of intracellular calcium is closely linked to glucose metabolism in J774 macrophages. Cell Calcium, *20*:361-371.

Forsyth, R.J., Bartlett, K., Burchell, A., Scott, H.M., and Eyre, J.A. (1993) Astrocytic glucose-6-phosphatase and the permeability of brain microsomes to glucose-6-phosphate. Biochem. J., *294*:145-151.

Fulceri, R., Bellomo, G., Gamberucci, A., Scott, H.M., Burchell, A., and Benedetti, A. (1992) Permeability of rat liver microsomal membrane to glucose 6-phosphate. Biochem. J., *286*:813-817.

Fulceri, R., Romani, A., Pompella, A., and Benedetti, A. (1990) Glucose 6-phosphate stimulation of MgATP-dependent Ca^{2+} uptake by rat kidney microsomes. Biochem. Biophys. Acta, *1022*:129-133.

Gitzelmann, R., and Bosshard, N.U. (1993) Defective neutrophil and monocyte functions in glycogen storage disease type Ib: a literature review. Eur. J. Pediatr., *152*:S33-S38.

Golovina, V.A., and Blaustein, M.P. (1997) Spatially and functionally distinct Ca2+ stores in sarcoplasmic and endoplasmic reticulum. Science, *275*:1643-1648.

Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem., *260*:3440-3450.

Inesi, G., and de Meis, L. (1989) Regulation of steady state filling in sarcoplasmic reticulum. roles of back-inhibition, leakage, and slippage of the calcium pump. J. Biol. Chem., *264*:5929-5936.

Kilpatrick, L., Garty, B.Z., Lundquist, K.F., Hunter, K., Stanley, C.A., Baker, L., Douglas, S.D., and Korchak, H.M. (1990) Impaired metabolic function and signaling defects in phagocytic cells in glycogen storage disease type 1b. J. Clin. Invest., *86*:196-202.

Korchak, H.M., Garty, B.Z., Stanley, C.A., Baker, L., Douglas, S.D., and Kilpatrick, L. (1993) Impairment of calcium mobilization in phagocytic cells in glycogen storage disease type 1b. Eur. J. Pediatr., *152*:S39-S43.

Luttrell, I. (1993) The biological relavance of the binding of calcium ions by inositol phosphates. J. Biol. Chem., *268(3)*:1521-1524

Lowry, O.H., and Passonneau, J.V. (1972) A flexible system of enzymatic analysis (New York and London: Academic Press).

Perez, M., and Hirschberg, C.B. (1987) Transport of sugar nucleotides into the lumen of vesicles derived from rat liver rough endoplasmic reticulum and golgi apparatus. Methods Enzymol. *138*:709-715.

Sung, S.S., and Silverstein, S.C. (1985) Role of 2-deoxy-D-glucose in the inhibition of phagocytosis by mouse peritoneal macrophage. Biochim. Biophys. Acta, *845*:204-215.

Veyna-Burke, N.A. (1996) Glycosylation and function of phosphoglucomutase in rat cerebral cortex. In Department of Cell Biology (Dissertation, University of Alabama at Birmingham).

INHIBITION OF CAPACITATIVE CALCIUM ENTRY BY GLYCOLYTIC INHIBITORS AND GLUCOSAMINE: INVOLVEMENT OF A CELLULAR METABOLITE DOWNSTREAM OF ATP GENERATION

by

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Submitted to Journal of Biological Chemistry

Format adapted for dissertation

SUMMARY

Capacitative Ca^{2+} entry pathway in J774 macrophages is inhibitied within 4 min of 2-deoxy-D-glucose (2dGlc) treatment or glucose deprivation (Darbha and Marchase. 1996). In this report we demonstrate that staurosporine, a microbial alkaloid that has been widely used as a broad range serine-threonine kinase inhibitor, reverses the 2dGlc-mediated inhibition of this Ca^{2+} influx pathway. However, the reversal appeared to be independent of PKC or PKA. Staurosporine was also able to reverse the inhibition in capacitative Ca^{2+} entry seen in the presence of cytochalasin B and iodoacetic acid. Although it appeared that staurosporine was able to overcome the requirement for ATP, by itself, it did not alter cellular ATP levels.

Glucosamine and mannosamine were also found to inhibit capacitative Ca^{2+} influx. However, the inhibitory action of these amino sugars appeared to be distinct from that of 2dGlc. This was based on our observations that the decrease in ATP levels following a 4 min incubation in these sugars was not as pronounced as that caused by 2dGlc. Also, the inhibition of capacitative Ca^{2+} influx in the presence of the amino sugars was not reversed by staurosporine.

INTRODUCTION

In macrophages and other non-excitable cells, Ca^{2+} influx across the plasma membrane is triggered following a release of Ca^{2+} from intracellular, IP_3-sensitive stores. The formation of IP_3 is not a prerequisite for this influx to be initiated (Kwan et al., 1990). A release of Ca^{2+} from intracellular stores by any number of ways, including an inhibition of the ER-Ca^{2+}-ATPase, can cause this influx pathway to be activated. Since the entry of Ca^{2+} appears to be regulated by the state of filling of the intracellular stores it

has been referred to as the capacitative influx of Ca^{2+} (Putney, 1986). Recently, the plasma membrane channel that allows such an influx of Ca^{2+} in mammalian cells has been shown to be homologous to the drosophila trp/trpl proteins (Philipp et al., 1996) and coexpression of these Drosophila proteins reconstitutes capacitative influx in Xenopus oocytes (Gillo et al., 1996).

The precise mechansim of activation of the capacitative Ca^{2+} entry is unkown. Several second messengers and protein kinases have been implicated in regulating the activation state of the capacitatvie influx pathway (for a review, see Berridge, 1995). These include a diffusible second messenger (Randriamampita and Tsien, 1993), cGMP (Bischof et al., 1995), tyrosine kinases (Sargeant et al., 1993), PKA (Chen et al., 1993), and PKC (Petersen and Berridge, 1994). G proteins, heterotrimeric (Jaconi et al., 1993) as well as small molecular weight G proteins (Fasolato et al., 1993), have been proposed to play regulatory roles in the capacitative entry pathway.

We have previously shown that 2-deoxy-D-glucose (2dGlc) treatment or glucose deprivation of J774 macrophages causes an inhibition in the capacitative entry of Ca^{2+} (Darbha and Marchase, 1996). 2dGlc is converted to 2dGlc-6-P by hexokinase but is not metabolized further by glycolytic enzymes. Our results are in agreement with those in MDCK cells (Lien et al., 1995). Lien et al. (1995) have shown that in these cells the capacitative influx of Ca^{2+} can be enhanced by the addition of extracellular glucose. In light of the reports that capacitative influx of Ca^{2+} is sensitive to cellular ATP levels (Gamberucci et al., 1994; Marriott and Mason, 1995), our results were not unexpected. However, it is worth noting that in J774 cells ATP levels following incubation with the mitochondrial inhibitor, rotenone, were similar to those seen under glucose-free

conditions. However, rotenone had little effect on the capacitative influx of Ca^{2+}. Thus, it appears plausible that in certain cells the capacitative influx of Ca^{2+} is exclusively dependent on the presence of glycolytic ATP. Alternatively, some other metabolite of glucose may be involved.

In an attempt to further elucidate these possibilities, two lines of investigation, were used. The first approach was based on the use of alternate monosaccharides that might exert the same inhibitory effects as 2dGlc. If a metabolite other than glycolytic ATP is involved, then the metabolism of these alternate sugars might shed light on the 2dGlc block.

Our second line of investigation was based on the assumption that 2dGlc-mediated inhibition of capacitative influx is a result of an inhibition of a specific signal transduction event. We, therefore, attempted to either mimic or reverse the 2dGlc block on capacitative influx using inhibitors/activators of protein kinases that have already been implicated in the regulation of this pathway. Here, we report the results of these investigations.

EXPERIMENTAL PROCEDURES

Cell Culture. J774 cells (American Type Culture Collection, Rockville, MD, USA) were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco-BRL, Gaithersburgh, MD, USA) and 1% penicillin/streptomycin.

Buffers and Stimulants. HEPES based saline solution (HBS): 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 10 mM $NaHCO_3$, 1 mM $CaCl_2$, 20 mM HEPES, pH 7.3.

Measurement of Cytoplasmic Ca^{2+} with Fura-2/AM. J774 cells were washed in serum-free DMEM and resuspended in DMEM containing 1 mg/ml BSA and 2.5 mM probenecid. Probenecid was added to decrease the leakage of Fura-2 (DiVirgilio, 1988). 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., S. Brunswick, NJ, USA) 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 500 nm with excitation wavelengths of 340 and 380 nm. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. (1985): $[Ca^{2+}]_i = K_d \times (R-R_{min})/ (R_{max}-R) \times S_{f2}/S_{b2}$, where K_d is the Fura-2 dissociation constant for Ca^{2+} (224 nM), R is the ratio of the intensities at 340 nm and 380 nm, and R_{min} and R_{max} are the R values at 0 and saturating levels of Ca^{2+} respectively. S_{f2}/S_{b2} is the ratio of the intensities at 380 nm excitation under R_{min} and R_{max} conditions.

Assay for Intracellular ATP Levels. Cellular ATP levels were determined using an ATP assay kit (Calbiochem, San Diego, CA, USA) based on firefly luciferase-catalyzed oxidation of d-luciferin. The emitted light was quantitated by luminometry (Analytical Luminescence Laboratory). Cells (10^6/ml) were incubated in HBS containing the indicated nutrients or inhibitors for 4 min at 37°C. The cells were then centrifuged and extracted with 1 M $HClO_4$ at 4°C. The extracts were neutralized with 5 M K_2CO_3. A 10 µl aliquot of the extract was added to 400 µl HBS buffer, and the reaction was initiated by addition of the luciferase.

RESULTS

Inhibition of Capacitative Ca^{2+} Influx by Glucosamine and Mannosamine. We have previously reported that 2dGlc inhibits the capacitative Ca^{2+} influx pathway in J774 macrophages. Here, we investigated the possibility that an alternate glucose analogue may have similar effects on Ca^{2+} signaling. To this end, several monosaccharides were tested. Under control conditions, addition of thapsigargin to fura-2-loaded J774 cells led to an activation of Ca^{2+} influx (Fig. 1). This irreversible inhibitor of the ER Ca^{2+}-ATPase causes the depletion of intracellular stores of Ca^{2+} and thereby the activation of the capacitative influx of Ca^{2+} (Kwan, 1990). The plateau value of Ca^{2+} presumably represents the balance between Ca^{2+}-extruding mechanisms active at the plasma membrane and the influx of Ca^{2+}. An elevated plateau value of $[Ca^{2+}]_i$ (~ 100 nM higher than the starting baseline under control conditions) is attributed to the sustained activation of the capacitative influx pathway. The activation of capacitative Ca^{2+} influx in the presence of thapsigargin was assessed following a 4 min incubation in the sugars indicated. As shown in Figure 1, galactose, mannose, N-acetyl glucosamine, and 3-0-methyl glucose were without effect. However, glucosamine and mannosamine caused an inhibition in the capacitative influx of Ca^{2+}.

Staurosporine Reverses the 2dGlc Block on Capacitative Ca^{2+} Influx. As mentioned in the introduction, we speculated that local changes in the metabolism of glucose could lead to an alteration in the activation state of a protein kinase thereby inhibiting capacitative Ca^{2+} entry. Staurosporine is a microbial alkaloid that was initially used as an inhibitor of PKC but has since been shown to be a broad range inhibitor of protein kinase activity (Meggio et al., 1995). It has been shown that staurosporine

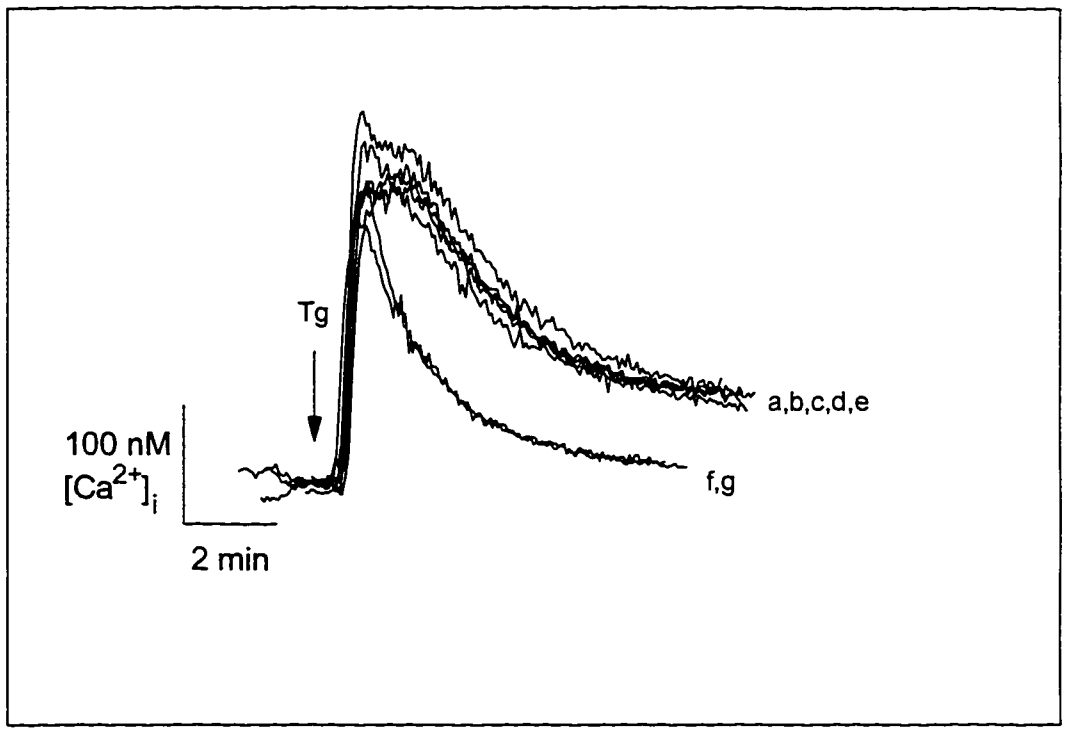


Fig. 1. Inhibition of capacitative Ca^{2+} entry by glucosamine and mannosamine. Fura-2-loaded J774 cells were suspended in HBS containing 5 mM Glc and 5 mM pyruvate. Incubation in the presence of various monosaccharides (25 mM) was performed for 5 min. Thapsigargin (Tg) was then added in order to deplete the intracellular Ca^{2+} stores and initiate Ca^{2+} influx via the capacitative pathway. a is control: b,c.d and e are responses in the presence of galactose, N-acetylglucosamine, mannose, and 3-O-methylglucose, respectively; f and g indicate the presence of glucosamine and mannosamine, respectively. The results shown are representative of three similar experiments.

augments capacitative Ca^{2+} influx in rat parotid acinar cells (Tojyo et al., 1995). Also, in Xenopus oocytes the half-time of inhibition of Ca^{2+} entry by GTPγS was found to be increased by staurosporine (Petersen and Berridge, 1995).

