0026-895X/04/6503-639–645$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2004 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 65:639–645, 2004

Vol. 65, No. 3
2939/1129859
Printed in U.S.A.

Dipyridamole Alters Cardiac Substrate Preference by Inducing Translocation of FAT/CD36, but Not That of GLUT4

Joost J. F. P. Luiken, Susan L. M. Coort, Jodil Willems, Will A. Coumans, Arend Bonen, and Jan F. C. Glatz

Department of Molecular Genetics, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, the Netherlands (J.J.F.P.L., S.L.M.C., J.W., W.A.C., J.F.C.G.); and Department of Human Biology and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada (A.R.)

Received September 3, 2003; accepted November 21, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

In cardiac myocytes, uptake rates of glucose and long-chain fatty acids (FA) are regulated by translocation of GLUT4 and FA translocase (FAT)/CD36, respectively, from intracellular stores to the sarcolemma. Insulin and contractions are two major physiological stimuli able to induce translocation of both transporters and therefore enhance the uptake of both substrates. Interestingly, the cardiovascular drug dipyridamole was able to enhance FA uptake but had no effect on glucose uptake. The selective stimulatory effect of dipyridamole on FA uptake was unrelated to its effects on phosphodiesterase inhibition and on nucleoside transport inhibition. However, dipyridamole-stimulated FA uptake was abolished in the presence of sulfo-*N*-succinimidylpalmitate, which indicated that FAT/CD36 is involved in the uptake process. Furthermore, the effect was additive to that of insulin but not to that of the AMP-elevating agent oligomycin, indicating that dipyridamole stimulates FAT/CD36-mediated FA uptake by activating the AMP-activated protein kinase (AMPK) signaling pathway. Dipyridamole, however, neither influenced the intracellular AMP content nor induced activation of AMPK. Finally, dipyridamole was able to induce FAT/CD36 translocation from intracellular storage sites to the sarcolemma but had no effect on the subcellular distribution of GLUT4. It is concluded that beyond AMP-activated protein kinase the contraction-induced and AMPK-mediated signal branches off into separate mobilization of GLUT4 and of FAT/CD36, and that dipyridamole activates a yet unidentified target in the FAT/CD36 mobilizing branch.

Dipyridamole is a cardiovascular drug that is currently being used in the clinic because of 1) its coronary dilatory properties and 2) its ability to inhibit platelet aggregation (Humphreys et al., 2002; MacWalter and Shirley, 2002; Pettigrew and Williams, 2002; De Schryver et al., 2003). This is based, at least in part, on some interesting pharmacological effects elicited by dipyridamole (DPY) on cardiac myocytes and other mammalian cells, in that it is recognized to be 1) an inhibitor of phosphodiesterases V and VI, thereby increasing intracellular levels of cyclic AMP, and even more potently cyclic GMP (Komas et al., 1991; Beavo, 1995), and 2) an inhibitor of nucleoside transport (Hammond et al., 1985). Furthermore, dipyridamole is known to alter cardiac utilization of long-chain fatty acids (FA). Its main action in this respect is the stimulation of uptake of FA by cardiac myocytes (Abdel-Aleem et al., 1999, Luiken et al., 2002c). The molecular mechanism by which dipyridamole stimulates FA uptake involves a translocation of the FA transporter FAT/CD36 to the sarcolemma (Luiken et al., 2002b). In contrast, the effect of dipyridamole on uptake of glucose, the other main substrate for cardiac myocytes, is less clear. It has been reported that dipyridamole inhibits glucose uptake (Steinfelder and Joost, 1988), stimulates glucose uptake (Mainwaring and Mentzer, 1986), or does not affect glucose uptake (Prasad et al., 1999). At the level of GLUT4, the main cardiac glucose transporter, dipyridamole has been found to inhibit its intrinsic activity via a direct interaction with the transporter at the sarcolemma (Steinfelder and Joost, 1988).

For the maintenance of mechanical activity, the heart is entirely dependent on the uptake of both glucose and FA. Glucose uptake by cardiac myocytes is completely accounted

This work was supported by the Netherlands Heart Foundation (grant 2000.156), the Heart and Stroke Foundation of Ontario, and the Netherlands Organization for Scientific Research (ZonMw grant 016.036.305 to J.J.F.P.L.). A.B. is a Canada Research Chair in Metabolism and Health. J.F.C.G. is Netherlands Heart Foundation Professor of Cardiac Metabolism.

This work was presented in abstract form at the FEBS Special Meeting 2003 on Signal Transduction, Brussels Belgium 3–8 July 2003 (*Eur J Biochem* **270** Suppl I:PS01–PS0191, 2003).

ABBREVIATIONS: DPY, dipyramidole; FA, long-chain fatty acids; FAT, fatty acid translocase; LY-294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PI3K, phosphatidylinositol-3 kinase; AMPK, P-activated protein kinase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; BSA, bovine serum albumin; SSP, sulfo-*N*-succinimidylpalmitate; DMSO, dimethyl sulfoxide; PM, plasma membrane fraction; LDM, low density microsomal fraction.

for by the facilitative action of both GLUT1 and GLUT4. Of these glucose transporters, GLUT4 is the predominant cardiac isoform; it is ~3-fold more abundantly expressed in the heart than is GLUT1 (Fischer et al., 1997). FA uptake by cardiac myocytes is mainly mediated by the facilitative action of FAT/CD36 (Coburn et al., 2001; Brinkmann et al., 2002). In nonstimulated cardiac myocytes, a substantial portion of FAT/CD36 is stored in intracellular membrane compartments. Insulin, which is known to translocate GLUT4 from intracellular compartments to the cell surface (Kandror and Pilch, 1996), has recently also been shown to induce the translocation of FAT/CD36 to the sarcolemma in different muscle tissues (Luiken et al., 2002a,b). This insulin-induced translocation is sensitive to inhibition by wortmannin and LY-294002, indicating a dependence on activated phosphatidylinositol-3 kinase (PI3K). Other factors, such as hypoxia/ischemia, mitochondrial inhibitors, and an increase in contractile activity, known to induce GLUT4 translocation, have all been found to activate a PI3K-independent pathway involving AMP-activated protein kinase (AMPK) (Hayashi et al., 1998; Winder, 2000; Hue et al., 2002). The involvement of AMPK in the translocation of GLUT4 has been further substantiated by using the cell-permeable adenosine analog AICAR. AICAR's phosphorylated product (ZMP) has been recognized as a suitable activator of AMPK; consequently, AMPK has been shown to induce GLUT4 translocation that is sensitive to inhibitors of adenosine kinase (Hayashi et al., 1998; Russell et al., 1999). Recently, we have found that contractions, AICAR and the mitochondrial inhibitor oligomycin all stimulate FA uptake via translocation of FAT/CD36 independently of PI3K (Luiken et al., 2003). Clearly, FAT/CD36 can be mobilized from intracellular stores by two independent pathways, one inducible by PI3K and the other by AMPK. Thus, regulation of FA uptake and regulation of glucose uptake share 1) a common mechanism (i.e., transporter translocation), 2) induction by the same physiological stimuli, and 3) common signaling components.

