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Expression of a bovine vesicular monoamine transporter in COS cells

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Abstract

Catecholamines are accumulated in vesicles by a proton gradient-dependent transport, which has mostly been studied in bovine chromaffin granules. The full sequence of a cDNA encoding a vesicular transporter from bovine chromaffin cells, $bVMAT_2$, was recently reported. We now present an analysis of $bVMAT_2$, expressed in transfected COS cells. Comparing the binding of a labelled ligand, [^{3}H]TBZOH, and the rate of uptake, we find a much lower molecular turnover number than in chromaffin granules, probably indicating that a majority of expressed transporters are correctly folded and possess the ligand binding site but cannot actively transport monoamines because they are located in compartments which do not possess a proton gradient. The substrate specificity of uptake and its pharmacological sensitivity to various inhibitors closely resemble those previously observed in chromaffin granules. These results suggest that $VMAT_2$ is the major transporter in bovine adrenal glands, and raise the question of the significance of the second related transporter, $VMAT_1$, which is also expressed in this tissue.

Key words: Chromaffin granule; Neurotransmitter transporter; COS cell

1. Introduction

In endocrine or neuronal monoaminergic cells, the monoamines (or their precursors) are transported and stored in vesicles prior to their release. The transport is catalyzed by a specific protein, the vesicular monoamine transporter, which utilizes the energy of the H^+ electrochemical gradient generated by an ATP-dependent H^+ pump of the V type [1–3]. The transporter has a low substrate specificity since it transports all monoamines as well as other non-physiological compounds, such as MIBG (*meta*-iodobenzylguanidine) [4] or MPP^+ (1-methyl-4-phenylpyridinium) [5].

The molecular properties of the vesicular monoamine transporter have been investigated by biochemical and pharmacological approaches. For the latter approach, several ligands were developed which bind specifically to the vesicular monoamine transporter [6]. Dihydrotetrabenazine (TBZOH) and ketanserin bind to the same site with dissociation constants in the nanomolar concentration range, and binding is unaffected by the H^+ electrochemical gradient. On the other hand, reserpine binding (K_D 30 pM) is dependent upon the proton gradient since it is strongly accelerated when the H^+ pump is activated, thus suggesting the existence of a different binding site.

These studies, which were performed mostly on the chromaffin granules of bovine adrenal medulla but also on various rodent tissues [7,8], did not suggest any heterogeneity of the vesicular monoamine transporter, for instance between neuronal and endocrine tissues. The vesicular monoamine transporter has recently been cloned in rat [9,10], bovine [11,12] and human [13]. Surprizingly, in rat two cDNA clones were described [9] which encode two related proteins. These proteins have the same general organization with 12 putative transmembrane domains, and a large intravesicular loop located between transmembrane segments 1 and 2. The first gene, $VMAT_1$ (formerly named CGAT) was reported to be expressed in adrenal medulla but not in brain, whereas the converse is true for the second one, $VMAT_2$ (formerly named SVAT). In transfected CHO cells, $VMAT_1$ induced an efficient transport activity. However, this transport was sensitive to TBZ only in the micromolar concentration range. Erickson et al. [10] independently obtained a clone identical to $VMAT_2$ and reported that the encoded transporter was more sensitive to TBZ.

The interpretation of the existence of two genes encoding VMAT has been complicated by the recent finding [11,12] that in bovine adrenal medulla a gene highly related to $VMAT_2$ was expressed at a high level. This gene, $bVMAT_2$, was assumed to encode the vesicular

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Abbreviations: bVMAT, bovine vesicular monoamine transporter; TBZ, tetrabenazine; TBZOH, 2-hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b (H)benzo[1]quinolizine; [^{3}H]TBZOH, [2-^{3}H]dihydrotetrabenazine; RES, reserpine; MPP^+, 1-methyl-4-phenylpyridinium; MIBG, *meta*-iodobenzylguanidine; CCCP, carbanylcyanide *m*-chlorophenylhydrazone.