The effect of staurosporine on capacitative Ca^{2+} influx in J774 macrophages was, therefore, tested. In control cells, the addition of thapsigargin caused a rapid increase in $[Ca^{2+}]_i$ which then equilibrated to a value that is ~ 100 nM higher than the initial, baseline value of $[Ca^{2+}]_i$. As shown previously (Darbha and Marchase, 1996), the capacitative Ca^{2+} influx, in response to the addition of thapsigargin, was markedly inhibited when these cells were pretreated with 2dGlc for 4 min. We found that a 5 min incubation with staurosporine, either prior to or after 2dGlc addition, was able to partially reverse the inhibition seen in the presence of 2dGlc alone (Fig. 2). However, unlike the finding in rat parotid acinar cells (Tojyo et al., 1995) staurosporine itself did not affect the capacitative Ca^{2+} entry pathway in J774 cells even at concentrations of up to 150 nM.

The inhibition of the capacitative Ca^{2+} influx by 2dGlc can also be demonstrated by its addition after the pathway has been activated. This is shown in Figure 3A. Here, fura-2-loaded cells were treated with thapsigargin; and once a plateau value for Ca^{2+} entry was established, 2dGlc was added. Within ~ 1 min of its addition Ca^{2+} influx decreased. The subsequent addition of 40 nM staurosporine caused the $[Ca^{2+}]_i$ to return to the initial, higher plateau value. Furthermore, the addition of staurosporine prior to 2dGlc prevented the drop in Ca^{2+} influx normally seen with 2dGlc (Fig. 3B). There was a slight decrease in $[Ca^{2+}]_i$ following 2dGlc addition, but this was only transient, and an influx of Ca^{2+} to normal levels was established.

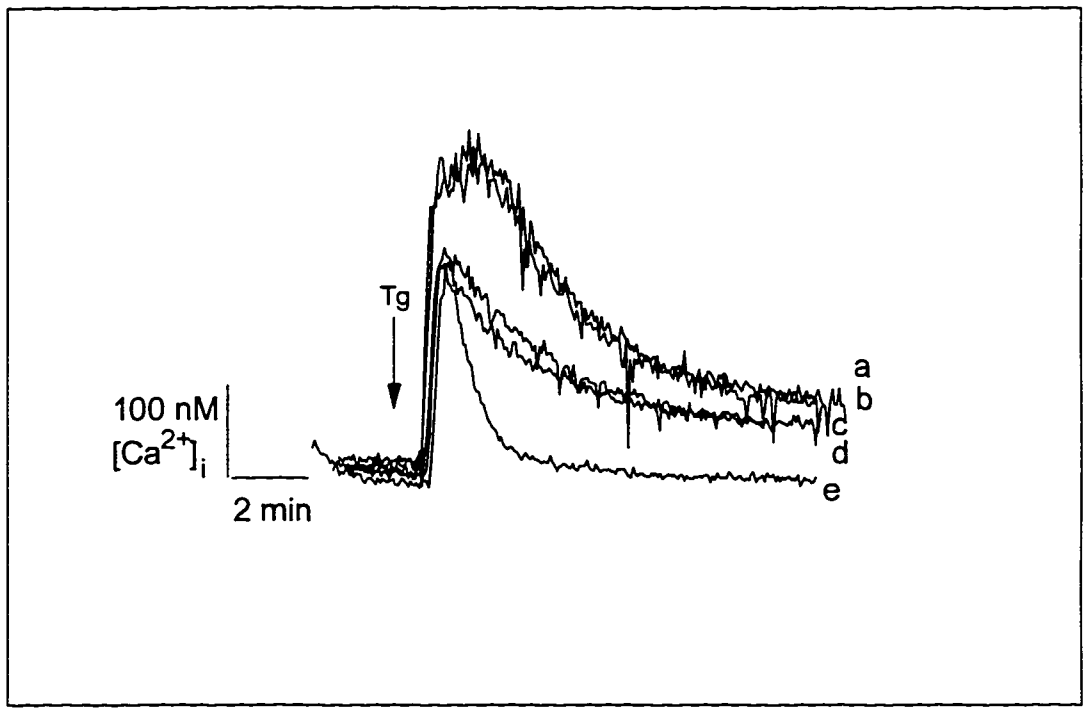


Fig. 2. Reversal of 2dGlc-mediated inhibition of capacitative Ca^{2+} influx by staurosporine. Fura-2 loaded cells were incubated in HBS containing 5 mM Glc and 5 mM Pyr. Prior to the addition of thapsigargin (Tg, 200nM), incubations were performed as follows. a: control; b: staurosporine (stp, 40 nM) for 4 min; c: stp for 4 min and an additional 4 min in 2dGlc (25 mM); d: 2dGlc for 4 min followed by stp for 4 min; and e: 2dGlc for 4 min. The data shown represents one of four similar experiments.

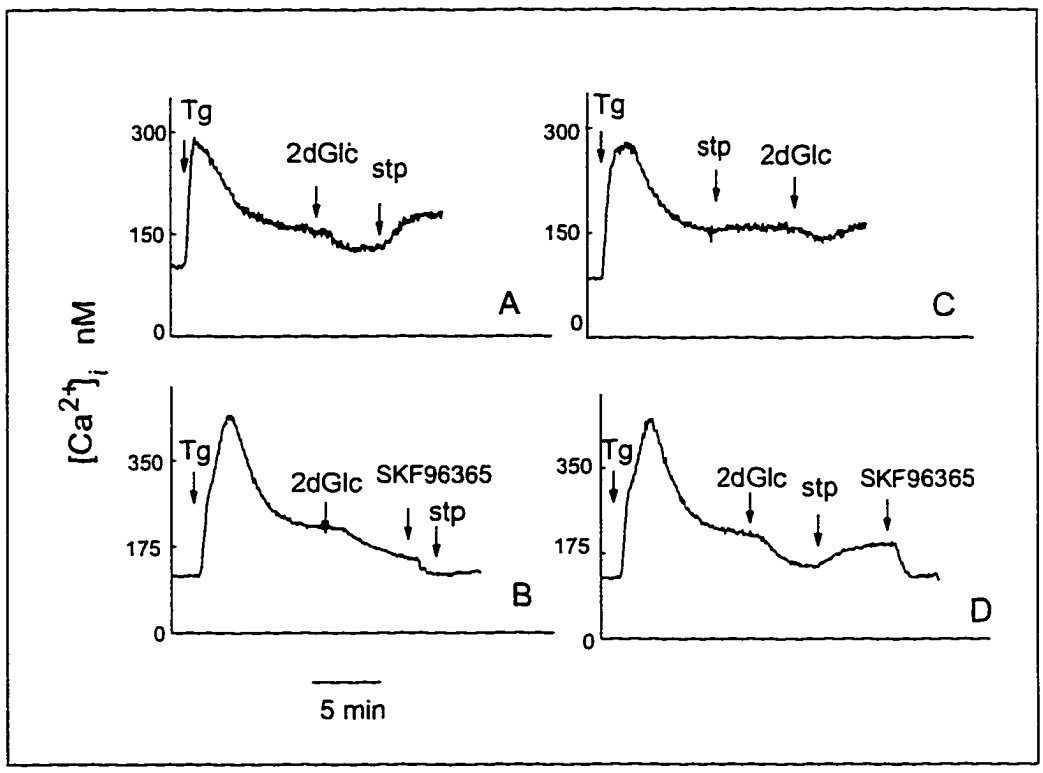


Fig. 3. SKF96365 blocks the ability of staurosporine to reverse 2dGlc-mediated inhibition in capacitative Ca^{2+} influx. Fura-2-loaded J774 cells were suspended in HBS containing 5 mM Glc and 5 mM Pyr. Thapsigargin (Tg, 200 nM) was added as indicated. Once a stable plateau value of $[Ca^{2+}]_i$ was reached 25 mM 2dGlc, 40 nM staurosporine (stp), and 20 μM SKF 96365 were added as indicated.

The restoration of Ca^{2+} influx by staurosporine was also examined in the presence of SKF 96365, a known inhibitor of Ca^{2+} influx via the capacitative entry pathway (Merritt et al., 1990). In the presence of this compound, staurosporine was not able to reverse the 2dGlc-mediated inhibition (Fig. 3C). Also, the addition of SKF 96365 following the recovery of Ca^{2+} influx by staurosporine caused the $[Ca^{2+}]_i$ to return to baseline (Fig. 3D). This, as well as the finding that staurosporine itself caused no increase over the normal capacitative Ca^{2+} plateau (Figs. 2 and 3C), suggests that the effect of staurosporine on Ca^{2+} influx is not due to an independent mechanism of Ca^{2+} entry.

Based on previous reports on the involvement of protein kinases in the capacitative pathway for Ca^{2+} entry, the most apparent targets for staurosporine are PKC and PKA (Petersen and Berridge, 1995; Parekh and Penner, 1995). To address if staurosporine is having its effect via inhibition of PKC, the effects of alternate PKC inhibitors as well as that of PMA, an activator of PKC, were examined. The PKC inhibitors, K252a, bisindolylmaleimide, calphostin C, and H-7, were all unable to mimic the effect of staurosporine (Fig. 4A and data not shown). If inhibition of PKC by staurosporine can account for the restoration of Ca^{2+} influx in 2dGlc-treated cells, then its activation by PMA might be expected to cause a decrease in Ca^{2+} influx. However, PMA did not have any modulatory effects on the capacitative influx pathway in these cells in the presence or absence of 2dGlc (Fig. 4A and data not shown). PMA has been previously shown to inhibit the Ca^{2+} response to IgG in these cells (DiVirgilio, 1988). The fact that in these experiments PMA is able to suppress the response to IgG excludes the possibility that under the conditions/concentrations used, the phorbol ester was ineffective in activating PKC (Fig. 4B).

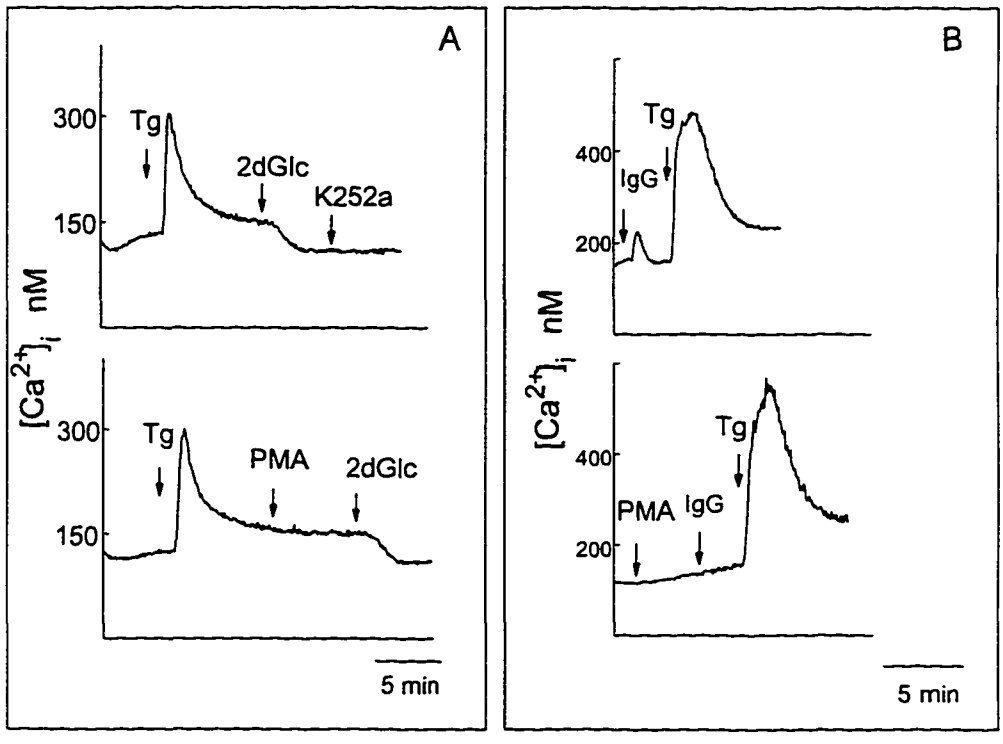


Fig. 4. Effects of PMA and K252a on capacitative Ca^{2+} entry. Fura-2 loaded cells were suspended in HBS containing 5 mM Glc and 5 mM Pyr. (A) Upper panel: thapsigargin (Tg) was added, as indicated. After a plateau value was reached, 25 mM 2dGlc was added. This was followed by the addition of 100 nM K252a. Lower panel: Tg was added followed by 60 nM PMA and 2dGlc, as indicated. (B) Upper panel: Aggregated IgG , prepared as previously described (Darbha and Marchase, 1996), was added followed by Tg. Lower panel: PMA was added followed by IgG and Tg.

