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# Transmitter Release from Presynaptic Terminals of Electric Organ: Inhibition by the Calcium Channel Antagonist Omega *Conus* Toxin

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**Cholinergic synaptosomes from electroplax of the ray *Ommata discopyge* release both ATP and ACh when depolarized with high $K^+$ concentration in the presence of $Ca^{2+}$. Others have shown that the ATP and ACh are released in the molar ratio found in isolated synaptic vesicles. Thus, it is assumed that the release of ATP reflects exocytosis of synaptic vesicles, and that transmitter release can be indirectly monitored by assaying ATP release. We present further evidence for this assumption and examine the effects of presynaptic neurotoxins on this ATP release. As expected for transmitter release, we find that depolarization-evoked ATP release is supported by $Sr^{2+}$ and $Ba^{2+}$ and is inhibited by the Ca channel antagonists $Co^{2+}$ and $Mn^{2+}$. Likewise, the presynaptic toxins ω-CmTX and ω-CgTX, omega peptides from the venom of the marine snails *Conus magus* and *Conus geographus*, respectively, inhibit 80% of the depolarization-evoked ATP release. Half-maximal inhibition of ATP release occurs with ~0.5 μM of either toxin. The toxins' effects are reversible, and when toxin is washed away, the time dependence of recovery of release is approximately first order and half complete within 40 min with ω-CmTX and 15 min with ω-CgTX. The $Ca^{2+}$ ionophore A23187 induces $Ca^{2+}$-dependent ATP release from resting synaptosomes. As would be expected of a Ca channel antagonist, ω-CmTX does not affect this ionophore-induced release. Leptinotarsin-d (LPTd), a putative Ca channel agonist from the Colorado potato beetle, evokes $Ca^{2+}$-dependent ATP release from resting synaptosomes. ω-CmTX does not block LPTd-evoked release of ATP, which suggests that ω-CmTX and LPTd act at different sites. These data are consistent with the hypothesis that ω-CmTX and ω-CgTX inhibit release of ATP from synaptosomes of ray electric organ by blocking $Ca^{2+}$ flux through voltage-gated Ca channels. These toxins are therefore potentially useful for identifying and purifying presynaptic Ca channels from this tissue.**

An essential step in synaptic transmission is the influx of calcium into presynaptic nerve terminals through voltage-gated calcium channels. The resulting elevation of the intracellular $Ca^{2+}$ concentration triggers the release of neurotransmitter. Calcium channels, therefore, play a pivotal role in excitation–secretion coupling (reviewed in Augustine et al., 1987).

Calcium channels are functionally heterogeneous and differ, from tissue to tissue, in almost every measurable property (Hagiwara and Byerly, 1981). For instance, *t*-tubule Ca channels from skeletal muscle, partially purified by Curtis and Catterall (1984), are blocked by dihydropyridine Ca channel antagonists, whereas synaptosomal Ca channels from mammalian CNS are relatively unaffected (Nachsen and Blaustein, 1979; Ebstein and Daly, 1982). In addition, Ca channels can be modulated in different tissues by neurotransmitters such as norepinephrine, serotonin, GABA, somatostatin, and enkephalins (Hagiwara and Byerly, 1981; Reuter, 1983; Tsien, 1983; Michaelson et al., 1984; Rane and Dunlap, 1986). In addition to demonstrating heterogeneity, the effects of these neurotransmitters and effectors suggest that presynaptic Ca channels represent an important potential site for the regulation of synaptic transmission. In order to characterize the full range of Ca channel regulatory mechanisms, it is necessary to study Ca channel function in a wide variety of tissues and cell types.

Recent advances in elucidating the structures of ion channels that are involved in signaling in the nervous system have been achieved through the use of toxins. Alpha bungarotoxin, for example, was instrumental in the purification of the ACh receptor (for a review, see Conti-Tronconi and Raftery, 1982); likewise, tetrodotoxin and scorpion toxins aided in the purification and characterization of the voltage-sensitive sodium channel (for a review, see Catterall, 1984). Because of the lack of specific toxins, however, biochemical analysis of presynaptic Ca channels has progressed slowly.

Olivera and his collaborators (reviewed in Olivera et al., 1985) have reported that the venom of the fish-eating marine snail *Conus geographus* contains a group of several neurotoxic peptides with different targets. One subgroup of these peptides, designated ω-*C. geographus* toxin (ω-CgTX), irreversibly inhibits transmitter release at the frog skeletal neuromuscular junction and blocks the Ca component of the action potential in chick dorsal root ganglion neurons (Kerr and Yoshikami, 1984).

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Both of these effects are due to blockade of $Ca^{2+}$ channels (Feldman and Yoshikami, 1985; Feldman et al., 1987). A related peptide from *C. magus,* designated ω-CmTX, causes paralysis and death when injected intraperitoneally into fish. Both toxins elicit shaking behavior in mice when injected intracerebrally, suggesting an effect on CNS Ca channels. Omega toxins from both species have been purified and sequenced: ω-CgTX peptides are a closely related family, 26–29 amino acids long; ω-CmTX is a 25-amino acid peptide with close sequence homology to the ω-CgTX peptides. In this study we present evidence consistent with the idea that omega *Conus* toxins block voltage-gated Ca channels in purely cholinergic synaptosomes from the electric organ of the ray *Ommata discopyge.* This block appears to result from reversible, high-affinity binding to a receptor, possibly a Ca channel itself, and leads to a maximal 80% inhibition of ATP release into the extracellular medium. Because of their blocking and apparent binding characteristics, should the toxins' receptor be the Ca channel itself, ω-conotoxins ought to prove useful in the affinity labeling and purification of presynaptic, voltage-sensitive Ca channels from this abundant, accessible, and well-defined tissue preparation.

