Effects of cAMP modulators on long-chain fatty-acid uptake and utilization by electrically stimulated rat cardiac myocytes

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Recently, we established that cellular contractions increase long-chain fatty-acid (FA) uptake by cardiac myocytes. This increase is dependent on the transport function of an 88 kDa membrane FA transporter, FA translocase (FAT/CD36), and, in analogy to skeletal muscle, is likely to involve its translocation from an intracellular pool to the sarcolemma. In the present study, we investigated whether cAMP-dependent signalling is involved in this translocation process. Isoproterenol, dibutyryl-cAMP and the phosphodiesterase (PDE) inhibitor, amrinone, which markedly raised the intracellular cAMP level, did not affect cellular FA uptake, but influenced the fate of intracellular FAs by directing these to mitochondrial oxidation in electrostimulated cardiac myocytes. The PDE inhibitors 3-isobutyl-1-methylxanthine, milrinone and dipyridamole each significantly stimulated FA uptake as well as intracellular cAMP levels, but these effects were quantitatively unrelated. The stimulatory effects of these PDE inhibitors were antagonized by sulpho-*N*-succinimidylpalmitate, indicating the involvement of FAT/CD36, albeit that the different PDE inhibitors use different molecular mechanisms to stimulate FAT/CD36-mediated FA uptake. Notably, 3-isobutyl-1-methylxanthine and milrinone increased the intrinsic activity of FAT/CD36, possibly through its covalent modification, and dipyridamole induces translocation of FAT/CD36 to the sarcolemma. Elevation of intracellular cGMP, but not of cAMP, by the PDE inhibitor zaprinast did not have any effect on FA uptake and metabolism by cardiac myocytes. The stimulatory effects of PDE inhibitors on cardiac FA uptake should be considered when applying these agents in clinical medicine.

Key words: cellular contractions, membrane fatty acid transporters, phosphodiesterase inhibitor, translocation of fatty acid translocase (FAT)/CD36.

INTRODUCTION

Evidence is accumulating that plasma membrane (PM) proteins are involved in bulk uptake of long-chain fatty acids (FAs) into mammalian cells. Three putative transporters have been postulated to function in transmembrane transport of FAs, and each is present in the sarcolemma of cardiac and skeletal muscle cells. These are 40 kDa PM FA-binding protein ('FABPpm'), 88 kDa FA translocase (FAT/CD36), and 62 kDa FA-transport protein ('FATP') [1–4]. The mechanism(s) by which these transporters facilitate cellular FA uptake is not known. Our recent studies [2,5–7] strongly indicate that FAT/CD36 plays a key role in the *trans*-sarcolemmal uptake of FA in heart and skeletal muscle. In these studies and others [5,6,8,9], we used sulpho-*N*-succinimidylesters of FAs as specific inhibitors of the FA-transport action of FAT/CD36.

When rat hindlimb muscles were electrically stimulated to contract, we found evidence for the novel concept that cellular FA uptake is regulated by FAT/CD36 translocation [6]. Muscle contractions resulted in simultaneous redistribution of FAT/CD36 and of GLUT4 from endosomes to the sarcolemma, leading to an increase in the quantity of surface transporters. More recently, we observed that FA uptake by isolated cardiac myocytes is also substantially increased by electrically induced contractions [10]. With 4 Hz stimulation, initial FA uptake was increased by 1.5-fold. The additional palmitate taken up by these electrically stimulated cells was channelled into mitochondrial β-oxidation for energy production to support contractile activity. Contraction-induced FA uptake was sensitive to sulpho-*N*-succinimidyloleate (SSO), indicating that FAT/CD36 is involved in mediating contraction-induced FA uptake. In analogy to electrostimulated skeletal muscle, it is likely that an increased workload in cardiac myocytes induces FAT/CD36 translocation to the PM. At present, the signalling pathway by which cellular contractions recruit intracellularly stored FAT/CD36 to the sarcolemma is unknown.

It is well documented that myocyte contractility is regulated by intracellular levels of cAMP. Effects of cAMP on contraction are mediated through protein kinase A-induced phosphorylation of proteins involved in myocardial Ca^{2+} regulation, such as the sarcolemmal L-type Ca^{2+} channel [11]. Manipulations to raise the intracellular cAMP concentration have an inotropic effect. For example, Kammermeier and co-workers [12] have demonstrated that the β-agonist isoproterenol enhances the amplitude of the contraction of isolated cardiac myocytes by up to 3-fold. This inotropic action of isoproterenol leads to increased energy demands, as can be deduced from a rise in O_2 consumption when this β-agonist was added to electrically stimulated cardiac myocytes.

