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# EVIDENCE FOR THE INVOLVEMENT OF DISTINCT VOLTAGE-SENSITIVE CALCIUM CHANNELS IN THE RELEASE OF $^3$H-DOPAMINE FROM PRIMARY CULTURES OF MESENCEPHALIC NEURONS

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**SUMMARY**: In ventral mesencephalic neurons cultured for five days the $K^+$-evoked $^3$H-dopamine release is mediated through activation of N-type $Ca^{2+}$ channels, while L- or T-type channels appear to be inactive. In contrast, veratridine-elicited release of $^3$H-dopamine that was attenuated by tetrodotoxin was not altered by N-, L-, nor T-type $Ca^{2+}$ channel blockers.

**KEYWORDS:** Dopamine release, $Ca^{2+}$ channels, membrane depolarization, primary cultures, ventral mesencephalic neurons.

During membrane depolarization, voltage-sensitive calcium channels open and allow $Ca^{2+}$ to enter and to diffuse in the cytoplasm. There $Ca^{2+}$ is either sequestered by proteins associated with vesicles and other cytoskeletal components or is operative in the activation of calcium-dependent enzymes. The activation of specific protein kinases by $Ca^{2+}$ was proposed to trigger vesicular exocytosis resulting in release of neurotransmitters from the cell (Llinas, McGuiness, Leonard, Sugimori and Greengard, 1985). Tsien and coworkers (1988) suggested the existence of at least three types of voltage-sensitive $Ca^{2+}$ channels (L-, N-, and T-types) that can be distinguished by the characteristics of gating duration, ionic conductance and pharmacological properties. Recently, another type of voltage-sensitive $Ca^{2+}$ channel referred to as "P" channel was described (Llinas, Sugimori, Lin and Cherksey, 1989) and was functionally linked to neurotransmitter release in giant squid synapse. Although the N-type $Ca^{2+}$ channels were implicated in controlling the release of neurotransmitter from cultured sympathetic neurons (Hirning et al., 1988), the physiological significance of the diverse types of calcium channels in neurotransmitter release process remains still to be elucidated. In this report we investigate the involvement of different types of voltage-sensitive $Ca^{2+}$ channels in controlling dopamine release elicited by different depolarizing agents in primary cultures of rat ventral mesencephalon.

## MATERIALS AND METHODS

Preparation of Cultures: Ventral mesencephalic cell cultures were prepared as described by Prochiantz and coworkers (1979). In brief, female Sprague-Dawley rats (Zivic Miller, Penna.), 14 days in gestation, were decapitated, the embryos were removed, the ventral mesencephali were dissected under sterile conditions and were mechanically dissociated in complete culture medium. The culture medium consisted of equal volumes of nutrient mixture F12 (Gibco) and Modified Minimum Essential Medium (Quality Biological) supplemented with 2 mM glutamine, 6 mg/ml glucose, a mixture of 0.5 units of Penicillin G and 0.5 mg of Streptomycin per ml and 15% heat inactivated horse serum (Hyclone). Cells were plated at a density of 30,000-50,000/cm$^2$ in multiwell plates (16 mm diam., Costar) previously coated with poly-D-lysine (10 µg/ml; Sigma) and were cultured for 5 days at 37°C in a water-saturated atmosphere of 95% air and 5% $CO_2$.

$^3$H-Dopamine release: The experiments were performed on neurons in culture for five days. Each culture dish was washed once with 1 ml Krebs-Ringer-Henseleit (KRH) buffer at 37°C. The monoamine stores in the neurons were labelled with $^3$H-dopamine by incubating them for 15 min. at 37°C with KRH-buffer containing 5 x $10^{-8}$M $^3$H-dopamine (New England Nuclear; spec. act. 5-10 Ci/mmol), and thereafter unbound radioactivity was removed by three washes with KRH buffer. The neuronal cultures were then incubated at 37°C for 5 min, with 0.5 ml KRH buffer to determine the basal release of $^3$H-dopamine followed by a 5 min period of incubation in the presence of 0.5 ml of KRH buffer containing a depolarizing agent. At the end of each incubation interval, the supernatants were collected and counted for radioactivity by liquid scintillation spectrometry. At the end of the experiment the residual intracellular radioactivity in the cells was determined. The composition of the standard KRH buffer was (mM): NaCl (136), KCl (5), $MgSO_4 \cdot 7H_2O$ (0.8), $NaHCO_3$ (2.6), $KH_2PO_4$ (0.4), $Na_2HPO_4 \cdot 7H_2O$ (0.34), Glucose (5.6), Hepes (15), $CaCl_2$ (1.3), Pargyline (0.1), and (0.01%) Ascorbic acid (pH 7.38).

