Blocking and isolation of a calcium channel from neurons in mammals and cephalopods utilizing a toxin fraction (FTX) from funnel-web spider poison

R. LLINÁS, M. SUGIMORI, J.-W. LIN, AND B. CHERKSEY

Department of Physiology and Biophysics, New York University Medical School, New York, NY 10016

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ABSTRACT A Ca\(^{2+}\)-channel blocker derived from funnel-web spider toxin (FTX) has made it possible to define and study the ionic channels responsible for the Ca\(^{2+}\) conductance in mammalian Purkinje cell neurons and the preterminal in squid giant synapse. In cerebellar slices, FTX blocked Ca\(^{2+}\)-dependent spikes in Purkinje cells, reduced the spike afterpotential hyperpolarization, and increased the Na\(^{+}\)-dependent plateau potential. In the squid giant synapse, FTX blocked synaptic transmission without affecting the presynaptic action potential. Presynaptic voltage-clamp results show blockade of the inward Ca\(^{2+}\) current and of transmitter release. FTX was used to isolate channels from cerebellum and squid optic lobe. The isolated product was incorporated into black lipid membranes and was analyzed by using patch-clamp techniques. The channel from cerebellum exhibited a 10- to 12-pS conductance in 80 mM Ba\(^{2+}\) and 5-8 pS in 100 mM Ca\(^{2+}\) with voltage-dependent open probabilities and kinetics. High Ba\(^{2+}\) concentrations at the cytoplasmic side of the channel increased the average open time from 1 to 3 msec to more than 1 sec. A similar channel was also isolated from squid optic lobe. However, its conductance was higher in Ba\(^{2+}\), and the maximum opening probability was about half of that derived from cerebellar tissue and also was sensitive to high cytoplasmic Ba\(^{2+}\). Both channels were blocked by FTX, Cd\(^{2+}\), and Co\(^{2+}\) but were not blocked by \(\omega\)-conotoxin or dihydropyridines. These results suggest that one of the main Ca\(^{2+}\)-conductances in mammalian neurons and in the squid preterminal represents the activation of a previously undefined class of Ca\(^{2+}\) channel. We propose that it be termed the "P" channel, as it was first described in Purkinje cells.

While different Ca\(^{2+}\)-channel blockers have been described in past years (1), a true blocker for the main Ca\(^{2+}\)-dependent action potential in mammalian and in some molluscan neurons had not been encountered. This lack of a suitable blocker suggested that at least one of the main Ca\(^{2+}\)-conductances present in the central nervous system (CNS) does not belong to the categories proposed by Tsien and co-workers (2, 3), as those channels respond quite specifically to dihydropyridines and \(\omega\)-conotoxins. The use of funnel-web spider toxin (FTX) as a CNS Ca\(^{2+}\)-channel blocker was first described in Purkinje cells, where it blocked dendritic spiking (4). At that time the fragment utilized was known as AG1 and was obtained from Bioactives (Salt Lake City, UT). Because of the variability in the blocking observed, we attempted to isolate the main factor involved in this blockade from crude venom. We report here on the specific neuronal Ca\(^{2+}\)-channel blocking properties of FTX and its use in the isolation of functional channels, which we have studied in the black lipid membranes. That the Ca\(^{2+}\)-channel blocking fraction, which we call FTX, is different from that initially described as AG1 relates to its molecular mass as determined chromatographically, which is one-fifth that of AG1, and its high affinity for Ca\(^{2+}\) conductance both in the mammalian CNS and the squid giant synapse. Some of these results have been presented as short communications (4-7).

MATERIALS AND METHODS

Electrophysiological Techniques. The methods utilized for the brain slice work on the cerebellum and for the giant synapse are the same as those described in previous papers from our laboratory (8, 9). Purkinje cell recordings were obtained from in vivo adult guinea pig cerebellar slices. These cells were impaled at either the soma or the dendrites. Electrical excitability of the cell was determined by direct stimulation of the neurons. Sodium conductances were blocked by using tetrodotoxin (TTX).