The ability of dipyridamole to stimulate FAT/CD36 translocation in combination with the fact that it has not been documented that dipyridamole influences GLUT4 recycling makes it a potentially interesting compound to separate FAT/CD36 translocation from that of GLUT4. For this, we investigated the parallel effects of dipyridamole on FA uptake/FAT/CD36 translocation and on glucose uptake/GLUT4 translocation in the absence and presence of physiological and pharmacological modulators of PI3K and AMPK signaling. We also included the inhibitory action of dipyridamole on nucleoside transport in these studies, because it has been shown that adenosine accumulates inside cardiac myocytes in the presence of this compound (Van Belle, 1993). Adenosine, through conversion into AMP by adenosine kinase, could theoretically activate AMPK, thereby offering a potential action mechanism for dipyridamole. The results show that dipyridamole stimulates FAT/CD36-mediated FA uptake by activating the AMPK signaling pathway without increasing GLUT4-mediated glucose uptake. These observations make dipyridamole an interesting tool to identify the molecular components uniquely involved in FAT/CD36 trafficking.

Materials and Methods

Isolation of Cardiac Myocytes. Cardiac myocytes were isolated from male Lewis rats (200–250 g) using a Langendorff perfusion system and a Krebs-Henseleit bicarbonate medium supplemented with 11 mM glucose, and equilibrated with a 95% O_2/5% CO_2 gas phase (medium A) at 37°C as described previously (Luiken et al., 1997). After isolation, the cells were washed twice with medium A supplemented with 1.0 mM $CaCl_2$ and 2% (w/v) BSA (medium B) and then suspended in 15 ml of medium B. The isolated cells were allowed to recover for approximately 2 h at room temperature. At the end of the recovery period, cells were washed and suspended in medium B. Only when >80% of the cells had a rod-shaped appearance and excluded trypan blue were they used for subsequent tracer uptake studies.

Substrate Utilization by Cardiac Myocytes. Cells (2.0 ml; 5–8 mg wet mass/ml), suspended in medium B without glucose, were preincubated in capped 20-ml incubation vials for 15 min at 37°C under continuous shaking. To study palmitate uptake, 0.5 ml of the [1-^{14}C]palmitate/BSA complex was added at the start of the incubations so that the final concentration of palmitate amounted to 100 μM with a corresponding palmitate/BSA ratio of 0.3. This palmitate/BSA complex was prepared as described previously (Luiken et al., 1997). To study deoxyglucose uptake, [^{3}H]deoxyglucose was added at the start of the incubations in 0.6 ml of medium B without glucose to a final concentration of 100 μM. Cellular uptake of palmitate (3-min incubation) and of deoxyglucose (3-min incubation) was determined upon washing the cells three times for 2 min at 100*g* in an ice-cold stop solution containing 0.2 mM phloretin as described previously (20). The washing procedure did not affect cellular integrity as evaluated by microscopic inspection.

Oligomycin (30 μM), insulin (10 nM), dipyridamole (≤500 μM), amrinone (500 μM), zaprinast (100 μM), nitrobenzylthioinosine (100 μM), wortmannin (200 nM), and adenosine (≤1.0 mM) were added to the cell incubations 15 min before addition of radiolabeled substrate. 5-Iodotubercidin (20 μM) was added 90 min before any other addition. Cell suspensions were incubated with sulfo-*N*-succinimidyl-palmitate (SSP; 400 μM) for 15 min, washed to remove unbound SSP, and resuspended in medium B before addition of radiolabel. Stock solutions of oligomycin, dipyridamole, amrinone, zaprinast, nitrobenzylthioinosine, wortmannin, SSP, and 5-iodotubercidin were prepared in DMSO, which never exceeded a final concentration of 0.5% in the cell suspensions. At this concentration, DMSO did not affect cellular substrate utilization. All agents were added at the minimal concentration at which they exerted the maximal effect. None of these agents, alone or in combination and including SSP, were found to affect the percentage of cells that 1) were rod-shaped and 2) excluded trypan blue, as parameters of cellular integrity.

Measurement of Adenosine Phosphates in Cardiac Myocytes. Cardiac myocytes (12–15 mg wet mass/ml) were incubated in medium B in the absence and presence of additions for 15 min. Then, they were centrifugated in a microcentrifuge at 2000 rpm. Upon removal of the supernatant, the cell pellet was extracted with 250 μl of 3 M perchloric acid and neutralized with 1 M $KHCO_3$. Thereafter, the cellular content of adenosine phosphates was determined by high-performance liquid chromatography as described previously (Van der Vusse et al., 1984).

Measurement of Activation of AMP Kinase. Cardiac myocytes (8–12 mg wet mass/ml) were incubated in medium B in the absence and presence of additions for 15 min. At the end of the incubation, an aliquot was quickly transferred to one third volume of sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2 mM EDTA, 20 mM dithiothreitol, and 7.5% (w/v) SDS, and used for SDS-polyacrylamide gel electrophoresis. Subsequently, Western blotting was performed with an antiserum specific for the serine79-phosphorylated acetyl-CoA carboxylase according to the manufacturer's instructions.

