Activation of polyphosphoinositide phospholipase C by fluoride in WRK1 cell membranes

Gilles Guillon, Bernard Mouillac and Marie-Noëlle Balestre

Centre CNRS-INSERM de Pharmacologie-Endocrinologie, Route de la Cardonille, 34094 Montpellier Cedex, France

Received 14 June 1986

Partially purified plasma membranes prepared from *myo*-[^{3}H]inositol-prelabeled WRK1 cells exhibit a phosphatidylinositol 4,5-bisphosphate (PIP_2) phospholipase C activity sensitive to NaF. NaF increased the production of IP_2 and IP_3 in a time- and concentration-dependent manner. The maximal increase in IP_2 and IP_3 production rates represented 400 ± 18 and 360 ± 40% of the basal production rate, respectively. Half-maximum stimulation was reached with 2–4 mM NaF. The observed effect was specific for F^-. Aluminium potentiated fluoride-induced IP_3 and IP_2 accumulation in a concentration-dependent manner. The effect of fluoride on the PIP_2 phospholipase C from WRK1 cell membranes appears to be similar to the well-documented effect of F^- on the well-characterized N_s, N_i and transducin GTP-binding proteins. This observation constitutes an additional argument to suggest that a GTP-binding protein is involved in the process of receptor-mediated activation of PIP_2 phospholipase C.

Sodium fluoride *Aluminum* *Phospholipase C* *(WRK1 cell)* *Inositol phosphate* *GTP-binding protein*

1. INTRODUCTION

Receptor-mediated activation of a PIP_2 phospholipase C is a common mechanism by which several hormones and neurotransmitters increase free cytosolic calcium within their target cells (reviews [1,2]). There is experimental evidence indicating that GTP is involved in the process of receptor-mediated PIP_2 breakdown. Non-hydrolyzable analogues of GTP mimic the effects of calcium-mobilizing agents when introduced into permeabilized mast cells and platelets [3,4]. These analogues also stimulate PIP_2 breakdown in membranes from rat liver [5–7], blow salivary glands [8], GH3 [9], and WRK1 cells [10]. In membranes from WRK1 cells, a vasopressin-sensitive cell line derived from a chemically induced mammary tumor in the rat [11], we recently showed that vasopressin stimulates PIP_2 breakdown in a strictly GTP-dependent manner [10]. Altogether these results suggest that a GTP-binding protein with a GTPase activity is involved in the functional coupling of several receptors to PIP_2 phospholipase C. This GTP-binding protein would therefore be homologous to the well-characterized N_s, N_i and transducin G-proteins. It was recently reported that AlF_4 mimics the effects of Ca^{2+}-mobilizing hormones on intact isolated hepatocytes [12] and that NaF stimulates calcium mobilization in human neutrophils [13]. This can be considered as further evidence for functional homology between the N_s, N_i and transducin G-proteins and the putative G-protein involved in receptor-mediated PIP_2 breakdown. Indeed, it is known that Al^{3+} in the presence of F^- is a potent modulator of N_s, N_i and transducin [14–16]. The active agent is thought to be AlF_4 which acts by mimicking the effect of the γ-phosphate of GTP on the α-subunits of these G-proteins [16]. Here,

Abbreviations: IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; GTPγS, guanosine 5′-*O*-thiotriphosphate; LVP, lysine vasopressin

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

we took advantage of the presence of a hormone-sensitive PIP_2 phospholipase C in broken cell preparations derived from *myo*-[^{3}H]inositol-prelabeled WRK1 cells [10] to investigate the effects of F^- on this PIP_2 phospholipase C.

2. MATERIALS AND METHODS

2.1. *Products*

GTP and GTPγS were purchased from Boehringer Mannheim, LVP from Bachem, *myo*-[^{3}H]inositol (16.5 Ci/mol) from New England Nuclear, Eagle's minimum essential medium (MEM) and calf fetal serum from Gibco, Dowex 1 × 10 (100–200 mesh, chloride form) from Fluka, NaF (suprapur) from Merck, L-*myo*-[^{14}C]inositol 1-phosphate and D-*myo*-[2-^{3}H]inositol 1,4,5-trisphosphate from Amersham.

