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Phosphorylation by protein kinase C and the responsiveness of Mg^{2+}-ATPase to Ca^{2+} of myofibrils isolated from stunned and non-stunned porcine myocardium

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Abstract

Previously we showed in an *in situ* porcine model that the thiadiazinone derivative [+]EMD 60263, a Ca^{2+} sensitizer without phosphodiesterase III inhibitory properties, increased contractility more profoundly in stunned than in non-stunned myocardium. This finding was consistent with the observed leftward shifts of the pCa^{2+}/Mg^{2+}-ATPase curves of isolated myofibrils induced by [+]EMD 60263. The aim of the present investigation was to study the possible involvement of protein kinase C in the mechanism of reduced Ca^{2+} responsiveness of myofilaments during stunning. No differences were observed in the maximal activity of the Ca^{2+}-stimulated Mg^{2+}-ATPase and in the pCa_{50} of myofibrils isolated from non-stunned and stunned myocardium. After phosphorylation with [gamma-^{32}P]-ATP and excess of purified rat brain protein kinase C, the myofibrils were separated on sodiumdodecylsulphate-polyacrylamide gelectrophoresis and the ^{32}P incorporation counted by the Molecular Imager. Ca^{2+}/phosphatidylserine/*sn*-1,2 diolein-dependent ^{32}P incorporation catalyzed by excess of purified rat brain protein kinase C in C-protein, TnT and TnI subunits did not show any differences between myofibrils from non-stunned and stunned myocardium. However, protein kinase C-induced phosphorylation of myofibrils isolated from ventricular myocardium of sham-operated pigs resulted in a marked leftward shift of the pCa_{50} from 6.03 ± 0.04 to 6.44 ± 0.06 ($p < 0.05$), while porcine heart cyclic AMP-dependent protein kinase-induced phosphorylation resulted in an expected small rightward shift to 5.97, although statistical significance was not reached. Protein kinase C-induced phosphorylation also stimulated (80%) the maximal myofibrillar Mg^{2+}-ATPase activity. [+]EMD 60263 (3 µM) produced a leftward shift of the myofibrillar pCa^{2+}/Mg^{2+}-ATPase curve which was unaffected by prior protein kinase C-induced phosphorylation. In conclusion, the findings with isolated myofibrils from myocardium of anaesthetized open-chest pigs indicate that protein kinase C might be involved in the mechanism of reduced Ca^{2+} responsiveness of myofilaments in stunned myocardium. However, at this stage no differences could be found between the maximal activity of the Ca^{2+}-stimulated Mg^{2+}-ATPase, the pCa_{50} and the degree of phosphorylation of myofibrils isolated from stunned and non-stunned myocardium. (Mol Cell Biochem **176:** 211–218, 1997)

Key words: cardiac myofibrils, cardiac sarcoplasmic reticulum, human, pig, Ca^{2+} stimulated Mg^{2+}-ATPase, thiadiazinone derivatives, stunning

Abbreviations: ATPase – Adenosine-5′-triphosphatase; EGTA – ethylene glycol bis(β-aminoethylether) N,N,N′,N′-tetraacetic acid; DTT – dithiothreitol; MOPS – 4-morpholino-propane sulfonic acid; P_i – inorganic phosphate; PS – phosphatidylserine; DG – *sn*-1,2 diolein; SDS-PAAGE – sodiumdodecylsulphate-polyacrylamide gelectrophoresis; PMSF – phenylmethanesulfonylfluoride; PDE III – phosphodiesterase III; LADCA – left anterior descending coronary artery; LCXCA – left circumflex coronary artery; TnC – troponin C; TnI – troponin I; TnT – troponin T; MLC – myosin light chain; PKC – protein kinase C; PKA – cyclic AMP dependent protein kinase; [+]EMD 60263 – 5-[1-(α-ethylimino-3,4-dimethoxybenzyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3, 6-dihydro-2H-1,3,4-thiadiazin-2-one