Subcellular Fractionation of Cardiac Myocytes. Cardiac myocytes (2.25 ml; 20–25 mg wet mass/ml) were incubated for 15 min

in medium B in the absence and presence of additions. At the end of the incubation, the total volume of cell incubations was quickly transferred to a tightly fitting 5-ml Potter-Elvejhem glass homogenizer on ice containing 1 ml of ice-cold H_2O, after which NaN_3 was added to a final concentration of 5 mM to stop ATP-dependent vesicular trafficking events. Immediately thereafter, cell suspensions were homogenized with 10 strokes. Subsequently, fractionation was carried out as described previously (Fischer et al., 1995; Luiken et al., 2002b). For determination of the GLUT4 and FAT/CD36 content in the plasma membrane (PM) and in low-density microsomes (LDM), aliquots of the membrane fractions were separated with SDS-polyacrylamide gel electrophoresis and Western blotting (Luiken et al., 2002b). To detect FAT/CD36, we used a monoclonal antibody (MO25) directed against human CD36; for detection of GLUT4, a polyclonal IgG antiserum was applied. Signals obtained by Western blotting were quantified by densitometry.

The purity of the fractions obtained by this fractionation procedure was previously checked (Fischer et al., 1995). Specifically, the PM fraction is 13.5-fold enriched with ouabain-sensitive *p*-nitrophenyl-phosphatase, whereas the specific activity of the sarcoplasmatic EGTA-sensitive Ca^{2+}-ATPase was 3.6-fold decreased. In addition, no activity of *p*-nitrophenyl-phosphatase or of Ca^{2+}-ATPase could be detected in the LDM fraction, indicating that this fraction was devoid of plasma membrane and of sarcoplasmic reticulum. Furthermore, caveolin-3 was found to be 2.9-fold more abundant in the PM fraction than in the LDM fraction (data not shown).

Other Procedures. Cellular wet mass was obtained from cell samples taken during the incubation period and determined after centrifugation for 2 to 3 s at maximal speed in a microcentrifuge and subsequent removal of the supernatant. Protein was quantified with the bicinchoninic acid protein assay (Pierce, Rockford, IL) according to manufacturer's instructions.

Materials. [1-^{14}C]palmitic acid was obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). BSA (fraction V, essentially FA free) phloretin, dipyridamole, nitrobenzylthioinosine, oligomycin, adenosine, insulin, and wortmannin were all obtained from Sigma (St. Louis, MO). 5-Iodotubercidin was purchased from Biomol (Plymouth Meeting, PA). Collagenase type 2 was purchased from Worthington (Lakewood, NJ). BCA protein assay reagent kit was from Pierce (Rockford, IL). Antibody MO25 was a gift from Dr. N. N. Tandon (Thrombosis and Vascular Biology Laboratory, Otsuka America Pharmaceutical, Inc., Rockville, MD). Antibodies directed against GLUT4 were obtained from Sanver Tech (Heerhugowaard, the Netherlands). Anti-phosphoacetyl-CoA carboxylase was obtained from Brunschwig Chemie (Amsterdam, the Netherlands). SSP is routinely synthesized in our laboratory, as has been described previously (Coort et al., 2002). Its purity was confirmed with infrared spectroscopy (kindly performed by Dr. van Genderen, Eindhoven Technical University).

Data Presentation and Statistics. All data are presented as means ± S.E.M. for the indicated number of myocyte preparations. Statistical difference between groups of observations was tested with a paired Student's *t* test. *P* values ≤ 0.05 were considered significant.

Results

Effects of Dipyridamole on Substrate Uptake. Dipyridamole has previously been shown to enhance FA uptake by cardiac myocytes (Abdel-Aleem et al., 1999; Luiken et al., 2002c). We have applied dipyridamole to cardiac myocytes at a variety of concentrations up to 500 μM. Within this concentration range, dipyridamole did not alter the number of cardiac myocytes that were rod-shaped or excluded trypan blue, indicating that cellular integrity was not affected (see *Materials and Methods*). When studying FA uptake as a function of the concentration of dipyridamole, this compound seemed to be optimally increasing FA uptake at 100 μM (Fig. 1). At this concentration of dipyridamole, the intracellular ATP concentration was measured to be 1.25 ± 0.35 μmol/g of wet mass, which is not significantly different from non-treated myocytes (ATP concentration, 1.47 ± 0.27 μmol/g of wet mass), indicating that cell viability remained unchanged.

At 100 μM, the stimulatory effect of dipyridamole on FA uptake amounted to 2.0-fold, whereas simultaneous uptake of deoxyglucose was slightly (32%) inhibited (Fig. 2). In contrast, other manipulations to stimulate FA uptake also stimulated deoxyglucose uptake (Fig. 2). In this respect, oligomycin stimulated FA uptake and deoxyglucose uptake to the same extent (2.0-fold), whereas insulin had a greater stimulatory effect on deoxyglucose uptake (2.7-fold stimulation) than on FA uptake (1.6-fold stimulation).

To explore the possibility that the stimulatory effect of dipyridamole on FA uptake was caused by its inhibitory action on phosphodiesterases and on nucleoside transport, it was determined whether amrinone (phosphodiesterase III inhibitor resulting in accumulation of cyclic AMP; Endoh et al., 1982), zaprinast (phosphodiesterase V inhibitor resulting in accumulation of cyclic GMP; Gong et al., 1997), adenosine (theoretical substrate for AMP formation resulting in AMPK activation), and nitrobenzylthioinosine (nucleoside transport inhibitor; Hammond et al., 1985) were able to influence FA uptake into cardiac myocytes (Fig. 3A). However, none of these agents had an effect on FA uptake.