## RESULTS

The depolarizing agents KCl (15 mM - 40 mM) and veratridine (1 μM - 5 μM) elicit a dose-dependent increase of $^3$H-dopamine release from ventral mesencephalic dopaminergic neurons cultured for five days. In contrast to the KCl-evoked $^3$H-dopamine release, that elicited by veratridine was completely abolished by the presence of 0.1 μM tetrodotoxin (Table 1). The KCl- and veratridine-evoked release of $^3$H-dopamine required the presence of calcium in the culture medium and was diminished when cells were incubated in calcium-free buffer supplemented with 0.5 mM EGTA (Table 1).

**Table 1. Requirement of Extracellular $Ca^{2+}$ For $^3$H-Dopamine Release Elicited by KCl and Veratridine in Primary Cultures of Mesencephalic Neurons**

| Addition to Incubation Medium | $^3$H-Dopamine Released (% of Total) | | |
|---|---|---|---|
| | Control | 20 mM KCl | 2 μM Veratridine |
| None | 7.3 | 20 | 19 |
| 0.1 uM Tetrodotoxin | 3.3 | 21 | 4.0 |
| 0 $Ca^{2+}$ + 0.5 mM EGTA | 4.5 | 11 | 8.5 |
| 10 uM $Zn^{2+}$ | 13 | 8.2 | 7.8 |

Each value represents the percentage of total $^3$H-dopamine taken up by neurons in each well and was derived from two determinations in triplicate. After loading the cells with $^3$H-dopamine, the *basal release of $^3$H-dopamine/5 min* was determined followed by measurements of the evoked *$^3$H-dopamine release/5 min* and measurements of the remaining $^3$H-dopamine in each well.

The veratridine- or KCl-evoked $^3$H-dopamine release was also blocked by the presence of 10 μM $Zn^{2+}$ that was reported to inhibit $Ca^{2+}$ dependent release of neurotransmitter by directly competing for $Ca^{2+}$ binding sites (Nishimura, 1987). Interestingly, 10 μM $Zn^{2+}$ in KRH buffer increased the basal release of $^3$H-dopamine by 78%, an effect that was also observed for acetylcholine release from mouse neuromuscular junction by Nishimura (1988). To characterize the type of $Ca^{2+}$ channel that may be involved in $^3$H-dopamine release we have tested compounds that were shown to interact with L-, T-, or N-type $Ca^{2+}$ channels. Omega-conotoxin GVIA (ω-CT), a peptide shown to block N- and L-type $Ca^{2+}$ channel (Tsien et al., 1988) inhibited, in a dose-dependent manner, the KCl-evoked release of $^3$H-dopamine (Fig. 1A). The release of $^3$H-dopamine elicited by 20 mM KCl was completely inhibited with 10 μM ω-CT, while the $IC_{50}$ was around 50 nM. In contrast, ω-CT failed to inhibit $^3$H-dopamine release elicited by 1 μM or 3 μM veratridine (Fig. 1B). Preincubation for 15 min with 10 μM ω-CT failed to inhibit the veratridine-evoked release of $^3$H-dopamine (data not shown).