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Introduction

Stunned myocardium is defined as reversible contractile dysfunction in heart muscle reperfused after a period of ischemia which does not produce irreversible damage [1]. Despite numerous efforts, the cellular mechanism of myocardial stunning is not fully understood [2–16]. Stunned myocardium remains responsive to positive inotropic agents such as catecholamines [2], increased extracellular Ca^{2+} concentration [3], phosphodiesterase III (PDE III) inhibitors (e.g. AR-L 57) [4] and the so-called Ca^{2+} sensitizers (e.g. thiadiazinone derivatives [+]EMD 57033 [5] and [+]EMD 60263[6]). In the past the origin of force impairment in stunned myocardium has been approached by studying e.g. the Ca^{2+} transient that occurs during each cardiac cycle, the responsiveness of the contractile apparatus to Ca^{2+} and the maximal Ca^{2+} activated force [7–10]. A common finding is that Ca^{2+} transients are not decreased in stunned myocardium [9]. Instead, based upon evidence obtained from studies using Ca^{2+} indicators [7, 11, 12], measuring the rate of the sarcoplasmic reticulum Ca^{2+} pump [13], administration of Ca^{2+} sensitizers [6, 14] and measuring the Ca^{2+} sensitivity of isometric tension in skinned myocytes [15, 16] more attention is now directed to determining the nature and the origin of decreased myofilament Ca^{2+} responsiveness in stunned myocardium. For instance, recently we showed in an *in situ* porcine model that the thiadiazinone derivative [+]EMD 60263 increased contractility more profoundly in stunned than in non-stunned myocardium [6]. Subsequently, we demonstrated that [+]EMD 602363, at concentrations (1–3 µM) close to the effective plasma concentrations in the *in situ* porcine model, sensitizes Mg^{2+}-ATPase of isolated porcine cardiac myofibrils to Ca^{2+} and had no effect on the Ca^{2+} stimulated Mg^{2+}-ATPase of isolated porcine cardiac SR membrane vesicles [17]. On the other hand, it has been shown that the maximal rate of myofibrillar ATP hydrolysis of isolated myofibrils is not affected during stunning in the rabbit heart [18].

Decreased Ca^{2+} responsiveness of myofilaments normally functions as a negative feedback mechanism and contributes to the increased relaxation rate of beating heart under the influence of β-adrenergic stimulation [19]. This effect occurs primarily through cyclic AMP-dependent protein kinase (PKA)-mediated phosphorylation of the myofibrillar troponin I (TnI) subunit resulting in a reduced affinity of troponin C (TnC) for Ca^{2+} [19]. Another important signalling enzyme, protein kinase C (PKC), also plays a role in cardiac physiology although its precise function is not fully understood. Controversial findings are reported concerning phorbolesters, potent and long acting PKC activators, which when applied to intact cells enhanced myofilament Ca^{2+} sensitivity [20, 21] but produced a decrease in permeabilized cardiac muscle [22]. Evidence is emerging that certain isoenzymes of PKC become activated during ischemia (and reperfusion) [23–25]. However, the complex molecular events by which PKC (or more precisely, one of the individual isozymes) modulates cardiac contractility remains unclear [26]. Potential phosphorylation targets for PKC are present in the contractile apparatus itself as in *in vitro* experiments the subunits C-protein, troponin T (TnT), TnI and myosin light chain-2 of myofibrils from rat and guinea pig myocardium have been shown to be substrates for PKC [26–30]. However, the consequences of these PKC-induced phosphorylations are currently unclear [27–29].

In the present study we first investigated the Ca^{2+} responsiveness of the Mg^{2+} ATPase of myofibrils isolated from stunned and adjacent non-stunned myocardium in the *in situ* porcine model. Subsequently, we tested the hypothesis that changes in Ca^{2+} responsiveness of the Mg^{2+} ATPase of myofilaments are related to the degree of PKC-induced phosphorylation. To examine this, we backphosphorylated the C-protein, TnT and TnI subunits of isolated myobrils from stunned and adjacent non-stunned porcine myocardium *in vitro* with excess amounts of PKC purified from rat brain in the presence of [gamma-^{32}P]-ATP and recorded the ^{32}P-incorporation in the electrophoretically separated bands. We also determined the effect of *in vitro* phosphorylation of isolated myofibrils by excess of PKC or PKA on the responsiveness of the Mg^{2+}-ATPase to Ca^{2+}. Moreover, we studied the effect of the [+]EMD 60263 on the responsiveness of myofibrillar Mg^{2+} -ATPase to Ca^{2+} before and after pretreatment with purified rat brain PKC.

Materials and methods

Materials

Leupeptin, aprotinin, pepstatin and the purified catalytic subunit of PKA (from porcine heart), phosphatidylserine PS and *sn*-1,2 diolein (DG) were obtained from Sigma Chemical Company (St Louis, USA). Purified rat brain PKC (0.08 U/ml) was obtained from Boehringer (Mannheim, Germany). Sodiumdodecylsulphate-polyacrylamide gelelectrophoresis (SDS-PAAGE) molecular weight standards were obtained from Bio-Rad Laboratories (California, USA). [Gamma-^{32}P]-ATP (150 Tbqmmol^{-1}) was obtained from Amersham International plc. (Amersham, UK). The pure enantiomer [+]EMD 60263 (5-[1-(α-ethylimino-3,4-dimethoxybenzyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one) was supplied by E. Merck, Darmstadt, Germany. Stock solutions (0.2 mM) of [+]EMD 60263 were made in distilled water and prepared on the day of the experiment. All other chemicals were obtained from either E. Merck (Darmstadt, Germany), Boehringer (Mannheim, Germany) or Sigma Chemical Company (St Louis, USA).