## Materials and Methods

### *Materials*

HEPES, firefly lantern extract, luciferin, and ATP were purchased from Sigma. A23187 was from Calbiochem. The luminometer, with an EMI S2 photomultiplier, was custom made by Biolog, Los Angeles, CA. Electric fish (*O. discopyge*) were imported from the Gulf of California, Mexico, by Marinus, Long Beach, CA, and were kept alive in artificial seawater until use. Omega-CgTX (GVIA) was purified from *Conus geographus* venom as previously described (Olivera et al., 1984). The ω-CmTX (MVIIA) was obtained by solid-phase peptide synthesis according to published procedures (Olivera et al., 1987). Leptinotarsin-d was prepared after the method of Koenig (1985) from *Leptinotarsa decemlineata* hemolymph, generously provided by T. H. Hsaio. All other reagents were of the highest grade available from commercial sources.

### *Methods*

*Preparation of synaptosomes.* Synaptosomes were prepared by a modification of the method of Miljanich et al. (1982). In a typical preparation, electric organs were dissected from 2 rays that had been stunned with 0.25 gm/liter tricaine HCl and cooled to 0°C immediately prior to dissection. All subsequent manipulations were carried out at 0–4°C whenever possible. Organs were diced and homogenized for 4 × 15 sec in a Waring blender with an equal weight of plain synaptosome buffer (PSB): 280 mM NaCl, 3 mM KCl, 1.8 mM $MgCl_2$, 300 mM urea, 100 mM sucrose, 5.5 mM glucose, 20 mM HEPES, pH 7.2), containing 5 mM Mg-EGTA. The homogenate was loaded into a 60 ml syringe, force-filtered through a 40-mesh stainless steel screen, and centrifuged at 30,400 × *g* for 15 min. The supernatant was discarded and the pellet taken up in 20 ml PSB. The resuspended pellet was further disrupted with 5 strokes of a loose-fitting Teflon pestle in a glass homogenizer at 400 rpm; the resulting suspension was centrifuged once more at 30,400 × *g* for 15 min. The supernatant was discarded and the pellet resuspended with the Teflon–glass homogenizer by 10 passes of the pestle at 400 rpm. This homogenate was layered onto six 32 ml 3–20% Ficoll gradients in PSB and centrifuged at 76,600 × *g* for 1 hr in a swinging bucket rotor. The synaptosome band (the first band below the buffer–gradient interface) of each gradient was aspirated off and diluted at more than 1:1 with PSB. The diluted synaptosome suspension was pelleted at 30,400 × *g* for 15 min and resuspended in 1 ml of PSB (with the inclusion, for some experiments, of 1% BSA to enhance stability of the synaptosomes). This final synaptosome preparation was stored at 0°C and used for ATP release experiments within 30 hr. Storage for longer periods resulted in the almost complete loss of depolarization-dependent ATP release activity.

*Luminometry.* Luminometry was performed by the method of Morel and Meunier (1981), as modified by Schweitzer (1987). Except where otherwise indicated, the following procedure was used: Into a 5 ml transparent polystyrene test tube were mixed 465 μl PSB, 5 μl of 5 μg/ml luciferin in PSB, 20 μl firefly lantern extract (1 Sigma FLE-50 bottle reconstituted in 1 ml PSB and spin-dialyzed through 3 ml of Sephadex G-25 preequilibrated in PSB), 5 μl 100 mM $CaCl_2$, and 5 μl synaptosome suspension (5–7 mg/ml protein, excluding BSA). The tube was placed in the chamber and the light output produced by extracellular ATP was continuously monitored by a chart recording of the voltage generated by the photomultiplier tube. Exocytotic release of ATP was evoked by injecting 0.5 ml of high $K^+$ buffer (PSB with $Na^+$ replaced by an equivalent amount of $K^+$) into the reaction mixture in the luminometer. ATP release was quantitated by comparing the peak heights of unknowns with the heights of peaks generated by ATP standards that were injected into each reaction mixture at the end of each trial. Over the range investigated, light output was linear with respect to the amount of ATP injected.

*Protein assays.* Protein was assayed by the method of Bradford (1976), using Bio-Rad protein reagent.

## Results

### *Depolarization-dependent ATP release from electric organ synaptosomes*

The amount of ATP release evoked by high $K^+$ concentration has already been shown to reflect accurately the exocytotic release of ACh from synaptic vesicles (Morel and Meunier, 1981; Schweitzer and Kelly, 1982; Schweitzer, 1987). Figure 1*a* represents a typical chart recording of the time course of light output that results when ATP release is evoked by depolarization of synaptosomes with 140 mM $K^+$. The responses generated by depolarization-evoked release have the same general shape as those resulting from *in situ* injection of ATP standards into the luminometric cocktail; hence, comparison of peak heights allows accurate quantitation of exocytotic ATP release and, by inference, release of ACh.

