Volume 333, number 1,2, 56–60 FEBS 13140 October 1993

rab3-Peptide stimulates exocytosis from mast cells via a pertussis toxin-sensitive mechanism

G.J. Law*, A.J. Northrop, W.T. Mason

Laboratory of Neural and Secretory Signalling, Department of Neurobiology, AFRC, Babraham Institute, Babraham, Cambridge CB2 4AT, UK

Received 8 September 1993

Synthetic peptides of the putative effector domain of members of the *rab3* gene family of small GTP-binding proteins have been shown to have potent actions on vesicular transport and exocytosis [1,2]. Here, we use similar rab3-effector domain peptides to study their role in intracellular signalling in mast cells. We find that rab3-like peptides stimulate exocytosis and decrease cyclic 3′,5′-AMP levels in these cells when applied extracellularly. Cells pretreated with pertussis toxin (PtX) to selectively uncouple α_i/α_o type G proteins from their biological activators, however, did not respond to rab3 peptides. rab3-like peptides also induce a Ca^{2+} transient in mast cells. These observations provide evidence for functional coupling between an effector domain peptide sequence of rab3 protein and a PtX-sensitive G protein substrate.

Exocytosis; rab3; GTP-binding protein; Pertussis toxin; Mast cell

1. INTRODUCTION

Synthetic peptides corresponding to a putative effector domain of the small GTP binding protein rab3 have been shown to stimulate exocytosis from chemically permeabilised pancreatic acinar cells [3], adrenal chromaffin cells [4], patch-clamped mast cells [2]; and in a cell-free assay system between pancreatic zymogen granules and plasma membrane [5]. How these peptides stimulate exocytosis is unknown but they are commonly used as biochemical tools to mimic the role of rab3 protein inside cells [1–4]. We report here that rab3-like peptides stimulate exocytosis from mast cells via a pertussis toxin (PtX) sensitive mechanism.

2. MATERIALS AND METHODS

Peptides were synthesised on a SMPS-A350 multiple peptide synthesis instrument utilising Fmoc chemistry (Zinsser Analytic, Maidenhead, UK). Purity was checked on reverse phase C18, HPLC (Waters, Milford, USA). Sequence analysis was performed by Edman degradation on an automated gas phase protein sequencer (Applied Biosystems, Warrington, UK). Mastoparan (*Vespula lewisii*) and pertussis toxin were purchased from Sigma Chemical Company, UK. Guanosine-5′-*O*-(3-thiotriphosphate) (GTPγs) was obtained from Boehringer Mannheim, UK. Streptolysin-O (SLO) was ordered from Wellcome Research, UK.

Rat peritoneal mast cells were prepared and purified by published procedures [6]. Experiments were performed at 30°C in the following buffer: 133 mM NaCl, 5 mM KCl, 0.9 mM NaH_2PO_4, 8 mM glucose, 1 g/l BSA, 10 μM $CaCl_2$, 20 mM HEPES at pH 7.4 set with NaOH. Secretory output of cell populations was determined by a sensitive assay for hexosaminidase enzyme activity [6]. This assay requires a fluorimetric substrate known as 4-methyl-umbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside obtained from Koch Light Laboratories, UK. Secretory output was also measured for histamine and heparin (data not shown); all three assays have reported similar qualitative changes in this work. Cellular cyclic 3′,5′ AMP concentrations were measured with a kit obtained from Amersham, UK. Changes in intracellular free calcium ion concentration $[Ca^{2+}]$ were calculated from fura2-loaded mast cells using quantitative fluorescence ratio-imaging as described in [7].

*Corresponding author. Fax: (44) (223) 836614.

3. RESULTS AND DISCUSSION

3.1. *rab3-Peptides stimulate release from intact mast cells*

Our initial experiments sought to confirm previous findings that the authentic peptide sequence of rab3 (VSTVGIDFKVKTIYRN), and a more potent two-amino acid substituted derivative rab3AL (VSALGIDFKVKTIYRN), induced secretion from streptolysin O (SLO)-permeabilised cells [3,4]. We unexpectedly found that rab3AL stimulated secretory output from intact mast cells and that this effect was attenuated upon cell permeabilisation (Fig. 1). This finding was not due to the lack of ability of permeabilised cells to respond because an increase of 750% was observed with 10 μM GTPγs (Fig. 1). rab3-like peptides behave like other polybasic compounds such as 48/80 and mastoparan which trigger degranulation from intact mast cells but are less effective upon cell permeabilisation [8–10].