Myofibrillar preparation

Regional stunned myocardium was induced in 4 anesthetized open-chest pigs (cross-bred Landrace × Yorkshire pigs of either sex) in the distribution territory of the left anterior descending coronary artery (LADCA) by two sequences of 10 min coronary artery occlusion and 30 min of reperfusion. After the second reperfusion period myofibrils were isolated from the stunned myocardium. Non-stunned myocardium was obtained from the distribution territory of the left circumflex coronary artery (LCXCA). For details on this *in situ* porcine model the reader is referred to [6]. For the experiments in which we examined the effect of PKC or PKA-induced phosphorylation on pCa/Mg^{2+}ATPase activity relationship, myofibrils were isolated from ventricular biopsies taken from sham-operated pigs. The cardiac muscle specimens (about 3 g) were minced, mixed with 4 volumes 10 mM $NaHCO_3$ and 1 mM dithiothreitol (DTT) and homogenized with a Polytron PTX 10 (Kinematica GmbH, Luzern, Switserland). The homogenate was centrifuged at 9000 g_{av} and 4°C for 20 min, after which the pellet was removed and the supernatant was centrifuged for another 20 min at 9000 g_{av}. From the combined pellets the myofibrils were purified according to the method described by Murphy and Solaro [31]. Briefly, the pellets were resuspended in 4 volumes solution containing 10 mM ethylene glycol bis(β-aminoethylether) N,N,N′,N′-tetraacetic acid (EGTA), 8.2 mM $MgCl_2$, 14.4 mM KCl, 60 mM imidazole (pH 7.0), 5.5 mM ATP, 22 mM creatinephosphate, 10 U.ml^{-1} creatine kinase, 1% Triton X-100, 5 µg.ml^{-1} leupeptin, 10 µg.ml^{-1} pepstatin, 10 µg.ml^{-1} aprotinin, 1.7 mg.ml^{-1} phenylmethane-sulfonylfluoride (PMSF) in a glass-Teflon homogenizer, and left on ice for 30 min before the suspension was centrifuged for 15 min at 1100 g_{av}. The supernatant was discarded and the myofibrillar pellet washed twice with 2 volumes of 30 mM KCl, 30 mM imidazole (pH 7.0) and 2 mM $MgCl_2$, before being resuspended in the washing-buffer containing additionally 50% (v/v) glycerol up to a protein concentration of 10 mg.ml^{-1}. The myofibrillar suspension was stored in aliquots at –80°C. Possible cross-contamination of isolated myofibrils by sarcoplasmic reticulum membranes was excluded by testing the effect of thapsigargin (1 µM), a specific inhibitor of the sarcoplasmic reticulum Ca^{2+} pump, on the Ca^{2+}-stimulated Mg^{2+}-ATPase [17]. Myofibrillar protein content (yield was about 20 mg protein/g myocardium) was determined with the method of Bradford [32].

Backphosphorylation of isolated myofibrils by excess of PKC and PKA

The reaction mixture for backphosphorylation of isolated myofibrils by PKC contained 50 mM Tris/HCl (pH 7.0), 10 mM $MgCl_2$, 10 mM DTT, 10 mM NaF (for inhibition of endogenous phosphoprotein-phosphatases), 1 mg.ml^{-1} bovine serum albumin, 500 µM $CaCl_2$, 1.6 mg.ml^{-1} PS, 0.4 mg.ml^{-1} DG, 0.2 mU.ml^{-1} purified rat brain PKC and 2–4 mg.ml^{-1} myofibrils. Myofibrils were pretreated with purified porcine heart PKA in the same reaction mixture after replacing Ca^{2+}, PS and DG by 20 µM cyclic AMP. The phosphorylation reactions were initiated by addition of 4 mM ATP and carried out at 30°C for 1 h. Every 15 min, additional 4 mM ATP was added to avoid depletion of ATP by the myofibrillar Mg^{2+}-ATPase. Control (not phosphorylated) myofibrils were treated similarly, except for the repeated additions of ATP. Reactions were terminated by washing the myofibrils twice (each time centrifuged at 1100 g_{av} at 4°C for 10 min) with resuspension buffer containing 50 mM Tris/HCl (pH 7.0), 10 mM $MgCl_2$, 10 mM DTT and 1 mM NaF. Finally, the myofibrils were resuspended in 1 ml resuspension buffer. The pCa/Mg^{2+}-ATPase activity relationship was always measured immediately after the phosphorylation.

