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Research Report

Pharmacological identification of a novel Ca^{2+} channel in chicken brain synaptosomes

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Abstract

Ca^{2+} influx was measured in rat and chicken brain synaptosomes in the presence of a number of pharmacological tools which have recently been used to define voltage-sensitive Ca^{2+}-channel (VSCC) types. In chicken brain synaptosomes, VSCCs which, because of their sensitivity to inhibition by omega-conotoxin (ω-CgTx), are thought to be exclusively N-type, the P-type VSCC polyamine inhibitor FTX (from *Agelenopsis aperta* venom; 1 μl/ml), its synthetic analogue, sFTX (1–5 mM) and the polypeptides AgaIVA (IC_{50} 0.29 μM) and ω-CgTx MVIIC (IC_{50} 0.0022 μM) inhibited 70–100% of the measurable K^+ stimulated Ca^{2+} influx. The prototypical N-channel VSCC inhibitor ω-CgTx GVIA (IC_{50} 0.014 μM), Cd^{2+} (50 μM) and diluted venom from *Hololena curta* (1:2,500) also caused complete or almost complete, inhibition of Ca^{2+} influx. In comparable studies using rat brain synaptosomes, sFTX (1–10 mM) caused a dose-dependant reduction of Ca^{2+} influx, while FTX (1 μl/ml) and AgaIVA (IC_{50} 0.02 μM) completely inhibited Ca^{2+} influx. Similar to the findings in chicken synaptosomes, Cd^{2+} (50 μM) and *H. curta* (1:2,500 dilution) both inhibited K^+ stimulated influx by > 80% whereas ω-CgTx (1 μM) only caused a maximum 25% inhibition. Both sFTX and its congener spermine, inhibited [^{125}I]ω-CgTx binding to rat and chicken synaptosomal membranes. These results strongly implicate P-type channels as the major VSCC in rat brain. The results also clearly demonstrate a heretofore unrecognized, novel, FTX/AgaIVA/ω-CgTx GVIA/ω-CgTx MVIIC-sensitive VSCC in chicken brain.

Key words: FTX; Ca^{2+}-channel; N-channel; Spermine; ω-Conotoxin; P-channel; Binding; AgaIVA; MVIIC; GVIA

1. Introduction

Voltage-sensitive Ca^{2+}-channels (VSCCs) are responsible, in large measure, for the depolarization induced increase in intraterminal free Ca^{2+} concentrations which initiate events such as neurotransmitter release [2]. It is now clear that there are several different VSCC-types, often located on the same neuronal population [39].

Three types of high threshold VSCC have been defined by pharmacological criteria and designated as the L-, the N- and the P-type channels. The L-channel is characterized by its sensitivity to inhibition by dihydropyridines [15,36]. It has been difficult to demonstrate a major role for this channel in controlling the Ca^{2+} necessary for governing neurotransmitter release because release from most neuronal preparations, including synaptosomes, has not usually been shown to be affected by reasonable concentrations of dihydropyridine agonists or antagonists [24,27]. The N-channel is defined by its sensitivity to inhibition by omega-conotoxin (ω-CgTx) [28] and has been thought to be exclusively responsible for regulating neurotransmitter linked Ca^{2+} influx in avian neurons [10] and synaptosomes [20,26,37,42,48]. These considerations suggest that chicken brain synaptosomes are an excellent functional model with which to study N-channels. It is important to note that this channel also plays a significant, albeit partial, role in governing Ca^{2+} influx and neurotransmitter release in mammalian brain synaptosomes [6,16,21,34,38,45,49]. The third high threshold channel, the P-channel, has been identified by its sensitivity to inhibition by natural or synthetic FTX (structural analogues of the polyamine spermine Fig. 1) [5] or the polypeptide, AgaIVA [26]. Although this channel is very closely related to the N-channel on the basis of electrophysiological parameters [14,19,47] it is not in-

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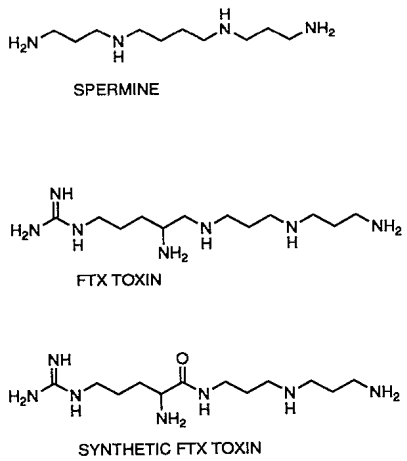

Fig. 1. Chemical structure of the three polyamine compounds studied for their activity on VSCCs. The proposed structure for authentic FTX and the structure of sFTX are reproduced from Llinás et al., 1992 [18].

hibited either by ω-CgTx or by the dihydropyridines but only by the polyamine and the polypeptide spider toxins described above.

