Interactions of the Neurotoxin Apamin with a Ca\textsuperscript{2+}-activated K\textsuperscript{+} Channel in Primary Neuronal Cultures*

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Mono\textsuperscript{[125I]}iodoapamin bound to specific sites on cultured rat embryonic neurons. The dissociation constant for the receptor-neurotoxin complex measured at equilibrium was 60–120 pM at pH 7.2 and 4 °C, with a maximal binding capacity of 3–8 fmol/mg of cell protein. Apamin inhibited calcium ionophore-induced \textsuperscript{86}Rb\textsuperscript{+} release from cell cultures. The dose effect curve for this pharmacological test corresponded closely to the displacement of \textsuperscript{125I}-apamin by native apamin in binding experiments. Formation of the \textsuperscript{125I}-apamin receptor complex requires exogenous K\textsuperscript{+}. Reduced binding in the absence of K\textsuperscript{+} was due to diminished binding capacity rather than a lower affinity. The apamin receptor seems to be associated with a cell surface K\textsuperscript{+} site which shows 50% occupancy at 1.6 mM, and which could be involved in the regulation of channel activity. Apamin sites were present at the earliest developmental stage tested and their number did not evolve during 8 days in culture. In the same period, however, \textalpha;scorpion toxin binding increased by a factor of 10. The ontogenesis of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels does not seem to occur in parallel with that of voltage-sensitive Na\textsuperscript{+} channels.

Outward potassium conductances gated by relatively small variations in cytoplasmic calcium concentration exist in a large variety of both excitable and nonexcitable cells (1, 2). Calcium influx via voltage or receptor-dependent channels activates the potassium channel leading to a long lasting hyperpolarization. In mammalian neurons, membrane hyperpolarization controls the frequency of trains of action potentials (3), and this is one of the means by which neurotransmitters can regulate the repetitive electrical activity of central neurons (4, 5).

Apamin, a 2000-dalton neurotoxic peptide from bee venom, first shown to be a specific blocker of the calcium-dependent potassium permeability in hepatocytes (6) has recently been used to explore this membrane channel in excitable cells. Calcium-activated slow potassium currents sensitive to apamin have been detected by electrophysiological techniques in NIE-115 mouse neuroblastoma cells (7) and in cultured muscle cells (8). The radioligand binding approach has shown the presence of high affinity \textsuperscript{125I}-apamin sites in membranes from the rat brain (9, 10), neuroblastoma (7), and muscle (8).

In this paper we describe \textsuperscript{125I}-apamin binding to intact rat embryonic neurons in culture and correlate a high affinity interaction with a pharmacological effect on \textsuperscript{86}Rb\textsuperscript{+} efflux. The in vitro ontogenesis of the apamin receptor is compared to that of \textalpha;scorpion toxin (11), which binds to a component of the voltage-sensitive sodium channel.

EXPERIMENTAL PROCEDURES

Neurotoxin Iodination—Apamin was purified from bee venom (12). 25 nmol of apamin were routinely reacted with 1 mCi of Na\textsuperscript{[125I]} (New England Nuclear Corp.) and 75 nmol of iodogen (Pierce Chemical Co.) and a pure mono\textsuperscript{[131I]}iodoapamin derivative. Uptake of 2000 Ci/mmol was separated by SP (sulfopropyl)-Sephadex C-25 chromatography as described (10). Toxin II from the venom of the scorpion Androctonus australis Hector was purified and radioiodinated with lactoperoxidase method (13) to a specific radioactivity of 1000 Ci/mmol.

Cell Culture—Brains from 15–17-day-old Wistar rat embryos were mechanically dissociated and seeded in 24-mm wells pretreated with poly 1-lysine (14) on multiwell plates (Flow Laboratories Inc.). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum (Gibco) and 1% streptomycin/penicillin (10,000 units/ml, Gibco). The medium was changed on the 5th and 9th day of culture. Cell cultures were used in binding or tracer experiments between the 6th and the 11th day unless otherwise stated.

During this period they can be considered as being a mixture of neuronal and glial cells but with a large majority of neurons.

