ELSEVIER

FEBS Letters 343 (1994) 37–41

FEBS LETTERS

FEBS 13903

Staurosporine induces hydrolysis of phosphatidyl inositol 4,5-bisphosphate in human platelets

James Turkson, Xue-Bin Li, Kenneth Wong*

Department of Pharmacology and Therapeutics, University of Calgary, and the Canadian Red Cross Blood Transfusion Centre, 13th Ave. S.W., Calgary, Alberta T2R 1J1, Canada

Received 2 March 1994

Abstract

Staurosporine in the micromolar range raised inositol trisphosphate in intact human platelets to levels comparable to that mediated by thrombin. This response was inhibited by neomycin, a phospholipase C antagonist. Staurosporine alone induced a weak, transient rise in cytosolic free calcium levels ($[Ca^{2+}]_i$) from release of internal Ca^{2+} stores but potentiated the effect induced by thrombin. Therefore, it is unlikely that this alkaloid suppressed inositol trisphosphate mobilization of Ca^{2+}. Additional studies show that staurosporine, 0.5–5 μM, stimulated GTPase activity in platelet membranes while 2 μM K252a and 20 μM H7 were inactive. Present results suggest that staurosporine may activate platelet phospholipase C at the level of G proteins or receptors.

Key words: Staurosporine; Platelet; Phospholipase C; Calcium; Inositol phosphate

1. Introduction

Staurosporine, a microbial alkaloid from *Streptomyces* sp. is a potent but nonspecific inhibitor of protein kinase C (PKC) (IC_{50} of 3 nM) [1]. This reagent has been used in numerous studies to inhibit PKC-dependent responses in platelets [3–6]. Recently we showed that staurosporine in the micromolar range causes a rapid and sustained elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in neutrophils via an indirect mechanism [7]. Subsequently, Himpens and coworkers [8] reported that staurosporine mobilizes intracellular Ca^{2+} stores and induces Ca^{2+} influx in cultured DDT1MF-2 smooth muscle cells. In both studies, the effect of staurosporine on $[Ca^{2+}]_i$ is not correlated with PKC inhibition or phospholipase C (PLC) activation. The present study was undertaken to investigate and compare the effect of staurosporine on $[Ca^{2+}]_i$ in human platelets. We report here that micromolar staurosporine induced significant hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP2) but weak transient elevations of $[Ca^{2+}]_i$.

*Corresponding author. Fax: (1) (403) 541 4466.

Abbreviations: PIP2, phosphatidylinositol 4,5-bisphosphate; $InsP_3$, inositol trisphosphate; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; PKC, protein kinase C; PLC, phospholipase C; G protein, heterotrimeric guanine nucleotide-binding regulatory protein; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; TX, thromboxane; Me_2SO, dimethyl sulfoxide.

2. Materials and methods

2.1. Materials

[^{3}H]Myo-inositol, (80–120 Ci/mmol) and a ligand binding assay kit for $Ins(1,4,5)P_3$ were from Amersham Canada Ltd. (Oakville, Ont.); human thrombin, apyrase, neomycin sulfate, and prostacyclin were from Sigma Chemical Co. (St. Louis, MO); staurosporine was obtained from Sigma and Boehringer Mannheim (Montreal, P.Q.); Fura-2 AM and ionomycin were from Calbiochem Corp. (San Diego, CA); Stock solutions of staurosporine and ionomycin were dissolved in dimethyl sulfoxide (Me_2SO) and stored at −80°C.

2.2. Preparation of platelet suspension

Blood was drawn by venipuncture from drug-free volunteers into 1/6 volume acid citrate dextrose. Platelet-rich plasma was obtained by centrifuging whole blood at $300 \times g$ at room temperature for 15 min. After careful removal, the plasma was further centrifuged at $2500 \times g$ at room temperature for 10 min to pellet the platelets. Platelets were resuspended in Tyrode-HEPES buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.36 mM NaH_2PO4, 1 mM $MgCl_2$, 5 mM HEPES, 5 mM glucose; pH 7.2) containing apyrase (0.6 ADPase U/ml) and 0.35% bovine serum albumin. For labeling platelets with [^{3}H]myo-inositol, the buffer also contained 1 mM EGTA and 500 ng/ml prostacyclin [9].