In addition to the three VSCCs described above,the possibility of a fourth channel appears to exist on many nerves. This latter channel which is resistant to inhibition by saturating concentrations of dihydropyridines and ω-CgTx, has been described in a number of electrophysiological [1,31,35] and biochemical studies [21,23,43]. It has not yet been sufficiently characterized to be classified as one of the known channel types, the most likely one being the P-channel. The possibility could not be ruled out that this channel might be a subtype of the N-channel or even a novel channel altogether, such as the recently described channel designated as the E-type, whose function is presently not clear [9].

The results presented here indicate that the portion of the Ca^{2+} influx in rat synaptosomes which is resistant to inhibitors of L- and N-channels, is sensitive to inhibition by sFTX, FTX and AgaIVA and is probably the P-channel. The results also indicate that the VSCC in chicken brain, previously thought to be exclusively the N-type based on its sensitivity to ω-CgTx, is instead a novel Ca^{2+}-channel type sensitive to both N- and P-type VSCC inhibitors.

2. Materials and methods

2.1. Preparation of synaptosomes

Rat cortex or chicken forebrain synaptosomes were prepared by homogenization in 0.32 M sucrose using a teflon/glass homogenizer. The homogenate was centrifuged at 4°C, 1,000 *g* for 10 min. The supernatant was decanted and centrifuged at 12,400 *g* for 25 min. The resulting pellet (P_2) was resuspended in a HEPES buffered physiological salt solution (PSS) of the following composition (mM): choline chloride, 132; KCl, 5.0; $MgCl_2$, 1.3; $CaCl_2$, 1.5; NaH_2PO_4, 1.2; D-glucose, 10; HEPES, 20 adjusted to pH 7.4 with Tris base. The protein concentration was adjusted to between 1.0 and 1.5 mg/ml with PSS.

2.2. Ca^{2+} influx

Ca^{2+} influx was carried out essentially as described by Blaustein [3] with minor modifications as outlined previously [22].

Substances to be examined for their ability to inhibit basal and K^+ evoked Ca^{2+} influx included ω-CgTx, *H. curta* venom, spermine, spermidine, synthetic FTX, authentic FTX, ω-AgaIVA and ω-CgTx MVIIC, prepared in glass distilled water. Aliquots of each, were added to synaptosomal preparations and pre-incubated in a microfuge tube in an Eppendorf thermostat-controlled water bath at 30°C for 15 min. At the end of this incubation period, a 100 μl aliquot of this synaptosomal suspension was injected into an equal volume of resting (5 mM K^+) or depolarizing PSS (20 mM K^+), containing 0.5 μCi $^{45}Ca^{2+}$ (New England Nuclear, Boston MA). Cd^{2+}, when used, was not pre-incubated with the tissue but was present only during depolarization. Ca^{2+} influx was allowed to proceed for 3 s at which time influx was stopped by rapid dilution with 4 ml of ice cold Ca^{2+} free PSS containing 4 mM EGTA (quench buffer) and rapidly filtered over Gelman GA-6 (0.45 μm) filters using a Hoeffer filtration apparatus (Hoeffer Scientific, San Francisco, CA). Filters were washed with 2×5 ml of resting PSS containing excess $CaCl_2$ (4 mM), dried, placed in scintillation fluid and counted on a Beckman LS9800 scintillation counter. Results are presented as K^+ stimulated minus basal Ca^{2+} influx.

2.3. [^{125}I]-ω-Conotoxin binding

Synaptosomes were prepared as described above with the following modifications: the P_2 pellet was washed once with ice-cold resting PSS of the following composition (mM): HEPES, 10; NaCl, 132; KCl, 5; D-glucose, 10 at pH 7.4. Ca^{2+} was omitted to minimize its interference with ω-CgTx binding [17].