Binding Experiments—The standard buffer contained 25 mM Hepes, \textsuperscript{3}10 mM glucose, 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\textsubscript{2}, 0.8 mM MgSO\textsubscript{4}, and 0.25% bovine serum albumin adjusted to pH 7.2 with Tris base. In the wash buffer, the Hepes concentration was reduced to 5 mM. For experiments with \textsuperscript{125I}-iodoapamin from A. australis Hector, NaCl was replaced by choline chloride. In typical experiments the culture medium was aspirated, the cell layer washed once with 1 ml of buffer, and the radioligand added at the required concentration in the presence or absence of other test substances in 0.4 ml of buffer. In experiments on the effect of cations, the cell layer was washed three times with 1 ml of buffer from which KCl, CaCl\textsubscript{2}, and MgSO\textsubscript{4} had been omitted before addition of the binding medium. At the indicated times, binding was terminated by aspiration of the medium and the cell layer was washed three times in 15 s with 1 ml of buffer at 4 °C. Cells were then collected in 1.5 ml of 0.1 M NaOH for γ counting, with 80% efficiency (Packard Auto-Gamma). Cell protein was assayed by a modified Lowry (15) method with bovine serum albumin as a standard. Experimental points are the mean of duplicate determinations/mg of cell protein.

\textsuperscript{86}Rb\textsuperscript{+} Release—Cells were incubated in 0.5 ml of standard buffer, from which divalent ions had been omitted, containing 1 μCi/ml of \textsuperscript{86}RbCl (C.E.A.) for 120 min at 37 °C. Uptake was stopped by aspiration and rapid washing. 0.7 ml of release medium at 37 °C was added, containing 2 mM A23187 (Calbiochem-Behring) dissolved in dimethyl sulfoxide (final concentration <1%) with or without apamin or quinidine in the standard buffer. Samples of the medium were taken at indicated times, and the experiment was terminated by washing the cell layer. The \textsuperscript{86}Rb\textsuperscript{+} activity present in medium samples and in the cell layer at the end of the experiment was measured by liquid scintillation spectrometry.

The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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RESULTS

125I-Apamin Binding in Physiological Conditions—Fig. 1A shows the results of an equilibrium binding experiment in which increasing concentrations of 125I-apamin were added to neuronal cells in the absence (total binding) or in the presence of a large excess of unlabeled apamin (nonspecific binding). The difference in cell-associated radioactivity gives the saturable binding component. Specific binding was detected at both 4 and 37 °C (Fig. 1B). However, as a number of experiments had indicated reduced binding at 37 °C, parameters were systematically estimated at 4 °C. A Scatchard plot of the specific component (Fig. 1C) shows a single class of sites with a dissociation constant \( K_d = 98 \text{ pM} \) and a binding capacity \( B_{	ext{max}} = 5.8 \text{ fmol/mg of cell protein} \) at 4 °C and pH 7.2.

Results from several experiments gave \( K_d \) values from 60 to 120 pM and \( B_{	ext{max}} \) in the 3-8 fmol/mg of protein range.

Native apamin competes with 125I-apamin for interaction with neuronal cells displacing 50% of the bound radioligand at 110 pM (Fig. 2) giving a calculated \( K_d \) of 90 pM.

The kinetics of 125I-apamin association to and dissociation from its receptor on intact cells at 4 °C are shown in Fig. 3. The slopes of the linear semilogarithmic plots (insets to Fig. 3) gave values for \( k_{\text{app}} \) and \( k_{-1} \) which when substituted into the equation

\[
k_{\text{app}} = k_1 [\text{125I-apamin}] + k_{-1}
\]

allowed calculation

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Fig. 1. 125I-apamin binding to neuronal cell cultures. A, cells were incubated with increasing concentrations of 125I-apamin in the presence (C, nonspecific binding) or the absence (O, total binding) of 0.4 \( \mu \text{M} \) unlabeled apamin. Cell-associated radioactivity was determined after 90 min at 4 °C. B, specific binding (■) is the difference between the total and the nonspecific components in A. A saturable specific component was also detectable after a 20-min incubation at 37 °C (□). C, Scatchard plot of specific binding at 4 °C. B, bound; \( F, \) free. Binding units are fmol/mg of cell protein.

