Reconstitution of skinned cardiac fibres with human recombinant cardiac troponin-I mutants and troponin-C

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Abstract **Troponin C (TnC) could be extracted from skinned porcine cardiac muscle fibres and their Ca^{2+} sensitivity restored by reconstitution with recombinant human cardiac TnC. After extraction of troponin I (TnI) and TnC using the vanadate treatment method of Strauss et al. [Strauss, J. D., Zeugner, C., Van Eyk, J.E., Bletz, C., Troschka, M. and Rüegg, J.C. (1992) FEBS Lett. 310, 229–234], skinned porcine cardiac muscle fibres were reconstituted with wild-type recombinant human cardiac TnC and either wild-type cardiac TnI or several mutant isoforms of human TnI. Reconstitution with wild-type proteins restored the Ca^{2+} sensitivity of the tissue and phosphorylation of the TnI with the catalytic subunit of protein kinase A reduced the Ca^{2+} sensitivity (i.e. $-\log[Ca^{2+}]$ for 50% of maximal force) as has been shown by others. However, reconstitution with the TnI mutant Ser-23Asp/Ser-24Asp mimicking the phosphorylated form of cardiac TnI, led to a reduced Ca^{2+} sensitivity compared with reconstitution with wild-type TnI, whereas the mutant Ser-23Ala/Ser-24Ala behaved as the dephosphorylated form of TnI. These data confirm the importance of negative charge in this region of the TnI molecule in altering the Ca^{2+} responsiveness in this system.**

Key words: Cardiac muscle; Phosphorylation; Protein engineering; Regulation; Skinned fibre; Troponin C; Troponin I

1. Introduction

Previous work with skinned muscle fibres has demonstrated that typical Ca^{2+} responsiveness in contraction is dependent upon an intact troponin–tropomyosin complex. The skinned fibre method is powerfull as it allows to extract and replace the native regulatory proteins by genetically engineered proteins or alternate isoforms in order to study structure–function relationship (cf. [1]). Different isoforms of the troponin subunits have been reported to affect the Ca^{2+} sensitivity (i.e. $-\log[Ca^{2+}]$ for 50% of maximal force) in a distinct manner [2]. For example, Ca^{2+} sensitivity of fast twitch skeletal muscle fibres is lowered after reconstitution with cardiac troponin C (TnC) and recombinant mutants [3]. Furthermore, different isoforms of troponin I (TnI) and troponin T [4–7] as well as phosphorylation of cardiac TnI correlated with different Ca^{2+} sensitivities [8,9] and with changes to the acidic pH-induced depression of contractile Ca^{2+} sensitivity in cardiotrabeculae [7,10].

By utilizing a method of treating skinned cardiac muscle fibres with vanadate which permits the extraction of TnI as well as some TnC [11], it is possible to investigate the function of structural changes in TnI by substitution with different TnI isoforms and mutants. Cardiac TnI differs from its skeletal muscle counterpart by possessing an N-terminal extension (30–33 amino acid residues in length depending on the species) that may be phosphorylated at adjacent serine residues [12,13]. Phosphorylation of TnI by protein kinase A (PKA) decreases the Ca^{2+} sensitivity of the myofilaments as indicated by the higher Ca^{2+} concentration required to produce 50% activation of the regulated actomyosin [14] and myofibrillar ATPases [15,16] and of force development in skinned fibres [8]. In the heart, phosphorylation of TnI by PKA occurs following β-adrenergic stimulation [17] and may be considered as an adaptive mechanism to prevent overstimulation. The phosphorylation of cardiac TnI has been shown to reduce the affinity of TnC for Ca^{2+} [18], thus, promoting more rapid relaxation [9].

Different TnI isoforms from various tissues have been overexpressed in bacterial systems [19,20] and a number of mutants have been produced in order to investigate the structure–function relationship in TnI [7,10,21–23]. Here, we report, for the first time, the reconstitution into skinned cardiac fibres of recombinant human cardiac TnC and TnI which will provide a convenient test system for investigating engineered forms of these proteins. We show that site-directed mutagenesis of serine residues 23 and 24 in human cardiac TnI to aspartate mimics the effect of TnI phosphorylation on Ca^{2+} sensitivity. On the other hand, mutating these residues to alanine caused the protein to behave like the dephosphorylated form. These experiments suggest that the net charge of the residues 23 and 24 of the cardiac TnI-N-terminus is an important determinant of Ca^{2+} sensitivity.

