# The recombinant GABA transporter GAT1 is downregulated upon activation of protein kinase C

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**Abstract Treatment of human embryonic kidney 293 cells expressing the rat γ-aminobutyric acid (GABA) transporter 1 (GAT1) with the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) was found to decrease the velocity of specific [$^3$H]GABA uptake. This downregulation varied with extracellular GABA concentration and was blocked by the PKC inhibitors 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H7) and staurosporine. An about 50% reduction of uptake velocity by PMA treatment was observed at GABA concentrations >1 μM, whereas only a minor effect was seen at low substrate concentrations. These data indicate that GAT1 activity is downregulated by PKC activation.**

*Key words:* GABA uptake; Neurotransmitter transporter; Protein kinase C; Rat

## 1. Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate central nervous system, where it activates a variety of GABA receptor subtypes. The actions of GABA released upon nerve stimulation are thought to be terminated by its rapid re-uptake via GABA transporters into presynaptic neurons and surrounding glial cells [1,2]. Recently, cDNAs encoding four different GABA transporters (GAT1–4; reviewed in [3]) have been isolated. The corresponding transcripts show distinct distribution patterns, and the respective proteins differ in their pharmacological characteristics. Thus, in addition to the known heterogeneity of GABA receptors, the various GATs contribute to the molecular heterogeneity of GABAergic synapses, and modulation of their activity could add another level of synaptic regulation.

Most neurotransmitter transporters contain putative phosphorylation sites [1], and some have been shown to be regulated via kinase systems [4–8]. In case of the major GABA transporter in mammalian brain, GAT1, the data reported sofar are controversial. Upon treatment of *Xenopus* oocytes expressing GAT1 with phorbol ester known to activate protein kinase C (PKC), Osawa and colleagues [9] observed a downregulation in GABA transport resulting from a decrease in substrate affinity. In contrast, when using the same heterologous expression system, Corey et al. [10] found an upregulation of GABA uptake caused by an increase in the maximal transport velocity ($V_{max}$) upon phorbol ester treatment. This discrepancy may reflect differences in drug application, since the former investigators added the ester to the incubation medium, whereas Corey et al. injected it directly into the oocytes. Here, we used a mammalian cell expression system to re-analyse PKC modulation of this transporter protein. The human embryonic kidney cell line 293 (HEK-293) has been widely employed to express brain membrane proteins [11,12], and we therefore transfected GAT1 cDNA into these cells for investigating the effect of phorbol ester. Our data show that the velocity of GABA uptake mediated by GAT1 is decreased upon activation of PKC.

## 2. Materials and methods

### 2.1. Materials

[$^3$H]GABA (86 Ci/mmol) was obtained from Amersham. Phorbol 12-myristate 13-acetate (PMA), staurosporine, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H7), amiloride, and dimethylamiloride all were purchased from Sigma.

### 2.2. Cloning of GAT1 cDNA

The cDNA coding for GAT1 was isolated by screening a λZAPII rat spinal cord library with a PCR fragment containing nucleotide positions 46–1773 of the GAT1 sequence [13]. This resulted in the isolation of a full-length cDNA encompassing all nucleotides of the published GAT1-coding sequence and 150 and 2104 bp of the 5′ and 3′ untranslated regions, respectively [13]. For heterologous expression, the coding sequence of GAT1, including 150 bp of 5′ untranslated sequence and 165 bp of 3′ untranslated sequence, was subcloned into the *Not*I/*Hpa*I sites of the eucaryotic expression vector pCIS [14]. The resulting clone, termed pGAT, was used throughout this study.

### 2.3. Cell transfection and [$^3$H]GABA uptake assays

HEK-293 cells (ATCC CRL 1573) were grown and transfected as described elsewhere [15,16]. For uptake measurements, cells were plated into 24 well dishes ($d$ = 2 cm) and transfected with 1 μg of DNA. 36–48 h after transfection, the culture medium was replaced by 0.2 ml of transport buffer (TB1: 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.5) containing radioactively labelled substrate. After incubation for 3 min (if not indicated otherwise ) at room temperature, the medium was removed by suction, and the cells were washed twice with TB1 and then extracted with 0.4 ml of 10% (w/v) sodium dodecylsulfate. Radioactivity was determined by scintillation counting using a Beckman LS60001IC scintillation counter. PMA treatment of the cultures was performed for 1 h before the determination of [$^3$H]GABA uptake, if not indicated otherwise. All data represent the means of quadruplicate deteminations. S.E. values (indicated by bars) were routinely < 10%. Protein concentrations were determined using the Detergent Compatible Protein Assay (BioRad), using bovine serum albumin as a standard.