A dose response with staurosporine (Fig. 5) also revealed that its effect is not likely to be due to PKC inhibition since the IC_{50} for PKC inhibition has been reported to be ~ 5 nM (Meggio et al., 1995), whereas a concentration of ~ 18 nM is reached before there is a half-maximal reversal of the inhibition of capacitative Ca^{2+} influx in our experiments.

It also appears unlikely that, under the conditions used, staurosporine is inhibiting PKA. At the concentrations used, K252a and H-7 also inhibit PKA. But these inhibitors had no modulatory effect on Ca^{2+} influx (Fig. 4A and data not shown). Also, activation of PKA by the addition of membrane permeant analogues of cAMP at a concentration of 5 μM did not inhibit the capacitative influx of Ca^{2+} (Fig. 6A). The subsequent addition of 2dGlc caused a decrease in Ca^{2+} influx.

When used at higher concentrations (0.5 mM) db- or 8CPT-cAMP caused an increase in Ca^{2+} influx (Fig. 6B). Several other investigators have noted this enhancement in Ca^{2+} influx by cAMP (Kass et al., 1994; Denning et al., 1994; Tornquist and Ahlstrom, 1993). Petersen and Berridge (1995) have shown that dbcAMP has a biphasic effect on the capacitative Ca^{2+} entry pathway, inhibiting influx at lower, micromolar concentrations and potentiating at higher, millimolar concentrations. The mechanism of this cAMP-mediated increase was not further investigated in these cells. However, it was observed that the decrease of Ca^{2+} influx by 2dGlc was not prevented by pretreatment with cAMP (Fig. 6A and B). The fact that in these cells, cGMP does not mimic the effect of cAMP(Fig. 6C) indicates that the effect of higher cAMP concentration is not due to a crosstalk between cAMP- and cGMP-dependent kinases.

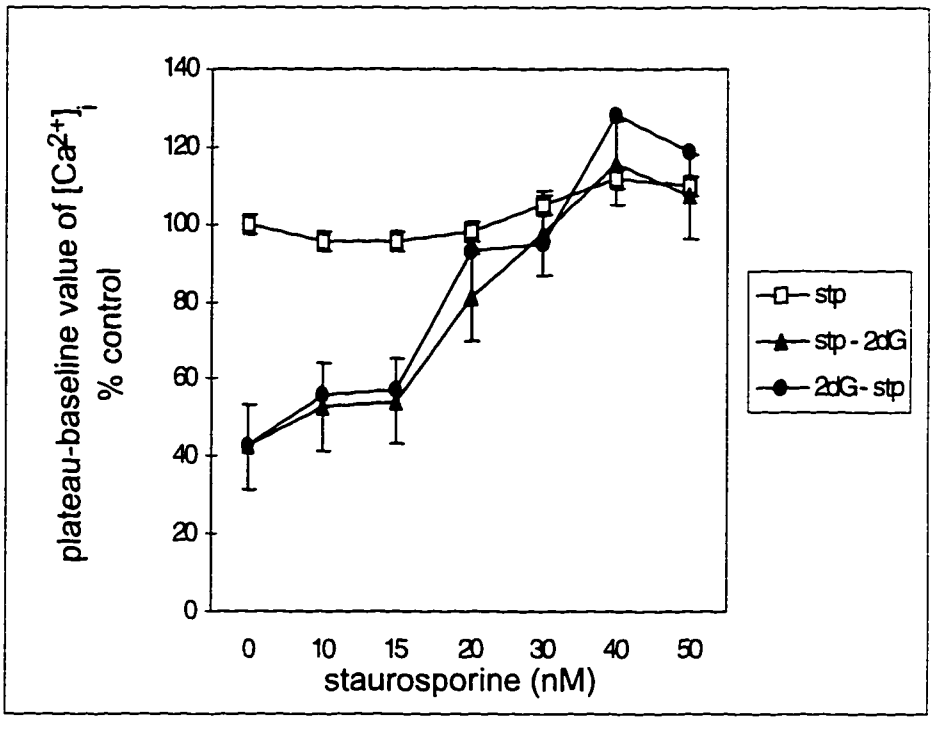


Fig. 5. Dose-dependent effect of staurosporine on capacitative Ca^{2+} influx. Thapsigargin was added to Fura-2-loaded cells suspended in HBS containing 5 mM Glc and 5 mM Pyr. At each of the stp concentrations used, the plateau value of Ca^{2+} was measured following the addition of Tg, stp, and 2dGlc, in the order indicated. (a) Tg alone ; (b) Tg, stp; (c) Tg, stp, 2dGlc; and (d) Tg, 2dGlc, stp. The values plotted (as %) are as follows. Stp: b/a; stp-2dGlc:c/a; 2dGlc-stp: d/a .

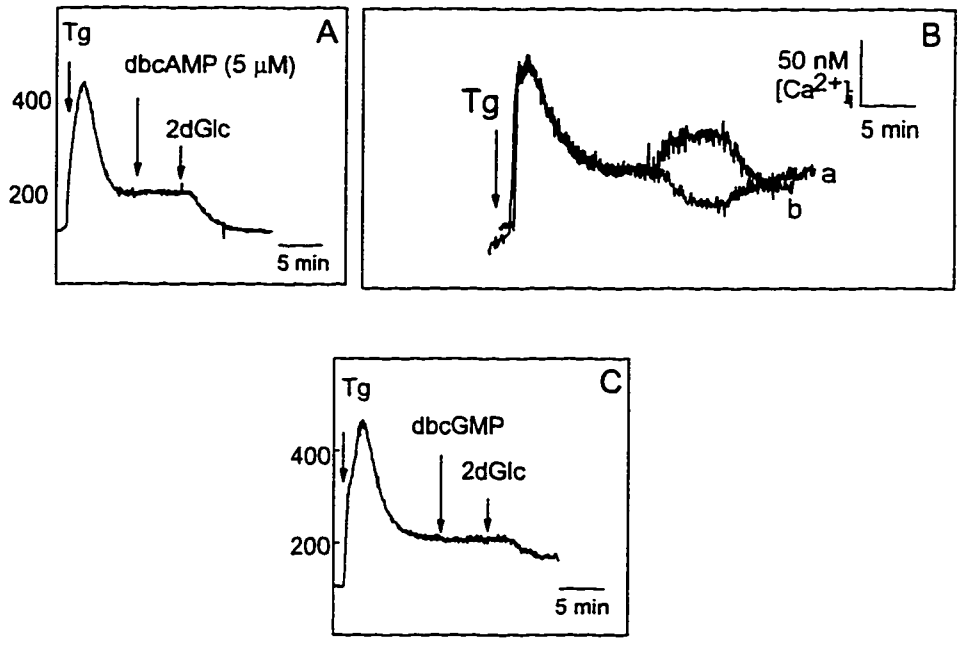


Fig. 6. Effects of membrane permeant analogues of cAMP and cGMP. Fura-2 loaded cells were suspended in HBS containing 5 mM Glc and 5 mM Pyr. (A) Thapsigargin (Tg) was added as indicated. This was followed by the addition of 5 μM dbcAMP and the subsequent addition of 25 mM 2dGlc. (B) Following the activation of capacitative Ca^{2+} influx in the presence of thapsigargin, 25 mM 2dGlc was added. This was followed by the addition of 0.5 mM 8CPTcAMP (curve a); in curve b the order of additions of 8CPT-cAMP and 2dGlc was reversed. (C) Thapsigargin, 0.5 mM dbcGMP, and 2dGlc were added, as indicated. Experiment shown is representative of three similar tracings.

Intracellular ATP Levels Are Not Altered by Staurosporine. The effect of staurosporine on cellular ATP levels was also investigated. As shown in Figure 7, staurosporine had no effect on ATP levels in the presence or absence of 2dGlc. Thus, staurosporine is not reversing the 2dGlc-mediated inhibition by countering the effect of 2dGlc on intracellular ATP levels.

This is in contrast to the effect of glucose on 2dGlc-mediated inhibition in capacitative Ca^{2+} influx. As shown in Figure 8, an inhibition of capacitative Ca^{2+} entry can also be affected by the addition of 5 mM 2dGlc to cells suspended in media 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 with an increase in cellular ATP levels (data not shown).

Staurosporine Does Not Reverse the Inhibition of Capacitative Ca^{2+} Entry by Glucosamine and Mannosamine. Although the mechanism of staurosporine reversal remained unclear, we asked if it could reverse the inhibition of the capacitative Ca^{2+} influx seen in the presence of amino sugars. As shown in Figure 9, staurosporine was unable to reverse the inhibition in capacitative Ca^{2+} influx that is brought about by the action of glucosamine and mannosamine.

Correlation Between ATP Decrease and Staurosporine Reversal. Measurement of ATP levels following a 4 min treatment with glucosamine or mannosamine indicated a decrease to 91-92% ($p > 0.1$) of control. This is in contrast to the much more substantial decreases obtained with 2dGlc (Fig. 7).

This suggested one of two possibilities:either the effect of staurosporine is limited to reversing only a 2dGlc-mediated inhibition, or staurosporine reversal is specific to

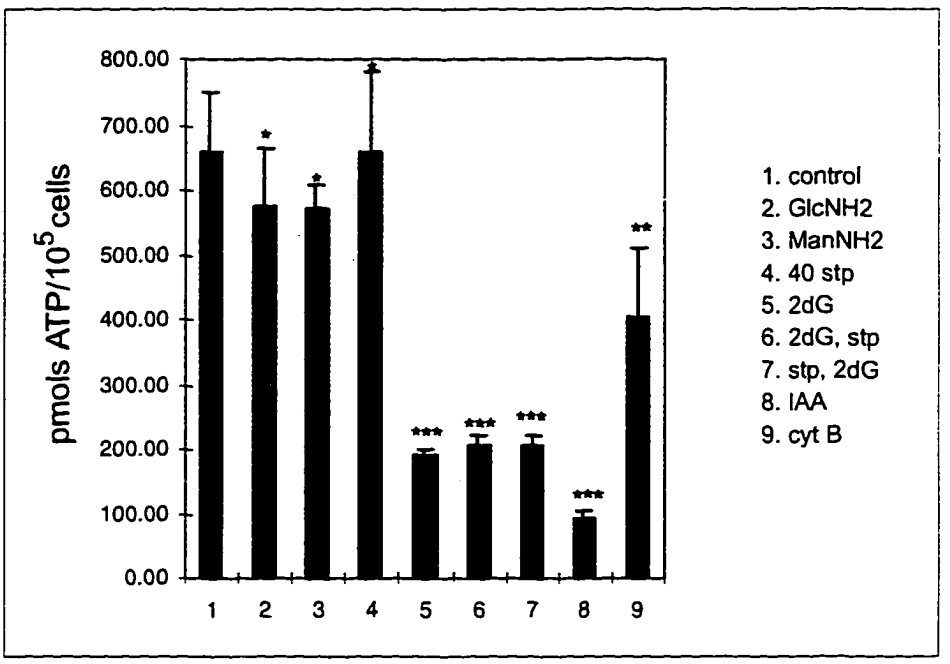


Fig. 7. Intracellular ATP measurements. ATP content was assayed as described under experimental procedures. The cells were incubated in HBS containing 5 mM Glc and 5 mM Pyr along with the indicated components. The concentrations used for the various additives are as follows. Glucosamaine and mannosamine: 25 mM; stp: 40 nM; 2dGlc: 25 mM; IAA: 1mM; cytB: 10 μM. The data obtained under test conditions were compared to control using the paired *t* test. * $p > 0.1$; ** $p > 0.02$; ***$p < 0.001$.

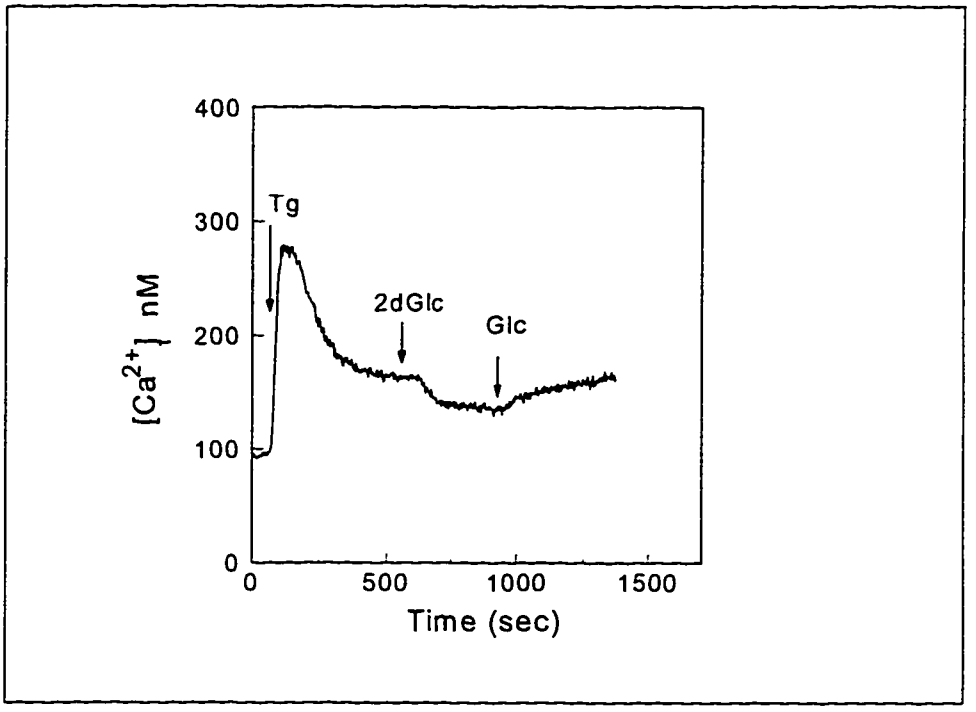


Fig. 8. Reversal of 2dGlc-mediated inhibition of capacitative Ca^{2+} influx by glucose. Fura-2 loaded cells were suspended in HBS containing 1 mM Glc and 5 mM Pyr. Thapsigargin was added as indicated. The addition of 5 mM 2dGlc leads to a decrease in Ca^{2+} influx. The subsequent addition of 10 mM glucose (Glc) causes the influx to return to normal.