3.2. *Stimulation of exocytosis is a specific action of rab3-like peptides*

To demonstrate that stimulation of secretion by a rab3-like peptide was a specific effect, we synthesised different peptides to equivalent regions of other small

GTP binding proteins, and to different regions of the rab3 class of proteins, and applied these to intact cells. We tested those peptides which have been previously used to show specificity of rab3 peptide [1]. Even when tested at the high concentration of 100 μM, only authentic rab3 and rab3AL were found to stimulate significant release of hexosaminidase (Fig. 2). rab3 had 60% of the activity associated with rab3AL, in agreement with previous work [1,3]. Stimulation of release appeared to be specific to this region of rab3 because the corresponding aligned peptide sequences of rab2 and arf, and C-terminal domains for rab3a and rab3b, were without effect. A 5-mer peptide (VSALG) corresponding to the N-terminal end of rab3AL also had no effect (Fig. 2); and no response was found at a higher concentration of 400 μM (data not shown). These results were obtained by measuring the effects of various peptides on hexosaminidase secretion from a population of mast cells but a similar profile of responses was also found for release of heparin and histamine (data not shown). Authentic rab3-peptide gives essentially the same experimental data as rab3AL but is less pronounced. For the purpose of clarity and brevity we only present data here for rab3AL.

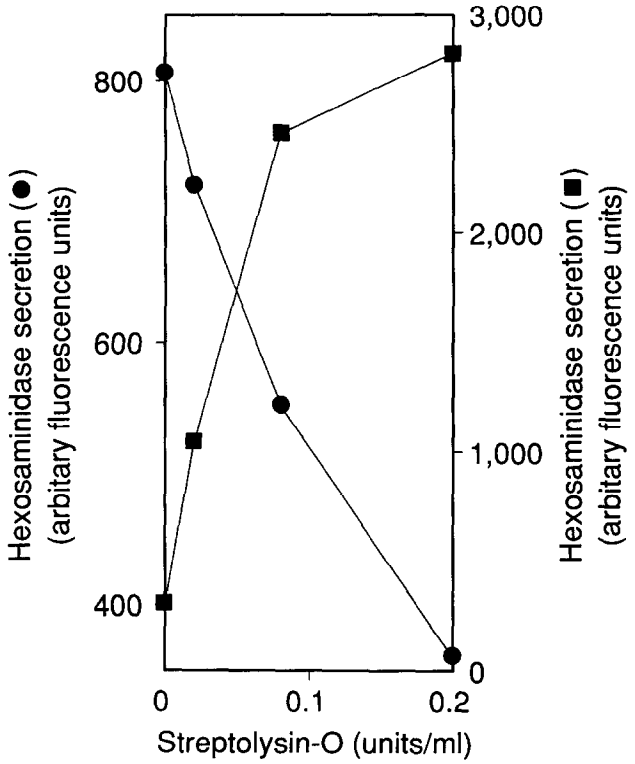

Fig. 1. Effect of rab3AL peptide, or GTPγs, on secretion from mast cells exposed to various concentrations of streptolysin-O. A typical response to 100 μM rab3AL, or 10 μM GTPγs. SLO was added for 5 min prior to addition of peptide, or GTPγs; 10 min later cells were pelleted and supernatant was assayed for release of hexosaminidase [6]. Similar responses have been found in 3 other experiments.