The experiments on the squid (Loligo Pealeii) giant synapse were performed at the Marine Biological Laboratory (Woods Hole, MA). Pre- and postsynaptic recordings from the giant synapse were utilized to test the action of FTX on synaptic transmission, and presynaptic voltage-clamp was used to determine the effect of the toxin fraction on the inward presynaptic Ca\(^{2+}\) current and on synaptic transmitter release in the absence of action potentials.

Toxin. Crude venom from American funnel-web spiders, including Agelenopsis aperta, Hololina curta, and Calilena, was obtained commercially (Spider Pharm, Black Canyon, AZ). FTX, a factor with a molecular mass in the 200- to 400-Da range, was purified chromatographically from the venom. We estimate the concentration of toxin used to block Ca\(^{2+}\) conductance to be submicromolar, based on the elution profile (OD\(_{280}\)) of the FTX fraction on column chromatography and the dilution factors of the chamber.

Affinity Gel Construction. Purified toxin, the activity of which had been previously verified, was coupled to Sepharose 4B via 1,4-butanediol diglycidyl ether. The purified toxin obtained from 100 \(\mu\)l of the A. aperta venom was treated with 50 ml of the spacer-attached gel to form the final affinity gel.

Biochemical Methods. Animals. Adult Hartley guinea pigs (400–600 g) were decapitated with a small-animal guillotine under sodium pentobarbital (Nembutal, 40 mg/kg, i.p.) anesthesia. A rapid craniotomy was performed to remove the squamous portion of the occipital bone. The cerebellum was then separated from the brainstem and placed in ice-cold 400 mM sucrose/5 mM Tris-HCl, pH 7.4/0.1% phenylmethylsulfonyl fluoride/0.1% bacitracin/5 mM EDTA containing approximately 2 units of aprotinin per ml.

Adult squid (Loligo Pealeii) with a 10- to 13-cm mantle size were decapitated, and the optic lobe was excised and rapidly frozen in liquid nitrogen.

Abbreviations: FTX, funnel-web spider toxin; TTX, tetrodotoxin.
Purification Procedure. Published protocols for the affinity purification of membrane proteins (10) were followed for the isolation of the toxin-binding components of the cerebellar and optic lobe membranes. The membrane pellet was resuspended to a concentration of 20 mg of protein per ml into 100 mM sodium citrate, pH 7.4/3% sodium cholate to solubilize the membrane protein in a Dounce homogenizer. The solution was stirred overnight at 4°C and centrifuged at 47,000 × g for 30 min; the supernatant was filtered under vacuum. The resulting filtrate was then subjected to affinity chromatography. The solution containing the solubilized membrane protein was allowed to react batchwise with 20 ml of the toxin-Sepharose gel by gently stirring overnight at 4°C. The gel was separated from the detergent solution by vacuum filtration. The resulting gel cake was resuspended into 20 ml of 1 M CaCl2/1% sodium cholate/10 mM Hepes, pH 7.4, and was stirred for 2 hr at 4°C. The suspension was then filtered, and the filtrate was retained. The elution procedure was repeated a second time, and the resulting filtrates were combined. The volume of the solution was reduced by dialysis against polyethylene glycol 35,000 (Merck). The protein-containing concentrate was desalted on a 1.0 × 25 cm column of Sephadex G-25 equilibrated with 100 mM Hepes (pH 7.4) and was dialyzed extensively against 400 mM sucrose/10 mM Hepes, pH 7.4. Samples were taken at each step of the procedure for determination of protein by the Bradford assay (11). The product obtained with this procedure typically had a final yield of 0.0005% or less, consistent with the purification of a membrane protein such as an ionic channel.