To determine the specific ^{32}P incorporations into C-protein, TnT and TnI subunits of myofibrils, reactions were run in parallel to those described in which every 15 min, instead of 4 mM ATP, 0.2 mM [gamma-^{32}P]-ATP (0.2 Tbqmmol^{-1}) was added. These reactions were terminated with stopmixture (0.3 M Tris/HCl (pH 7.0), 5 % SDS, 20% glycerol, 2.5 M β-mercaptoethanol and 0.006% phenolred as tracking dye for the electrophoresis) and the labelled myofibrillar proteins (6 µg/well) were separated on 10–20% gradient SDS-PAAGE as described in ref. [33] and the ^{32}P incorporation into C-protein, TnT and TnI bands on the vacuum-dried gel counted in the Molecular Imaging System (GS-363) (BioRad Laboratories, California, USA). Due to the low amount of PKC-induced incorporation, particularly into TnT and TnI, large quantities of myofibrillar protein had to be poured into the electrophoresis-wells for accurate determination of ^{32}P incorporation which resulted in more diffuse bands.

Assay of the Mg^{2+}-ATPase

The ATPase activities of phosphorylated or not-phosphorylated myofibrils were determined by measuring formation of P_i according to the method of Lanzetta *et al.* [34]. Briefly, aliquots of the myofibrillar suspension were thawed and the glycerol-containing storage buffer was removed by centrifugation at 2000 g_{av} and 4°C for 15 min. The myofibrillar pellet was washed twice with 60 mM KCl, 30 mM imidazole and 2 mM $MgCl_2$, pH 7.0 and finally resuspended in a solution containing 60 mM KCl, 30 mM imidazole (pH 7.0), 2 mM $MgCl_2$, 1 mM DTT and 1.7 mg.ml^{-1} PMSF. Myofibrils (40 µg protein) were incubated at 30°C in a total volume of 200 µl solution containing 60 mM KCl, 2.5 mM $MgCl_2$, 1 mM

DTT, 25 mM MOPS, pH 7.0, 2 mM EGTA, 2 mM ATP, 5 mM NaN_3 and various amounts of Ca^{2+}. Different levels of free Ca^{2+} were achieved by varying the Ca^{2+}/EGTA ratio, keeping the total EGTA concentration constant. Free Ca^{2+} in the buffer was calculated using Fabiato's SPECS computer program [35, 36].

Statistics

The results are presented as mean ± SEM. The pCa-Mg^{2+} ATPase data were fitted to a sigmoid function by nonlinear regression analysis. The data were normalized to maximum activity, after subtracting basal ATPase activity and fitted to the Hill equation ($P = P_o/(1 + Q/[Ca^{2+}]^n)$) in which P_o is the maximal Ca^{2+}-stimulated Mg^{2+}-ATPase, P the level of Ca^{2+}-stimulated Mg^{2+}-ATPase less than maximum, Q a constant and n_H is the Hill value [17, 37, 38]. Only data points which fulfilled the condition $0.1\ P_o \leq P \leq 0.9\ P_o$ were used to solve the Hill equation. The pCa_{50} (at 50% of the maximal Ca^{2+}-stimulated Mg^{2+}-ATPase activity), was determined, by using n_H and the Q calculated from the Hill equation [17, 37]. The pCa (i.e. $-\log[Ca^{2+}]$) corresponding to 50% activation of Ca^{2+}-stimulated Mg^{2+}-ATPase was calculated as $-(1/n)\log Q$. Data were evaluated for statistical significance by the Student *t*-test and significance was accepted at $p < 0.05$.

Results

pCa^{2+}/Mg^{2+}-ATPase relationships of myofibrils from non-stunned and stunned myocardium

Four experiments were carried out with anesthetized open-chest pigs, in which the distribution territory of the LADCA was stunned by 2 sequences of 10 min coronary artery occlusion and 30 min reperfusion. From each pig myofibrils were isolated from the non-stunned (distribution territory of the not-occluded LCXCA) and stunned myocardium. The myofibrils were isolated in the presence of a mixture of protease inhibitors to preserve their intactness. The normalized pCa^{2+}/Mg^{2+}-ATPase activity curves, obtained from the myofibrils of non-stunned and stunned myocardium were fitted to the Hill equation to calculate the pCa^{2+} corresponding to 50% activation of the Ca^{2+}-stimulated Mg^{2+}-ATPase and the Hill value (n_H). No significant differences between the maximal activity of the Ca^{2+}-stimulated Mg^{2+}-ATPase and the pCa_{50}'s of the myofibrils from non-stunned and stunned myocardium were observed (Table 1). In 3 out of the 4 pigs a rightward shift of the pCa^{2+}/Mg^{2+}-ATPase curves of myofibrils from stunned comparing to non-stunned was observed as the mean pCa_{50} of stunned myocardium slightly decreased (Table 1). Thus, more experiments have to be carried out to establish whether or not the tendency of myofibrils from stunned myocardium to desensitize towards Ca^{2+} activation is a reproducible finding.