Evoked release of transmitter is $Ca^{2+}$-dependent in this system (Michaelson and Sokolovsky, 1978), and, as expected, ATP release was also dependent on the presence of external $Ca^{2+}$. Half-maximal release of ATP occurred at 0.5 mM $Ca^{2+}$; release was maximal by approximately 2 mM (Fig. 2).

The extent of ATP release also depended upon the degree of depolarization produced by elevating the external $K^+$ concentration. Figure 3 shows the dependence of ATP release on external $K^+$ concentration in the presence of 0.5 mM $Ca^{2+}$. Saturation was apparently not reached within the range of concentrations tested. For convenience, 140 mM $K^+$ was used in all subsequent experiments.

*$Ca^{2+}$ dependence of ATP release.* Hagiwara and Byerly (1981) outlined several criteria that help establish the functional presence of Ca channels in a system. These criteria specify the ion selectivity which should be present in a biological system whose function is mediated by $Ca^{2+}$ channels. Accordingly, substitution of 0.5 mM $Ca^{2+}$ with equimolar $Sr^{2+}$ or $Ba^{2+}$ was performed, and depolarization-evoked ATP release measured. Consistent with the mediation of exocytosis by voltage-sensitive $Ca^{2+}$ channels, both of these divalent cations supported release. Indeed, $Ba^{2+}$ and $Ca^{2+}$, at 0.5 mM, were equally effective in supporting ATP release, while $Sr^{2+}$ at the same concentration effected 50% of the release seen with $Ca^{2+}$ or $Ba^{2+}$. When higher concentrations of $Sr^{2+}$ were tested, the extent of depolarization-evoked ATP release surpassed that mediated by 0.5 mM $Ca^{2+}$.

Several investigators have observed that $Co^{2+}$ and $Mn^{2+}$ block $Ca^{2+}$ channels in all systems that contain them (Hagiwara and Byerly, 1981). Figure 4 confirms that $Co^{2+}$ inhibits the depolarization-evoked release of ATP in the presence of 0.5 mM $Ca^{2+}$ in a dose-dependent manner. Blockade of release in the

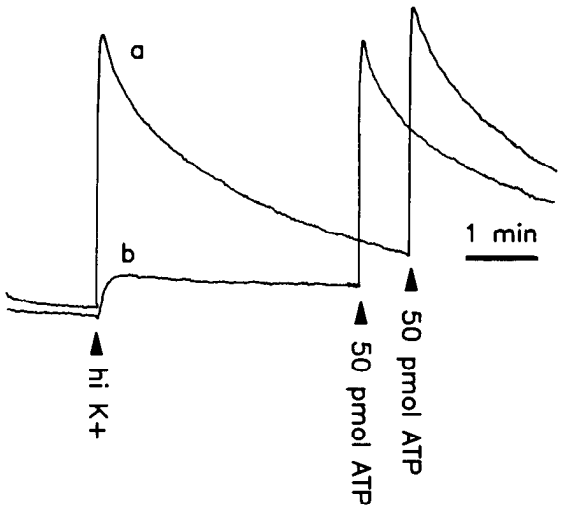


*Figure 1.* Luminometric traces of ATP release from electric organ synaptosomes in the absence (*a*) and the presence (*b*) of ω-CmTX. Synaptosomes were prepared as described in Methods. Five microliter aliquots of synaptosomes were incubated for 10 min at 25°C either in the absence (*a*) or presence (*b*) of 40 μM ω-CmTX. Each sample was then diluted with 465 μl of PSB, 5 μl of luciferin (5 μg/ml), 20 μl of firefly lantern extract, and 5 μl of 100 mM $CaCl_2$ (final concentration, 0.5 mM). Immediately thereafter, samples were placed in the luminometer and the light output (*ordinate*) was monitored while 500 μl of high $K^+$ buffer was injected (*first triangle*). Following each response due to ATP release by exocytosis, a calibration standard (50 pmol) of ATP was injected (*second and third triangles*).

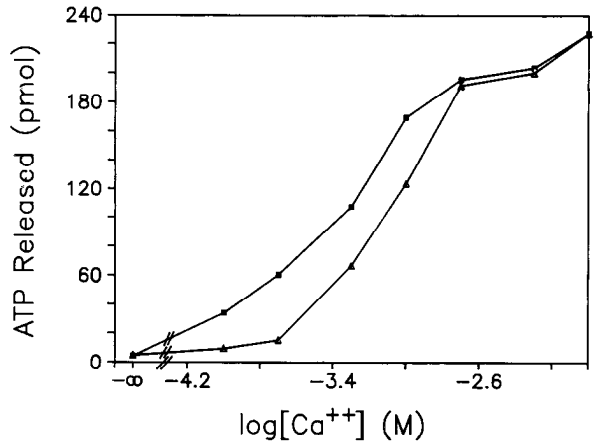


*Figure 2.* Calcium dependence of ATP release from electric organ synaptosomes. Synaptosomes were prepared as in Methods and assayed as described in Figure 1, with the indicated concentrations of $Ca^{2+}$ in the absence (■) and the presence (▲) of 0.5 mM $CoCl_2$.

