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Apamin as a selective blocker of the calcium-dependent potassium channel in neuroblastoma cells: Voltage-clamp and biochemical characterization of the toxin receptor

(receptor binding/neuroblastoma cell differentiation)

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ABSTRACT **This paper describes the interaction of apamin, a bee venom neurotoxin, with the mouse neuroblastoma cell membrane. Voltage-clamp analyses have shown that apamin at low concentrations specifically blocks the Ca^{2+}-dependent K^+ channel in differentiated neuroblastoma cells. Binding experiments with highly radiolabeled toxin indicate that the dissociation constant of the apamin–receptor complex in differentiated neuroblastoma cells is 15–22 pM and the maximal binding capacity is 12 fmol/mg of protein. The receptor is destroyed by proteases, suggesting that it is a protein. The binding capacity of neuroblastoma cells for radiolabeled apamin dramatically increases during the transition from the nondifferentiated to the differentiated state. The number of Ca^{2+}-dependent K^+ channels appears to be at most 1/5th the number of fast Na^+ channels in differentiated neuroblastoma. The binding of radiolabeled apamin to its receptor is antagonized by monovalent and divalent cations. Na^+ inhibition of the binding of ^{125}I-labeled apamin is of the competitive type ($K_{d(Na^+)}$ = 44 mM). Guanidinium and guanidinated compounds such as amiloride or neurotensin prevent binding of ^{125}I-labeled apamin, the best antagonist being neurotensin.**

In recent years neurotoxins have become essential tools in neurobiological studies (1, 2). Apamin is a bee venom polypeptide of 18 amino acids with two disulfide bridges (3). It is the only polypeptide neurotoxin, as far as we know, that passes the blood–brain barrier. Arg-13 and Arg-14 are in the active site of the toxin (4). Apamin does not seem to interact with receptors of the most classical neurotransmitters (5). Recent K^+ flux studies have suggested that it blocks a Ca^{2+}-dependent K^+ channel (6, 7, 8).

Moolenaar and Spector (8, 9) have reported that the action potential of N1E 115 mouse neuroblastoma cells in solutions containing a high Ca^{2+} concentration was followed by a long-lasting after-hyperpolarization (a.h.p.). This a.h.p. was inferred to be mediated by the activation of a Ca^{2+}-dependent K^+ conductance that is voltage dependent and tetraethylammonium (Et_4N^+) insensitive. The existence of a Ca^{2+}-dependent K^+ conductance has also been demonstrated in a variety of excitable cells such as vertebrate motoneurons, cardiac Purkinje fibers, smooth muscles (10), skeletal myotube cultures (11), etc. It is likely that the Ca^{2+}-dependent K^+ conductance plays a major role in the regulation of the repetitive firing frequency (11).

Two approaches have been used in this paper to study the mode of action of apamin on neuroblastoma cells: first, an electrophysiological approach to determine the specificity of action of apamin on the Ca^{2+}-dependent K^+ conductance; second, a biochemical approach to investigate the properties of the specific binding of apamin to the cell membranes.

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MATERIALS AND METHODS

Cultures of Neuroblastoma Cells. Neuroblastoma cells (clone N1E 115) were grown as described (12); they were induced to differentiate in the presence of 1% fetal calf serum and 1.5% dimethyl sulfoxide.

Iodination of Apamin. Apamin was purified as described (13). There is no tyrosine residue in the sequence, but there is histidine residue that is not essential for activity (4) and that can be iodinated. It has been found that the procedure previously described to prepare ^{125}I-labeled apamin (^{125}I-apamin) (14) could not be used for the characterization of the ^{125}I-apamin receptor on neuroblastoma cells. The labeled apamin obtained by this technique has a specific radioactivity of only 200–500 Ci/mmol (1 Ci = 3.7×10^{10} becquerels). Apamin (100 μg) was incubated with 2 mCi of $Na^{125}I$ (IMS.30, Amersham) in a 10 mM Tris·HCl buffer at pH 8.6 in a final volume of 200 μl. Four 3-μl portions of 10 mM chloramine-T (Merck) were added at 30-sec intervals. After the last addition of chloramine-T, the mixture was acidified to pH 6 with 0.1 M HCl and the monoiodo derivative was purified on a SP-Sephadex C-25 (Pharmacia) column (0.6 × 21 cm) equilibrated with a 100 mM NaCl/50 mM NaH_2PO_4 buffer at pH 6. The column was eluted first with the equilibration buffer (10 ml) then with a 50 mM NaH_2PO_4 buffer at pH 6 containing 300 mM NaCl. Fractions were 1 ml. The monoiodo derivative was eluted in fraction 32. The specific radioactivity of the monoiodo derivative was 2000 Ci/mmol.