Ca2+-Channel Reconstitution and Recording. The purified protein was reconstituted into lipid vesicles by using a 4:1 mixture of phosphatidylethanolamine/phosphatidylincholine (Sigma) in 400 mM sucrose formed by the sonication–dialysis procedure of Racker (12, 13). After vesicle formation, the dialysate was applied to a 1.0 × 35 cm column of Sephadex G-25, and the void volume was collected. The resulting vesicle suspension was used for the bilayer studies.

The functional activity of the reconstituted protein was studied by using the lipid bilayer technique. Vesicles containing affinity-gel-processed protein were fused to a planar lipid bilayer, and the electrical activity was determined. Bilayers were formed on two-pull micropipettes with opening diameters near 1 μm by using a lipid solution composed of a 3:1 mixture of phosphatidylcholine/phosphatidylethanolamine (Avanti Polar Lipids). Voltage was applied via the micropipette by using a Dagan 8900 patch/whole-cell clamp with a 10-GΩ head stage (Dagan Instruments, Minneapolis).

The bathing solution was held at ground. Data obtained in the channel studies were amplified to a level of 500 mV/pA, and the membrane current was recorded on an HP 3960 FM instrumentation recorder for subsequent analysis. Data were filtered at 1 kHz and were digitized at 2500 or 5000 samples per sec. The digitized records could then be viewed on a computer-generated display, and amplitude and open-time and closed-time distributions were obtained.

RESULTS

The Effect of FTX on the Intact Mouse. The activity of the toxin fraction was tested on mice. The results indicate that for a 30-g mouse, a 5-μl i.p. injection of a solution diluted 1:9 from the spider venom fraction produced death in 15–20 min, apparently by respiratory failure. No effect was observed on neuromuscular transmission. With half of the lethal dose, the animal became sleepy and rarely responded to auditory, visual, or tactile stimuli; when present, the response was phasic and short-lasting, emulating abrupt awakening from deep sleep. The results suggest that FTX crosses the brain–blood barrier, directly affecting the CNS. This is in contrast with results reported for the dihydropyridines (14, 15) and conotoxins (16, 17), which have little effect on mammalian CNS.

The Effect of FTX on Purkinje Cells. Intracellular recordings from Purkinje cells were obtained from cerebellar slices (4, 8). The two types of spike responses typically recorded from these neurons under control conditions are shown in Fig. 1. Depolarization of the neuron by square current pulses produced rapid sodium-dependent firing, which culminated in a set of long, low-amplitude, repetitive Ca2+-dependent spikes (Fig. 1A). After bath application of FTX, the Ca2+-dependent spikes disappeared, and the duration of the afterpotential hyperpolarization was reduced (Fig. 1B). This blocking occurred 5–10 min after bath application of the toxin at a calculated 0.5 μM final concentration and was partly reversible after washing for about 30 min. In addition to blocking the Ca2+-dependent action potentials, FTX increased the likelihood of plateau potentials (Fig. 1B), which are produced by the activation of the persistent Na+ conductance (8, 9). Both of these plateau potentials and the initial fast spikes were blocked by the addition of TTX (10⁻⁶ g/ml) to the bath (Fig. 1C). Nearly identical results were obtained with FTX from the three different types of funnel-web spiders tested.

In a second set of experiments, recordings were made from the Purkinje cell soma after TTX administration. Under these

![Fig. 1](image-url)  
**Fig. 1.** (A) Intracellular recording from a Purkinje cell shows fast Na+-dependent spikes and Ca2+-dependent action potentials (arrows). (B) Five minutes after application of FTX, Ca2+-spiking disappeared, and a prolonged Na+-dependent plateau potential is observed that far outlasts the duration of the stimulation (lower trace). (C) Addition of TTX to the bath blocks the fast action potentials and the plateau potentials shown in B. (D) Ca2+-dependent slow action potential is generated by direct stimulation of the Purkinje cell in the presence of TTX. (E) Addition of FTX to the bath produces a blockage of the voltage-dependent Ca2+ electroresponsiveness.
conditions, Purkinje cell stimulation is known to elicit a slow 
Ca\(^{2+}\)-dependent plateau potential (8, 9). One such response 
is illustrated in Fig. 1D. In this case, a short, direct depolar-
ization of the soma initiated a prolonged dendritic potential 
that outlasted the pulse by about 20 msec. However, after 
application of FTX (=0.5 μM), this Ca\(^{2+}\)-dependent conduc-
tance was blocked (Fig. 1E).