presence of 0.5 mM $Ca^{2+}$ was substantial at 5 mM $Co^{2+}$, well within the range cited in the Hagiwara and Byerly (1981) criteria. Approximately 30% of ATP release was refractory to $Co^{2+}$ inhibition at $Co^{2+}$ concentrations up to 10 mM, although the amount of this residual ATP release varied between 15 and 30% from one experiment to the next. $Mn^{2+}$ effected a similar dose-dependent block in this system (not shown). Figure 2 illustrates the competitive nature of $Co^{2+}$ inhibition of $Ca^{2+}$-supported release. $Co^{2+}$ (0.5 mM) shifted the $Ca^{2+}$ dose–response curve of ATP release to the right by a factor of 2, but above 2 mM $Ca^{2+}$ the same maximal value for ATP release was obtained whether or not $Co^{2+}$ was present. $Cd^{2+}$ also inhibited depolarization-evoked ATP release in the presence of $Ca^{2+}$, but inhibition of the luciferase component of the ATP assay system by $Cd^{2+}$ rendered quantitation of this effect unreliable (data not shown).

*Effects of nitrendipine and tetrodotoxin.* Nitrendipine is a dihydropyridine that has been shown to block $Ca^{2+}$ channels in many cell types. Its efficacy in blocking CNS nerve terminal $Ca^{2+}$ channels is subject to controversy (Nachsen and Blaustein, 1979; Ebstein and Daly, 1982; Miller and Freedman, 1984; Turner and Goldin, 1985). Nitrendipine, at concentrations of up to 10 μM, did not block depolarization-evoked ATP release in the presence of $Ca^{2+}$ from the synaptosomes used in the present investigations (not shown).

To detect a possible role for voltage-sensitive $Na^+$ channels in the process of exocytosis in electric organ synaptosomes, the specific $Na^+$ channel antagonist, TTX, was tested as an inhibitor of the voltage-sensitive, $Ca^{2+}$-dependent release of ATP. At 1 μM, TTX had no effect on ATP release evoked by $K^+$ depolarization. It should be noted that, in these synaptosomes, the Na channel agonist, veratridine, was not effective in causing ATP release in the presence of $Na^+$ and $Ca^{2+}$ (data not shown). The lack of agonist effects of this toxin could possibly be explained by the absence of functional, voltage-sensitive $Na^+$ channels in *Ommata* electric organ synaptosomes.

*Inhibition by ω-conotoxins.* Having established the presence of functional presynaptic, voltage-sensitive $Ca^{2+}$ channels in the electric organ synaptosome preparation, we investigated the effect of ω-CmTX and ω-CgTX on these channels. To establish, through biochemical methods, the mechanism of action of ω-conotoxins and to find a well-defined and easily accessible system that is sensitive to these toxins, the effects of ω-CmTX and ω-CgTX on exocytosis and $Ca^{2+}$ flux in *Ommata* electric organ synaptosomes were investigated.

To establish the efficacy of ω-CmTx on the nerve terminal preparation, it was shown that the toxin prevented the release of ATP from electric organ synaptosomes upon depolarization with 140 mM $K^+$. Figure 1 demonstrates, by luminometry, that ATP release was attenuated by approximately 80% by 40 μM ω-CmTX. A small, slow phase of release could not be blocked by 40 μM ω-CmTX, the maximum toxin concentration tested. Likewise, $Co^{2+}$ and TTX were both ineffective in blocking this residual depolarization-evoked, $Ca^{2+}$-dependent ATP release.

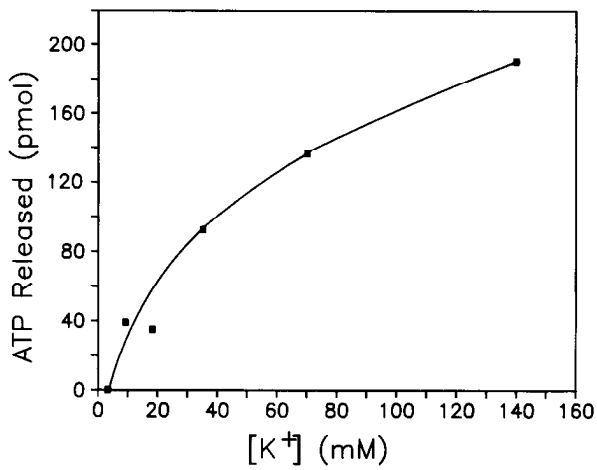


*Figure 3.* Potassium-induced ATP release from electric organ synaptosomes. Synaptosomes were prepared and assayed as described in Methods. Potassium content of the depolarization buffer was varied by replacement with sodium to yield the final potassium concentrations shown.

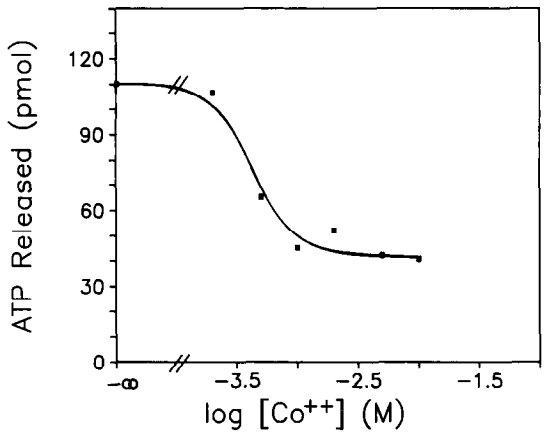


*Figure 4.* Inhibition of ATP release from electric organ synaptosomes by $Co^{2+}$. Synaptosomes were prepared and assayed as described in Methods, in the presence of 0.5 mM $Ca^{2+}$ and the indicated concentrations of $Co^{2+}$. *Line through the data points* is a visual estimate of the best fit.