2. Materials and methods

2.1. Skinned fibres

We used skinned rabbit psoas fibres and cardiac muscle fibres prepared from porcine trabecula septomarginalia from the right ventricle. Fibre bundles were extracted overnight at 4°C in a solution containing 50% glycerol and 50% buffer containing 20 mM imidazole, 10 mM NaN_3, 5 mM ATP, 5 mM $MgCl_2$, 4 mM EGTA, 2 mM dithioerythritol (DTE), 1% Triton X-100, adjusted to pH 7.0 with HCl. Subsequently, cardiac and psoas preparations were stored at −20°C in a similar solution but without Triton X-100. Just before use, small fibre bundles, ~200 μm in diameter, termed 'fibres', were dissected for the assays.

2.2. Solutions

Normal relaxation solution contained 30 mM imidazole, 10 mM ATP, 10 mM creatine phosphate, 5 mM NaN_3, 5 mM EGTA, 12.5 mM $MgCl_2$ and creatine kinase 380 U/ml. The pH was adjusted to 6.7 with KOH. Maximum Ca^{2+} activating solution (contraction solution) contained also 5 mM $CaCl_2$ (pCa = 4.34), ionic strength (120 mM) was adjusted with KCl.

The composition of the solution was calculated using a computer program similar to that described by Fabiato and Fabiato (1979) [24] using the stability constants given in Fabiato (1981) [25]. The pCa

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($-\log[Ca^{2+}]$) was varied by mixing contraction and relaxation solutions as appropriate.

2.3. Force measurements

For force measurements, fibres were mounted isometrically between an AME 801 strain gauge (SensoNor, 3191 Horton, Norway) and a rigid post attached to a micrometer for length adjustment [26], sarcomere length was ~2.04 ± 0.07 μm (determined by laser diffraction). All measurements were carried out at 22°C.

2.4. Source of recombinant human cardiac TnI isoforms and recombinant human cardiac TnC

All mutants were constructed using mega-primer oligonucleotide-directed mutagenesis by the polymerase chain reaction [27]. Two double mutants were made: Ser-23Ala/Ser-24Ala and Ser-23Asp/Ser-24Asp. To synthesize Ser-23Ala/Ser-24Ala, the following oligonucleotide was made: 5′-GCG GTA GTT AGC CGC GCG GCG TCT GAT-3′; this placed two alanine residues at positions 23 and 24. To synthesize Ser-23Asp/Ser-24Asp, the following oligonucleotide was used: 5′-GCG GTA GTT ATC GTC GCG GCG TCT GAT-3′;this introduced aspartic acid residues at positions 23 and 24 to replace the serines. Both genes were cloned into the pET11c vector as previously described [19]. The DNA sequence of the two mutants was checked by the dideoxy chain termination method [28]. These were subsequently used to transform competent BL21(DE3) cells. Protein expression as described by Al-Hillawi et al. (1994) [19].The two mutant proteins were purified using ion-exchange chromatography [19] except that a 0–0.5 M NaCl gradient was used to elute the protein of the CM-Sepharose fast flow column.

The overexpression and purification of the human cardiac TnC was exactly as described by Al-Hillawi et al. (1994) [19].

Recombinant TnI and TnC were combined in ~1:1 ratio in 6 M urea, 50 mM Tris, 50 mM EDTA, 0.1 M dithiothreitol (DTT), adjusted to pH 8.8 with HCl and dialyzed against 3× the volume of 20 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 1 mM EDTA and 5 mM DTT for at least 6 h (changing of the dialysate approximately 5 times) to obtain the TnI–TnC complex.

2.5. TnC extraction and reconstitution with human cTnC

To remove the TnC, cardiac fibres were incubated for 2 h with an ice-cold solution containing 2 mM imidazole-HCl, 2 mM *trans*-1,2-diaminocyclohexane *N,N,N′,N′*-tetraacetic-acid (CDTA), 0.1 mM trifluoperazine (TFP), pH 7.8. Fibres were then reconstituted with human cardiac TnC (110 μM) for 60 min (cf. Fig. 1A).