2.3. Assay for Inositol phosphates

Inositol phosphate formation in platelets was assayed according to procedures described by Lapetina and Siess [9]. Briefly, platelets prelabeled with [^{3}H]myo-inositol at a density of 8×10^8 cells/ml Tyrode-HEPES were incubated with agonists then stopped with chloroform/methanol/HCl. The neutralized aqueous phases were applied to Dowex 1-X8 anion-exchange columns (formate form) and radio-labelled inositol mono-, bis-, and trisphosphates ($InsP_3$) were eluted by washing columns with ammonium formate/formic acid.

Inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) was quantitated by a competitive ligand-binding assay previously applied to neutrophil studies in this laboratory [7,10]. Platelet suspensions ($5–6 \times 10^9$/ml) were treated with trichloroacetic acid and $Ins(1,4,5)P_3$ levels in supernatant extracts were quantitated according to the protocol supplied by Amersham. The major cross reactivities were 0.22% and 6.4% for D-myo-

SSDI 0014-5793(94)00288-7

inositol 1,3,4-trisphosphate and D-myo-inositol 1,3,4,5-tetrakisphosphate respectively.

2.4. Measurement of $[Ca^{2+}]_i$

Platelets (8×10^8/ml) were loaded with fura-2 by incubation at 37°C with 1 μM Fura-2 AM for 45 min [11]. Washed platelets (2.0 ml at 3×10^8/ml) were measured for changes in fluorescence at monochromator settings of 339 nm (excitation) and 505 nm (emission). $[Ca^{2+}]_i$ was calculated as described previously [7,10].

2.5. GTPase assay for platelet membranes

Lysates of human platelets were obtained by rapid freezing and thawing in 10 mM triethanolamine-HCl, pH 7.4, containing 5 mM EDTA. Lysed platelets were centrifuged for 20 min at $30,000 \times g$ at 4°C. The pellet was washed twice in the same medium and finally suspended in 10 mM triethanolamine-HCl, pH 7.4, without EDTA. GTPase activity was measured according to previously published procedures [12,13]. Reactions were carried out in 100 μl, 50 mM triethanolamine-HCl, pH 7.4, containing the following: 0.1–0.3 μM [γ-^{32}P]GTP, (0.1 μCi/tube); 0.1 mM ATP; 0.1 mM EGTA; 2 mM $MgCl_2$; 1 mM dithiothreitol, 5 mM creatine phosphate; 0.4 mg/ml creatine kinase; 2 mg/ml bovine serum albumin. Reactions were started by adding platelet membranes (5–10 μg protein/tube) and stopped by the addition of 0.5 ml ice-cold sodium phosphate buffer (20 mM), pH 7.0, containing 5% (w/v) activated charcoal. High-affinity GTPase activity was calculated from the difference between total $^{32}P_i$ released in the presence of 0.3 μM GTP and that formed in the presence of 50 μM GTP. All reactions were done in triplicate.

3. Results

In studies with platelets suspended in Ca^{2+}-free medium, staurosporine, up to 4 μM, induced weak transient rises in $[Ca^{2+}]_i$ (Fig. 1A, traces a,b) which was not obtained with Me_2SO alone (trace c). Subsequent addition of Ca^{2+} failed to affect $[Ca^{2+}]_i$. The apparent increase in the signal was due to Ca^{2+} complexing with small amounts of extracellular fura-2 since Tris-EGTA depressed it quickly (traces b,c). In the same traces, thrombin elevated $[Ca^{2+}]_i$ in platelets from about 0.1 μM to > 0.5 μM (trace a). In most cases, the rise in $[Ca^{2+}]_i$ peaked at less than 10–15 s and declined gradually to basal levels over several min. When platelets were suspended in medium with 1 mM Ca^{2+}, staurosporine produced an equally transient increase in $[Ca^{2+}]_i$ (trace d). Here, pretreatment with staurosporine potentiated the effect of thrombin on platelet $[Ca^{2+}]_i$. The extent and duration of the rise in $[Ca^{2+}]_i$ was increased compared with the response induced in cells treated with thrombin alone (trace e).