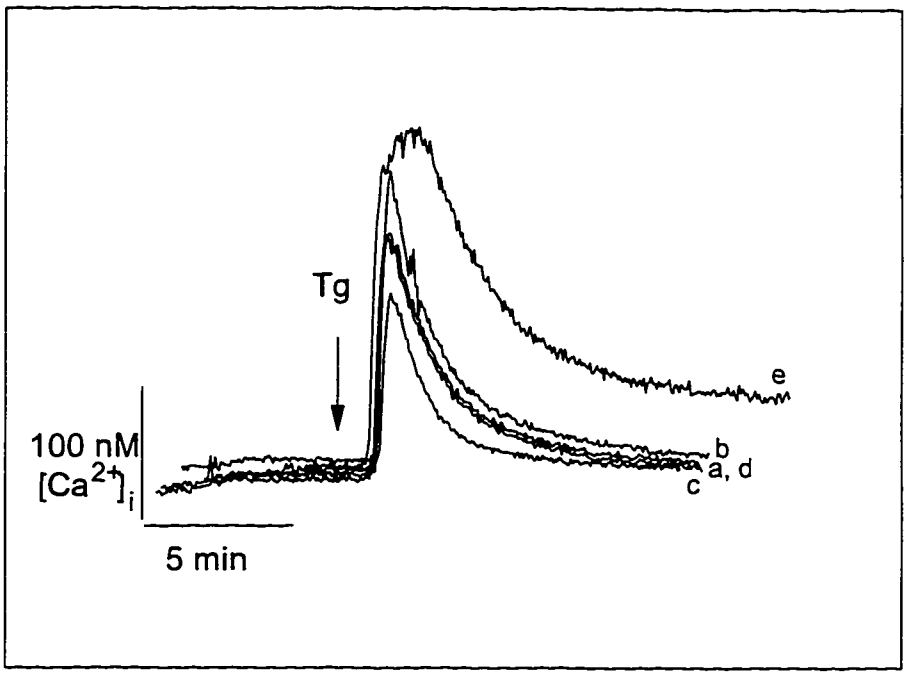


Fig. 9. Effect of staurosporine on capacitative Ca^{2+} influx in the presence of amino sugars.
Fura-2 loaded cells were suspended in HBS containing 5 mM Glc and 5 mM Pyr. Thapsigargin was added, as indicated. The cells were pretreated in the following manner: (a) 4 min in 25 mM glucosamine; (b) 4 min in 25 mM glucosamine followed by 4 min in stp; (c) 4 min in 25 mM mannosamine; (d) 4 min in mannosamine followed by 4 min in stp; and (e) no addition.

conditions accompanied by decreased ATP levels. In order to distinguish these possibilities we tried alternate ways of inhibiting glycolysis. As shown in Figure 10A. addition of cytochalasin B, an inhibitor of glucose transporter (Salter and Weber, 1979). caused a decrease in Ca^{2+} influx similar to that seen upon the addition of 2dGlc. This is in agreement with our previous results indicating that glucose deprivation also inhibits the influx of Ca^{2+} via the capacitative entry pathway. The subsequent addition of staurosporine restored the influx to normal. Dihydrocytochalasin B was used as a control. At concentrations of 1-10 μM, this compound has effects similar to cytochalasin B on the cytoskeleton but does not interfere with glucose transport (Atlas and Lin, 1978). As shown in Figure 10B, the addition of dihydrocytochalsin B had no effect on capacitative Ca^{2+} influx. As an additional means of inhibiting glycolysis, iodoacetic acid was used. As shown in Figure 10C, the addition of iodoacetic also caused a decrease in Ca^{2+} influx which was reversed by the subsequent addition of staurosporine. Thus, it appears that staurosporine is able to reverse compromised capacitative Ca^{2+} entry that is accompanied (or caused) by a decrease in cellular ATP levels.

DISCUSSION

We have previously shown an inhibition of capacitative Ca^{2+} influx within 4 min of 2dGlc treatment or glucose deprivation in J774 macrophages. Here, the mechanism responsible for the sensitivity of this pathway to glucose was investigated. We found that staurosporine, a microbial alkaloid that has been widely used as a broad range serine-threonine kinase inhibitor, is able to reverse the 2dGlc-mediated inhibition of the capacitative influx pathway. The decreases in influx, following alternate ways of inhibiting glycolysis were also reversed by staurosporine. The identity of staurosporine's

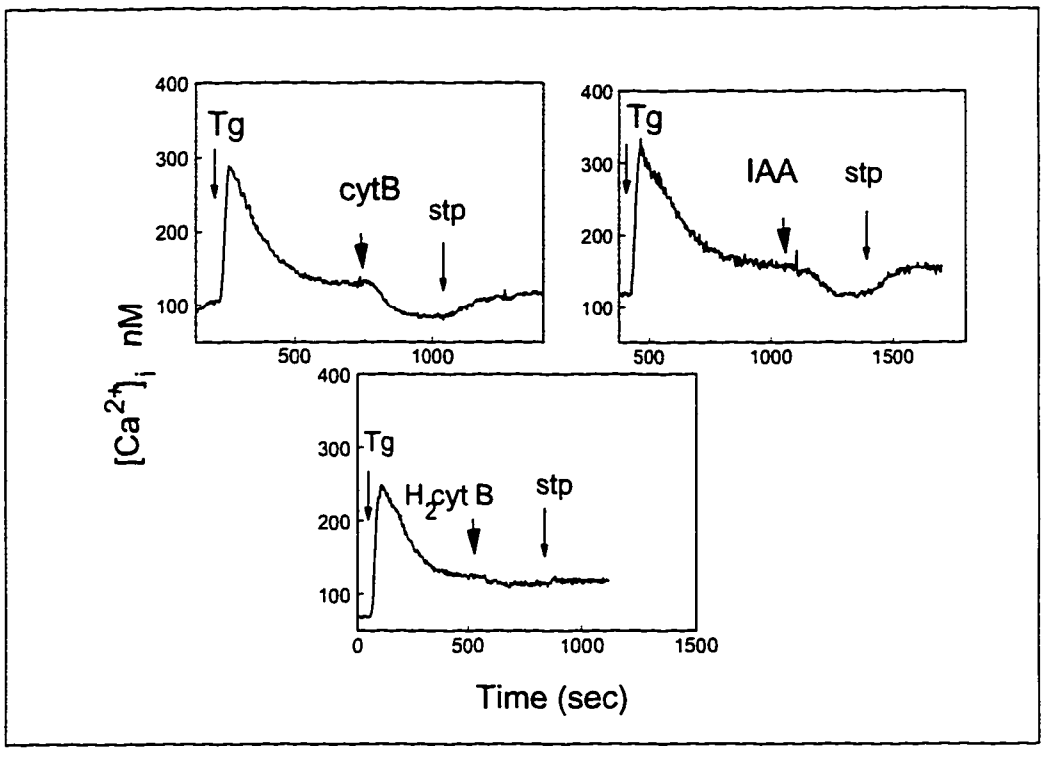


Fig.10. Inhibition of capacitative Ca^{2+} influx by cytochalasin B and iodoacetic acid: reversal by staurosporine. Fura-2-loaded cells were suspended in HBS containing 5 mM Glc and 5 mM Pyr. Thapsigargin was added as indicated. Cytochalasin B (cytB, 10 μM), dihydrocytochalasinB (10 μM), iodoacetic acid (IAA, 1mM), and staurosporine (stp, 40 nM) were added where indicated. The experiment was repeated three times with similar results.

target remains unclear. PKC and PKA are two serine-threonine kinases that have been found to have regulatory effects on capacitative Ca^{2+} influx. However, based on our efforts to define a role for these kinases in regulation of the capacitative Ca^{2+} influx in J774 cells, it appears that staurosporine is not relieving the 2dGlc-mediated inhibition by inactivation of PKC or PKA.

We also found that the amino sugars glucosamine and mannosamine inhibited capacitative Ca^{2+} influx. However, the inhibitory action of these amino sugars appears to be distinct from that of 2dGlc. This is based on our observations that in the presence of these sugars, inhibition of calcium entry could not be reversed by staurosporine. Also, the decrease in ATP levels in the presence of glucosamine and mannosamine was not as pronounced as with 2dGlc.

There appears to be a correlation between the ability of staurosporine to restore Ca^{2+} influx and decreased intracellular ATP levels. Thus, in all cases where an inhibition of capacitative pathway was accompanied by a decrease in cellular ATP, the addition of staurosporine was able to reverse the inhibition. In contrast, the inhibitory effects of glucosamine and mannosamine, which are not accompained by marked decrease in ATP levels, were not reversed by staurosporine.

Although it appeared that staurosporine was able to overcome the requirement for ATP, by itself, it did not alter cellular ATP levels. Thus, it is unlikely that ATP itself is the key molecule. This is also indicated by observations in RBL-2H3 cells. Parekh and Penner (1995) have shown that the activation of Ca^{2+} influx via the capacitative pathway was not inhibited in the absence of ATP. Furthermore, microinjection of 2 mM ATP in these cells led to an inactivation of this Ca^{2+} influx pathway. They suggest that the

decrease in Ca^{2+} influx involves activation of a protein kinase. There is an apparent discrepancy between these observations and those reported here in J774 cells. In RBL-2H3 cells, addition of ATP causes an inactivation of Ca^{2+} influx via the capacitative entry pathway, whereas the results reported here indicate that capacitative Ca^{2+} influx is inhibited following decreased ATP levels. In the experiments reported by Parekh and Penner (1995), the transition is from a complete absence of ATP to 2 mM ATP. In contrast, in our experiments, the comparison is between approximately 3.5 mM ATP, under control conditions [assuming a diameter of 15μm], to approximately 1.5 mM - 2.5 mM ATP in 2dGlc-treated cells or glucose-deprived cells, respectively. Since most of the known kinases have afffinities for ATP in the micromolar range, it is unlikely that the decreased ATP levels are directly inactivating or activating any kinase activity.

It is conceivable, though, that a cellular metabolite other than ATP is involved, the decrease of which is closely linked to that of ATP. As suggested by Gamberucci et al., alteration in GTP levels and possibly a small molecular weight G protein may be involved. In relevance to this, microinjection of GTPγS has been shown to inhibit capacitative Ca^{2+} entry. It is not clear whether this is via a small molecular weight G protein or a heterotrimeric G protein. Interestingly, it has been shown that, in an in vitro assay, staurosporine can enhance GTPase activity of pertussis toxin-sensitive G proteins isolated from neutrophils (Kanaho et al., 1992). Our data does not exclude the involvement of a G protein.

Based on the effects of glucosamine, a role for UTP or a pyrimidine nucleotide-sugar is likely. It is of interest to note here that microinjection of CDP-Glc or UDP-Glc in Xenopus oocytes has been reported to cause a release of intracellular Ca^{2+} as well as

activation of Ca^{2+} influx. Furthermore, in their efforts to isolate CIF, Kim et al. (1996) detected the presence of pyrimidine nucleotide-sugar derivatives in partially purified extracts from Jurkat lymphocytes. Thus, the interference with pyrimidine nucleotide-sugars might account for the inhibitory effects of the amino sugars on capacitative Ca^{2+} entry.

REFERENCES

Atlas, S.J., and Lin, S. (1978) Dihydrocytochalasin B: Biological effects and binding to 3T3 cells. J. Cell Biol., *76*:360-370.

Berridge, M.J. (1995) Capacitative calcium entry. Biochem. J., *312*:1-11.

Bischof, G., Brenman, J., Bredt, D.S., and Machen, T.E. (1995) Possible regulation of capacitative Ca2+ entry into colonic epithelial cells by NO and cGMP. Cell Calcium. *17*:250-262.

Chen, Y., Pollock, J.D., Wang, Y., DePaoli-Roach, A.A., and Yu, L. (1993) Protein kinase A modulates endogenous calcium channel, but not the calcium-activated chloride channel, in Xenopus oocytes. FEBS Lett., *336*:191-196.

Darbha, S., and Marchase, R. B. (1996) Regulation of intracellular calcium is closely linked to glucose metabolism in J774 macrophages. Cell Calcium, *20*:361-371.

Denning, G.M., Clark, R.A., and Welsh, M.J. (1994) Cyclic AMP and inositol 1,4,5 trisphosphate increase Ca^{2+} in HT-29 cells by activating different Ca^{2+} influx pathways. Am. J. Physiol., *267*:C776-C783.

DiVirgilio, F., Meyer, B.S., Greenberg, S., and Silverstein, S.C. (1988) F_c receptor-mediated phagocytosis occurs in macrophages at exceedingly low cytosolic Ca^{2+} levels. J. Cell Biol., *106*:657-666.

Fasolato, C., Hoth, M., and Penner, R. (1993) A GTP-dependent step in the activation mechanism of capacitative calcium influx. J. Biol. Chem., *268*:20737-20740.

Gamberucci, A., Innocenti, B., Fulceri, R., Banhegyi, G., Guinti, R., Pozzan, T., and Benedetti, A. (1994) Modulation of Ca^{2+} influx dependent on store depletion by intracellular adenine-guanine nucleotide levels. J. Biol. Chem., *269*:23597-23602.

Gillo, B., Chorna, I., Cohen, H., Cook, B., Manistersky, I., Chorev, M., Arnon, A., Pollock, J.A., Selinger, Z., and Minke, B. (1996) Coexpression of Drosophila TRP and TRP-like proteins in Xenopus oocytes reconstitutes capacitative Ca^{2+} entry. Proc.Natl. Acad. Sci., *93*:14146-14151.

Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem., *260*:3440-3450.

Jaconi, M.E.E., Theler, J.M., Schlegel, W., and Lew, P.D. (1993) Cytosolic free Ca^{2+} signals in single adherent human neutrophils: generation and functional role. Eur. J. Pediatr., *152*:S26-S32.

