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Involvement of *rho* in GTPγS-induced enhancement of phosphorylation of 20 kDa myosin light chain in vascular smooth muscle cells: inhibition of phosphatase activity

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Abstract In β-escin-permeabilized cultured pig aortic smooth muscle cells GTPγS dose-dependently enhances Ca^{2+}-induced, wortmannin-sensitive phosphorylation of 20 kDa myosin light chain (MLC_{20}). GTPγS does not potentiate thiophosphorylation of MLC_{20}, but does inhibit its dephosphorylation. Pretreatment with *C. botulinum* exotoxin C_3, which specifically ADP-ribosylates and inactivates the *rho* family of the small molecular weight G proteins, completely abolishes the effects of GTPγS. These results indicate that *rho* is involved in the GTPγS-induced enhancement of Ca^{2+}-dependent MLC_{20} phosphorylation in aortic smooth muscle cells, and strongly suggest that this effect of *rho* is due to inhibition of protein phosphatase activity toward MLC_{20}.

Key words: Rho; Myosin light chain phosphatase; GTPγS; Smooth muscle

1. Introduction

Vasoconstrictor stimulation of smooth muscle induces a rise in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and activation of the Ca^{2+}, calmodulin-dependent enzyme, myosin light chain kinase (MLCK) [1,2]. It is now generally accepted that the resultant phosphorylation of the 20 kDa myosin light chain (MLC_{20}) leads to the interaction of actin with myosin, triggering a contractile response [2]. Recent studies have shown that the $[Ca^{2+}]_i$ is not the sole determinant for the extent of phosphorylation of MLC_{20} achieved by agonist stimulation [3–7]. Thus, as compared to KCl depolarization, excitatory agonist consistently produces larger increases in both MLC_{20} phosphorylation and tension at a given level of the $[Ca^{2+}]_i$, implying that agonists somehow enhance the Ca^{2+} sensitivity of both MLC_{20} phosphorylation and contraction [3,4,6]. Indeed, it has recently been demonstrated in skinned smooth muscle fibers that, at a fixed level of $[Ca^{2+}]_i$, an agonist causes increases in the phosphorylation level of MLC_{20} and tension in a GTP-dependent manner, providing evidence for the involvement of a GTP binding protein (G protein) in receptor-mediated sensitization of Ca^{2+}-dependent MLC_{20} phosphorylation [5,7,8]. To investigate in depth the molecular mechanism of G protein-induced potentiation of MLC_{20} phosphorylation, we have developed a useful cultured cell system of vascular smooth muscle, in which GTPγS potentiates Ca^{2+}-dependent phosphorylation of MLC_{20}. By using the bacterial exoenzyme C_3 which ADP-ribosylates and inactivates *rho* proteins [9,10], we have found that the small molecular weight G protein *rho* mediates GTPγS enhancement of Ca^{2+}-dependent MLC_{20} phosphorylation, and that *rho* acts to inhibit MLC_{20} phosphatase activity.

2. Materials and methods

2.1. Cell culture, permeabilization and determination of Ca^{2+}-dependent MLC_{20} phosphorylation

Pig aortic smooth muscle cells were obtained by the explant method [11] and were used between the 5th and the 15th passages. Before each experiment, confluent cells were deprived of serum for 24 h. Cells were washed with Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS) and permeabilized with 20 μM of β-escin in buffer A comprising 130 mM potassium glutamate, 4 mM $MgCl_2$, 2 mM EGTA, 2 mM ATP and 20 mM HEPES (pH 7.2) at 28°C for 10 min. After washing with buffer A without β-escin, phosphorylation of cellular myosin was started by adding buffer A containing 0.3 μM bovine calmodulin and various concentrations of $CaCl_2$ to give a desired concentration of free Ca^{2+}, with or without guanosine 5′(3-*O*-thio)triphosphate (GTPγS) or other nucleotides (phosphorylation buffer). Where indicated, permeabilized cells were pretreated with or without C_3 in the presence of NAD as described below and then phosphorylation of cellular myosin was started. Free Ca^{2+} concentrations were calculated as described previously [12]. Under this experimental condition phosphorylation levels of MLC_{20} were found to be maintained for at least 60 min. The inclusion of 1 μM ionomycin in both the permeabilization and the phosphorylation buffers did not alter the results. The extent of MLC_{20} phosphorylation was determined by glycerol–urea gel electrophoresis followed by quantitation of the relative amounts of non-phosphorylated and phosphorylated forms of MLC_{20} with Western blot technique [13]. Densities of bands corresponding to non-phosphorylated, monophosphorylated and diphosphorylated forms of MLC_{20} were quantitated by using a scanning densitometer (The Discovery Series, PDI). Percent values of monophosphorylated form, diphosphorylated form and the sum of both forms of total MLC_{20} were calculated.