2.2. *Membrane preparation*

WRK1 cells were grown in monolayer culture for 5 days in MEM containing 5% fetal calf serum, 2% rat serum, glutamine (290 mg/l), penicillin (100 U/ml), streptomycin (100 mg/ml) and *myo*-[^{3}H]inositol (1.5 μCi/ml) (for details see [17]). The culture medium was renewed 2 days after seeding. Half an hour before the experiment, the culture medium was removed and replaced by the same medium depleted of *myo*-[^{3}H]inositol and serum. Cells were washed 3 times at 0°C with phosphate-buffered saline (PBS) without calcium and magnesium, and suspended in a homogenization medium composed of 10 mM LiCl, 0.5 mM EDTA, 10 mM Tris-HCl (pH 8.0) and 0.1 mM ATP. Cells were disrupted at 4°C using a Dounce homogenizer equipped with a loose pestle (15 strokes). Partially disrupted cells were eliminated by a rapid centrifugation (100 × *g* at 0°C for 5 min). The supernatant was subjected to a second centrifugation (30000 × *g* at 0°C for 15 min). The final pellet was resuspended in the homogenization buffer and used immediately. We previously showed that this preparation contains the bulk of plasma membranes from disrupted cells [10]. For the sake of simplicity, this preparation is designated as WRK1 cell membranes. Protein content of each preparation was determined by the method of Lowry et al. using bovine serum albumin as a standard.

2.3. *Assay of phospholipase C activity*

Membranes prepared from *myo*-[^{3}H]inositol-prelabeled WRK1 cells (40–90 μg protein per assay) were incubated at 37°C essentially as described [10] with minor modifications. Briefly, the incubation medium (300 μl) was composed of 10 mM LiCl, 0.1 mM ATP, 0.25 mM EDTA, 10 mM Tris-HCl (pH 8.0), 1 μM $CaCl_2$ and 5 mM $MgCl_2$ and the agents to be tested. When the effects of Al^{3+} were tested, special attention was paid to avoid any contact of the different solutions and membrane preparations used with glassware from which Al^{3+} could have been released [14]. The reaction was stopped by the addition of 300 μl $HClO_4$ (10%) and 100 μl bovine serum albumin (20 mg/ml) and cooling the tube to 0°C. The pH of the extract was adjusted to 7.0 by adding 1.5 M KOH. $KClO_4$ was eliminated by centrifugation. Labeled IP_1, IP_2 and IP_3 present in the supernatant were separated by chromatography on Dowex 1 × 10 (100–200 mesh, formate form) columns (4.0 × 0.6 cm) as described by Berridge et al. [18] with minor modifications [19]. This protocol was established using purified labeled IP_1 and IP_3 obtained from Amersham. Labeled inositol lipids were determined after chloroform/methanol/HCl (100:100:1) extraction and deacylation in the presence of NaOH as described by Creba et al. [20]. Radioactivity found in the IP_1, IP_2 and IP_3 fractions was measured by liquid scintillation spectrometry. All results were corrected for quenching and expressed in dpm. All determinations were performed in triplicates.

3. RESULTS AND DISCUSSION

The relative proportions of PI, PIP and PIP_2 in WRK1 cell membranes (94.6 ± 0.5; 2.7 ± 0.5; 2.8 ± 0.2% of total inositol lipids, respectively, means ± SE of 5 experiments) were close to those determined in intact prelabeled cells (93.7 ± 0.8; 3.3 ± 0.7; 3.0 ± 0.1%, means ± SE of 5 experiments). This observation indicates that no preferential hydrolysis of one class of inositol lipid occurred during the process of membrane preparation. During the course of a 20 min incubation period, the total content of labeled inositol lipids of non-stimulated membranes remained fairly stable. A slight and transient increase in PIP and PIP_2 contents was observed, suggesting the

presence in the membrane preparation of enzymatic activities enabling the phosphorylation of PI and PIP (fig.1). During the first 3 min of incubation, the concentration of labeled IP_1, IP_2 and IP_3 in the incubation medium increased slightly. Thereafter the IP_1 content remained stable while a moderate linear accumulation of IP_2 and IP_3 was observed. NaF (20 mM) increased the accumulation of IP_3 and IP_2 but did not affect the IP_1 content of the incubation medium (fig.1). The rate of fluoride-stimulated IP_3 and IP_2 accumulation was not linear with time, indicating the transient nature of the observed effect. The fluoride-induced accumulation of $IP_3 + IP_2$ followed an exponential time course, suggesting that the effect of fluoride was to liberate inositol phosphate from a precursor pool, the size and half-life of which were estimated to be 2150 dpm/mg membrane protein and 8.4 min, respectively. The size of this pool represents 5% of the PI pool. Indeed, there was no significant effect of fluoride on the size of the PI pool. Conversely, the total amount of inositol phosphates produced under the influence of NaF is roughly equivalent to the size of the PIP and PIP_2 pools. In addition, at all time points tested NaF