A further attempt to unravel the mechanism of dipyridamole's stimulatory action on FA uptake was carried out by investigating its effect in combination with other compounds that influence FAT/CD36-mediated FA uptake (Fig. 3B). Addition of the specific FAT/CD36 inhibitor SSP completely prevented the stimulatory effect of dipyridamole on FA uptake. Pharmacological activation of AMPK signaling by oli-

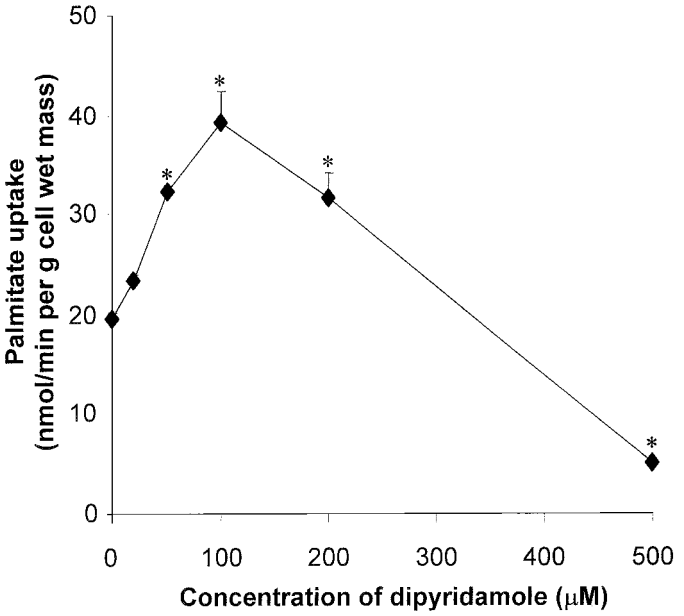

Fig. 1. Dose-dependent effect of dipyridamole on FA uptake by cardiac myocytes. Cardiac myocytes were incubated for 15 min with various concentrations of dipyridamole. Data are means ± S.E.M. of three experiments carried out with different cardiomyocyte preparations. *, $P < 0.05$; significantly different from myocytes without additions.

gomycin, a potent inhibitor of mitochondrial F_1F_0-ATPase, resulted in a 2.0-fold increase in FA uptake, but the addition of dipyridamole did not further increase FA uptake rates. In contrast, whereas insulin stimulated FA uptake (~2-fold), the addition of DPY stimulated FA uptake further by 1.6-fold. In fact, the absolute stimulatory action of dipyridamole on FA uptake was similar in the absence (+18.6 nmol/min/g of cell wet mass) and presence (+22.5 nmol/min/g of cell wet mass) of insulin (Fig. 3B). Blockade of PI3K activity upon the addition of wortmannin neither affected FA uptake into cardiac myocytes under nonstimulated conditions nor affected dipyridamole-stimulated FA uptake. The adenosine kinase inhibitor 5-iodotubercidin neither influenced FA uptake under nonstimulated conditions nor inhibited the stimulatory action of dipyridamole (Fig. 3B). In parallel cardiac myocyte incubations, we found 5-iodotubercidin able to completely block the effect of AICAR on AMPK activation (see next section), indicating that AICAR was not converted into ZMP and that adenosine kinase was indeed not active.

Effects of Dipyridamole on Intracellular AMP/ATP Ratio and on AMPK Activation. The nonadditivity of the effect of dipyridamole with oligomycin on FA uptake into cardiac myocytes indicates that dipyridamole acts on a component in the AMPK signaling pathway. Therefore, we tested the ability of dipyridamole to alter the intracellular AMP/ATP ratio and to induce phosphorylation of acetyl-CoA carboxylase, which is a sensitive marker of activation of AMPK activation (Park et al., 2002). Similar to insulin (but in contrast to oligomycin, which enhanced the AMP/ATP ratio by 2.4-fold and the phosphorylation of acetyl-CoA carboxylase by 8.6-fold), dipyridamole had no effect on both parameters of contraction signaling (Fig. 4).

To verify that 5-iodotubercidin was blocking adenosine kinase but not influencing FA uptake, we could demonstrate in parallel incubations that AICAR was able to induce phosphorylation of acetyl-CoA carboxylase by 5.7 ± 1.6-fold ($P < 0.05$) in the absence of 5-iodotubercidin and had a minor and nonsignificant effect on phosphorylation of this enzyme in the presence of 5-iodotubercidin (increase 1.3 ± 0.2-fold) ($n = 4$).

Effects of Dipyridamole on Transporter Translocation. Despite the fact that dipyridamole does not induce the activation of AMPK, the nonadditivity of the effects of dipyridamole and oligomycin on FA uptake into cardiac myocytes suggests that dipyridamole, just like oligomycin (Luiken et al., 2003), induces translocation of FAT/CD36 from a contraction-responsive storage compartment to the sarcolemma. In-

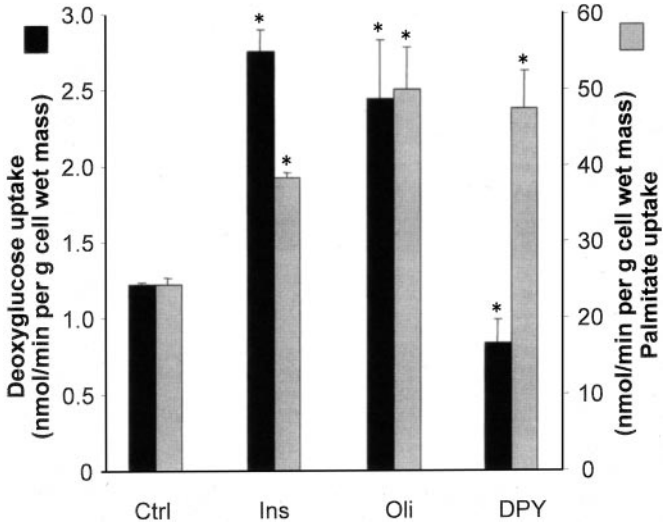

Fig. 2. Effects of insulin, oligomycin and dipyridamole on substrate uptake by cardiac myocytes. Cell suspensions were incubated in the absence of additions (Ctrl) or presence of 10 nM insulin, 30 μM oligomycin (Oli), or 100 μM DPY for 15 min before execution of palmitate uptake studies (3 min) or deoxyglucose uptake studies (3 min). Data are means ± S.E.M. of four to six experiments carried out with different cardiomyocyte preparations. *, significantly different from myocytes without additions (Ctrl).

