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Characterization and regulation of the expression of scyllatoxin (Leiurotoxin I) receptors in the human neuroblastoma cell line NB-OK 1

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^{125}I-[Tyr^2]scyllatoxin allowed to label a single class of high-affinity receptors in membranes from the human neuroblastoma cell line NB-OK 1. The K_d of these receptors was 60 pM for scyllatoxin (Leiurotoxin I) and 20 pM for apamin and the B_{max} was low (3.8 fmol/mg membrane protein). K^+ increased toxin binding at low concentrations but exerted opposite effects at high concentrations. Ca^{2+}, guanidinium and Na^+ exerted only inhibitory effects on binding. Scyllatoxin binding sites were overexpressed 2.5-fold after a 24-h cell pretreatment with 2 mM butyrate. This effect was suppressed by cycloheximide.

Scyllatoxin (Leiurotoxin I); Apamin; Ca^{2+}-activated K^+ channel; Butyrate; Human neuroblastoma cell line NB-OK 1

1. INTRODUCTION

Apamin, a neurotoxin from bee venom, is a highly basic octadecapeptide that binds with high affinity to a protein present in low abundance in membranes from the central nervous system, skeletal, cardiac and smooth muscles, and hepatocytes, thereby blocking SK 'small' Ca^{2+}-activated K^+ channels [1–6]. Apamin-sensitive SK channels have a low conductance (5–20 pS) and are responsible for the prolonged after-hyperpolarization that follows Na- and/or Ca-dependent action potentials in nerve and muscle cells [7,8]. Scyllatoxin (Leiurotoxin I), a 31-amino-acid peptide from the venom of the scorpion *Leiurus quinquestriatus hebraeus* is also a blocker of these channels. Despite the fact that scyllatoxin shares no obvious sequence homology with apamin (apart from high basicity and sulfur content and a C-terminal amidated His residue: Table I), it inhibits apamin binding to its receptors, alters smooth muscle contraction, and blocks apamin-sensitive SK channels in guinea pig hepatocytes [9–12]. We report here the characterization of scyllatoxin binding sites in the human neuroblastoma cell line NB-OK 1 and the overexpression of these binding sites in the presence of butyrate.

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Abbreviations: K_d, concentration required for half-maximal occupancy of binding sites with tracer; IC_{50}; concentration required for half-maximal inhibition of tracer binding

2. MATERIALS AND METHODS

2.1. *Cell culture and membrane preparation*

The NB-OK 1 cells were cultured in RPM 1 1640 medium enriched with 10% foetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. At confluence, the cells were subcultured using a trypsin-EDTA solution for detaching cells. In case of cell pretreatment, the culture medium of preconfluent cells was renewed with fresh medium supplemented with the drug to be tested. At confluence, the cells were dislodged with a rubber policeman, centrifuged for 5 min at 100 × g, rinsed with fresh culture medium, lyzed in 1 mM $NaHCO_3$ and quickly frozen in liquid N_2. The lysate was defrosted and centrifuged at 4°C for 10 min at 100 × g. The resulting supernatant was centrifuged at 20 000 × g for 20 min at 4°C. This crude membrane pellet was re-homogenized in 50 mM Tris-HCl, pH 8.0, containing 2 mM KCl, 2 mM EDTA and 0.5% bovine serum albumin.

2.2. *Radioiodination and purification of ^{125}I-[Tyr^2]scyllatoxin*

We used a synthetic scyllatoxin analogue with a Tyr residue in position 2 (to permit iodination on that residue rather than on His^{31}) with binding properties similar to those of scyllatoxin [10]. [Tyr^2]scyllatoxin (15 μg = 4.5 nmol) was incubated with IODO-GEN (2 μg = 4.5 nmol) and Na^{125}I (2 mCi = 1 nmol) in 100 μl of 200 mM potassium phosphate buffer, pH 7.4. After 15 min at room temperature, the reaction mixture was transferred, adjusted to 1 ml with 0.1% TFA–5% CH_3CN and loaded into a μBondapak C_{18} column (3.9 × 300 mm) equipped with a guard column equilibrated with 0.1% TFA–5% CH_3CN. The column was eluted (in 60 min) with a linear gradient of CH_3CN (8–32%) in 0.1% TFA. HPLC was monitored at 280 nm and radioactivity measured in a gamma counter. Fractions of interest were checked for their binding capacity on neuroblastoma cell membranes (see below). In this system, ^{125}I-

Table I

Amino acid sequences of (A) scyllatoxin (Leiurotoxin I) from *Leiurus quinquestriatus hebraeus* and (B) apamin from *Apis mellifera*

	1 5 10 15 20 25 30
A/	A-F-C-N-L-R-M-C-Q-L-S-C-R-S-L-G-L-L-G-K-C-I-G-D-K-C-E-C-V-K-H-NH$_2$
	1 5 10 15
B/	C-N-C-K-A-P-E-T-A-L-C-A-R-R-C-Q-Q-H-NH$_2$

[Tyr^2]scyllatoxin eluted with 21% CH_3CN in 0.1% TFA (as compared to 20% CH_3CN for [Tyr^2]scyllatoxin) and its specific radioactivity was 2 mCi/nmol.