## 3. Results

### 3.1. [$^3$H]GABA uptake by recombinant GAT1

Functional expression of GAT1 was achieved by transient

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*Abbreviations*: GABA, γ-aminobutyric acid; GAT, GABA transporter; H7, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine; HEK cell, human embryonic kidney cell; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; SERT, serotonin transporter; TPA, 12-0-tetradecanoylphorbol-13-acetate; PDD, 4α-phorbol-12,13-didecanoate.


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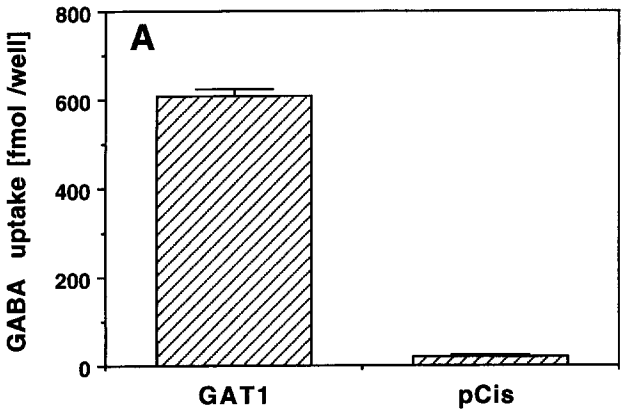


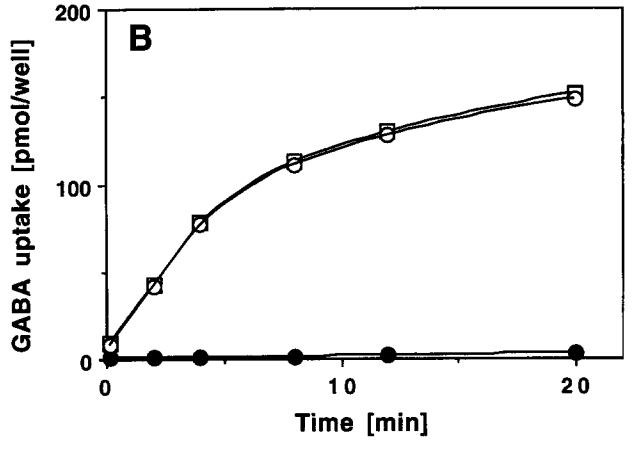




Fig. 1. (A) [$^3$H]GABA uptake into HEK-293 cells. Cells transfected with pGAT1 or pCis were incubated with 60 nM [$^3$H]GABA. [$^3$H]GABA uptake was determined after 12 min. (B) Time Course of [$^3$H]GABA uptake into HEK-293 cells. Cells transfected with pGAT1 (□) or pCis (●) were incubated with 1 $\mu$M GABA containing 10 nM [$^3$H]GABA as tracer, and [$^3$H]GABA uptake was monitored at the time points indicated. ○, GAT1-mediated uptake.

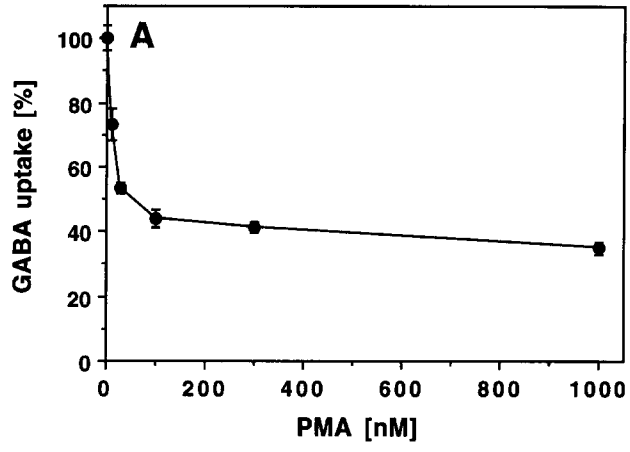
transfection of HEK-293 cells with pGAT cDNA. 36–48 h after transfection, the cells were incubated with [$^3$H]GABA, and its uptake was determined as described under section 2. Compared to HEK-293 cells transfected with insert-less vector (control), pGAT-transfected cells showed an about 30× higher uptake of the radiolabelled neurotransmitter (Fig. 1A). The very low uptake seen with control cells indicates that HEK-293 cells have no endogenous high-affinity GABA uptake system.

