FEBS Letters 350 (1994) 130–134

FEBS 14365

Presence of protein phosphatase type 1 and its involvement in temperature-dependent flagellar movement of fowl spermatozoa

Koji Ashizawa[a,*], Graham J. Wishart[b], Hiroshi Tomonaga[a], Kohfumi Nishinakama[a], Yasuhiro Tsuzuki[a]

[a]*Laboratory of Animal Reproduction, Faculty of Agriculture, Miyazaki University, Miyazaki 889-21, Japan*
[b]*Department of Molecular and Life Sciences, University of Abertay Dundee, Dundee DD1 1HG, UK*

Received 3 June 1994

Abstract

Even in the presence of ATP, the motility of demembranated fowl spermatozoa was negligible at the avian body temperature of 40°C. Motility could be restored by the addition of calyculin A, okadaic acid, specific inhibitors of phosphatase type 1 (PP1) and PP-2A, and inhibitor 1 or inhibitor 2, which are specific inhibitors of protein phosphatase type 1 (PP1). Demembranated spermatozoa, stimulated by calyculin A or okadaic acid, lost their motility following the addition of 1 mM $CaCl_2$, but this was restored gradually by the stepwise addition of EGTA. Immunoblotting of sperm extract using an antibody to PP1 revealed a major cross-reacting protein of 36–37 kDa, which corresponded to the molecular weight of the known catalytic subunit of PP1. These results suggest that PP1 present in the fowl sperm axoneme may be involved in the inhibition of fowl sperm motility at 40°C via Ca^{2+}-dependent regulatory systems.

Key words: Phosphorylation/dephosphorylation; Protein phosphatase; Okadaic acid; Calyculin A; Motility; Spermatozoa

1. Introduction

It is well known that flagellar movement of spermatozoa is based on the active sliding of microtubules as a result of ATP hydrolysis by dynein ATPase (reviewed in [1–3]). Although this basic axonemal mechanism is fairly well accepted, much remains to be learned about how sliding is controlled. To investigate this regulatory mechanism, fowl spermatozoa provide an excellent model, since they display the unique phenomenon of reversible temperature-dependent immobilization: in simple salt solutions they become immotile at the avian body temperature of 40–41°C, but motility is restored by decreasing the temperature [4–9]. The axoneme itself appears to be directly involved in this regulatory system, since the motility of demembranated spermatozoa is, as with intact spermatozoa, negligible at 40°C and restored at 30°C [10,11]. However, the intracellular molecular mechanisms involved in the immobilization and restoration of motility have remained unsolved.

There is substantial evidence that protein phosphorylation by cAMP-dependent protein kinase plays a primary role in the second messenger regulatory mechanisms of flagellar axoneme-based movement (reviewed in [1–3,12,13]). For example, phosphorylation by cAMP-dependent protein kinase of a 15 kDa protein is indispensable for the initiation of trout sperm motility [14]; the phosphorylation of a 55 kDa protein is apparently related to the motility state of bovine spermatozoa [15]; and axokinin, a soluble 56 kDa phosphoprotein, seems to play a key role in mediating the cAMP response in dog spermatozoa [16,17]. On the other hand, cAMP-independent phosphoproteins have also been identified in human [18], rat [19], dog [20] and fowl [21] spermatozoa.

If phosphorylation is required for the activation of sperm flagellar motility, dephosphorylation by protein phosphatases in the axoneme should also affect motility. Serine/threonine protein phosphatases are classified into four main enzymes; type 1 (PP1), type 2A (PP2A), type 2B (PP2B) and type 2C (PP2C) [22]. However, limited information is available which enzymes are involved in the regulation of sperm flagellar motility [23]. We report here that PP1 might be dominant in the involvement for the temperature-dependent flagellar movement of fowl spermatozoa, since in addition to calyculin A and okadaic acid, the PP1-specific inhibitors 1 and 2 also stimulated the motility of demembranated spermatozoa at 40°C.