Kanaho, Y., Takahashi, K., Tomita, U., Iiri, T., Katada, T., Ui, M., and Nozawa, Y. (1992) A protein kinase C inhibitor, staurosporine, activates phospholipase D via a pertussis toxin-sensitive GTP-binding protein in rabbit peritoneal neutrophils. J. Biol. Chem., *267*:23554-23559.

Kass, G.E.N., Gahm, A., and Llopis, J. (1994) Cyclic AMP stimulates Ca^{2+} entry in rat hepatocytes by interacting with the plasma membrane carriers involved in receptor - mediated Ca^{2+} influx. Cellular Signalling, *6*:493-501.

Kim, H.Y., Thomas, D., and Hanley, M.R. (1996) Stimulation of Ca(2+)-dependent membrane currents in Xenopus oocytes by microinjection of pyrimidine nucleotide-glucose conjugates. Mol. Pharmacol. *49*:360-364.

Kwan, C. Y., Takemura, H., Obie, J.F., Thastrup, O., and Putney, J.W.,Jr. (1990) Effects of MeCh, thapsigargin, and La^{3+} on plasmalemmal and intracellular Ca^{2+} transport in lacrimal acinar cells. Amer. J. Physiol, *258*:C1006-C1015.

Lien, Y.-H.H., Wang, X., Gillies, R.J., and Martinex-Zaguilan, R. (1995). Modulation of intracellular Ca^{2+} by glucose in MDCK cells: role of endoplasmic reticulum Ca^{2+}-ATPase. Am. J. Physiol., *268*:F671-F679.

Marriott, I., and Mason, M.J. (1995) ATP depletion inhibits capacitative Ca^{2+} entry in rat thymic lymphocytes. Am. J. Physiol., *269*:C766-C774.

Meggio, F., Donella, D.A., Ruzzene, M., Brunati, A.M., and Pinna, L. (1995) Different susceptibility of protein kinases to staurosporine inhibition. Eur. J. Biochem., *234*:317-322.

Merritt, J.E., Armstrong, W.P., Benham, C.D., Hallam, T.J., Jacob, R., Jaxa-Chamiec, A., Leigh, B.K., and McCarthy, S.A. (1990) SKF 96365, a novel inhibitor of receptor-mediated calcium entry. Biochem. J., *271*:515-522.

Parekh, A.B., and Penner, R. (1995) Depletion-activated calcium current is inhibited by protein kinase in RBL-2H3 cells. Proc. Natl. Acad. Sci. USA, *92*:7907-7911.

Petersen, C.C.H., and Berridge, M.J. (1994) The regulation of capacitative calcium entry by calcium and protein kinase C in Xenopus oocytes. J. Biol. Chem., *269*:32246-32253.

Petersen, C.C.H., and Berridge, M.J. (1995) G-protein regulation of capacitative calcium entry may be mediated by protein kinases A and C in *Xenopus* oocytes. Biochem. J., *307*:663-668.

Philipp, S., Cavalie, A., Freichel, M., Wissenbach, U., Zimmer, S., Trost, C., Marquart, A., Murakami, M., and Flockerzi, V. (1996) A mammalian capacitative calcium entry channel homologous to Drosophila TRP and TRPL. EMBO J., *15*:6166-6171.

Putney, J., Jr (1986) A model for receptor-regulated calcium entry. Cell Calcium, *7*:1-12.

Randriamampita, C., and Tsien, R.Y. (1993) Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimlates Ca^{2+} influx. Nature, *364*:809-813.

Salter, D.W., and Weber, M.J. (1979) Glucose-specific cytochalasin B binding is increased in chicken embryo fibroblasts transformed by Rous sarcoma virus. J. Biol. Chem., *254*:3554-3561.

Sargeant, P., Farndale, R.W., and Sage, S.O. (1993) The tyrosine kinase inhibitors methyl 2,5-dihydroxycinnamate and genistein reduce thrombin-evoked tyrosine phosphorylation and Ca^{2+} entry in human platelets. FEBS Lett., *315*:242-246.

Tojyo, Y., Tanimura, A., Matsumoto, Y., and Sugiya, H. (1995) Staurosporine enhances Ca^{2+} entry induced by depletion of intracellular Ca^{2+} stores in rat parotid acinar cells. Cell Calcium, *17*:32-40.

Tornquist, K., and Ahlstrom, M. (1993) Modulatory effect of cyclic AMP on calcium fluxes in FRTL-5 cells. J. Cell. Physiol., *157*:625-630.

DISCUSSION

The central aim of this dissertation has been to explore the mechanisms through which glucose metabolism is linked to Ca^{2+} regulation in J774 macrophages. In this concluding section, the findings of the study are discussed in light of the immediate questions they raise and the implications these have on physiological and pathological phenomena.

Conclusions From the Study

2dGlc treatment or glucose deprivation of J774 cells for 4 min caused a partial loss of intracellular Ca^{2+} stores. Under these conditions, capacitative Ca^{2+} influx was also inhibited. As a result of this disruption in Ca^{2+} regulation, the generation of Ca^{2+} signals in response to IgG, ATP, and PAF were inhibited. None of these effects were observed when the cells were treated with rotenone, a mitochondrial inhibitor.

The partial loss of intracellular Ca^{2+} stores in 2dGlc-treated or glucose-deprived cells does not appear to be directly related to decreased Glc-6-P levels under these conditions. This is based on observations in permeabilized J774 cells and J774 microsomes where Glc-6-P was unable to enhance ATP dependent sequestration of Ca^{2+}. These observations were in contrast to those in liver microsomes where Glc-6-P caused an increased sequestration of Ca^{2+}.

Further investigations into the decreased capacitative Ca^{2+} entry in 2dGlc-treated cells revealed that the entry of Ca^{2+} could be restored by the addition of staurosporine.

This effect of staurosporine could not be explained based on altered cellular ATP levels or inhibition of PKC/PKA. We also made the novel observation that capacitative Ca^{2+} influx is inhibited in the presence of glucosamine and mannosamine. This effect appeared to be distinct from that seen in 2dGlc-treated or glucose-deprived cells. Cellular ATP levels were not appreciably altered by the amino sugars. Also, staurosporine was unable to reverse the inhibition in capacitative Ca^{2+} influx mediated by glucosamine or mannosamine. These results suggest the involvement of a glucose metabolite, other than glycolytic ATP, in the activation of capacitative Ca^{2+} entry.

Significance of the Findings

Inhibition of agonist-induced Ca^{2+} signals by 2dGlc. It has been previously shown that 2dGlc inhibits F_c receptor-mediated phagocytosis in macrophages and neutrophils (Michl et al., 1976; Boxer et al., 1976). The mechanism of this 2dGlc-mediated inhibition has remained elusive and appears to be unrelated to the effect of 2dGlc on cellular ATP levels and protein/glycoprotein synthesis (Sung and Silverstein, 1985).

The engulfment of an IgG-coated particle is preceded by a transient increase in cytoplasmic Ca^{2+} (Hishikawa et al., 1991). Although the precise role of Ca^{2+} signals in phagocytosis remains to be elucidated, the occurrence of these signals has been widely noted. Our observations indicate that within 4 min of 2dGlc treatment or glucose deprivation, Ca^{2+} signals in response to IgG are completely abolished. This lack of Ca^{2+} signals in 2dGlc-treated cells suggests a mechanism for the 2dGlc-mediated inhibition of phagocytosis.

In the case of F_c receptor-mediated phagocytosis it has been shown that the ratio between 2dGlc and glucose determines the efficacy with which 2dGlc can inhibit this

process (Boxer et al., 1976; Michl et al., 1976). It is of interest to note that in our studies the ratio at which 2dGlc inhibited IgG-induced Ca^{2+} signals (5 2dGlc:1 glucose) was the same as that reported earlier for the inhibition of phagocytosis.

The inhibition of agonist-induced Ca^{2+} responses in the presence of 2dGlc is directly related to our findings that 2dGlc releases Ca^{2+} from intracellular thapsigargin-sensitive Ca^{2+} stores and that the capacitative influx of Ca^{2+} is inhibited under the experimental conditions of 2dGlc treatment or glucose deprivation.

A "glucose-sensitive" pool of intracellular Ca^{2+}. The inhibition of Ca^{2+} signals by 2dGlc is preceded by a partial release of Ca^{2+} from intracellular thapsigargin-sensitive stores. Previous investigators have suggested that the maintenance of intracellular Ca^{2+} stores requires the presence of glycolytic ATP (Zhang and Paul, 1994). However, the release of only a portion of the intracellular Ca^{2+} stores by 2dGlc, such as was shown in this study, has not been previously demonstrated. Our result suggests that only a portion of the intracellular Ca^{2+} stores is dependent on the presence of extracellular glucose.

The inhibition of Ca^{2+} signaling occurs despite the fact that most of the thapsigargin-sensitive Ca^{2+} pools remain intact following 2dGlc treatment. This suggests that the maintenance of the "glucose-sensitive" portion of the ER Ca^{2+} pool is essential for the generation of IP_3-induced Ca^{2+} release. It is, as yet, not clear whether in non-excitable cells there are two pools within the thapsigargin-sensitive Ca^{2+} stores, one sensitive and the other insensitive to IP_3. If this is the case, then it is conceivable that the "glucose-sensitive" pool of intracellular Ca^{2+} which is lost upon 2dGlc treatment coincides with IP_3-sensitive pools. The issue of heterogeneous pools of intracellular Ca^{2+} is addressed later in this chapter.

2dGlc and glucosamine as inhibitors of capacitative Ca^{2+} entry. Recent reports (Gamberucci et al., 1994; Marriott and Mason, 1995) have indicated that a decrease in cellular ATP content leads to a reduction in capacitative influx of Ca^{2+}. Gamberucci et al. (1994) noted an inhibition of Ca^{2+} influx in the presence of rotenone, although the effect appeared to vary among the cell types that were examined. They noted that in ETCs and in HeLa cells, rotenone was able to inhibit capacitative Ca^{2+} influx more effectively when the cells were suspended in glucose-free media, whereas in hepatocytes and Jurkat T cells, the effect of rotenone was independent of the presence of extracellular glucose. Our observations in J774 cells are that mitochondrial inhibition by rotenone does not cause the significant change in capacitative influx seen in the presence of 2dGlc or upon glucose removal. It is possible that J774 macrophages rely exclusively on glycolytic ATP for optimal activation of the capacitative entry pathway or that some other glucose metabolite is involved.

Our observation that glucosamine and mannosamine inhibit capacitative Ca^{2+} entry is novel. It is important to note that the inhibition in the presence of these amino sugars occurs without an appreciable decrease in cellular ATP levels. This result suggests a role for a metabolite of glucose other than glycolytic ATP.

It is not clear whether 2dGlc and glucosamine inhibit capacitative Ca^{2+} entry at a common step. The effects of glucosamine differ from those of 2dGlc in two ways. First, the decrease in ATP levels with glucosamine is not as significant as with 2dGlc. Second, as mentioned later, staurosporine reverses the 2dGlc-mediated inhibition of capacitative Ca^{2+} entry but not the glucosamine-mediated inhibition.

Reversal by staurosporine. The effects of staurosporine on capacitative Ca^{2+} influx in J774 cells appear to be specific in three ways. First, staurosporine is able to reverse the inhibition of capacitative influx in the presence of glycolytic inhibitors but not the inhibition seen in the presence of glucosamine or mannosamine. Second, the microbial alkaloid K252a is unable to mimic staurosporine's effect. K252a, like staurosporine, is also an indolo [2,3-a] carbazole derivative (see Appendix A). Third, staurosporine by itself has no modulatory effects on capacitative Ca^{2+} influx or on cellular ATP levels. It should also be noted that the ability of staurosporine to reverse 2dGlc-mediated inhibition was observed in other cell types as well (see Appendix B). Such a specificity for staurosporine action has not been previously observed.

Our observation that staurosporine reverses the decrease in capacitative Ca^{2+} influx caused by glycolytic inhibitors indicates that the dependence of this Ca^{2+} entry pathway on extracellular glucose is at a specific step. Thus, inhibition of capacitative Ca^{2+} influx in the absence of glycolysis is not a result of a general metabolic poisoning.

Future Work

Based on the investigations described here, it is apparent that in glucose-deprived or 2dGlc-treated cells Ca^{2+} signaling is inhibited due to compromised Ca^{2+} stores and decreased capacitative Ca^{2+} entry. The plausible mechanisms underlying these effects are discussed here.

Mechanism of ER Ca^{2+} release. It is clear from the observations described in Chapter 1 that 2dGlc treatment or glucose deprivation of J774 macrophages causes the loss of a portion of the ER Ca^{2+} stores. Without doubt, the inability to maintain all of the Ca^{2+} stores under these conditions is due to a suboptimal functioning of the ER Ca^{2+}-

ATPase. However, the mechanism of this is not clear. As shown in Chapter 2, an effect of Glc-6-P on Ca^{2+} sequestration was not detectable in these cells. Thus, the explanation based on a cooperation between the Glc-6-phosphatase and the Ca^{2+}-ATPase systems in these cells might not be valid. The alternate explanation, which is based on the compartmentation of glycolysis and the dependence of ER Ca^{2+}-ATPase on glycolytic ATP, is also not sufficient. As explained in the second section, 2dGlc releases only a portion of the ER Ca^{2+} stores in J774 cells, while rotenone has no effect. If all of the Ca^{2+} stores depend on the presence of glycolytic ATP, then the release with 2dGlc should be complete. On the other hand, if some of the stores depend on glycolytic ATP while others utilize mitochondrial ATP, then in the presence of rotenone there should be a detectable loss of the ER Ca^{2+} stores. The two most likely possibilities that would sufficiently explain the release of intracellular Ca^{2+} by 2dGlc are shown in Figure 5A. The first is based on the existence of two distinct pools of Ca^{2+} [as opposed to the presence of distinct pools of ATP]. Thus, while one pool of Ca^{2+} can be maintained effectively by either mitochondrial or glycolytic ATP generation, the other might require the presence of glycolytic ATP. In the presence of 2dGlc, this latter pool of Ca^{2+} would be lost. Testing this model might require Ca^{2+} imaging (with fura-2) in 2dGlc-treated cells using, for example, confocal laser scanning microscopy. Local changes in cytoplasmic Ca^{2+} may then be detected. Alternatively, dyes that can be loaded into the ER may be used. In this case, a loss of intensity upon 2dGlc-treatment might be apparent only at specific loci of the ER.