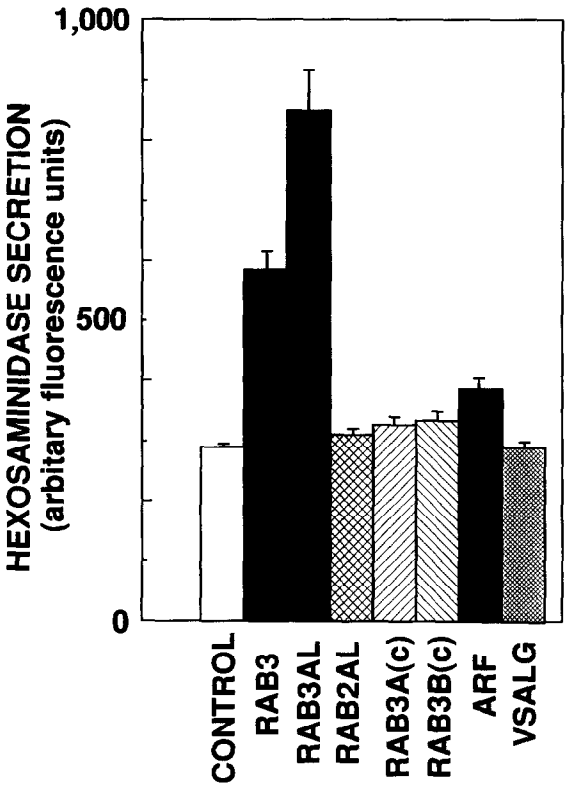

Fig. 2. Effect of peptides derived from small GTP binding on hexosaminidase release from intact mast cells. Each peptide was added at 100 μM and their effect over 30 min on secretory output determined. For peptide sequences see [5]. ARF is a peptide sequence derived from ADP-ribosylation factor another small GTP-binding protein. VSALG is derived from the N-terminal end of rab3AL. Data is shown as the mean value ± S.E.M. of a typical result of one of 4 separate experiments.

3.3. *rab3AL stimulates exocytosis by activating a PtX-sensitive G protein*

To investigate whether a G protein might transduce the rab3AL-triggered degranulation event, mast cells were pretreated with PtX to inactivate heterotrimeric G proteins known to be vital for mast cell secretion induced by other polybasic agents such as mastoparan, 48/80, and substance P [8–10]. rab3AL had no effect on secretion from PtX-treated cells (Fig. 3A), and in addition, PtX treatment blocked the ability of rab3AL to reduce cellular cAMP levels (Fig. 3B). Similar responses were found with mastoparan (Fig. 3A,B). Reduction of cellular cAMP levels by rab3AL and mastoparan indicates that both of these peptides may act via a G-protein of the $G\alpha_{i/o}$ class which is linked to inhibition of adenylate cyclase activity. PtX-sensitive G proteins activated by mastoparan are also linked to phospholipase C activation and inositol 1,4,5 trisphosphate (IP3) production in mast cells [10]. We have found that rab3AL stimulates a transient increase in the intracellular free calcium ion concentration in mast cells (Fig. 4); and this effect could be due to stimulation of IP3 production by

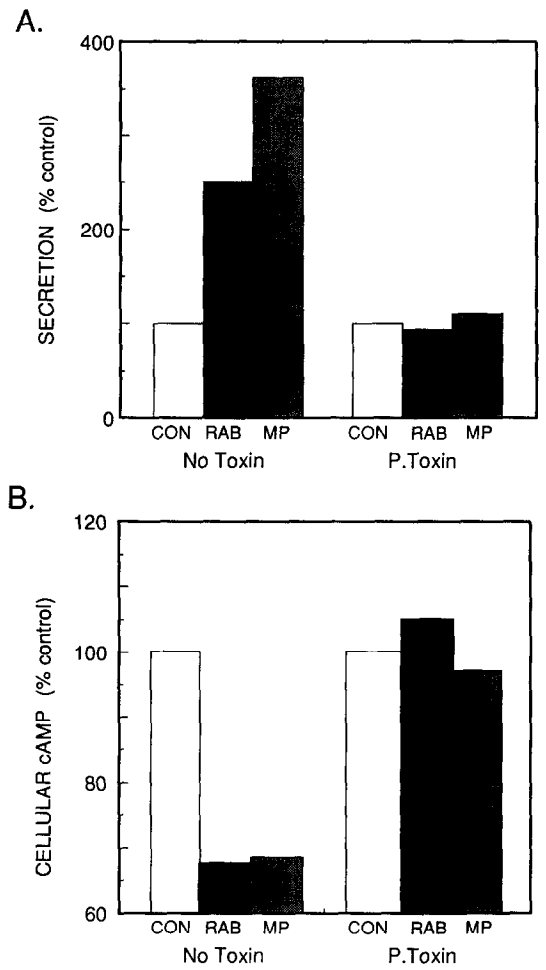

Fig. 3. Effect of 100 μM rab3AL (RAB), or 10 μM mastoparan (MP), on cells pre-treated with PtX (P. Toxin) (2 μg/ml for 5 h). (A) secretory output of hexosaminidase was collected for 10 min. (B) cellular cAMP levels for part (A). Data were averaged from 6 separate experiment and expressed as a % of their control value. Error bars are too small to depict in this histogram.

rab3AL. However, recent evidence also suggests that there is an additional PtX-sensitive G protein which acts downstream of phospholipase C to trigger degranulation [11]. Details about the nature of the PtX-sensitive G protein which could account for rab3AL-induced degranulation remain to be established.