2. Materials and methods

2.1. Chemicals

Calyculin A and okadaic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Activated, i.e. phosphorylated, inhibitor 1, and inhibitor 2 were kindly provided by Professor P. Cohen (University of Dundee, UK). Adenosine 5′-triphosphate (ATP), bovine serum albumin (BSA), 3, 3′-diaminobenzidine tetrahydrochloride (DAB), dithiothreitol, *p*-nitrophenyl phosphate, potassium glutamate, *N*-Tris-[hydroxymethyl]-methyl-2-aminoethanesulphonic acid (TES) and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). Avidin-biotin peroxidase complex (ABC) was obtained from Vector Laboratories (Burlingame, CA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were purchased from Life Technologies, Inc. (Gaithersburg, MD). Other chemicals were of reagent grade from Nacalai Tesque, Inc. (Kyoto, Japan).

*Corresponding author. Fax: (81) (985) 58 2884.

SSDI 0014-5793(94)00752-7

2.2. Antisera

Immune rabbit serum against a synthetic peptide corresponding to the C-terminal amino acid residues 316–330 of human PP1α conjugated to keyhole limpet hemocyanin (a polyclonal IgG) was purchased from Upstate Biotechnology Incorporated (Lake Placid, NY). Biotinylated anti-rabbit immunoglobulins goat serum was obtained from Dako Japan (Kyoto, Japan).

2.3. Preparation of spermatozoa

Ejaculated spermatozoa from commercial White Leghorn roosters were diluted approximately 10-fold in 150 mM NaCl with 20 mM TES at pH 7.4 and centrifuged at 700 × *g* for 13 min at room temperature (20–25°C). The washed spermatozoa were reconstituted in the same buffer to give a final concentration of approximately 1×10^9 cells/ml. Samples of 3–4 ml were poured into 30 ml Erlenmeyer flasks with a screw cap.

2.4. Analysis of demembranated sperm motility

Demembranation and reactivation of spermatozoa were performed according to the method described previously [11]. The extraction medium used consisted of 0.1% Triton X-100, 200 mM sucrose, 25 mM potassium glutamate, 1 mM $MgSO_4$, 1 mM dithiothreitol and 20 mM Tris-HCl buffer (pH 7.9). The reactivation medium consisted of 0.5 mM ATP, 200 mM sucrose, 25 mM potassium glutamate, 1.5 mM $MgSO_4$, 1 mM dithiothreitol and 20 mM Tris-HCl buffer (pH 7.9). To examine the effects of protein phosphatase inhibitors, various concentrations of calyculin A or okadaic acid were added to the reactivation medium. Addition of inhibitor 1, inhibitor 2 or *p*-nitrophenyl phosphate to demembranated spermatozoa was also performed. When Ca^{2+} concentrations in the reactivation medium were changed, appropriate proportions of EGTA and 1 mM $CaCl_2$ were added. Equilibrium constants for binding of Ca^{2+} and Mg^{2+} by ATP and EGTA were obtained from published work [24]. The reactivation medium, containing demembranated spermatozoa was incubated in a water bath at 40°C for 5 min. The suspension of demembranated spermatozoa was then placed into a microscope slide chamber (Sekisui Chemical Co., Ltd., UR-157 type, Tokyo, Japan) on a thermostatically-controlled warm plate, and the motility of spermatozoa was recorded by videomicroscopy (magnification on the 12-inch black and white monitor was approximately × 600) at 40°C [25]. Measurements were made on a total of 200–300 spermatozoa, distributed uniformly among the three or more fields, to determine the percent of motile spermatozoa.

2.5. Western immunoblot analysis of PP1

Washed demembranated spermatozoa described above were mixed with equal volumes of concentrated (two-fold) Laemmli sample buffer [26] and were boiled for 5 min. Samples containing approximately 15 μg protein were loaded on to 10% SDS-polyacrylamide slab gel, and electrophoresed. Western blotting was performed basically according to the protocol of Towbin et al. [27], but with some modifications. Briefly, the proteins were transferred electrophoretically to an polyvinylidene difluoride membrane sheet (Atto Co., Ltd., Tokyo, AE-6660). Following transfer, nonspecific sites on the membranes were blocked by incubating them for 1 h at room temperature (20–25°C) in phosphate-buffered saline (PBS) containing 5% skimmed milk powder. The blots were then incubated overnight at 4°C with antibody to PP1α (1:100 dilution with 1% BSA in PBS, 14 μg IgG/ml). For control, the blots were incubated in PBS containing 1% BSA alone. The blots were further incubated for 1 h at 37°C with biotinylated anti-rabbit immunoglobulins goat serum (1:100 dilution with 1% BSA in PBS, 14 μg immunoglobulins/ml) and then avidin–biotin peroxidase complex. Following each incubation, the membranes were rinsed extensively in PBS containing 0.05% Tween-20. Finally, 0.05% DAB/0.01% H_2O_2 solutions in 50 mM Tris-HCl buffer (pH 7.5) were applied to visualize the immunoreactive materials.