2.6. TnI extraction and reconstitution

Test contraction–relaxation cycles and responses to different Ca^{2+} concentrations up to pCa = 4.34 were performed in all fibres to determine the Ca^{2+}–force relation in each preparation. Fibres were incubated over 10 min in relaxation solution (pCa = 8) containing 10 mM vanadate. As described by Strauss et al. (1992) [11], it is possible to extract up to 90% of TnI and a part of TnC using this vanadate incubation procedure.

To remove the vanadate, fibres were transferred to fresh Ca^{2+}-free solution in which they contracted Ca^{2+}-independently and subsequently to contraction solution to check for the loss of Ca^{2+}-dependent force.

After transferring fibres to relaxation solution containing 50 mM 2,3-butanedionemonoxime (BDM), they relaxed and were then incubated over night at 0°C in relaxation solution without BDM containing in addition ~70 μM TnI and 80 μM TnC, 5 mM DTT and 10% glycerol. Subsequently, fibres were immersed into TnI- and TnC-free relaxation solution containing 50 mM BDM, to ensure complete relaxation (since BDM is known to dissociate actin myosin linkages (cf. [29]). Fibres were then transferred into BDM-free relaxation solution (in which they contracted slightly) and then in contraction solution (pCa = 4.34). Reconstitution was considered successful when the ratio of force development with and without Ca^{2+} was ~0.2 or less.

2.7. Phosphorylation of TnI in skinned cardiac fibres with the catalytic subunit of PKA

Phosphorylation of TnI by PKA was achieved and monitored by incubation of myofibrils or reconstituted skinned fibres with the catalytic subunit of PKA (500 U/ml relaxation solution containing [γ-^{32}P]ATP (specific activity = 50 μCi/μM) for 45 min), based on a method described in [8]. The skinned fibre proteins were then denatured with 20% TCA (v/v) and 2% sodium pyrophosphate, dissolved in sodium dodecyl sulphate (SDS) and electrophoresed on SDS-polyacrylamide gels by the method of Laemmli [30] using 10–16% gradient gels. ^{32}P incorporation was visualized by autoradiography as described [8].

2.8. Statistics

The normalized tension pCa relationships were fitted to the Hill equation:

$$\text{Fractional force} = \frac{[Ca_x]^n}{Ca_{50}{}^n + [Ca_x]^n}$$

where Ca_{50} is the Ca^{2+} concentration needed to obtain half-maximal tension, Ca_x is the Ca^{2+} concentration and n is the Hill coefficient.

All numbers are mean values ± S.E.M. Significance ($P < 0.05$) was determined by using the unpaired Student's t test.

3. Results and discussion

3.1. TnC extraction and replacement

Fig. 1A shows the response of a skinned fibre to Ca^{2+} (pCa = 4.34) before and after extraction of TnC. Following TnC extraction with a solution of CDTA and TFP, only a small residual force could be elicited by Ca^{2+} in the fibres indicating that the vast majority of the TnC had been removed (Fig. 1A). Replacement with recombinant human cardiac TnC restored 69% of the original force. The Ca^{2+}–force relationship and the