Fig. 2. Competition for binding between 125I-apamin and native apamin. 125I-apamin (10 pM) was added to cell cultures in the presence of increasing concentrations of unlabeled apamin. Cell-bound radioactivity was determined after 90 min at 4 °C. B, bound; \( B_0, \) bound in the absence of native apamin.

Fig. 3. 125I-apamin binding kinetics. A, association kinetics: 125I-apamin (0.1 nM) was added to neuronal cultures and the cell-bound radioactivity was determined after the indicated time at 4 °C. Nonspecific binding, estimated in the presence of 0.4 \( \mu \text{M} \) unlabeled apamin was subtracted. \( B, \) dissociation kinetics: at equilibrium, 0.4 \( \mu \text{M} \) unlabeled apamin was added to the wells, and the remaining specific cell-bound radioactivity at the indicated times was measured. Insets, linear semilogarithmic plots of kinetic data. B, bound; \( B_{\text{eq}}, \) bound at equilibrium.
of \( k_1 \). The association rate constant was \( k_1 = 4.1 \times 10^6 \, \text{M}^{-1} \, \text{s}^{-1} \) and the dissociation rate constant \( k_{-1} = 3.3 \times 10^{-4} \, \text{s}^{-1} \). The dissociation constant \( K_d \) calculated from the kinetic data (\( K_d = k_{-1}/k_1 \)) was 80 pm at 4°C and pH 7.2, in good agreement with results obtained at equilibrium.

**Apamin Inhibition of Ca\(^{2+}\): Ionophore-induced \( ^{86}\text{Rb}^+ \) Release**—Cells were loaded with \(^{86}\text{Rb}^+\), a convenient K\(^+\) tracer, and then rapidly washed and the tracer efflux into the incubation medium was followed. Addition of A23187, a divalent ionophore, which presumably increases the cytosolic Ca\(^{2+}\) concentration (16), increased \(^{86}\text{Rb}^+\) release from neuronal cells. This ionophore-dependent \(^{86}\text{Rb}^+\) loss was inhibited by apamin (Fig. 4B). The blocking effect was only partial with a maximum of about a 15–30% reduction occurring with 10 nM apamin. A23187 seems to induce a Ca\(^{2+}\)-dependent effect as it did not notably increase \(^{86}\text{Rb}^+\) efflux when added in a low Ca\(^{2+}\) medium (100 \( \mu \text{M} \)) (Fig. 4A). Furthermore, apamin did not inhibit the basal efflux measured in these conditions (not shown).

The dose-effect curve for apamin in Fig. 4C shows an EC\(_{50}\) of about 300 \( \mu \text{M} \) and the pharmacological response superimposes fairly well on the competition curve obtained from binding experiments in the same conditions. Quinidine also inhibits \(^{86}\text{Rb}^+\) release (Fig. 4C) and displaces \(^{125}\text{I}\)-apamin from its receptor in the same micromolar concentration range. There is therefore good correlation between high affinity apamin-binding and a pharmacological effect on Ca\(^{2+}\)-induced cation release.

**Effects of Cations on \(^{125}\text{I}\)-Apamin Binding**—The role of several monovalent cations in the specific interaction of apamin with its receptor was determined at constant ionic strength by substituting test ions for Na\(^+\). A clear requirement for K\(^+\) was observed. As can be seen in Fig. 5, binding increased by a factor of three between 100 \( \mu \text{M} \) and 10 mM K\(^+\) with 50% of the stimulatory effect occurring at 1.6 mM K\(^+\).

The results of Scatchard analysis of equilibrium binding data obtained in the presence of 10 mM K\(^+\) or without added K\(^+\) are shown in the inset to Fig. 5. Enhanced binding in the presence of K\(^+\) is essentially due to increased capacity with little modification of ligand affinity. This suggests that occupancy of a K\(^+\) site which saturates in a physiological concentration range, and is accessible to the extracellular medium is essential for formation of the apamin-receptor complex.