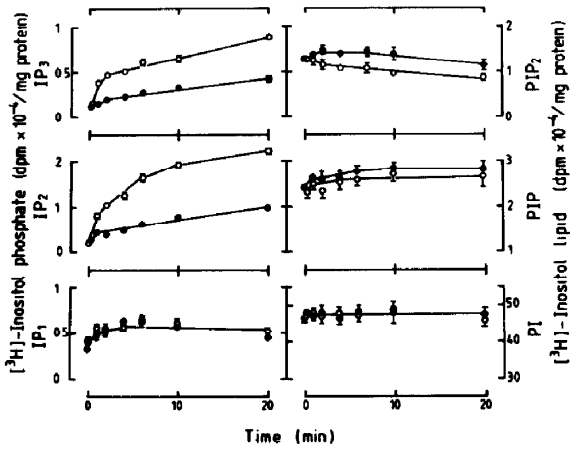

Fig.1. Effects of NaF on inositol lipid breakdown in WRK1 cell membranes: time dependency. Membranes from prelabeled WRK1 cells (90 μg/assay) were incubated at 37°C for the indicated periods of time in either the absence (●) or presence of 20 mM NaF (○). Inositol phosphates (left) and inositol lipids (right) were measured as indicated in section 2. Values are means ± SE of 3 determinations. When no error bars are shown, the SE is contained within the symbol.

slightly reduced PIP and PIP_2 pools as compared to the corresponding values determined on non-stimulated membranes. Altogether, these results suggest that: (i) the putative precursor pool(s) of IP_3 and IP_2 are renewed from PI; (ii) the effect of NaF on IP_3 and IP_2 accumulation cannot be accounted for by an increase in the size of the PIP_2 and PIP pools or an inhibition of the IP_3 and IP_2 phosphatases but probably reflects an activation of PIP_2 phospholipase C. The progressive fading of the fluoride response might reflect the fact that in WRK1 cells the larger part of the hormone-sensitive PIP_2 pool derives from a minor PI pool which, according to Koreh and Monaco [21], represents about 17% of the total PI pool. This value is close to the fraction of labeled PI (11.5%) recovered as the sum of IP_3, IP_2, PIP_2 and PIP at the end of a 20 min incubation period.

Fig.2 illustrates the dose dependency of NaF-induced stimulation of IP_3 and IP_2 production. IP_3 and IP_2 production was measured at the end of either a 2 or 6 min incubation period in the presence of NaF, i.e. in a situation where the size of the NaF-sensitive pool was not limiting (cf. fig.1 and above discussion). The maximal NaF effect was observed at 20 mM NaF. At higher concentrations a reduction in the magnitude of the NaF response was observed. The EC_{50} values were not markedly dependent on the experimental conditions used (2 or 6 min incubation period). For a 6 min incubation period, the means ± SE (6 determinations) of EC_{50} values were 5.4 ± 1 and 4.8 ± 1 mM for NaF-stimulated IP_3 and IP_2 production, respectively. These values resemble those for adenylate cyclase activation by NaF [22,23].

The data in table 1 indicate that the maximal effects of NaF and vasopressin plus GTP on inositol phosphate production were of similar magnitude. A 2 min preincubation period at 37°C of WRK1 cell membranes followed by washing, centrifugation and resuspension in fresh medium reduced but did not abolish the ability of these membranes to respond to NaF or vasopressin stimulation by increased inositol phosphate production during a final 2 min post-incubation at 37°C. When the preincubation was performed in the presence of NaF or vasopressin, no residual activation of inositol phosphate production was observable during the post-incubation period, suggesting that fluoride and vasopressin stimulation was reversed