The washed P_2 pellet was then suspended in PSS to achieve a protein concentration of 40–80 μg/ml (2–4 μg protein per assay tube) for use in the binding assay. [^{125}I]-ω-conotoxin (specific activity 2200 Ci/mmol, NEN) was dissolved in distilled water. Dissociation curves were obtained by incubating unlabelled spermine or sFTX at 37°C with 20–30 pmol ω-CgTx. The reaction was terminated using ice cold PSS containing 0.2% BSA and 200 mM NaCl. Membranes were collected on GFC filters using a Brandel tissue harvester, washed twice with quench buffer and counted in a scintillation counter. Non-specific binding was determined in the presence of 100 nM unlabelled ω-CgTx.

Curves were fit to the binding data using non-linear least squares fitting techniques (GraphPad). Statistical analyses (ANOVA and multiple regression comparisons) were performed using RS/1.

2.4. Chemicals

Drugs and chemicals used in this study and their sources are as follows: spermine and spermidine HCl (Sigma Chemical); *H. curta* venom (Spider Pharm); ω-CgTx (Peninsula Laboratories); $^{45}CaCl_2$ (New England Nuclear). Authentic FTX and synthetic FTX [*N*-(7-amino-4-azaheptyl)-L-arginamide] were generous gifts from Drs. R. Llinás and B. Cherksey of New York University. ω-CgTx MVIIC was purchased from Bachem (Torrance, CA) and AgaIVA from Peptides International (Louisville, KY).

2.5. Protein determination

Protein content of synaptosomes and of spider venom were measured by the method of Bradford [4] using Coomassie protein assay reagents from Bio-Rad (Richmond, CA).

3. Results

K^+ induced Ca^{2+} influx was measured in the presence of substances which have the ability to inhibit pharmacologically defined VSCC-types. These compounds include: ω-CgTx, an inhibitor which defines the N-type channel [28,37,47]; Cd^{2+}, a non-specific inhibitor of VSCCs [44]; and the venom of the spider *H. curta* which blocks Ca^{2+} influx in rat synaptosomes at a site which is insensitive to both dihydropyridines and ω-CgTx [22]. These substances were utilized for comparative purposes or illustration of the type of Ca^{2+}-channels likely targeted by the compounds sFTX, FTX and their polyamine congeners (spermine and spermidine).

Results showing the ability of these various Ca^{2+} channel blockers to inhibit channels in rat brain synaptosomes are presented in Fig. 2. Cd^{2+} (50 μM) inhibited the great majority of the total measurable Ca^{2+} influx while the relative ineffectiveness of ω-CgTx on influx is also shown. *H. curta* venom inhibited more than 80% of measurable influx at a dilution of 1:2,500 (results not shown, see [22]). Spermine caused a concentration dependant reduction in Ca^{2+} influx which was virtually complete at concentrations higher than 5 mM. Spermidine caused similar but somewhat less potent effects on the influx which are not shown in Fig. 2.

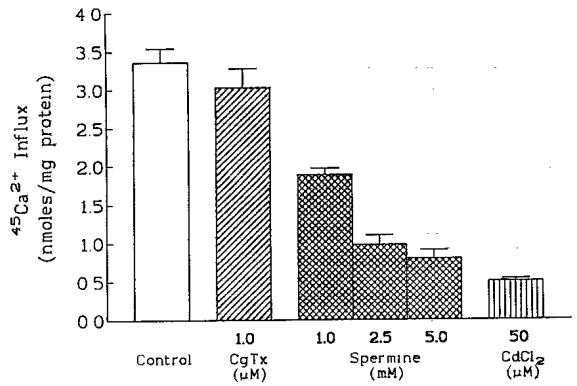

Fig. 2. The effects of VSCC inhibitors and spermine on K^+ stimulated Ca^{2+} influx in rat brain synaptosomes. Influx was allowed to proceed for 3 s. Spermine, spermidine (not shown) or ω-CgTx were pre-incubated in the synaptosomal preparation for 15 min before initiating Ca^{2+} influx in resting K^+ (5 mM) or depolarizing PSS (K^+ 20 mM). Cd^{2+} was not pre-incubated and was only present during resting or depolarization periods. Results represent means ± S.E.M obtained in 3–9 experiments, carried out in triplicate.