Fig. 1B shows the kinetics of [$^3$H]GABA uptake. At a [$^3$H]GABA concentration of 1 $\mu$M, uptake was linear for up to 4 min. In addition, uptake was also linear over this time period at other (5 nM-500 $\mu$M) GABA concentrations (data not shown). Thus, uptake was routinely measured after a 3-min incubation. Competition studies with increasing concentrations of unlabelled substrate revealed a saturable specific GABA uptake with a $V_{max}$ value of 275 ± 59 pmol/min/well and a $K_m$ value of 15 ± 5.4 $\mu$M ($n$ = 2).

### 3.2. *PMA treatment decreases GAT1-mediated [$^3$H]GABA uptake*

In order to investigate the role of PKC in GABA transport regulation, pGAT1-transfected HEK-293 cells were incubated with medium containing increasing concentrations of the phorbol ester PMA for 1 h before measuring [$^3$H]GABA transport. This resulted in a decline of GAT1-mediated [$^3$H]GABA uptake, which reached a plateau at about 100 nM PMA (Fig. 2A). Fig. 2B shows the time dependence of PMA treatment at a concentration of 100 nM. Specific uptake was reduced to about 50% after 1 h of incubation. Thus, in all following experiments preincubation with PMA was performed routinely at a concentration of 100 nM for 1 h.

To confirm that the decrease of GAT1-specific [$^3$H]GABA uptake induced by PMA was indeed caused by PKC activation, we pretreated the transfected cells with inhibitors of the enzyme and also used the inactive phorbol ester 4α-phorbol-12,13-didecanoate (PDD). The latter compound did not induce a detecta-



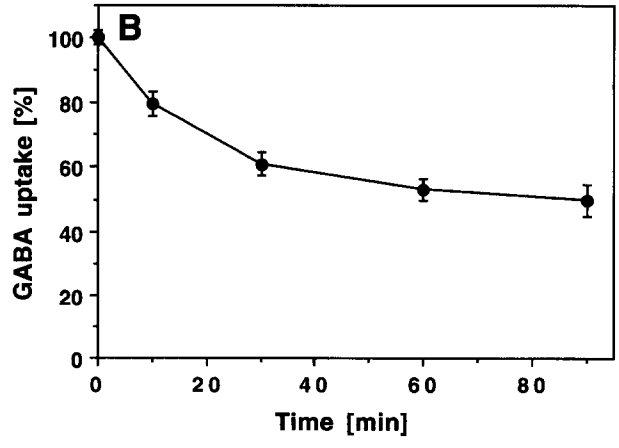




Fig. 2. Effects of PMA on [$^3$H]GABA uptake. (A) Concentration dependence of PMA. Cells were incubated for 1 h at the indicated concentrations of PMA before measuring [$^3$H]GABA uptake at 40 $\mu$M GABA containing 10 nM [$^3$H]GABA as tracer. (B) Time course of PMA effect on [$^3$H]GABA uptake. Cells were incubated in 100 nM PMA for the indicated time period, and GABA uptake determined as in (A).

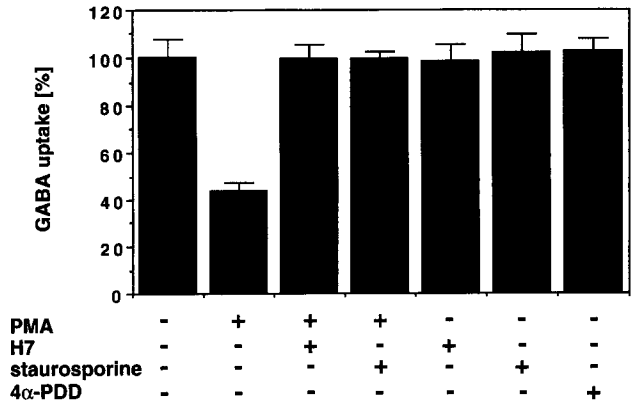



Fig. 3. Effects of PKC inhibitors on PMA-modulated [$^3$H]GABA uptake. Cells were incubated in the presence or absence of H7 (100 $\mu$M) or staurosporine (0.3 $\mu$M) for 1 h. Then 100 nM PMA, or 4$\alpha$-PDD, was added for another hour as indicated, and the specific uptake of [$^3$H]GABA was determined at 40 $\mu$M GABA containing 10 nM [$^3$H]GABA as tracer.

ble decrease of [$^3$H]GABA uptake (Fig. 3). The PKC inhibitors staurosporine (0.3 $\mu$M) and H7 (100 $\mu$M) both caused a complete block of the PMA-induced downregulation of GAT1 activity (Fig. 3). At the indicated concentrations, both kinase inhibitors did not affect GAT1-mediated GABA uptake when applied alone (Fig. 3).