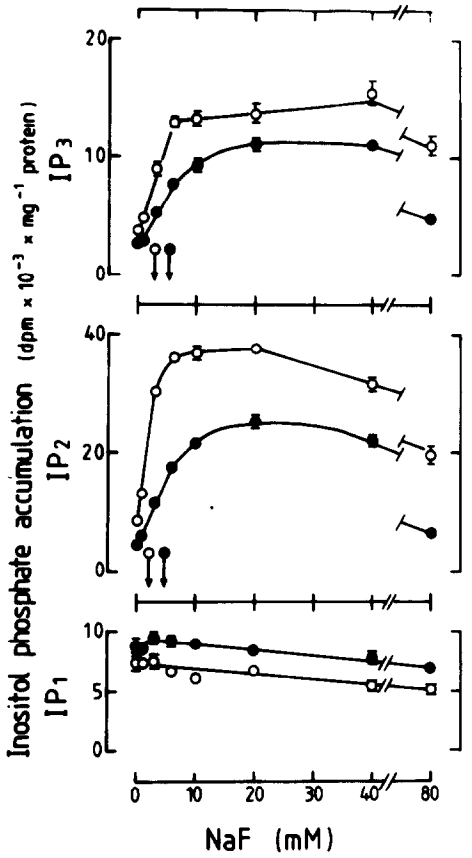

Fig.2. Dose-dependent activation of PIP_2 phospholipase C by NaF. WRK1 cell membranes (50 μg protein) were incubated at 37°C for 2 (●) or 6 min (○) in the presence of NaF added at the indicated concentrations. Inositol phosphates liberated in the incubation medium were determined as indicated in section 2. Results expressed are means ± SE of 3 determinations. When no error bars are shown, the SE is contained within the symbol. Arrows on the graphs correspond to the apparent EC_{50} values for NaF-induced IP_3 and IP_2 accumulation.

during the process of membrane washing and centrifugation. Prestimulated washed membranes were able to respond to NaF or vasopressin stimulation although less efficiently than non-prestimulated membranes. This would suggest that fluoride activation of inositol phosphate production is not a consequence of NaF-induced removal from the membranes of some substance(s) inhibiting inositol lipid breakdown. The NaF sensitivity of inositol phosphate production by WRK1 cell membranes would thus appear to be intrinsic to the process controlling inositol lipid metabolism in these membranes.

The effect of NaF on inositol phosphate production by WRK1 cell membranes was specific for F^-. It could not be reproduced by NaCl, NaBr or NaBr. The $IP_2 + IP_3$ production in the presence of these salts used at 10 mM represented 111 ± 8, 106 ± 3, and 113 ± 3% of the basal production respectively (means ± SE of 6 determinations). As shown in fig.3, the effects of maximally active NaF and vasopressin concentrations on inositol phosphate production were not additive. This non-additivity could not be accounted for by the maximal activity of PIP_2 phospholipase C nor by the size of the inositol phosphate precursor pool. Indeed the effect of GTPγ was of a higher magnitude than that of vasopressin or NaF tested alone or in combination. The observation that NaF did not potentiate the vasopressin effect on inositol phosphate production excludes the possibility that NaF had affected a rate-limiting step in inositol phosphate production other than the vasopressin-sensitive step. These results suggest that these two agents affect some common step in the activation of PIP_2 phospholipase C. This common step might be the activation of GTP-binding protein involved in receptor-PIP_2 phospholipase C coupling.

Under experimental conditions where the contact of WRK1 cell membranes with glassware was avoided (except at the homogenisation step), potentiation by $AlCl_3$ of the NaF-induced inositol phosphate production could be demonstrated (fig.4). The $AlCl_3$ effect was concentration-dependent with a maximal effect (1.5 ± 0.2-fold increase in the response to NaF, mean ± SE of values derived from 3 experiments) obtained at 10 μM $AlCl_3$ and with an apparent EC_{50} of 1 μM. The latter value falls within the range of $AlCl_3$ concentrations active in the NaF-induced activation of adenylate cyclase [14] or inhibition of cGMP phospholipase C from the rod outer segment in retina [16]. Autoinhibition of the $AlCl_3$ effect on WRK1 cell membranes was observed at concentrations higher than 10 μM (fig.4). It is tempting to consider that the effect of $AlCl_3$ on NaF-induced inositol phosphate production by WRK1 cell membranes is homologous to its well-documented effect on N_s and transducin, i.e. AlF_4^- mimicking the γ-phosphate group of GTP at its binding site on the α-subunit [17].