Mn^{2+} entry into platelets and consequent quenching of fura-2 fluorescence was employed as an indirect measurement of Ca^{2+} influx (Fig. 1B) [7,10]. Mn^{2+} added to resting platelets produced a basal rate of quenching due to entry via leak channels. Thrombin activation of cells accelerated Mn^{2+} entry in agreement with previous reports [14]. Staurosporine, 2 μM, on the other hand, neither affected the basal rate nor interfered with Mn^{2+} entry mediated by ionomycin. The results in Fig. 1A and B indicate that staurosporine induced a limited release

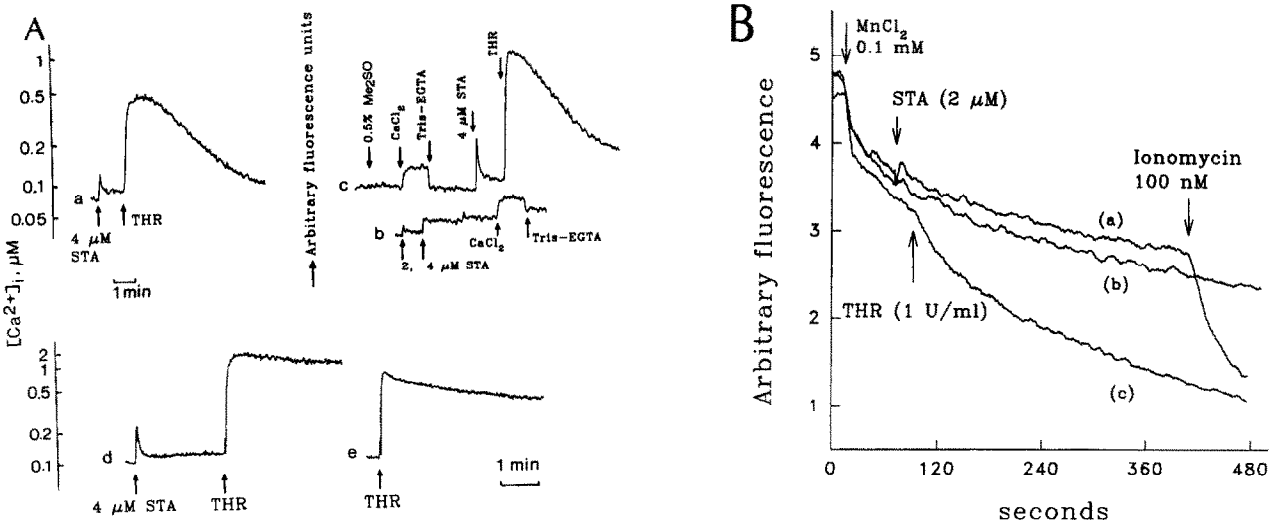

Fig. 1. (A) The effect of staurosporine on $[Ca^{2+}]_i$ of human platelets. Human platelets preloaded with fura-2 and resuspended in Tyrode-HEPES buffer were treated with staurosporine (STA) and reagents in the order shown in the figure. Final concentrations were 1 U/ml thrombin (THR), 1 mM $CaCl_2$, 25 mM Tris-base and 2 mM ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA). Fluorescence measurements and calculation of $[Ca^{2+}]_i$ (except for traces b and c) were carried out as outlined in section 2. In traces (d) and (e), the medium was supplemented with 1 mM $CaCl_2$. Results are representative of four experiments. (B) Kinetics of Mn^{2+} quenching of fluorescence in thrombin- and staurosporine-treated platelets. Fura-2 loaded platelets were stimulated with reagents in the following order: trace (a) Mn^{2+}, staurosporine, ionomycin; trace (b), Mn^{2+}; trace (c) Mn^{2+}, thrombin. The excitation wavelength used was 360 nm (isobestic wavelength for Ca^{2+}). Results are representative of three experiments.