Binding Assays under Various Standard Conditions. Neuroblastoma cells were scraped from culture dishes in an ice-cold medium consisting of 20 mM Tris·HCl, 0.25 M sucrose, and 1 mM EDTA at pH 7.5, centrifuged for 5 min at 1000 × g, resuspended in the same buffer (11–18 mg of protein per ml), divided into aliquots, and stored in liquid nitrogen. The concentration of protein was measured by Hartree's method (15), using bovine serum albumin as a standard. Frozen N1E 115 cells kept their apamin receptor in a stable form. Homogenates from the neuroblastoma cells were obtained with a Potter homogenizer (900 rpm, five strokes). The standard incubation medium for binding experiments consisted of a 20 mM Tris·HCl buffer at pH 7.5 containing bovine serum albumin at 0.5 mg/ml and 5.4 mM KCl.

Cellulose acetate filters (Sartorius, SM 11107, 0.2 μm pore size) used in binding experiments were incubated in 10 mM Tris·HCl (pH 7.5) and 0.1% serum albumin for 1 hr and then washed once with 5 ml of the same buffer at 0°C just before use.

(i) *Kinetics of association and dissociation of ^{125}I-apamin to neuroblastoma cells.* N1E 115 homogenates (0.3 mg of protein per ml) were incubated in the standard medium at 0°C. The

Abbreviations: a.h.p., long-lasting after-hyperpolarization; Et_4N^+, tetraethylammonium.

onset of binding was studied by adding ^{125}I-apamin at 40 pM. Aliquots (0.8 ml) were taken at different times and filtered under reduced pressure. Filters were rapidly washed twice with 5 ml of the washing buffer containing 10 mM Tris·HCl (pH 7.5) and 0.1% bovine serum albumin. Radioactivity bound to filters was measured with an Intertechnique CG 4000 gamma counter.

After 75 min of association the amount of specifically bound ^{125}I-apamin reached a plateau value. At that time, a large excess of unlabeled apamin (1 μM) was added to the incubation medium, thereby displacing the ^{125}I-apamin associated with the receptor. Dissociation kinetics were followed by measuring the decrease in bound ^{125}I-apamin with the filtration technique described above.

A series of experiments has also been carried out in the physiological buffer for neuroblastoma cells: 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.3 mM $MgSO_4$, 140 mM NaCl buffered by 20 mM Tris·HCl at pH 7.5.

(ii) *Equilibrium binding experiments.* N1E 115 homogenate (0.3 mg/ml) was incubated with ^{125}I-apamin at increasing concentrations for 60 min at 0°C. Duplicate aliquots (0.8 ml) were then filtered and the bound radioactivity was measured as described above. Nonspecific binding was determined in parallel experiments in the presence of an excess of unlabeled toxin (1 μM).

(iii) *Competition experiments between labeled and unlabeled apamin.* N1E 115 homogenates (0.3 mg/ml) were incubated for 60 min at 0°C with a fixed concentration of labeled iodotoxin (3.3 pM) and various concentrations of unlabeled toxin in 2 ml of the standard or of the physiological incubation medium. The amount of labeled iodotoxin that remained bound to neuroblastoma cells in the presence of the unlabeled toxin was estimated as described in *ii*.

(iv) *Competition experiments between labeled apamin and monovalent cations, divalent cations, and other drugs.* Experiments were carried out as described in *iii*.

(v) *Protease digestion of the apamin receptor.* N1E 115 homogenates (18 mg/ml) were incubated for 1 hr at 25°C in the presence of the different proteases in 20 mM Tris·HCl (pH 7.5) containing 1 mM EDTA and 0.25 M sucrose. For papain the digestion was carried out in 6.6 mM dithiothreitol and 1.6 mM cysteine.

Electrophysiological Experiments. Culture dishes containing N1E 115 neuroblastoma cells were used directly for electrophysiological analysis after replacing the culture medium with a modified Earle's medium (90 mM NaCl/5.4 mM KCl/25 mM $CaCl_2$/0.8 mM $MgSO_4$/25 mM Hepes–Tris/25 mM Et_4N^+/5 mM glucose) buffered at pH 7.4. Choline cations were used as a substitute for Na^+ in Na^+-free solution. The culture dish was placed on the warm stage of an inverted microscope (Leitz Diavert) and the temperature was maintained at about 30°C. Experiments were performed by using a suction pipette method which combines internal perfusion with voltage clamp of isolated cells (16–19). The suction pipette had a tip diameter of about 10 μm. The measured tip resistance ranged between 200 and 300 kΩ; the ionic composition of the solution used for internal perfusion was 10 mM NaH_2PO_4, 1 mM $MgCl_2$, 115 mM glutamic acid adjusted to pH 7.1 with KOH and to the osmotic pressure of the external solution (305 milliosmolar) with sucrose.