2.3. *Binding studies*

The standard incubation medium consisted of 50 mM Tris-HCl, pH 8.0, containing 2 mM KCl, 2 mM EDTA, 0.5% bovine serum albumin, the indicated unlabelled peptide concentrations, ^{125}I-[Tyr^2]scyllatoxin and 150 μg membrane protein in a final volume of 120 μl. After incubation at 0°C, the reaction was terminated by adding 2 ml ice-cold 50 mM sodium phosphate buffer, pH 7.4, followed by rapid filtration through glass-fiber filter (GF/C, Whatman, Maidstone, UK) presoaked for 24 h in 0.1% poly(ethyleneimine) in order to reduce non-specific binding. The filter was then rinsed 3× with 2 ml of the same buffer and the radioactivity measured. Except when otherwise indicated, non-specific binding was determined in the presence of 1 μM apamin and accounted for approximatively 25% of total binding. Specific binding was defined as total binding minus non-specific binding and always represented less than 15% of the total radioactivity offered.

2.4. *Chemicals*

Carrier-free Na^{125}I (IMS 300, 600–800 mCi/ml) came from Amersham Corp. (Bucks, UK) and IODO-GEN from Pierce (Oud-Beijerland, Netherlands). Foetal calf serum and medium for cell cultures were from Gibco Europe (Gent, Belgium). HPLC materials were from Waters Associates (Milford, MA). Butyrate and analogues were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Highly purified natural apamin was from Serva Feinbiochemica (Heidelberg, Germany).

3. RESULTS

Specific ^{125}I-[Tyr^2]scyllatoxin binding to NB-OK 1 membranes was rapid, reversible and saturable. At 0°C, an apparent binding equilibrium was attained after 20 min (Fig. 1A). Tracer bound after 45 min dissociated with a $t_{1/2}$ of 5 min at 0°C, as studied by isotopic and chemical dilution, i.e. rapidly, considering the temperature (Fig. 1B). Saturation curves were obtained with various concentrations of ^{125}I-[Tyr^2]scyllatoxin (10–300 pM) and their Scatchard transformation was compatible with the existence of one class of high-affinity binding sites (K_d of 40 ± 8 pM) with low maximal binding capacity (3.8 ± 0.6 fmol/mg membrane protein) (Fig. 1C).

Competition curves showed that unlabelled [Tyr^2]scyllatoxin inhibited tracer binding with an IC_{50} of 0.15 nM, i.e. a K_d of 60 pM, while apamin was 3 times more potent with a K_d of 20 pM (Fig. 2).

As previously described for [^{125}I]apamin binding sites [13–15], K^+ ions increased ^{125}I-[Tyr^2]scyllatoxin binding at low concentration (by a factor of 1.3 at 1 × 10^{-3} mM) while higher concentrations fully inhibited ligand binding with an IC_{50} at 40 mM (Fig. 3A). Three other cations were only inhibitory with IC_{50} values at 4 mM for Ca^{2+}, 15 mM for guanidinium, and 60 mM for Na^+ (Fig. 3B). ATP, ATP S, GTP, GppNHp and GTP S, when tested in the $3 \cdot 10^{-6}$–$3 \cdot 10^{-4}$ M concentration range, exerted no effect on tracer binding.

A 24-h preincubation of NB-OK 1 cells with 2 mM butyrate increased 2- to 3-fold the number of scyllatoxin receptors and Scatchard plots were compatible with a general 2-fold increase in K_d or, more likely so, with the appearance of a second subpopulation of receptors with a 4- to 5-fold lowered affinity (Fig. 4 and see

