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Isolation and characterization of a fraction from brain that inhibits 1,4-[^{3}H]dihydropyridine binding and L-type calcium channel current

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Bovine brain was subjected to acid extraction and several purification steps. A fraction from brain that eluted from C18 reverse-phase columns at 30–35% acetonitrile inhibited [^{3}H]nitrendipine binding to cardiac membranes. Further purification of this fraction on a sizing column in the presence of 30% acetonitrile yielded a low molecular mass fraction (< 1 kDa) that produced a time- and voltage-dependent inhibition of L-type (but not T-type) Ca^{2+}-channel current in GH_3 cells. The results suggest that this fraction contains an endogenous substance that binds directly to slowly-inactivating Ca^{2+} channels and thereby inhibits current flow.

Endogenous ligand; Modulator; 1,4-Dihydropyridine; Nitrendipine; Ca^{2+} antagonist; Receptor binding; (Brain)

1. INTRODUCTION

There are several sites on L-type Ca^{2+} channels at which antagonist drugs bind stereoselectively with high affinity [1,2]. The density of these sites can be modulated by treating animals chronically with various Ca^{2+}-channel ligands [2–4]. Synthetic pathways have been proposed that may occur spontaneously in vivo to produce DHPs [5]. These findings, along with the existence of BAY K8644 and related drugs that 'activate' Ca^{2+} channels, support the idea that endogenous substances analogous to DHPs or related drugs may exist [6,7].

A few preliminary reports suggest the existence of endogenous substances that modify DHP binding [8–11] (for review, see [2]). Recently, brain fractions that inhibit [^{3}H]DHP binding and ^{45}Ca influx have been reported [10,11]. In contrast to previous reports, we describe the isolation of a fraction from brain that exerts specific, reversible, and voltage-dependent inhibition of L-type Ca^{2+}-channel current under voltage-clamp conditions.

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Abbreviations: DHP, 1,4-dihydropyridine-type Ca^{2+}-channel antagonist; FBI, <1 kDa inhibitory fraction from brain; HPLC, high-performance liquid chromatography; MBP, myelin basic protein; SEC, size exclusion chromatography; TFA, trifluoroacetic acid

2. MATERIALS AND METHODS

2.1. Purification procedures

Fresh bovine brains were obtained from a local slaughterhouse and lyophilized bovine brain from Burlington Bio-Medical Corp., NY. Typically, 10–15 kg of fresh or 0.1–1 kg of lyophilized calf brain was homogenized (Polytron, Brinkman) in acid by the method of Bennett [12] as modified by Quirion et al. [13]. The homogenate was clarified by centrifugation (16000 × g for 45 min) and the supernatant was extracted with 2 vols of petroleum ether. The aqueous phase was adjusted to pH 2 with NaOH and subjected to ultrafiltration on a Pellicon apparatus (Millipore) equipped with a ≅30 kDa cut-off membrane. The ultrafiltrate was then subjected to preparative reverse-phase HPLC on an octadecylsilane column (5.7 × 30 cm) employing a gradient of 0–70% acetonitrile in

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0.1% TFA over 1.5 h at a flow rate of 150 ml/min. Fractions were collected, aliquots were dried (Speed Vac, Savant) and tested for their ability to inhibit the specific binding of [^{3}H]DHP to rat cardiac membranes. Fractionation of the active region from this column was performed on a BioSil TSK-250 column (7.5 × 300 mm; BioRad) utilizing a mobile phase of 40% acetonitrile/0.1% TFA at a flow rate of 0.5 ml/min. Aliquots were dried and assayed for [^{3}H]DHP binding inhibitory activity. The activity that elutes with an apparent molecular mass of <1 kDa is herein denoted as FBI.

2.2. *Ligand binding*

Inhibition of binding of [^{3}H]DHPs (nitrendipine or PN 200-110; New England Nuclear, Boston) was carried out at 25°C in 0.25 to 2.5 ml assay volumes as described previously [8]. The percent inhibition of binding was independent of the ^{3}H-ligand used. Protease inhibitors (1 μM aprotinin; 50 μM benzamidine; 50 μM leupeptin) were added in some assays (see section 3). [^{3}H]Ouabain and [^{3}H]saxitoxin binding were determined as previously described [14,15].