RESULTS

Voltage-clamp analyses of differentiated N1E 115 neuroblastoma cells have revealed the existence of four voltage-dependent membrane currents: a fast inward Na^+ current blocked by tetrodotoxin, a delayed outward Et_4N^+-sensitive K^+ current, a slow inward Ca^{2+} current, and a slow outward Ca^{2+}-dependent Et_4N^+-insensitive K^+ current (9, 19). In our experiments, only a fraction (about 20%) of the cells bathed in a solution containing 25 mM Ca^{2+} and 20 mM Et_4N^+ displayed an a.h.p. following the spike. Fig. 1 *A* and *B* (*Left*) presents typical a.h.p. obtained by anodal break stimulation when the cells were bathed either in a 90 mM Na^+/25 mM Ca^{2+}/20 mM Et_4N^+ solution or in a 25 mM Ca^{2+}/20 mM Et_4N^+ Na^+-free solution.

In a Na^+-free external solution containing 20 mM Et_4N^+ and 25 mM Ca^{2+}, the only measurable currents in voltage-clamp experiments upon prolonged step depolarizations were the inward Ca^{2+} current and the slow Ca^{2+}-dependent outward K^+ current (Fig. 1*C*, *Left*). The slow outward current was inhibited by Ca^{2+} channel blockers such as La^{3+} (2 mM) (Fig. 2*A*) and Co^{2+} (10 mM) (Fig. 2*B*). For a fixed level of depolarization (+20 mV in the experiment shown in Fig. 2*C*), the maximal intensity of the slow outward current was dependent on the degree of inactivation of the Ca^{2+} channel (Fig. 2*C*). Furthermore, the slow outward current was greatly reduced by internal perfusion with the Ca^{2+}-chelating agent EGTA (1 mM) (Fig. 2*D*). These results are additional indications supporting the view (9) that the slow outward current is due to an internal free Ca^{2+}-mediated K^+ channel activation in neuroblastoma cells.

The only effect of apamin (0.1 μM) was to suppress the a.h.p.