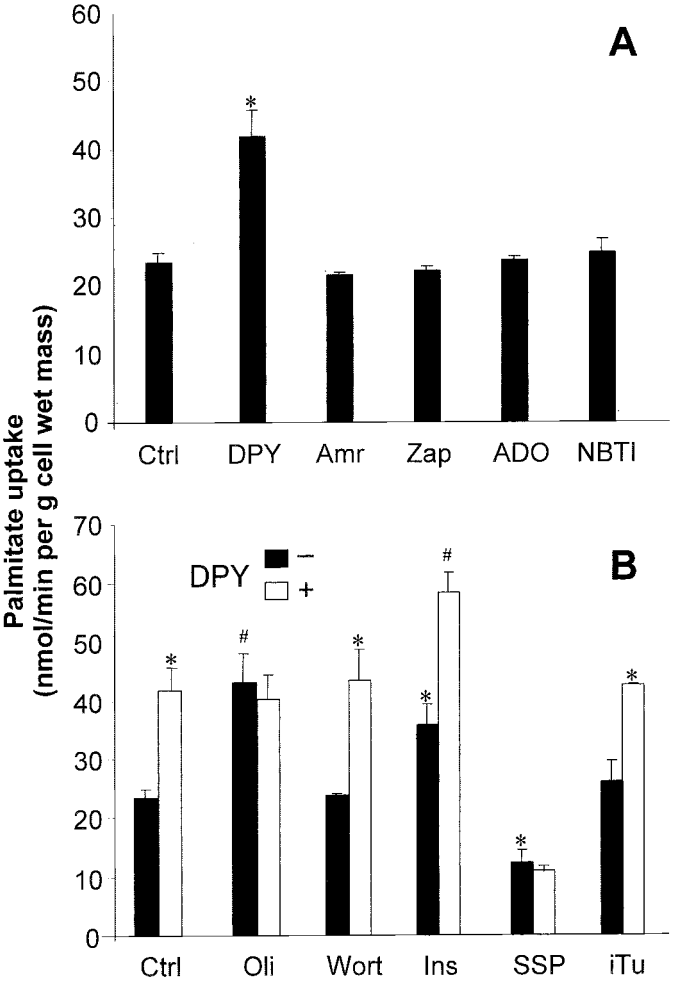

Fig. 3. Onto the mechanism of the effect of dipyridamole on FA uptake into cardiac myocytes. A, effect of phosphodiesterase inhibitors, adenosine, and a nucleoside transport inhibitor on FA uptake by cardiac myocytes. Before determination of FA uptake, cell suspensions were incubated for 15 min in the absence of additions (Ctrl) or presence of 100 μM DPY, 500 μM amrinone (Amr), 100 μM zaprinast (Zap), 300 μM adenosine (ADO) or 100 μM nitrobenzylthioinosine (NBTI) before execution of palmitate uptake studies (3 min). B, modulation of the stimulatory effect of dipyridamole on FA uptake by insulin, oligomycin, and protein inhibitors. In the case of SSP, cell suspensions were preincubated with DMSO (basal) or with 400 μM SSP (dissolved in DMSO), after which the cells were washed twice with medium B. In the case of 5-iodotubercidin (iTu), cells were suspended in DMSO (basal) or 10 μM iTu (dissolved in DMSO) for 90 min before preincubation with 100 μM DPY. In the case of the other compounds used in combination with dipyridamole [i.e., 30 μM oligomycin (Oli), 200 μM wortmannin (Wort), or 10 nM insulin (Ins)], cells were incubated in the absence or presence of DPY before determination of palmitate uptake (3 min). Data are means ± S.E.M. of four to eight experiments carried out with different cardiomyocyte preparations. *, $P < 0.05$; significantly different from myocytes without additions (Ctrl). **, $P < 0.05$; significantly different from corresponding basal myocytes.

cubation of cardiac myocytes for 15 min in the presence of oligomycin and dipyridamole decreased the content of FAT/CD36 in the LDM fraction by 42% and 46%, respectively. These agents simultaneously increased the content of FAT/CD36 in the PM fraction to a similar magnitude of 1.5-fold (Fig. 5). Oligomycin had comparable effects on subcellular distribution of GLUT4 (i.e., a decrease by 54% in the LDM fraction and an increase by 1.7-fold in the PM fraction). However, dipyridamole, unlike its stimulation of translocation of FAT/CD36 to the sarcolemma, was unable to modulate the subcellular distribution of GLUT4 (Fig. 5).

Discussion

The main purpose of this study was to assess the use of dipyridamole as a tool to examine the regulation of FAT/CD36 translocation and its relationship with GLUT4 translocation. The novel findings of this study are that 1) dipyridamole stimulates FA uptake by inducing contraction-inducible, but not insulin-inducible, FAT/CD36 translocation through interaction with an unknown component downstream from AMPK, and 2) dipyridamole is able to divorce FAT/CD36 translocation from GLUT4 translocation. These findings indicate the potential of dipyridamole to study the regulation of cardiac substrate preference at the level of trafficking of substrate transporters.

Action of Dipyridamole on Contraction-Inducible FAT/CD36 Translocation. To unravel the molecular mechanism by which dipyridamole stimulates FA uptake, we investigated whether the known ability of dipyridamole to block phosphodiesterases and nucleoside transporters was involved in stimulating FA uptake. Cyclic AMP elevation by amrinone and cyclic GMP elevation by zaprinast did not influence FA uptake into cardiac myocytes, indicating that the stimulatory action of dipyridamole on FA uptake is not related to inhibition of phosphodiesterases. Inhibition of nucleoside transport and concomitant intracellular accumulation of adenosine was ruled out as a contributing factor to dipyridamole-induced FA uptake on basis of three lines of evidence: 1) the well-established nucleoside transport inhibitor nitrobenzylthioinosine, which, like dipyridamole, causes adenosine accumulation in cardiac myocytes (Kalsi et al., 1998), did not influence FA uptake; 2) adenosine added at concentrations up to 1 mM did not stimulate FA uptake; 3) inhibition of conversion of adenosine into AMP by inclusion of the adenosine kinase inhibitor 5-iodotubercidin did not inhibit dipyridamole-induced FA uptake.