2.3. *Electrophysiology*

Electrophysiological effects of fractions on GH_3 cells were determined by the methods of Cohen and McCarthy previously used to study the effect of nimodipine in our laboratory [16]. Fractions were added either extracellularly or into the dialysis solution of the recording pipette. The whole cell variant of the patch-electrode voltage-clamp technique was used. Tail-current analysis with curve peeling was used to separate the contributions of the two types of Ca^{2+} channels.

2.4. *Other methods*

Inorganic phosphate and lipids were determined by published methods [17,18]. 1 pU activity is defined as that amount of activity which inhibits the specific binding of [^{3}H]DHP (0.2 nM [^{3}H]PN 200-110 or 1 nM [^{3}H]nitrendipine) to rat cardiac membranes by 50% in a 0.25 ml assay volume. Unlabeled nimodipine was the standard displacer.

3. RESULTS AND DISCUSSION

Fractions eluting at 30–35% acetonitrile from the preparative reverse-phase C18 column inhibited [^{3}H]DHP binding (fig.1). Electrophysiological measurements were subsequently performed to more stringently address the question of whether the activity was DHP-like in nature. Active fractions upon intracellular dialysis in the pipette solution produced time- and voltage-dependent block of L-type Ca^{2+}-channel current in GH_3 cells. The characteristics of this inhibition were consistent with those seen for DHP as determined by tail-current analysis. However, the fraction was inactive when added to the extracellular bathing solution.

The fraction produced a concentration-dependent inhibition of [^{3}H]DHP binding to cardiac and brain membranes. Polyacrylamide electrophoresis in the presence of SDS showed the presence of two bands with relative molecular masses of 18 and 20 kDa. Amino acid analysis and tryptic maps of the 18 kDa protein matched that of MBP. The IC_{50} value for inhibition of [^{3}H]DHP binding to cardiac membranes of the 18 kDa protein was $\cong 4\ \mu$M, the same as that observed for commercially available preparations of MBP. Tryptic mapping confirmed that most of the 18 kDa protein was MBP. Commercially available MBP inhibited [^{3}H]DHP binding but did not block the L-type Ca^{2+}-channel current, suggesting that some other substance was co-purifying with MBP. Indeed, further purification of this fraction by SEC in the presence of 40% acetonitrile revealed the presence of two inhibitors of DHP binding. One was MBP and the other, a small molecular mass fraction, FBI, with an apparent size of <1 kDa (fig.2). This low molecular mass fraction, in contrast to MBP, completely inhibited [^{3}H]DHP binding. The inhibition curve obtained had an apparent Hill slope of >2, suggesting that the inhibition was not due to a simple 1:1 direct binding to the DHP recognition site. FBI but not the MBP-containing fraction, was capable of blocking the L-type Ca^{2+}-channel current. Thus, MBP dissociated from FBI by SEC, was now inactive on Ca^{2+}-channel current as previously noted for commercial preparations of MBP. In addition, SEC-purified FBI was capable of blocking L-type Ca^{2+}-channel current when applied extracellularly (fig.3). A prepulse of variable length at −40 mV from a hyperpolarized potential (−90 mV) was followed by a strong depolarization to assay for current through available L-type channels upon repolarization: ≅10 pU of FBI activity was applied extracellularly. The time constant for the decrease in current was 47.8 s for the control and 8.45 s for the FBI-treated GH_3 cells.

The chemical nature of FBI was explored by analysis for amino acids, fatty acids and certain other lipids. High-sensitivity amino acid analysis failed to detect amino acids in this fraction. Based on the amount of activity used, it can be concluded that either the sample is free of amino acids, or it contains a peptide that is considerably more potent than nimodipine. Those fatty acids that are known to inhibit [^{3}H]DHP binding at 100 to 200 μM ([18] and unpublished) were not present in this fraction.