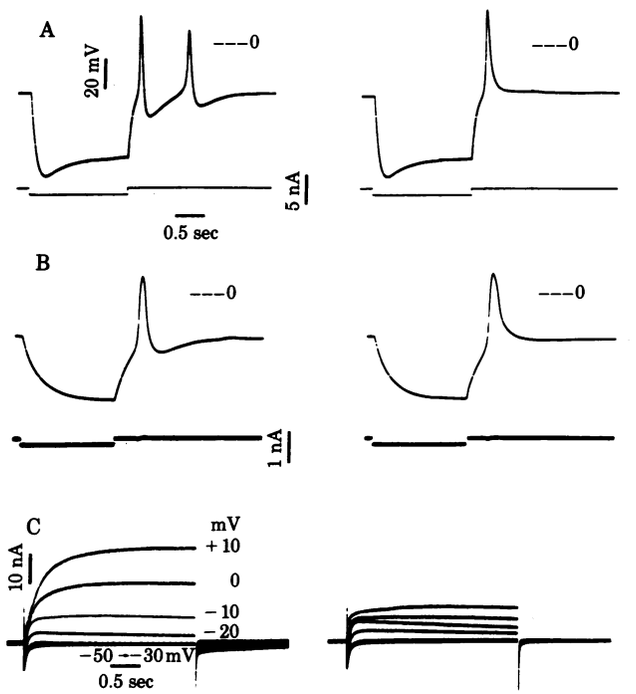

FIG. 1. Selective block by apamin of the a.h.p. following the action potentials of N1E 115 neuroblastoma cells bathed in solutions containing a high concentration of Ca^{2+} (25 mM); voltage-clamp analysis. (*A*) (*Left*) Multiple spike response evoked by anodal break stimulation in a 25 mM Ca^{2+}/90 mM Na^+ solution containing 25 mM Et_4N^+. The a.h.p. following the first spike has partially reactivated the Na^+ conductance, allowing the initiation of a second spike. (*Right*) Same cell, 2 min after the application of 0.1 μM apamin. Note the absence of a.h.p.. (*B*) (*Left*) Ca^{2+} action potential and a.h.p. evoked by anodal break stimulation in a 25 mM Ca^{2+}, Na^+-free solution containing 25 mM Et_4N^+. (*Right*) Suppression of the a.h.p. after a 2-min application of 0.1 μM apamin. In *A* and *B*, the zero voltage line is indicated. (*C*) Voltage-clamp analysis of the effect of apamin on neuroblastoma cells bathed in a 25 mM Ca^{2+}, Na^+-free solution containing 25 mM Et_4N^+. Families of membrane currents associated with different step depolarizations from a holding potential (V_H) of −90 mV. (*Left*) Control currents. (*Right*) Currents 5 min after the addition of 0.1 μM apamin. The Ca^{2+} current was not affected; the Ca^{2+}-dependent slow outward current was strongly depressed.

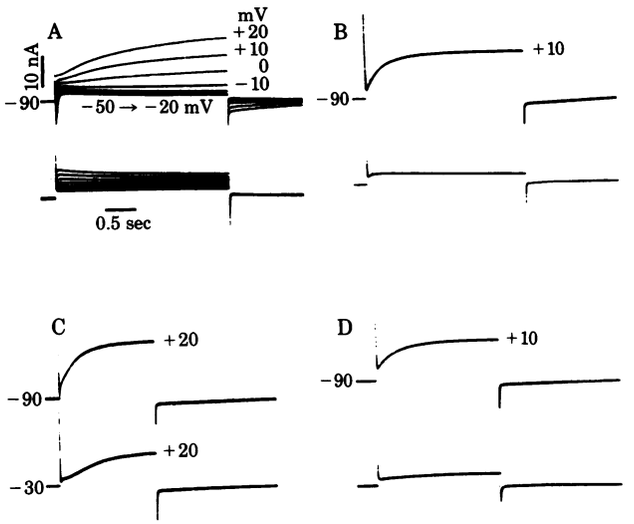

FIG. 2. Evidence for a Ca^{2+}-dependent slow outward current in N1E 115 neuroblastoma cells; voltage-clamp analysis. Inhibition of the slow outward current by Ca^{2+} blockers such as La^{3+} (*A*) or Co^{2+} (*B*), by inactivation of the Ca^{2+} current (*C*) and by internal perfusion with EGTA (*D*). (*A*) Families of membrane currents associated with different step depolarizations from $V_H = -90$ mV. Upper traces, control currents. Lower traces, 2 min after the addition of 2 mM La^{3+}. Note that both the Ca^{2+} inward current and the slow outward current were blocked. (*B*) Upper trace, control current associated with a step depolarization from $V_H = -90$ mV to +10 mV. Lower trace, 5 min after the addition of 10 mM Co^{2+}, the slow outward current was blocked. (*C*) Upper trace, control current associated with a step depolarization from $V_H = -90$ mV to +20 mV. Lower trace, membrane current associated with a step depolarization from $V_H = -30$ mV to +20 mV, 1.5-sec duration followed by a step repolarization to −90 mV. Note the significant reduction of the slow outward current. (*D*) Upper trace, control current associated with a step depolarization from $V_H = -90$ mV to +10 mV. Lower trace, 10 min after the normal internal perfusion solution has been switched to a solution containing 1 mM EGTA. Note the large reduction of the slow outward current.

(Fig. 1 *A* and *B*, *Right*). Voltage-clamp analyses showed that apamin (0.1 μM) is a specific blocker of the slow outward current. The remaining current after 0.1 μM apamin treatment (Fig. 1*C*, *Right*) or after treatment with Ca^{2+} blockers (Fig. 2 *A* and *B*) can be attributed to a nonlinear leakage current; i.e., the leakage conductance is higher in depolarization than around the holding potential (9). The concentration of apamin that blocked 50% of the slow outward current was around 10 nM.

Independent voltage-clamp analyses of the tetrodotoxin-sensitive fast Na^+ current and of the Et_4N^+-sensitive K^+ current showed that the corresponding ionic conductances were unaltered by apamin.

Association and Dissociation Kinetics of the Interaction Between ^{125}I-Apamin and Neuroblastoma Cell Membranes. Typical kinetics of association between ^{125}I-apamin and neuroblastoma cell homogenates are represented in Fig. 3*A*. These experiments were carried out with an ^{125}I-apamin concentration of 40 pM. The concentration of specifically bound ^{125}I-apamin that corresponds to 100% in Fig. 3*A* was 2.28 pM. The free ^{125}I-apamin concentration varied less than 6% during the course of the association kinetics. Fig. 3*A Inset* shows that a semilogarithmic plot of the data is linear, which is expected for a pseudo-first-order reaction. The rate constant of the association is then $k = k_a[^{125}\text{I-apamin}] + k_d$, in which k_a and k_d represent the rate constants of association and dissociation, respectively, of the ^{125}I-apamin–receptor complex. The value of k under conditions used in Fig. 3*A* is 1.3×10^{-3} sec^{-1}.

