Conventional protein kinase C isoforms regulate human dopamine transporter activity in *Xenopus* oocytes

Suzanne Doolen*, Nancy R. Zahniser

Department of Pharmacology and Neuroscience Program, C-236, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262, USA

Received 11 January 2002; revised 28 February 2002; accepted 28 February 2002

First published online 13 March 2002

Edited by Maurice Montal

Abstract The hypothesis that specific protein kinase C (PKC) isoforms regulate dopamine transporter (DAT) function was tested in *Xenopus laevis* oocytes expressing human (h)DAT. Activation of conventional PKCs (cPKCs) and novel PKCs (nPKCs) using 10 nM phorbol 12-myristate 13-acetate (PMA) significantly inhibited DAT-associated transport currents. This effect was reversed by isoform-non-selective PKC inhibitors, selective inhibitors of cPKCs and δPKC, and by Ca^{2+} chelation. By contrast, the εPKC translocation inhibitor peptide had no effect on PMA-induced inhibition of hDAT transport-associated currents. Thus, the primary mechanism by which PMA regulates hDAT expressed in oocytes appears to be by activating cPKC(s).

Key words: Electrophysiology; Neurotransmitter; Transporter regulation; Trafficking

1. Introduction

Dopamine (DA) transporter (DAT) belongs to a superfamily of Na^+/Cl^--dependent neurotransmitter transporters that also includes transporters for serotonin, norepinephrine, and γ-aminobutyric acid (GABA) [23]. DAT is crucial for clearing DA from the extracellular space. DAT transports substrates such as DA against their electrochemical gradient by coupling to the inward transport of DA^+ with two Na^+ and one Cl^- ions [5,10,12]. Thus, at negative membrane potentials (−120 to −20 mV) a net positive inward current is produced by substrate transport. In *Xenopus* oocytes expressing human (h)DAT, this inward current reflects real-time measurements of transporter function [18].

It has been well established that protein kinase C (PKC) activation inhibits DAT function by decreasing the number of functional transporters on the cell surface. In support of this concept, functional studies have consistently demonstrated PKC activation causes a decrease in DAT maximal transport velocity (V_{max}) and DAT-associated transport currents with no change in substrate affinity (K_M) [1,2,6,9,13,16,20,24,25]. Furthermore, several experimental approaches have been used to show a PKC-induced redistribution of DAT away from the cell surface membrane [2,13,16,24].

There are at least 12 PKC isoforms of PKC that are divided into three subfamilies based on structure and function [14]. Conventional PKC (cPKC) isoforms (α, βI, βII, γ) have a putative Ca^{2+}-binding site and are Ca^{2+}-sensitive. Novel PKC (nPKC) isoforms (δ, ε, η, θ) lack a putative Ca^{2+}-binding site and are Ca^{2+}-independent. Both cPKCs and nPKCs are activated by phorbol esters such as phorbol 12-myristate 13-acetate (PMA). Atypical PKC isoforms (ζ, ι, λ, μ) are Ca^{2+}-insensitive and are not activated by phorbol esters. Several pharmacological inhibitors have been developed to investigate the role of PKC experimentally. For example, chelerythrine and bisindolylmaleimide (BIS) I are structurally dissimilar, and both inhibit all PKC isoforms. Gö 6979 selectively inhibits the Ca^{2+}-sensitive cPKC isoforms (IC_{50} = 2.3–7.9 nM) [11]. Rottlerin is thought to be a selective inhibitor for δPKC (IC_{50} = 3–6 μM). However, rottlerin also inhibits Ca^{2+}/calmodulin kinase III at similar concentrations (IC_{50} = 5.3 μM) and is active at 10-fold higher concentrations against recombinant cPKC isoforms (IC_{50} = 30–42 μM) [4]. PKC subcellular localization and function involve the binding of PKC to isoform-specific receptors for activated C kinases (RACKs) [17]. Inhibition of this interaction disrupts substrate phosphorylation and function of PKC isoforms. For example, the εPKC translocation inhibitor peptide binds the εPKC-specific RACK and inhibits PMA-induced εPKC activity [8].

