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MONOCLONAL ANTIBODIES REACTIVE WITH A MONOAMINE TRANSPORTER PREPARATION PURIFIED FROM BOVINE ADRENAL CHROMAFFIN GRANULE MEMBRANES

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Abstract—There have been no reports of monoclonal antibodies reactive with vesicular monoamine transporters from any source. Western blotting and ELISA data obtained using polyclonal serum from a mouse immunized with a highly purified bovine chromaffin granule monoamine transporter preparation yielded data consistent with the presence of antibodies to the transporter. Hybridomas produced by polyethylene glycol fusion of spleen cells from the mouse with X63/Ag8.653 myeloma cells were screened in an ELISA using partially purified transporter as coating agent. Of the 1142 wells containing colonies, 14 were positive in the initial screen. Hybridomas from wells testing positive were transferred to 24-well plates, grown up, and rescreened. Those still testing positive were subcloned, and the resulting positive wells containing single colonies were grown up and stocked. Of the 6 positive clones that tolerated freeze/thaw (0.5% of the wells tested), 1 was IgG1 κ, 2 were IgG2aκ, and 3 were IgG2bκ isotypes. Ascites fluid was generated in pristane-primed BALB/c mice using hybridomas that had been cloned 2–3 times, and antibodies purified on immobilized Protein A. Immunoreactivity with a mixture of these antibodies, or with only one of them, coincided with dihydrotetrabenazine (TBZOH) binding activity in fractions eluted from all columns employed in the transporter purification. Antibody from at least one of the clones was capable of removing [^{3}H]TBZOH binding activity from a partially purified preparation of transporter. Monoclonal antibodies exhibiting these properties have not been reported previously.

Key words: monoclonal antibodies; bovine adrenal; monoamine transporter; chromaffin granule; immunoprecipitation; immunoaffinity chromatography

The chromaffin granule isolated from the bovine adrenal medulla has long served as a model system for the study of vesicular storage. Catecholamines and serotonin are concentrated in storage granules and vesicles by a transport system driven by an outwardly-oriented proton electrochemical gradient (see Refs. 1–3 for reviews). The ability of the transporter to bind radiolabeled analogs of the inhibitors reserpine, TBZOH† and 7-azido-8-iodo ketanserin has been employed by three different groups to purify a 70–85 kDa glycoprotein from chromaffin granule membranes [4–6].

Although production of monoclonal antibodies against the transporter has not been reported, such antibodies are desirable for a number of reasons. An unlimited amount of antibody can be produced, and all preparations of a particular monoclonal can be expected to display identical specificities. In addition, the unique specificity of each antibody for a single epitope limits cross-reactivity with related antigens, and can be useful in studies of the structure and membrane orientation of the antigen. In this paper, we describe the production of a bank of monoclonal antibodies using a bovine chromaffin granule monoamine transporter preparation as immunogen. Results of ELISA and immunoprecipitation assays are consistent with the interpretation that at least one of these antibodies is reactive with the monoamine transporter.

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† Abbreviations: TBZOH, dihydrotetrabenazine (2-hydroxy - 3 - isobutyl - 9,10-dimethoxy-1,2,3,4,6,7 - hexa - hydro-11b*H*-benzo[*a*]quinolizine); and WGA, wheat germ agglutinin.

MATERIALS AND METHODS

Purification of monoamine transporter from chromaffin granules. Purified monoamine transporter used for immunizations, and partially purified transporter employed for screening hybridomas and characterization of antibodies, were prepared as described previously [6]. Briefly, 18–20 g medullary tissue was dissected and homogenized, and membranes were prepared by differential and sucrose density gradient centrifugation as described [7]. The chromaffin granule membrane enriched fraction was solubilized in a total of 100 mL solubilization buffer, 1% (w/v) cholate, 0.2% lecithin, 150 mM KCl, 10 mM HEPES, pH 7.6. After centrifugation, the supernatant was applied to a WGA-Sepharose 6MB column, the column was washed with 20 mL of the same buffer, and it was eluted with a 50-mL linear gradient (0–250 mM) of *N*-acetyl glucosamine. Fractions exhibiting [^{3}H]TBZOH binding activity were pooled, solid KCl was added to a final

