Biochimica et Biophysica Acta, 673 (1981) 303—311

BBA 29545

IMMUNOCYTOCHEMICAL AND BIOCHEMICAL EVIDENCE FOR THE PRESENCE OF CALMODULIN IN BULL SPERM FLAGELLUM

ISOLATION AND CHARACTERIZATION OF SPERM CALMODULIN

JACQUELINE FEINBERG [a], JACQUELINE WEINMAN [a], SERGE WEINMAN [a], MICHAEL P. WALSH [b], MARIE CECILE HARRICANE [b**], JACQUELINE GABRION [b**] and JACQUES G. DEMAILLE [b*]

UER Biomédicale des Saints-Pères, 45 rue des Saints-Pères, 75006 Paris and Faculté de Médecine, Unité INSERM and Centre de Recherches de Biochimie Macromoléculaire du CNRS, BP 5051, 34033-Montpellier (France)

(Received August 28th, 1980)

Key words: Calmodulin; Ca^{2+} binding; Motility; Immunofluorescence; (Bull sperm flagellum)

Summary

Upon fluorescent staining with a goat antibody anti-ram testis calmodulin, washed bull sperm appears to contain calmodulin in the acrosome, in the post acrosomal region, in the neck region probably associated with the implantation plates and thin laminated fibers, and in a sheath around the upper part of the flagellum. Heads and midpieces + tails were separated by elutriation of sonicated sperm. Immunofluorescent labeling of fragments confirms the presence of calmodulin in implantation plates, where sonication disrupted heads from midpieces, and in a sheath around the midpiece and the upper part of the principal piece.

These results were confirmed by electrophoretic and radioenzymatic assays of calmodulin in the fragments, using calmodulin-deficient Ca^{2+}/calmodulin-dependent myosin light chain kinase. Small but significant amounts (approx. 3 μg per 10^{10} sperm) are found in midpieces + tails vs. approx. 280 μg in the same number of heads. These results are in agreement with a recent report from Jones et al. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 2772—2776.

Sperm calmodulin was purified from a whole sperm 1 M KCl extract and found to exhibit the same characteristics as other mammalian calmodulins isolated so far in terms of ultraviolet absorption spectrum and amino acid composition, including one residue of ϵ-*N*-trimethyllysine. Its behavior upon SDS-

* To whom correspondence should be addressed

** Permanent address: Laboratoire d'Histologie, Faculté de Médecine de Montpellier.

Abbreviations: buffer 1, 0.15 M NaCl/5 mM Tris-HCl, pH 7.5; SDS, sodium dodecyl sulfate.

polyacrylamide gel electrophoresis was dependent on the presence or absence of Ca^{2+}. The high performance liquid chromatography tryptic peptide maps were similar, if not identical, to mammalian calmodulin maps (Autric et al. (1980) Biochim. Biophys. Acta 631, 139—147). Sperm calmodulin is therefore probably identical to the somatic cell protein.

Introduction

Many of the calcium effects in eukaryotic cells are thought to be mediated by calmodulin, the ubiquitous and multifunctional calcium dependent regulator (see Ref. 1 and 2 for reviews).

The role of calmodulin in the triggering of smooth muscle [3] and non muscle cells [4,5] actin-myosin interaction via the myosin light chain kinase system is now firmly established. In contrast, there is as yet no evidence for such an involvement in the Ca^{2+}-regulation of another motile apparatus, namely the dynein-tubulin system of cilia and flagella. A recent report [6] suggests however that calmodulin may play a role in the control of ciliary motility in *Tetrahymena* spp. Similarly, an acidic calcium binding protein that is able to form calcium-dependent complexes with troponin I, was isolated from this ciliate [7]. In contrast, calmodulin could not be detected in tail and midpiece extracts of rabbit sperm by using the cyclic nucleotide phosphodiesterase assay [8]. The same authors however recently detected calmodulin at the tail base and tip using the immunofluorescence localization technique, suggesting that calmodulin may be involved in axonemal assembly/disassembly [9].

We show in this report that calmodulin is mostly present in sperm heads. A small but significant fraction was found to be associated with the midpiece + tail. When purified, sperm calmodulin appeared to be essentially identical to the somatic mammalian homologous proteins.