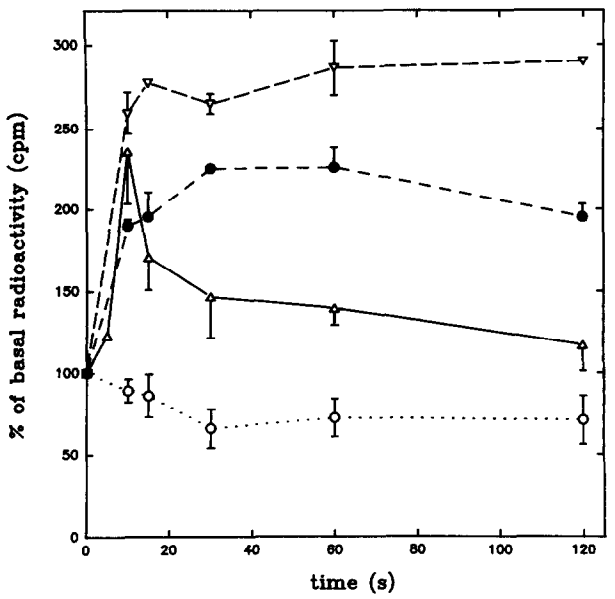

Fig. 2. Time course of [^{3}H]InsP$_3$ formation in human platelets treated with thrombin or staurosporine. Human platelets prelabeled with [^{3}H]myo-inositol were incubated with thrombin, 2 U/ml (△), staurosporine, 1.0 μM (●), 2.0 μM (▽) or Me_2SO (○), for up to 120 s. Reactions were stopped and inositol phosphate levels determined as described in section 2. Results were calculated as % of basal radioactivity in Me_2SO-treated cells at 0 time. Each point represents the mean ± S.E.M. of four separate experiments.

of intracellular Ca^{2+} without an accompanying influx of cations.

PLC activity in platelets prelabeled with [^{3}H]myo-inositol was measured as rises in [^{3}H]-(InsP$_3$). (InsP$_3$ isomers were not resolved by anion-exchange chromatography.) Positive controls show that thrombin induced rapid hydrolysis of PIP2; InsP$_3$ levels were maximally elevated at the earliest time sampled (10 s) after thrombin addition to platelets. Thereafter, InsP$_3$ activity declined towards basal levels (Fig. 2). Staurosporine, 1–2 μM, also induced rapid formation of InsP$_3$ which plateaued at 15–20 s but remained elevated after 2 min. The maximal levels of InsP$_3$ detected were similar to that found for thrombin. This response was dose-dependent with significant InsP$_3$ formation occurring at submicromolar concentrations of staurosporine (Fig. 3). Staurosporine-dependent InsP$_3$ formation was abolished when platelets were treated with 20 mM neomycin, an inhibitor of phosphoinositide signalling [15]. This result among others suggests that staurosporine increased InsP$_3$ levels by activation of the PLC-PIP2 hydrolysis pathway.

The product of the preceding pathway, Ins(1,4,5)P$_3$, was specifically quantitated using a competitive binding assay for this second messenger. Results show that 2 μM staurosporine elevated Ins(1,4,5)P$_3$ to about 50% of levels induced by thrombin (Fig. 4). The lower amount of Ins(1,4,5)P$_3$ detected by this method compared with results obtained by anion-exchange chromatography may arise from the presence of species other than Ins(1,4,5)P$_3$ in the InsP$_3$ fraction eluted from columns. Besides InsP$_3$ isomers, this fraction contains contaminating [^{3}H]inositol mono- and bisphosphates [9].