**Single-Channel Properties.** The functional activity of the 
isolated protein was assessed by the lipid-bilayer technique. 
Electrical activity was measured in asymmetric solutions 
containing 80 mM BaCl\(_2\) and 10 mM Hepes (pH 7.4) on the 
cis side and 120 mM CsCl, 1 mM MgCl\(_2\), and 10 mM Hepes 
(pH 7.4) on the trans side or in the patch pipette. Recordings 
were also obtained with the solutions reversed. When vesicles 
containing the purified protein were added to the bilayer 
chamber, an increase in the conductance of the bilayer was 
usually detected within 10 min. In general, it was difficult to 
obtain recordings of only a single channel when vesicles were 
added to the Ba\(^{2+}\)-containing solutions because of the fusion-
promoting effect of divalent cations.

Two types of single-channel activity were found. The first 
was seen when Ba\(^{2+}\) was present on the extracellular face of 
the channel protein and was characterized by short-duration 
openings. Fig. 2 A–C illustrates this activity from the cere-
bellar preparation at holding potentials of −45, −30, and −15 
mV. The voltage dependence of the open probability is shown in 
Fig. 2F. The channel was closed at −60 mV and 
expanded to exhibit openings as depolarizing potentials were 
applied. The maximum open probability, 0.6–0.65, occurred 
at holding potentials greater than 0 mV. The mean open time 
was also found to be voltage-dependent and ranged from less 
than 0.6 msec to 5 msec. The \(E-V\) relationship constructed 
from six experiments is nonlinear as shown in Fig. 2E, where 
each point represents the mean of the current at each voltage 
step. Voltage steps above 0 mV generated unitary currents 
too low to be measured reliably (Figs. 2E and 4 F and G). A 
conductance of 10–12 pS in Ba\(^{2+}\) and 6–8 pS in 100 mM Ca\(^{2+}\) 
was obtained from these data. The extrapolated reversal 
potential was between −90 and −120 mV, which, to the limit 
of the accuracy of the measurements, is the theoretical value 
for a Ba\(^{2+}\) permeable channel.

A second type of channel-like activity was found when the 
solutions were reversed so that the high-Ba\(^{2+}\) solution was on 
the cytoplasmic face of the channel. Typical recordings are 
shown in Fig. 2D, which were made at holding potentials of 
−45, −30, and −15 mV. In this particular experiment, three 
channels with identical conductances had fused with the bilayer. 
An unexpected characteristic was the predominance of 
openings longer than 1 sec. Also present are rapid openings 
with durations of less than 100 msec. From these data we 
estimated a unitary channel conductance of 20 pS.

The measured Ca\(^{2+}\) currents represent contributions from 
both the individual currents and the opening probabilities of 
the channels. Thus, it should be possible to approximate the 
macroscopic current by multiplying the single-channel cur-
rents by the opening probabilities at each potential as has 
been done for the “fast” Ca\(^{2+}\) channel (18). The voltage and 
time dependence of the current obtained in this way is 
comparable to the results from current clamp experiments in 
the dendrites of Purkinje cells (8). However, in the absence 
of Ca\(^{2+}\)-current measurements for Purkinje cell dendrites, 
only a qualitative statement can be offered. The effect of 
known blockers of the neuronal Ca\(^{2+}\) channels was deter-
ded for the reconstituted channels. Single channels were 
blocked by both Cd\(^{2+}\) and Co\(^{2+}\) at concentrations of less than 
100 μM in a manner consistent with a fast-block mechanism 
(18). The single channels were also blocked by the microliter 
addition of a 1:10 dilution of FTX.