2.6. Statistical analysis

Statistical comparisons were performed using Student's *t*-test.

Table 1
Effects of protein phosphatase inhibitors and substrate on the motility of demembranated fowl spermatozoa at 40°C

Inhibitor	Concentration (μM)	Motility (%)
None (control)		0.0 ± 0.0[a]
Calyculin A	0.1	62.0 ± 4.0[b]
Okadaic acid	1.0	45.3 ± 6.2[b]
Inhibitor 1	2.0	61.3 ± 9.4[b]
Inhibitor 2	2.0	55.6 ± 2.7[b]
p-Nitrophenyl phosphate	10,000	64.4 ± 0.9[b]

Each value represents the mean (± S.E.M.) of five samples of spermatozoa. Values with different superscripts differ significantly ($P < 0.01$) from each other.

3. Results

3.1. Effects of protein phosphatases inhibitors on the motility of demembranated fowl spermatozoa

Reactivated movement of fowl spermatozoa without the addition of inhibitors was negligible at 40°C. In contrast, the presence of 1–1000 nM calyculin A, which is a specific inhibitor of PP1 and PP2A, permitted reactivation of sperm motility in a dose-dependent manner. Okadaic acid also stimulated the motility of demembranated spermatozoa, although calyculin A appeared to be more potent, stimulating motility to maximum of 72% at 100 nM, compared with 45% at 1000 nM for okadaic acid (Fig. 1). The addition of inhibitor 1 or inhibitor 2, specific heat-stable protein inhibitors for PP1, and *p*-nitrophenyl phosphate, a non-specific phosphatase substrate, also stimulated the motility of demembranated spermatozoa at 40°C (Table 1).

Demembranated spermatozoa, stimulated by calyculin A or okadaic acid at 40°C, lost their motility following the addition of 10^{-3} M Ca^{2+} to the medium. When the Ca^{2+} concentrations were varied from 10^{-8}–10^{-4} M by the subsequent addition of various concentrations of EGTA, the motility was restored in inverse proportion to the Ca^{2+}concentrations (Fig. 2A,B). In the presence of 2 μM inhibitor 1 or inhibitor 2, the motility of demembranated spermatozoa was also inhibited by the addition of 10^{-3} M Ca^{2+}, as well as those in calyculin A or okadaic acid. However, spermatozoa regained their motility at 10^{-8} M Ca^{2+} (60.2 ± 4.8% in inhibitor 1 treatment and 53.7 ± 3.5% in inhibitor 2 treatment, mean ± S.E.M. of five samples).

3.2. Immunoblot identification of PP1 in fowl spermatozoa

Non-specific bands of 30 kDa and 31 kDa proteins were detected in both control and anti-PP1α antibody-treated lanes. In addition, a 36–37 kDa protein was specifically recognized by the anti-PP1α antibody (denoted by arrow), whereas no appreciable immunoreactive band on this molecular weight was detected in the control (Fig. 3).

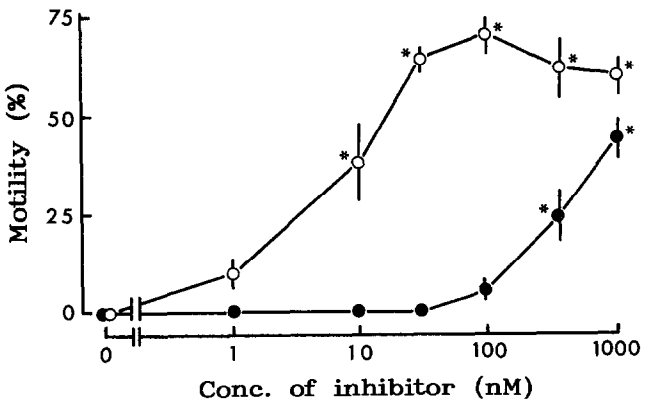

Fig. 1. The motility of demembranated fowl spermatozoa in the reactivation medium at 40°C after addition of various concentrations of okadaic acid (●) or calyculin A (○). Each point represents the mean (± S.E.M.) of five samples of spermatozoa. *$P < 0.01$ compared with value at 0 nM (control).