Further studies show that staurosporine increased the linear rate of GTP hydrolysis in crude platelet membranes. As the results in Fig. 5 indicate, staurosporine increased the GTPase activity in a dose-dependent manner; 5 μM staurosporine was as potent as thrombin in stimulating GTPase. By comparison, the kinase inhibitors, K252a (2 μM) (structurally related to staurosporine [1]), and H7 (20 μM) did not significantly affect this activity at the concentrations tested.

4. Discussion

Based on results of assays for inositol phosphates, we conclude that micromolar concentrations of staurosporine activated the PIP2/PLC pathway in human platelets. The suppression of InsP$_3$ formation by neomycin, a known inhibitor of phosphoinositide signalling, further implicates the involvement of PLC (Fig. 3). Unlike thrombin which caused a transient rise in InsP$_3$, staurosporine produced a sustained elevation if InsP$_3$ (Fig. 2). This may be due partly to concurrent inhibition of PKC-dependent reactions which convert Ins(1,4,5)P$_3$ to other inositol phosphates. Previously, King and Rittenhouse [3] found that PKC promotes the metabolism of Ins(1,4,5)P$_3$ to other inositol phosphates by enhancing the activities of InsP$_3$ 5-phosphatase and 3-kinase.

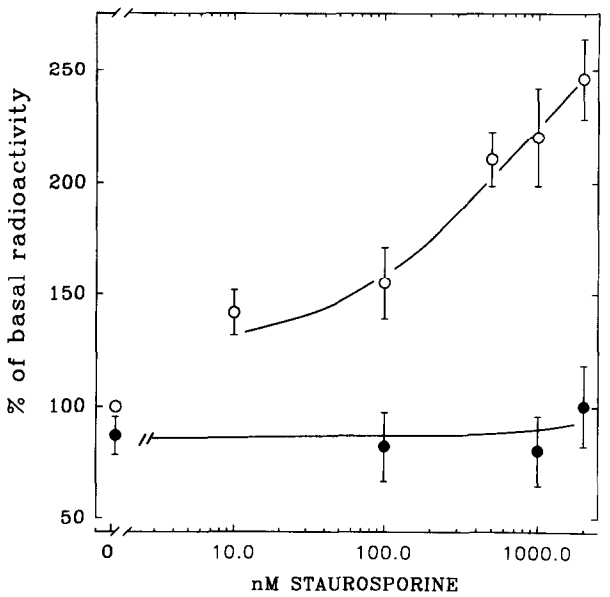

Fig. 3. Dose–response effect of staurosporine on platelet InsP$_3$ and the effect of neomycin. [^{3}H]InsP$_3$recovered from platelets after 60 s incubation with staurosporine (○–○) or staurosporine plus 20 mM neomycin (●–●) were calculated as % of basal radioactivity in Me_2SO-treated cells. Results represent the mean ± S.E.M. of 4 (○) or 3 (●) separate experiments.

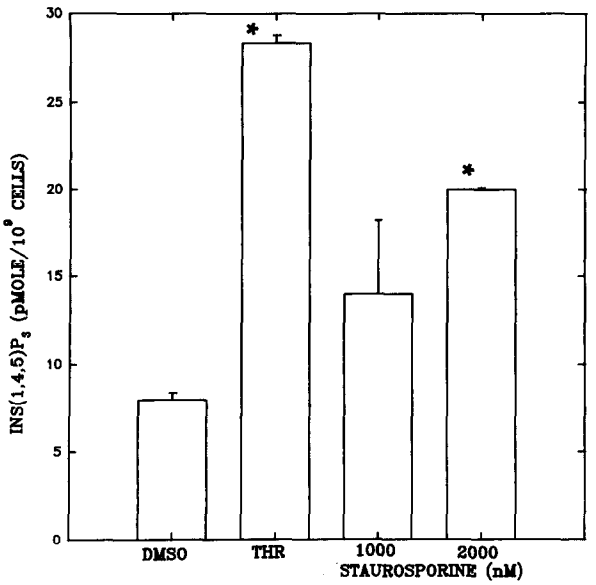

Fig. 4. Ins(1,4,5)P_3 formation in staurosporine- or thrombin-treated platelets. Ins(1,4,5)P_3 levels in platelets incubated for 15 s with thrombin, staurosporine or Me_2SO were quantified by the Ins(1,4,5)P_3 ligand binding assay as described in section 2. Values are the mean ± S.E.M. of three separate experiments. *Significantly different compared with Me_2SO control values ($P < 0.05$) by the Student's paired t-test.