The intracellular concentration of cAMP can be increased in different manners: (i) by using cell-permeant cAMP analogues, (ii) by stimulation with β-adrenergic hormones, (iii) by inhibition of cAMP breakdown through phosphodiesterase (PDE) inhibitors, and (iv) by increasing intracellular [Ca^{2+}]. Abdel-Aleem and Frangakis [13] have investigated the possibility that cAMP-elevating agents could enhance FA oxidation by cardiac myocytes. In incubations of quiescent myocytes in suspension, a cell-permeant cAMP analogue dibutyryl cAMP (db-cAMP), isoproterenol and the Ca^{2+} ionophores ionomycin and A23187

Abbreviations used: db-cAMP, dibutyryl-cAMP; FA, long-chain fatty acid; FAT/CD36, fatty acid translocase; IBMX, 3-isobutyl-1-methylxanthine; LDM, low-density microsomal; PDE, phosphodiesterase; PM, plasma membrane; SSO, sulpho-*N*-succinimidyloleate; SSP, sulpho-*N*-succinimidylpalmitate.

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all failed to increase both FA uptake and oxidation. However, these same researchers [13,14] have shown that the PDE inhibitors enoximone (PDE III inhibitor) and dipyridamole (an inhibitor of PDE V and PDE VII) [15,16] both markedly increased FA oxidation by cardiac myocytes. Based on the lack of an effect on FA uptake and oxidation with β-adrenergic stimuli, the effect of the PDE inhibitors must be independent of the increase in intracellular cAMP. However, these PDE inhibitors were not effective in stimulating the oxidation of acyl-CoA and of acylcarnitine in cell homogenates, whereas they did stimulate initial uptake of FA into intact cells [13]. These results suggested that the increase in FA oxidation by both agents was secondary to an increase in uptake of these substrates, implying that these agents stimulated FA utilization at the level of the sarcolemma in a cAMP-independent manner.

In the light of the novel evidence that cellular FA uptake is regulated by the subcellular distribution of FAT/CD36, the first aim of the present study was to investigate the influence of cAMP-elevating agents on SSO-sensitive FA uptake and on FAT/CD36 translocation in cardiac myocytes. Furthermore, since the inotropic effect of an elevation in cAMP cannot be manifested in quiescent myocytes, the second aim of the study was to re-evaluate the effects of cAMP-elevating agents on FA uptake and metabolism by contracting myocytes, using the technique of electrical field stimulation of cardiac myocytes in suspension. Finally, we aimed to assess the possible cAMP-unrelated effects of PDE inhibitors at the level of the sarcolemma using heart giant sarcolemmal vesicles allowing the study of FA uptake in the absence of subsequent FA metabolism. In these investigations, we used the following tools: (i) isoproterenol, db-cAMP, and the PDE inhibitors 3-isobutyl-1-methylxanthine (IBMX; inhibits all PDE isoforms [17]), amrinone and milrinone (inhibits PDE III [18]), zaprinast (inhibits PDE V and VI [19]) and dipyridamole.

The main findings of the present study were that elevated intracellular levels of cAMP did not affect cellular uptake of FAs by cardiac myocytes. However, cAMP induction caused a contraction-dependent channelling of incoming FAs into mitochondrial β-oxidation. An unexpected outcome of the present study was that all tested PDE inhibitors increased FA uptake, not in a manner related to intracellular cAMP levels, but rather by enhancing the flux through FAT/CD36 at the cell surface.

EXPERIMENTAL

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 and 5% CO_2 gas phase (medium A) at 37 °C, as described by Fischer et al. [20], which has been described previously [21]. 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 approx. 2 h at 20 °C. 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.

Electrical stimulation of cardiac myocytes in suspension

Electrical stimulation of cardiac myocytes in suspension has been described in detail elsewhere [10]. Briefly, cell suspensions were subjected to an electric field via two platinum electrodes that were connected to a pulse generator. The duration of a monophasic pulse was set at 100 μs, and the time interval between the monophasic components before reversal of the voltage was fixed at 10 μs. The voltage was set at 200 V and the frequency at 4 Hz.

Substrate utilization by cardiac myocytes

Cells (1.8 ml; 5–8 mg of wet mass/ml), suspended in medium B, were preincubated in capped 20-ml incubation vials for 15 min at 37 °C under continuous shaking. At the start of the incubations, 0.6 ml of the [1-^{14}C]palmitate–BSA complex was added so that the final concentration of palmitate was 100 μM, with a corresponding palmitate/BSA ratio of 0.3. This palmitate–BSA complex was prepared as described previously [21]. Palmitate uptake (3-min incubation period), oxidation (measured as production of $^{14}CO_2$ after 20 min of incubation) and esterification (measured as incorporation of the radiolabel into phospholipids and triacylglycerols after 20 min of incubation) were determined as described previously [21]. Uptake of 100 μM 2-deoxy-D-[1-^{3}H]glucose by cardiac myocytes after 3 min of incubation was also measured as described previously [21].