Hence, this slow phase of release does not seem to result from the opening of a voltage-gated $Na^+$ or $Ca^{2+}$ channel. Figure 5 shows the concentration dependence on ω-CmTX of ATP release from synaptosomes depolarized with 140 μM $K^+$ in the presence of 0.5 mM $Ca^{2+}$. Half-maximal inhibition of release occurred at approximately 0.5 μM CmTX; the blockade of release approached saturation by about 5 μM ω-CmTX although, as noted, a residual release of approximately 15–20% remained that could not be abolished at the highest concentration tested. In most experiments spontaneous, or background, ATP release was observed in normal, low $K^+$ buffer prior to depolarization of the synaptosomes. This resting background release was also blocked by ω-CmTX in the same concentration range that blocked depolarization-dependent release.

A closely related toxin, ω-CgTX, from *C. geographus,* yielded a similar dose–response curve, with half-maximal and maximal inhibitory concentrations of essentially the same values as those obtained for the toxin from *C. magus* (not shown).

The divalent cations $Ba^{2+}$ and $Sr^{2+}$, which substitute for $Ca^{2+}$

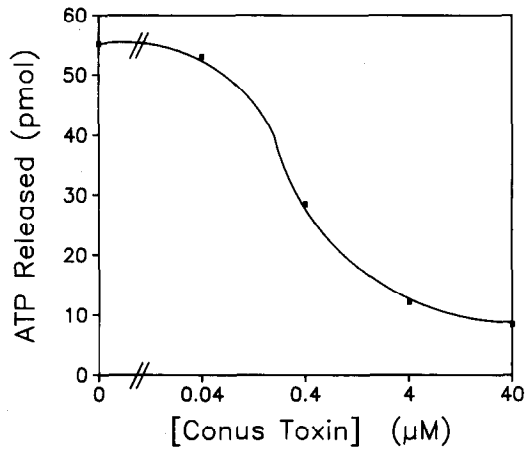


*Figure 5.* Inhibition of ATP release from electric organ synaptosomes by ω-CmTX. Synaptosomes were prepared as described in Methods, and were incubated with the indicated concentrations of ω-CmTX, according to the procedure given in the legend to Figure 1. Luminometry was performed, as in Figure 1, in the presence of 0.5 mM $Ca^{2+}$. *Line through the data points* is a visual estimate of the best fit.

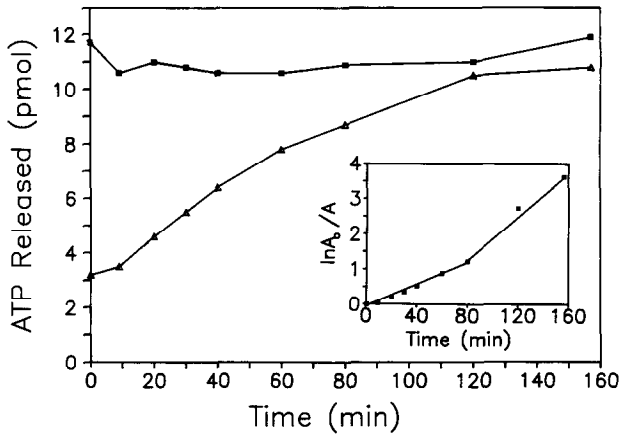


*Figure 6.* Apparent off-rate of ω-CmTX from electric organ synaptosomes. Synaptosomes were prepared as described in Methods. Fifty microliters of synaptosomes were combined with either 50 μl of 2 μM ω-CmTX in PSB (▲) or 50 μl of 0.02 μM ω-CmTX in PSB (■). The mixtures were incubated for 20 min at 25°C, then diluted with 4950 μl each of luminometric assay cocktail, as described in Methods. At the time points indicated, 500 μl aliquots of each diluted mixture were assayed for $K^+$-induced ATP release, as described in Figure 1. *Points* are the averages of triplicate determinations. *Inset,* Logarithmic plot of $A_0/A$ versus time, where $A_0$ = the difference between ATP release from synaptosomes exposed to 0.01 μM CmTX at time 0 and ATP release from synaptosomes exposed to 1 μM CmTX at time 0, and $A$ = the difference in ATP release from synaptosomes exposed to 0.01 μM CmTX at the time point indicated, and synaptosomes exposed to 1 μM CmTX at the same time point.

in mediating ATP release from the *Ommata* electric organ synaptosome system are, like $Ca^{2+}$, ineffective mediators of exocytosis in the presence of ω-CmTX or ω-CgTX. The maximal inhibition of release in the presence of either of these ions was 80%, virtually the same value as that observed with $Ca^{2+}$ as the supporting divalent cation (data not shown).