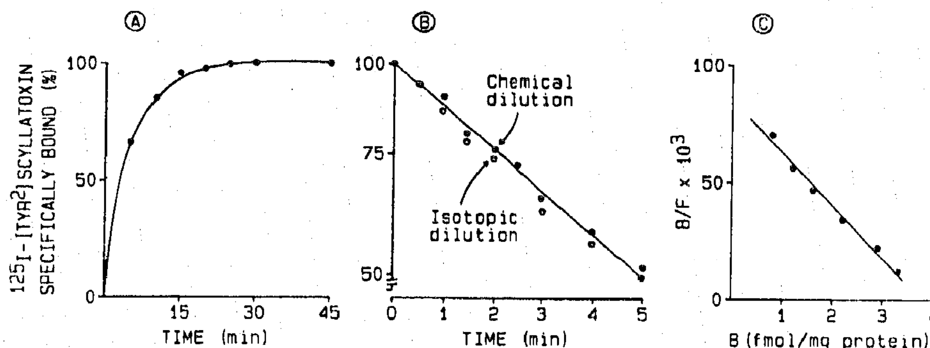

Fig. 1. (A) Kinetics of specific ^{125}I-[Tyr^2]scyllatoxin binding to NB-OK 1 membranes at 0°C (in % of equilibrium binding). (B) Dissociation kinetics of prebound ^{125}I-[Tyr^2]scyllatoxin at 0°C. After 45 min incubation with tracer at 0°C, 1 μM unlabelled [Tyr^2]scyllatoxin (●) or 10 ml cold standard incubation medium (○) was added to induce dissociation. (C) Scatchard representation of a saturation curve of ^{125}I-[Tyr^2]scyllatoxin binding. Results were the means of experiments performed on three different membrane preparations.

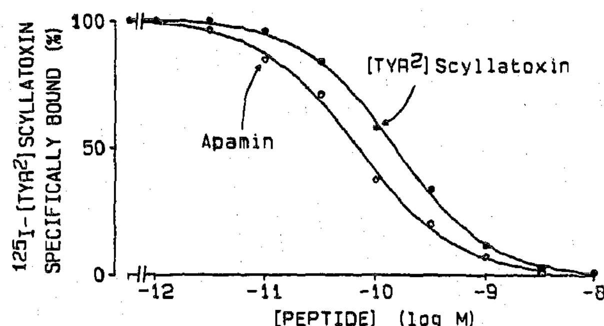

Fig. 2. Inhibition of ^{125}I-[Tyr^2]scyllatoxin binding to NB-OK 1 membranes by unlabelled [Tyr^2]scyllatoxin (●) and apamin (○). Membranes were incubated for 45 min at 0°C in the presence of 60 pM tracer and increasing concentrations of unlabelled peptide. The results were expressed in percent of tracer specifically bound in the absence of unlabelled peptide and were the means of experiments performed in duplicate on three different membrane preparations.

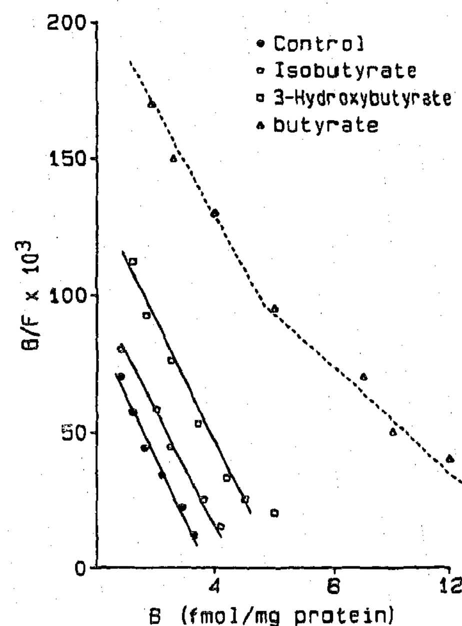

Fig. 4. Scatchard representation of ^{125}I-[Tyr^2]scyllatoxin binding to membranes from control NB-OK 1 cells (●) and cells pretreated with 2 mM butyrate (Δ), 2 mM isobutyrate (○), or 2 mM 3-hydroxybutyrate (□) for 24 h. These results illustrate one experiment performed in duplicate that was representative of two others.

discussion). The presence of 3.6 μM cycloheximide, together with butyrate, prevented this overexpression (data not shown). These alterations were reproduced very partially with 2 mM 3-hydroxybutyrate but not with 2 mM isobutyrate, 1 mM carbamylcholine, 100 nM 12-*O*-tetradecanoylphorbol 13-acetate (TPA), 2 mM 8-bromo-cAMP, or the two latter agents added in combination. Under these experimental conditions the number and protein content of NB-OK 1 cells were not significantly affected after 24 h.

