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The Ca^{2+}-dependent slow K^+ conductance in cultured rat muscle cells: characterization with apamin

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The interaction of apamin, a bee venom neurotoxin, with rat skeletal muscle cell membranes has been followed using both an electrophysiological and a biochemical approach. Voltage-clamp analyses have shown that apamin, at low concentrations, specifically blocks the Ca^{2+}-dependent slow K^+ conductance in rat myotubes and myosacs. A specific binding site for apamin in rat muscle cell membranes has been characterized with the use of a highly radiolabelled apamin derivative ([^{125}I]apamin). The dissociation constant for the apamin-receptor complex is 36–60 pM and the maximal binding capacity is 3.5 fmol/mg of protein. [^{125}I]Apamin binding to rat muscle membranes is displaced by quinine and quinidine with $K_{0.5}$ values of 110 μM and 200 μM, respectively.

Key words: apamin/Ca^{2+}-dependent K^+ conductance/quinine/rat muscle cell/receptor binding

Introduction

The presence of Ca^{2+}-dependent K^+ conductances in a variety of excitable tissues is now widely accepted (Meech, 1978). This K^+ conductance system seems to play an important role in regulating repetitive activity in both vertebrate and invertebrate excitable cells (Meech, 1978). A Ca^{2+}-dependent slow K^+ conductance has been particularly well studied in rat skeletal muscle cells in culture (Barrett *et al.*, 1981). The activation of such a conductance by Ca^{2+} entry during the action potential leads to a long-lasting after-hyperpolarization (a.h.p.). Since the development of patch-clamp techniques, single-channel recordings of Ca^{2+}-activated K^+ currents have been analyzed in rat myotubes (Pallotta *et al.*, 1981) and in rabbit T-tubule membrane fragments reconstituted into planar lipid bilayers (Latorre *et al.*, 1982).

In both systems the channel shows a large conductance highly selective for K^+ ions. The opening frequency and the life-time of these single channels depend on both membrane potential and the internal Ca^{2+} concentration.

Apamin, a bee venom polypeptide of 18 amino acids with two disulfide bridges, has recently been shown to block specifically the Ca^{2+}-dependent slow K^+ conductance in neuroblastoma cells (Hugues *et al.* 1982c). Other studies have shown that apamin blocks the Ca^{2+}-dependent channel in mammalian hepatocytes but not the Ca^{2+}-dependent K^+ permeability in red blood cells, suggesting that there are at least two types of Ca^{2+}-dependent K^+ conductances (Burgess *et al.*, 1981).

Two approaches have been used in the present work to study the mode of action of apamin on rat skeletal muscle cells in culture: (1) an electrophysiological approach to determine the specificity of the action of apamin on the Ca^{2+}-dependent slow K^+ channel and (2) a biochemical approach to analyse the properties of the specific binding of apamin to the cell membranes.