2.2. ADP-ribosylation of endogenous rho protein

Permeabilized cells were preincubated in buffer A containing 10 μM NAD with or without 1 μg/ml C_3 at 28°C for 20 min. After a wash with buffer A, cells were then incubated in buffer A containing 10 μM ^{32}P-NAD (Dupont-NEN) in the presence or absence of 1 μg/ml C_3 at 28°C for 20 min. Cellular proteins were separated on SDS-polyacrylamide gel electrophoresis (15%), followed by autoradiography. C_3 exoenzyme was purified from culture supernatant of *Clostridium botulinum* type D South African (DSA) as described previously [14].

2.3. Dephosphorylation experiment of phosphorylated MLC_{20}

Permeabilized cells were pretreated with 1 μg/ml C_3 or left untreated for 25 min and then exposed to the phosphorylation buffer containing 0.1 μM free Ca^{2+} with or without 30 μM GTPγS for 15 min. After washing once with the Ca^{2+}-free phosphorylation buffer containing

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2 mM EGTA, cells were incubated in dephosphorylation buffer of the same composition containing 50 μM wortmannin for indicated time periods and the phosphorylation level of MLC_{20} was determined as described above. Values were expressed as percent of the initial MLC_{20} phosphorylation level at time zero.

2.4. Materials

GTPγS, guanosine 5′*O*-(β-thio)diphosphate (GDPβS) and other nucleotides were purchased from Boehringer-Mannheim. β-Escin, bovine calmodulin, NAD and anti-MLC_{20} antibody were purchased from Sigma. Biotinylated secondary antibody and streptoavidin-conjugated alkaline phosphatase were bought from Zymed. Anti-*rho*A antibody was purchased from Santa Cruz.

3. Results

In β-escin-permeabilized pig aortic smooth muscle cells, raising the ambient free Ca^{2+} concentration causes a dose-dependent increase in the extent of MLC_{20} phosphorylation (Fig. 1). The addition of the MLCK inhibitor wortmannin [15] completely inhibits Ca^{2+}-induced MLC_{20} phosphorylation, suggesting that Ca^{2+}-induced phosphorylation of MLC_{20} is mediated by MLCK. The addition of GTPγS (30 μM) potently enhances Ca^{2+}-induced MLC_{20} phosphorylation: GTPγS causes an increase in the level of monophosphorylated MLC_{20} and appearance of diphosphorylated MLC_{20} (see Figs. 1 and 2A). GTPγS shifts the Ca^{2+}-MLC_{20} phosphorylation curve to the left by approximately one order of magnitude and also slightly increases the maximal level of MLC_{20} phosphorylation. The addition of 100 μM of GTP also causes an increase in MLC_{20} phosphorylation at 0.1 μM Ca^{2+}, although to a lesser extent as compared to 30 μM of GTPγS. Thus, the non-hydrolyzable GTP analogue GTPγS appears to be more potent than GTP. In contrast, both CTP and UTP at 100 μM are totally ineffective. Either GDP or GDPβS has no effect on Ca^{2+}-induced MLC_{20} phosphorylation. When GDPβS (1 mM) is added before GTP or GTPγS, it completely inhibits the enhancing effects of these guanine nucleotides.