Materials and Methods

Materials

Fresh bull sperm was obtained from the Centre d' Insémination Artificielle de l'Aigle, and frozen bull sperm was from the Union Coopérative d'Elevage de Francheville.

Ram testis calmodulin was isolated as previously described [10]; IgG directed against calmodulin were elicited in goats by repeated injections of the performic acid oxidized ram testis protein emulsified in Freund's complete adjuvant. Antibodies were purified by affinity chromatography on calmodulin-Sepharose from which they were eluted by a 0.2 M glycine (HCl) buffer, pH 2.8 (Demaille, J.G., unpublished data). Fluorescein-conjugated rabbit anti goat IgG was purchased from Nordic. Calmodulin-free rabbit skeletal or bovine cardiac myosin regulatory light chains were prepared essentially according to Nairn and Perry [11]. Bovine cardiac myosin light chain kinase was purified as previously described [12].

Methods

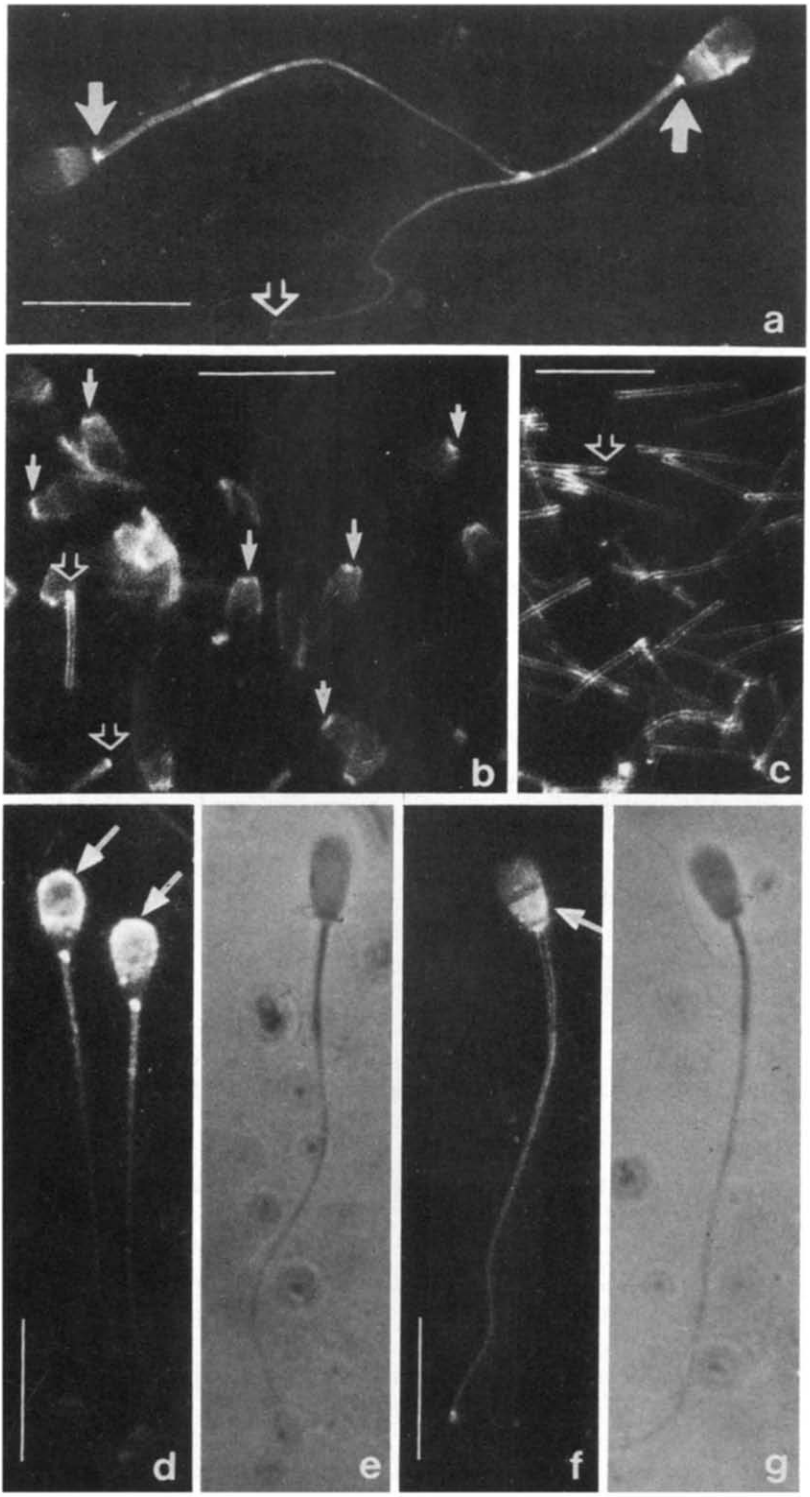
Fresh bull spermatozoa were freed from seminal plasma [13] and fractionated into sperm heads and flagellar fragments (midpieces + tails and part of the principal + terminal piece) by sonication and elutriation as described elsewhere (Weinman, S., Weinman, J., Escaig, F., Pariset, C. and Feinberg, J., unpublished data). Sperm suspensions (5 ml containing $1.2—1.5 \cdot 10^{10}$ elements) were sonicated for 10 s in 1 inch diameter glass tubes at 0°C, by using a Branson sonifier Model B 12 fitted with a 1/8 inch microprobe, with 40 W power.