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monoamine transporter since the protein sequence derived from bVMAT$_2$ contained peptides determined by sequencing the bovine protein.

In the present communication, we describe the expression of bovine VMAT$_2$ in COS cells and we characterize the corresponding activity by assaying ATP-dependent [^{3}H]noradrenaline uptake and [^{3}H]TBZOH binding.

2. Materials and methods

2.1. Transfections

The complete bVMAT$_2$ clone [11] was subcloned into the pCDM8 expression vector. The corresponding nucleotide data sequence was registered in the EMBL, Genbank and DDBJ Nucleotide sequence Databases under accession number X76380. Transfections in COS-7 cells were performed as described [14].

2.2. [^{3}H]Noradrenaline uptake and [^{3}H]TBZOH binding

Three days after transfection, cells were scraped into ice-cold 0.3 M sucrose/10 mM HEPES (Na^+) buffer, pH 7.6, containing 1 mM EGTA, 5 μg/ml aprotinin and 6 μg/ml leupeptin. The suspension was homogenized with a glass–Teflon homogenizer and centrifuged for 5 min at 1,500 × *g* to remove cell debris. Homogenates were used within 24 h of their preparation. Noradrenaline uptake was measured as described previously [15], using undiluted [^{3}H]noradrenaline (16 Ci/mmol) from Amersham and HAWP filters (Millipore). For competition experiments, different procedures were followed for substrates (catecholamines, serotonin, MPP^+ and MIBG) and for inhibitors (RES, TBZ and ketanserin), in order to reach steady-state conditions in all cases. For transport experiments, the homogenate was preincubated for 15 min in the presence of ATP-Mg, and the uptake was initiated by the simultaneous addition of [^{3}H]noradrenaline (0.08 μM final concentration) and the competitor. In the second case, the homogenate was preincubated for 90 min in the presence of ATP-Mg and of the inhibitor. In both cases, uptake was stopped by dilution with ice-cold buffer.

TBZOH binding was measured as described [16], using [^{3}H]TBZOH (11.4 Ci/mmol) from C.E.A. (Saclay, France) and HAWP filters (Millipore). In competition experiments, homogenates were incubated for 9 h in the presence of 4.8 nM [^{3}H]TBZOH and various concentrations of competitor.

3. Results

COS cells were transfected with bVMAT$_2$ inserted into the pCDM-8 vector [17], as described previously [14]. After 3 days, the cells were homogenized and the homogenate was incubated with [^{3}H]noradrenaline, in the presence of ATP. We observed an uptake of radioactivity (Fig. 1A). [^{3}H]Noradrenaline could be released by osmotic shock, thus indicating that it was accumulated in closed membrane structures. As in the case of chromaffin granules, this process was ATP-dependent and driven by an H^+ electrochemical gradient, presumably generated by a V-type proton pump since it was inhibited by the H^+ ionophore, CCCP (Fig. 1A, inset). The uptake was blocked by 20 μM TBZ and 2 μM RES. The TBZ-sensitive [^{3}H]noradrenaline uptake was not observed in non-transfected COS cells, in cells treated without DNA, or in cells transfected with the vector alone (see Fig. 1B).

In the presence of ATP, the TBZ-sensitive [^{3}H]noradrenaline uptake was linear for about 5 min (Fig. 1A). The transport was saturable (Fig. 1B) and linearization of the saturation isotherm (Fig. 1B, inset) indicated a Michaëlis constant, K_M, of 1.9 μM and a maximal velocity, V_{max},