4. DISCUSSION

Scyllatoxin binding sites were identified in human neuroblastoma NB-OK 1 membranes with ^{125}I-[Tyr^2]-scyllatoxin, an analogue where the substitution of Phe by Tyr in position 2 does not change the toxin affinity for binding sites in rat brain membranes [9]. In the present study, a single class of such binding sites was characterized in membranes from human neuroblastoma NB-OK 1 cells. The K_d of 40 pM was in good

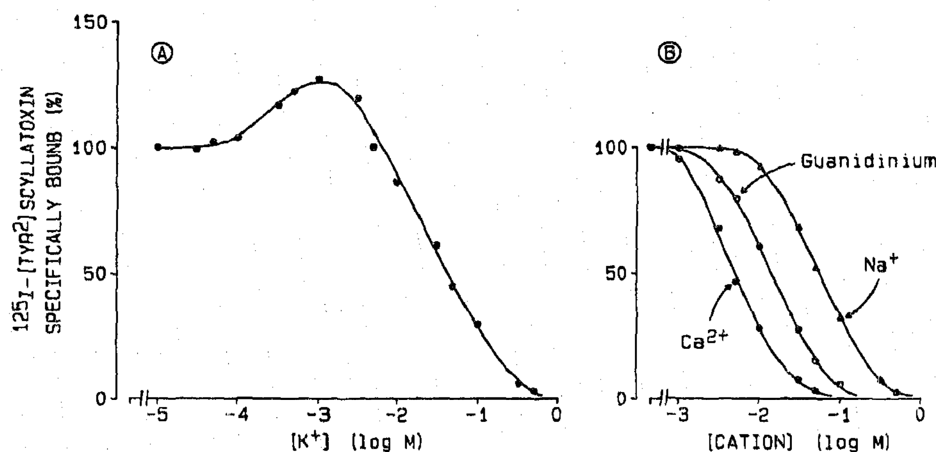

Fig. 3. (A) Effects of K$^+$ on ^{125}I-[Tyr^2]scyllatoxin binding to NB-OK 1 membranes. Various KCl concentrations were added to a standard incubation medium (free of KCl). Membranes were incubated for 45 min at 0°C in the presence of 60 pM tracer. (B) Effects of Ca^{2+}, guanidinium and Na$^+$ on ^{125}I-[Tyr^2]scyllatoxin binding to NB-OK 1 membranes. The standard incubation medium (see section 2) was supplemented with various concentrations of free Ca^{2+} (●), of guanidinium-HCl (○) or of NaCl (Δ). Membranes were incubated for 45 min at 0°C in the presence of 120 pM tracer. Results were expressed in percentage of maximal binding measured with a KCl-free medium (panel A) or with the standard unsupplemented incubation medium (panel B) and were the means of experiments performed in duplicate on three different membrane preparations.

agreement with that found for rat brain membranes (K_d 80 pM) [9]. Apamin competitively inhibited ^{125}I-[Tyr^2]scyllatoxin binding (Fig. 2).

As for ^{125}I-[Tyr^2]scyllatoxin and [^{125}I]apamin binding to rat brain membranes, we observed that ^{125}I-[Tyr^2]scyllatoxin binding was enhanced at low K^+ concentration but inhibited at higher K^+ concentrations. Ca^{2+}, guanidinium and Na^+ were only inhibitory. This variety of cationic effects has been explained previously in terms of two sites: (a) an allosteric stimulatory K^+ (and Rb^+) specific site, and (b) a second, inhibitory anionic site, which binds the cationic groups of scyllatoxin and apamin [14,16].

Apamin receptors are present in very small amounts (1–30 fmol/mg protein) in all cell types and excitable membranes investigated until now (for references see [17]). For example, there are 125 times more tetrodotoxin binding sites than apamin binding sites in synaptosomal membranes [14]. Higher levels of [^{125}I]apamin binding sites (600 fmol/mg protein) have been reported in the undifferentiated rat pheochromocytoma cell line PC 12 but, when treated with nerve growth factor, these cells differentiate into a more neuronal-like type and show a reduced number of apamin receptors [17].

Due to the very low density of scyllatoxin binding sites in the present human neuroblastoma cell line NB-OK 1, and to the usual contribution of SK channels to membrane repolarization, it is of interest to note that our cell line is endowed with muscarinic M_1 receptors that are susceptible to stimulate PIP_2 hydrolysis and Ca^{2+} movements ([18] and data not shown).

Butyrate produces pleiotropic effects on cells in culture that are often associated with histone hyperacetylation and gene transcription (for review see [19–22]). In the present case, butyrate provoked a cycloheximide-inhibited overexpression of scyllatoxin receptors with an affinity likely to be somewhat reduced as compared to control receptors (Fig. 4). Isobutyrate, which has also been reported to inhibit histone deacetylase [22], did not increase the number of scyllatoxin receptors but this might be due to distinct NB-OK 1 cell permeability to short chain fatty acids or to their distinct intracellular metabolism. The present data show the interest to elucidate the possible role of scyllatoxin binding sites (SK channels) as regulators of proliferation, differentiation and biological activation in this neuronal cell line.

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