Western blot analysis using anti-*rho*A antibody demonstrates that aortic smooth muscle cells contain abundant *rho* proteins

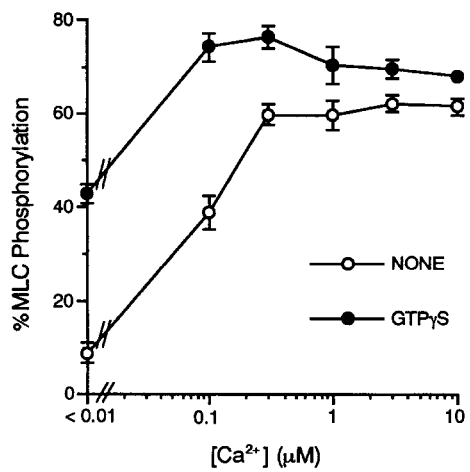

Fig. 1. Ca^{2+}-MLC_{20} phosphorylation relationship in the presence and absence of GTPγS in β-escin-permeabilized pig aortic smooth muscle cells. Permeabilized cells were incubated in various concentrations of free Ca^{2+} with or without 30 μM GTPγS for 15 min. The level of MLC_{20} phosphorylation was determined as described in section 2. Each value represents the mean ± S.E.M. of four determinations.

and that the permeabilization procedure does not cause any detectable loss of *rho* proteins out of cells. In order to examine the role of *rho* in GTPγS-induced enhancement of MLC_{20} phosphorylation, we investigated the effect of the botulinum exoenzyme C_3 [9,10]. As shown in Fig. 2A and B, pretreatment of permeabilized cells with C_3 (1.0 μg/ml) for 20 min completely inhibits GTPγS enhancement of Ca^{2+}-induced MLC_{20} phosphorylation. In the absence of GTPγS, C_3 is without effect on Ca^{2+}-induced phosphorylation of MLC_{20}. The effectiveness of the C_3 activity in permeabilized cells is proven by the fact shown in Fig. 2C that a protein with apparent M_r of 23 kDa in permeabilized cells is fully ADP-ribosylated by the incubation with C_3 and [^{32}P]NAD. Thus, when cells are first pretreated with C_3 and non-radiolabeled NAD for 20 min, the following second incubation with C_3 and [^{32}P]NAD does not cause any further ADP-ribosylation of the 23 kDa protein, indicating that the first incubation with C_3 causes full ADP-ribosylation of *rho*. It has been reported that ADP-ribosylation of purified 21 kDa *rho* protein causes decreased mobility on electrophoresis [16].

GTPγS-induced enhancement of MLC_{20} phosphorylation could result from GTPγS-mediated potentiation of MLCK activity, or alternatively, inhibition of protein phosphatase activity toward MLC_{20}. To discriminate between the two possibilities, we conducted following experiments. First, we studied whether GTPγS increases thiophosphorylation of MLC_{20}. Thiophosphorylated MLC_{20} is a very poor substrate for phosphatase [17]. As shown in Fig. 3A, when ATP is included as substrate in the absence of GTPγS, the addition of 0.1 μM Ca^{2+} causes a rapid increase in the phosphorylation level of MLC_{20} up to about 50% of total MLC_{20} by 2 min, which is maintained for 10 min. In the presence of GTPγS, the extent of MLC_{20} phosphorylation is further potentiated at every time point examined. When ATPγS is used as substrate in stead of ATP (Fig. 3B), the level of MLC_{20} thiophosphorylation continues to rise for up to 10 min and reaches higher levels than those achieved with ATP (compare Figs. 3A and B). Importantly, GTPγS does not cause any further increase in the thiophosphorylation level of MLC_{20} at any time point. These results indicate that GTPγS does not potentiate the activity of MLCK.