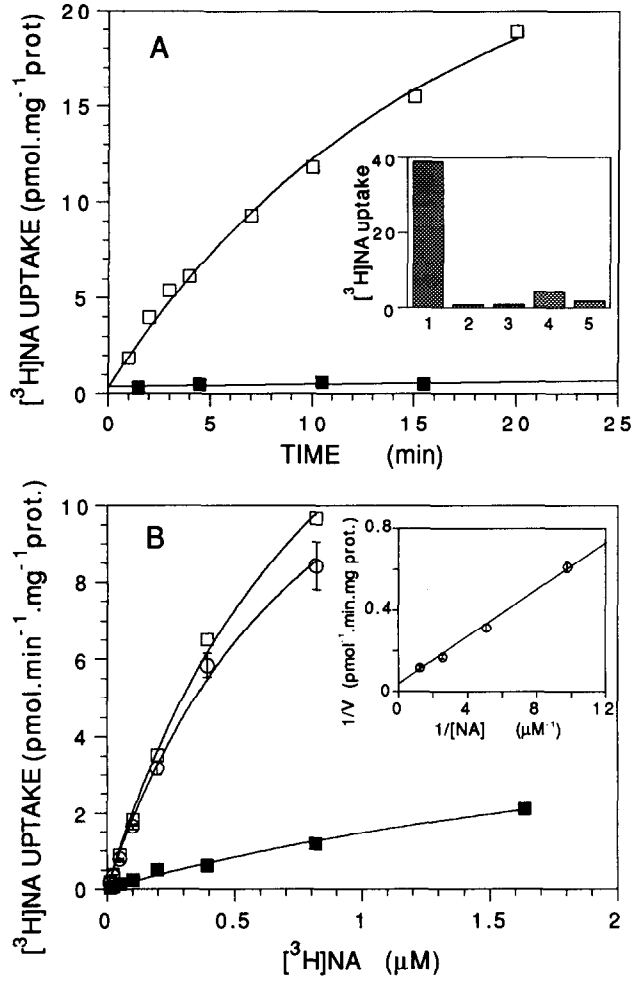

Fig. 1. [^{3}H]Noradrenaline transport in bVMAT$_2$-transfected COS cells. (A) Accumulation of noradrenaline as a function of time. The cell homogenate (0.5 mg of protein/ml) was preincubated for 15 min at 28°C in 0.3 M sucrose, 2.5 mM ATP, 1.25 mM $MgSO_4$, 10 mM HEPES (Na^+) buffer pH 7.6, with (■) or without (□) 5 μM TBZ. Uptake was initiated by the addition of 0.1 μM [^{3}H]noradrenaline. Non-linear regression analysis of the data gave an exponential curve with $t_{1/2}$ = 10.5 min (r = 0.977). (Inset) The cell homogenate was preincubated in the presence of ATP-Mg (1–4), 20 μM TBZ (2), 2 μM RES (3) or 10 μM CCCP (4) and incubated for 30 min with [^{3}H]noradrenaline (1–5). In (5), ATP was omitted. (B) Saturation isotherm. Uptake was measured after a 4-min incubation at different [^{3}H]noradrenaline concentrations. The results are the means of three determinations. The TBZ-sensitive uptake (○) was obtained as the difference between transport in the absence (□) and in the presence (■) of TBZ. No TBZ-sensitive uptake could be detected in homogenates from untreated COS cells or cells treated in the absence of DNA, which gave results similar to those observed in the presence of TBZ for bVMAT$_2$-transfected cells. (Inset) Lineweaver–Burke plot of the data. Linear regression indicated a V_{max} value of 32 pmol/min/mg of protein and K_M value of 1.9 μM (r = 0.998). Similar values were obtained in an independent transfection experiment.