Table 1. Maximal activity and pCa_{50} of Mg^{2+}-ATPase measured in myofibrils isolated from non-stunned and stunned myocardium of open-chest anesthetized pigs

	Non-stunned (n = 4)*	Stunned (n = 4)
Maximal Mg^{2+}-ATPase (nmol $P_i.mg^{-1}.min^{-1}$)	35 ± 4	37 ± 3
pCa_{50} (-logM)	6.01 ± 0.08	5.94 ± 0.09

Stunned myocardium was obtained from the distribution territory of the LADCA which was stunned by two sequences of 10 min coronary artery occlusion and 30 min of reperfusion. Non-stunned myocardium was obtained from the distribution territory of the LCXCA. The normalized Mg^{2+}-ATPase activities measured at various pCa^{2+} values as chosen in [17] and Fig. 2, were fitted to a sigmoid function by nonlinear regression analysis. The ATPase activities were normalized to maximum activity and fitted to the Hill equation as described in Materials and methods, and when analyzed gave the calculated pCa_{50} values. *number of pigs.

In vitro *back-phosphorylation of myofibrils from non-stunned and stunned myocardium*

The myofibrils were also used for backphosphorylation by activated purified rat brain PKC of various intrinsic subunit-proteins. Figure 1 illustrates a representative ^{32}P-incorporation pattern of myofibrils from non-stunned and stunned myocardium and the strong stimulation of PKC action in the presence of the known activators Ca^{2+}, PS and DG. Three proteins, C-protein (150 kDa), TnT (43 kDa) and TnI (30 kD) were identified by the parallel running molecular weight marker proteins and the Coomassie Blue staining (not shown). Phosphorylation of the three myofibrillar subunit-proteins was maximal after a 60 min incubation at 30°C. Counting the ^{32}P-incorporation into each of these proteins by the Molecular Imaging System revealed that there was no difference in the degree of phosphorylation of non-stunned and stunned myocardium (Table 2).

In vitro *backphosphorylation by PKC and the myofibrillar pCa^{2+}/Mg^{2+}-ATPase relationship*

Myofibrils prepared from ventricular myocardium of sham-operated pigs were *in vitro* phosphorylated with excess amounts of purified rat brain PKC in the presence of the activators Ca^{2+}, PS and DG after which the myofibrils were washed and the pCa^{2+}/Mg^{2+}ATPase was determined immediately. As can be seen from Fig. 2, the Mg^{2+}-ATPase was increased at each pCa^{2+} value without a significant change in the slope (n_H) but with a marked increase in pCa_{50} (not-

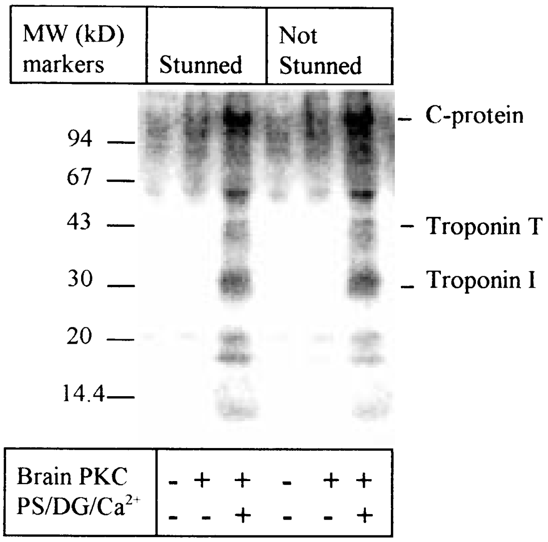

Fig. 1. Representative autoradiographic image of *in vitro* ^{32}P-phosphorylated and SDS-PAAGE separated myofibrils isolated from stunned and non-stunned myocardium of four open-chest anesthetized pigs. The myofibrils were incubated for 60 min at 30°C in the presence or absence of purified rat brain PKC, activated or not activated by the addition of Ca^{2+}, PS and DG. After termination of the phosphorylation reaction, the myofibrils were separated on SDS-PAAGE and the dried gel was autoradiographically imaged with a Molecular Imager. For further details the reader is referred to Materials and methods. The kit of marker proteins for the molecular weight (MW in kilodaltons (kD)) determinations, as indicated on the left part of the figure, was obtained from Bio-Rad Laboratories. Due to the low amount of PKC-induced ^{32}P-incorporation, particularly into TnT and TnI, larger quantities of myofibrillar protein had to be poured into the electrophoresis-wells for accurate determination of ^{32}P incorporation which resulted in more diffuse bands.