Isoproterenol (final concentration of 1 μM), insulin (10 nM), db-cAMP (100 μM), IBMX (500 μM), amrinone (500 μM), milrinone (500 μM), zaprinast (100 μM), dipyridamole (100 μM) and wortmannin (200 nM) were added to the cell incubations 15 min prior to addition of radiolabelled palmitate. To test the involvement of FAT/CD36, cell suspensions were incubated with sulpho-*N*-succinimidylpalmitate (SSP; 400 μM) for 15 min, and subsequently washed to remove unbound SSP, and resuspended in medium B prior to addition of the radiolabel. Stock solutions of db-cAMP, IBMX, milrinone, dipyridamole, wortmannin and SSP 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 (i) were rod-shaped and (ii) excluded Trypan Blue, as parameters of cellular integrity. It must be stressed that at the indicated concentrations, these agents did not significantly affect the ratio of cellular protein content over cell wet mass (results not shown; for description of measurement of protein and cell wet mass, see below), amounting to 0.18 under basal conditions [21]. Hence, their observed effects on parameters of palmitate utilization cannot be explained by alterations in cell volume.

Isolation of giant sarcolemmal vesicles from heart for determination of palmitate uptake

Giant vesicles were isolated from heart muscle, and palmitate uptake rates by these vesicles were determined as described previously [9]. cAMP-elevating agents were added 15 min prior to the measurement of palmitate uptake at the same concentrations as applied to cardiac myocytes (see above).

cAMP accumulation in cardiac myocytes

Cells (2.0 ml; 5–8 mg of wet mass/ml) were preincubated in medium B under identical conditions as described for incubations with [1-^{14}C]palmitate. Agonists were added to the cell suspensions at the concentrations indicated above. After 15 min, 100–200 μl of cell sample was centrifuged in a microcentrifuge at 200 ***g*** for 1 min. The pellet was used for determination of intracellular cAMP levels using an enzyme-immunoassay kit from Amersham

Life Sciences (Little Chalfont, Bucks., U.K.), in accordance with the manufacturer's instructions, without acetylation.

Subcellular fractionation of cardiac myocytes

Cardiac myocytes (2.25 ml; 20–25 mg of wet mass/ml) were incubated in medium B in the absence and presence of PDE inhibitors for 15 min. At the end of the incubation, the total cell suspension was diluted with 1.0 ml of water, and NaN_3 was added to a final concentration of 5 mM in order to stop ATP-dependent vesicular trafficking events, such as GLUT4 translocation. Immediately thereafter, cell suspensions were homogenized in a tightly fitting 10-ml Potter–Elvejhem glass homogenizer with 10 strokes, and frozen in liquid N_2. Subsequently, fractionation was carried out as described by Fischer et al. [22]. For determination of the FAT/CD36 content in PM and low-density microsomal (LDM) fraction, aliquots of the membrane fractions were separated with SDS/PAGE and Western blotting using the monoclonal antibody MO25, as described previously [6]. Signals obtained by Western blotting were quantified by densitometry.

Other procedures

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

Materials

[1-^{14}C]Palmitic acid and 2-deoxy-D-[1-^{3}H]glucose were obtained from Amersham Life Sciences. BSA (fraction V, essentially FA free) and phloretin were obtained from Sigma (St. Louis, MO, U.S.A.). In addition, all cAMP-elevating agents were obtained from Sigma. Collagenase type II was purchased from Worthington (Lakewood, NJ, U.S.A.). SSO and SSP were routinely synthesized in our laboratory, as has been described previously [23]. Purity of these compounds was confirmed with infrared spectroscopy (kindly performed by Dr van Genderen, Eindhoven Technical University, Eindhoven, The Netherlands).