The mechanism by which staurosporine activated PLC in platelets remains to be elucidated. Indirect activation via thromboxane A_2 (TXA_2) generation appears unlikely as assays for its metabolite, TXB_2, by a radioimmunoassay method (NEN, Dupont) failed to detect significant amounts of this agent (results not shown). It is possible that staurosporine interacted directly with a receptor or a G protein coupled to PLC. The latter conjecture is supported by Kanaho and coworkers' experiments showing that staurosporine activates purified G_{i1} reconstituted into phospholipid vesicles [16]. Also in keeping with this hypothesis is the finding that staurosporine stimulated GTPase activity in platelet membranes (Fig. 5).

There is evidence that PIP2 hydrolysis in platelets may also be mediated by a tyrosine kinase-activated phospholipase C-gamma [17]. However it is unlikely that staurosporine activated this pathway since it inhibits tyrosine kinase activities [17].

Additional studies found that micromolar staurosporine did not induce platelets to aggregate or to secrete 5-hydroxytryptamine (results not shown). These results are not surprising since staurosporine itself inhibited PKC and caused only a weak rise in $[Ca^{2+}]_i$ - two important components for signal transduction in platelets. These negative results agree with findings reported by various groups which have used up to 10 μM staurosporine to inhibit platelet PKC. Heretofore, control assays showed that staurosporine on its own does not elicit platelet responses such as aggregation and degranulation [2–5]. We note that Murphy et al. [4] reported no effect for 1 μM staurosporine on Ins(1,4,5)P_3 in rabbit platelets. Species differences may account for the difference in results. Besides its stimulation of PLC described here, staurosporine has been shown to both inhibit and activate subsets of serine/threonine kinases in platelets [18].

A puzzling finding of present experiments was that staurosporine induced a weak response on platelet $[Ca^{2+}]_i$ compared with its effect on $InsP_3$. Staurosporine inhibition of Ca^{2+} mobilization by Ins(1,4,5)P3 is unlikely since it potentiated rather than inhibited the effect of thrombin on platelet $[Ca^{2+}]_i$ (Fig. 1). Other factors may account for its weak effect on $[Ca^{2+}]_i$. Firstly, average changes in cellular $InsP_3$ are not necessarily correlated with increases in $[Ca^{2+}]_i$ [19]. Secondly, initial increases in $[Ca^{2+}]_i$ triggered by platelet agonists are amplified by phospholipase A_2 activation and TXA_2 formation [20–22]. It is possible that staurosporine failed to activate the amplification pathway or that it blocks amplification at an early step. Direct staurosporine inhibition of TXA_2 synthesis can be ruled out since 1 μM staurosporine has been shown to enhance thromboxane formation in platelets stimulated by platelet activating factor [4].

Acknowledgements: Present studies were funded by grants from the Medical Research Council of Canada and the Betty Bedford Boyd Endowment Fund.