3.4. *rab3AL peptide has a structure and function similar to mastoparan*

Previous work has shown that mastoparan binds to phospholipid bilayers and causes a direct stimulation of $G\alpha_{i/o}$ activity [8,9]. Our data (Fig. 3) suggest that rab3AL might act in a similar manner. Alpha-helical wheel projections reveal striking structural similarities between the hydrophobic domains and charge distribution of these two peptides (Fig. 5). This compelling structural (Fig. 5) and functional (Fig. 3) similarity suggest that signal transduction of rab3 peptide, like mastoparan, is probably due to a direct action on a PtX sensitive G-protein. Our data do not rule out the possibility that mast cells have a unique receptor on their cell surface to respond to rab3AL. But no specific cell-surface receptors have been found yet for other polybasic peptides on mast cells [10]. Mastoparan does, however, bind directly to G proteins [9].

3.5. *rab3AL is a polybasic peptide which can activate G proteins*

This paper clearly shows that externally applied rab3AL stimulates exocytosis from mast cells via a PtX-sensitive mechanism. A family of polybasic compounds like 48/80, mastoparan, and substance P, appear to stimulate exocytosis from mast cells in manner similar to rab3AL; and so it was imperative to investigate whether the positive charged amino acids of rab3AL were important for activity. To investigate the effect of charge each lysine and arginine was systematically replaced with the neutral amino acid residue alanine and the results are shown in Fig. 6. A marked reduction in activity was apparent when the C-terminal arginine residue was substituted with alanine. Complete loss of activity was obtained when a second alanine substitution was made for a lysine residue in position 11 of the linear sequence of rab3AL to produce a net neutral charged peptide (see Fig. 6). We conclude that basic amino acid residues are necessary to confer activity to rab3AL

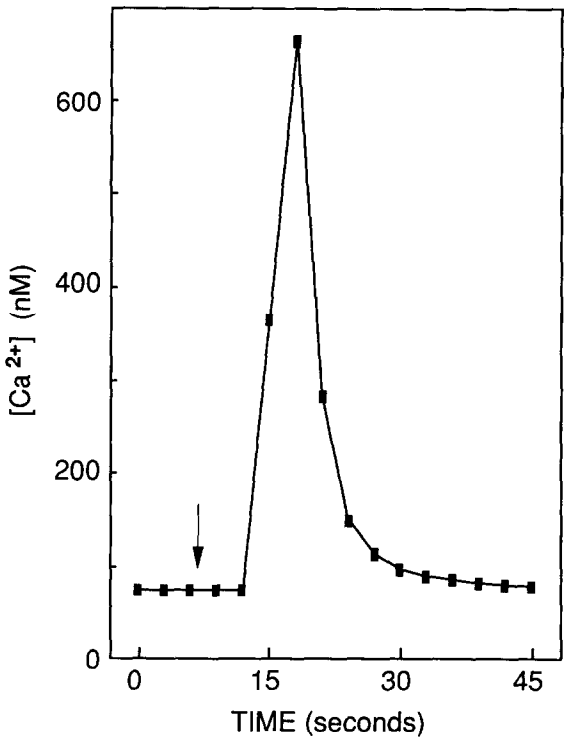

Fig. 4. Effect of 100 μM rab3AL on changes in the intracellular free calcium ion concentration [Ca^{2+}] measured by fura-2 and digital ratio-imaging [7]. Arrow marks addition of rab3AL to bath. 1.8 mM $CaCl_2$ was added to the medium prior to the experiment. Ca^{2+} transients have been recorded in 32/36 cells.