Calmodulin localization by indirect immunofluorescence. Fixation was either performed with 4% paraformaldehyde in phosphate-buffered saline (room temperature, 10—20 min), or with acetone (—20°C, 10 min), or with 4% paraformaldehyde in phosphate-buffered saline, followed by acetone at —20°C. After fixation, preparations were treated for 10 min with a 1/20th dilution of normal rabbit serum in buffer 1, rinsed and incubated overnight at 4°C with the anticalmodulin IgG (50 μg/ml of buffer 1). After 1 h rinse with buffer 1, slides were incubated for 45 min at room temperature with a 1/40th dilution of the fluorescein conjugated rabbit anti-goat IgG. After exhaustive washes samples were covered with a drop of 90% glycerol in buffer 1 then with a glass coverslip. Cells were examined with a Zeiss microscope equipped with epifluorescence illumination, and interference filters (blue combination). Control reactions were performed either by omitting the purified antibodies vs. calmodulin or by using these purified antibodies premixed with 60 μM native calmodulin.

Extraction of sperm cells. Heads and flagellar fractions were suspended in 2 vols. of 1 mM EDTA/10 mM Tris-HCl, pH 8.0, freeze-thawed and sonicated. The sonicate was centrifuged at 27 000 × *g* for 15 min. The supernatant will be referred to as the 'cold extract'. Subsequently, the pellet was extracted with the same buffer, at 85°C for 3 min, then centrifuged (27 000 × *g*, 15 min). The supernatant will be referred to as the 'hot extract' [8]. Both extracts were dialyzed vs. 20 μM $CaCl_2$/65 mM NH_4HCO_3 and lyophilized.

Calmodulin assays. Calmodulin concentration was determined by the activation of calmodulin-deficient myosin light chain kinase as previously described [14] and by gel electrophoresis followed by elution of the protein-bound Coomassie blue as described by Grand and Perry [15]. Cell extracts were also analyzed by 0.1% SDS-14—25% polyacrylamide gradient gel electrophoresis, using the discontinuous buffer system of Laemmli [16]. Either 10 mM $CaCl_2$ or 5 mM EDTA were added to the sample to identify calmodulin by its different migration rates in the presence or absence of divalent cations [17].