### 3.3. Saturation analysis of [$^3$H]GABA uptake

To examine the mechanism of GAT1 downregulation by PMA, we determined [$^3$H]GABA uptake at different substrate concentrations before and after phorbol ester treatment. An Eadie–Hofstee analysis [17] showed that pretreatment with PMA reduced the $V_{max}$ from 275 ± 59 pmol/well/min to 118 ± 0.01 pmol/well/min without significantly affecting its $K_m$ value (control, 15 ± 5.4 $\mu$M; PMA-treated, 9.4 ± 2.7 $\mu$M; $n$ = 2; Fig. 5). Determination of the protein contents in control and treated wells confirmed that PMA treatment did not change the total protein content of the cultures (data not shown).

### 3.4. Reduction of [$^3$H]GABA uptake by PMA varies with external GABA concentration

When measuring the PMA-induced reduction of [$^3$H]GABA uptake at different external GABA concentrations, high GABA concentrations always gave a larger reduction of GABA uptake by PMA than observed at lower substrate concentrations. To corroborate this finding, we determined the extent of PMA-induced inhibition over a wide range of external GABA concentrations. Fig. 5A shows that at GABA concentrations < 500 nM, inhibition was about 25% and largely independent of the substrate concentration. At GABA concentrations > 500 nM, however, inhibition decreased to ≈ 50% inhibition at 50 $\mu$M, i.e. a concentration at which GAT1-mediated transport is saturated (cf. Fig. 4).

### 3.5. PMA does not affect the $Na^+/H^+$ exchange system

Treatment of some cell types with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is known to activate an amiloride-sensitive $Na^+/H^+$ exchange system, which results in alkalization and an increase in intracellular sodium concentration [18,19]. This TPA-induced $Na^+/H^+$ exchange system is inhibited by amiloride and dimethylamiloride, a more potent derivatives [18,19]. To investigate the possible involvement of this exchange system in the reduction of [$^3$H]GABA uptake seen upon PMA treatment, we analysed GAT1-mediated uptake in the presence of saturating concentrations of amiloride and dimethylamiloride, respectively. At both 10 and 100 $\mu$M, neither of these drugs affected the PMA-mediated decrease in [$^3$H]GABA uptake seen in GAT1-transfected cells (data not shown). Therefore, the reduction of GAT1-mediated [$^3$H]GABA uptake seen upon PMA treatment is not due to PKC effects on the $Na^+/H^+$ exchange system.

## 4. Discussion

In the present study we show that activation of PKC causes a downregulation of recombinant rat GAT1 expressed in HEK-293 cells. Treatment of the GAT1 expressing cells with low doses of the phorbol ester PMA resulted in a reduction of the velocity of [$^3$H]GABA uptake without affecting the substrate affinity of the transporter. Preincubation with the PKC inhibitors staurosporine and H7 caused a complete block of the PMA-induced downregulation of GAT1 activity, whereas the inactive phorbol ester 4$\alpha$-PDD had no effect. Thus, the decrease of specific GAT1-mediated [$^3$H]GABA uptake induced by PMA is due to PKC activation. However, wether this regulation results from a direct phosphorylation of the transporter molecule or involves indirect mechanisms, e.g. activation of another signaling system that regulates GAT1 activity, is not clear. Also, we do not know whether the actual number of GAT1 molecules on the cell surface is altered upon PMA treatment, since high-affinity ligands or specific antibodies are not available for this transporter. Our observation that the extent of PMA-induced reduction of GAT1 activity correlates with extracellular substrate concentration, is consistent with the idea that not the number of GAT1 molecules on the cell surface, but their transport properties are changed upon PKC activation. Interestingly, a maximal reduction of [$^3$H]GABA uptake by PMA was observed at extracellular GABA concentrations be-