Fig. 3*B* demonstrates that ^{125}I-apamin bound to neuroblastoma cells can be displaced by unlabeled apamin. Because the presence of a large excess of unlabeled apamin (1 μM) prevents the reassociation of ^{125}I-apamin with neuroblastoma cells, the dissociation process should be first-order with k_d as rate constant. As expected, the semilogarithmic representation of the dissociation data is linear (Fig. 3*B Inset*). The calculated value of k_d from data in Fig. 3*B Inset* is 2×10^{-4} sec^{-1}. The value of k_a calculated from k, k_d, and the ^{125}I-apamin concentration is 2.7×10^7 M^{-1} sec^{-1}. These kinetic data enable us to calculate the dissociation constant of the ^{125}I-apamin–receptor complex: $K_d^* = k_d/k_a = 7.3$ pM.

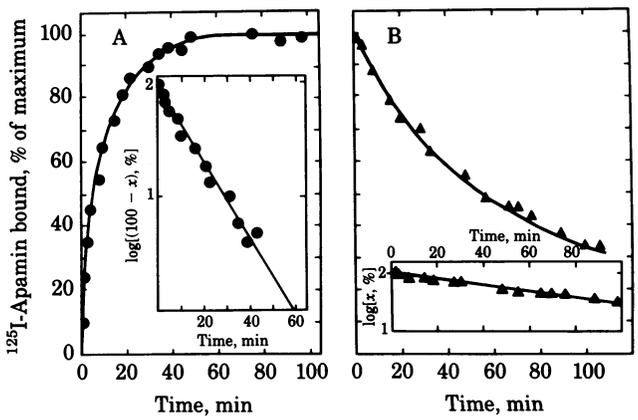

FIG. 3. Association and dissociation kinetics for the binding of ^{125}I-apamin to N1E 115 homogenate. (*A*) Association kinetics of ^{125}I-apamin (40 pM) binding at 0°C to N1E 115 homogenate (0.3 mg/ml). (*Inset*) Semilogarithmic representation of the data; x represents the percentage of maximal ^{125}I-apamin bound. The plateau value (100%) corresponds to 7.6 fmol of ^{125}I-apamin bound per mg of protein. (*B*) Dissociation kinetics of ^{125}I-apamin initiated by addition of 1 μM unlabeled apamin. (*Inset*) Semilogarithmic representation of the data.

Equilibrium Binding of ^{125}I-Apamin to its Receptor in Neuroblastoma Cells. Fig. 4*A* shows typical results of binding experiments in which increasing concentrations of ^{125}I-apamin are added to a fixed concentration of a homogenate of differentiated N1E 115 cells. The specific binding is represented by the difference between total and nonspecific binding. Linearity of the Scatchard plot (Fig. 4*B*) demonstrates that ^{125}I-apamin binds to a single class of noninteracting sites. The dissociation constant, K_d^*, of the complex formed between ^{125}I-apamin and neuroblastoma cells is 22 pM and the maximal binding capacity is 12 fmol/mg of protein.

The Scatchard plot obtained with nondifferentiated neuroblastoma (Fig. 4*B*) indicates a dissociation constant of 32 pM and a maximal binding capacity of 3.2 fmol/mg of protein.

Apamin Receptor Is Degraded by Proteases. Fig. 5 shows drastically decreased levels of ^{125}I-apamin binding to its receptor after treatment of the neuroblastoma cell homogenate with trypsin, chymotrypsin, Pronase, or papain.

Competition Between Unlabeled Apamin and ^{125}I-Apamin. Fig. 6*A* shows that increasing concentrations of unlabeled apamin gradually inhibit ^{125}I-apamin binding to the specific toxin receptor. Half-maximal inhibition of ^{125}I-apamin binding to neuroblastoma cells is at $K_{0.5} = 18$ pM. The equation for $K_{0.5}$ is: $K_{0.5} = K_d[1 + ([^{125}\text{I-apamin}]_{0.5}/K_d^*)]$, in which $[^{125}\text{I-apamin}]_{0.5}$ is the concentration of free labeled ligand at half-displacement. K_d^* and K_d are the dissociation constants of complexes formed between neuroblastoma and ^{125}I-apamin or unlabeled apamin, respectively. K_d^* is 22 pM (see above) and $[^{125}\text{I-apamin}]_{0.5}$ is 3 pM under the experimental conditions of Fig. 6*A*. K_d is then 16 pM.