It is not yet known which of these PKC isoform(s) contribute(s) to DAT regulation. In the present study, pharmacological and biochemical approaches were used to investigate this question in hDAT-expressing oocytes. Western blot analysis using *Xenopus* oocytes has demonstrated the presence of cPKC and nPKC isoforms including α-, βI-, βII-, γ-, δ-, and εPKC [7,21]. The atypical PKC isoforms were not considered in this study because phorbol esters that mediate hDAT regulation do not activate these isoforms.

2. Materials and methods

2.1. Materials

BIS I (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3yl)-maleimide), BIS V (2,3-bis(1*H*-indol-3-yl)-*N*-methylmaleimide), chelerythrine chloride, Gö 6979 (12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-a)pyrrolo(3,4-c)-carbazole), PMA, εPKC translocation inhibitor peptide and rottlerin were purchased

*Corresponding author. Fax: (1)-303-315 7097.
E-mail address: suzanne.doolen@uchsc.edu (S. Doolen).

Abbreviations: BIS, bisindolylmaleimide; cPKC, conventional PKC; DA, dopamine; DAT, DA transporter; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; FRB, frog Ringer's buffer; GABA, γ-aminobutyric acid; hDAT, human DAT; PKC, protein kinase C; nPKC, novel PKC; PMA, β-phorbol 12-myristate 13-acetate; RACK, receptors for activated C kinase

0014-5793/02/$22.00
PII: S0014-5793(02)02554-1

from Calbiochem (La Jolla, CA, USA). DA was purchased from Sigma (St. Louis, MO, USA).

2.2. Animals and oocyte preparation

Oocyte positive *Xenopus laevis* frogs were purchased from *Xenopus* I (Ann Arbor, MI, USA). All animal use procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee, University of Colorado Health Sciences Center. Stage V and VI oocytes were defolliculated by gentle shaking in OR2 buffer (82 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 5 mM HEPES, pH 7.5) containing 1.4–2.0 mg/ml collagenase B (Boehringer Mannheim, Indianapolis, IN, USA) for approximately 2 h at room temperature.

2.3. DAT expression

Capped cRNA was transcribed from a linear oocyte expression vector pOTV containing the 1.9 kb hDAT cDNA insert [18] using mMessage mMachine with T7 polymerase (Ambion, Austin, TX, USA). Oocytes were injected with water-diluted cRNA (~10 ng) and maintained at room temperature in frog Ringer's buffer (FRB; 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.5) supplemented with 2.5 mM sodium pyruvate, 0.5 mM theophylline, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin for 3–4 days.

2.4. Two-electrode voltage-clamp electrophysiology

Currents were measured in oocytes using two-electrode voltage-clamp. Microelectrodes were filled with 3 M KCl [18]. A Warner OC-725B amplifier (Warner Instruments, Hamden, CT, USA) was used with a DigiData 1200 interface. pClamp6 software (Axon Instruments, Foster City, CA, USA) was used to control stimulation parameters, for data acquisition and for analysis. MacLab data acquisition software (AD Instruments, Castle Hill, Australia) and a MacLab/2e interface were used to monitor experiments. Currents were low-pass-filtered at 100 Hz and digitized at 2048 Hz.