Table 1

Reversal of NaF and vasopressin-activation of PIP_2 phospholipase C from WRK1 cell membranes

Added during preincubation	Added during post-incubation		
	None	NaF	LVP + GTP
None	6155 ± 316 (7)	11574 ± 1492 (7)	13370 ± 236 (4)
NaF	6668 ± 247 (11)	10122 ± 310 (11)	–
LVP + GTP	6846 ± 227 (4)	–	11121 ± 326 (4)
No preincubation	6712 ± 756 (7)	18786 ± 984 (7)	18774 ± 575 (4)

WRK1 cell membranes were incubated for 2 min at 37°C in the presence of the indicated effectors. NaF, vasopressin (LVP) and GTP were used at 20 mM, 1 μM and 100 μM, respectively. At the end of the preincubation period, the membrane suspensions were diluted by adding an 11-fold excess of ice-fold 10 mM Tris-HCl buffer (pH 8). They were centrifuged for 15 min at 20000 × g at 0°C and resuspended in the ice-cold buffer. Non-preincubated membranes were used as controls. Preincubated and control membranes were then incubated for 2 min at 37°C in the presence of the indicated effectors. Values are means ± SE (number of determinations in parentheses) of the sum of labeled IP_3 and IP_2 (dpm/mg protein) recovered at the end of the final incubation period

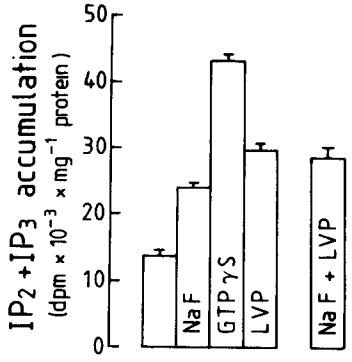

Fig.3. Effects of NaF, GTPγS, and vasopressin on inositol phosphate production by WRK1 cell membranes. WRK1 cell membranes (50 μg) were incubated for 4 min at 37°C in the presence of the indicated effectors used at the following concentrations: 10 mM NaF, 10 μM GTPγS, and 1 μM LVP. LVP was tested in the presence of 100 μM GTP (GTP is inactive per se but is required for the expression of the LVP effect [10]). The open bar on the left corresponds to the control conditions. Results are expressed as the sum of IP_2 + IP_3 recovered at the end of the incubation period and are means ± SE of 3 determinations.

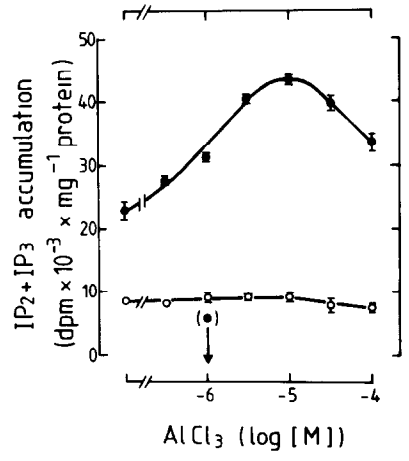

Fig.4. Potentiation by $AlCl_3$ of NaF-induced inositol phosphate production by WRK1 cell membranes. For these experiments contact of the membrane preparation and all solutions used with glassware was avoided. (●) Membranes were incubated for 4 min at 37°C in the presence of NaF (5 mM) and $AlCl_3$ at the indicated concentrations. (○) Control membranes incubated in the absence of NaF. Results are expressed as the sum of IP_2 and IP_3 recovered at the end of the incubation period and are means ± SE of 3 determinations. The arrow on the graph corresponds to the apparent EC_{50} values for the $AlCl_3$ effect on NaF-induced $IP_3 + IP_2$ accumulation.

In conclusion, our results show that NaF (AlF_4^- possibly being the active species) increased labeled IP_2 and IP_3 formation by *myo*-[^{3}H]inositol-prelabeled WRK1 cell membranes. This effect is homologous to (and non-additive with) the vasopressin receptor-mediated and GTP-dependent activation of a PIP_2 phospholipase C in these membranes. Activation of PIP_2 phospholipase C by fluoride can be considered as an additional argument favoring the hypothesis that a GTP-binding protein homologous with the well-characterized N_s, N_i and transducin proteins is involved in the functional coupling of vasopressin receptors to PIP_2 phospholipase C in WRK1 cells. There are contradictory reports on the possible mediation by N_i of PIP_2 phospholipase C activation. Inactivation of N_i by pertussis toxin (IAP) abolishes the Ca^{2+}-dependent responses and PIP_2 breakdown in neutrophils and mast cells [24,27] but not in other systems studied so far; hepatocytes, 3T3 fibroblasts, GH3 and chick heart cells [7,28–30]. Preliminary results indicate that the GTP-binding protein involved in the activation of PIP_2 phospholipase C in WRK1 cells resembles those present in hepatocytes, 3T3, and GH3 cells. Indeed vasopressin-activated inositol phosphate production in these cells appeared insensitive to IAP treatment (Guillon, G., in preparation).