Table 2. In vitro back-phosphorylation by purified rat brain PKC of myofibrils isolated from non-stunned and stunned myocardium of open-chest anesthetized pigs

Protein subunit of myofibrils	Ca^{2+}/PS/DG-dependent ^{32}P-incorporation (counts × 10^{-6}.mg^{-1})	
	Non-stunned (n = 4)	Stunned (n = 4)
C-protein	3.52 ± 0.21	4.30 ± 0.45
TnT	0.95 ± 0.18	1.04 ± 0.06
TnI	1.55 ± 0.15	1.48 ± 0.13

Stunned and non-stunned myocardium was obtained from anesthetized open-chest pigs in which the distribution territory of the LADCA was stunned by two sequences of 10 min coronary artery occlusion and 30 min reperfusion. The non-stunned myocardium was obtained from the distribution territory of the LCXCA. Ca^{2+}/PS/DG-dependent ^{32}P incorporation in protein subunits of myofibrils was determined by SDS-PAAGE and autoradiographic imaging (Molecular Imager). A typical autoradiogram is shown in Fig. 1.

phosphorylated versus PKC-phosphorylated respectively 6.03 ± 0.04 versus 6.44 ± 0.06 ($p < 0.05$)). Thus, next to an increase of Ca^{2+} sensitivity PKC enhanced (80%) the maximal activity of the Ca^{2+}-stimulated Mg^{2+}-ATPase. This effect of PKC depended on the presence of ATP as well as the activators Ca^{2+}/PS/DG (results not shown) The latter finding indicates, according to the results illustrated in Fig. 1, that increased phosphate incorporation into C-protein, TnC and TnI correlates with the alteration of properties of the myofibrillar ATPase. As a control we measured the effect of pretreatment of myofibrils with catalytic subunit of porcine heart PKA, ATP and cyclic AMP on the myofilament Ca^{2+} sensitivity. Similar as found by others (e.g. ref [19]), we observed a slight, but not significant, decrease in Ca^{2+} sensitivity when TnI was phosphorylated.

The effect of [+]EMD 60263 on not-phosphorylated and PKC phosphorylated myofibrils

Previously we demonstrated convincingly that 3 µM of the Ca^{2+} sensitizer [+]EMD 60263 induced a leftward shift of the pCa^{2+}/Mg^{2+}-ATPase activity curve and an increase of the maximal Ca^{2+}-stimulated Mg^{2+}-ATPase of isolated myofibrils [17]. Because PKC-induced phosphorylation produced a stimulation pattern similar to [+]EMD 60263, it was wondered whether the sites of action of PKC-induced phosphorylation and [+]EMD 60263 are similarly localized on the myofibrils and mutually influencing. In two additional experiments we tested this hypothesis (Table 3). However, when the myofibrils were phosphorylated by PKC, [+]EMD 60263 still produced a clearcut increase of the pCa_{50} indicative for independent actions of PKC and [+]EMD 60263 on the responsiveness of myofibrillar Mg^{2+}-ATPase to Ca^{2+}.

Discussion

Although previous hypotheses regarding the pathogenesis of contractile function in stunning focussed on a decrease on cytoplasmic activator Ca^{2+} as initial mechanism [38, 39], more recently considerable evidence has been provided which shifted the focus to an abnormal responsiveness of contractile proteins to Ca^{2+} [6, 7, 9, 11, 15–18]. Most of the latter studies used intact ventricular preparations, skinned cardiac muscles or isolated cardiomyocytes but only a few used isolated myofibrillar preparations. It is well known that the activity of the actomyosin Ca^{2+} stimulated Mg^{2+}-ATPase correlates directly with Ca^{2+} stimulated tension development in skinned cardiac fibers [40]. Krause was the first to show that the Ca^{2+} dependency of ATP hydrolysis of myofibrils isolated from stunned rabbit heart remained unchanged and found that the maximal rate of myofibrillar ATP hydrolysis was also not affected [18]. However, Andres *et al.* observed that the Ca^{2+} responsiveness of isolated myofibrils from globally stunned rabbit myocardium shifted to higher Ca^{2+} concentrations, i.e. a decrease in Ca^{2+} sensitivity [41].

Table 3. Effect of 3 µM [+]EMD 60263 on the maximal Ca^{2+}-stimulated Mg^{2+}-ATPase and pCa^{2+}/Mg^{2+}-ATPase relationship of porcine ventricular myofibrils without and after phosphorylation by excess of rat brain PKC.