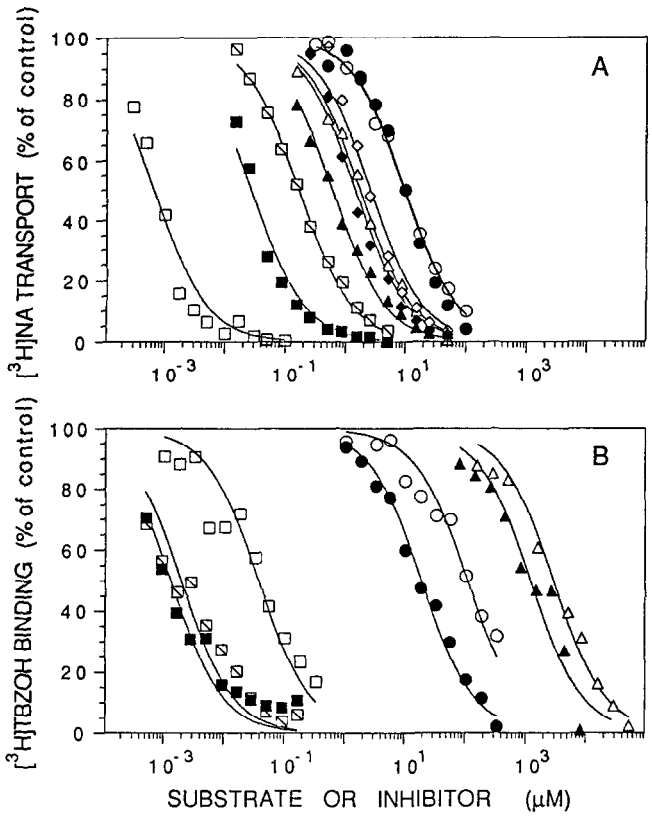

Fig. 2. Pharmacological profiles of [^{3}H]noradrenaline uptake and [^{3}H]TBZOH binding to homogenates of transfected COS cells expressing bVMAT$_2$. (A) [^{3}H]Noradrenaline uptake. (B) [^{3}H]TBZOH binding. Both activities were assayed in parallel experiments on the same homogenate at 0.4 mg of protein/ml in 0.3 M sucrose, 10 mM HEPES (Na^+), pH 7.6, containing 2.5 mM ATP, 1.3 $MgSO_4$, and various concentrations of the competitors, at 26°C. The results are expressed as percentage of the control in the absence of competitor. The following competitors were used: RES (□), TBZ (■), ketanserin (□), noradrenaline (△), adrenaline (◇), dopamine (◆), serotonin (▲), MPP^+ (○) and MIBG (●).

of 32 pmol/min/mg of protein (pH 7.6, 28°C). The affinity of the uptake system for various substrates was determined by competition experiments (Fig. 2). IC_{50} values derived from these experiments are very similar to those obtained previously for bovine chromaffin granules (Table 1).

We also observed a saturable binding of the tritiated derivative TBZOH (Fig. 3A). The amount of bound ligand was larger than expected from the V_{max} of uptake (see section 4). From a Scatchard plot of a saturation isotherm experiment (Fig. 3B), we obtained an equilibrium dissociation constant (K_D) of 6.7 nM and a number of binding sites (B_{max}) of 4.5 pmol/mg protein (pH 7.6, 28°C). The pharmacological profile of the TBZ binding sites was defined by displacement experiments (Fig. 2B). The corresponding IC_{50} values (Table 1) are in reasonably good agreement with data previously reported for bovine chromaffin granules.

4. Discussion

Transfection of COS cells with bVMAT$_2$ resulted in the expression of an ATP-dependent noradrenaline uptake activity in membranes derived from the transfected cells. This result shows clearly that bVMAT$_2$ encodes the vesicular monoamine transporter. This result also shows that, in the non-endocrine COS cells, the vesicular monoamine transporter is, at least partially, addressed to a cellular compartment possessing an ATP-dependent H^+ pump, since the observed [^{3}H]noradrenaline uptake is ATP-dependent and is blocked by the H^+ ionophore, CCCP. A similar conclusion has been reached for rat [9,10] and human [13] vesicular transporters (rVMAT$_2$ and hVMAT$_2$), which were expressed in chinese hamster ovary and CV_1 cells, respectively.