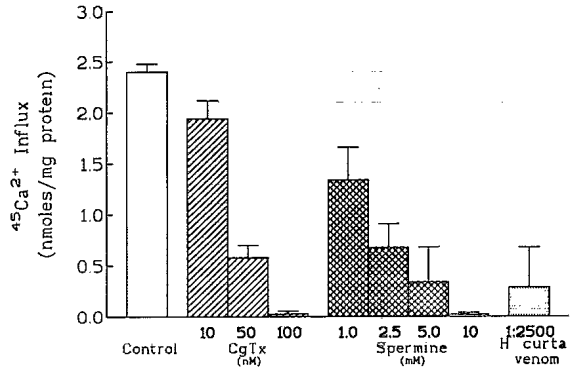

Fig. 3. Effects of spermine and other VSCC inhibitors on K^+ stimulated Ca^{2+} influx in chicken brain synaptosomes. Influx was allowed to proceed for 3 s following pre-incubation of the tissue with either spermine, ω-CgTx or *H. curta* venom. Each bar represents results obtained, in triplicate, from 3–10 tissue preparations ± S.E.M. Details are as described in *Methods*.

Similar experiments were repeated on synaptosomes prepared from chicken forebrain and the results are shown in Fig. 3. Activities of Ca^{2+}-channel inhibitors were initially thought to be somewhat easier to interpret in this preparation as compared to mammalian tissue, because all of the measurable Ca^{2+} influx in this tissue is sensitive to inhibition by ω-CgTx and therefore, by definition, occurs via N-type Ca^{2+}-channels. ω-CgTx potently and completely inhibited influx (> 95%) in a concentration dependent manner, in contrast to its relatively minor effects in rat brain. Spermine, *H. curta* venom and Cd^{2+} (not shown) all caused either major or complete reduction of Ca^{2+} influx. As was the case in rat synaptosomes, spermidine was somewhat less potent in reducing Ca^{2+} influx than was spermine.

Influx experiments were repeated in both rat and chicken brain synaptosomal preparations to examine the effects of authentic and synthetic FTX and spermine, at similar concentrations. sFTX and spermine caused concentration dependent decreases in Ca^{2+} influx in rat brain synaptosomes (Fig. 4). Authentic FTX, at a dilution of 1:1,000 was found to completely inhibit Ca^{2+} influx in the rat brain preparation in agreement with a previous report [46]. Spermine caused a similar degree of inhibition to that resulting from sFTX, at similar concentrations, while spermidine although it expressed full efficacy, was again less potent than either sFTX or spermine (results not shown). Cd^{2+} produced the expected degree of influx inhibition (i.e., > 80%).

In chicken brain synaptosomes the inhibition of Ca^{2+} influx obtained following spermine, sFTX and FTX pretreatment of tissue (Fig. 5) was quantitatively similar to that reported above in rat synaptosomes. Spermine and sFTX inhibited Ca^{2+} influx by greater than 80% at concentrations similar to each other and

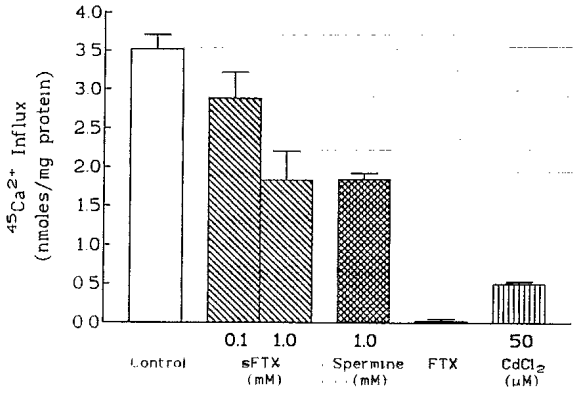

Fig. 4. Comparative effects of spermine, s-FTX and FTX on K^+ stimulated Ca^{2+} influx in rat brain synaptosomes. Authentic FTX was used at a final venom dilution of 1:1,000. The synaptosomes were pre-incubated with the Ca^{2+} channel inhibitors (except in the case of Cd^{2+} for 15 min). Each bar represents influx during 3 s and represents the mean influx obtained in 3–8 tissue preparations carried out in triplicate ± S.E.M.