Purification of calmodulin. Frozen bull sperm ($25 \cdot 10^{11}$ cells) were thawed and washed extensively with 0.15 M NaCl. Cells were treated by ethanol/diethyl ether according to Fine et al. [18]. The resulting powder (60 g) was air dried overnight, then extracted with 1.2 l of 10 mM 2-mercaptoethanol/1 M KCl adjusted to pH 7.0 with Tris, for 16 h at 4°C. The suspension was centrifuged for 30 min at 10 000 × *g* and the supernatant brought to 70°C in a boiling water bath, kept at this temperature for one minute further and chilled rapidly on ice. The resulting suspension was centrifuged for 30 min at 100 000 × *g*. The supernatant was dialyzed vs. 0.2 M KCl/10 mM 2-mercaptoethanol and fractionated by $(NH_4)_2SO_4$ according to Hartshorne and Mueller [19]. The precipitate obtained at 75% saturation was dialyzed vs. 20 μM $CaCl_2$/


a
b
c
d
e
f
g

65 mM NH_4HCO_3 and lyophilized. This fraction, initially prepared for the purpose of identifying troponin C-like proteins, appeared to contain calmodulin. The next steps, DEAE-cellulose chromatography and gel filtration on Sephacryl S-200 were performed essentially as previously described [10].

Similarly, characterization of sperm calmodulin was carried out using the already described techniques [10].

Results

Immunocytochemical localization of calmodulin

Indirect immunofluorescent labeling of intact bull sperm shows calmodulin to be present not only in heads where it appears mostly in the post acrosomal region, but also as a bright spot or an arch in the neck region and as a fluorescent sheath all along the midpiece and most of the principal piece of the tail (Fig. 1a). Under our observation conditions, no labeling of the terminal part of the tail was visible.

These results were confirmed and extended by a similar study performed on fragments generated by sonication. When the whole sonicate was labeled, the fracture zone between head and midpiece was fluorescent on both, with labeling of the base of the head, presumably at the implantation plate, and of the top part of the midpiece, presumably at the thin laminated fibers (Fig. 1b). The fluorescent sheath was visible all along the midpiece as also shown by Fig. 1c, which depicts the labeling of a tail + midpiece fraction purified by elutriation. Since the plasma membrane was lost during this procedure, the peripheral stain does not identify membrane-bound calmodulin, but may rather show its presence either on dense fibers or on mitochondria. Other experiments performed on principal and terminal pieces purified by elutriation failed to show any calmodulin. No staining was ever obtained when anticalmodulin antibodies were substituted for either by a non immune serum or by calmodulin-depleted specific antibodies (not shown).

The type of fixation used proved to be critical for the calmodulin localization experiments. As shown by staining of intact cells, calmodulin appeared mostly localized as a cap over the anterior region of the head when fixed with acetone only (Fig. 1d). By contrast, paraformaldehyde fixation resulted into the loss of apical fluorescence and most of the label was found to be present in the post acrosomal region (Fig. 1f).

Fig. 1. Immunofluorescent localization of calmodulin in bull sperm. (Bar = 20 μM). (a) Immunofluorescent staining of calmodulin in intact bull sperm. Note the fluorescent line and associated spot at the neck region (↓) and the staining sheath all along the midpiece and most part of the principal piece. Under our conditions no fluorescence could be detected at the tip of the tail (⇓). (b) Immunofluorescent staining of calmodulin in sonicated bull sperm. Note the repartition of the label between the head (fluorescent line at the base of the head (↓) and the base of the midpiece (fluorescent spot (⇓)). A bright sheath is also visible all along the midpiece. (c) Immunofluorescent staining of calmodulin in purified midpiece + tail fraction. Note the intense fluorescence associated with peripheral structures of the flagellum (⇓). (d) Effect of acetone fixation on immunofluorescent labelling of intact bull sperm. Note a more positive staining over the anterior part of the head (↙). (e) Phase contrast micrograph of acetone-fixed bull sperm. (f) Effect of paraformaldehyde fixation on immunofluorescent labelling of intact bull sperm. Note the fluorescence associated with the post acrosomal region of the head (↖). No fluorescence was detected on the acrosomal region. (g) Phase contrast micrograph of paraformaldehyde-fixed bull sperm.

The fluorescence associated with implantation plates and midpieces was found to be less sensitive to the fixation procedure. Sperm heads exhibited the same morphological pattern when observed by phase contrast microscopy irrespective of the fixation technique (Fig. 1e and 1g). In particular, no difference in the occurrence or extent of the acrosomal reaction could be noticed.

Biochemical analysis of calmodulin distribution.