The stimulatory action of dipyridamole on FA uptake occurred at the level of FAT/CD36, based on the ability of the specific FAT/CD36 inhibitor SSP (Coort et al., 2002) to block dipyridamole-inducible FA uptake (Fig. 3B; see also Luiken et al., 2002c). By using heart-derived, giant sarcolemmal vesicles, we were previously able to exclude a direct interaction of dipyridamole with FAT/CD36. In this preparation, in which the amount of FAT/CD36 at the surface is fixed and not subject to regulation, FA uptake was not inducible by dipyridamole (Luiken et al., 2002c).

The following observations were instrumental in pinpointing the effect of dipyridamole on FA uptake to its ability to induce translocation of FAT/CD36 from contraction-inducible stores: 1) in the presence of oligomycin (Fig. 3B) or AICAR (data not shown), dipyridamole was not effective, indicating

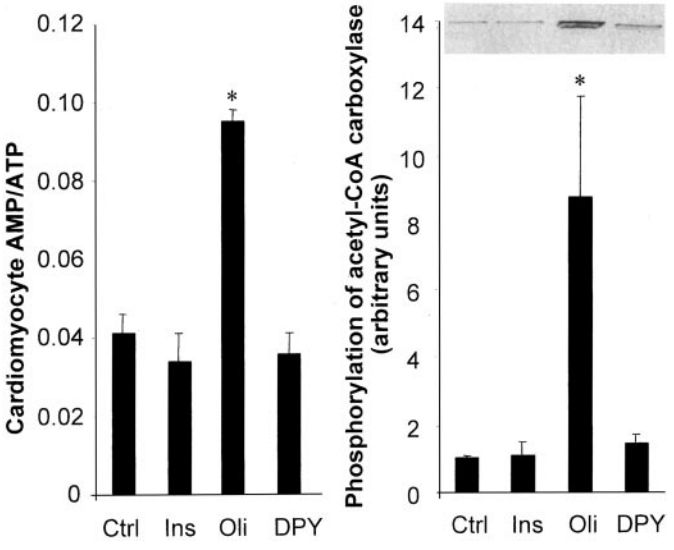

Fig. 4. Influence of insulin, oligomycin, and dipyridamole on intracellular nucleoside phosphates in and AMPK activation in cardiac myocytes. Cardiac myocytes were incubated for 15 min in the absence (Ctrl) or presence of 10 nM insulin (Ins), 30 μM oligomycin (Oli), or 100 μM dipyridamole (DPY). Intracellular levels of AMP and ATP were determined as described under *Materials and Methods*, from which the AMP/ATP ratio was calculated (left). Phosphorylation of acetyl-CoA carboxylase was detected at 280 kDa (i.e., the major isoform of this enzyme in heart) (right). However, in the oligomycin-treated cells, a second lower band is apparent, corresponding to the 265-kDa isoform. Data are means ± S.E.M. of three to six experiments carried out with different cardiomyocyte preparations. *, $P < 0.05$; significantly different from myocytes without additions (Ctrl).

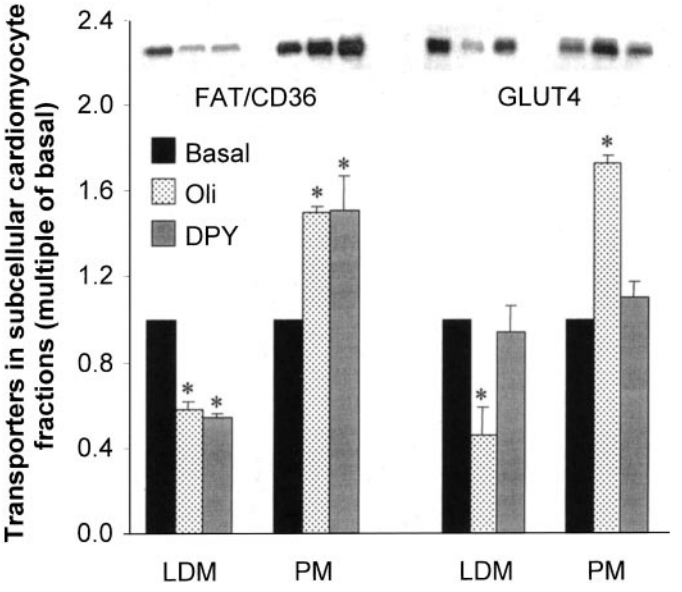

Fig. 5. Effect of oligomycin and dipyridamole on subcellular distribution of FAT/CD36 and GLUT4 in cardiac myocytes. Cardiac myocytes were incubated for 15 min in the absence (Basal) and presence of 30 μM oligomycin (Oli) or 100 μM DPY, after which NaN_3 was added to a final concentration of 5 mM to stop ATP-demanding processes. Immediately hereafter, cells were frozen in liquid nitrogen, and upon thawing, were subjected to subcellular fractionation. The collected fractions were analyzed on the relative contents of GLUT4 (45 kDa) and FAT/CD36 (88 kDa). Transporter content was expressed as multiple of control (Basal) in the corresponding fraction. Data are means ± S.E.M. of four experiments carried out with different cardiomyocyte preparations. Representative Western blots are shown. *, $P < 0.05$; significantly different from myocytes in the absence of additions (Basal).