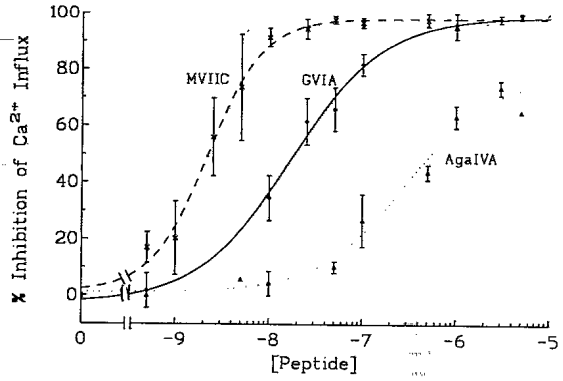

Fig. 6. Effect of AgaIVA, ω-CgTx GVIA and ω-CgTx MVIIC on Ca^{2+} influx in chicken synaptosomes. Various concentrations of AgaIVA (△), ω-CgTx (◇) and ω-CgTx MVIIC (×) were pre-incubated for 15 min with chicken brain synaptosomes. $^{45}Ca^{2+}$ influx was then measured for 3 s following depolarization with K^+ (20 mM) (see Materials and Methods). Values are means ± S.E.M. from 4–7 experiments performed in triplicate.

similar to those employed in the rat synaptosomes. In these latter experiments the inhibitory properties of authentic FTX (diluted 1 : 1,000) were slightly less potent than those effects demonstrated in rat brain (at an FTX dilution of 1 : 1,000), while Cd^{2+}, *H. curta* venom and ω-CgTx all produced the expected degree of inhibition.

Fig. 6 shows the effect of 3 peptides which have been used to define VSCC-types, on the K^+ stimulated Ca^{2+} influx in chicken brain. As expected from previous work, ω-CgTx caused a complete and dose-related inhibition of Ca^{2+} influx with an IC_{50} of 0.014 μM. The N- and P-channel antagonist MVIIC also potently inhibited Ca^{2+} influx with an IC_{50} of 0.0022 μM, a concentration less than that required of ω-CgTx. The funnel web spider toxin AgaIVA, although it did not produce complete inhibition, certainly blocked a majority (> 75%) of the Ca^{2+} influx through this nominally

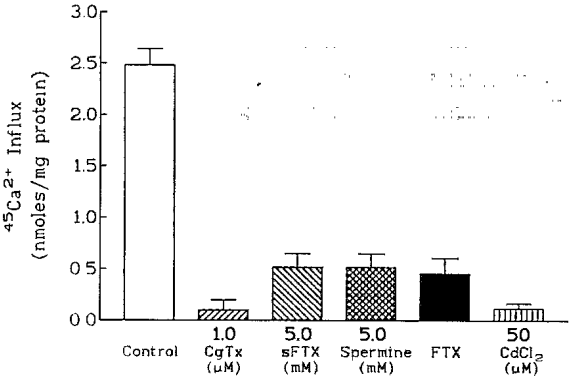

Fig. 5. Effects of spermine (5 mM), FTX (1 : 1,000) and sFTX (5 mM) on K^+ stimulated Ca^{2+} influx in chicken brain synaptosomes. As described previously, the toxins (except Cd^{2+}) were pre-incubated (15 min) in the synaptosomal suspension before addition of an aliquot of the tissue to resting (5 mM K^+) or depolarizing buffer (20 mM K^+). The Ca^{2+} accumulation by the synaptosomes was allowed to proceed for 3 sec. Values are means ± S.E.M. from 4–8 experiments in triplicate.

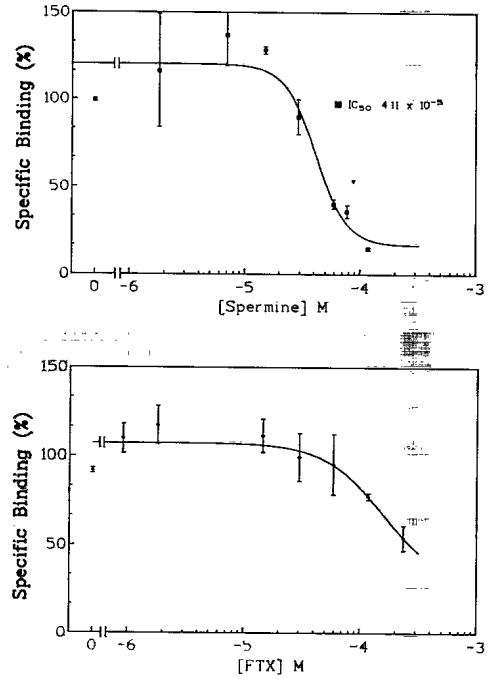

Fig. 7. Effect of spermine (top) and sFTX (bottom) on [^{125}I]-ω-conotoxin binding to rat brain synaptosomes. The synaptosomes were incubated with the polyamines and [^{125}I]-ω-conotoxin for 60 min. Non-specific binding was determined in the presence of 100 nM unlabelled ω-CgTx The data presented represent the results of one of three experiments performed in triplicate. Other details are described in Materials and Methods.