The above results were confirmed by two independent approaches on intact sperm and elutriated fragments (Table I). Quantitation of the Coomassie-blue stained calmodulin after extraction of sperm and of midpieces + tails by two different techniques, shows that most of the calmodulin is present in heads with only approx. 2.5% present in the midpiece + tail region. The difference in calmodulin content was even more obvious when the specific myosin light chain kinase assay was used. Most of the sperm calmodulin was only extracted at high temperature, whereas the small but significant fraction (approx. 1%) present in the midpiece + tail fragments was easily extractable (Table I).

Isolation and characterization of sperm calmodulin

From $25 \cdot 10^{11}$ sperm cells that contained 69—175 mg calmodulin depending on the quantitation technique (see Table I), 8 mg pure calmodulin was obtained. The yield was therefore 5—11%, as expected from the multistep isolation procedure. The protein appeared homogeneous after SDS-polyacrylamide gel electrophoresis with the usual pattern in the presence of EDTA, where it migrates as a single band of M_r 19 000 and in the presence of Ca^{2+}, where it migrates as a doublet with a major faster band of M_r 17 000 (not shown). As shown in Fig. 2, the ultraviolet absorption spectrum shows the characteristic vibronic structures of phenylalanyl residues at 253.5, 259.5, 265.5 and 269 nm and the tyrosine peak at 277 nm. The absorbance ratio of the 277 nm peak to the 251 nm trough was 1.5.

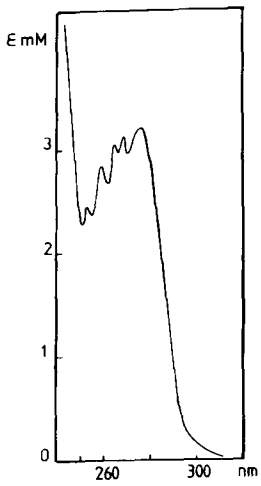
The amino acid composition (Table II) is very close to that of bovine brain calmodulin [20] and none of the differences observed can be considered as significant. Sperm calmodulin contains, like its somatic counterpart, a unique residue of ϵ-*N*-trimethyllysine. The similarity of previously isolated mam-

TABLE I

CALMODULIN CONTENT OF BULL SPERM CELLS AND SUBCELLULAR FRACTIONS

For electrophoresis extraction was carried out with 2 vols of 1 mM EDTA/10 mM Tris-HCl, pH 8, sonication and heat step. The supernatant was fractionated by ammonium sulphate precipitation according to Teo et al. [24]. MLCK, myosin light chain kinase.

Fraction	Calmodulin (μg per $1 \cdot 10^{10}$ elements)				Calmodulin (% of heads + midpieces and tails)	
	Determined by the MLCK assay			Determined by electrophoresis (16)	MLCK	Electrophoresis
	Cold extract	Hot extract	Total			
Whole spermatozoa	8	695	703	460	—	—
Heads	—	—	284	595	99	97.5
Midpieces + tails	2.5	0.8	3.3	15	1	2.5



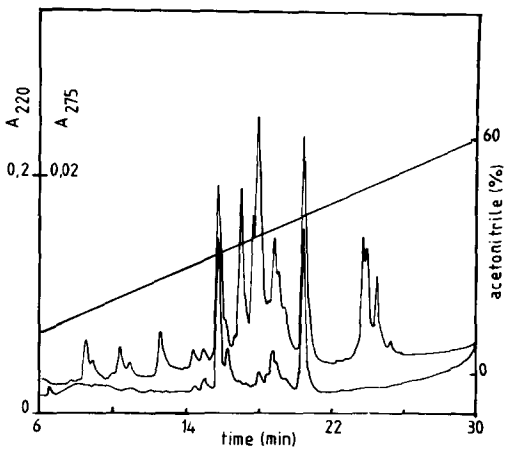


Fig. 2. Ultraviolet absorption spectrum of bull sperm calmodulin. The protein was dissolved in 20 μM $CaCl_2$/65 mM NH_4HCO_3, pH 8.