Fig. 6*A* also presents a competition experiment under conditions in which the physiological buffer is used instead of the

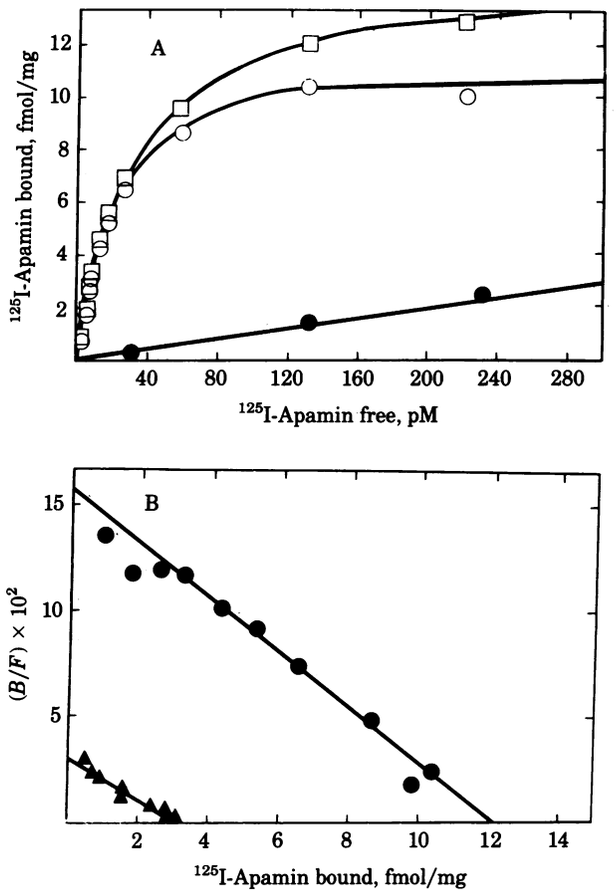

FIG. 4. Binding of ^{125}I-apamin to N1E 115 homogenate under standard conditions. (*A*) N1E 115 homogenate (0.3 mg/ml) was incubated with increasing concentrations of ^{125}I-apamin. □, Total binding. Nonspecific binding (●) was determined in the presence of a large excess of unlabeled toxin (1 μM). Specific binding (○) is the difference between total binding and nonspecific binding. (*B*) Scatchard plot of the data. *B*, bound; *F*, free. Data from differentiated (●) and nondifferentiated (▲) N1E 115 neuroblastoma cells.

standard buffer. In this case, $K_{0.5}$ = 75 pM and K_d = 49 pM. The nontoxic apamin derivative in which Arg-13 and Arg-14 have been modified by 1,2-cyclohexanedione (4) was unable to prevent ^{125}I-apamin binding to its receptor at concentration up to 0.1 μM (Fig. 6*A*).

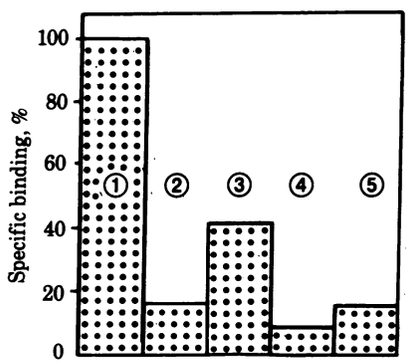

FIG. 5. Protease degradation of the apamin receptor. The homogenate (18 mg/ml) was incubated in 20 mM Tris·HCl/1 mM EDTA/0.25 M sucrose (pH 7.5) for 1 hr at 25°C in the absence of protease (bar 1) or in the presence of trypsin at 0.1 mg/ml (bar 2), chymotrypsin at 0.5 mg/ml (bar 3), Pronase at 0.1 mg/ml (bar 4), or papain at 0.1 mg/ml (bar 5). Proteolysis was stopped by washing the homogenate with ice-cold buffer (20 vol) three times, then specific binding of ^{125}I-apamin (3.3 pM) to each homogenate was measured.