Calculations and statistics

All results are expressed as the means $\pm$ S.E.M. for the indicated number of myocyte and giant vesicle preparations. Statistical difference between groups of observations was tested with a paired Student's t test. P values equal to or less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Effects of isoproterenol and db-cAMP on FA utilization by cardiac myocytes

In quiescent cardiac myocytes the intracellular cAMP level was increased by 3.7- and 13.3-fold upon a 15-min exposure to isoproterenol and db-cAMP respectively (Table 1). In contrast, initial palmitate uptake in quiescent cardiac myocytes was not increased by isoproterenol or db-cAMP (Table 1). When electrically stimulating cells at 4 Hz, the induction of cellular contractions did not by itself increase intracellular cAMP concentrations, but was able to enhance FA uptake by 1.5-fold (Table 1). In addition, electrical stimulation was without effect on cAMP in the presence of isoproterenol and db-cAMP, and neither isoproterenol or db-cAMP affected the contraction-induced uptake of palmitate (Table 1). Overall, these findings strongly suggest that alterations in intracellular cAMP are not involved in regulation of FA uptake by cardiac myocytes. Hence, it is highly unlikely that the FA uptake process is under control of protein kinase A-dependent signal-transduction pathways. However, a permissive action of (a low concentration of) cAMP cannot be excluded.

Effects of PDE inhibitors on FA uptake by cardiac myocytes

The non-selective PDE inhibitor IBMX had the greatest effect on accumulation of cAMP in cardiac myocytes. Furthermore, the cAMP-elevating effect of milrinone (2.8-fold) was greater than that of dipyridamole (1.9-fold), which is in agreement with the notion that PDE III is the major PDE isofom in heart [24]. Amrinone, similar to milrinone, is a selective inhibitor of PDE III. Accordingly, the cAMP-stimulatory effect of amrinone in cardiac myocytes is reported to be very similar to that of milrinone [25–27]. On the other hand, zaprinast, a selective inhibitor of cGMP-specific PDEs, does not affect intracellular cAMP in cardiac myocytes, but enhances cGMP levels by 2-fold [28,29]. Electrically induced contractions only had a significant stimulatory effect on cAMP accumulation in the presence of IBMX (Table 1).

Considering the effects of the selected PDE inhibitors on FA uptake by cardiac myocytes, it was observed that IBMX, milrinone and dipyridamole enhanced FA uptake by 1.5-fold, 1.3-fold and 2.0-fold respectively, whereas amrinone and zaprinast were without effect (Table 1). Thus, in the absence of a general stimulatory effect of PDE inhibitors, it is clear that the stimulatory effect of IBMX, milrinone and dipyridamole on FA uptake is unrelated to PDE activity. Moreover, this stimulatory effect of IBMX, milrinone and dipyridamole on FA uptake is also not proportional to their effect on cAMP levels (Table 1), again arguing against a role of cAMP in regulation of FA uptake.

Studying the effects of PDE inhibitors in 4 Hz-stimulated cells, the contractions of which by themselves enhanced FA uptake by 1.5-fold (see above), revealed that the stimulatory effects of IBMX and of milrinone were additive to that of electrostimulation ($P < 0.05$), whereas amrinone, zaprinast and dipyridamole were without effect (Table 1). These surprising findings allow the speculation that the mechanism by which IBMX and milrinone increase FA uptake is different from that of dipyridamole. This notion is bolstered by the observation that the stimulation of FA uptake by IBMX was additive to that of dipyridamole (Figure 1).

To study in further detail the mechanism by which IBMX, milrinone and dipyridamole stimulated FA uptake, we used SSP, which specifically inhibits FAT/CD36 without affecting PM FA-binding protein and FA-transport proteins (discussed in detail in [10]). Preincubation of quiescent cardiac myocytes with SSP reduced palmitate uptake by 50 % (Figure 1). In the presence of this FA-transport inhibitor, cellular FA uptake is not enhanced by cellular contractions or by any of the selected PDE inhibitors (Figure 1). Hence, inhibition of FAT/CD36 abolishes contraction-induced- and PDE-inhibitor-induced FA uptake completely.

The only other reports on the influence of PDE inhibitors on FA utilization by cardiac myocytes are from Abdel-Aleem and co-workers [13,14,30], and suggest that PDE inhibitors directly stimulate the FA-uptake process at the level of the sarcolemma. The ability of SSP to antagonize the stimulatory effects of IBMX, milrinone and dipyridamole on FA uptake in the present study, indeed, pinpoints the effects of the PDE inhibitors at the level of the membrane-associated FAT/CD36, albeit that FAT/

Table 1 Effect of cAMP-elevating agents on intracellular cAMP levels and FA uptake into quiescent and electrically stimulated cardiac myocytes

To determine intracellular cAMP levels, cell suspensions were incubated for 15 min followed by pelleting and measurement of intracellular cAMP. To determine FA uptake, cell suspensions were incubated for 15 min prior to execution of palmitate uptake studies (3 min). Electrical stimulation was at 200 V and 4 Hz. Basal, cardiac myocytes incubated in the absence of additions; n.m., not measured. Results are expressed as the means ± S.E.M. for 3–5 experiments (cAMP content) or for 4–8 experiments (FA uptake). *Significantly different from control (Basal). †Significantly different from corresponding quiescent myocytes (−).