Fig. 3. HPLC peptide maps of bull sperm calmodulin (5 nmol) after tryptic digestion. Upper trace: absorbance of the eluate at 220 nm, 0.4 absorbance unit full scale. Lower trace: absorbance of the eluate at 275 nm, 0.04 absorbance unitl full scale. Lower and upper traces were obtained on different and consecutive runs. In the illustrated part of the run (6—30 min), the acetonitrile gradient (———) was from 12 to 60%.

TABLE II

AMINO ACID COMPOSITION OF BULL SPERM CALMODULIN

Values are expressed as residues per mol after triplicate analyses of 24, 48 and 72 h hydrolyzates. The composition of the bovine brain calmodulin was from Ref. 20.

Residue	Bull sperm calmodulin		Bovine brain calmodulin
	Found	Integer	
Asx	24.4	24	23
Thr [a]	13.2	13	12
Ser [a]	4.0	4	4
Glx	29.1	29	27
Pro	2.3	2	2
Gly	11.0	11	11
Ala	10.7	11	11
Cys	0	0	0
Val [b]	6.9	7	7
Met	8.3	8	9
Ile [b]	7.9	8	8
Leu	8.8	9	9
Tyr	1.8	2	2
Phe	8.1	8	8
His	1.0	1	1
TML [c]	0.9	1	1
Lys	6.7	7	7
Arg	6.1	6	6
Trp [d]	0	0	0

[a] After extrapolation at zero time of hydrolysis.
[b] From the 72 h hydrolysis values.
[c] TML: ε-*N*-trimethyllysine.
[d] From spectroscopic evidence (see Figure 2).

malian calmodulins and the one described in this report is further documented by the tryptic peptide map obtained by high performance liquid chromatography.

As shown in Fig. 3, the peptide elution pattern, monitored at 220 nm, is strikingly similar to those previously reported for bovine brain and ram testis calmodulins [10]. Moreover, the two tyrosine containing peptides, presumably peptides 91—106 and 127—148 [20], elute at exactly the same time along the gradient (16.15 and 20.9 min, respectively [10]).

Discussion

Both biochemical and immunocytochemical localization techniques point to the localization of the major part (at least 97%) of bull sperm calmodulin in heads, in agreement with previous reports [8]. Since ejaculated bull sperm has under gone the acrosomal reaction, our study cannot evaluate the importance of the acrosomal vs. post acrosomal localization in heads. Also we have conclusively shown that the type of fixation is of major importance in the repartition of the label within sperm heads. When acetone was used, most of the label formed a bright cap at the anterior part of the head. Such a stain is no longer visible after paraformaldehyde fixation as if calmodulin were released under these conditions. Similarly, purified heads obtained by elutriation of sonicated sperm exhibit a poor staining, in line with a poor recovery of calmodulin as measured by the myosin light chain kinase assay (Table I).

More important perhaps is the evidence for the presence of flagellar calmodulin. Even though calmodulin was not initially detectable in flagella [8], further work by the same group showed the presence of calmodulin associated with the basal bodies and at the tip of the tail [9]. It is clearly of crucial importance to determine whether Ca^{2+}-effects on the dynein-tubulin system are mediated by calmodulin or not. This was suggested by recent studies from Jamieson et al. [6] and Suzuki et al. [7] on *Tetrahymena piriformis* cilia. If such an assumption were true, one would expect calmodulin staining to parallel the distribution of tubulin all along the axoneme.This was not found to be true either in this report or in Ref. 9. Calmodulin appears rather as a peripheral component of the first part of the flagellum. The level of resolution afforded by optic observations does not of course permit the assignment of calmodulin stain to either the coarse outer fibers or mitochondria. Plasma membranes, that are no longer present in elutriated midpieces, are not involved in this peripheral labeling. High local concentrations of calmodulin are also found at the point of the neck that is fractured by sonication, leaving intensely labeled implantation plates on the head fragments and a bright stain of what we suppose to be thin laminated fibers. Here too, there is as yet no hint as to the role of calmodulin.

Finally, although calmodulin is now firmly established to be present in the midpiece + tail region of sperm albeit at low concentrations, there is no positive evidence for its involvement in any specific function of the cell and in particular in flagellar motility.