In the second set of experiments, we examined whether GTPγS affects dephosphorylation of phosphorylated MLC_{20} and, if so, whether C_3 pretreatment inhibits the effect of GTPγS on MLC_{20} dephosphorylation in permeabilized cells. After cells were preincubated with or without C_3, MLC_{20} was made phosphorylated by introducing Ca^{2+} (0.1 μM) alone or together with GTPγS (30 μM). Cells were then transferred to the dephosphorylation buffer containing 2 mM EGTA and the MLCK inhibitor wortmannin, and MLC_{20} dephosphorylation started. As shown in Fig. 4, in control cells without C_3 pretreatment the phosphorylation level of MLC_{20} rapidly falls down to 33% of the initial level by 4 min. GTPγS potently inhibits dephosphorylation of MLC_{20}, and the phosphorylation level at 4 min is maintained at 53% of the initial level. C_3 pretreatment completely abolishes this effect of GTPγS and causes the MLC_{20} phosphorylation level to decrease to the level of the no treatment group. The MLC_{20} phosphorylation level is similar between the no treatment group and the C_3 plus GTPγS group except at the time point of 4 min, where the difference between both groups is significant ($P<0.05$). These results strongly suggest that GTPγS inhibits the MLC_{20} phosphatase activity and that this action of GTPγS is mediated by *rho*.