**Cephalopod Ca\(^{2+}\) Channels.** A set of experiments similar to 
those described for the Purkinje cell were carried out at the 
squid giant synapse. Two experimental paradigms were 
followed. Pre- and postsynaptic intracellular recordings were 
obtained, and synaptic transmission was tested by electrical 
activation of the presynaptic fiber. Presynaptic stimulation 
generated a presynaptic action potential that served as a 
trigger for transmitter release and the generation of a postsyn-

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**Fig. 2.** Functional properties of channels isolated from guinea pig cerebellum by an FTX affinity gel. (A–C) Single-channel recording with 
80 mM external Ba\(^{2+}\) obtained for protein reconstituted in lipid vesicles and fused with lipid bilayer. Holding potentials: −45 mV (A), −30 mV 
(B), and −15 mV (C). (D) Single-channel recordings obtained with 80 mM Ba\(^{2+}\) on the cytoplasmic face of the channel. Holding potentials of 
−45, −30, and −15 mV are shown. In this particular experiment, three identical channels had fused with the bilayer. The long duration of the 
open time seems to be an effect of the high amount of internal Ba\(^{2+}\), as it can be converted into the short-duration channel (A–C) when Ba\(^{2+}\) 
is removed from the cytoplasmic side. (E) \(E-V\) curve in 80 mM Ba\(^{2+}\) for the "fast" channel of A–C constructed from the single-channel events 
obtained from six different experiments. Each point represents the mean current from 200 or more events at each voltage. (F) Plot of open 
probability vs. applied voltage showing the voltage dependence of the channel, which reaches a maximum value of 0.6–0.65 above 0 mV. (G) 
Values from \(E-F\) multiplied to give an approximation of the macroscopic current.
Fig. 3. Action of FTX on synaptic transmission at the squid giant synapse. (A–C) Effect of FTX on synaptic transmission evoked by presynaptic action potentials. Pre- (first arrow) and postsynaptic (second arrow) action potentials were evoked after direct stimulation of the presynaptic fiber (A). Five minutes after the application of FTX, a total block of synaptic transmission was observed without affecting significantly the presynaptic action potential (B). Superimposition of the records in A and B (C) shows the small reduction in the amplitude of the afterpotential hyperpolarization after application of FTX. (D–F) Presynaptic voltage-clamp records and postsynaptic recording. Traces: upper, voltage step; middle, postsynaptic response; lower, inward calcium current. Results are shown before (D) and 10 min after (E) the application of FTX. Note the block of the calcium current and the postsynaptic response. Superimposition of records in D and E is shown in F.

aptic response (Fig. 3A). After administration of FTX in submicromolar concentrations, synaptic transmission was blocked within 5–8 min, and the presynaptic afterpotential hyperpolarization was slightly reduced (Fig. 3B). In a second set of experiments, the presynaptic terminal was voltage-clamped, and the relation between the presynaptic Ca\(^{2+}\) current and transmitter release was studied as shown in Fig. 3D. Addition of FTX at similar concentrations to that used in the experiment illustrated in Fig. 3E blocked the Ca\(^{2+}\) current within a few minutes. This was accompanied by a block of transmitter release, as judged by the absence of a postsynaptic response. The toxin fraction did not have a direct effect on postsynaptic channels, since no reduction of the postsynaptic response to pressure-injected glutamic acid was observed after the synaptic transmission was blocked by the toxin (not shown).

Isolation of Ca\(^{2+}\) Channels from the Squid Optic Lobe. Channel protein derived from the optic lobe was extracted and studied in the black lipid membranes. In Fig. 4 examples of the single-channel currents are illustrated. In the same asymmetric solutions used for the cerebellum, two types of channel-like activity were found. The first, shown at three holding potentials in Fig. 4 A–C was characterized by voltage-dependent openings with a mean duration of 1–3 mV.