Table I lists the results of replacing 100 mM Na\(^+\) with the same concentration of other monovalent cations in the presence of 5.4 mM K\(^+\). Specific binding while not significantly modified by Li\(^+\) was reduced by both choline and guanidinium.

Increasing the divalent ion concentration also inhibited binding, which in this case was probably due to raising the ionic strength. Cell associated \(^{125}\text{I}\)-apamin was diminished by 50% in 56 mM Ca\(^{2+}\).

**Comparative Ontogenesis of Apamin and \( \alpha \)-Scorpion Toxin Receptors**—If one assumes that apamin binds to a component of the Ca\(^{2+}\)-activated K\(^+\) channel, it is interesting to compare the evolution of the number of apamin sites during neuronal differentiation in culture with that of other structures involved in electrical excitability. Fig. 6 represents an experiment in which apamin binding was followed during the first 8 days in culture. Apamin sites were present on day 1 and their number remained fairly constant up until day 8. The \( K_d \) of the apamin-receptor complex as determined by equilibrium binding did not vary during this period.

The receptor sites for an \( \alpha \)-scorpion toxin (\( ^{125}\text{I}\)iodotoxin II from \( A. \) australis Hector), which interacts with the voltage-sensitive Na\(^+\) channel, were titrated in parallel. A greater than 10-fold increase in receptors occurred between day 1 and 5, as previously described in mouse neuronal cultures (17). The \( K_d \) for the \(^{125}\text{I}\)iodotoxin II from \( A. \) australis Hector-receptor complex in rat neuronal cells was constant at 200 pm throughout this period.

![Fig. 4](http://www.jbc.org/)
Apamin Interactions with Neuronal Cells

**Fig. 5.** The effect of K⁺ on ³²P-apamin binding. Neuronal cells were incubated with ³²P-apamin (0.1 nM) in the presence of increasing concentrations of K⁺. Constant ionic strength was maintained by substituting K⁺ for Na⁺ in the absence of divalent ions. Cell-associated radioactivity was measured after 90 min at 4 °C. Nonspecific binding measured with 0.4 μM unlabeled spamin has been subtracted. Inset, the results of ³²P-apamin equilibrium binding experiments in 10 mM K⁺ (○) or without added K⁺ (△) are represented in a Scatchard plot. B₀ bound; F, free; Bₙ bound in the presence of 100 mM K⁺.

**TABLE I**

Effect of monovalent cations on ³²P-apamin binding

<table>
<thead>
<tr>
<th>Cation</th>
<th>³²P-Apamin bound (fmol/mg protein)</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>2.2 ± 0.2</td>
<td>100 ± 11</td>
</tr>
<tr>
<td>Li⁺</td>
<td>2.4 ± 0.1</td>
<td>111 ± 6</td>
</tr>
<tr>
<td>Choline⁺</td>
<td>1.3 ± 0.1</td>
<td>60 ± 7</td>
</tr>
<tr>
<td>Guanidinium⁺</td>
<td>1.1 ± 0.1</td>
<td>49 ± 6</td>
</tr>
</tbody>
</table>

These observations indicate that during ion channel ontogenesis in developing embryonic neurons, the appearance of Ca²⁺-activated K⁺ channels is separated in time from that of voltage-sensitive Na⁺ channels.

**DISCUSSION**

³²P-Apamin associates with high affinity to a small number of receptor sites on intact cultured neuronal cells. This is the first demonstration of apamin binding to intact excitable cells. The fact that specific binding sites are present on neurons in this culture system has been confirmed by scanning electron microscope visualization of apamin-hemocyanin conjugates. This technique showed a homogeneous distribution of receptors on all observed neuronal cell bodies. The receptor capacity calculated from the radioligand binding data corresponds to about 1000 sites/cell. This estimation is quite close to that reported in the only other study on whole cells to date: 1500 sites/cell in hepatocyte suspensions (18).