Oocytes were superfused in a 0.5 ml bath at approximately 2 ml/min with normal FRB (unless otherwise indicated) at room temperature. Oocytes were voltage-clamped at −60 mV and then subjected to a series of 400 ms steps in membrane potential ranging from −120 mV to +40 mV in 10 mV increments. Currents were recorded before and again after a 1 min superfusion with 10 μM DA. Steady-state currents were measured as the average of the last 100 ms of each voltage step. Inward currents were calculated by performing off-line subtraction of currents measured in the presence of DA from current measured in the absence of DA ($I_{DA}-I_{buffer}$). Effects of PKC activation on DA-induced currents were determined after superfusing oocytes with FRB containing 10 nM PMA for 3 min followed by a 10 min wash with FRB. In some oocytes superfusion with PMA was preceded by a 3 min superfusion with a PKC inhibitor, and the inhibitor was present during PMA superfusion. Subsets of oocytes were injected with 32 nl of selective PKC inhibitor to achieve the intracellular concentration indicated in Section 3, assuming the volume of an oocyte to be 1000 nl. Injected oocytes were allowed to recover until the resting membrane potential stabilized (approximately 10 min) before measuring hDAT-mediated currents. These injections had no effect on DA-induced currents.

2.5. Data analysis

Currents from each oocyte were normalized to the control hDAT-mediated current measured at −120 mV. For statistical analysis, transport-associated currents were compared at −60 mV. This membrane potential was chosen to approximate the resting potential of a neuron. Statistical significance was determined by unpaired Student's *t*-test with a significance criterion of $P < 0.05$.

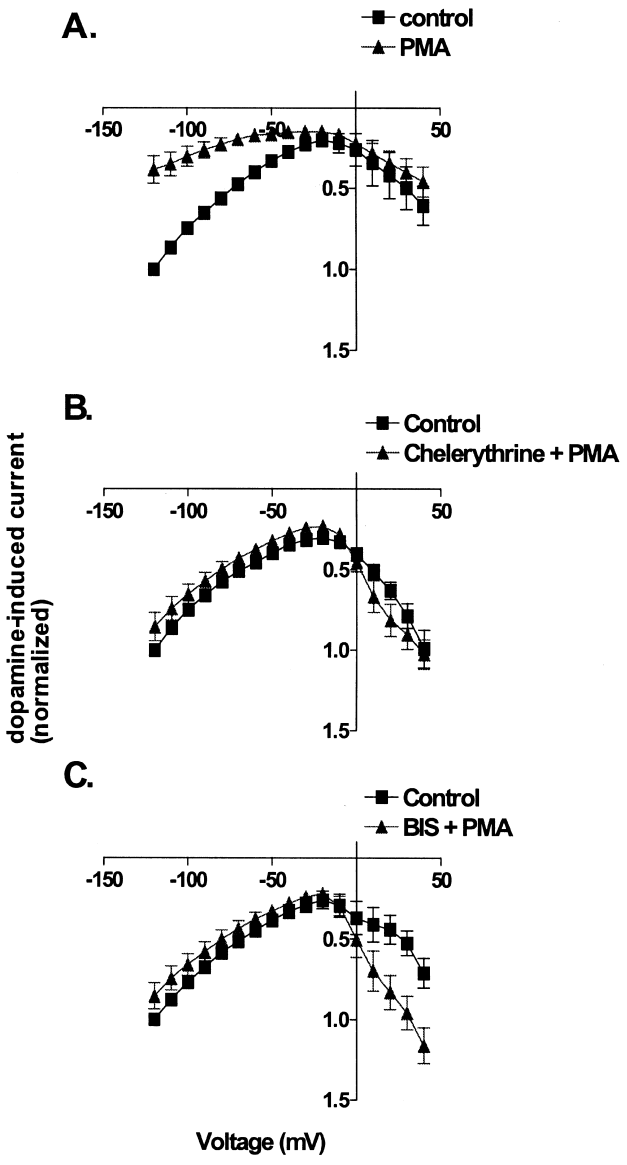

Fig. 1. Effect of PMA and PKC inhibitor pretreatment on hDAT-mediated currents. Two-electrode voltage-clamp recording was used to measure hDAT-associated currents as described in Section 2. Current–voltage (*I–V*) relationships are shown for hDAT-mediated currents elicited by superfusion with a maximally effective concentration of the hDAT substrate DA (10 μM). After control DA-induced currents were measured, oocytes were superfused with FRB containing drugs: (A) 10 nM PMA, (B) 1 μM chelerythrine followed by 10 nM PMA, or (C) 1 μM BIS I followed by 10 nM PMA. Oocytes were superfused with PKC inhibitors for 3 min and then inhibitor plus PMA for 3 min. DA-induced currents were measured again 10 min following drug treatment. Data represent mean ± S.E.M. for seven to nine oocytes from three batches of oocytes. At −60 mV, unpaired *t*-tests showed $P < 0.05$ for control vs. PMA only in A.