Another major problem is the eventual existence of different genes coding for calmodulin in germinal vs. somatic cells. This was suggested by the fact that *Xenopus* oocyte calmodulin exhibited slight but significant differences in

amino acid composition and tryptic peptide maps when compared to mammalian calmodulins [21]. Since bovine brain calmodulin structure is now available [20], bull sperm offered the opportunity to check whether different calmodulins were coded for in the course of development. Results presented herein point to a negative answer. Sperm and somatic proteins are indeed indistinguishable by the usual analytical techniques. Such a finding is not surprising in view of the highly conserved structure of calmodulin [22,23].

Acknowledgments

The authors are indebted to Dr. J.C. Cavadore for helpful advice and criticism and to Ms. D. Waeckerlé for expert editorial assistance. This work was supported in part by grants to S.W. from the Univeristy of Paris VI and CNRS (L.A. 87) and to J.G.D. from Faculté de Médecine, CNRS (ATP Modulation de l'action des hormones au niveau cellulaire), DGRST (ACC Biologie et Fonction du Myocarde et Parois artérielles), INSERM (CRL 78.4.086.1. and ATP 63.78.95 Fibre musculaire cardiaque), NATO (grant 1688) and Fondation pour la Recherche Médicale Française.

References

1 Cheung, M.Y. (1980) Science 207, 19—27
2 Means, A.R. and Dedman, J.R. (1980) Nature 285, 73—77
3 Dabrowska, R., Sherry, J.M.F., Aromatorio, D.K. and Hartshorne, D.J. (1978) Biochemistry 17, 253—258
4 Dabrowska, R. and Hartshorne, D.J. (1978) Biochem. Biophys. Res. Commun. 85, 1352—1359
5 Hathaway, D.R. and Adelstein, R.S. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1653—1657
6 Jamieson, G.A., Vanaman, T.C. and Blum, J.J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 6471—6475
7 Suzuki, Y., Hirabayashi, T. and Watanabe, Y. (1979) Biochem. Biophys. Res. Commun. 90, 253—260
8 Jones, H.P., Bradford, M.M.; McRorie, R.A. and Cormier, M.J. (1978) Biochem. Biophys. Res. Commun. 82, 1264—1272
9 Jones, H.P., Lenz, R.W., Palevitz, B.A. and Cormier, M.J. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 2772—2776
10 Autric, F., Ferraz, C., Kilhoffer, M.C., Cavadore, J.C. and Demaille, J.G. (1980) Biochim. Biophys. Acta 631, 139—147
11 Nairn, A.C. and Perry, S.V. (1979) Biochem. J. 179, 89—97
12 Walsh, M.P., Vallet, B., Autric, F. and Demaille, J.G. (1979) J. Biol. Chem. 254, 12136—12144
13 Harrison, R.A.P. (1976) J. Reprod. Fert. 48, 347—353
14 Le Peuch, C.J., Ferraz, C., Walsh, M.P., Demaille, J.G. and Fischer, E.H. (1979) Biochemistry 18, 5267—5273
15 Grand, R.J.A. and Perry, S.V. (1979) Biochem. J. 183, 285—295
16 Laemmli, U.K. (1970) Nature (London) 227, 680—685
17 Klee, C.B. (1977) Biochemistry 16, 1017—1024
18 Fine, R.E., Blitz, A.L., Hitchcock, S.E. and Kaminer, B. (1973) Nature New Biol. 245, 182—186
19 Hartshorne, D.J. and Mueller, H. (1969) Biochim. Biophys. Acta 175, 301—319
20 Watterson, D.M., Sharief, F. and Vanaman, T.C. (1980) J. Biol. Chem. 255, 962—975
21 Cartaud, A., Ozon, R., Walsh, M.P., Haiech, J. and Demaille, J.G. (1980) J. Biol. Chem. 255, 9404—9408
22 Watterson, D.M., Mendel, P.A. and Vanaman, T.C. (1980) Biochemistry 19, 2672—2676
23 Demaille, J.G., Haiech, J. and Goodman, M. (1980) Protides of the Biological Fluids 28, 95—98
24 Teo, T.S., Wang, T.H. and Wang, J.H. (1973) J. Biol. Chem. 248, 588—595