ACKNOWLEDGEMENTS

We thank Professor S. Jard for many stimulating discussions and Mrs M. Paolucci for secretarial assistance.

REFERENCES

[1] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
[2] Michel, R.H. (1975) Biochim. Biophys. Acta 415, 81–147.
[3] Haslam, R.J. and Davidson, H.L. (1984) J. Receptor Res. 4, 605–629.
[4] Gomperts, B.D. (1983) Nature 306, 64–66.
[5] Wallace, M.A. and Fain, J.N. (1985) J. Biol. Chem. 260, 9527–9530.
[6] Uhing, R.J., Jiang, H., Prpic, V. and Exton, J.H. (1985) FEBS Lett. 188, 317–320.
[7] Uhing, R.J., Prpic, V., Jiang, H. and Exton, J.H. (1986) J. Biol. Chem. 261, 2140–2146.
[8] Litosch, I., Wallis, C. and Fain, J. (1985) J. Biol. Chem. 260, 5464–5471.
[9] Straub, R.E. and Gershengorn (1986) J. Biol. Chem. 261, 2712–2717.
[10] Guillon, G., Balestre, M.N., Mouillac, B. and Devilliers, G. (1986) FEBS Lett. 196, 155–159.
[11] Monaco, M.E., Kidwell, W.R. and Lipman, M.E. (1980) Biochem. J. 188, 437–441.
[12] Blackmore, P.F., Bocckino, S.B., Waynick, L.E. and Exton, J. (1985) J. Biol. Chem. 260, 14477–14483.
[13] Strnard, C.F. and Wong, K. (1985) Biochem. Biophys. Res. Commun. 133, 161–167.
[14] Sternweiss, P.C. and Gilman, A.G. (1982) Proc. Natl. Acad. Sci. USA 79, 4888–4891.
[15] Katada, T., Northup, J.K., Bokoch, G.N., Ui, M. and Gilman, A.G. (1984) J. Biol. Chem. 259, 3578–3585.
[16] Bigay, J., Deterre, P., Pfister, C. and Chabre, M. (1985) FEBS Lett. 191, 181–185.
[17] Monaco, M.E., Kidwell, W.R. and Lippman, M.E. (1978) Cancer Res. 38, 4101–4104.
[18] Berridge, N.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P. and Irvine, R.F. (1983) Biochem. J. 212, 473–483.
[19] Guillon, G. and Gallo-Payet, N. (1986) Biochem. J. 235, 209–214.
[20] Creba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H. and Kirk, C.J. (1983) Biochem. J. 212n, 733–747.
[21] Koreh, K. and Monaco, M.E. (1986) J. Biol. Chem. 261, 88–91.
[22] Perkins, J.H. and Moore, M.M. (1971) J. Biol. Chem. 246, 62–68.
[23] Brostrom, C.O., Brostrom, M.A. and Wolff, D.J. (1977) J. Biol. Chem. 252, 5677–5685.
[24] Brandt, S.J., Dougherty, R.W., Lapetina, E.G. and Niedel, J.E. (1985) Proc. Natl. Acad. Sci. USA 82, 3277–3280.
[25] Nakamura, T. and Ui, M. (1985) J. Biol. Chem. 260, 3584–3593.
[26] Krause, K.H., Schlegel, W., Wolheim, C.B., Anderson, T., Waldvogel, F.A. and Low, P.D. (1985) J. Clin. Invest. 76, 1348–1354.
[27] Verghese, M.W., Smith, C.D. and Snyderman, R. (1985) Biochem. Biophys. Res. Commun. 127, 450–457.
[28] Murayama, T. and Ui, M. (1985) J. Biol. Chem. 260, 7226–7233.
[29] Martin, T.F.J., Lucas, D.O., Bajjalich, S.M. and Kowalchyk, J.A. (1986) J. Biol. Chem. 261, 2918–2927.
[30] Masters, S.B., Martin, H.W., Harden, T.K. and Brown, J.H. (1985) Biochem. J. 227, 933–937.