pure N-channel with an IC_{50} of 0.29 μM which is about one order of magnitude larger than the concentration required to inhibit influx in rat preparations (Lundy et al., unpublished results). It should be noted that the addition of small amounts (i.e., 20 nM) of ω-CgTx to the chicken synaptosomes after AgaIVA had produced its maximal effect, resulted in a further reduction of Ca^{2+} influx (i.e., ω-CgTx inhibited the 20% or so of AgaIVA resistant K^+ stimulated Ca^{2+} influx).

The inhibitory properties of both spermine and sFTX on [^{125}I]-ω-conotoxin binding to rat brain membranes are shown in Fig. 7. Both polyamines inhibited the binding in a concentration dependent manner. The spermine IC_{50} was about 40 μM and concentrations of 100 μM or higher resulted in nearly complete inhibition of [^{125}I]-ω-conotoxin binding. In order to conserve our limited supply of sFTX, only a partial inhibition curve was constructed. Inspection of the curve suggests the IC_{50} to be about 250 μM. The effect of sFTX on ω-CgTx binding was somewhat less potent than previously reported for arginine polyamine which appears structurally identical to sFTX [40,41]. The differences between the activity of sFTX on the [^{125}I]-ω-conotoxin binding found in the present experiments and the recent report of Scott et al. [40] appear likely due to the considerable differences in the methodology used for tissue preparation in the two studies. However, the results were similar qualitatively.

4. Discussion

The low molecular weight venom fraction obtained from funnel web spiders, known as FTX and its analogue, synthetic FTX, have been used interchangeably as specific inhibitors of the P-type Ca^{2+} channel [7,18,19]. P-channels are by definition, unaffected by ω-CgTx, the specific N-type channel inhibitor or by the dihydropyridine type of L-channel inhibitors [19,26,35, 47]. The P-channels, originally identified on cerebellar Purkinje neurons, have now been identified at a number of central [12,18,26,25] and peripheral sites [8,25,46], using FTX and sFTX or the peptide inhibitor AgaIVA [26] as definitive inhibitors. A number of studies have confirmed the existence of important Ca^{2+}-channels in rat brain which are neither N- nor L-type VSCCs as determined by their resistance to inhibition by both ω-CgTx and DHPs [16,21,31,32,35]. These resistant channels now appear, with some degree of certainty, to be P-channels because of their sensitivity to sFTX, FTX and AgaIVA as shown here and to AgaIVA as shown elsewhere [26]. These results presented in this communication provide some interesting considerations regarding the classification of VSCC-type based on sensitivity to pharmacological tools and offer substantial evidence for a novel Ca^{2+}-channel in chicken brain, perhaps best described as an NP-type, because it is inhibited by those toxins previously used to designate the N- and P-type of VSCC.