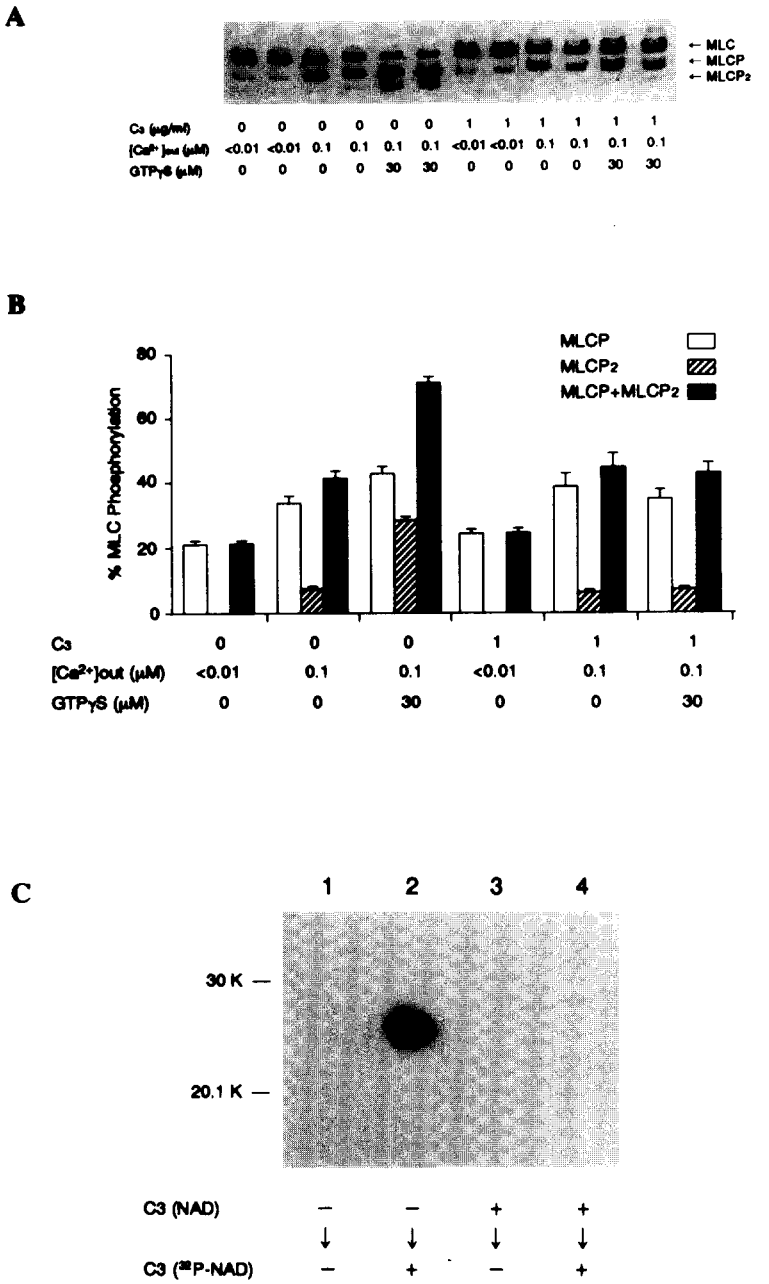

Fig. 2. Inhibition by botulinum exoenzyme C_3 of GTPγS-induced enhancement of MLC_{20} phosphorylation. Permeabilized cells were pretreated with C_3 (1 μg/ml) for 20 min or left untreated, and then incubated in 0.1 μM Ca^{2+} with or without 30 μM GTPγS for 15 min. The level of MLC_{20} phosphorylation was determined as described in section 2. MLCP and $MLCP_2$, monophosphorylated and diphosphorylated forms of MLC_{20}. (A) Representative Western blot. (B) Quantitative summary of MLCP level, $MLCP_2$ level and the sum of MLCP level plus $MLCP_2$ level. C_3 treatment causes complete inhibition of GTPγS-induced enhancement of MLC_{20} phosphorylation. Each value represents the mean ± S.E.M. of four to six determinations. (C) Validity of C_3 treatment was confirmed in parallel dishes, in which treatment with C_3 (1 μg/ml) for 20 min had achieved the full extent of ADP-ribosylation of an endogenous protein of approximately 23 kDa. See text for detail.

4. Discussion

The results in the present study demonstrate that in permeabilized pig aortic smooth muscle cells the botulinum exoenzyme C_3, which is known to specifically ADP-ribosylate and to inactivate members of the *rho* family [9,10], completely abolishes enhancement by GTPγS of Ca^{2+}-induced MLC_{20} phosphorylation. Under the same experimental condition C_3 causes complete ADP-ribosylation of an endogenous 23 kDa protein in permeabilized cells. These results strongly suggest that *rho* is involved in GTPγS-induced Ca^{2+} sensitization of MLC_{20} phosphorylation in vascular smooth muscle cells. In accordance with previous reports for skinned vascular smooth muscle fiber and tracheal smooth muscle homogenates [18,19], GTPγS causes inhibition of dephosphorylation of phosphorylated MLC_{20}, but does not potentiate thiophosphorylation of MLC_{20} in β-escin permeabilized cells. The present study further shows that this GTPγS effect on dephosphorylation of MLC_{20} is to-

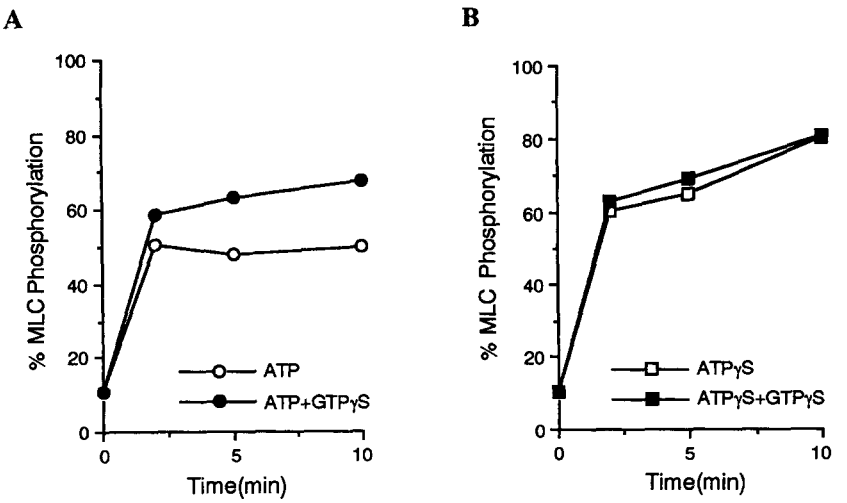

Fig. 3. GTPγS does not potentiate thiophosphorylation of MLC_{20}. Permeabilized cells were incubated in 0.1 μM Ca^{2+} in the presence of 2 mM ATP (A) or ATPγS (B) with or without 30 μM GTPγS for indicated time periods. The level of MLC_{20} phosphorylation was determined as described in section 2. Each value is the mean of duplicate determinations and representative of two separate experiments.

tally abolished in C_3-pretreated permeabilized cells. These results suggest that the site of action of *rho* resides in regulation of protein phosphatase activity toward MLC_{20}. Our results, together with a previous report of skinned smooth muscle that *rho* is involved in GTPγS enhancement of Ca^{2+}-induced contraction [20], strongly suggest that *rho* plays an important regulatory role in agonist-induced MLC_{20} phosphorylation and contraction.