The alternate explanation is based on existence of a cellular metabolite; the presence of which is required for an optimal functioning of the ER Ca^{2+}-ATPase (Fig. 5B). The assumption here is that, the absence of glucose is accompanied by decreased levels of

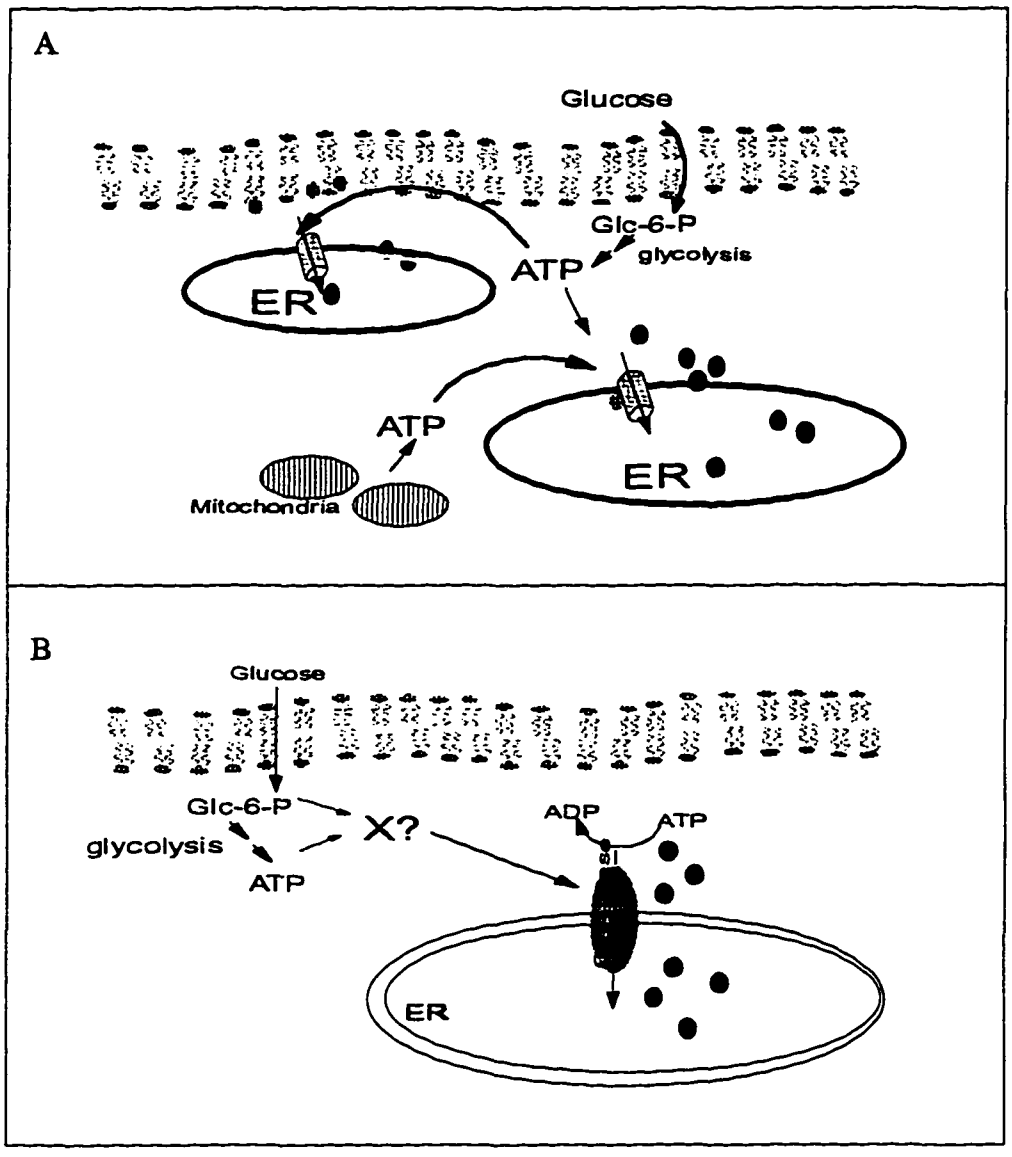


Fig. 5. Models for the release of Ca^{2+} from intracellular stores in glucose-deprived cells.

this metabolite. This model is similar to the 'Glc-6-phosphatase hypothesis' in that, along with ATP, the involvement of an additional cellular metabolite is proposed.

Relevant to these models is our observation (Chapter 1, Fig. 3) that when platelet activating factor (PAF) is present there is a recovery of IgG response in 2dGlc-treated cells. Thus, it is possible that a second messenger, which is generated in the presence of PAF, aids in the reuptake of Ca^{2+} into the ER stores which are lost upon 2dGlc-treatment. This would explain the subsequent recovery of IgG response. Such an activity for cAMP and cGMP has been proposed in human platelets (Brune and Ullrich, 1992).

Inhibition of capacitative Ca^{2+} entry by glucosamine. Based on the metabolism of glucosamine, two possibilities exist for its inhibitory effect on the capacitative Ca^{2+} influx (Fig. 6). Glucosamine may cause a decrease in cellular UTP and UDP-Glc levels. Alternatively, accumulation of UDP-N-acetylglucosamine is likely. A measurement of these metabolite levels in glucosamine-treated cells is, therefore, necessary.

How might each of these situations lead to the inhibition of capacitative Ca^{2+} entry? It is possible that the presence of UDP-Glc is required for the synthesis of CIF, the putative diffusible messenger that activates Ca^{2+} entry via capacitative Ca^{2+} channel. Decreased UDP-Glc in the presence of glucosamine might, therefore, inhibit CIF synthesis. This possibility is currently being tested in our laboratory by pretreating J774 cells with glucosamine and then preparing cellular extracts. The level of CIF activity in partially purified extracts can then be determined by injecting these extracts into Xenopus oocytes. Another approach might involve the pretreatment of cells with 6-azauridine, which inhibits de novo synthesis of uridine and leads to decreased UTP and UDP-Glc levels.

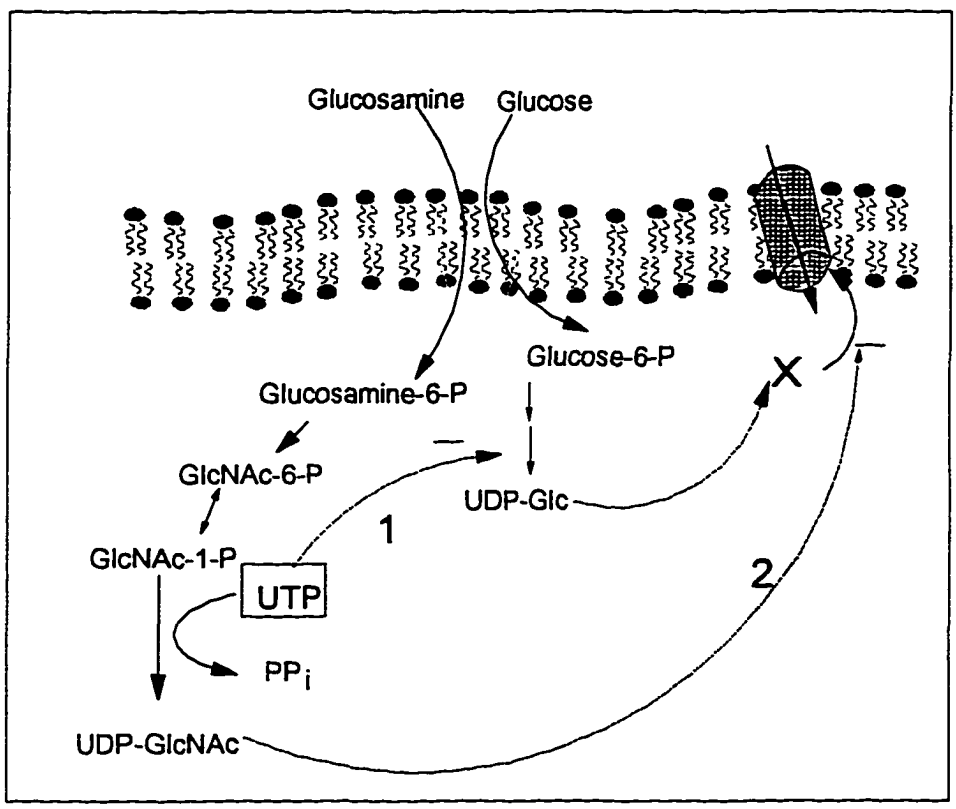


Fig. 6. Plausible mechanisms for the inhibition of capacitative Ca^{2+} entry by glucosamine.

The alternate mechanism might involve the accumulation of UDP-N-acetyl-glucosamine (UDP-GlcNAc). UDP-GlcNAc may directly inhibit Ca^{2+} entry by acting at the capacitative Ca^{2+} channel. This can be easily tested by co-injecting UDP-GlcNAc with extracts containing CIF activity.

Our observations regarding the inhibitory effects of glucosamine are supported by two recent reports. First, Kim et al. (1996) detected the presence of a pyrimidine nucleotide-sugar group in partially purified Jurkat cell extracts possessing CIF activity. Second, the microbial product adenophostin A was reported to stimulate Ca^{2+} influx in Xenopus oocyte. Adenophostin A is known to be a potent agonist of the IP_3 receptor. However, DeLisle et al. (1997) have observed that at lower concentrations it can stimulate Ca^{2+} influx without depleting the IP_3-sensitive Ca^{2+} stores. It is of interest to note that adenophostin A is a nucleotide-glucose conjugate (see Appendix A).

Mechanism of staurosporine action. As noted earlier, staurosporine is able to reverse the inhibition in capacitative Ca^{2+} influx in the presence of glycolytic inhibitors but not the amino sugar-mediated inhibition of Ca^{2+} influx. This suggests one of two possibilities. First, inhibition by glucosamine might occur at a step downstream of where staurosporine acts. As proposed earlier, UDP-GlcNAc might directly block the entry of Ca^{2+}. Thus, in the presence of UDP-GlcNAc, staurosporine might be unable to reverse the inhibition.

The alternate possibility is that the effects of 2dGlc and glucosamine reflect the requirement for two separate metabolites for the activation of capacitative Ca^{2+} channel (Fig. 7). Thus, it is possible that in the presence of glycolytic inhibitors, decreased cellular ATP leads to an inhibition in capacitative Ca^{2+} entry. For instance, this might happen by a

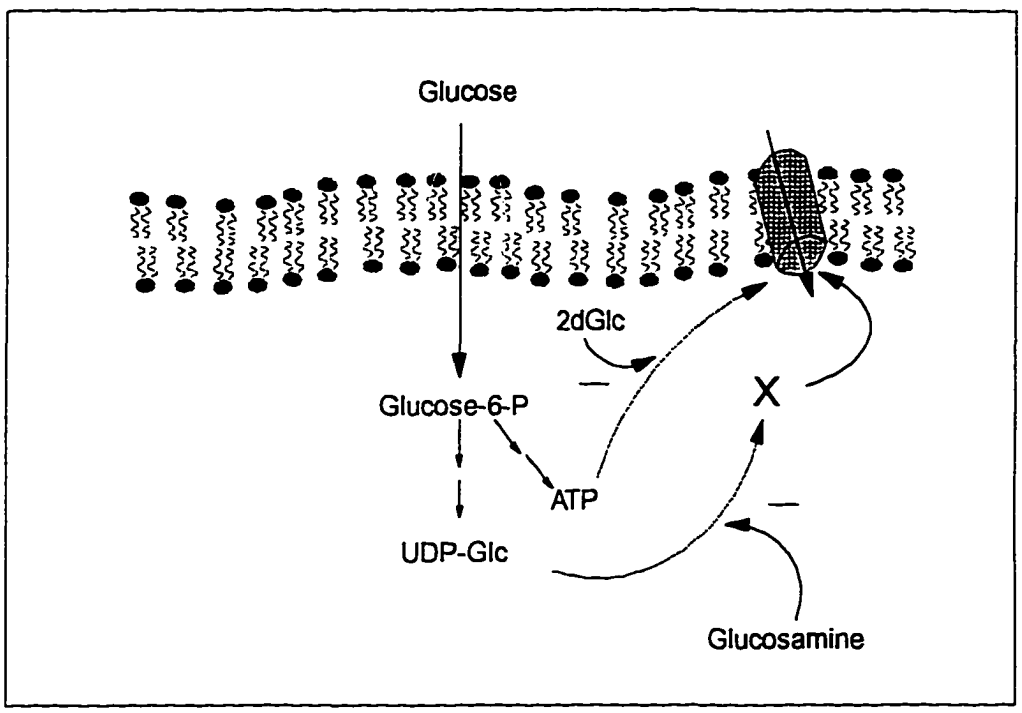


Fig. 7. A model for the activation of capacitative Ca^{2+} entry based on the requirement for glycolytic ATP and UDP-Glc.

direct binding of ATP to the Ca^{2+} channel. The second step at which glucose might be important is in providing the precursor for CIF synthesis and this might be affected in the presence of glucosamine. A possible role for UDP-Glc, in this manner, has already been discussed. In the presence of glucosamine then although ATP levels are not altered, the precursor for CIF synthesis might be decreased. If this model were to hold true, then it is likely that staurosporine can overcome the inhibition that arises due to decreased glycolytic ATP but not the block in CIF synthesis.