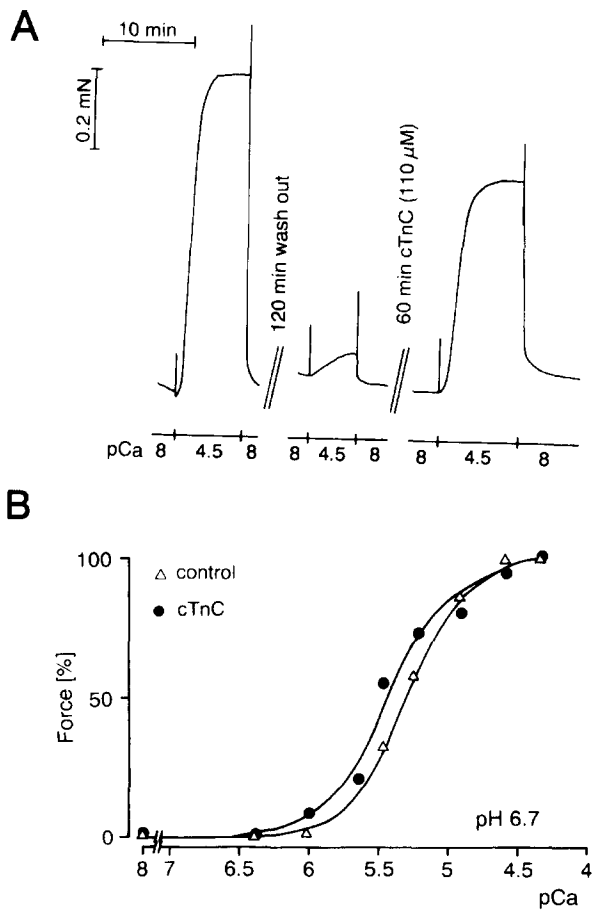

Fig. 1. (A) Force development of skinned trabecula before and after extraction and reconstitution with human cardiac TnC. To remove the TnC, skinned porcine ventricle fibres were incubated for 2 h with an ice-cold solution containing 2 mM imidazole-HCl, 2 mM CDTA, 0.1 mM TFP, pH 7.8, and reconstituted with human cardiac TnC (110 μM) for 60 min. (B) Force–pCa relationship after replacement with human cardiacTnC in porcine cardiac muscle.

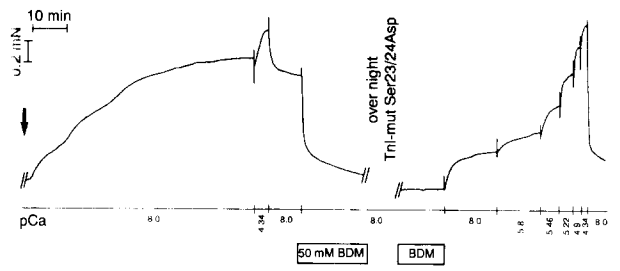

Fig. 2. Troponin extraction and reconstitution in skinned fibres. After extraction of TnI with vanadate (not shown), vanadate was washed out with relaxation solution (at arrow). Note contraction at pCa = 8 and 4.34 and relaxation induced by BDM (50 mM). After reconstitution with mutant-TnI and TnC overnight (as described in methods), a cumulative increase in Ca^{2+} concentration (from pCa = 8 to pCa = 4.34) caused a stepwise increase in force.

Ca^{2+} sensitivity of the reconstituted porcine ventricular fibre (pCa_{50} = ~5.42) were similar to that observed in the fibre prior to reconstitution but it was consistently found that there was a small increase in the Ca^{2+} sensitivity of the fibre when the human cardiac protein replaced the pig isoform fibres (Fig. 1B). These data demonstrate that human cardiac TnC works well in the porcine skinned fibre system.

3.2. *Effect of extraction and reconstitution of TnI and TnC*

All fibres used in this study were from the same heart preparation and selected to have a similar Ca^{2+} sensitivity (pCa_{50} = 5.46 ± 0.01, n = 14 prior to reconstitution with the TnI wild-type or with TnI mutants and a similar Hill coefficient (2.45 ± 0.16, n = 14) to facilitate comparison of fibres reconstituted with wild-type and mutant TnI. A typical reconstitution experiment is shown in Fig. 2. Treatment with vanadate extracted TnI and relaxed the skinned fibres as described in [11]. After washing out the vanadate solution, the fibres slowly developed Ca^{2+}-independent force (see left hand side of Fig. 2) which was 86.9 ± 2.6% (n = 14) of the initial force. In the experiment shown in Fig. 2, there was still a small increase in force after transferring the fibres to a solution of high Ca^{2+} concentration (pCa = 4.34) but usually there was no change or even a small decrease of force after TnI extraction.

After addition of 50 mM BDM, the fibres relaxed and were then reconstituted overnight with the TnI–TnC complex (without BDM) as described in methods. An example of reconstitution using the Ser-23Asp/Ser-24Asp double mutant is also shown in Fig. 2. The addition of recombinant TnI and TnC in Ca^{2+}-free solution resulted in the relaxation of the fibre overnight. After transferring into relaxation solution containing 50 mM BDM, the fibre remained fully relaxed but contracted only slightly after washout of BDM, indicating that reconstitution with TnI was fairly (but not quite) complete. Cumulative increases in the Ca^{2+} concentration led to a stepwise increase in force (up to 85% of the original force, see right hand side of Fig. 2).