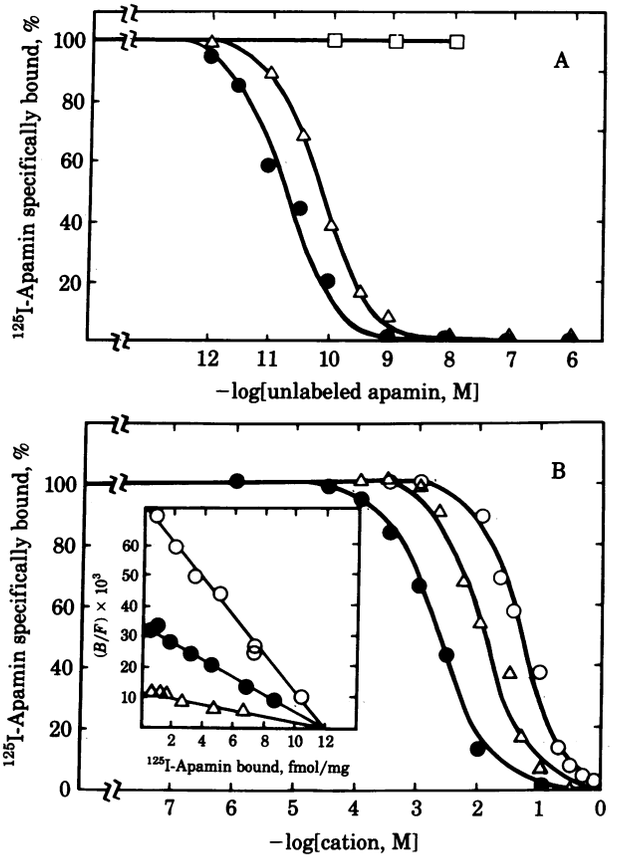

FIG. 6. (*A*) Competition between ^{125}I-apamin and unlabeled apamin in standard conditions (●) and in physiological buffer (△). Competition between ^{125}I-apamin and apamin modified on Arg-13 and Arg-14 by 1,2-cyclohexanedione (□). The concentrations of ^{125}I-apamin were 3.3 pM (●, □) and 26 pM (△). (*B*) Competition in standard conditions between ^{125}I-apamin (3.3 pM) and Ca^{2+} (●), Na^+ (○), and guanidinium (△). (*Inset*) Scatchard plots of the data obtained from binding of ^{125}I-apamin to N1E 115 homogenate (0.15 mg/ml) in the presence of 0 Na^+ (○), 50 mM Na^+ (●), or 100 mM Na^+ (△).

Effect of Monovalent and Divalent Cations on ^{125}I-Apamin Binding to its Receptor. In all these experiments N1E 115 homogenates were incubated under standard conditions (20 mM Tris·HCl/5.4 mM KCl, pH 7.5) in the presence of ^{125}I-apamin (3.3 pM) and at different concentrations of mono- and divalent cations. Na^+, guanidinium, and Ca^{2+} inhibit ^{125}I-apamin binding (Fig. 6*B*). Fifty percent inhibition ($K_{0.5}$) is observed at 2 mM Ca^{2+}, 44 mM Na^+, and 12 mM guanidinium. Choline inhibits ^{125}I-apamin binding like Na^+ or guanidinium, with a $K_{0.5}$ value of 25 mM. Et_4N^+ inhibits ^{125}I-apamin binding with a $K_{0.5}$ value of 6 mM.

The large differences in affinity of apamin for its receptor measured by electrophysiology on one hand and by binding experiments with ^{125}I-apamin on the other hand are explained by the experimental conditions required in the voltage-clamp approach—i.e., high Ca^{2+} (25 mM), choline (90 mM), and Et_4N^+ (20 mM) concentrations.

Scatchard plots of ^{125}I-apamin binding (Fig. 6*B*, *Inset*) show that increasing Na^+ concentrations do not change the maximal binding capacity of neuroblastoma membranes, they only change the apparent dissociation constant, which increases from 25 pM at 0 mM Na^+ to 150 pM at 100 mM Na^+. This is the typical indication that Na^+ is a competitive inhibitor of ^{125}I-apamin binding to its receptor.

Effects on ^{125}I-Apamin Binding of Neurotransmitters, Neuropeptides, and Drugs Acting on Transmitter Receptors or Ionic Channels. Histamine (10 μM), γ-aminobutyric acid (10 μM), glutamic acid (10 μM), epinephrine (1 μM), norepinephrine (1 μM), acetylcholine (1 μM), glycine (1 μM), serotonin (1 μM), and dopamine (1 μM) are without effect on ^{125}I-apamin binding. Diazepam (1 μM), lysergic acid diethyl amide (1 μM), strychnine (100 μM), and isoproterenol (1 μM) are also without effect. Toxins specific for the fast Na^+ channel (1, 2), such as tetrodotoxin, veratridine, sea anemone toxin II, or pure scorpion toxins from *Androctonus*, *Tityus*, or *Centruroides* venom are also without effect at concentrations as high as 1 μM.