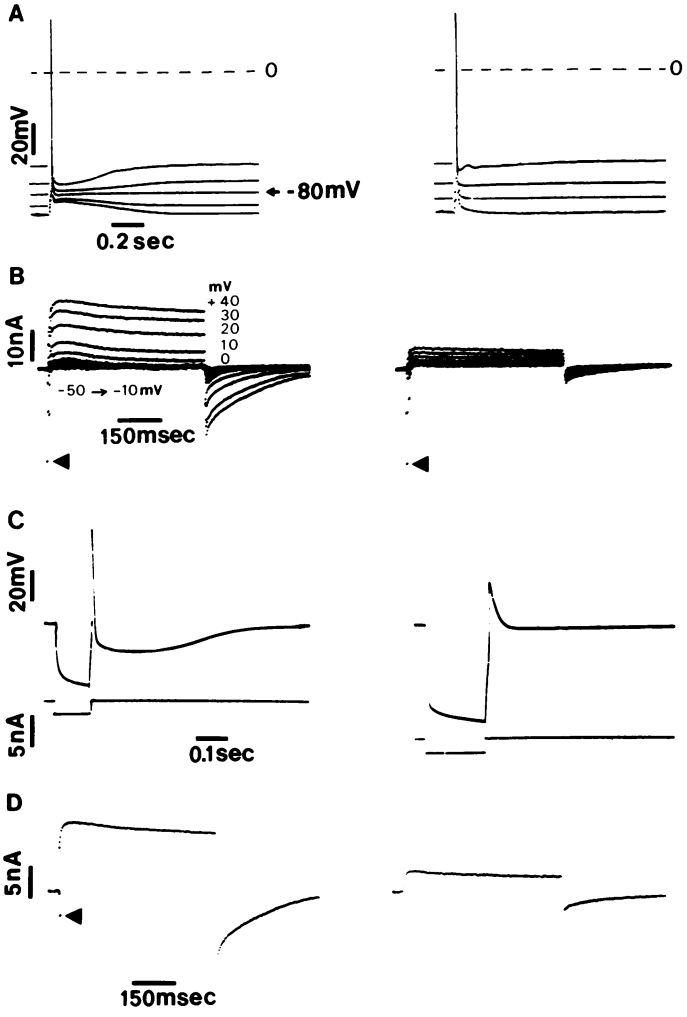

Fig. 1. A (Left): reversal of the slow a.h.p. The superimposed voltage traces show action potentials and after-potentials evoked in a rat myosac during the passage of a steady hyperpolarizing current of increasing intensity. The a.h.p. reverses at −80 mV (arrow). **(Right)**: same myosac, selective block of the a.h.p. after 10 min incubation with 10 nM apamin. The zero voltage line is indicated. **B**: voltage-clamp analysis of the effect of apamin on rat myosacs. Families of membrane currents associated with different step depolarizations from a holding potential (V_H) of −90 mV. **(Left)**: control currents. **(Right)**: currents 10 min after the addition of 10 nM apamin. The fast inward current is not affected; the slow outward current is strongly depressed. **C (Left)**: action potential and a.h.p. evoked by anodal break stimulation. **(Right)**: suppression of the a.h.p. and reduction of the spike amplitude after a 5-min application of 200 μM quinidine. The zero voltage line is indicated. **D (Left)**: control current associated with a step depolarization from V_H = −90 mV to +10 mV. **(Right)**: 10 min after the addition of 200 μM quinidine, the fast inward current and the slow outward current are blocked. In **B** and **D**, the peak inward Na^+ current is indicated (◀).

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Results

We have confirmed the observation that action potentials in rat myotubes or myosacs are always followed by a long-lasting a.h.p. mediated by the activation of a Ca^{2+}-dependent slow K^+ conductance, which is insensitive to tetraethylammonium (TEA) (Barrett *et al.*, 1981). In current-clamp experiments we adjusted the membrane potential of rat myosacs at different levels by injecting constant hyperpolarizing currents; action potentials were evoked by brief depolarizing pulses of current. Figure 1A (left) shows superimposed a.h.p. recorded at various levels of hyperpolarizing current. Reversal of the a.h.p. occurs at about −80 mV, which is the expected value for a K^+ system. As shown in Figure 1A (right), the a.h.p. are suppressed within a few minutes of the application of 10 nM apamin. Voltage-clamp data were also obtained from rat myosacs. The experiments were performed in the presence of 20 mM TEA in the incubation medium in order to block K^+ currents other than the Ca^{2+}-dependent slow K^+ current. The experiment presented in Figure 1B shows that apamin (10 nM) is a specific blocker of the slow outward current. Quinine and quinidine are commonly used to inhibit the Ca^{2+}-dependent K^+ conductance (Burgess *et al.*, 1981; Fishman and Spector, 1981). In the experiment illustrated by Figure 1C, quinidine (200 μM) blocked the a.h.p. obtained by anodal break stimulation and reduced the amplitude of the action potential. In voltage-clamp experiments both the slow outward current and the fast inward transient current were inhibited by 200 μM quinidine (Figure 1D). Therefore, quinine and quinidine, unlike apamin, are not specific for the Ca^{2+}-dependent K^+ conductance; they block other K^+ conductances and also the fast Na^+ conductance, as has also been shown in other excitable systems (Yeh and Narahashi, 1976; Wong, 1981).