Implications of the Results

Relevance to cellular processes. As discussed elsewhere in this dissertation (see Introduction and Chapter 1), a number of Ca^{2+}-related cellular processes have been reported to be dependent on glucose. These include phagocytosis, muscle contraction, and immune cell function. In the case of phagocytosis although several reports have described the inhibitory effects of 2dGlc on this process, the mechanism of the inhibition has remained elusive. Based on the investigations described in the preceding sections, it is likely that the inhibition of phagocytosis in the presence of 2dGlc is a consequence of its deleterious effects on Ca^{2+} signaling.

The precise role of Ca^{2+} signals in phagocytosis remains unclear. There has been considerable controversy regarding the requirement for Ca^{2+} in the process of engulfment or cytoskeletal rearrangements during phagocytosis. However, in the case of T cells requirement for Ca^{2+} signals is more apparent. In these cells, the role of Ca^{2+} in IL-2 production has been well-defined (Luo et al., 1996). Other investigators in our laboratory are, therefore, examining the effects of 2dGlc and glucosamine in these cells.

Pathological and physiological consequences. Under physiological conditions, the effect of glucose on Ca^{2+} signaling is most relevant in the case of pancreatic β cells. The secretion of insulin by these cells, in response to glucose, has been reported to be mediated via alteration in cytoplasmic Ca^{2+} levels (Dukes et al., 1994).

Other cell types do not respond to glucose as an 'agonist' but require the presence of glucose for optimal cellular functioning. In these cells and tissues, disruption of Ca^{2+} regulation due to altered extracellular glucose is relevant under pathological situations accompanied by hyper- or hypo-glycemia. An example of hypoglycemia and its relation to altered Ca^{2+} signaling is found in glycogen storage disease. As mentioned in the preceding sections, in this disease, the liver is unable to mobilize glycogen effectively. As a result, in these patients, there is a failure to maintain blood glucose levels in the unfed state. Frequent hypoglycemic episodes in these patients have, therefore, been noted. As mentioned in Chapter 2, neutrophils from patients suffering from a subtype of this disease are defective in their ability to generate Ca^{2+} signals.

Another pathological situation where glucose metabolism is known to be altered is neoplasia. In tumor cells, an overexpression of glucose transporters has been noted. Thus, the relative efficiency with which glucose analogues such as 2dGlc and glucosamine will be taken up by tumor cells would be much greater compared to normal cells. The possibility that 2dGlc and glucosamine would be selectively toxic to tumor cells has, therefore, been investigated (Bekesi and Winzler, 1970; Molnar and Bekesi, 1972). It is of interest to note here the effects of genistein, a naturally occurring isoflavone found in soybean. Genistein has been attributed with the ability to decrease the incidence of cancer (Lamartiniere et al., 1995). This compound has also been used as a broad range tyrosine

kinase inhibitor. Recently, genistein has been shown to bind to glucose transporter and inhibit glucose transport into the cell (Vera et al., 1996). In agreement with this latter effect of genistein, we found that it was able to inhibit capacitative Ca^{2+} influx in J774 cells (Appendix C).

Glucosamine has also been suggested as a therapeutic agent in inflammatory bowel disease and in osteoarthritis (Setnikar et al., 1991; Reichelt et al., 1994). Based on the investigations described here, it is possible that part of the anti-inflammatory effects of glucosamine are due to its ability to interfere with Ca^{2+} signaling.

Cell survival and disruption of Ca^{2+} homeostasis. Prolonged periods of glucose deprivation can, no doubt, lead to the disruption of a variety of cellular processes. Interestingly, in the case of glucose regulated proteins (GRPs), a marked correlation is seen between glucose metabolism and Ca^{2+} regulation. There is an overexpression of these proteins following prolonged glucose deprivation, 2dGlc treatment, or glucosamine treatment. Such an overexpression of GRPs can also be observed in the presence of the Ca^{2+} ionophore, ionomycin (Lee, 1992).

However, in all of the investigations described in the preceding chapters, the exposure of J774 cells to glucose deprivation and 2dGlc or glucosamine treatments was limited to ~ 5 min. Also, all of the observed inhibitory effects of 2dGlc could be reversed by the addition of glucose. Thus, it is clear that under these conditions, 2dGlc is not irreversibly affecting these cells. However, our observations can still be interpreted in the light of cell survival mechanisms. It is possible that upon glucose deprivation, the cell down-regulates Ca^{2+} signaling and Ca^{2+} influx pathways in an attempt to avoid unduly high cytoplasmic Ca^{2+} concentrations. In a state of limited supply of energy, this would be to

the advantage of the cell since uncontrolled increase in cytoplasmic Ca^{2+} levels can lead to cellular death.

In summary, there is considerable evidence that alterations in glucose metabolism can lead to improper regulation of intracellular Ca^{2+}. Other investigators have suggested that the link between these two processes can be explained solely on the basis of glycolytically generated ATP. The findings of this study complement those reported elsewhere in the following ways. First, glucose deprivation for brief periods of time appears to affect only a selected few of the various Ca^{2+} regulatory processes that are present in cells. Second, the dependence of Ca^{2+} regulatory processes on the continuous presence of extracellular glucose might reflect the requirement for not only ATP but also for an additional cellular metabolite.

GENERAL LIST OF REFERENCES

Bekesi, J.G., and Winzler, R.J. (1970) Inhibitory effects of D-glucosamine on the growth of Walker 256 carcinosarcoma and on protein, RNA, and DNA synthesis. Cancer Research, *30*:2905-12.

Benedetti, A., Fulceri, R., and Comporti, M. (1985) Calcium sequestration activity in rat liver microsomes. Evidence for a cooperation of calcium transport with glucose-6-phosphatase. Biochim. Biophys. Acta., *816*:267-277.

Berridge, M. (1993) Inositol trisphosphate and calcium signalling. Nature, *361*:315-325.

Berridge, M.J. (1995) Capacitative calcium entry. Biochem. J., *312*:1-11.

Bischof, G., Brenman, J., Bredt, D.S., and Machen, T.E. (1995) Possible regulation of capacitative Ca^{2+} entry into colonic epithelial cells by NO and cGMP. Cell Calcium, *17*:250-262.

Boxer, L.A., Baehner, R.L., and Davis, J. (1976) The effect of 2-deoxyglucose on guinea pig polymorphonuclear leukocyte phagocytosis. Cell. Physiol., *91*:89-102.

Bright, R.T., Salvaterra, C.G., Rubin, L.J., and Yuan, X.-J. (1995) Inhibition of glycolysis by 2-DG increases $[Ca^{2+}]_i$ in pulmonary arterial smooth muscle cells. Am. J. Physiol., *269*:L203-L208.

Brune, B., and Ullrich, V. (1992) Cyclic nucleotides and intracellular-calcium homeostasis in human platelets. Eur. J. Biochem., *207*:607-613.

Burchell, A., and Waddell, I.D. (1991) The molecular basis of the hepatic microsomal glucose-6-phosphatase system. Biochim. Biophys. Acta, *1092*:129-137.

Carafoli, E. (1982) Membrane transport of Calcium, E. Carafoli, ed. (London: Academic Press).

Chen, Y., Pollock, J.D., Wang, Y., DePaoli-Roach, A.A., and Yu, L. (1993) Protein kinase A modulates endogenous calcium channel, but not the calcium-activated chloride channel, in Xenopus oocytes. FEBS Lett., *336*:191-196.

Clapham, D.E. (1995) Calcium Signaling. Cell, *80*:259-268.

DeLisle, S., Marksberry. E.W., Bonnett, C., Jenkins, D.J., Potter, B.V.L., Takahashi, M., and Tanzawa, K. (1997) Adenophostin A can stimulate Ca^{2+} influx without depleting the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores in the Xenopus oocyte. J. Biol. Chem., *272*:9956-9961.

DiVirgilio, F., Meyer, B.S., Greenberg, S., and Silverstein, S.C. (1988) F_c receptor-mediated phagocytosis occurs in macrophages at exceedingly low cytosolic Ca^{2+} levels. J. Cell Biol., *106*:657-666.

Dukes, I.D., McIntyre, M.S., Mertz, R.J., Philipson, L.H., Roe, M.W., Spencer, B., and Worley, J.F,I. (1994) Dependence on NADH produced during glycolysis for β-cell glucose signaling. J. Biol. Chem., *269*:10979-10982.

Fasolato, C., Hoth, M., and Penner, R. (1993) A GTP-dependent step in the activation mechanism of capacitative calcium influx. J. Biol. Chem. *268*:20737-20740.

Fulceri, R., Romani, A., Pompella, A., and Benedetti, A. (1990) Glucose 6-phosphate stimulation of MgATP-dependent Ca^{2+} uptake by rat kidney microsomes. Biochim. Biophys. Acta, *1022*:129-133.

Gamberucci, A., Innocenti, B., Fulceri, R., Banhegyi, G., Guinti, R., Pozzan, T., and Benedetti, A. (1994) Modulation of Ca^{2+} influx dependent on store depletion by intracellular adenine-guanine nucleotide levels. J. Biol. Chem., *269*:23597-23602.

Gillo, B., Chorna, I., Cohen, H., Cook, B., Manistersky, I., Chorev, M., Arnon, A., Pollock, J.A., Selinger, Z., and Minke, B. (1996) Coexpression of Drosophila TRP and TRP-like proteins in Xenopus oocytes reconstitutes capacitative Ca^{2+} entry. Proc. Natl. Acad. Sci. USA, *93*:14146-14151.

Gitzelmann, R., and Bosshard, N.U. (1993) Defective neutrophil and monocyte functions in glycogen storage disease type Ib: a literature review. Eur. J. Pediatr., *152*:S33-S38.

Hawkins, M., Angelow, I., Liu, R., Barzilai, N., and Rossetti, L. (1997) The tissue concentration of UDP-N-acetylglucosamine modulates the stimulatory effect of insulin on skeletal muscle glucose uptake. J. Biol. Chem., *272*:4889-4895.

Hishikawa, T., Cheung, J.Y., Yelamarty, R.V., and Knutson, D.W. (1991) Calcium transients during Fc receptor-mediated and nonspecific phagocytosis by murine peritoneal macrophages. J. Cell Biol., *115*:59-66.

Irvine, R.F. (1990) 'Quantal' Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates-a possible mechanism. FEBS Lett., *263*:5-9.

Jaconi, M.E.E., Theler, J.M., Schlegel, W., and Lew, P.D. (1993) Cytosolic free Ca^{2+} signals in single adherent human neutrophils: generation and functional role. Eur. J. Pediatr., *152*:S26-S32.

Kilpatrick, L., Garty, B.Z., Lundquist, K.F., Hunter, K., Stanley, C.A., Baker, L., Douglas, S.D., and Korchak, H.M. (1990) Impaired metabolic function and signaling defects in phagocytic cells in glycogen storage disease type 1b. J. Clin. Invest., *86*:196-202.

Kim, H.Y., Thomas, D., and Hanley, M.R. (1996) Stimulation of Ca(2+)-dependent membrane currents in Xenopus oocytes by microinjection of pyrimidine nucleotide-glucose conjugates. Mol. Pharmacol., *49*:360-364.

Korchak, H.M., Garty, B.Z., Stanley, C.A., Baker, L., Douglas, S.D., and Kilpatrick, L. (1993) Impairment of calcium mobilization in phagocytic cells in glycogen storage disease type 1b. Eur. J. Pediatr., *152*:S39-S43.

Kwan, C.Y., Takemura, H., Obie, J.F., Thastrup, O., and Putney, J.W.,Jr. (1990) Effects of MeCh, thapsigargin, and La^{3+} on plasmalemmal and intracellular Ca^{2+} transport in lacrimal acinar cells. Am. J. Physiol., *258*:C1006-C1015.

Lamartiniere, C.A., Moore, J.B., Brown, N.M., Thompson, R., Hardin, M.J., and Barnes, S. (1995) Genistein suppresses mammary cancer in rats. Carcinogenesis, *16*:2833-2840.

Lee, A.S. (1992) Mammalian stress response:induction of the glucose-regulated protein family. Curr. Op. Cell Bio., *4*:267-273.

Lien, Y.-H.H., Wang, X., Gillies, R.J., and Martinex-Zaguilan, R. (1995) Modulation of intracellular Ca^{2+} by glucose in MDCK cells: role of endoplasmic reticulum Ca^{2+}-ATPase. Am. J. Physiol., *268*:F671-F679.

Louzao, M.C., Ribeiro, C.M.P., Bird, G.S.,Jr., and Putney, J.W.Jr. (1996) Cell type-specific modes of feedback regulation of capacitative calcium entry. J. Biol. Chem., *271*:14807-14813.

Luo, C., Burgeon, E., Carew, J.A., McCaffrey, P.G., Badalian, T.M., Lane, W.S., Hogan, P.G., and Rao, A. (1996) Recombinant NFAT1 (NFATp) is regulated by calcineurin in T cells and mediates transcription of several cytokine genes. Mol. Cell. Biol., *16*:3955-3966.

Marriott, I., and Mason, M.J. (1995) ATP depletion inhibits capacitative Ca^{2+} entry in rat thymic lymphocytes. Am. J. Physiol., *269*:C766-774.

Michl, J., Ohlbaum, D.J., and Silverstein, S.C. (1976) 2-Deoxyglucose selectively inhibits Fc and complement receptor-mediated phagocytosis in mouse peritoneal macrophages. J. Exp. Med., *144*:1465-1483.

Molnar, Z., and Bekesi, J.G. (1972) Effects of D-glucosamine, D-mannosamine, and 2-deoxy-D-glucose on the ultrastructure of ascites tumor cells in vitro. Cancer Res., *32*:380-389.

Parekh, A.B., and Penner, R. (1995) Depletion-activated calcium current is inhibited by protein kinase in RBL-2H3 cells. Proc. Natl. Acad. Sci. USA, *92*:7907-7911.

Petersen, C.C.H., and Berridge, M.J. (1994) The regulation of capacitative calcium entry by calcium and protein kinase C in Xenopus oocytes. J. Biol. Chem., *269*:32246-32253.

Philipp, S., Cavalie, A., Freichel, M., Wissenbach, U., Zimmer, S., Trost, C., Marquart, A., Murakami, M., and Flockerzi, V. (1996) A mammalian capacitative calcium entry channel homologous to Drosophila TRP and TRPL. EMBO J., *15*:6166-6171.