4. Discussion

It has been reported that the addition of a crude or partially purified protein phosphatase preparation from bovine cardiac muscle or rabbit skeletal muscle inhibits the motility of demembranated sea urchin spermatozoa, presumably due to its ability to cause dephosphorylation of axonemal proteins [28,29]. However, it has not been clear what kind of protein phosphatases are involved. Okadaic acid is a very potent inhibitor of PP1 and PP2A, two of the four major serine/threonine protein phosphatases. Of the other two, the Ca^{2+}/calmodulin-dependent protein phosphatase (PP2B) is far less sensitive to okadaic acid, whilst the Mg^{2+}-dependent protein phosphatase 2C (PP2C) is unaffected. In addition, neither protein tyrosine phosphatase, nor acid or alkaline phosphatases and neither cAMP or Ca^{2+}/calmodulin-dependent protein kinases, nor protein kinase C are inhibited by okadaic acid [30]. Calyculin A has a potency similar to that of okadaic acid as an inhibitor of PP2A, but is 10–100-fold more effective as an inhibitor of PP1 [31]. The present study showed that temperature-dependent immobilization of demembranated fowl spermatozoa at 40°C was reversed by the addition of okadaic acid or calyculin A, and that calyculin A was effective at 10–100-fold lower concentrations than okadaic acid. Furthermore, sperm motility was also stimulated by the addition of small heat-stable proteins inhibitors 1 and 2, which inhibit PP1 activity, but are insensitive to PP2A [22,32]. These findings strongly suggest that PP1, rather than PP2A, plays an important role in regulating fowl sperm movement at 40°C.

Two full-length clones encoding PP1 catalytic subunits have been isolated from a rabbit skeletal muscle cDNA library. One of them, termed PP1α, codes for a 330-residue 37.5 kDa protein [33]. A cDNA clone encoding PP1α was also isolated from a *Drosophila* head library and the deduced amino acid sequence (302 residues) showed 92% identity with PP1α from skeletal muscle, demonstrating that the structure of PP1 is remarkably conserved [34]. With regard to spermatozoa, from invertebrates to mammals, the presence of PP1 and its function in flagellar movement have not been determined previously. Thus, the data reported here represent the first description of the presence of PP1 in the sperm axoneme, since immunoblotting of sperm extract using an antibody to PP1α revealed a major cross-reacting protein of 36–37 kDa which corresponds to the molecular weight of the known catalytic subunit of PP1 (PP1α). In addition, immunogold particles which react with the anti-PP1α antibody were observed around/on the axoneme at the ultrastructural level (Ashizawa et al., unpublished observations). It is therefore proposed that an endogenous PP1 is present in the fowl sperm axoneme.

Although the above evidence clearly indicates the presence and function of PP1 in the fowl sperm axoneme, the further finding that Ca^{2+} inhibits the phosphatase-in-