Characterization of the apamin receptor in rat muscle cell membranes was carried out with the use of a highly radiolabelled monoiodo-apamin derivative ([^{125}I]apamin) with a specific radioactivity of 2000 Ci/mmol (Hugues *et al.*, 1982a). Figure 2A shows typical kinetics of association of [^{125}I]apamin to its receptor in rat myotube homogenate and dissociation of the [^{125}I]apamin-receptor complex. Figure 2B shows that semilogarithmic plots of the data are linear. The association process follows pseudo first-order kinetics with a rate of association $k = (k_a[^{125}\text{I}]\text{apamin} + k_d)$, where k_a and k_d represent the rate constants of formation and dissociation of the [^{125}I]apamin-receptor complex. The dissociation of the [^{125}I]apamin-receptor complex is a first-order process with k_d as rate constant. The values of k and k_d calculated from Figure 2B are 1.0×10^{-3}/s and 1.5×10^{-4}/s, respectively. Therefore, the value of k_a is 7.2×10^6/M/s and the dissociation constant of the [^{125}I]apamin-receptor complex K_d^* ($= k_d/k_a$) is 22 pM.

Figure 3A shows the results of equilibrium-binding experi-

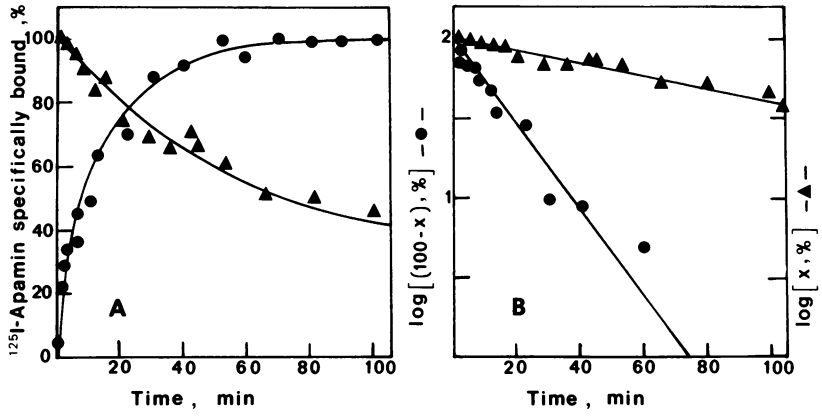

Fig. 2. Association and dissociation kinetics for the binding of [^{125}I]apamin to rat muscle homogenate. **A** (●) Association kinetics of [^{125}I]apamin (128 pM) at 0°C to rat muscle homogenate (0.3 mg protein/ml). (▲) Dissociation kinetics of [^{125}I]apamin from its receptor initiated by addition of 1 μM unlabelled apamin. **B.** Semilogarithmic representation of the data. X represents the percentage of [^{125}I]apamin bound. The concentration of specifically bound [^{125}I]apamin that corresponds to 100% was 0.75 pM so that the free [^{125}I]apamin concentration varied <1% during the course of the association kinetics.

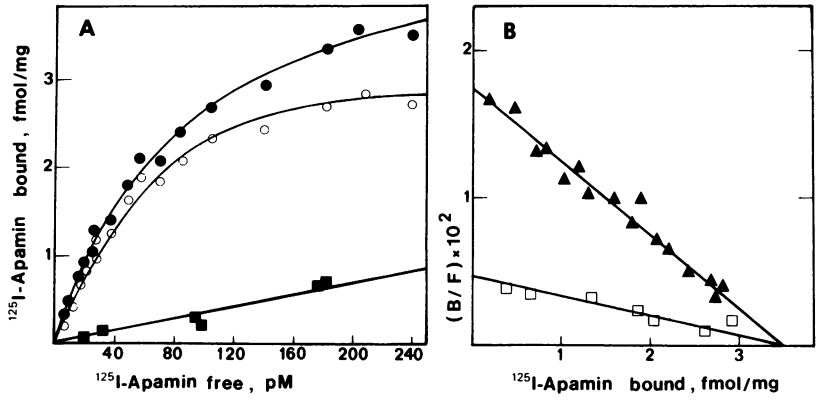

Fig. 3. Binding of [^{125}I]apamin to rat muscle cell homogenate. **A** (●) Total binding; (■) non-specific binding was determined in the presence of a large excess of unlabelled toxin (1 μM). (○) Specific binding is the difference between total binding and non-specific binding,. **B** Scatchard plot of the data. B, bound; F, free. Data obtained in standard (▲) and physiological (□) medium.

ments in which various concentrations of [^{125}I]apamin were added to a fixed amount of rat myotube homogenate (0.3 mg protein/ml) in a standard buffer. The linearity of the Scatchard plot of the data (Figure 3B) demonstrates that [^{125}I]apamin binds to a single class of non-interacting sites. The dissociation constant, K_d*, was 60 pM, not very different from that measured from the values of k_a and k_d, and the maximal binding capacity was 3.5 fmol/mg of protein.