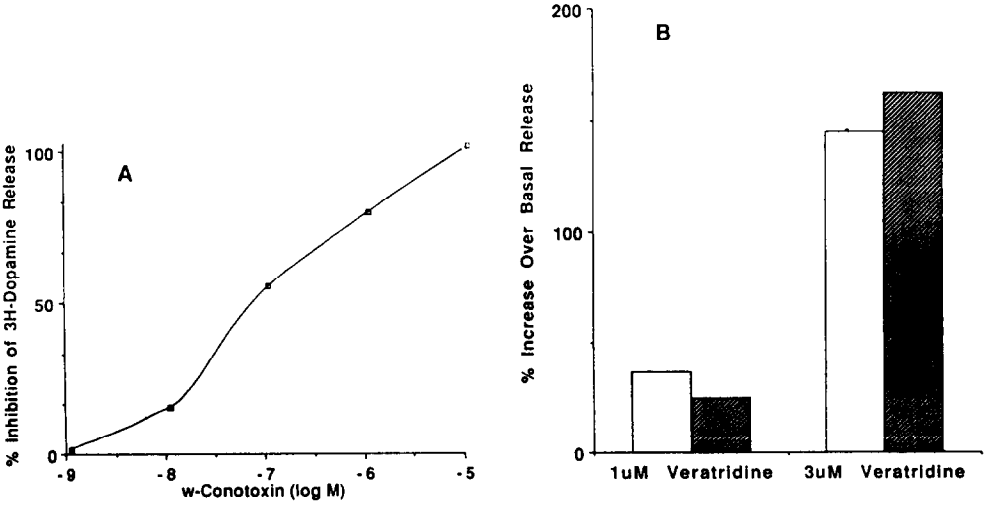


**Figure 1.** (A) Dose response relationship of ω-CT and percentage of inhibition of KCl-evoked $^3$H-dopamine release. Basal $^3$H-dopamine release was 7.1 ± 1.1 (% of total) and that elicited by 20 mM KCl was 20 ± 3.0 (% of total). Results are expressed as % of inhibition of $^3$H-dopamine release elicited by different concentrations of ω-conotoxin. (B) Effect of ω-conotoxin (10 μM) on the veratridine-evoked $^3$H-dopamine release. Results are expressed as percent increase over basal $^3$H-dopamine release in absence (open bar) and presence (hatched bar) of 10 μM ω-CT. The values are the mean derived from two different experiments in triplicate.

The data shown in Table 2 document that neither L- nor T-type $Ca^{2+}$ channels are involved in the depolarization-dependent release of $^3H$-dopamine. Nifedipine, a specific inhibitor of the L-type $Ca^{2+}$ channel and an endogenous modulator for L- and T-type channels, that was isolated from rat brain (Callewaert, Hanbauer and Morad, 1989) failed to modify $^3H$-dopamine release elicited by KCl or veratridine (Table 2). Amiloride, a $K^+$-sparing drug that was shown to inhibit the T-type $Ca^{2+}$ channels (Tang et al., 1988), also failed to alter $^3H$-dopamine release elicited by KCl or veratridine (Table 2).

**Table 2. The Effect of L- and T-type $Ca^{2+}$ Channel Inhibitors on the KCl- and Veratridine-Elicited $^3H$-Dopamine Release in Primary Cultures of Mesencephalic Neurons**

| $Ca^{2+}$ Channel Inhibitor (M) | Type of Channel | %-Increase over Basal $^3H$-Dopamine Release | |
|---|---|---|---|
| | | 20 mM KCl | 2 μM Veratridine |
| None | | 97 | 204 |
| Nifedipine ($5 \times 10^{-8}$) | L | 86 | 198 |
| Nifedipine ($5 \times 10^{-7}$) | L | 86 | 180 |
| E.M.*(1.0 unit) | L and T | 97 | 229 |
| Amiloride ($10^{-4}$) | T | 111 | 198 |

Each value is the mean of two determinations in triplicate and represents the percentage of increase over basal $^3H$-dopamine release. The $Ca^{2+}$ channel inhibitors were added 5 min before addition of depolarizing agents. *E.M. = Endogenous $Ca^{2+}$ channel modulator. 1 unit causes 50% inhibition of $^3H$-nitrendipine binding.

## DISCUSSION

The role of different types of voltage-sensitive $Ca^{2+}$ channels in $^3H$-dopamine release was studied in primary cultures of rat ventral mesencephalon. This type of preparation allows one to study neurotransmitter release in intact and living neurons and not in severed axons as is the case in slices or synaptosomes prepared from adult rat brain. These results clearly showed that although KCl- and veratridine-evoked release of $^3H$-dopamine requires extracellular $Ca^{2+}$, they are operative through different $Ca^{2+}$ channels. The KCl-evoked release of $^3H$-dopamine was mediated through activation of N-type $Ca^{2+}$ channels because ω-CT completely blocked this response. Inhibition of L- or T-type $Ca^{2+}$ channels failed to alter KCl-evoked $^3H$-dopamine release. These findings are in line with previous reports suggesting that L-type $Ca^{2+}$ channels occur at the soma (Sanna, Head and Hanbauer, 1986) and N-type channels are located on presynaptic nerve terminals (Miller, 1987). Our results also show that the veratridine-evoked release of $^3H$-dopamine was not altered by L-, T-, or N-type $Ca^{2+}$ channel blockers. Since veratridine elicits membrane depolarization by prolonging the opening time of voltage-sensitive $Na^+$ channels it is possible that $Ca^{2+}$ could enter the cell along with $Na^+$. In fact, inactivation of $Na^+$ channels by tetrodotoxin that was shown to block the veratridine-evoked $^{45}Ca^{2+}$-influx in striatal slices (Sanna et al., 1986) attenuated $^3H$-dopamine release in cultured mesencephalic cells. This suggestion does not exclude the possibility that veratridine may also activiate an as yet undefined voltage-sensitive $Ca^{2+}$ channel. The possibility that the "P" channel may play such a role is being investigated.

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