	Not-phosphorylated				Phosphorylated by PKC			
	–EMD		+EMD		–EMD		+EMD	
	1	2	1	2	1	2	1	2
Maximal Ca^{2+},Mg^{2+}-ATPase (nmol P_i.mg^{-1}.min^{-1})	35	50	59	92	53	65	49	78
pCa_{50} (-logM)	6.25	6.01	6.37	6.37	6.50	6.27	6.69	6.47

Porcine ventricular myofibrils were first phosphorylated by excess rat brain PKC or not phosphorylated (complete phosphorylation reaction carried out in the absence of ATP). The maximal Ca^{2+} stimulated Mg^{2+}-ATPase was measured at pCa 5 (compare also Fig. 2). The normalized pCa/Mg^{2+}-ATPase activity curves in the absence and presence of 3 µM [+]EMD 60263 measured at pCa^{2+} values as these were chosen in [17], Table 2 and Fig. 2, were fitted to a sigmoid function by nonlinear regression analysis. The ATPase activity as normalized to maximum activity were fitted to the Hill equation as described in Materials and methods, and from which pCa_{50} values were calculated. Results represent absolute values determined in two separate experiments 1 and 2.

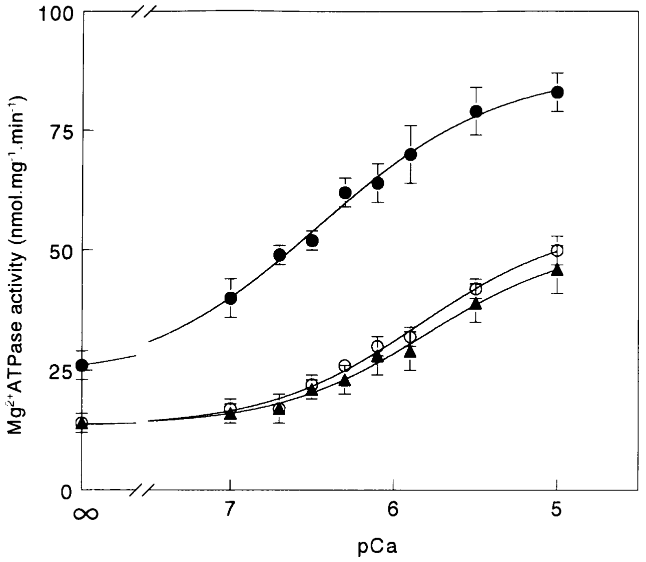

Fig. 2. Graphs showing the relation between pCa^{2+} (-logM) and Mg^{2+}-ATPase activity (in nmol P_i.mg $protein^{-1}$.min^{-1}) of porcine ventricle myofibrils after phosphorylation with purified rat brain PKC (●), purified porcine heart catalytic subunit of PKA (▲), or not phosphorylated (○). For further details the reader is referred to Materials and methods. The symbols represent the mean ± SEM of 4, 5 and 10 experiments with respectively PKC-, PKA- and not-phosphorylated myofibrils. After subtracting the basal Mg^{2+}-ATPase activities, the data were normalized to maximum activity and fitted to the Hill equation, yielding the following parameters: PKC-phosphorylated myofibrils, $pCa_{50} = 6.44 \pm 0.06$, $n_H = 0.94 \pm 0\ 07$; PKA-phosphorylated myofibrils, $pCa_{50} = 5.97 \pm 0.03$, $n_H = 1.24 + 0.09$; not-phosphorylated myofibrils, $pCa_{50} = 6.03 \pm 0.04$, $n_H = 1.22 \pm 0.08$. The pCa_{50} values of PKC phosphorylated myofibrils differed significantly from the PKA- and not-phosphorylated myofibrils.

Furthermore, the latter investigators explored the possibility that altered phosphorylation degree of TnI or myosin-light-chains causes the observed Ca^{2+} desensitization of the myofibrils, but could not find any changes in phosphorylation [41]. These controversial findings [18, 41] led us to examine these aspects in post-ischemic myocardium obtained from anesthetized open-chest pigs. *In situ* myocardial stunning was induced by two cycles of 10 min of LADCA occlusion separated by 30 min of reperfusion. This protocol caused a prolonged depression of regional myocardial function without myocardial necrosis [6, 13, 14]. In this model we showed previously that the thiadiazinone-derived [+]EMD 60263 increased contractile function more profoundly in stunned myocardium than that of non-stunned myocardium lending support to the hypothesis that Ca^{2+} desensitization of the myofibrils is involved in myocardial stunning. The present finding that pCa_{50} (-logM) of Ca^{2+}-stimulated Mg^{2+}-ATPase of myofibrils is similar for non-stunned and stunned myocardium (respectively 6.01 ± 0.08 and 5.94 ± 0.09) does not support the 'Ca^{2+} desensitization hypothesis'. It should be noted that the comparison of stunned and non-stunned myocardium in the same heart may not be valid because comparative data of sham-operated pigs are still lacking. However, previously we showed already in pigs, that had not undergone the stunning protocol, that [+]EMD 60263 only slightly increased contractile function in the distribution area of the LADCA [6].

