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Dendrotoxin and charybdotoxin increase the cytosolic concentration of free Ca^{2+} in cerebrocortical synaptosomes: an effect not shared by apamin

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Nanomolar concentrations of charybdotoxin or dendrotoxin increase the cytoplasmic free Ca^{2+} concentration in isolated central nerve terminals. The effects of the two toxins, normally considered to be blockers of K^+ channels controlled by voltage in a Ca^{2+}-sensitive or -insensitive manner, respectively, show only marginal additivity. Apamin, an inhibitor of low conductance Ca^{2+}-activated K^+ channels, was without effect in either the absence or presence of dendrotoxin. The effect of charybdotoxin on polarized, isolated central nerve terminals seems to be mediated largely by a block of K^+ channels sensitive to dendrotoxin. Apparently, these voltage-operated K^+ channels make a more significant contribution to maintaining the polarized potential of synaptosomes than do those activated by Ca^{2+}.

Dendrotoxin; Charybdotoxin; Apamin; K^+ channel; Ca^{2+}, cytosolic free fraction; Nerve terminal

1. INTRODUCTION

Dendrotoxin (DTX) and 4-aminopyridine (4-AP) increase the spontaneous excitability of synaptosomes [1-3] as a consequence of a reduction in the K^+ permeability [3-5]. Rapidly-activating, voltage-dependent, Ca^{2+}-insensitive K^+ channels inhibited by both ligands have been characterized [6,7].

Charybdotoxin has been identified as a blocker of the large and intermediate conductance, Ca^{2+}-sensitive, voltage-activated K^+ channels from skeletal muscle [8,9] and the mammalian central nervous system (CNS) [10,11]; these channels have been implicated in repolarization following an action potential in both the amphibian peripheral nervous system (PNS) [13] and mammalian CNS [14,15]. Apamin inhibits the small conductance, Ca^{2+}-dependent, voltage-insensitive K^+ channel [12] which appears to underlie the slow after-hyperpolarization that controls repetitive firing in the PNS [16,17]. By analogy, the slow after-hyperpolarization in the CNS may also be mediated by such low conductance channels although no apamin sensitivity has yet been reported therein ([14,18]; but see [19]). Thus, it was of interest to ascertain if either of these toxins could modulate the excitability of isolated nerve terminals either independently or synergistically with that of DTX.

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Abbreviations: 4-AP, 4-aminopyridine; DTX, dendrotoxin; CTX, charybdotoxin; fura-2/AM, fura-2 acetoxymethyl ester; Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]-amino)-ethane sulphonate; $[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration; CNS, central nervous system; PNS, peripheral nervous system

2. EXPERIMENTAL

Synaptosomes were prepared by discontinuous Ficoll gradient centrifugation [1] and stored on ice after pelleting from 250 mM sucrose buffered with Na-Tes to pH 7.4. $[Ca^{2+}]_c$ was determined using fura-2 acetoxymethyl ester (fura-2/AM) as described previously [2]. Briefly, pellets were resuspended in incubation medium (122 mM NaCl, 3.1 mM KCl, 0.4 mM KH_2PO_4, 5 mM

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$NaHCO_3$, 20 mM Tes-Na, 1.2 mM $MgSO_4$, 5 mM glucose and 1 mg/ml bovine serum albumin, pH 7.4) to a concentration of 1.34 mg synaptosomal protein/ml and incubated at 37°C for 35 min in the presence of 5 μM fura-2/AM. The synaptosomes were pelleted (Eppendorf 5412 microfuge for 60 s), resuspended in fresh incubation medium and transferred to continuously stirred cuvettes in Perkin-Elmer LS-5B fluorimeters interfaced, with IMB-PC compatible computers. The excitation wavelength was cycled between 340 and 380 nm under software control and emission recorded at 505 nm. Maximum and minimum 340 nm/380 nm ratios were determined on the addition of SDS to 0.3% (w/v), then 7.5 mM EGTA, pH 8 (adjusted with 3 M Tris base). Data analysis was performed in Lotus 123 using a dissociation constant for fura-2 of 224 nM. DTX [6] and CTX [20] were isolated according to the general principles of previously published methods; purified apamin was donated by R. Shipolini.