It was recently demonstrated that the major smooth muscle myosin phosphatase belongs to protein phosphatase 1 (PP1) and is composed of three subunits, a 37 kDa catalytic subunit (PP1 isozyme) and 110 kDa and 20 kDa regulatory subunits [21]. It was also demonstrated that the regulatory components function to target the catalytic subunit of the smooth muscle protein phosphatase to myosin filament and to increase the phosphatase activity toward myosin. In liver and skeletal muscle, PP1 with the subunit structure similar to the smooth muscle myosin-associated phosphatase is found to associate with glycogen particles, where it regulates activities of enzymes involved in glycogen metabolism through dephosphorylation [22]. It is known for the glycogen particle-associated PP1 that the activity of the catalytic subunit is regulated through covalent modification of the regulatory subunit and interaction with allosteric effectors [22,23]. For example, adrenalin induces activation of cyclic AMP-dependent protein kinase, which phosphorylates the regulatory subunit of glycogen-associated PP1 and inhibits the phosphatase activity. Hepatic glycogen-associated PP1 is also inhibited allosterically by the phosphorylated form of phosphorylase. By analogy with this, it is an intriguing possibility that activated *rho* inhibits the smooth muscle myosin-associated PP1 through a change in phosphorylation state of the regulatory subunit. In this regard, it should be noted that *rac*, a small molecular weight G protein closely related to *rho*, activates a cytosolic serine/threonine protein kinase [24]. Alternatively, it might be possible that a complex of activated *rho* and its downstream effector induces a change in the phosphatase activity by allosteric interaction with the regulatory subunit.

In a number of smooth muscle types excitatory agonists bind to a rhodopsin type of membrane receptors and activate effector molecules such as phospholipase C and phospholipase A_2 via heterotrimeric G proteins. However, the mechanisms by which this class of agonists activate low molecular weight G protein(s) are not well understood. There are several known regulatory molecules for the *rho* family members including smgGDS, *rho*GDS, *rho*GDI and *rho*GAP [25,26]. An agonist may alter activities of these regulatory molecules through activation of a heterotrimeric G protein to bring about a change in the *rho* activity. It remains to be clarified whether and how an agonist exactly activates the *rho* family G proteins. We expect that the cultured smooth muscle cell system we have

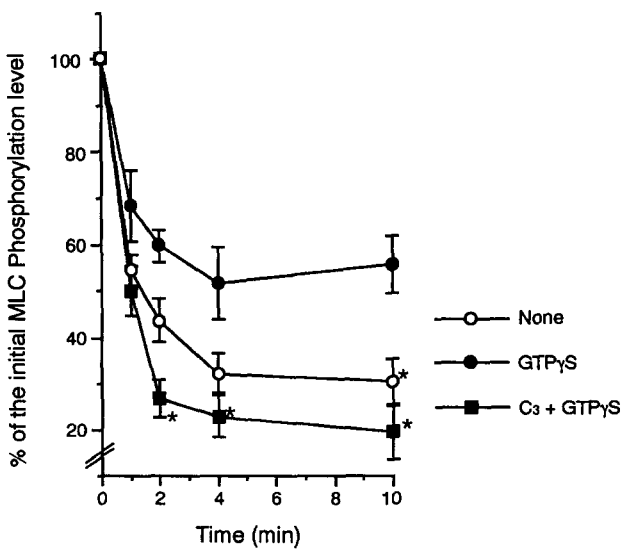

Fig. 4. GTPγS inhibits dephosphorylation of MLC_{20} in a C_3-sensitive manner. Permeabilized cells which had been pretreated with C_3 (1 μg/ml) or left untreated were incubated in the dephosphorylation buffer for indicated time periods. The level of MLC_{20} phosphorylation was determined as described in section 2, and the values were expressed as percent of the initial MLC_{20} phosphorylation level at time zero. Each value represents the mean ± S.E.M. of four determinations. The asterisk (*) denotes a significant ($P<0.05$) difference as compared with the values of the 'GTPγS' group.

developed would provide a useful model for further biochemical investigation on the role of *rho* in the regulation of MLC_{20} phosphorylation.