3. Results and discussion

3.1. PMA reduces hDAT-mediated currents via PKC activation

It has been well documented that PMA decreases DAT function via redistribution of the transporter away from the cell surface [1,2,6,9,13,16,20,24,25]. These previous studies include one from our laboratory that showed that PMA exposure to hDAT-expressing oocytes results in attenuation of hDAT-mediated currents [25]. In the present study, the PKC isoform(s) involved in hDAT regulation were investigated. Oocytes expressing hDAT were superfused for 3 min with a near-maximal PMA concentration (10 nM) [25] and then washed with FRB for 10 min. DAT substrate transport-associated currents predominate at −120 to −20 mV while a DAT-associated leak current is evident at −20 to

+40 mV [18]. This leak current shows more variable effects and will not be addressed here. Similar to our previous observations, PMA treatment decreased hDAT transport-associated currents induced by a maximally effective DA concentration (10 μM) (Fig. 1A). For example, at −60 mV the hDAT-associated current after PMA treatment was 42.5% of the control current (Fig. 1A).

Two structurally dissimilar isoform-non-selective PKC inhibitors, namely chelerythrine and BIS I, were used in conjunction with PMA to confirm that PMA-mediated induced attenuation of hDAT transport-associated currents was occurring via PKC activation. In oocytes pretreated with either 1 μM chelerythrine or 1 μM BIS I followed by PMA, the PMA-induced attenuation of hDAT transport-associated currents was abolished, compared to PMA pretreatment alone (Fig. 1B,C). An inactive analog of BIS I, BIS V, had no effect on hDAT-mediated currents, suggesting BIS I blocks the effects of PMA by selectively inhibiting PKC activation (data not shown). None of the inhibitors when used alone in this study affected the oocyte resting membrane potential or control currents (data not shown).

3.2. Effects of cPKC inhibition on PMA-induced attenuation of hDAT-mediated currents

Several lines of evidence suggest that Ca^{2+}-dependent cPKCs contribute to PMA-mediated hDAT regulation. First,