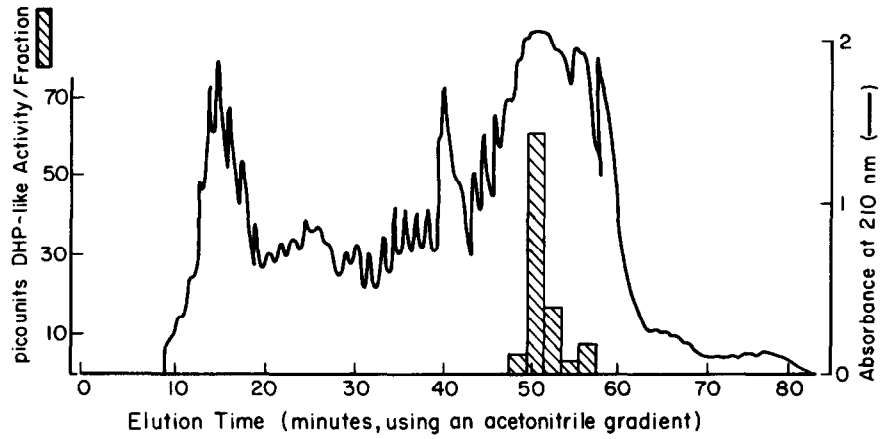

Fig.1. Preparative reverse-phase chromatography of a preparation from an acid extract of fresh calf brains. The hatch bars denote the region containing FBI and MBP as determined by [^{3}H]DHP binding and subsequent SEC.

FBI did not inhibit [^{3}H]ouabain or [^{3}H]saxitoxin binding to brain membranes in amounts that inhibit [^{3}H]DHP binding. The lack of effect on [^{3}H]ouabain binding is of particular interest because it indicates that certain nonesterified fatty acids and lysophospholipids which are known to inhibit ouabain binding [20,21], are not present in significant amounts in FBI.

FBI appears to be different from the other brain fractions that have been reported to inhibit [^{3}H]DHP binding. The 1.2 kDa fraction of Ebersole et al. [11] did not inhibit [^{3}H]DHP binding to heart membranes, the fraction of Sanna and Hanbauer [12] inhibited both L- and T-type Ca^{2+} channels, and is likely to be an acidic peptide (personal communication), and that of Thayer et al. [12] was reported to be of 5 to 10 kDa. These characteristics

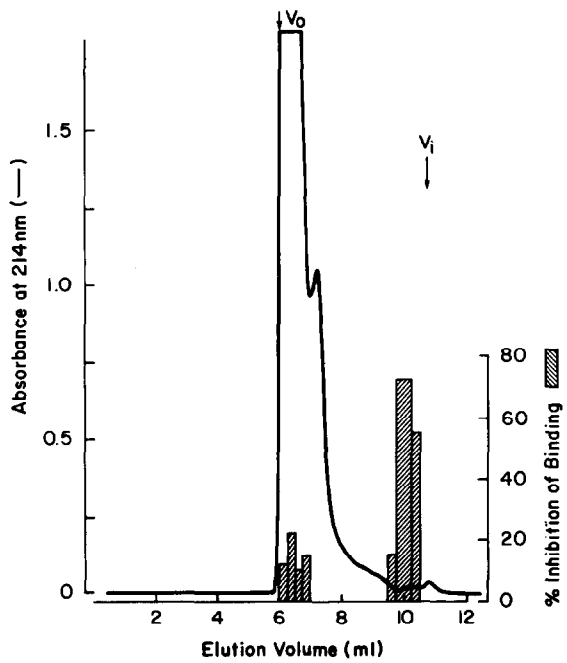

Fig.2. Separation of the low molecular mass inhibitory fraction, FBI, from myelin basic protein by size exclusion chromatography.

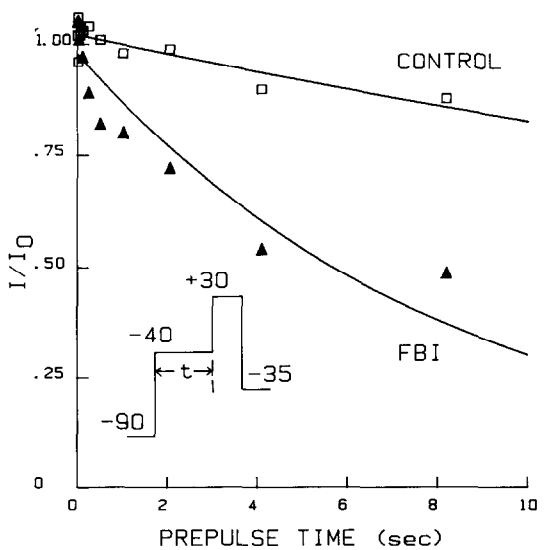

Fig.3. L-type calcium-channel tail currents in GH_3 cells exhibit time- and voltage-dependent onset of block by FBI at −40 mV.

differentiate FBI from the other factors. In summary, we report for the first time the isolation of a < 1 kDa fraction that specifically inhibits the L-type Ca^{2+}-channel current and [^{3}H]DHP binding.

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