In the present report, however, the membranes of transfected cells have been tested not only for [^{3}H]noradrenaline uptake, but also for [^{3}H]TBZOH binding. [^{3}H]TBZOH binding does not require an H^+ electrochemical gradient and it detects transporter molecules irrespective of their vectorial transport activity [6]. For instance, this ligand binds to detergent-solubilized transporter [18]. The number of binding sites measured in COS cells homogenates, B_{max}, was 4.5 pmol/mg of protein, i.e. analogous to that obtained for homogenates of adrenal medulla or chromaffin cells, thus indicating a high level of expression. When ATP-dependent [^{3}H]noradrenaline uptake was measured on the same preparation and under the same conditions, a figure of 32 pmol/min/mg of protein was derived for V_{max}. This figure indicates a comparatively low transport activity since the derived molecular turn-over number of the transfected transporter (V_{max}/B_{max} = 7 molecules/min at 25°C) is much lower than that obtained with intact chromaffin

Table 1
Pharmacological profiles of [^{3}H]noradrenaline uptake and [^{3}H]TBZOH binding in homogenates of transfected COS cells expressing bVMAT$_2$

Compounds	IC_{50} (μM)			
	Noradrenaline uptake		TBZOH binding	
	bVMAT$_2$	Chromaffin granules	bVMAT$_2$	Chromaffin granules
RES	0.0007	0.005	0.038	0.300
TBZ	0.027	0.012	0.0013	0.0027
Ketanserin	0.170	0.07	0.002	0.055
Serotonin	0.59	0.4	1200	330
Dopamine	1.4	–	n.d.	–
Noradrenaline	1.7	1.4	2800	1,000
Adrenaline	2.5	1.4	n.d.	1,000
MPP^+	9.7	1.5	110	70
MIBG	9.4	10	19	26

The data derived from Fig. 2 are compared to those previously obtained in our laboratory for bovine chromaffin granules membrane vesicles, according to [21] for ketanserin, [5] for MPP^+ and B. Gasnier (Ph.D. Thesis) for the others, n.d., not determined.

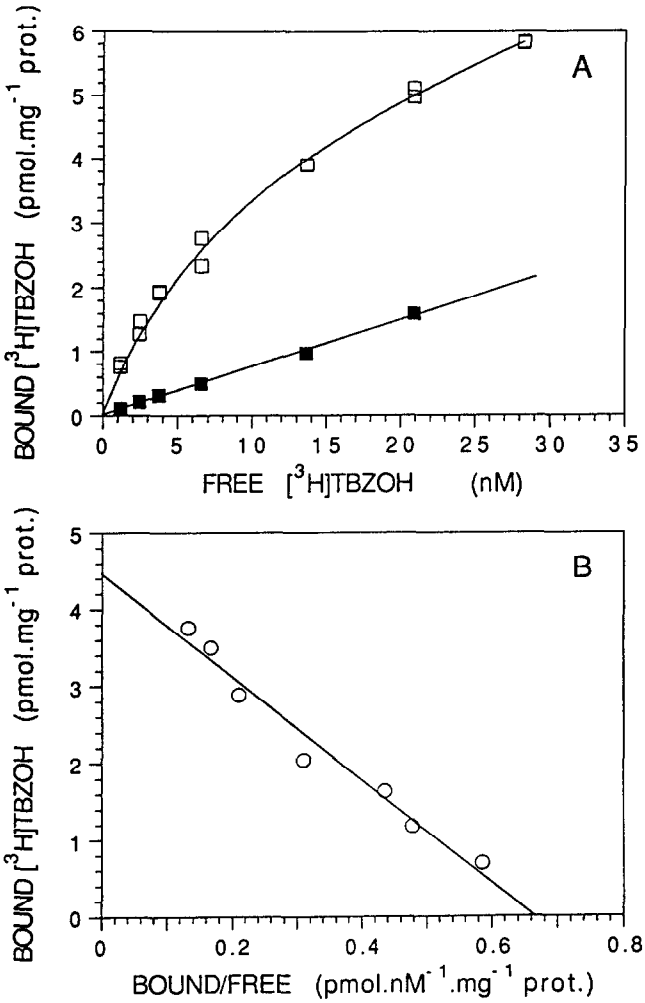

Fig. 3. Saturation isotherm of [^{3}H]TBZOH binding to an homogenate of bVMAT$_2$-transfected COS cells. (A) The homogenate was incubated for 3 h at 28°C with various concentrations of [^{3}H]TBZOH in the absence (□) or in the presence (■) of 5 μM TBZ. These experiments were performed with the same homogenate as those shown in Fig. 1. (B) Scatchard plot of specific [^{3}H]TBZOH binding. The results are the means of two determinations. A K_D value of 6.7 nM and a B_{max} value of 4.5 pmol/mg of protein were derived from these data (r = 0.985). Similar values were obtained in an independent transfection experiment.