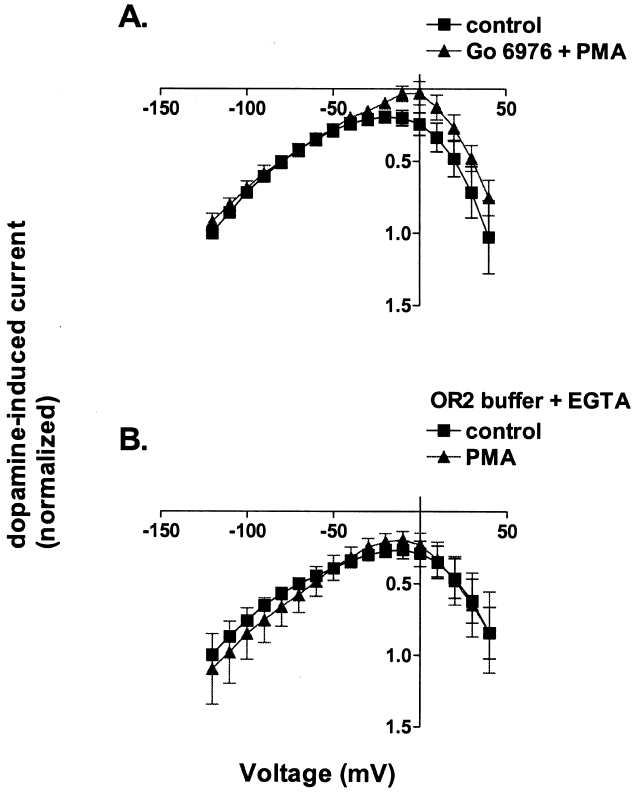

Fig. 2. Effect of cPKC inhibition on PMA-induced inhibition of hDAT-mediated currents. See Fig. 1 for experimental details. DA-induced currents are shown before (control) and after 3 min superfusion with 10 nM PMA. See Fig. 1A for control response to PMA alone. A: Gö 6976 (32 nl; final concentration 100 nM) was injected directly into oocytes before current measurements. B: Oocytes were superfused with Ca^{2+}-free OR2 buffer containing 0.5 mM EGTA throughout current measurements. Data represent mean ± S.E.M. for six to nine oocytes from three batches of oocytes.

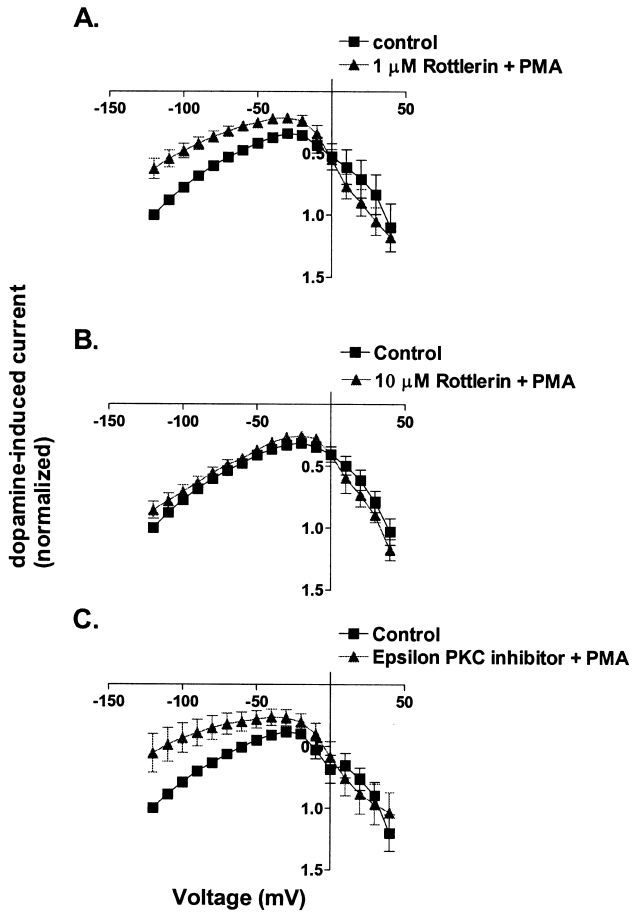

Fig. 3. Effect of nPKC inhibitors on PMA-induced inhibition of hDAT transport-associated currents. See Fig. 1 for experimental details. DA-induced currents are shown before (control) and after 3 min superfusion with 10 nM PMA. See Fig. 1A for control response to PMA alone. In A and B, after control DA-induced currents were measured, oocytes were superfused with FRB containing (A) 1 μM or (B) 10 μM rottlerin followed by PMA. Drug treatment included superfusion with rottlerin for 3 min followed by a 3 min exposure to PMA and rottlerin. DA-induced currents were measured again 10 min following drug treatment. C: 32 nl (8 ng) εPKC translocation inhibitor peptide was injected directly into hDAT-expressing oocytes prior to current measurements. Data represent mean ± S.E.M. for seven to nine oocytes from three batches of oocytes. At −60 mV, unpaired *t*-tests showed $P < 0.05$ for control vs. PMA in A and C.

the selective cPKC inhibitor, Gö 6976, blocked inhibition of hDAT transport-associated currents by PMA. Gö 6976 (32 nl; final concentration 100 nM) was injected directly into oocytes, and the oocytes were allowed to recover until the resting potential stabilized before currents were recorded. As seen in Fig. 2A, the effect of PMA was abolished in Gö 6976 pretreated oocytes compared with oocytes in the absence of Gö 6979 (see Fig. 1A). At −60 mV the hDAT-associated current was 97.2% of control current. Gö 6979 was more effective when injected directly into the oocyte compared with superfusion, suggesting limited permeability into the oocyte (data not shown).