The results of competition experiments between unlabelled apamin and [^{125}I]apamin are shown in Figure 4. The concentration of apamin required to produce half-maximal inhibition ($K_{0.5}$) of binding of [^{125}I]apamin was 40 pM. The dissociation constant of the apamin-receptor complex (K_d) can be calculated from

$$K_{0.5} = K_d\,[1 + ([^{125}\text{I}]\text{apamin})_{0.5}/K_d^*)$$

where $([^{125}\text{I}]\text{apamin})_{0.5}$ is the concentration of free labelled ligand at half-displacement. The latter value was 7 pM and K_D* was 60 pM, so that K_d was 36 pM under the standard conditions. Quinine and quinidine were much less effective inhibitors of [^{125}I]apamin binding, their $K_{0.5}$ values being 110 μM and 200 μM, respectively (Figure 4 inset).

Various monovalent and divalent cations interfere with the binding of [^{125}I]apamin to its receptor so that the binding is particularly sensitive to the prevailing ionic conditions (Hugues *et al.*, 1982c). Hence, replacement of the standard incubation medium with a physiological medium containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, and 20 mM Hepes-Tris (pH 7.5) caused the values of K_d* (Figure 3B) and K_d (Figure 4) to increase to 230 pM and 190 pM, respectively.

Discussion

In the current- and voltage-clamp experiments with rat skeletal myosacs in culture described above, apamin was found to block selectively the Ca^{2+}-dependent slow K^+ conductance described by Barrett *et al.* (1981). The use of a highly radiolabelled derivative of apamin, [^{125}I]apamin, permitted a detailed analysis of the interaction of the toxin with its receptor in rat skeletal myotubes in culture. The binding is saturable and specific. An interesting property of this Ca^{2+}-dependent K^+ channel in these cells is its low concentration, only 3.5 fmol/mg protein, which is 1600 times lower than that of the (Na^+,K^+)ATPase (2.1 pmol/mg of protein, Vigne *et al.*, 1982) and seven times lower than the density of Na^+ channels (25 fmol/mg of protein) in chick myotubes in culture (Frelin *et al.*, 1981).

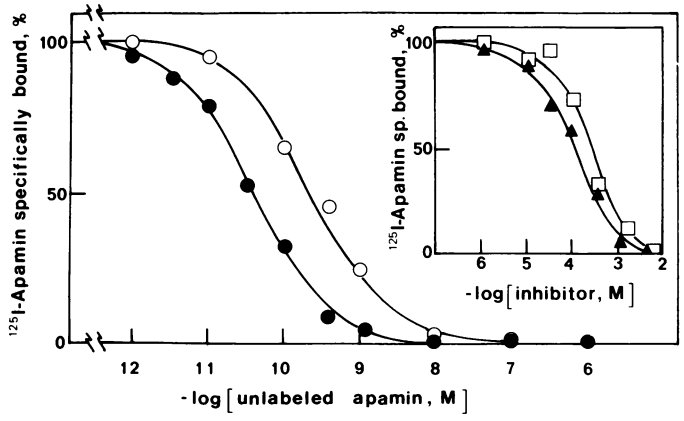

Fig. 4. Competition between [^{125}I]apamin and unlabelled apamin in standard conditions (●) and in physiological medium (○). **(Inset)**: competition between [^{125}I]apamin and quinine (▲) and quinidine (□).

Using the extracellular patch-clamp technique on rat myotubes in culture, Pallotta *et al.* (1981) have observed single channel currents that would give rise to this Ca^{2+}-dependent slow K^+ conductance. The fact that this channel has a conductance of 130–180 pS (Pallotta *et al.*, 1981), *i.e.*, at least 10 times higher than that of the classic voltage-activated Na^+ and K^+ channels (Sigworth and Neher, 1980; Conti and Neher, 1980), may compensate for its low density.