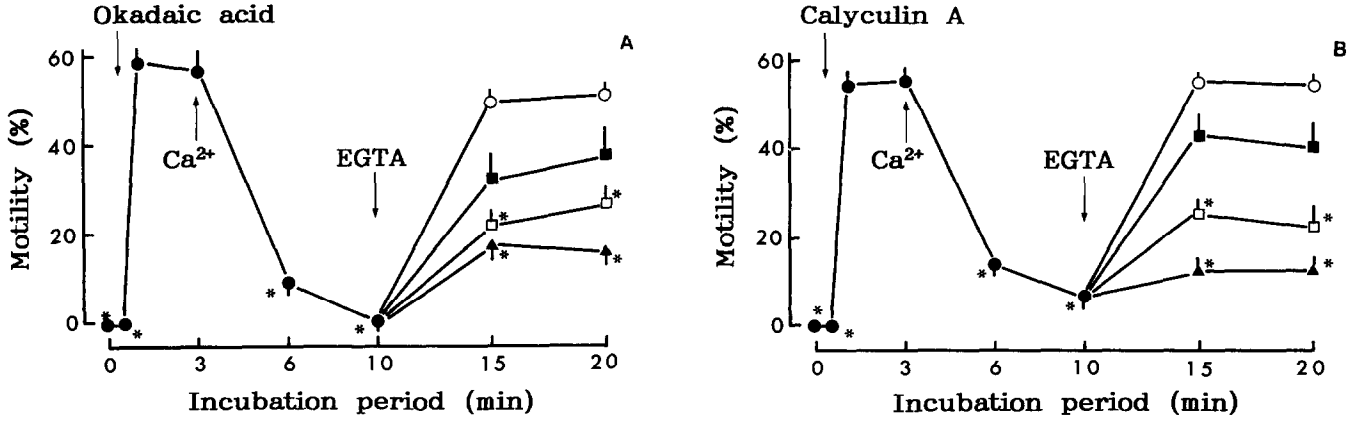

Fig. 2. The time course of motility of demembranated fowl spermatozoa in the reactivation medium at 40°C following addition of 1000 nM okadaic acid (A) or 100 nM calyculin A (B), 1 mM $CaCl_2$ and various concentrations of EGTA (pCa 4, ▲; 6, □; 7, ■; 8, ○). Each point represents the mean (± S.E.M.) of five examples of spermatozoa. *$P < 0.01$ compared with value at 3 min.

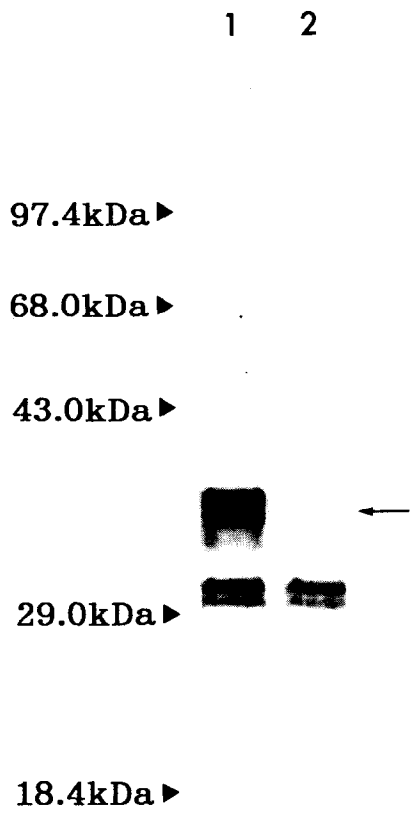

Fig. 3. The Immunoblot analysis of fowl sperm protein phosphatase type 1 catalytic subunit (PP1α). Lane 1: incubation with antibody against a synthetic 316–330 amino acid peptide of PP1α; lane 2: control (see section 2).

duced movement of demembranated fowl spermatozoa, and that this inhibition is released by EGTA, is at odds with the generally-accepted idea that PP1 is active towards most substrates in the absence of divalent cations such as Ca^{2+} [34]. Indeed PP2B is the only protein phosphatase known to have a requirement for Ca^{2+} and this enzyme has been suggested to play a major role in the Ca^{2+}-dependent regulation of dog sperm motility [23]. However, since the inhibitor responsiveness and immunological specificity of the fowl axonemal phosphatase is clearly that of PP1, we may assume that either this phosphatase is unusual in its Ca^{2+} requirement, or that the Ca^{2+} acts at a location 'downstream' of the activity of PP1.

According to a recent hypothesis on the mechanisms of smooth muscle contraction, myosin light chain phosphatase (MLCP), which is regarded as PP1 [34], rather than myosin light chain kinase (MLCK), may be involved more actively in the regulation of contraction [35]: that is, inactivation of an endogenous protein phosphatase inhibitor, such as inhibitor 1, perhaps via dephosphorylation by PP2B, results in increased MLCP activity and a phasic reduction in force. Conversely, activation of the inhibitor by phosphorylation inhibits the MLCP activity and causes potentiation of the force response to Ca^{2+}. Presumably, similar mechanisms may be present in the reversible inactivation of fowl spermatozoa at 40°C, although the precise mechanisms of action of Ca^{2+} in the regulation of sperm motility remain to be elucidated. Furthermore, an important point to be established is the target of the action of PP1 in the axoneme.

Acknowledgements: We thank Professor Philip Cohen, University of Dundee, UK, for a generous gift of inhibitors 1 and 2. G. J. Wishart was supported by the Royal Society of Edinburgh, U.K. This study was supported by a grant from the Ministry of Education, Science and Culture, Japan (No. 06660360).