Second, the attenuation on hDAT transport-associated currents by PMA was reversed in the presence of EGTA (ethylenebis(oxyethylenenitrilo)tetraacetic acid), a Ca^{2+} chelator (Fig. 2B), suggesting that the effect of PMA on hDAT func-

tion is Ca^{2+}-dependent. The fact that the effect of PMA was completely reversed in the presence of the cPKC inhibitor Gö 6976 or in the absence of Ca^{2+} suggests not only that cPKC isoforms are involved in PMA-mediated hDAT regulation, but also that they may be exclusively responsible for PMA-mediated decrease in hDAT transport-associated currents.

3.3. Effects of nPKC inhibitors on PMA-induced attenuation of hDAT-mediated currents

Rottlerin is thought to selectively inhibit the δPKC isoform [4]. In oocytes that were pretreated with rottlerin followed by PMA, the PMA-induced attenuation of hDAT transport-associated currents was reversed in a concentration-dependent manner, and the effect of PMA was completely blocked by 10 μM rottlerin (Fig. 3A,B; refer to Fig. 1A for control response to PMA). These results suggest the δPKC isoform also contributes to PMA-mediated hDAT regulation. However, this may not be the case as several groups have reported that rottlerin inhibits other kinases including cPKCs [3,4,22]. It is important to note that inhibitor selectivity studies were performed in purified bovine and human PKC preparations while the present experiments were performed in *Xenopus* oocytes expressing native amphibian PKC isoforms. Thus, the potency of these inhibitors may differ from that reported in the literature, and it is plausible that rottlerin inhibits cPKC isoforms in this preparation. Furthermore, the fact that two different methods used to inhibit cPKC activity reversed PMA-mediated hDAT inhibition suggests that δPKC cannot be completely responsible for hDAT regulation. Thus, one interpretation is that rottlerin reversed the effect of PMA on hDAT-associated currents by inhibiting cPKC isoforms. It is also possible that both cPKC and δPKC isoforms act as a cascade, but it seems much less likely that a PKC substrate would phosphorylate another PKC isoform. It is not plausible that the cPKC and δPKC isoforms substitute for one another or that either may be part of cascades that converge. If this were the case, activation of cPKCs and nPKCs followed by selective blockade would not have resulted in complete reversal of PKC activation.

Activation and translocation of PKC can occur via the binding of the PKC isoform to specific RACKs. When a εPKC translocation inhibitor peptide (8 ng) was injected directly into hDAT-expressing oocytes, PMA-mediated hDAT transport-associated currents were unaltered, suggesting that εPKC does not contribute to PKC regulation of hDAT (Fig. 3C).

Western blot analysis has confirmed the presence of cPKCs (α, βI, βII, and γ) in dopaminergic brain regions in rat including the nucleus accumbens, striatum, substantia nigra, and ventral tegmental area [19]. Immunostaining has revealed δPKC expression in rat substantia nigra [15]. Thus, it is reasonable that these PKC isoforms regulate DAT function and therefore synaptic DA levels in the brain. More selective inhibitors are needed before it can be determined precisely which cPKC isoform(s) regulate(s) DAT. It is also interesting to note that Na^+/Cl^--dependent transporters for serotonin, norepinephrine, and GABA are inhibited by PKC activation [23]. Future studies should reveal if the same PKC isoforms that regulate DAT function also regulate these transporters or if distinct isoforms regulate each transporter.

Acknowledgements: We would like to thank Dr. Eric Nelson for helpful discussions and critically reading of this manuscript. We gratefully acknowledge support from the National Institute on Drug Abuse to N.R.Z. DA04216, Alexander Sorkin DA14204 and N.R.Z. DA00174.