concentration of 750 mM, and the sample was applied to a phenyl-Sepharose CL-4B column (HR 10/10). The binding activity was eluted with a 20-mL linear KCl gradient (750 to 10 mM), followed by 20 mL elution buffer. The phenyl-Sepharose fractions with binding activity were pooled, diluted with 1 vol. of 10 mM KCl, 10 mM HEPES, pH 7.6, and injected onto a Mono Q HR 5/5 column that had been prewashed with 2 vol. of water. The column was washed with 5 mL of 0.5% sodium cholate, 0.1% lecithin, 10 mM KCl, 10 mM HEPES, pH 7.6, and eluted with a 20-mL linear gradient of KCl (10 to 750 mM) in the same buffer. Two peaks of binding activity were observed. The second peak fraction eluting at higher KCl concentration was diluted with 0.5% sodium cholate, 0.1% lecithin, 10 mM HEPES, pH 7.6, to give a KCl concentration of 100 mM and injected again onto the column. After washing with starting buffer until 10 mL of cholate-containing buffer had been loaded, binding activity was eluted with the same gradient used for the first Mono Q column. Hydroxylapatite in an HR 5/10 column was washed with several volumes of water, the Mono Q peak fraction was injected, and the column was washed with 1% sodium cholate, 0.2% lecithin, 10 mM KCl, 10 mM HEPES, pH 7.6 (input buffer), until the absorbance at 280 nm returned to baseline. The column was washed with 2 mL of 1.1 M $MgCl_2$ in input buffer, by injecting the solution and washing with input buffer until the peak cleared. In the same fashion, the column was washed with 2 mL of 1.1 M KCl in input buffer. After washing with 7 mL of 10 mM K_2HPO_4 in input buffer, binding activity was eluted with a 20-mL linear gradient to 300 mM K_2HPO_4 in the same buffer. Immunoreactivity of fractions was determined by ELISA as described below after 10- to 50-fold dilution in PBS.

Purification of [^{3}H]*reserpine-labeled transporter.* Chromaffin granule membranes were prepared from fresh bovine adrenals and labeled with [^{3}H]reserpine as described by Stern-Bach *et al.* [4]. Most of the nonspecifically bound radioactivity was removed from the membranes by the method of Rudnick *et al.* [8] using Sephadex LH-20. The membranes were then centrifuged (34,000 *g*, 30 min), the pellet was resuspended in 0.3 M sucrose, 10 mM potassium·HEPES, pH 7.4, and Triton X-100 was added to a final concentration of 1.5% (w/v). After incubation for 15 min at 4°, the mixture was centrifuged for 1 hr at 100,000 *g*. The supernatant was employed in immunoaffinity chromatography experiments described below, and for further purification of the transporter on DEAE-52, hydroxylapatite, and phosphocellulose columns by the method of Stern-Bach *et al.* [4]. Fractions were assayed for immunoreactivity by ELISA after 10- or 50-fold dilution in PBS.

Immunizations. A 5½-month-old, female BALB/c mouse was given an intraperitoneal injection of an emulsion of 150 μL of Hunter's TiterMax® (CytRx Corp., Norcross, GA) containing 4 μg of the vesicular monoamine transporter purified from bovine adrenal medulla as described above. Comparable booster immunizations were administered 5 and 12 weeks later.

Fusion. Three days after the last boost the mouse was euthanized by cervical dislocation, and spleen cells were obtained. These were fused with X63/Ag8.653 myeloma cells using procedures and cell culture medium previously reported [9].

Coating of immunoplates. Alternate rows of 96-well immunoplates were coated for 16–18 hr at 4° with 50 μL of partially purified transporter. The transporter preparation used was purified through the WGA-Sepharose step from frozen bovine adrenal medulla [6], and 50 μL contained approximately 100 ng of transporter calculated from the binding capacity for dihydrotetrabenazine and assuming a molecular weight of 70,000. The remaining rows were coated under identical conditions with 50 μL of buffer. After washing the plates once with 100 μL of Triton/PBS (0.04% Triton X-100 in PBS) at room temperature, they were blocked with 150 μL casein (0.1% casein in 0.01 M sodium phosphate, pH 7.0) for 1 hr at 4°. The plates were then washed twice with Triton/PBS and used immediately.