granules (140 molecules/min) [8,19]. The low turn-over number in COS cells can be interpreted in several ways. In the first interpretation, the COS cells contain an homogenous population of low activity transporters. Each individual transporter molecule expressed in COS cells would function at only 5% of the rate of the chromaffin cells transporters. This could result from an incomplete processing of the transporter in COS cells. On the other hand, if we assume that the transporter functions as a dimer, the high turn-over number in bovine chromaffin granules might reflect the activity of bVMAT$_1$/bVMAT$_2$ hetero-oligomers, while bVMAT$_2$ homo-oligomers would possess a much lower activity.

In the second interpretation, the COS cells contain a majority of inactive VMAT protein and few (5%) fully active transporters. This second hypothesis appears more likely, and it would explain the apparent discrepancy between the pharmacological inhibition of binding and of uptake by TBZ and ketanserin (see below). The presence of a large pool of non-translocating transporter could result from incomplete processing, or from inadequate subcellular localization, or both. As discussed above, most of the synthesized transporter molecules, although they could bind [^{3}H]TBZOH, would remain inactive because transport activity would require post-translational modifications occurring in the biosynthetic compartments (endoplasmic reticulum, Golgi network) or in the acidic final compartment. It may be noted that the transporter is glycosylated and that it has several putative phosphorylation sites [11]. We may also imagine that all transporters are able to transport monoamines, but that most of them are localized in a compartment which cannot generate an H^+ electrochemical gradient. ATP-dependent noradrenaline uptake may only occur when the monoamine transporter is addressed to a cellular compartment equipped with a V-type ATPase. Along the secretory pathway, the V-type ATPase is present in the trans-Golgi network and in secretory vesicles; it is also present along the endocytotic pathway. Accumulation of the expressed protein in the endoplasmic reticulum, possibly resulting from saturation of the secretory pathway because of protein over-production, might thus explain the low transport/binding ratio.

In endocrine cells, the vesicular monoamine transporter might be present on Golgi-derived secretory granules (e.g. chromaffin granules) or on synaptic-like microvesicles which are sorted from endosomes [20]. At the present time, it is not known whether the two types of organelles utilize the same transporter or are equipped with two different proteins resulting from the expression of the two VMAT genes. The latter hypothesis would imply some sorting mechanism, the efficiency of which would depend upon the type of cell used for the transfection. It will therefore be interesting to express bVMAT$_2$ in endocrine cells.

From a practical point of view, our results show that, in transfected cells, transport assays are not necessarily more sensitive than ligand binding assays, although they benefit from a time-dependent amplification factor. This should be considered when designing strategies for expression cloning of transport proteins.

4.1. *Pharmacological characterization of the expressed bVMAT$_2$*

The pharmacological profiles of both ATP-dependent [^{3}H]noradrenaline uptake and [^{3}H]TBZOH binding have been analyzed (Table 1). They agree reasonably well with those previously reported for chromaffin granules. It may be noted that the affinity for TBZ is in the nanomolar concentration range and that this drug blocks [^{3}H]nor-

adrenaline uptake in the same range. This result differs from that previously reported for $rVMAT_1$-transfected cells, in which the uptake was more resistant to this drug by several orders of magnitude [9]. The affinity for the substrates (5-HT > Dopamine ~ Noradrenaline ~ Adrenaline > MPP^+), as estimated from competition experiments, can be considered to be similar to that reported for rat and human $VMAT_2$ in vivo [13]. It is difficult to appreciate whether this is significantly different from that reported for $rVMAT_1$ [9], for which the rank of order was 5-HT > Adrenaline > Dopamine > Noradrenaline.