Reconstitution of the porcine cardiac fibres with wild-type human recombinant TnI and TnC resulted in a small decrease in the Ca^{2+} sensitivity (initial pCa_{50} = 5.44 ± 0.01, after reconstitution pCa_{50} = 5.35 ± 0.015, n = 5) with a reduced Hill coefficient (1.5 ± 0.08, n = 5) (Fig. 3A). These changes in the Hill coefficient are consistent with those observed by Strauss et al. [11] in their reconstitution experiments. Incubation of the cardiac fibres reconstituted with wild-type TnI with PKA resulted in the phosphorylation of the TnI (cf. also [31–34]). Successful phosphorylation was demonstrated by incubating the fibres with PKA and [γ-^{32}P]ATP and showing incorporation of the label into TnI as described in [8] (data not shown). Fig. 3A shows that after PKA treatment the Ca^{2+} sensitivity of the fibres reconstituted with wild-type TnI was significantly reduced by 0.22 pCa units (from 5.35 ± 0.015 to 5.13 ± 0.04, n = 5). This change in Ca^{2+} sensitvity induced by PKA was similar to that observed by others [9].

Serine to aspartate mutations at putative phosphorylation sites have been shown to imitate the phosphorylated form of serine residues in a number of proteins (for examples, see [35,36]). Here we have used the TnI Ser-23Asp/Ser-24Asp mutant to investigate the possibility of it mimicking the phosphorylated wild-type TnI. As a control, we used wild-type TnI but also a Ser-23Ala/Ser-24Ala mutant which was incapable of being phosphorylated at these sites. Fig. 3B shows that fibre population reconstituted with the Ser-23Asp/Ser-24Asp mutant had a much lower Ca^{2+} sensitivity (pCa_{50} = 5.19 ± 0.02) compared with that of the wild-type (ΔpCa = 0.16). Prior to reconstitution the Ca^{2+} sensitivity was identical (before reconstitution with TnI wild-type pCa_{50} = 5.44 ± 0.01, n = 5 and be-

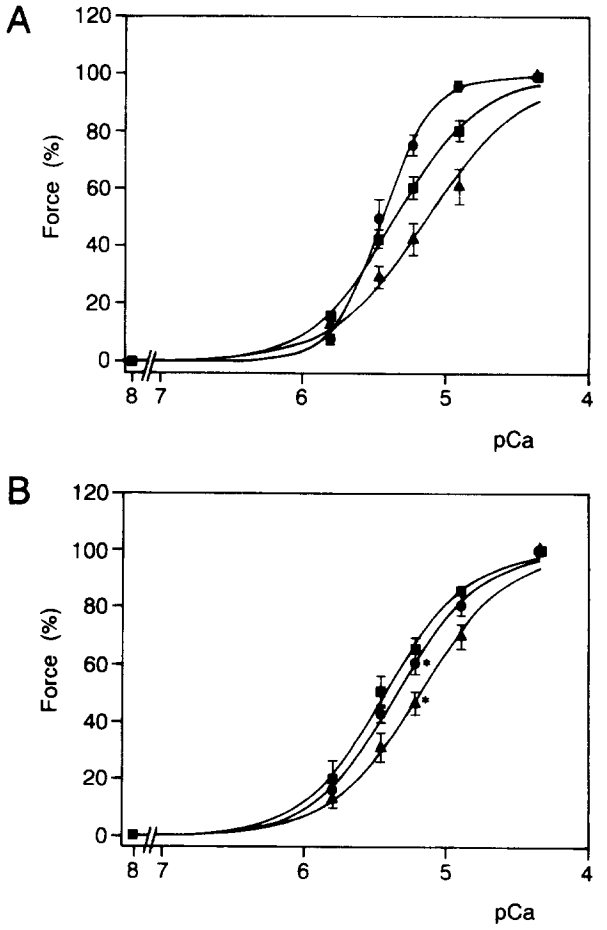

Fig. 3. Calcium responsiveness in skinned trabecula. (A) Force–pCa relationship before reconstitution (●), after reconstitution with wild-type TnI (■) and after incubation over 45 min with the catalytic subunit of PKA (▲) (n = 5). (B) Force–pCa relationship after reconstitution with TnI mutant Ser-23Ala/Ser-24Ala (■, n = 4), wild-type TnI (●, n = 5) and TnI mutant Ser-23Asp/Ser-24Asp (▲, n = 5). *Significantly different (P < 0.05).