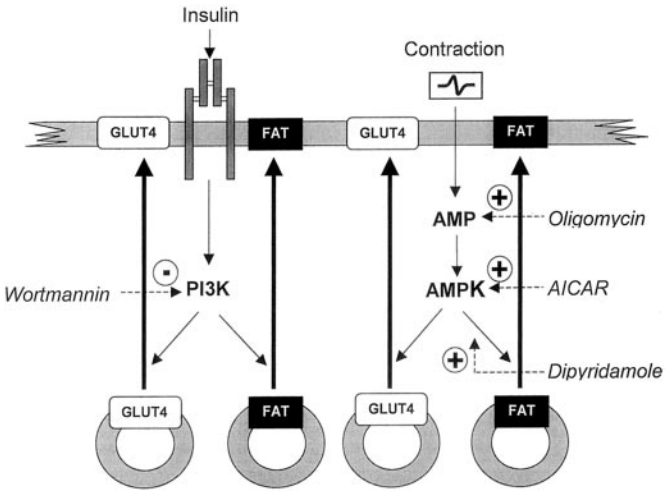

Fig. 6. Schematic presentation of signaling pathways involved in mobilization of FAT/CD36 and GLUT4 in cardiac myocytes. Intracellular FAT/CD36 can be mobilized by both insulin and cellular contractions. Insulin activates its receptor at the sarcolemma leading via a well defined pathway to activation of wortmannin-sensitive PI3K. The sequence of events downstream of PI3K is not completely elucidated but leads to mobilization of separate GLUT4- and FAT/CD36 stores. Cellular contractions induce ATP utilization concomitant with an elevation of intracellular AMP. This causes activation of AMPK. In addition to PI3K, signaling downstream of AMPK is not well defined but mobilizes both GLUT4 and FAT/CD36. Contraction-induced transporter mobilization is insensitive to wortmannin and can be activated by oligomycin, an inhibitor of mitochondrial ATPase. Contraction-induced transporter mobilization can also be activated by the cell-permeable AMP analog AICAR, which directly activates AMPK. Dipyridamole also mobilizes FAT/CD36 via the contraction pathway but does not affect GLUT4 mobilization. The site of action of dipyridamole is not yet identified but has to be downstream of AMP kinase.

that this compound is activating AMPK signaling; 2) dipyridamole, like oligomycin and insulin, is able to decrease the FAT/CD36 content in intracellular membrane compartments and simultaneously increase its content in the sarcolemma, indicating that this compound operates through induction of translocation of FAT/CD36. A possible interaction of dipyridamole with insulin-inducible FAT/CD36 translocation was excluded by the observations that 1) dipyridamole retained its full stimulatory effect on FA uptake in the presence of insulin and 2) inhibition of PI3K, a key enzyme in the insulin signaling pathway, did not block dipyridamole-inducible FA uptake.

The next goal was to locate the action of dipyridamole along the AMPK signaling pathway. The observations that dipyridamole neither elevated the intracellular AMP/ATP ratio nor enhanced the phosphorylation state of acetyl-CoA carboxylase indicated that the action of dipyridamole on contraction signaling is downstream of AMPK. Because the signaling events downstream of AMPK leading to translocation of FAT/CD36 are at present completely unknown, there is no list of candidate proteins at hand that could serve as target for dipyridamole to induce FAT/CD36 translocation.

Mechanism of Separation of FAT/CD36 Translocation from GLUT4 Migration by Dipyridamole. Unlike its stimulatory action on FA uptake and FAT/CD36 translocation, dipyridamole neither stimulates glucose uptake nor induces GLUT4 translocation. The slight inhibitory effect of dipyridamole on glucose uptake is in line with an earlier observation made in adipocytes that dipyridamole is directly interacting with glucose transporters, thereby decreasing their intrinsic activity (Steinfelder and Joost, 1988). The selective recruitment of FAT/CD36 suggests that 1) FAT/CD36 and GLUT4 are stored in distinct intracellular compartments or, on the other hand, that 2) FAT/CD36 and GLUT4 are stored in the same compartment but recruited by different signaling mechanisms. In the latter case, there must be a sorting mechanism present that is able to excise either GLUT4- or FAT/CD36-containing transport vesicles from the storage compartment shared by FAT/CD36 and GLUT4.

Because dipyridamole acts downstream of AMPK and at the same time is selective for FAT/CD36 recruitment, it follows that downstream of AMPK the contraction-induced signal transduction pathways branches off into two pathways, one leading to mobilization of intracellularly stored GLUT4 and the other to mobilization of intracellularly stored FAT/CD36. It also pinpoints the intracellular target of dipyridamole not only downstream of AMPK but also downstream of this branchpoint in AMPK signaling (i.e., at the pathway specifically leading to FAT/CD36 translocation). These novel insights are schematically depicted in Fig. 6.

Conclusion. The ability of dipyridamole to selectively recruit FAT/CD36 to the sarcolemma may help to unravel signaling proteins downstream of AMPK that are solely dedicated to FAT/CD36 trafficking without affecting GLUT4. Furthermore, this ability may make dipyridamole of potential clinical interest. For instance, it is conceivable that in cases in which AMPK signaling is impaired (by genetic or other causes), enhanced cardiac pump function would not be efficiently accompanied by an increase in substrate utilization. Then, dipyridamole could be useful as therapeutic agent to bypass the defect in AMPK activity and restore cardiac FA uptake.

Acknowledgments

Antibody MO25 was kindly provided by Dr. N. N. Tandon, (Thrombosis and Vascular Biology Laboratory, Otsuka America Pharmaceutical, Inc., Rockville, MD).

References

Abdel-Aleem S, El-Guindy N, Sallam TI, Hughes GC and Lowe JE (1999) Stimulation of long-chain fatty acid uptake by dipyridamole in isolated myocytes. *J Cardiovasc Pharmacol* **33:**43–48.

Beavo JA (1995) Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol Rev* **75:**725–748.

Brinkmann JF, Abumrad NA, Ibrahimi A, van der Vusse GJ, and Glatz JFC (2002) New insights into long-chain fatty acid uptake by heart muscle: a crucial role for fatty acid translocase/CD36. *Biochem J* **367:**561–570.

Coburn CT, Hajri T, Ibrahimi A, and Abumrad NA (2001) Role of CD36 in membrane transport and utilization of long-chain fatty acids by different tissues. *J Mol Neurosci* **16:**117–121.

Coort SLM, Willems J, Coumans WA, van der Vusse GJ, Bonen A, Glatz JFC, and Luiken JJFP (2002) Sulfo-N-succinimidyl esters of long chain fatty acids specifically inhibit fatty acid translocase (FAT/CD36)-mediated cellular fatty acid uptake. *Mol Cell Biochem* **239:**213–219.

De Schryver EL, Algra A, and van Gijn J (2003) Cochrane review: dipyridamole for preventing major vascular events in patients with vascular disease. *Stroke* **34:**2072–2080.

Endoh M, Yamashita S, and Taira N (1982) Positive inotropic effect of amrinone in relation to cyclic nucleotide metabolism in the canine ventricular muscle. *J Pharmacol Exp Ther* **221:**775–783.