In Table 1, the comparison between the pharmacological data obtained for noradrenaline uptake and [^{3}H]TBZOH binding deserves some comment. It is somehow surprising that for the inhibitors TBZ and ketanserin, which bind to the same site, larger concentrations are required to inhibit [^{3}H]noradrenaline uptake than to displace bound [^{3}H]TBZOH. The data of Fig. 2B show, for instance, that ketanserin at 20 nM fully occupies its site without any inhibition of ATP-dependent noradrenaline uptake. This is in contrast with the correlation, previously reported, between the occupancy of TBZ binding sites and the inhibition of monoamine uptake [16]. The most likely explanation is an heterogeneity in the transporter molecules. The active molecules, capable of noradrenaline uptake, represent only a minor population, as previously discussed. The characteristics of TBZ and ketanserin binding to this population would be different from those observed on the major population, because of either a different structure or a different environment.

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References

[1] Njus, D., Kelley, P.M. and Harnadek, G.J. (1986) Biochim. Biophys. Acta 853, 237–265.
[2] Johnson, R.G. (1988) Physiol. Rev. 68, 232–307.
[3] Kanner, B. and Schuldiner, S. (1987) CRC Crit. Rev. Biochem. 22, 1–38.
[4] Gasnier, B., Roisin, M.P., Scherman, D., Coornaert, S., Desplanches, G. and Henry, J.P. (1986) Mol. Pharmacol. 29, 275–280.
[5] Darchen, F., Scherman, D., Desnos, C. and Henry, J.P. (1988) Biochem. Pharmacol. 37, 4381–4387.
[6] Henry, J.P. and Scherman, D. (1989) Biochem. Pharmacol. 38, 2395–2404.
[7] Scherman, D., Boschi, G., Rips, R. and Henry, J.P. (1986) Brain Res. 370, 176–181.
[8] Scherman, D. and Boschi, G. (1988) Neuroscience 27, 1029–1035.
[9] Liu, Y., Peter, D., Roghani, A., Schuldiner, S., Privé, G.G., Eisenberg, D., Brecha, N. and Edwards, R.H. (1992) Cell 70, 539–551.
[10] Erickson, J.D., Eiden, L.E. and Hoffman, B.J. (1992) Proc. Natl. Acad. Sci. USA 89, 10993–10997.
[11] Krejci, E., Gasnier, B., Botton, D., Isambert, M.F., Sagné, C., Gagnon, J., Massoulié, J. and Henry, J.P. (1993) FEBS Lett. 335, 27–32.
[12] Howell, M., Shirvan, A., Stern-Bach, Y., Steiner-Mordoch, S., Strasser, J.E., Dean, G.E. and Schuldiner, S. (1994) FEBS Lett. (in press).
[13] Erickson, J.D. and Eiden, L.E. (1993) J. Neurochem. 61, 2314–2317.
[14] Duval, N., Massoulié, J. and Bon, S. (1992) J. Cell. Biol. 118, 641–653.
[15] Scherman, D. and Henry, J.P. (1981) Eur. J. Biochem. 116, 535–539.
[16] Scherman, D., Jaudon, P. and Henry, J.P. (1983) Proc. Natl. Acad. Sci. USA 80, 584–588.
[17] Arrufo, A. and Seed, B. (1987) Proc. Natl. Acad. Sci. USA 84, 8573–8577.
[18] Scherman, D. and Henry, J.P. (1983) Biochemistry 22, 2805–2810.
[19] Gasnier, B., Scherman, D. and Henry, J.P. (1987) FEBS Lett. 222, 215–219.
[20] Régnier-Vigouroux, A., Tooze, S.A. and Huttner, W. (1991) EMBO J. 10, 3589–3601.
[21] Darchen, F., Scherman, D., Laduron, P. and Henry, J.P. (1988) Mol. Pharmacol. 33, 672–677.