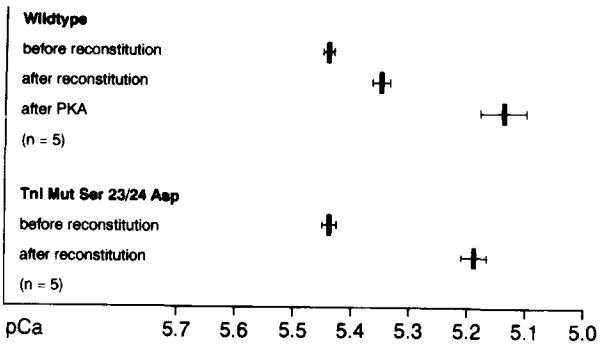

Fig. 4. Calcium sensitivity (pCa_{50}) before and after reconstitution with wild-type TnI and TnI mutant Ser-23Asp/Ser-24Asp (shown as Ser23/24Asp on the figure) ($n = 5$). Also shown is the effect of phosphorylation with the catalytic subunit of PKA after reconstitution with wild-type TnI on the calcium sensitivity ($n = 5$).

fore reconstitution with the Ser-23Asp/Ser-24Asp mutant $pCa_{50} = 5.44 \pm 0.01$, $n = 5$). The Hill coefficient and, thus, the cooperativity of the fibres reconstituted with the Ser-23Asp/Ser-24Asp mutant (Hill coefficient = 1.42 ± 0.12) was similar to that observed after reconstitution with wild-type TnI.

It can be seen in Fig. 3B that, at pCa = 5.22, the mutant-TnI reconstituted fibres developed a significantly lower force ($46.9 \pm 4.0\%$ of maximal force, $n = 5$) than those fibres reconstituted with the wild-type protein ($60.9 \pm 4.0\%$, $n = 5$). Note that the reduced Ca^{2+} sensitivity of the double Ser-23Asp/Ser-24Asp mutant was close to that observed following phosphorylation of the fibres reconstituted with wild-type protein (Fig. 3A). On the other hand, reconstitution of the fibres with the Ser-23Ala/Ser-24Ala mutant resulted in a Ca^{2+} sensitivity similar to that observed in the non-phosphorylated fibre containing the wild-type protein ($pCa_{50} = 5.38 \pm 0.02$, $n = 4$). The Hill coefficient was similar. Phosphorylation of fibres reconstituted with either the double Ser-23Asp/Ser-24Asp mutant or double Ser-23Ala/Ser-24Ala mutant had only a small effect on the Ca^{2+} sensitivity reflecting the phosphorylation of a small portion of endogenous TnI still remaining in the fibres after vanadate extraction (data not shown).

In conjunction, these observations re-enforce the view that Ca^{2+} sensitivity of heart muscle is dependent on the phosphorylation state of the Ser-23 and Ser-24 residues. The results summarized in Fig. 4 show that the exchange of only two amino acid residues in TnI (residues Ser-23 and Ser-24) to Asp has a significant effect on the Ca^{2+} sensitivity of the myofilament, in which the pCa_{50} for force decreased by 0.16 U. This mutagenesis of TnI mimicks the effect of phosphorylation of Ser-23 and 24 by PKA, suggesting that the net charge on the N-terminal region of the TnI molecule is an important determinant of Ca^{2+} responsiveness. Various phosphorylation states of TnI are possible in vivo; two monophosphorylated forms, the bisphosphorylated form and the dephosphorylated form. In future experiments, it is therefore necessary to undertake further mutagenesis of the TnI in order to investigate whether each of the two serine residues has a different role in the regulation of cardiac muscle contraction.