If the Ca^{2+} desensitization hypothesis is correct the mechanism underlying the decreased responsiveness of the contractile apparatus to Ca^{2+} after ischemia and reperfusion has to be resolved. It is likely that ischemia and/or reperfusion induces changes in the PKA- and/or PKC-induced phosphorylation status of certain subunit proteins of the contractile apparatus such C-protein, TnT, TnI or myosin-light-chains. There is abundant evidence that PKA and PKC have phosphorylation sites in the various subunits of the contractile apparatus, but the functional consequences of these phosphorylations are, except for PKA-induced phosphorylation of TnI [19], currently unclear [20–30]. In order to determine the functional consequence of PKC-induced phosphorylation of C-protein, TnT and TnI, we studied the ^{32}P-incorporation into these subunit proteins as well as the pCa^{2+}/Mg^{2+}-ATPase relationship after treatment (60 min at 30°C) of myofibrils with an excess of purified rat brain PKC. On the other hand, the PKC pretreatment produced a dramatic leftward shift in pCa^{2+}/Mg^{2+}-ATPase curve and an increase of the maximal Ca^{2+}-stimulated ATPase in myofibrils isolated from myocardium from sham-operated pigs. Our findings are consistent with the results from Clement *et al.*, who provided additional evidence that PKC works most effectively in combination with Ca^{2+}-calmodulin-dependent myosin-light-chain kinase [29]. In contrast, Noland and Kuo [27, 28] reported that PKC-induced phosphorylation of C-protein, TnI, and TnT decreased Ca^{2+} sensitivity of myofibrils. Moreover, the effects of the potent PKC activators, phorbolesters, on Ca^{2+} sensitivity of myofilaments in intact cardiomyocytes, are conflicting [20–22]. Nevertheless, based upon the effects of PKC on the Mg^{2+}-ATPase of myofibrils isolated from sham-operated pigs, this protein kinase remains a good candidate to account for the changes in Ca^{2+} sensitivity of myofilaments obtained from stunned myocardium. An argument against this view are our findings on ATPase characteristics properties and ^{32}P-incorporation into subunits proteins of myofibrils isolated from non-stunned comparing to stunned myocardium. However, rapid dephosphorylation by endogenous phosphoprotein phosphatases during the isolation of myofibrils can have caused the disappearance of the *in vivo* existing differences in Ca^{2+} sensitivity of the Mg^{2+}-ATPase and the PKC-induced ^{32}P-incorporation into subunit proteins between myofibrils isolated from non-stunned and stunned myocardium. In some control tests, carried out prior to the present investigation, we checked the influence of the presence of the phoshoprotein phosphatase inhibitor NaF (25 mM) during the isolation of the myofibrils. This intervention did, however, not affect the PKC-induced ^{32}P-incorporation patterns (results not shown) which finding indicates that the endogenous phosphoprotein phosphatases are highly active.

In our previous studies we showed that the positive inotropic action of [+]EMD 60263 *in situ* as well as the [+]EMD 60263-induced leftward shift of pCa^{2+}/Mg^{2+}-ATPase activity curve in isolated myofibrils were more pronounced in stunned than in non-stunned myocardium. Thus when one assumes that decrease of PKC activity contributes to the decreased responsiveness of contractile proteins to Ca^{2+} in stunning, PKC- and [+]EMD 60263 effects on myofibrillar Ca^{2+}-stimulated Mg^{2+}-ATPase might be antagonistic. Although we carried out only two experiments, the results proved that the effects of PKC and [+]EMD 60263 are additive. If PKC indeed plays a role *in vivo* in the change of responsiveness of myofibrils to Ca^{2+} during stunning, like its influence *in vitro* on myofibrils of control myocardium demonstrated by the data in Fig. 2, its target proteins in the contractile apparatus are expected to be less phosphorylated in stunned myocardium. Therefore, the proposed reduced phosphorylation state of the myofibrils during stunning can not explain our previous finding that [+]EMD 60263 produced a larger leftward shift in the pCa^{2+}/Mg^{2+}ATPase curve of myofibrils isolated from stunned myocardium.

In conclusion, our findings with isolated myofibrils obtained from anesthetized openchest pigs indicate that alteration of the activity of PKC isoenzymes may be involved in the mechanism of reduced responsiveness of myofilaments to Ca^{2+} in stunned myocardium, but that differences in phosphorylation degree of its target proteins may have disappeared during the isolation of the myofibrils due to the abundant activity of endogenous phosphoprotein-phosphatases.

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