		cAMP content (nmol/g of wet mass)		Palmitate uptake rate (nmol/g of wet mass per min)	
Parameter	Electrical stimulation ...	−	+	−	+
Basal		0.30 ± 0.07	0.39 ± 0.13	21.1 ± 1.4	31.1 ± 0.6†
Isoproterenol (1 μM)		1.08 ± 0.17*	0.92 ± 0.25*	20.1 ± 0.3	31.8 ± 1.6†
db-cAMP (100 μM)		3.93 ± 0.61*	3.94 ± 0.60*	19.9 ± 0.4	31.0 ± 1.9†
IBMX (500 μM)		1.75 ± 0.23*	2.49 ± 0.39*†	31.2 ± 2.0*	43.1 ± 3.5†
Amrinone (500 μM)		n.m.	n.m.	18.9 ± 0.1	27.5 ± 1.6†
Milrinone (500 μM)		0.85 ± 0.23*	1.26 ± 0.54	26.0 ± 0.6*	34.0 ± 0.7†
Zaprinast (100 μM)		n.m.	n.m.	19.4 ± 0.3	28.4 ± 1.3†
Dipyridamole (100 μM)		0.57 ± 0.18*	0.42 ± 0.11	42.4 ± 4.3*	43.3 ± 3.0

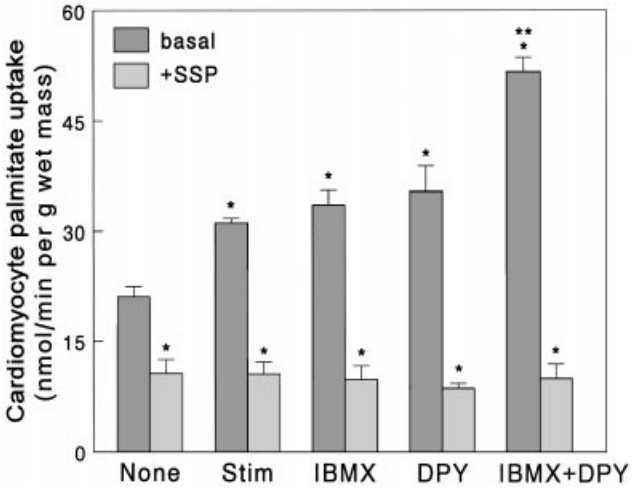

Figure 1 Modulation of effects of cAMP-elevating agents and electrostimulation on palmitate uptake by SSP

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. Subsequently, cells were incubated in the absence of additions (None), or in the absence of additions but in the presence of electrical stimulation at 200 V and 4 Hz (Stim), 100 μM IBMX and 500 μM dipyridamole (DPY) prior to determination of palmitate uptake (3 min). Results are expressed as the means ± S.E.M. for 4–6 experiments carried out with different cardiomyocyte preparations. *Significantly different from myocytes without additions (basal, none). **Significantly different from IBMX and from DPY (only calculated for IBMX + DPY) ($P < 0.05$).

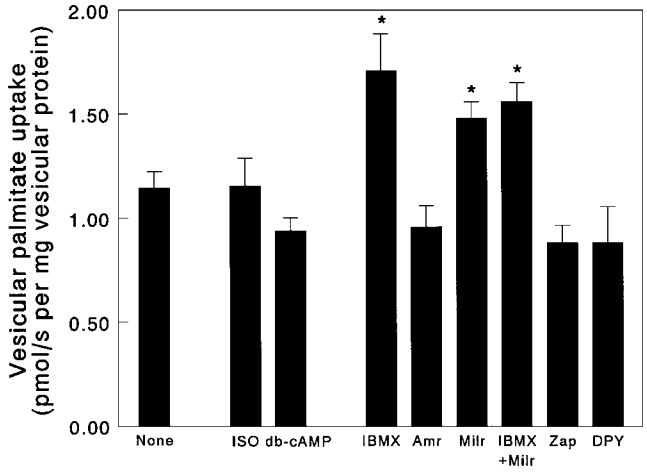

Figure 2 Effect of cAMP-elevating agents on palmitate uptake by heart giant vesicles

Vesicle suspensions were incubated for 15 min in the absence of additions (None), or with isoproterenol (ISO), db-cAMP, IBMX, amrinone (Amr), milrinone (Milr), IBMX plus Milr, zaprinast (Zap) or dipyridamole (DPY) at the concentrations described in the Experimental section and in Table 1 prior to determination of palmitate uptake (15 s). Results are expressed as the means ± S.E.M. for 4–10 experiments carried out with different giant vesicle preparations. *Significantly different from no additions (None) ($P < 0.05$).