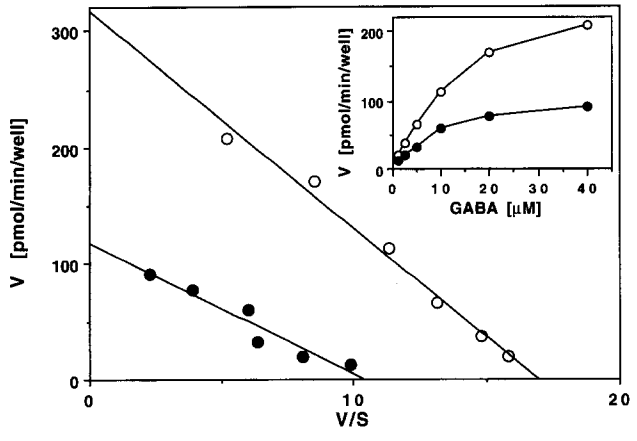



Fig. 4. Inhibition of [$^3$H]GABA uptake by PMA. Saturation characteristics were determined by the addition of unlabelled GABA to 10 nM [$^3$H]GABA using both PMA-treated (●) and untreated cells (○). Transport kinetic values were calculated using the Eadie–Hofstee transformation. $K_m$ and $V_{max}$ values obtained in this particular experiment were 18.7 $\mu$M and 317 pmol/min/well (control cells) and 11.3 $\mu$M and 118 pmol/min/well (PMA-treated cells). The inset shows the respective saturation curves.

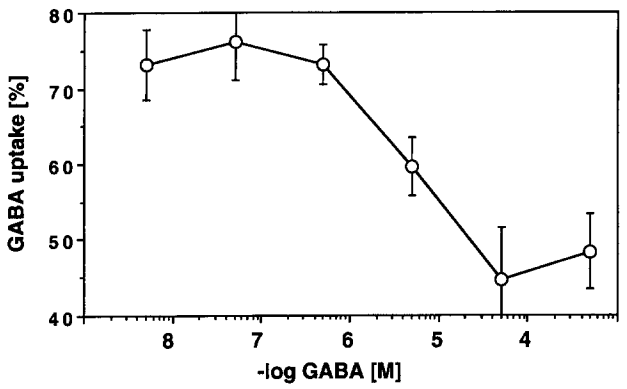



Fig. 5. Effects of PMA at different extracellular GABA concentrations. Cells were incubated in the presence or absence of 100 nM PMA for 1 h before determining specific [$^3$H]GABA uptake at the indicated GABA concentrations.

tween 1 and ≈ 50 $\mu$M, i.e. in a concentration range that is close to the KD values of both $GABA_A$ receptor binding [20] and GAT1-mediated transport. Thus, PKC-induced changes of GAT1 activity could contribute to the control of synaptic efficacy in GABAergic systems.

Regulation of recombinant GAT1 function by PKC phosphorylation has been previously analysed by expressing GAT1 in *Xenopus* oocytes. However, the results obtained were contradictory, since one study reported a PMA-induced GAT1 downregulation resulting from a decrease of transporter affinity [9], whereas the other found enhanced [$^3$H]GABA transport caused by an increase in $V_{max}$ value [10]. PKC is known to comprise a multigene family that encodes several distinct isoforms [21]. Upon activation by phorbol esters, these isoenzymes are specifically translocated to particular subcellular structures. This suggests that PKC substrate specificity in vivo is critically dependent on the respective cellular localization of distinct isoenzymes [22]. In addition, it has been shown that closely related isoforms of PKC can cause reciprocal effects upon activation by phorbol esters [23]. This may explain how different forms of application of phorbol esters (bath application and slow diffusion versus direct cytosolic injection) could activate different PKC isoforms, which in turn produce opposite effects. The observation that removal of all consensus PKC sites did not affect the modulation of GAT1 by PMA [10] implies that PKC modulation in PMA injected oocytes involves an indirect mechanism. In contrast, the data of Osawa et al. [9] may reflect direct phosphorylation and consequently a distinct regulatory mechanism.