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References

[1] Moss, R.L. (1992) Circ. Res. 70, 865–884.
[2] Gulati, J. and Babu, A. (1989) FEBS Lett. 245, 279–282.
[3] Morimoto, S. and Ohtsuki, I. (1994) J. Biochem. 115, 144–146.
[4] McAuliffe, J.J., Lizhu, G. and Solaro, R.J. (1990) Circ. Res. 66, 1204–1216.
[5] Solaro, R.J., Kumar, P., Blanchard, E.M. and Martin, A.F. (1986) Circ. Res. 58, 721–729.
[6] Nassar, R., Malouf, N.N. and Kelly, M.B. (1991) Circ. Res. 69, 1470–1475.
[7] Ding, X.L., Babu, A. and Gulati, J. (1995) Biochem. J. 34, 2309–2316.
[8] Herzig, J.W., Köhler, G., Pfitzer, G., Rüegg, J.C. and Wölffle, G. (1981) Pflügers Arch. 391, 208–212.
[9] Zhang, R., Zhao, J., Mandveno, A. and Potter, J.D. (1995) Circ. Res. 76, 1028–1035.
[10] Ghuo, X., Wattanapermpool, J., Palmiter, K.J., Murphy, A.M. and Solaro, R.J. (1994) J. Biol. Chem. 269, 15210–15216.
[11] Strauss, J.D., Zeugner, C., Van Eyk, J.E., Bletz, C., Troschka, M. and Rüegg, J.C. (1992) FEBS Lett. 310, 229–234.
[12] Swiderek, K., Jaquet, K., Meyer, H.E. and Heilmeyer, L.M.G. (1988) Eur. J. Biochem. 176, 335–342.
[13] Mittmann, K., Jaquet, K. and Heilmeyer, L.M.G. (1992) FEBS Lett. 302, 133–137.
[14] England, P.J. (1976) Biochem. J. 160, 295–302.
[15] Ray, K.P. and England, P.J. (1976) FEBS Lett. 70, 11–16.
[16] Holroyde, M.J., Howe, E. and Solaro, R.J. (1979) Biochem. Biophys. Acta 586, 63–9.
[17] Solaro, R.J, Moir, A.G.J. and Perry, S.V. (1976) Nature (London) 262, 615–617.
[18] Al-Hillawi, E., Bhandari, D.G., Trayer, H.R. and Trayer, I.P. (1995) Eur. J. Biochem. 228, 962–970.
[19] Al-Hillawi, E., Minchin, S.D. and Trayer, I.P. (1994) Eur. J. Biochem. 225, 1195–1201.
[20] Kluwe, L., Maéda, K. and Maéda, Y. (1993) FEBS Lett. 325, 83–88.
[21] Wiesner, R.J., Strauss, J.D., Maéda, Y., Kluwe, L. and Rüegg, J.C. (1993) Pflügers Arch. Suppl. 1 422, R 258.
[22] Potter, J.D., Sheng, Z., Pan, B.S. and Zhao, J. (1995) J. Biol. Chem. 270, 2557–2562.
[23] Farah, C.S., Miyamato, C.A., Ramos, C.H.I., da Silva, A.C.R., Quaggio, R.B., Fujimori, K., Smillie, L.B. and Reinach, F.C. (1994) J. Biol. Chem. 269, 5230–5240.
[24] Fabiato, A. and Fabiato, F. (1979) J. Physiol. (Paris) 75, 463–505.
[25] Fabiato, A. (1981) J. Gen. Physiol. 78, 457–497.
[26] Rüegg, J.C., Zeugner, C., Van Eyk, J.E., Kay, C.M. and Hodges, R.S. (1989) Pflügers Arch. 407, 552–557.
[27] Perrin, S. and Gilliland, G. (1990) Nucl. Acid. Res. 18, 7433–7439.
[28] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[29] Gwathmey, J.K., Hajjar, R.J. and Solaro, R.J. (1991) Circ. Res. 69, 1280–1292.
[30] Laemmli, U.K. (1970) Nature (London) 227, 680–685.
[31] Bailey, C. and Villar-Palasi, C. (1971) Fed. Proc. 30, 1147.
[32] Perry, S.V. and Cole, H.A. (1974) Biochem. J. 141, 733–743.
[33] Cole, H.E. and Perry, S.V. (1975) Biochem. J. 149, 525–533.
[34] Wattanapermpool, J., Guo, X. and Solaro, R.J. (1995) J. Mol. Cell Cardiol. 27, 1383–1391.
[35] Apodaca, G. and Mostov, K.E. (1993) J. Biol. Chem. 268, 23712–23719.
[36] Huang, B.D. and Erikson, R.L. (1994) Proc. Natl. Acad. Sci. USA 91, 8960–8963.