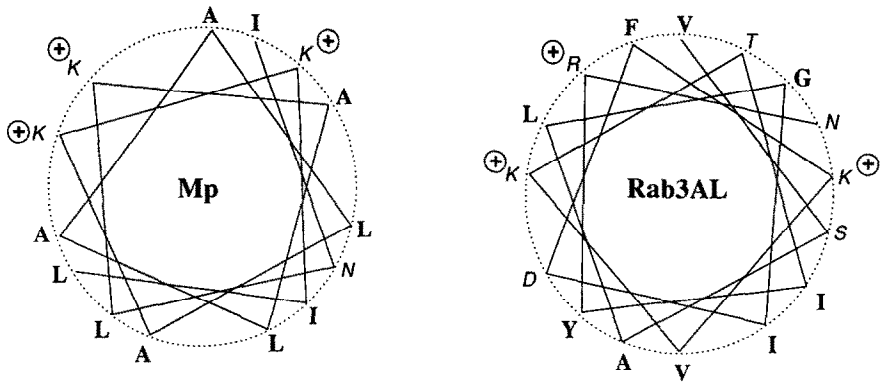

Fig. 5. Alpha-helical wheel projection for rab3AL and mastoparan (Mp). Bold type denotes hydrophobic amino acids. Positive charged residues are also marked (+).

when applied extracellularly, but hydrophobic residues might also have a function. This work does not distinguish between the possibility that binding and effector parts of rab3AL are one of the same, or different. Fig. 2 shows that VSALG has no effect on release from intact mast cells and lack of activity for this peptide has been recently confirmed in an in vitro membrane fusion assay that responds to rab3AL [12]. Both of these results can be explained by a requirement for polybasic amino acids. VSALG triggered degranulation in patch-clamped mast cells [2] showing that fragments of rab3AL can have non-polybasic effects – a point discussed below.

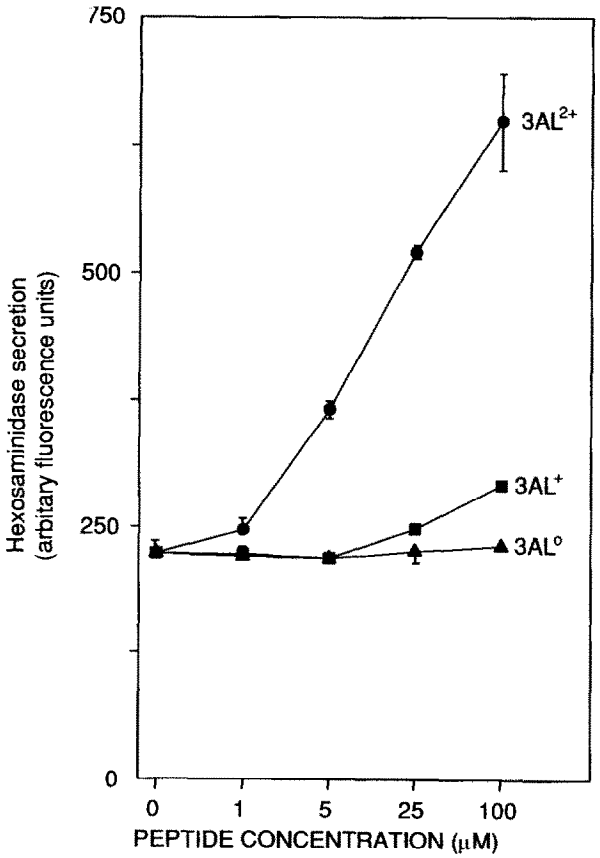

Fig. 6. Secretory response to charge neutralised derivatives of rab3AL. $3AL^{2+}$ is normal rab3AL. $3AL^{+}$ has the arginine residue of rab3AL changed to alanine. $3AL^{0}$ has a second alanine substituted for the lysine in position 11 of the linear sequence of rab3AL. Data is expressed as mean value ± S.E.M.

3.6. *Does regulation of a G protein by rab3AL explain other effects of this peptide?*

We previously reported a difference between the effect of GTPγS and rab3AL on an in vitro membrane fusion assay [5]. GTPγS stimulated membrane fusion of secretory granules to one another but rab3AL did not show this effect [5]. rab3AL, however, did stimulate membrane fusion between secretory granules and plasma membrane, an effect similar to that seen with GTPγS [5].

GDPβS has been recently found to inhibit rab3AL stimulation of exocytotic rate in patch-clamped melanotrophs (Rupnik, Zorec and Law, unpublished data). Upon guanine nucleotide exchange, the slowly hydrolysable analog (GDPβS) is expected to bind to a GTP binding protein (monomeric or heterotrimeric) and to stabilise it in an inactive state. These findings are consistent with our hypothesis that one mechanism of action of rab3AL is mediated via regulation of a G-protein on the plasma membrane.