The apamin receptor is present at all stages of development of the muscle cells *in vitro*. Its stoichiometry measured with [^{125}I]apamin in myoblasts before fusion into myotubes is 1.3 fmol/mg of protein, only 2.7-fold less than in myotubes. Another interesting property of this Ca^{2+}-dependent K^+ channel in rat skeletal myotubes in culture is its high affinity for apamin (k_d = 36–60 pM). This affinity is similar to those found for the Ca^{2+}-dependent slow K^+ channel in synaptic terminals (Hugues *et al.*, 1982a), neuroblastoma cells (Hugues *et al.*, 1982c), and smooth muscle membranes (Hugues *et al.*, 1982b). The situation is therefore different from that observed for the interaction between the Na^+ channel and tetrodotoxin. The dissociation constant of the complex formed between the Na^+ channel and tetrodotoxin is close to 1 nM for neuroblastoma cells and synaptosomes, whereas it is only 1 μM for rat skeletal muscle cells in culture (Lombet *et al.*, 1982). The low affinity of tetrodotoxin for rat skeletal muscle cells in culture is believed to be due to the absence of innervation. Denervation of the mammalian muscle transforms high affinity sites for tetrodotoxin into low affinity sites (Pappone, 1980). Muscle innervation seems to play no role in the regulation of the apamin affinity for the Ca^{2+}-dependent K^+ channel.

In conclusion, the work presented in this paper shows clearly that apamin blocks Ca^{2+}-dependent slow K^+ channels in skeletal muscle as well as in nervous tissues (Hugues *et al.*, 1982c). This property establishes the toxin as a general tool for studying this conductance both by electrophysiological and by biochemical techniques. Apamin will probably turn out to be as useful for investigating this K^+ channel as tetrodotoxin is for studying the Na^+ channel.

Materials and methods

Cell cultures

Primary cultures of thigh muscle of new-born rats were prepared as described previously (Lombet *et al.*, 1982). After one week of growth in Ham's F12 culture medium supplemented with 10% fetal calf serum and 10% horse serum, muscle cell cultures were transferred to Dulbecco modified Eagle's medium supplemented with 5% fetal calf serum. Under these conditions the fusion of myoblasts into myotubes occurs within 3–4 days. Myosacs were prepared by exposure of 2-day-old myotubes to 0.1 μM vinblastine.

Electrophysiological experiments

Culture dishes containing rat myosacs were used directly after the culture medium had been replaced by the physiological medium containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose, buffered by 20 mM Hepes-Tris at pH 7.5. The culture dish was placed on the warm stage of an inverted microscope (Leitz-Diavert) and the temperature was maintained at ~34°C. Experiments were performed with the use of a suction pipette method as described previously (Hugues *et al.*, 1982c).

Iodination of apamin

Apamin was purified as described by Gauldie *et al.* (1976). The monoiodoapamin derivative with a specific radioactivity of 2000 Ci/mmol was prepared as described by Hugues *et al.* (1982a).

Binding assays under standard conditions

Rat muscle 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 (10–15 mg protein/ml), divided into aliquots, and stored in liquid nitrogen. The concentration of protein was measured by Hartree's method (Hartree, 1972) using bovine serum albumin (BSA) as a standard. Homogenates from rat muscle cells were prepared with a Potter homogenizer (900 r.p.m., five strokes). The standard incubation medium for binding experiments consisted of a 20 mM Tris-HCl buffer at pH 7.5 containing BSA 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% BSA for 1 h and then washed once with 5 ml of the same buffer at 0°C just before use.

Kinetics of association of [^{125}I]apamin to and its dissociation from rat muscle cells. Rat muscle cell homogenates (0.3 mg protein/ml) were incubated in the standard medium at 0°C. The onset of binding was studied by adding [^{125}I]apamin at 128 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% BSA. Radioactivity bound to filters was measured with an Intertechnique CG 4000 gamma counter.

After 70 min of association the amount of specifically bound [^{125}I]apamin reached a plateau value. At that time, a large excess of unlabelled 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 were carried out in the physiological medium.

Equilibrium binding experiments. Rat muscle cell homogenate (0.3 mg/ml) was incubated with various concentrations of [^{125}I]apamin for 60 min at 0°C. Duplicate aliquots (0.8 ml) were then filtered and the bound radioactivity was measured as described above. Non-specific binding was determined in parallel experiments in the presence of an excess of unlabelled toxin (1 μM).

Competition experiments between [^{125}I]apamin and unlabelled apamin, quinine, and quinidine. Rat muscle cell homogenate (0.3 mg/ml) was incubated for 60 min at 0°C with a fixed concentration of [^{125}I]apamin (7 pM in the standard medium and 24 pM in the physiological medium) and various concentrations of apamin, quinine, or quinidine. The amount of labelled apamin that remained bound to rat muscle cells in the presence of unlabelled molecules was estimated as described above.

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