Inhibition of transmitter release from frog neuromuscular junctions by ω-CgTX, as well as binding to chick brain synaptosomes by labeled toxin, has been reported to be irreversible (Kerr and Yoshikami, 1984; Cruz and Olivera, 1986). In contrast, inhibition of ATP release by both ω-CgTX and ω-CmTX from *Ommata* electric organ synaptosomes is reversible, with $IC_{50}$s of approximately 0.5 μM. The off-rates for each toxin were measured directly by incubation of synaptosomes with half-maximal inhibitory toxin concentrations for 15 min at room temperature, followed by 100-fold dilution with PSB. At various times after the dilution, aliquots of the incubation mixture were assayed for ATP release activity. As judged by recovery from inhibition of the depolarization-evoked ATP release activity, effective dissociation of each toxin was apparently first order for the initial 80 min. The half-time for functional dissociation of CmTX from the synaptosomes was approximately 40 min (Fig. 6); CgTX was half-dissociated within 15 min (data not shown). Apparent first-order off-rates were 0.017 $min^{-1}$ for CmTX and 0.047 $min^{-1}$ for CgTX.

*Effects of ionophores and leptinotarsin.* Although the electrophysiological experiments (Kerr and Yoshikami, 1984) suggest there is a direct blockade of voltage-gated $Ca^{2+}$ channels by ω-CgTX, it is possible that omega toxins have other actions on electric organ synaptosomes. For example, omega *Conus* toxins could exert their inhibitory effects on exocytotic release at a step in the presynaptic signal-transduction process distal to entry of $Ca^{2+}$. If this were the case, exocytosis resulting from $Ca^{2+}$ entry

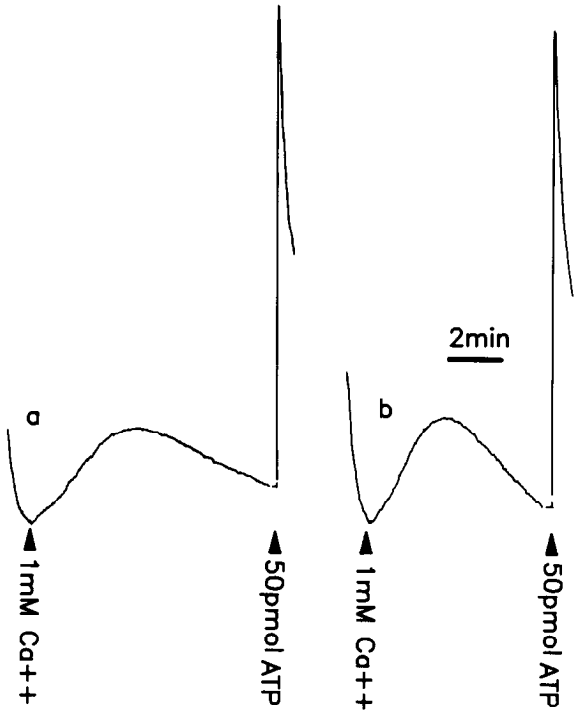


*Figure 7.* Luminometric traces of $Ca^{2+}$/A23187-mediated ATP release from electric organ synaptosomes in the presence (*a*) and the absence (*b*) of ω-CmTX. Synaptosomes were prepared as described in Methods. Five microliter aliquots of synaptosomes were incubated for 15 min at 25°C either in the presence of 8 μM ω-CmTX (*a*) or in its absence (*b*). Five minutes into the incubations, A23187 in $H_2O$/DMSO was added to a concentration of 0.2 μg/ml (DMSO concentration was 0.03% in the incubations, 0.0006% in assays). After the incubations, samples were assayed by luminometry for ATP, during which time $Ca^{2+}$ (final concentration 1 mM) was injected (*triangles*). The detailed time course of release varied somewhat from sample to sample, but no consistent effect of ω-CmTX on this release was noted (see Results).

via exogenously supplied ionophores would also be blocked by the ω-conotoxins. To test this hypothesis, the $Ca^{2+}$ ionophore A23187 was incorporated into the synaptosome system, and $Ca^{2+}$-dependent ATP release was monitored both in the absence and the presence of 8 μM ω-CmTX (Fig. 7). With A23187 incorporated into the synaptosomal membranes, addition of $Ca^{2+}$ alone, without high $K^+$ concentration, was capable of evoking ATP release. The rate of ATP release varied somewhat among determinations, but no consistent effect of ω-CmTX on this rate was noted. This variability was likely due to the variable efficiency of dispersion of the ionophore. Complete dispersion of the ionophore into its aqueous stock solution immediately prior to addition to the synaptosomes was essential to the success of $Ca^{2+}$-dependent ATP release. Alone, the dispersing agent, dimethyl sulfoxide (DMSO), failed to cause ATP release. Moreover, equimolar replacement of $Ca^{2+}$ with $Mg^{2+}$ elicited no measurable ATP release in the presence of A23187, although the affinities of the 2 cations for the ionophore are virtually the same. This experiment demonstrates that the ATP release evoked by A23187-mediated $Ca^{2+}$ entry was not at all diminished by ω-CmTX at a concentration sufficient to block depolarization-evoked ATP release maximally. Thus, ω-CmTX exerts its inhibitory effect on exocytosis at the level of $Ca^{2+}$ entry through voltage-sensitive $Ca^{2+}$ channels, and not at a subsequent step in the signal-transduction process. This result is consistent with the observation that ω-CgTX does not block spontaneous min-