Screen of hybridoma tissue culture supernatants. Hybridoma supernatants were screened for anti-transporter antibodies following the same procedure used previously to detect bradykinin antibodies [9], except that incubations were not terminated with the addition of 2 N sodium hydroxide and plates were read using a Bio-Tek EL 309 dual wavelength microplate reader set to record the difference in absorbance at 405 and 490 nm. A positive result in this assay was defined as one in which absorbance measurements for wells coated with transporter were at least 0.1 greater than those for wells coated with buffer, which in turn were comparable to those obtained when substrate was incubated under assay conditions in the absence of alkaline phosphatase. Hybridomas from wells testing positive were transferred to 24-well plates, grown up, and rescreened. Those still testing positive were subcloned. Two 96-well plates were seeded with either an average of 3 cells per well or an average of 1 cell per well. Positive wells containing single colonies were grown up and stocked. Ascites fluid was generated in pristane-primed BALB/c mice using hybridomas that had been cloned 2–3 times. Antibodies were purified from ascites fluid on Protein A affinity columns (2 mL bed volume) according to the instructions provided by the supplier (Pierce, Rockford, IL).

Isotyping. Cloned hybridomas were isotyped as previously described [9].

Immunoaffinity chromatography of reserpine-labeled transporter. Antibodies were immobilized (1.0 to 2.5 mg/mL gel) on CarboLink Coupling Gel (Pierce) using the method recommended in the supplier's literature. Disposable columns (5 mL) from the same source were packed with 2 mL of immobilized antibody, washed with 10 mL starting buffer (0.75% Triton X-100 in PBS), 20 mL elution buffer, and then 20 mL starting buffer immediately prior to use. [^{3}H]Reserpine-labeled chromaffin granule membranes, solubilized as described above, were diluted 25-fold with 0.75% (w/v) Triton X-100 in PBS, and approximately 150,000 cpm (0.15 mg protein) were applied to each column in a total volume of 7 mL at room temperature. When 2-fold

Table 1. Isotypes of monoclonal antibodies reactive with monoamine transporter preparation

Antibody	Isotype
6F7	IgG2bκ
6F11	IgG2bκ
10H8	IgG2bκ
15C8	IgG2aκ
16C9	IgG2aκ
16H9	IgG1κ

dilutions were employed, the samples were diluted with PBS alone, to give a final detergent concentration of 0.75% (w/v). Columns were washed with 20–30 mL of the starting buffer and then eluted with 0.75% Triton X-100, 0.1 M glycine, pH 2.5. Fractions (2 mL) were collected and neutralized with 0.2 mL Tris·HCl, pH 9.0, and aliquots were assayed for radioactivity by liquid scintillation counting.

Immunoprecipitation of dihydrotetrabenazine binding activity. The cholate solubilized transporter, purified through the WGA-Sepharose step as described previously [6] and containing 45–50 pmol TBZOH binding activity per mL, was diluted with 4 vol. of 150 mM KCl, 10 mM HEPES, pH 7.6. Monoclonal antibody was added to a final concentration of 8 or 80 nM. The mixture was incubated at room temperature for 2 hr with gentle agitation; then 50 μL/mL of a 50% (v/v) suspension of Protein A-agarose (Pierce) was added and incubated for a further 3 hr. The mixture was centrifuged in a microfuge for 5 min, and the pellet and supernatant were assayed for [^{3}H]TBZOH binding activity by the filter binding assay described previously [10]. In some cases, antibodies coupled directly to Carbolink gel were employed. In these experiments, 100 μL of a 50% suspension of the immobilized antibody was added per mL of diluted protein. The mixture was agitated at room temperature for 5 hr and then centrifuged, and fractions were assayed as described above.

RESULTS

To assess the feasibility of preparing monoclonal antibodies to the transporter, one mouse was immunized with the purified protein, and serum samples were subjected to ELISA using partially and completely purified transporter coated on polystyrene 96-well plates. A positive response was observed with both transporter preparations, and Western blots showed labeling of a protein of the correct size on SDS–PAGE. A fusion was performed with the spleen from this mouse, resulting cells were plated on 25 96-well plates, and wells with colonies were screened using partially purified bovine chromaffin granule amine transporter as antigen. Of the 1142 wells containing colonies, 14 were positive in the initial screen. Cells from the 14 positive wells were grown up and cloned out. Six positive clones remained after subcloning, and these survived being frozen and thawed. Isotypes of these antibodies are given in Table 1.

All of the monoclonals were reactive with the vesicular monoamine transporter purified from bovine adrenal by two different methods [4, 6]. Moreover, all fractions from all steps of the purification procedure of Vincent and Near [6] that exhibited TBZOH binding activity also were positive when tested in the ELISA against a mixture of these antibodies, while those that showed no binding did not contain immunoreactivity. Similar results were obtained when just one of the antibodies, 15C8, was used. It is interesting to note that immunoreactivity coeluted with both major charge forms of the transporter during ion exchange chromatography (Fig. 1). The transporter preparation employed to generate the antibodies had been purified from the second peak of binding activity, i.e. the form eluting at lower salt concentration was not present in the immunogen.