Different lines of evidence indicate that the activity of most neurotransmitter transporter proteins is subject to PKC regulation. Stimulation of PKC has been shown to downregulate transporter-mediated neurotransmitter uptake into different subcellular fractions and cellular systems, including (i) glycine uptake in human placental choriocarcinoma, C6 glioblastoma cells and mammalian cells expressing a recombinant glycine transporter [7,8,24], (ii) high-affinity GABA uptake in rat cortical synaptosomes, primary neurons and glial cells from rat brain cortex [9,25], (iii) serotonin uptake into human platelets [6,26], and (iv) dopamine uptake into COS cells expressing a recombinant dopamine transporter [5]. In case of serotonin and dopamine uptake, it could be shown that the observed reduction in $V_{max}$ values did not result from a decreased number of transporter molecules on the cell surface, since PMA treatment had no effect on the $B_{max}$ values of radiolabelled antagonists [5,6,26]. Currently, it is not known wether a similar PKC-driven mechanism causes the downregulation of GABA transporter activity reported here.

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## References

[1] Clark, J.A. and Amara, S.G. (1993) BioEssays 15, 323–332.
[2] Bennett, J.P., Mulder, A.H. and Snyder, S.H. (1974) Life Sci. 15, 1045–1056.
[3] Schloss, P., Püschel, A.W. and Betz, H. (1994) Curr. Opin. Cell Biol. 6, 595–599.
[4] Casado, M., Bendahan, A., Zafra, F., Danbolt, N.C., Aragon, C., Gilmenez, C. and Kanner, B. (1993) J. Biol. Chem. 268, 27313–27317.
[5] Kitayama, S., Dohi, T. and Uhl, G.R. (1994) Eur. J. Pharmacol. 268, 115–119.
[6] Anderson, G.M. and Horne, W.C. (1992) Biochem. Biophys. Acta 1137, 331–337.
[7] Gomeza, J., Zafra, F., Olivares, L., Giménez, C. and Aragon, C. (1995) Biochim. Biophys. Acta 1233, 41–46.
[8] Sato, K., Adams, R., Betz, H. and Schloss, P. (1995) J. Neurochem., in press.
[9] Osawa, I., Saito, N., Koga, T. and Tanaka, C. (1994) Neurosci. Res. 19, 287–293.
[10] Corey, J.L., Davidson, N., Lester, H.A., Brecha, N. and Quick, M. (1994) J. Biol. Chem. 269, 14759–14767.
[11] Cammack, J.N., Rakhilin, S.V. and Schwartz, E.A. (1994) Neuron 13, 949–960.
[12] Schloss, P. and Betz, H. (1995) Biochemistry, in press.
[13] Guastella, J., Nelson, N., Nelson, H., Czyzyk, L., Keynan, S., Miedel, M.C., Davidson, N., Lester, H.A. and Kanner, B.I. (1990) Science 249, 1303–1306.
[14] Gormann, C.M., Gies, D., McCray, G. and Huang, M. (1989) Virology 171, 377–385.
[15] Sontheimer, H., Becker, C.-M., Prichett, D.B., Schofield, P.R., Grenningloh, G., Kettenmann, H., Betz, H. and Seeburg, P. (1989) Neuron 2, 1491–1497.
[16] Chen, C. and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2751.
[17] Connors, K.A. (1987) Binding constants: the measurement of molecular complex stability. Wiley, New York, NY.
[18] Besterman, J.M., May, Jr., W.S., LeVine, III, H., Cragoe, Jr., E.J. and Cuatrecasas, P. (1985) J. Biol. Chem. 260, 1155–1159.
[19] Wiener, E., Dubyak, G. and Scarpa, A. (1986) J. Biol. Chem. 261, 4529–4534.
[20] Krishek, B.J., Xie, X., Blackstone, C., Huganir, R.L., Moss, S.J. and Smart, T.G. (1994) Neuron 12, 1081–1095.
[21] Nishizuka, Y. (1992) Science 258, 607–614.
[22] Goodnight, J., Mischak, H., Kolch, W. and Mushinski, J.F. (1995) J. Biol. Chem. 270, 9991–10001.
[23] Borner, C., Ueffing, M., Jaken, S., Parker, P.J. and Weinstein, I.B. (1995) J. Biol. Chem. 270, 78–86.
[24] Liu, W., Leibach, F.H. and Ganapathy, V. (1994) Biochim. Biophys. Acta 1194, 176–184.
[25] Gomeza, J., Casado, M., Giménez, C. and Aragon, C. (1991) Biochem. J. 275, 435–439.
[26] Ahtee, L., Briley, M., Raisman, R., Lebrec, D. and Langer, S.Z. (1981) Life Sci. 29, 2323–2329.