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References

[1] Rasmussen, H., Takuwa, Y. and Park, S. (1987) FASEB J. 1, 177–185.
[2] Kamm, K.E. and Stull, J.T. (1985) Annu. Rev. Pharmacol. Toxicol. 25, 593–620.
[3] Rembold, C.M. and Murphy, R.A (1988) Circ. Res. 63, 593–603.
[4] Jiang, M.J. and Morgan, K.G. (1989) Pflügers Arch 413, 637–643.
[5] Kitazawa, T., Gaylinn, B.D., Denney, G.H. and Somlyo, A.P. (1991) J. Biol. Chem. 266, 1708–1715.
[6] Tang, D.C., Stull, J.T., Kubota, Y. and Kamm, K.E. (1992) J. Biol. Chem. 267, 11839–11845.
[7] Moreland, S., Nishimura, J., van Breemen, C., Ahn, H.Y., and Moreland, R.S. (1992) Am. J. Physiol. 263, C540–C544.
[8] Kitazawa, T. and Somlyo, A.P. (1990) Biochem. Biophys. Res. Commun. 172, 1291–1297.
[9] Kikuchi, A., Yamamoto, K., Fujita, T. and Takai, Y. (1988) J. Biol. Chem. 263, 16303–16308.
[10] Narumiya, S., Sekine, A. and Fujiwara, M. (1988) J. Biol. Chem. 263, 17255–17257.
[11] Chamley-Campbell, J., Campbell, G.R. and Ross, R. (1979) Physiol. Rev. 59, 1–61.
[12] Waisman, D.M., Gimble, J.M., Goodman, D.B.P. and Rasmussen, H. (1981) J. Biol. Chem. 267, 409–414.
[13] Hathaway, D.R. and Haeberle, J.R. (1985) Am. J. Physiol. 249, C345–C351.
[14] Moriishi, K., Syuto, B., Yokosawa, N., Ogura, K. and Saito, M. (1991) J. Bacteriol. 173, 6025–6029.
[15] Nakanishi, S., Kakita, S., Takahashi, I., Kawahara, K., Tsukuda, E., Sano, T., Yamada, K., Yoshida, M., Kase, H., Matsuda, Y., Hashimoto, Y. and Nonomura, Y. (1992) J. Biol. Chem. 267, 2157–2163.
[16] Morii, N., Sekine, A., Ohashi, Y., Nakao, K., Imura, H., Fujiwara, M. and Narumiya, S. (1988) J. Biol. Chem. 263, 12420–12426.
[17] Cassidy, P., Hoar, P. E. and Kerrick, W.G.L. (1979) J. Biol. Chem. 254, 11148–11153.
[18] Kitazawa, T., Masuo, M. and Somlyo, A.P. (1991) Proc. Natl. Acad, Sci. USA 88, 9307–9310.
[19] Kubota, Y., Nomura, M., Kamm, K.E., Mumby, M.C. and Stull, J.T. (1992) Am. J. Physiol. 262, C405–C-410.
[20] Hirata, K., Kikuchi, A., Sasaki, T., Kuroda, S., Kaibuchi, K., Matsuura, Y., Seki, H., Saida, K. and Takai, Y. (1992) J. Biol. Chem. 267, 8719–8722.
[21] Alesso, D., Macdougall, L.K., Sola, M.M., Ikebe, M. and Cohen, P. (1992) Eur. J. Biochem. 210, 1023–1035.
[22] Hubbard, M.J. and Cohen, P. (1993) Trend Biochem. Sci. 18, 172–177.
[23] Hubbard, M.J. and Cohen, P. (1989) Eur. J. Biochem. 186, 701–709.
[24] Manser, E., Leuing, T., Salihuddin, H., Zhao, Z. and Lim, L. (1994) Nature 367, 40–46.
[25] Takai, Y., Kaibuchi, K., Kikuchi, A. and Kawata, M. (1992) Int. Rev. Cytol. 133, 587–654.
[26] Boguski, M.S. and McCormick, F. (1993) Nature 366, 643–654.