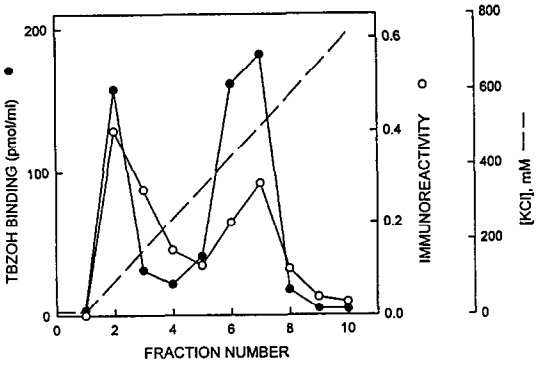

Fig. 1. Elution profiles of TBZOH binding activity and immunoreactivity from a Mono-Q anion exchange column. The starting material, chromatography conditions and TBZOH binding assay were as described previously [6]. For TBZOH binding, each point is the mean of two determinations with the standard deviation less than 8% of the indicated value. Immunoreactivity of each fraction (diluted 10-fold in PBS before coating on plates) was determined by ELISA with a mixture of Protein A purified monoclonals as described in Materials and Methods. Each point is the mean of three determinations with background determined in the presence of buffer alone subtracted, and standard deviations were less than 0.03. Data shown are from one of a total of three separate experiments, with similar results.

The 6 monoclonals were grown up in ascites, affinity purified on Protein A, and immobilized. When Triton X-100 extracts of [^{3}H]reserpine-labeled chromaffin granule membranes were diluted 25-fold and passed through columns of the immobilized antibodies, 6.5% of the original specifically bound radioactivity eluted at pH 2.5 from the column derivatized with 15C8 (Fig. 2). Radioactivity eluted from columns prepared with the other five antibodies ranged from 0.9 to 1.8% of the applied material. Immunoreactivity was detected in both the flow through and eluate fractions of all columns, although eluates from the 15C8 column gave the highest signals. TBZOH binding activity in the eluates could not be determined, as the elution conditions destroy the activity. Dilution of the applied sample had a profound effect on the amount of labeled material that bound and eluted from the columns. In similar experiments performed with more concentrated extracts (2-fold dilution), labeled material bound only to the column derivatized with 15C8, and 1%

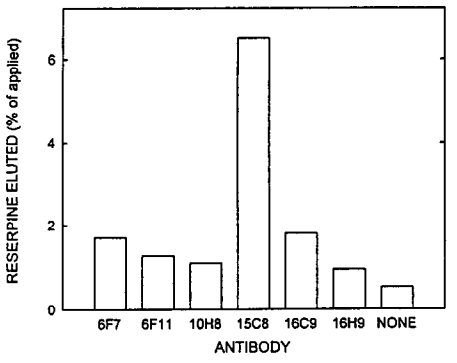

Fig. 2. Specifically bound reserpine eluted from Carbolink immunoaffinity columns. Chromaffin granule membranes were labeled with [^{3}H]reserpine, solubilized, diluted 25-fold in PBS containing 0.75% (w/v) Triton X-100, and subjected to immunoaffinity chromatography as described in Materials and Methods. Each value is the sum of the total radioactivity present in all eluted fractions from a single column, expressed as percent of applied radioactivity (150,000 cpm). Two other experiments performed under identical conditions gave qualitatively similar results.

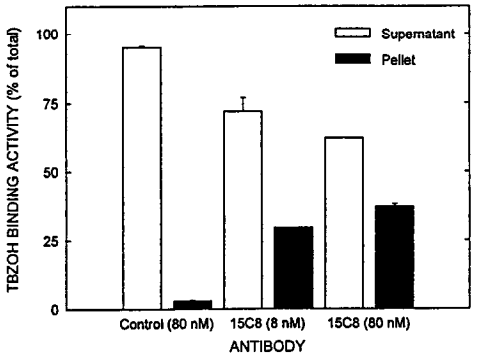

Fig. 3. Immunoprecipitation of TBZOH binding activity. Cholate extracts of chromaffin granule membranes purified through the WGA-Sepharose step were incubated with soluble antibodies, followed by incubation with Protein A-agarose as described in Materials and Methods. For the control, a monoclonal antibody with the same isotype as 15C8 raised against an unrelated antigen was employed. Values are expressed as percent of specific binding activity present in the starting material (105,000 cpm). Each point is the mean of three determinations ±SD. Data were taken from one of five separate experiments with similar results.

of the applied radioactivity was eluted at pH 4.0 from this column.