CD36 is differentially involved in the stimulatory effects of IBMX and milrinone compared with that of dipyridamole.

Effects of cAMP-elevating agents on FA uptake by heart giant vesicles

Giant vesicles prepared from heart were used to examine more specifically whether the effects of cAMP-elevating agents on FA uptake were exerted at the level of the sarcolemma. Only IBMX (+49%, $P < 0.05$) and milrinone (+29%, $P < 0.05$) were observed to increase plasmalemmal FA transport (Figure 2). The effects of milrinone and IBMX were not additive, indicating that both compounds stimulated FA uptake through an identical mechanism of action. It should be noted that this increase in FA uptake cannot be caused by a change in sarcolemmal FAT/CD36 content, because these vesicles lack intracellular organelles, such as the endosomal-recycling compartment [9,31] and, hence, translocation of FAT/CD36 cannot occur. The ability of IBMX and milrinone to activate FA uptake into giant vesicles is most likely to be caused by an enhancement of the intrinsic activity of FAT/CD36, since, as already mentioned, FAT/CD36 in giant vesicles is solely present at the vesicular surface. Notably, the magnitude of stimulation of FA uptake by IBMX or by milrinone is similar for cardiac myocytes (IBMX, +48%; milrinone, +23%; Table 1) and for giant vesicles (IBMX, +49%; milrinone +29%; Figure 2), indicating that in cardiac myocytes the stimulatory action of these compounds can be entirely ascribed to an increase in intrinsic activity of FAT/CD36. In

accordance with this notion, evidence that CD36 from platelets contains potential phosphorylation sites [32] allows to us to speculate that cardiac FAT/CD36 is activated by phosphorylation to enhance FA uptake.

Effects of cAMP-elevating agents on subcellular distribution of FAT/CD36 in cardiac myocytes

The differential effects of PDE inhibitors on FAT/CD36-mediated FA uptake were further studied by subcellular fractionation of cardiac myocytes and assessment of the subcellular localization of FAT/CD36. Previously, we have demonstrated that electrical stimulation increases FA uptake by cardiac myocytes through translocation of FAT/CD36 from intracellular stores to the sarcolemma [10]. The non-additivity of the stimulatory effect of dipyridamole on FA uptake with that of electrostimulation, also suggests that dipyridamole stimulates FA uptake through translocation of FAT/CD36. On the other hand, FAT/CD36 translocation seems not to be involved in the stimulatory effects of IBMX. These subcellular fractionation studies showed that IBMX did not influence the distribution of FAT/CD36 between a PM fraction enriched with ouabain-sensitive *p*-nitrophenyl-phosphatase, and an LDM fraction enriched with EGTA-sensitive Ca^{2+}-ATPase [22]. In view of the similar effects of IBMX and milrinone on FA uptake by giant vesicles, it was inferred that also milrinone does not affect the subcellular distribution of FAT/CD36. In contrast, addition of dipyridamole decreased the abundance of FAT/CD36 in the LDM fraction by 45 % and simultaneously increased its abundance in the PM fraction by 1.5-fold (Figure 3). Hence, dipyridamole, but not IBMX, stimulates FA uptake through translocation of FAT/CD36 to the PM from an intracellular storage compartment that is also used for recruitment of FAT/CD36 during cellular contractions.

Enhanced intracellular cAMP levels are associated with channelling of FA into β-oxidation

The possibility still remains that cAMP is regulating cardiac FA utilization at a site beyond *trans*-sarcolemmal transport, which may indirectly alter FA uptake. Since the inotropic effects of β-adrenergic stimulation are known to increase oxidative energy metabolism [12,33,34] to support enhanced contractile activity, the possiblity that intracellular cAMP regulates FA utilization by cardiac myocytes at the level of β-oxidation was investigated in the present study.