Fig. 4. Single-channel properties of protein from squid optic lobe. (A–C) Single-channel recordings at potentials of −10, −30, and −50 mV were obtained with 80 mM Ba\(^{2+}\) on the external face of the protein. (D) Opening probability of the channel reaches a maximum of 0.32–0.35 at 0 mV and above, exhibiting a clear voltage dependence (n = 6). (E) Mean open time vs. potential shows the voltage dependence of the length of time the channel remains open as the holding potential is varied (n = 6). (F and G) I–V curves obtained in 80 mM Ba\(^{2+}\) (F) and 100 mM Ca\(^{2+}\) (G). The conductance of the channel is 20 pS in Ba\(^{2+}\) and 8 pS in Ca\(^{2+}\) (200+ events at each potential). (H) Current obtained by multiplying values in D and G to approximate macroscopic behavior; ■, actual values for \(I_{Ca}\) in squid as previously published (9).
However, short, presynaptic the is the

Neither low-threshold the spike

Some computational and experimental evidence suggests that the

**DISCUSSION**

The present paper indicates that FTX, a fraction of funnel-web spider venom, has a molecular mass of 200–400 Da, blocks Ca2+ channels both in mammalian Purkinje cells and at the presynaptic terminal of the squid giant synapse. Because this channel has unique pharmacological properties and kinetics that are somewhat different from those described for the L, N, and T channels (2, 3), we propose that it should be referred to as the "P" channel, since it was first described in Purkinje cells. Whether the P channel observed in Purkinje cells and in the squid giant synapse are indeed the same is difficult to say at this time. From an electrophysiological point of view, the macroscopic Ca2+ currents obtained from presynaptic voltage clamp studies (18, 19) and those obtained from black lipid membrane recordings of channels isolated from cerebellum show a similar I-V relation. Because reliable voltage-clamping of Purkinje cells so far has not been technically feasible, we cannot compare the computed I-V relation obtained from single Ca2+-channel recordings with macroscopic I_{Ca}. However, the similarity between the kinetics of those observed in squid and Purkinje cells suggests that these two channels are similar.

A comparison of the P channel with previously described Ca2+ channels (2, 3) indicates clear differences. Indeed, the electrophysiological properties of both the squid giant synapse presynaptic spike as well as the action potential recorded from Purkinje cell dendrites are quite different from the low-threshold calcium spike observed in central neurons (20), which corresponds to the activation of the T channels (2, 3). Neither can the P channel be considered to be similar to the L channel, since the voltage-dependence of this channel is far more negative (~60 mV) (19, 20) than that reported for the L channel (2, 3). In addition, at least in the squid presynaptic terminal, a degree of inactivation may be observed. If anything, our channel may resemble the N channel. However, the P channel is not blocked by ω-conotoxin. In short, we must conclude on pharmacological and electrophysiological criteria that there exists yet another Ca2+ channel. This P channel is, in our estimation, one of a large variety of Ca2+ channels that will probably be encountered in the years to come.

In addition to the differences in pharmacology, single-channel conductances, and voltage-dependence kinetics, it is possible that the different channels have fundamentally different properties from a biochemical point of view as well. One of the interesting aspects of the present results relates to the action of calmodulin kinase II (CaMKII) on Ca2+ channels. This enzyme is known to phosphorylate the L-type Ca2+ channels, increasing their average open times, resulting in an increased Ca2+ current when these channels are phosphorylated (21). In the squid giant synapse, presynaptic injection of CaMKII has been shown to increase transmitter release as much as 7-fold without an observable effect on the Ca2+ current (22), indicating that CaMKII may modulate transmitter release by phosphorylation of synapsin I at site 2 (23, 24), which would increase vesicular availability at the presynaptic release site (22). Thus, specific biochemical modulation of the different Ca2+ channels may be a fundamental variable in their role in nervous system function.

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