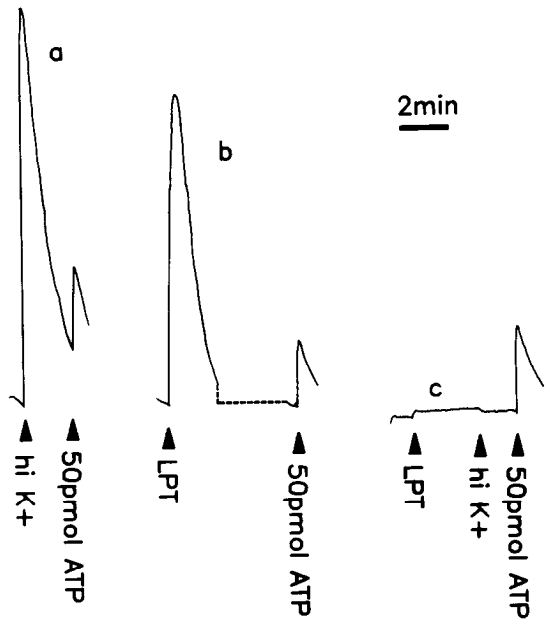


*Figure 8.* Potassium- and LPTd-evoked ATP release from electric organ synaptosomes in the presence and in the absence of $Ca^{2+}$. Synaptosomes were prepared as described in Methods. Five microliters of synaptosomes were combined with 465 μl of luciferin (5 μg/ml), 20 μl of firefly lantern extract, and either 5 μl of 100 mM $CaCl_2$ (*a,b*), or no added $CaCl_2$ (*c*). Samples were then immediately inserted into the luminometer at 25°C with no preincubation, and either 500 μl of high $K^+$ synaptosome buffer (*a,c*), or 10 μl of a solution of partially purified LPTd (*b,c*) was injected.

iature endplate potentials at the frog neuromuscular junction (Kerr and Yoshikami, 1984).

Leptinotarsin-d (LPTd), a peptide toxin that was suggested by McClure et al. (1980) as being an agonist of Ca channels, was also employed to explore the mechanism of action of ω-CmTX. Although LPTd has not generally been found to have effects in nonmammalian systems (Koenig, 1985), it does evoke $Ca^{2+}$-dependent release of ATP from elasmobranch synaptosomes used in this report (Fig. 8). As observed in other suscep-

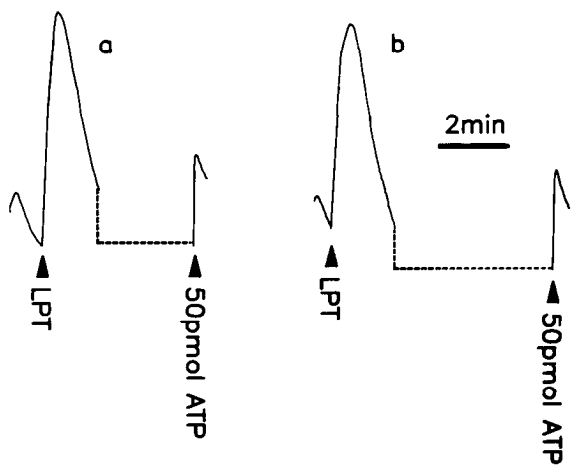


*Figure 9.* Effect of ω-CmTX on $Ca^{2+}$-dependent ATP release from electric organ synaptosomes evoked by LPTd. Synaptosomes were prepared as described in Methods. Five microliter aliquots of synaptosomes were incubated for 10 min at 25°C in the absence (*a*) or the presence (*b*) of 8 μM ω-CmTX, then assayed for ATP release in the presence of 1 mM $Ca^{2+}$ while 10 μl of partially purified LPTd was injected.

tible systems, the effect here does not require depolarization (R. Yeager, Z. Unver, T. Hsaio, and G. Miljanich, unpublished observations). Preincubation with ω-CmTX at the maximally effective concentration of 8 μM failed to block LPTd-evoked ATP release (Fig. 9).

## Discussion

The exocytotic corelease of ATP with ACh from electric organ synaptosomes is a well-documented phenomenon (e.g., see Wagner et al., 1978; Morel and Meunier, 1981; Schweitzer and Kelly, 1982; Schweitzer, 1987). Likewise, there is good evidence for the presence of voltage-sensitive calcium channels in this sort of preparation (e.g., see Michaelson and Sokolovsky, 1978). By means of a simple and rapid luminometric method, depolarization-dependent, calcium-mediated ATP release from electric ray electric organ synaptosomes was confirmed in the present work. Thus, the electrophysiological and biochemical properties of this presynaptic system provide a useful model with which to examine the mechanism of action of calcium channel toxins such as the ω-conotoxins.