It was observed that isoproterenol and db-cAMP, both of which did not increase the rates of FA uptake in either quiescent or contracting cardiac myocytes, also failed to alter the rates of palmitate oxidation in quiescent myocytes. When investigating the effects of these cAMP-elevating agents in cardiac myocytes exposed to electrical stimulation, which by itself increased palmitate oxidation by 2.7-fold (Figure 4A), addition of isoproterenol to these contracting myocytes further stimulated palmitate oxidation by 1.7-fold, whereas db-cAMP caused an 1.9-fold increase (Figure 4A). Thus, the stimulatory effect of an elevation of intracellular cAMP on FA oxidation can only be appreciated in contracting cardiac myocytes, because cAMP-elevating agents cause an additional positive inotropic effect on these cardiac myocytes when forced to contract by electrical stimulation. This explains the difference in experimental outcome between our present study and that of Abdel-Aleem and Frangakis [13], who studied quiescent cardiac myocytes and observed no stimulating effect of isoproterenol and db-cAMP on the rate of FA oxidation. Notably, their findings [13] were

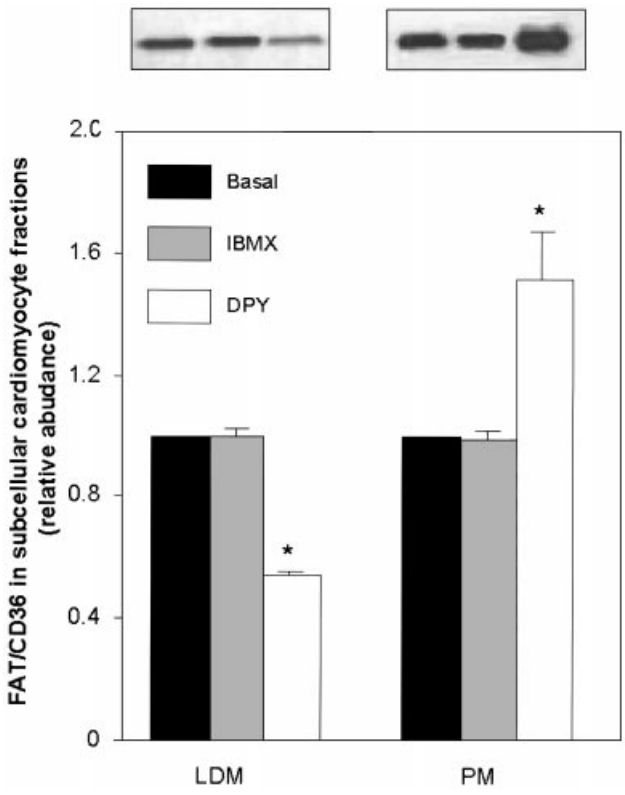

Figure 3 Effect of cAMP-elevating agents on subcellular distribution of FAT/CD36 in cardiac myocytes

Cardiac myocytes were incubated for 15 min in the absence (Basal) and presence of 500 μM IBMX or 100 μM dipyridamole (DPY), after which NaN_3 was added to a final concentration of 5 mM to stop ATP-demanding processes. Immediately thereafter, cells were frozen in liquid N_2, and upon thawing, subjected to subcellular fractionation. The collected fractions were subjected to electrophoresis and Western blotting. Signals were quantified by densitometry. FAT/CD36 content is expressed as a multiple of control (basal) in the corresponding fraction. Results are expressed as the means $\pm$ S.E.M. for four experiments carried out with different cardiomyocyte preparations. Representative Western blots are shown. FAT/CD36 was detected at 88 kDa. *Significantly different from the control (no additions; $P < 0.05$)

confirmed in the present study. It should be stressed that the quiescent state of cardiac myocytes in suspension is an artificial condition and that *in vivo* contractile performance is a permanent manifestation of cardiac myocytes.

When studying the effect of isoproterenol and db-cAMP on palmitate esterification into cellular lipid, it appeared that in quiescent cells these agents did not have an effect. In contracting cells, in which 4 Hz stimulation by itself was ineffective (indicating that the extra palmitate taken up in the presence of mechanical activity is completely utilized for β-oxidation), palmitate esterification was reduced in the presence of isoproterenol ($-30\,\%$, $P < 0.05$) or of db-cAMP ($-46\,\%$, $P < 0.05$) (Figure 4). It seems that in contracting cells with cAMP-induced higher energy demands, but in the absence of augmented influx of FA, incoming FA are diverted away from storage into triacylglycerols and directly channelled into mitochondrial β-oxidation.