Fischer Y, Thomas J, Rosen P, and Kammermeier H (1995) Action of metformin on glucose transport and glucose transporter GLUT1 and GLUT4 in heart muscle cells from healthy and diabetic rats. *Endocrinology* **136:**412–420.

Fischer Y, Thomas J, Sevilla L, Munoz P, Becker C, Holman G, Kozka IJ, Palacin M, Testar X, Kammermeier H, and Zorzano A (1997) Insulin-induced recruitment of glucose transporter 4 (GLUT4) and GLUT1 in isolated rat cardiac myocytes. Evidence of the existence of different intracellular GLUT4 vesicle populations. *J Biol Chem* **272:**7085–7092.

Gong GX, Weiss HR, Tse J, and Scholz PM (1997) Cyclic GMP decreases cardiac myocyte oxygen consumption to a greater extent under conditions of increased metabolism. *J Cardiovasc Pharmacol* **30:**537–543.

Hammond JR, Williams EF, and Clanachan AS (1985) Affinity of calcium channel inhibitors, benzodiazepines and other vasoactive compounds for the nucleoside transport system. *Can J Physiol Pharmacol* **63:**1302–1307.
Hayashi T, Hirshman MF, Kurth EJ, Winder WW, and Goodyear LJ (1998) Evidence for 5′ AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes* **47:**1369–1373.
Hue L, Beauloye C, Marsin AS, Bertrand L, Horman S, and Rider MH (2002) Insulin and ischemia stimulate glycolysis by acting on the same targets through different and opposing signaling pathways. *J Mol Cell Cardiol* **34:**1091–1097.
Humphreys DM, Street J, Schumacher H, Bertrand-Hardy JM, and Palluk R (2002) Dipyridamole may be used safely in patients with ischaemic heart disease. *Int J Clin Pract* **56:**121–127.
Kalsi KK, Smolenski RT, and Yacoub MH (1998) Effects of nucleoside transport inhibitors and adenine/ribose supply on ATP concentration and adenosine production in cardiac myocytes. *Mol Cell Biochem* **180:**193–199.
Kandror KV and Pilch PF (1996) Compartmentalization of protein traffic in insulin-sensitive cells. *Am J Physiol* **271:**E1–E14.
Komas N, Le Bec A, Stoclet JC, and Lugnier C (1991) Cardiac cGMP-stimulated cyclic nucleotide phosphodiesterases: effects of cGMP analogues and drugs. *Eur J Pharmacol* **206:**5–13.
Luiken JJFP, van Nieuwenhoven FA, America G, van der Vusse GJ, and Glatz JFC (1997) Uptake and metabolism of palmitate by isolated cardiac myocytes from adult rats: involvement of sarcolemmal proteins. *J Lipid Res* **38:**745–758.
Luiken JJFP, Dyck DJ, Han XX, Tandon NN, Arumugam Y, Glatz JFC, and Bonen A (2002a) Insulin induces the translocation of the fatty acid transporter FAT/CD36 to the plasma membrane. *Am J Physiol* **282:**E491–E495.
Luiken JJFP, Koonen DPY, Willems J, Zorzano A, Becker C, Fischer Y, Tandon NN, Van Der Vusse GJ, Bonen A, and Glatz JFC (2002b) Insulin stimulates long-chain fatty acid utilization by rat cardiac myocytes through cellular redistribution of FAT/CD36. *Diabetes* **51:**3113–3119.
Luiken JJFP, Willems J, Coort SLM, Coumans WA, Bonen A, Van Der Vusse GJ, and Glatz JFC (2002c) Effects of cAMP modulators on long-chain fatty-acid uptake and utilization by electrically stimulated rat cardiac myocytes. *Biochem J* **367:**881–887.
Luiken JJFP, Coort SLM, Willems J, Coumans WA, Bonen A, van der Vusse GJ, and Glatz JFC (2003) Contraction-induced FAT/CD36 translocation in rat cardiac myocytes is mediated through AMP-activated protein kinase signaling. *Diabetes* **52:**1627–1634.
MacWalter RS and Shirley CP (2002) A benefit-risk assessment of agents used in the secondary prevention of stroke. *Drug Saf* **25:**943–963.
Mainwaring RD and Mentzer RM Jr (1986) Effects of dipyridamole on myocardial glucose uptake in the newborn lamb. *J Surg Res* **40:**528–533.
Park SH, Gammon SR, Knippers JD, Paulsen SR, Rubink DS, and Winder WW (2002) Phosphorylation-activity relationships of AMPK and acetyl-CoA carboxylase in muscle. *J Appl Physiol* **92:**2475–2482.
Pettigrew LC and Williams SC (2002) Antithrombotic drugs for prevention of recurrent stroke. *J Ky Med Assoc* **100:**184–193.
Prasad RK, Behrooz A, and Ismail-Beigi F (1999) LY-83583 stimulates glucose transporter-1-mediated glucose transport independent of changes in cGMP levels. *Eur J Pharmacol* **366:**101–109.
Russell RR 3rd, Bergeron R, Shulman GI, and Young LH (1999). Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *Am J Physiol* **277:**H643–H649.
Steinfelder HJ and Joost HG (1988) Inhibition of insulin-stimulated glucose transport in rat adipocytes by nucleoside transport inhibitors. *FEBS Lett* **227:**215–219.
Van Belle H (1993) Nucleoside transport inhibition: a therapeutic approach to cardioprotection via adenosine? *Cardiovasc Res* **27:**68–76.
Van der Vusse GJ, Coumans WA, van der Veen E, Drake AJ, Flameng W, and Suy R (1984) ATP, creatine phosphate and glycogen content in human myocardial biopsies: markers for the efficacy of cardioprotection during aorto-coronary bypass surgery. *Vasc Surg* **18:**127–134.
Winder WW (2000) AMP-activated protein kinase: possible target for treatment of type 2 diabetes. *Diabetes Technol Ther* **2:**441–448.

Address correspondence to: Dr. Joost J. F. P. Luiken, Department of Molecular Genetics, CARIM, Maastricht University, P.O. Box 616, NL-6200 MD Maastricht. E-mail: j.luiken@gen.unimaas.nl