TBZOH binding is another characteristic activity that was used to determine the ability of the antibodies to interact with the transporter. Cholate extracts of chromaffin granule membranes that had been purified through the WGA-Sepharose step were incubated with 15C8. Depending upon the particular antibody used, and the incubation conditions, 30–40% of TBZOH binding activity was precipitated by subsequent incubation with Protein A coupled to Sepharose (Fig. 3). When antibody was omitted, or a monoclonal of the same isotype raised against an unrelated antigen was substituted, only 2–4% of the binding activity was precipitated on immobilized Protein A. In similar experiments employing directly immobilized antibodies, the amount of TBZOH binding activity in pellets and supernatants was not distinguishable from control values (not shown).

DISCUSSION

Six monoclonal antibodies reactive with preparations containing the bovine adrenal chromaffin granule monoamine transporter were obtained using the purified transporter as immunogen. A positive reaction in the ELISA was obtained for all fractions from two purification procedures that exhibited labeled inhibitor binding activity, a characteristic feature of the transporter. In an earlier report, we described the separation of two forms of TBZOH binding activity by anion exchange chromatography [6]. Stern-Bach *et al.* [4] likewise observed two charge forms of tritiated reserpine binding activity by isoelectric focusing and ion exchange chromatography of material from the same source. However, Isambert *et al.* [5] detected only one diffuse spot of [^{125}I]iodoazido ketanserin-labeled material on two-dimensional gels of chromaffin granule membranes, and concluded that the multiple forms described by others may have been due to endogenous desialylase or protease activity, or artifactual detergent effects. Regardless of the provenance of the more basic form of inhibitor binding activity, the antibodies described here were raised against the more acidic form, but immunoreactivity coeluted with both forms during anion exchange chromatography. This finding is consistent with the notion that the antibodies are reactive with the transporter rather than a contaminant, as it is unlikely that a contaminant would be present in both regions of the chromatogram.

Stern-Bach *et al.* [11] have described two polyclonal antibodies raised against the purified transporter and a synthetic peptide constructed using N-terminal sequencing data. Both antibodies were reactive with purified transporter, as determined by western blotting and immunoprecipitation assays. Reactivity of these antibodies with the more basic form of reserpine binding activity described in their previous work [4] was not addressed specifically, but fractions from various steps of their purification procedure were analyzed by western blotting. The fraction expected to contain such a form, the void volume from the first chromatographic step, exhibited little or no immunoreactivity. However, the amount of transporter present on the blot was much lower than for those fractions giving a positive result. Thus, it is not possible to reach any firm conclusion regarding the reactivity of their antibodies with the more basic form of the transporter.

Stern-Bach *et al.* [11] were able to immunoprecipitate almost all of the iodinated transporter from a highly purified preparation using polyclonal antibodies raised against the purified transporter, but the utility of their antibodies for purification of the transporter from cruder preparations was not assessed. As shown in Fig. 3, the monoclonal antibody 15C8 was capable of immunoprecipitating approximately 40% of TBZOH binding activity from chromaffin granule membrane extracts that had been

partially purified by lectin affinity chromatography. In control experiments, a monoclonal antibody of the same isotype raised against an unrelated immunogen removed 2–4% of the binding activity. Approximately 3% of the protein present in the lectin affinity column eluate is transporter, calculated on the basis of 1:1 stoichiometry for TBZOH binding and a molecular weight of 70,000. The ability of at least one of our monoclonals to immunoprecipitate inhibitor binding activity from such a crude preparation suggests that immunoaffinity chromatography could play an important role in a more rapid and efficient purification procedure for the transporter.