We also investigated the effect of PDE inhibitors on FA oxidation. In quiescent myocytes, amrinone and zaprinast were without effect, whereas IBMX and milrinone both stimulated FA oxidation by 1.3-fold, and dipyridamole by 2.0-fold (Figure 4B), in agreement with earlier observations of Abdel-Aleem et al. [14]. It is of interest to note that IBMX, amrinone, milrinone and

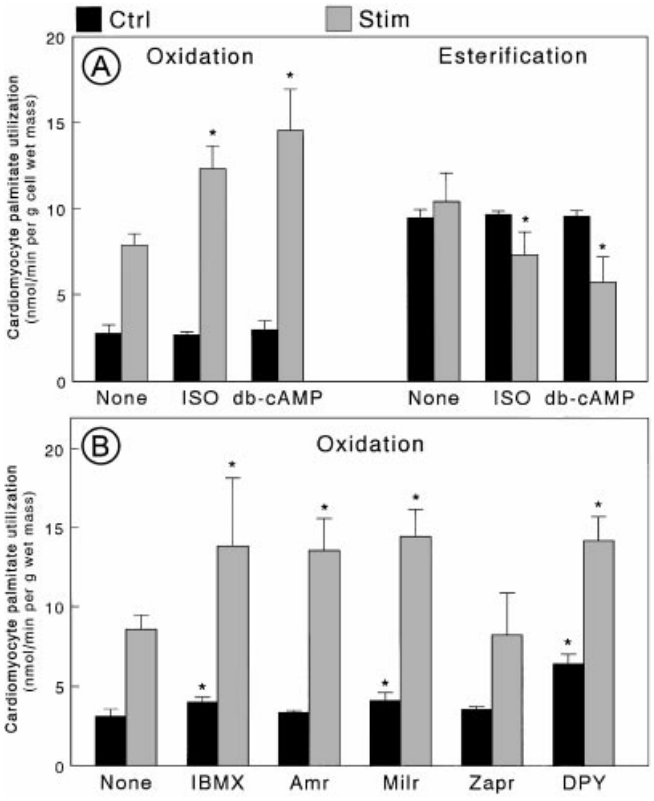

Figure 4 Effect of cAMP-elevating agents on palmitate metabolism by quiescent and electrically stimulated cardiac myocytes

Cell suspensions were incubated for 15 min in the absence of additions (None), or with 1 μM isoproterenol (ISO), or 100 μM db-cAMP (**A**), or the PDE inhibitors IBMX, amrinone (Amr), milrinone (Milr), Zaprinast (Zapr) or dipyridamole (DPY) (**B**) at the concentrations indicated in the Experimental section and in Table 1. Oxidation and esterification of palmitate were determined in the absence and presence of 4 Hz electrical stimulation at 200 V during 30 min after addition of radiolabelled palmitate. Results are expressed as the means $\pm$ S.E.M. for 4–6 experiments carried out with different cardiomyocyte preparations. *Significantly different from corresponding cardiac myocytes without additions (None; $P < 0.05$).

dipyridamole all stimulate the rate of FA oxidation in contracting cardiac myocytes to a similar extent, i.e. in a narrow range from 1.6- to 1.7-fold. In addition, these effects are of the same order of magnitude as observed in isoproterenol- and db-cAMP-treated contracting cardiac myocytes. In contrast, zaprinast, which elevates intracellular cGMP rather than cAMP, failed to stimulate FA oxidation in contracting cells. The collected findings indicate that the cAMP-elevating PDE inhibitors mimic the effect of isoproterenol and db-cAMP in channelling FA towards oxidation in electrically stimulated cardiac myocytes, irrespective of their effects on FA uptake.

In summary, we conclude that PDE inhibitors can affect FA utilization by cardiac myocytes at two levels: at the level of FAT/CD36-mediated FA uptake, which is independent of cAMP, and at the level of channelling into β-oxidation, which is dependent on cAMP and can only be appreciated in contracting myocytes.

Concluding remarks

In the present study, we have demonstrated that cAMP-dependent signal transduction is not involved in the regulation of FA uptake by cardiac myocytes. In contrast, intracellular cAMP is controlling the intracellular fate of FA following their uptake by directing the intracellular FA flux towards β-oxidation in contracting cardiac myocytes. Surprisingly, certain PDE inhibitors, used to manipulate intracellular cAMP levels, directly enhance cellular uptake, namely through molecular mechanisms involving FAT/CD36 and independent of PDE inhibition. Notably, dipyridamole is likely to induce the translocation of FAT/CD36 via signalling pathways also used by electrostimulation. Furthermore, IBMX and milrinone increase the intrinsic activity of FAT/CD36, probably through its covalent modification.

Although many PDE inhibitors are currently being used as cardiotonic agents for heart-failure patients [35–39], negative side effects have been documented, and their long-term use could eventually lead to increased mortality [40,41]. The ability of IBMX, milrinone and dipyridamole to increase FAT/CD36-mediated FA uptake, as observed in the present study, may suggest that prolonged use of these agents induces a chronic shift from glucose to FA utilization. It has been suggested that such a shift in substrate utilization could lead to the development of cardiomyopathies, as seen under diabetic conditions [42].

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