Plagemann, P.G.W., and Erbe, J. (1973) Transport and metabolism of glucosamine by cultured Novikoff rat hepatoma cells and effects on nucleotide pools. Cancer Res., *33*:482-492.

Putney, J.,Jr (1986) A model for receptor-regulated calcium entry. Cell Calcium, *7*:1-12.

Randriamampita, C., and Tsien, R.Y. (1993) Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimlates Ca^{2+} influx. Nature, *364*:809-813.

Reichelt, A., Forster, K.K., Fischer, M., Rovati, L.C., and Setnikar, I. (1994) Efficacy and safety of intramuscular glucosamine sulfate in osteoarthritis of the knee. Arzneim.-Forsch./Drug Res., *44*:75-80.

Roe, M.W., Mertz, R.J., Lancaster, M.E., Worley J.F., III, and Dukes, I.D. (1994) Thapsigargin inhibits the glucose-induced decrease of intracellular Ca^{2+} in mouse islets of Langerhans. Am. J. Physiol., *266*:E852-E862.

Sargeant, P., Farndale, R.W., and Sage, S.O. (1993) The tyrosine kinase inhibitors methyl 2,5-dihydroxycinnamate and genistein reduce thrombin-evoked tyrosine phosphorylation and Ca2+ entry in human platelets. FEBS Lett., *315*:242-246.

Setnikar, I., Cereda, R., Pacini, M.A., and Revel, L. (1991) Antireactive properties of glucosamine sulfate. Drug Res., *41*(1):157-161.

Sung, S.S., and Silverstein, S.C. (1985) Role of 2-deoxy-D-glucose in the inhibition of phagocytosis by mouse peritoneal macrophage. Biochim. Biophys. Acta, *845*:204-215.

Vera, J.C., Reyes, A.M., Carcamo, J.G., Velasquez, F.V., Rivas, C.I., Zhang, R.H., Strobel, P., Iribarren, R., Scher, H.I., Slebe, J.C., and Golde, D.W. (1996) Genistein is a

natural inhibitor of hexose and dehydroascorbic acid transport through the glucose transporter, GLUT1. J. Biol. Chem., *271*:8719-8724.

Veyna-Burke, N.A. (1996) Glycosylation and function of phosphoglucomutase in rat cerebral cortex. In Department of Cell Biology (Dissertation, University of Alabama at Birmingham).

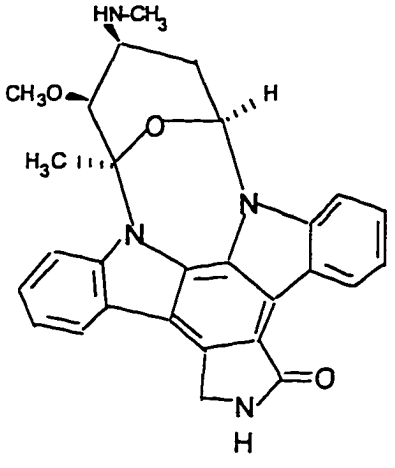
Wolf, B.A., Colca, J.R., Comens, P.G., Turk, J., and McDaniel, M.L. (1986) Glucose-6-phosphate regulates Ca^{2+} steady state in endoplasmic reticulum of islets. J. Biol. Chem.. *261*:16284-16287.

Zhang, C., and Paul, R.J. (1994) Excitation-contraction coupling and relaxation in porcine carotid arteries are specifically dependent on glucose. Am. J. Physiol., *36*:1996-2004.

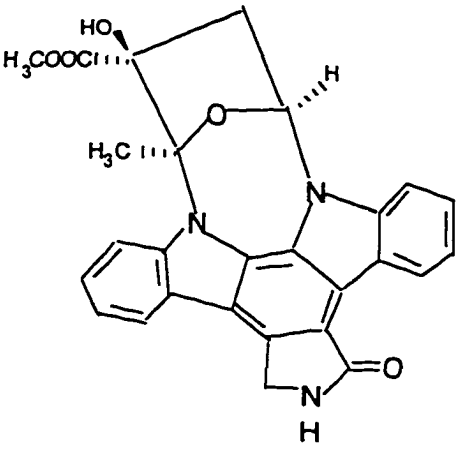
Ziegelstein, R.C., Cheng, L., Aversano, T., Ouyang, P., Lakatta, E.G., and Silverman. H.S. (1994) Increase in rat aortic endothelial free calcium mediated by metabolically sensitive calcium release from endoplasmic reticulum. Cardiovasc. Res., *28*:1433-1439.

APPENDIX A

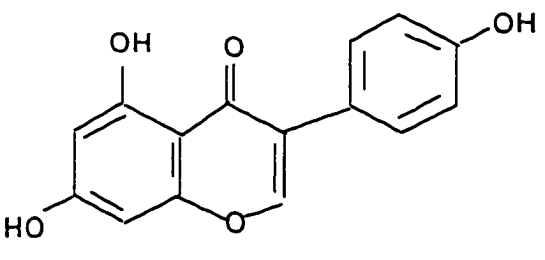
CHEMICAL STRUCTURES OF SOME COMPOUNDS



Staurosporine

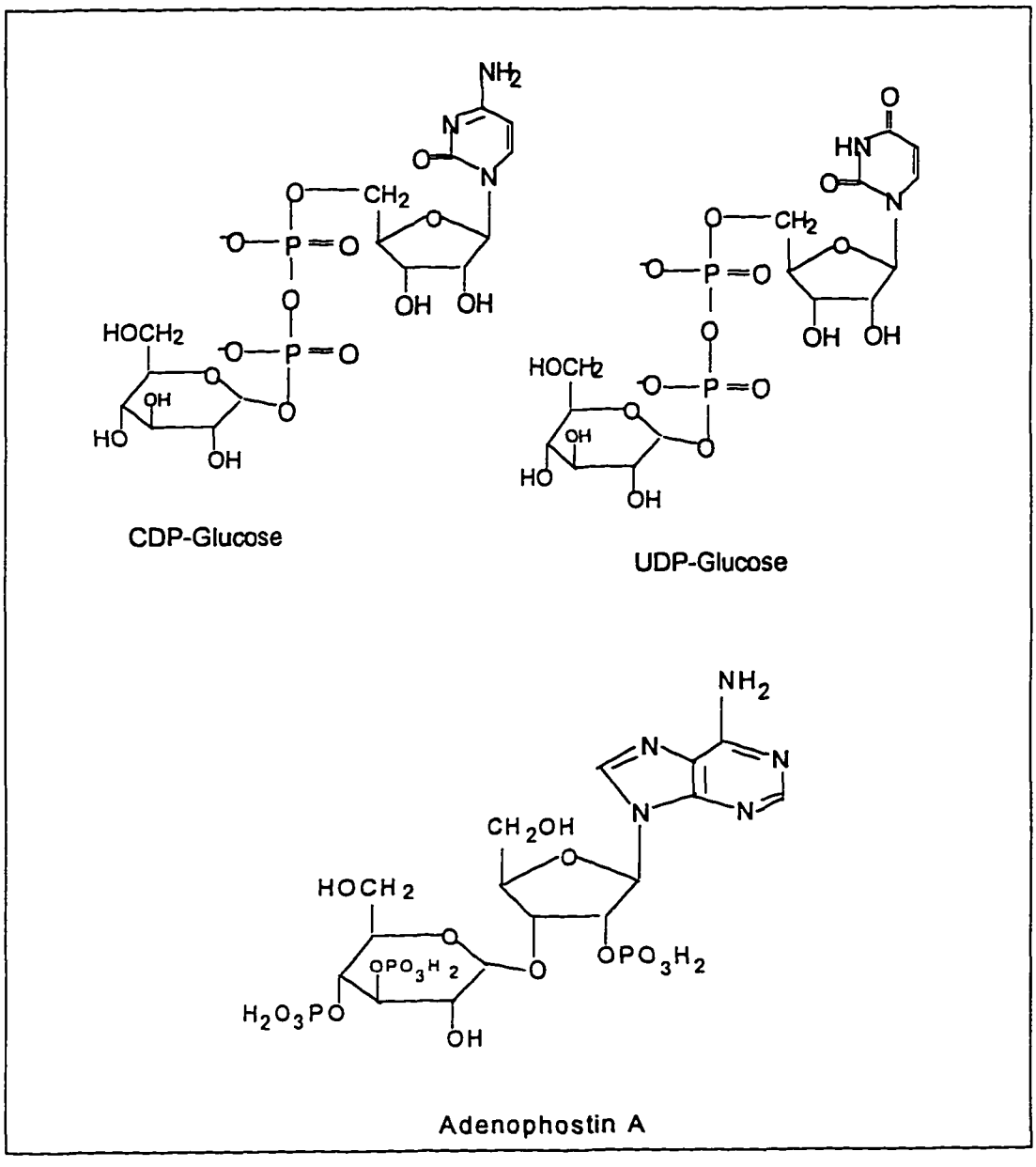


K252a



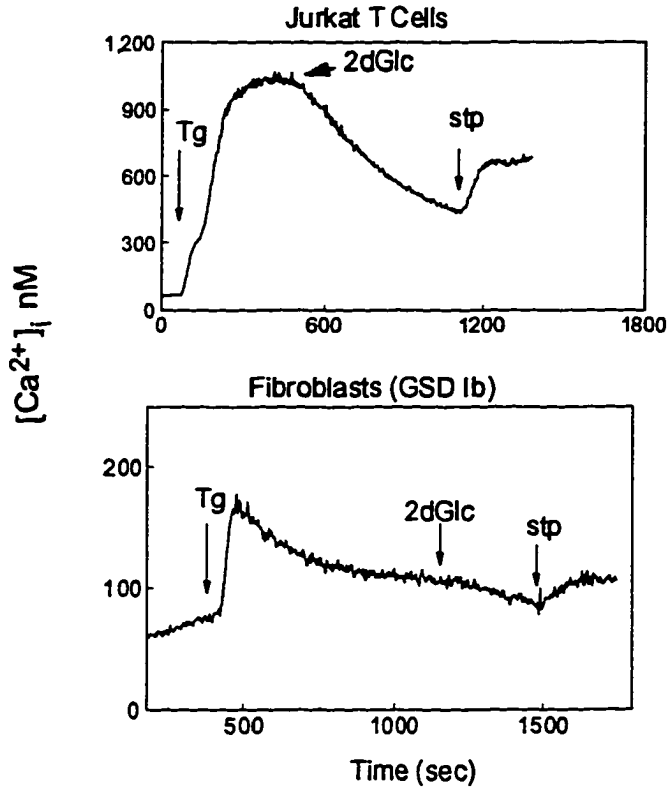
Genistein

4',5,7-trihydroyisoflavone



APPENDIX B

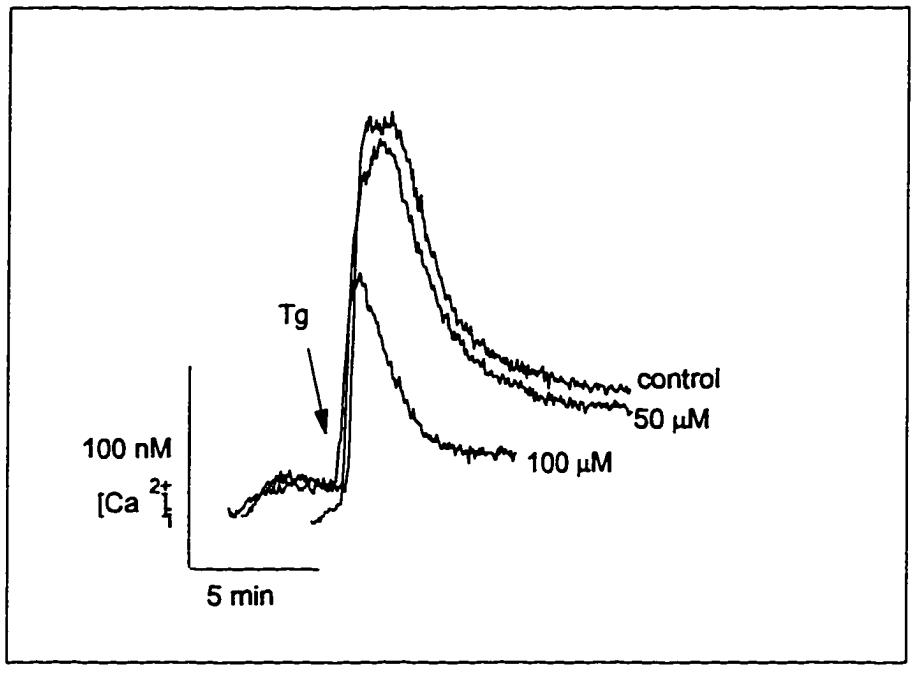
EFFECTS OF 2-DEOXY-D-GLUCOSE AND STAUROSPORINE ON OTHER CELL TYPES



The inhibition of capacitative Ca^{2+} influx following the addition of 2dGlc is also seen in Jurkat cells and fibroblasts. Fura-2-loaded cells were suspended in HBS containing 5 mM Glc and 5 mM Pyr. Thapsigargin (Tg, 200 nM) was added, as indicated. 2dGlc was added to a final concentration of 25 mM. Staurosporine (stp) was used at 40 nM. In both of these cell types, staurosporine was able to reverse the 2dGlc-mediated inhibiton. In Jurkat cells, the reversal is not complete.

APPENDIX C

INHIBITION OF CAPACITATIVE CALCIUM ENTRY BY GENISTEIN



Inhibition of capacitative Ca^{2+} influx by genistein appears to be due to its ability to block glucose transport . ATP levels in the presence of genistein were as follows. Control: 657 +/- 92; 50 μM genistein: 620 +/- 24; 100μM genistein: 296 +/- 86.

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Major Subject Cell Biology

Title of Dissertation Coupling Mechanisms Between Glucose Metabolism and Calcium Signaling in J774 Macrophages

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