Immunoaffinity resins were relatively ineffective in removing inhibitor binding activities from soluble transporter preparations. In contrast, treatment of solubilized transporter with soluble antibodies followed by addition of immobilized Protein A led to immunoprecipitation of a large fraction of the TBZOH binding activity present in such preparations. Although much more antibody was present in experiments using immunoaffinity resins, the amount of active antibody coupled to the resins could not be determined. The coupling method used relies on the formation of hydrozone bonds between hydrazide moieties on the gel and reactive aldehydes present in the carbohydrate portion of the periodate-oxidized antibody. While comparison of untreated and periodate-treated antibodies in the ELISA ruled out the possibility that the antibodies were inactivated during the oxidation step, immobilization of the antibody may have rendered it incapable of combining with antigen due to steric hindrance. Aggregation of the transporter, or the presence of an inhibitor of antibody binding in the transporter preparation, could also be involved. In the experiment for Fig. 2, the transporter preparation was diluted 25-fold before application to the immunoaffinity resins, whereas lower dilution factors resulted in less efficient removal of reserpine-labeled transporter. A parallel situation appears to prevail in the ELISA assay, where dilutions of 100-fold or more are required to give the highest signal for some fractions obtained from the conventional purification method. In general, it appears that the concentrations of buffer or detergent cannot account for this effect, but purer fractions containing a higher concentration of transporter require more dilution to achieve the highest signal. These observations, coupled with the propensity of the transporter to aggregate noted previously by ourselves and others [4, 6], suggest that concentration-dependent aggregation may obscure the relevant epitopes and block antibody binding. However, our current data does not rule out the possibility that another protein or proteins may block antibody binding.

Although a number of questions remain to be answered, the demonstration that the monoclonals react with a highly purified transporter preparation, that immunoreactivity coelutes with inhibitor binding activity in all steps of two different purification procedures, and that at least one of the immobilized antibodies can remove a portion of inhibitor binding activity from detergent extracts suggest that we have succeeded in generating monoclonal antibodies to the transporter. Labeling of a protein with the correct molecular weight on western blots would provide more compelling evidence for this conclusion. Unfortunately, we have been unable to detect labeling of any bands with monoclonal antibodies on western blots of transporter preparations, suggesting that the antibodies interact with conformational epitopes that are destroyed by denaturation. Definitive proof that these monoclonal antibodies bind the transporter could come from a demonstration of their reactivity with one or more of the monoamine transporters that have been cloned and expressed recently. Further characterization of their specificities along these lines, and evaluation of their usefulness for large scale immunoaffinity purification of the transporter, are subjects of current investigations.

REFERENCES

1. Johnson RG, Accumulation of biological amines into chromaffin granules: A model for hormone and neurotransmitter transport. *Physiol Rev* **68**: 232–307, 1988.
2. Kanner BI and Schuldiner S, Mechanism of transport and storage of neurotransmitters. *Crit Rev Biochem* **22**: 1–38, 1987.
3. Njus D, Kelley PM and Harnadek GJ, Bioenergetics of secretory vesicles. *Biochim Biophys Acta* **853**: 237–265, 1986.
4. Stern-Bach Y, Greenberg-Ofrath N, Flechner I and Schuldiner S, Identification and purification of a functional amine transporter from bovine chromaffin granules. *J Biol Chem* **265**: 3961–3966, 1990.
5. Isambert M-F, Gasnier B, Botton D and Henry J-P, Characterization and purification of the monoamine transporter of bovine chromaffin granules. *Biochemistry* **31**: 1980–1986, 1992.
6. Vincent MS and Near JA, Purification of a [^{3}H]-dihydrotetrabenazine-binding protein from bovine adrenal medulla. *Mol Pharmacol* **40**: 889–894, 1991.
7. Vincent MS and Near JA, Partial purification of dihydrotetrabenazine binding activity from bovine adrenal. *Biochem Pharmacol* **39**: 1823–1825, 1990.
8. Rudnick G, Steiner-Mordoch SS, Fishkes H, Stern-Bach Y and Schuldiner S, Energetics of reserpine binding and occlusion by the chromaffin granule biogenic amine transporter. *Biochemistry* **29**: 603–608, 1990.
9. Odya CE and Lee C-H, Enzyme-linked immunosorbent assays for kinins using high-affinity monoclonal kinin antibodies. *Biochem Pharmacol* **40**: 245–251, 1990.
10. Vincent MS and Near JA, Interaction of dihydrotetrabenazine binding activity with immobilized lectins. *Eur J Pharmacol* **172**: 317–319, 1989.
11. Stern-Bach Y, Keen JN, Bejerano M, Steiner-Mordoch S, Wallach M, Findlay JBC and Schuldiner S, Homology of a vesicular amine transporter to a gene conferring resistance to 1-methyl-4-phenylpyridinium. *Proc Natl Acad Sci USA* **89**: 9730–9733, 1992.