Other experiments using patch-clamped mast cells found GDPβS-accelerated degranulation to be stimulated by either rab3AL, or it's truncated form VSALG [2]. The effect of GDPβS is likely to be non-selective and cytosolic, where there is the potential to modify one or more GTP binding proteins. Glucagon activation of adenylate cyclase via a G protein is potentiated in the presence of GDPβS even though GDPβS inhibited the effect of a non-hydrolysable GTP analog [13]. GDPβS can act as a partial agonist and in certain cases will support hormone-receptor mediated stimulation of a G

protein [13] and this activity may account for it's effect reported in [2].

The accelerated response to GDPβS was however considered to be due to competition of rab3 peptides with endogenous rab3 proteins for a binding site on an unknown target effector protein [2]. However, patch-clamped mast cells were reported to respond to VSALG, VSTVG, ISTIG and ISALG nearly as well as rab3AL, calling into question the specificity of the rab3-like effect of 5-mer peptides. These 5-mer peptides have no net positive charge and their effects are clearly unrelated to the one shown in Fig. 6, where charge is important for activity. Furthermore, VSALG has no effect on intact mast cells (Fig. 2), cell-free membrane fusion [12], and patch-clamped melanotrophs (data not shown). rab3AL may act on more than one GTP binding protein inside the cell as seems to be the case for mastoparan [8,14]. Stimulation of mast cell degranulation by a PtX-sensitive G protein is thus evidently required. Our observation that rab3AL stimulates exocytotic release from mast cells via a PTX-sensitive G protein is consistent with other recent findings which report that rab3AL stimulates inositol 1,4,5-trisphosphate production in pancreatic acini [15]. Clearly evidence is accumulating to support the view that an isolated effector domain of rab3 regulates heterotrimeric G proteins and possibly other downstream events.

3.7. *Conclusion*

This study demonstrates that rab3-like peptides stimulate exocytosis via a PtX-sensitive G protein when added to intact mast cells. This potent action of rab3AL is probably due to the well known property of polybasic peptides to directly regulate activity of G proteins. Data obtained with rab3-like peptides alone however is not sufficient to prove that a GTP-bound rab3 protein works by a similar mechanism, or is even restricted to such a mechanism.

Acknowledgments: This work was supported by a project grant from the AFRC awarded to G.J.L. We thank Chris J. Littlewood of Zinsser Analytic, Maidenhead, UK for synthesis of the peptides.

REFERENCES

[1] Plutner, H., Schwaninger, R., Pind, S. and Balch, W.E. (1990) EMBO J. 9, 2375–2383
[2] Oberhauser A.F., Monck, J.R., Balch, W.E. and Fernandez, J.M. (1992) Nature 360, 270–273
[3] Padfield, P.J., Balch, W.E. and Jamieson, J.D. (1992) Proc. Natl. Acad. Sci. USA 89, 1656–1660
[4] Senyshyn, J., Balch, W.E. and Holz, R.W. (1992) FEBS Lett. 309, 41–46
[5] Edwardson, J.M., MacLean, C.M. and Law, G.J. (1993) FEBS Lett. 320, 52–56
[6] Lillie, T.H.W. and Gomperts, B.D. (1992) Biochem. J. 228, 181–187
[7] Law, G.J. and O'Brien, W. (1993) Fluorescent and Luminescent Probes for Biological Activity, Academic Press, pp. 196–203
[8] Higashijima, T., Burnier, J. and Ross, E.M. (1990) J. Biol. Chem. 265, 14176–14186
[9] Weingarten R., Ransnas L., Mueller, H., Sklar, L.A. and Bokoch, G.M. (1990) 265, 11044–11049
[10] Mousli, M., Bueb, J.-L., Bronner, C., Rouot, B. and Landry, Y. (1990) Trends Pharm. 11, 358–362
[11] Aridor, M. and Sagi-Eisenberg, R. (1990) J. Cell Biol. 111, 2885–2891
[12] MacLean, C.M., Law, G.J. and Edwardson, J.M. (1993) Biochem. J. 294, 325–328.
[13] Eckstein, F., Cassel, D., Levkovitz, H., Lowe, M. Selinger, Z. (1979) J. Biol. Chem. 254, 9829–9834
[14] Koch, G., Haberman, B., Mohr, C., Just, I. and Aktories, K. (1991) FEBS Lett. 2, 336–340
[15] Piiper, A., Stryjek-Kaminska, D., Stein, J., Caspary, W.F. and Zeuzem, S. (1993) Biochem. Biophys. Res. Commun. 192, 1030–1036