Amiloride, a specific blocker of epithelial Na^+ channels (20), inhibits ^{125}I-apamin binding to its receptor with a $K_{0.5}$ of 0.25 mM. Verapamil, a blocker of the Ca^{2+} channel, also inhibits ^{125}I-apamin binding with a $K_{0.5}$ of 0.10 mM. The most active molecule in inhibiting ^{125}I-apamin binding is the polypeptide neurotensin. The $K_{0.5}$ value in that case is 0.4 μM.

DISCUSSION

This paper demonstrates by using voltage-clamp techniques that apamin blocks a Ca^{2+}-dependent K^+ conductance. This blockade is very selective, because apamin is without effect on other ionic channels such as the fast Na^+ channel, the Et_4N^+-sensitive K^+ channel, or the slow Ca^{2+} channel. Under the particular conditions used for the electrophysiological study of the Ca^{2+}-dependent K^+ conductance (25 mM Ca^{2+}), a complete block of the channel is observed at 100 nM apamin.

The use of a very highly radiolabeled derivative of apamin, ^{125}I-apamin, permitted a detailed study of the interaction of the toxin with its receptor in neuroblastoma cells. The binding is saturable and specific. Nontoxic derivatives modified on Arg-13 and Arg-14 (4) do not prevent ^{125}I-apamin binding to its receptor. The interaction of apamin with its receptor has the following properties: (*i*) the dissociation of the toxin from its receptor is slow, with a half-life that may be as low as 58 min at 0°C and pH 7.5; (*ii*) the K_d value of the apamin–receptor complex is 16–22 pM under standard conditions; (*iii*) the apamin receptor is destroyed by proteases (trypsin, chymotrypsin, Pronase, papain) and is therefore probably a protein.

The maximal number of binding sites found on differentiated neuroblastoma cells is 12 fmol/mg of protein; it is about 1/5th of that found for fast Na^+ channels in the same preparation using a radiolabeled tetrodotoxin derivative [58 fmol/mg of protein (21)].

One of the interesting properties of the Ca^{2+}-dependent K^+ channel is that it develops during differentiation in low serum concentrations and in the presence of dimethyl sulfoxide. Nondifferentiated neuroblastoma cells do not have Ca^{2+}-dependent K^+ conductances that can be detected electrophysiologically. Moreover, comparable titrations of the apamin receptor in undifferentiated and in differentiated cells show an increase in number of apamin sites by a factor of about 4, without modification of the receptor properties since the dissociation constant remains essentially unchanged. Because a small percentage of cells are already morphologically differentiated before treatment with dimethyl sulfoxide and because all of the cells are not differentiated after treatment with low serum concentration plus dimethyl sulfoxide, it is possible that Ca^{2+}-dependent K^+ conductances are completely absent in undifferentiated neuroblastoma cells, whereas they are fully developed after differentiation.

Ionic conditions are of particular importance for the binding of apamin to its receptor (14). Various monovalent and divalent cations prevent ^{125}I-apamin binding to its receptor (Na^+, guanidinium, choline, Ca^{2+}). Because of these inhibitory effects of cations, under physiological conditions (5.4 mM K^+/2.8 mM Ca^{2+}/1.3 mM Mg^{2+}/100 mM Na^+/20 mM Tris·HCl, pH 7.5), the K_d value of apamin for its receptor is shifted to 71 pM (Fig. 6A).

The competition of guanidinium ions for ^{125}I-apamin binding is probably the result of the fact that the active site of the toxin contains two guanidinium groups, on Arg-13 and Arg-14. Moreover, amiloride, which is also a guanidinated molecule, also prevents ^{125}I-apamin binding. However the most active compound in preventing ^{125}I-apamin association to its receptor is neurotensin. This neuropeptide of 13 amino acids has two contiguous arginines in its active site (22).† Other charged molecules like verapamil also interfere with ^{125}I-apamin binding to the neuroblastoma membrane.

Since Ca^{2+}-dependent K^+ channels are now found in a large number of excitable cells preparations, we feel that apamin will play a role for this channel analogous to that played by the different toxins specific for the fast Na^+ channel (1).

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† Whereas neurotensin prevents ^{125}I-apamin binding, apamin does not prevent [^{3}H]neurotensin binding to its receptor (P. Kitabgi, personal communication). This shows that the receptors for the two peptides are completely distinct.