Omega conotoxins from the fish-hunting marine snails *C. magus* and *C. geographus* blocked exocytotic ATP release from electric organ synaptosomes prepared from the electric ray *O. discopyge*. Inhibition of ATP release appeared to be reversible, with $IC_{50}$s in the range of 0.5 μM for both ω-CmTX and ω-CgTX. Synaptosomes preincubated with 1 μM ω-CmTX or ω-CgTX recovered depolarization-dependent ATP release activity with half-times of 40 min and 15 min, respectively. In light of the relatively slow apparent off-rate of ω-CmTX, it is noteworthy that synaptosomes pretreated with ω-CmTX and thereafter exposed to LPTd, a putative $Ca^{2+}$ channel agonist from the hemolymph of *Leptinotarsa decemlineata,* manifested, within seconds, $Ca^{2+}$-dependent ATP release that was undiminished relative to controls that had not been pretreated with ω-CmTX. The lack of inhibition of LPTd's effect after ω-CmTX pretreatment suggests an absence of competition between their respective sites of action, and thus the possibility that the ω-CmTX and LPTd bind to distinct, noninteracting sites associated with one or more $Ca^{2+}$ channels in electric organ nerve terminals. Alternatively, LPTd could act on synaptosomes as a $Ca^{2+}$-dependent ionophore. Madeddu et al. (1985) reported that crude (1% pure) leptinotarsin-h (LPT) inserted into artificial planar lipid bilayers in the presence of $Ca^{2+}$ upon application of a transpositive transmembrane voltage. However, the impurity of the LPT used, and the physicochemical differences between artifical bilayers and synaptosomes, casts doubt on the physiological relevance of this phenomenon. In contrast to their finding of a voltage dependence for functional insertion of LPT into bilayers, we have found that LPTd mediates a robust ATP release even from electric organ synaptosomes that have been completely depolarized with high $K^+$ concentration (Yeager et al., 1986; R. E. Yeager, Z. Unver, T. Hsaio, and G. P. Miljanich, unpublished observations).

The calcium ionophore A23187 mediates the transfer of $Ca^{2+}$ across lipid bilayers of biological membranes. This property of the ionophore was exploited to pinpoint the site of action of CmTX. Since $Ca^{2+}$, in the presence of A23187, led to ATP release even when CmTX was present at concentrations that maximally inhibited depolarization-evoked ATP release, ω-CmTX apparently exerts its toxic effect by inhibiting the transfer of $Ca^{2+}$ into synaptosomes via endogenous, voltage-gated $Ca^{2+}$ channels.

In addition to depolarization-dependent ATP release from synaptosomes, a small amount of $Ca^{2+}$-dependent release also occurs "spontaneously," i.e., in the absence of depolarization. $Co^{2+}$ and ω-CmTX prevent this "spontaneous" ATP release to about the same extent that they inhibit depolarization-evoked release. Depolarization-independent release may reflect spontaneous activity of $Ca^{2+}$ channels that can be blocked by $Co^{2+}$ and ω-CmTX. The hypothesis that ω-conotoxins block voltage-gated $Ca^{2+}$ channels is further evidenced by preliminary studies indicating that both depolarization-evoked and "spontaneous" $^{45}Ca^{2+}$ fluxes are blocked by ω-CmTX and ω-CgTX, as well as by $Co^{2+}$ (Yeager et al., 1986; R. E. Yeager, J. Rivier, and G. P. Miljanich, unpublished observations).

In the present investigation, $Ba^{2+}$ was as effective as $Ca^{2+}$ in eliciting ATP release from synaptosomes. Augustine and Eckert (1984), on the other hand, found that $Ca^{2+}$ is much more effective than $Ba^{2+}$ in supporting transmitter release during single action potentials in spite of the greater permeability of $Ba^{2+}$ compared to that of $Ca^{2+}$ (Hagiwara and Byerly, 1981; Hess and Tsien, 1984). However, the luminometric assay used in the present work measured ATP release during prolonged depolarizations. Thus, the lower efficiency of $Ba^{2+}$ in activating the release mechanism may have been offset by less efficient clearance of this ion from the cytosol over the 0.1–0.5 min time course of the luminometric assay. The increase in the apparent potency of $Ba^{2+}$ in long-term transmitter release assays has been observed by others. This phenomenon is reviewed in Silinsky (1985).

Our results indicate that both ω-conotoxins (ω-CmTx and ω-CgTx) inhibit ATP release in electric organ presynaptic terminals with comparable potencies. By contrast, both electrophysiological effects and binding appear to be irreversible for ω-CgTx targets in higher vertebrates (Kerr and Yoshikami, 1984; Cruz and Olivera, 1986). The readily reversible binding with apparent $K_D$s in the micromolar range for the elasmobranch preparation is consistent with the hypothesis that significant structural divergence has occurred among $Ca^{2+}$ channels. Other channels may be more conserved: The binding characteristics of μ-conotoxins, which inhibit muscle-type Na channels, do not vary significantly between electric eel and rat muscle (Moczydlowski et al., 1986). Thus, ω-CgTx and ω-CmTx may be useful tools in analyzing the evolutionary divergence of $Ca^{2+}$ channels.

In the elasmobranch electric organ, a highly ramified network of nerve terminals innervates virtually the entire ventral surface of each electrocyte and represents more than 5% of the total tissue mass. In addition, kilograms of electric organ can be obtained from a single individual of the larger species of electric ray. As is evident from this, as well as previous reports, there are voltage-gated $Ca^{2+}$ channels in electric organ nerve terminals and this tissue appears to be an appropriate starting material for the purification of presynaptic $Ca^{2+}$ channels. Because the toxins used in the present studies appear to block these presynaptic voltage-sensitive $Ca^{2+}$ channels, they may prove to be useful tools in the labeling, purification, and functional characterization of this critical component of the process of synaptic transmission.

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