As mentioned above, sFTX, FTX and AgaIVA all blocked the ω-CgTx, dihydropyridine resistant portion of Ca^{2+} influx [16,22,35,43] in rat synaptosomes suggesting that these resistant channels should be classified as P-type [7,25,26]. However, it is worth noting that these agents blocked all of the Ca^{2+} influx, including the 20–40% [21,23,38,43,49] generally characterized as being ω-CgTx-sensitive and also that the inhibitory potency as well as the total percentage of Ca^{2+} influx blocked by AgaIVA decreased as the K^+ stimulus was increased to 60 mM (Lundy et al., unpublished observations). When these P-channel antagonists sFTX, FTX and AgaIVA were studied in chicken brain synaptosomes, a heretofore classical N-type preparation [20,22,26,27,42], all three were able to inhibit at least 80% of the K^+ induced Ca^{2+} influx. The polyamines spermine and spermidine were also able to inhibit Ca^{2+} influx in chicken brain, suggesting that these compounds along with sFTX and FTX might possess considerable N- (i.e., ω-CgTx-sensitive) channel blocking activity. This consideration received support from previous studies in tissues which showed that these toxins suppressed a variety of voltage and ligand activated Ca^{2+} currents [40,41] and the studies here in rat and chicken showing the displacement of specifically bound [^{125}I]ω-CgTx from synaptosomes. AgaIVA, on the other hand, which blocked about 80% of the Ca^{2+} influx in chicken brain at the highest concentration tested, did not displace high affinity ω-CgTx binding from rat or chicken membranes [26] and, after extensive studies in our laboratory, has failed to reveal any effect on the N-type channel of autonomic nerves. AgaIVA also does not compete for binding sites with MVIIC, the most potent inhibitor of the proposed NP channel in chicken brain [13]. It appears, therefore, as if sFTX and spermine block Ca^{2+} influx in chicken brain in part due to their P-channel blocking activity, in part due to their N-channel blocking activity (suggested by the displacement of [^{125}I]ω-CgTx binding) and also, perhaps, in part due to some recently described non-specific ionic membrane properties [40,41]. However, the exact contribution and nature of each of these factors to the inhibition of Ca^{2+} influx is presently unclear.

This study has shown that all three of the proposed P-channel blockers, along with ω-CgTx inhibited all or most of the K^+ stimulated Ca^{2+} influx in chicken brain. This strongly suggests the existence of a novel Ca^{2+} channel since this pattern of inhibition has not been previously described. This novel channel appears to consist of two distinct domains: one is sensitive to N-channel inhibitors like ω-CgTx and the other P-

channel blockers. It also appears that these two domains are functionally linked together in some fashion, such that inhibition of one site 'shuts down' the other. Therefore, small concentrations of ω-CgTx blocked all the K^+ stimulated Ca^{2+} influx including that 80% which was sensitive to AgaIVA or the polyamines. Similarly, AgaIVA and FTX inhibited about 80% of the total Ca^{2+} influx, which it has just been noted, is also completely ω-CgTx-sensitive. In this regard, our results with MVIIC are interesting. This peptide has been reported to block both N- and P-channels in mammalian brain [13]. In the present study, MVIIC blocked all of the K^+ stimulated Ca^{2+} influx at very low (IC_{50} 2.2 nM) concentrations. This could be interpreted as a pure N-channel effect, but the results obtained in mammalian brain where MVIIC also potently blocks Ca^{2+} influx but ω-CgTx does so only weakly [13], argue against this possibility. More likely, MVIIC blocks both the N- and the P-sensitive domains on the 'NP' Ca^{2+} channel of chicken brain and this activity may be reflected in its increased potency relative to ω-CgTx. It is interesting that the pharmacological profile of what we refer to as the NP channel is not accurately reflected by any of the cloned α_1 sub-units recently reviewed by Zhang and co-workers [50]. The fact that conotoxin MVIIC was identified from cDNA from *Conus magus* suggests that the toxin evolved to target a specific channel in its prey. This channel may be similar or identical to the channel we have described here in chicken brain and future studies may uncover its existence elsewhere. The results also offer ample evidence for caution against classification of a channel based on its sensitivity to a single blocking agent.

This study suggests that the ω-CgTx, dihydropyridine insensitive Ca^{2+} influx in mammalian brain occurs in large part through P-type VSCCs. The results also suggest that sFTX and FTX have some degree of N-channel blocking activity. Taken together, the evidence strongly suggests the existence of a novel Ca^{2+} channel in chicken brain for the following reasons: (a) it is sensitive to the P-channel inhibitors sFTX, FTX and AgaIVA; (b) it is sensitive to the N-channel inhibitor ω-CgTx; and (c) it is most sensitive to the NP channel inhibitor MVIIC. The channel could be considered to be a sub-type of the N- or the P-channel, but perhaps may best be classified as above to fully account for its sensitivity to these various Ca^{2+}-channel defining agents.

5. Acknowledgements

The authors are grateful to Drs. R. Llinás and B. Cherksey of New York University, for the generous supply of both authentic and synthetic FTX.

6. Abbreviations

AgaIVA	ω-Agatoxin IVA
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid
FTX	funnel web spider toxin
GVIA	ω-conotoxin GVIA
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
MVIIC	ω-conotoxin MVIIC
sFTX	synthetic funnel web spider toxin
VSCC	voltage-sensitive Ca^{2+}-channel

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