References

[1] Copeland, B.J., Vogelsberg, V., Neff, N.H. and Hadjiconstantinou, M. (1996) J. Pharmacol. Exp. Ther. 277, 1527–1532.
[2] Daniels, G.M. and Amara, S.G. (1999) J. Biol. Chem. 274, 35794–35801.
[3] Davies, S.P., Reddy, H., Caivano, M. and Cohen, P. (2000) Biochem. J. 351, 95–105.
[4] Gschwendt, M., Muller, H.J., Kielbassa, K., Zang, R., Kittstein, W., Rincke, G. and Marks, F. (1994) Biochem. Biophys. Res. Commun. 199, 93–98.
[5] Gu, H., Wall, S.C. and Rudnick, G. (1994) J. Biol. Chem. 269, 7124–7130.
[6] Huff, R.A., Vaughn, R.A., Kuhar, M.J. and Uhl, G.R. (1997) J. Neurochem. 68, 225–232.
[7] Johnson, J. and Capco, D.G. (1997) Mech. Dev. 67, 215–226.
[8] Johnson, J.A., Gray, M.O., Chen, C.H. and Mochly-Rosen, D. (1996) J. Biol. Chem. 271, 24962–24966.
[9] Kitayama, S., Dohi, T. and Uhl, G.R. (1994) Eur. J. Pharmacol. 268, 115–119.
[10] Krueger, B.K. (1990) J. Neurochem. 55, 260–267.
[11] Martiny-Baron, G., Kazanietz, M.G., Mischak, H., Blumberg, P.M., Kochs, G., Hug, H., Marme, D. and Schachtele, C. (1993) J. Biol. Chem. 13, 9194–9197.
[12] McElvain, J.S. and Schenk, J.O. (1992) Biochem. Pharmacol. 43, 2189–2199.
[13] Melikian, H.E. and Buckley, K.M. (1999) J. Neurosci. 19, 7699–7710.
[14] Mellor, H. and Parker, P.J. (1998) Biochem. J. 332, 281–292.
[15] Merchenthaler, I., Liposits, Z., Reid, J.J. and Wetsel, W.C. (1993) J. Comp. Neurol. 336, 378–399.
[16] Pristupa, Z.B., McConkey, F., Liu, F., Man, H.Y., Lee, F.J., Wang, Y.T. and Niznik, H.B. (1998) Synapse 30, 79–87.
[17] Ron, D., Chen, C.H., Caldwell, J., Jamieson, L., Orr, E. and Mochly-Rosen, D. (1994) Proc. Natl. Acad. Sci. USA 91, 839–843.
[18] Sonders, M.S., Zhu, S.-J., Zahniser, N.R., Kavanaugh, M.P. and Amara, S.G. (1997) J. Neurosci. 17, 960–974.
[19] Steketee, J.D., Rowe, L.A. and Chandler, L.J. (1998) Neuropharmacology 37, 339–347.
[20] Vaughan, R.A., Huff, R.A., Uhl, G.R. and Kuhar, M.J. (1997) J. Biol. Chem. 272, 15541–15546.
[21] Xiao, G.Q., Qu, Y., Sun, Z.Q., Mochly-Rosen, D. and Boutjdir, M. (2001) Am. J. Physiol. Cell Physiol. 281, c1477–c1486.
[22] Way, K.J., Chou, E. and King, G.L. (2000) Trends Pharmacol. Sci. 21, 181–187.
[23] Zahniser, N.R. and Doolen, S. (2001) Pharmacol. Ther. 92, 21–55.
[24] Zhang, L. and Reith, M.E. (1996) Eur. J. Pharmacol. 21, 345–354.
[25] Zhu, S.J., Kavanaugh, M.P., Sonders, M.S., Amara, S.G. and Zahniser, N.R. (1997) J. Pharmacol. Exp. Ther. 282, 1358–1365.