A comparison, in neuronal cultures, of ³²P-apamin and ³²P-α-scorpion toxin site capacity permits a tentative estimation of the Ca²⁺-activated K⁺ channel/Na⁺ channel ratio which is about 1/500, assuming one neurotoxin receptor/channel.

The occupancy of apamin sites indicated in binding experiments corresponds closely to an inhibition of the ²³Rb⁺ loss induced by a calcium ionophore. The fact that only part of the Ca²⁺ dependent loss was sensitive to apamin remains unexplained. The apamin-insensitive component was equally insensitive to 2 μM tetrodotoxin (not shown) ruling out an effect related to a depolarization induced opening of Na⁺ channels. Quinidine also blocks the A23187-activated ⁸⁶Rb⁺ permeability and displaces ³²P-apamin from its receptor. It is not, however, a specific inhibitor and is known to modify other ion permeabilities, notably the voltage dependent Na⁺ (8) and K⁺ (3) channels.

From the functional point of view the most interesting characteristic of apamin binding is its dependence on the presence of K⁺. This cation requirement has been pointed out in work on synaptosomal membranes which indicated that K⁺ induces a biphasic effect, increasing ³²P-apamins affinity for its receptor at low concentrations while decreasing the affinity at concentrations higher than 5 mM (10). This latter effect, probably being due to increasing ionic strength, was not observed in our conditions where K⁺ was substituted for Na⁺.

Furthermore, our results on intact neuronal cells which should be more significant physiologically show that K⁺ modulates ³²P-apamin binding capacity and not its binding affinity. This implies that for apamin to form a complex with its receptor, the latter must assume a conformation induced by K⁺ to be accessible to the extracellular medium, and that its occupancy varies between 0.1 and 10 mM K⁺, which corresponds to the physiological concentration range of the extracellular medium.

The presence of an extracellular K⁺ site involved in the regulation of the state of the Ca²⁺-dependent K⁺ channel has been deduced from flux studies in erythrocytes (2). Similarly, single channel recordings from membrane patches of chromaffin cells have shown outward Ca²⁺-activated K⁺ currents modulated by internal Na⁺ and external K⁺ (19). It is tempt-
Apamin Interactions with Neuronal Cells

1495

...ing therefore to suggest that the apamin receptor is in some way related to a regulatory cell surface K+ site of this kind.

Another possibility that cannot as yet be ruled out is that as modification of the extracellular K+ concentration changes the cellular membrane potential, apamin binding could be voltage depen dent rather than directly K+ dependent. This suggests that the ontogenesis of Ca2+-dependent K+ channels is also K+ dependent in membrane preparations that are presumably nonpolarized (10).3

125I-Apamin receptors were detected on cultured neuronal cells at the earliest developmental stages tested, that is 1 h after seeding dispersed brain cells from 16-day-old rat embryos. At this stage, cells are round and cell processes are absent. Considerable neurite growth occurs during the first 5–6 days in culture. This morphological differentiation is accompanied by the appearance of voltage-sensitive Na+ channels reflected in a 10-fold increase in α-scorpion toxin receptors, while apamin binding capacity remains stable. This suggests that the ontogenesis of Ca2+-dependent K+ channels and that of Na+ channels are independent processes which are not programmed in parallel.

No attempt has yet been made to determine whether apamin receptors increase in number at a later time in vitro. The present culture system results after about 8 days in increasing neuronal death together with glial cell proliferation. This last fact should be underlined as in studies with glial cell cultures we have detected high affinity apamin sites on astrocytes, although with a maximal binding capacity about five times less than in neurons.4 However, given the lower site density and the relatively small number of glial cells present in neuronal cultures, the contribution of astrocytes to the observed 125I-apamin binding is negligible up until days 11 and 12. Another argument in favor of neuronal binding is that the global binding capacity remains constant over a period of 1–8 days in vitro, which corresponds to a phase of glial cell proliferation. After this date, the presence of astrocytes should be taken into account, which would raise obvious difficulties in the interpretation of developmental phenomena.

Apamin, which can be considered as the only high affinity pharmacological probe specific for a K+ channel yet discovered, should prove to be a useful tool for physiological and developmental studies in excitable cell culture.

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