References

[1] Tash, J.S. and Means, A.R. (1983) Biol. Reprod. 28, 75–104.
[2] Lindemann, C.B. and Kanous, K.S. (1989) Arch. Androl. 23, 1–22.
[3] Mohri, H. (1993) Mol. Reprod. Dev. 36, 221–223.
[4] Munro, S.S. (1938) Q. J. Exp. Physiol. 27, 281–287.
[5] Ashizawa, K. and Nishiyama, H. (1978) Jpn. Poult. Sci. 15, 264–266.
[6] Ashizawa, K. and Wishart, G.J. (1987) J. Reprod. Fertil. 81, 495–499.
[7] Wishart, G.J. and Ashizawa, K. (1987) J. Reprod. Fertil. 80, 607–611.
[8] Thomson, M.F. and Wishart, G.J. (1989) Br. Poult. Sci. 30, 687–692.
[9] Thomson, M.F. and Wishart, G.J. (1991) J. Reprod. Fertil. 93, 385–391.
[10] Ashizawa, K., Maeda, S. and Okauchi, K. (1989) J. Reprod. Fertil. 86, 271–276.
[11] Ashizawa, K., Suzuki, Y. and Okauchi, K. (1989) J. Reprod. Fertil. 86, 263–270.
[12] Brokaw, C.J. (1987) J. Cell. Biochem. 35, 175–184.
[13] Majumder, G.C., Dey, C.S., Haldar, S. and Barua, M. (1990) Arch. Androl. 24, 287–303.
[14] Morisawa, M. and Hayashi, H. (1985) Biomedical Res. 6, 181–184.
[15] Brandt, H. and Hoskins, D.D. (1980) J. Biol. Chem. 255, 982–987.
[16] Tash, J.S., Kakar, S.S. and Means, A.R. (1984) Cell 38, 551–559.
[17] Tash, J.S., Hidaka, H. and Means, A.R. (1986) J. Cell Biol. 103, 649–655.
[18] Huacuja, L., Delgado, N.M., Merchant, H., Pancardo, R.M. and Rosado, A. (1977) Biol. Reprod. 17, 89–96.
[19] Chulavatnatol, M., Panyim, S. and Wititsuwannkul, D. (1982) Biol. Reprod. 26, 197–207.
[20] Tash, J.S. and Means, A.R. (1982) Biol. Reprod. 26, 745–763.
[21] Ashizawa, K., Katayama, S. and Tsuzuki, Y. (1992) Biochem. Biophys. Res. Commun. 185, 740–745.
[22] Ingebritsen, T.S. and Cohen, P. (1983) Science 221, 331–338.
[23] Tash, J.S., Krinks, M., Patel, J., Means, R.L., Klee, C.B. and Means, A.R. (1988) J. Cell Biol. 106, 1625–1633.
[24] Sillèn, L.G. and Martell, A.E. (1971) Stability Constants of Metal Ion Complexes, 2nd edition, Burlington House, London.
[25] Katz, D.F. and Overstreet, J.W. (1981) Fertil. Steril. 35, 188–193.
[26] Laemmli, U.K. (1970) Nature 227, 680–685.
[27] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.

[28] Takahashi, D., Murofushi, H., Ishiguro, K., Ikeda, J. and Sakai, H. (1985) Cell Struct. Funct. 10, 327–337.
[29] Brokaw, C.J. (1987) J. Cell Biol. 105, 1789–1798.
[30] Cohen, P., Holmes, C.F.B. and Tsukitani, Y. (1990) Trends Biochem. Sci. 15, 98–102.
[31] Ishihara, H., Martin, B.L., Brautigan, D.L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, K., Uemura, D. and Hartshorne, D.J. (1989) Biochem. Biophys. Res. Commun. 159, 871–877.
[32] Ingebritsen, T.S. and Cohen, P. (1983) Eur. J. Biochem. 132, 255–261.
[33] Cohen, P.T.W. (1988) FEBS Lett. 232, 17–23.
[34] Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508.
[35] Somlyo, A.P., Kitazawa, T., Himpens, B., Matthijs, G., Horiuti, K., Kobayashi, S., Goldman, Y.E. and Somlyo, A.V. (1989) in: Advances in Protein Phosphatases (Merlevede, W. and Di Salvo, J. Eds.) vol. 5, pp. 181–195, Leuven University Press, Leuven.