3. RESULTS

Fig.1 shows that DTX causes a rapid and saturatable elevation of the synaptosomal $[Ca^{2+}]_c$. At a toxin concentration of 316 nM, the $[Ca^{2+}]_c$ was increased by 71 ± 2 nM (mean ± SE, $n = 20$), with an EC_{50} of 2.1 nM. This correlates closely with the effect of the toxin on the release of endogenous neurotransmitter glutamate (EC_{50} of 1.7 nM) [1], though it is somewhat higher than its high affinity binding constant (K_D of 0.23 nM) in this preparation [1]. This toxin-induced increase in $[Ca^{2+}]_c$ is lower than that which could be achieved in parallel assays with 1 mM 4-AP (163 ± 11 nM; mean ± SE, $n = 19$).

Fig.2A shows that CTX induces an elevation of the $[Ca^{2+}]_c$ that is at least as extensive as that

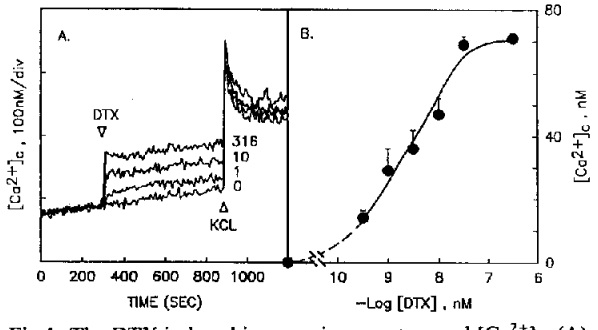

Fig.1. The DTX induced increase in synaptosomal $[Ca^{2+}]_c$. (A) Where indicated DTX was added to final toxin concentrations of 1, 10 or 316 nM (0, no toxin addition). KCl was added to 30 mM. In this and subsequent figures the data presentation begins 5 min after $CaCl_2$ addition. (B) The mean increase (± SE) in $[Ca^{2+}]_c$ by varying concentrations of DTX determined in 4–20 separate experiments. The resting $[Ca^{2+}]_c$ in these synaptosomes was 240 ± 5 nM (mean ± SE).

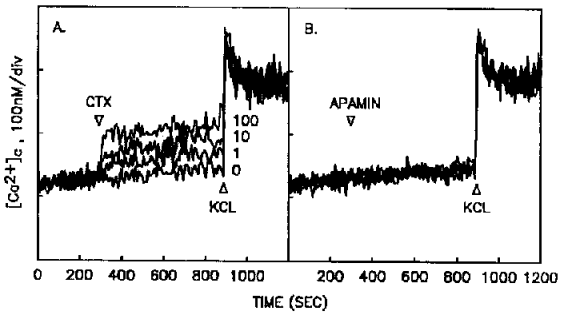

Fig.2. CTX but not apamin elevates the $[Ca^{2+}]_c$ of polarized synaptosomes. (A) CTX additions were to final toxin concentrations of 0, 1, 10 and 100 nM (as indicated). (B) Traces were strictly superimposable with a control when apamin was added to 1 nM, 100 nM or 10 μM. KCl was added to 30 mM.

elicited by DTX. Although a full dose response curve could not be constructed due to the limited amount of CTX available, data from a number of independent experiments indicated that a rise in $[Ca^{2+}]_c$ equivalent to the half-maximal increase elicited by DTX (35 nM Ca^{2+} at 2.1 nM toxin) was achieved by 1–10 nM CTX (e.g. fig.2A); 100 nM CTX increased $[Ca^{2+}]_c$ by 78 ± 10 nM (mean ± SE, $n = 3$). In contrast, fig.2B demonstrates that concentrations of apamin ranging from 1 nM to 10 μM produced no change in the $[Ca^{2+}]_c$. As with 4-AP [2], DTX, CTX or apamin failed to affect the subsequent elevation in $[Ca^{2+}]_c$ induced by raising the external $[K^+]$.

In the presence of a saturating concentration of DTX the subsequent addition of CTX resulted in only partial additivity (fig.3), a result apparent at all concentrations of CTX tested. Although slight, the further increase in $[Ca^{2+}]_c$ elicited by CTX in the presence of a saturating concentration of DTX accords with the somewhat more extensive increase in $[Ca^{2+}]_c$ induced by CTX alone. Incomplete additivity does not reflect a limitation in the maximal intra-terminal $[Ca^{2+}]_c$ that may be detected; a more pronounced increase in $[Ca^{2+}]_c$ is obtained in the presence of 4-AP (see above) or 30 mM KCl (figs 1–3). In parallel experiments, the addition of apamin did not alter the response of synaptosomal $[Ca^{2+}]_c$ to DTX added before or after the bee venom toxin.