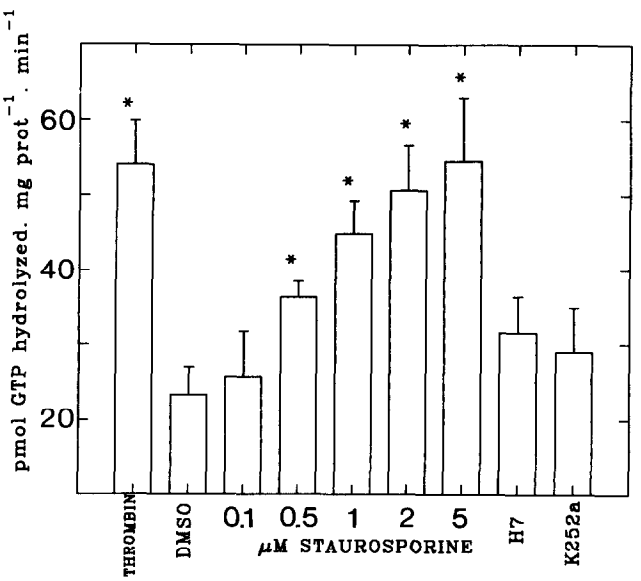

Fig. 5. GTPase activity of platelet membranes treated with staurosporine. Platelet membranes were prepared and incubated at room temperature for 10 min in the presence of thrombin, 2 U/ml; vehicle control, 0.4% Me_2SO; staurosporine, 0.1 to 5 μM; K252a, 2 μM; and H7, 20 μM. Results are the average (± S.E.M.) of 4 experiments. *Significantly different from vehicle control at $P < 0.05$.

References

[1] Rüegg, U.T. and Burgess, G.M. (1989) Trends Pharmacol. Sci. 10, 218–220.
[2] Watson, S.P., McNally, J., Shipman, L.J. and Godfrey, P.P. (1988) Biochem. J., 249, 345–350.
[3] King, W.G. and Rittenhouse, S.E. (1989) J. Biol. Chem. 264, 6070–6074.
[4] Murphy, C.T., Elmore, M., Kellie, S. and Westwick, J. (1991) Biochem. J. 278, 255–261.
[5] Wheeler-Jones, C.P., Saermark, T., Kakkar, V.V. and Authi, K.S. (1992) Biochem. J. 281, 465–472.
[6] Yatomi, Y., Ozaki, Y., Koike, Y., Satoh, K. and Kume, S. (1993) Biochem. Biophys. Res. Commun. 191, 453–458.
[7] Wong, K., Kwan-Yeung, L. and Turkson, J. (1992) Biochem. J. 283, 499–505.
[8] Himpens, B., De-Smedt, H. and Casteels, R. (1993) Am. J. Physiol. 264, C544-C551.
[9] Lapetina, E.G. and Siess, W. (1987) Methods Enzymol. 141, 176–193
[10] Wong, K., Parente, J., Prasad, K.V.S. and Ng, D. (1990) J. Biol. Chem. 265, 21454–21461.
[11] Pollock, W.K., Rink, T.J. and Irvine, R.F. (1986) Biochem. J. 235, 869–877.
[12] Gawler, D. and Houslay, M.D. (1987) FEBS Lett. 216, 94–97.
[13] Herman, E. and Jakobs, K.H. (1988) FEBS Lett. 229, 49–53.
[14] Rink, T.J. and Sage, S.O. (1990) Annu. Rev. Physiol. 52, 431–449.
[15] Bishop, W.R., August, J., Petrin, J.M. and Pai, J. (1990) Biochem. J. 269, 465–473.
[16] Kanaho, Y, Takahashi, K., Tomita, U., Iiri, T., Katada, T., Ui, M. and Nozawa, Y. (1992) J. Biol. Chem. 267, 23554–23559.
[17] Yatomi, Y., Ozaki, Y., Satoh, K. and Kume S. (1993) FEBS Lett. 322, 285–290.
[18] Kocher, M. and Clemetson, K.J. (1991) Biochem. J. 275, 301–306.
[19] Meyer, T., Holowka, D. and Stryer, L. (1988) Science 240, 653–656.
[20] Siess, W. (1989) Physiol. Rev. 69, 58–178.
[21] Hourani, S.M.O. and Cusack, N.J. (1991) Pharmacol. Rev. 43, 243–298.
[22] Knezevic, I., Dieter, J.P. and Le-Breton, G.C. (1992) J. Pharmacol. Exp. Ther. 260, 947–955.