4. DISCUSSION

Inhibition of voltage-sensitive K^+ channels by

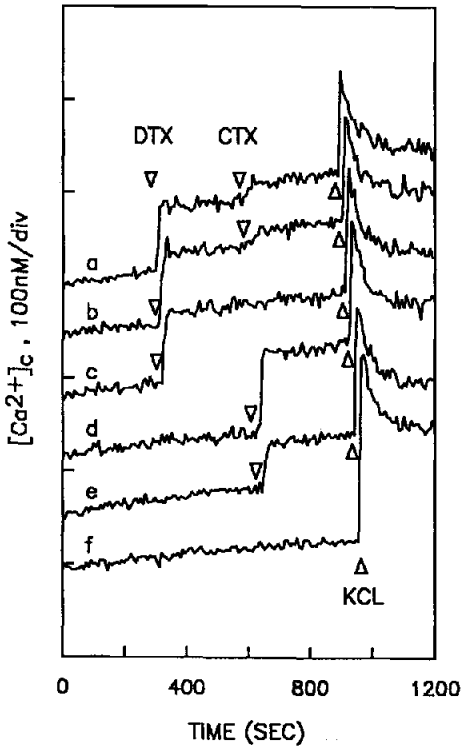

Fig.3. CTX induces only a small elevation in $[Ca^{2+}]_c$ in synaptosomes pre-intoxicated with a saturating concentration of DTX. Synaptosomes were exposed to the following concentrations of DTX and/or CTX added at the times indicated: (a) 316 nM DTX, 100 nM CTX; (b) 316 nM DTX, 32 nM CTX; (c) 316 nM DTX; (d) 100 nM CTX; (e) 32 nM CTX; (f) control. KCl was added to a final concentration of 30 mM. For clarity, traces have been X,Y shifted from a common origin.

DTX (or 4-AP) elicits a sustained increase in $[Ca^{2+}]_c$ despite the averaged membrane potential remaining above the threshold for activation of synaptosomal voltage-activated Ca^{2+} channels [1,21]. This reflects the induction of spontaneous Na^+ channel mediated 'action potentials' in the terminal membrane [1-3]. As previously observed with glutamate efflux [1], 4-AP elicits a more extensive increase in $[Ca^{2+}]_c$ than DTX, indicative of its blockade of a wider spectrum of K^+ channels [22]. In such a model, attenuation of a membrane conductance that contributes to repolarization or limitation of spike frequency (e.g. Ca^{2+}-activated K^+ channels; see section 1) should have no effect on the polarized nerve terminal but ought to yield a synergistic increase in $[Ca^{2+}]_c$ in the presence of a blocker of voltage-sensitive K^+ channels concerned with maintenance of the resting potential. To address this, the effects of the Ca^{2+}-activated K^+ channel blockers, apamin and CTX, following exposure to DTX were considered. Surprisingly, CTX alone was able to increase $[Ca^{2+}]_c$ more extensively than DTX. Nominally, this result would implicate an essential contribution of a Ca^{2+}-activated K^+ current to the suppression of spontaneous depolarization in synaptosomes. However, the marginal additivity observed when both toxins were present implies that CTX and DTX reduce the same K^+ conductance, in addition to a lesser component sensitive to CTX alone. Other lines of evidence support this deduction: (i) in an oocyte expression system mRNA from either rat brain or Shaker cDNA clones give rise to voltage-activated, Ca^{2+}-insensitive K^+ channels that are blocked by low concentrations of DTX or CTX [23,24] and (ii) CTX is able to suppress a voltage-activated, Ca^{2+}-insensitive flux of $^{86}Rb^+$ from synaptosomes evoked by strong depolarization [25].

Unlike CTX, apamin had no discernible effect on the $[Ca^{2+}]_c$ of either polarized or DTX-intoxicated synaptosomes. This apparent absence of the apamin-sensitive, low conductance, Ca^{2+}-activated K^+ channel accords with the ineffectiveness of apamin in the mammalian hippocampus [14,18], as distinct from the PNS of both amphibians and mammals [16,17].

Our results are consistent with the ability of low concentrations of CTX to inhibit mammalian central nerve terminal Ca^{2+}-insensitive, voltage-activated K^+ channels which are sensitive to DTX. The limited additivity of CTX and DTX, together with the lack of effect of apamin suggests that Ca^{2+}-activated K^+ channels are either absent or of relatively minor importance in controlling the activity of the central nerve terminal; this would contrast with rat sympathetic neurones where they may play a dominant role in determining the plasma membrane potential [26].

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