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Inhibition by ethaverine of catecholamine secretion through blocking L-type Ca^{2+} channels in PC12 cells

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Abstract—Ethaverine, a derivative of papaverine, is used as a vasodilator and antispasmodic drug. We have investigated the effects of ethaverine on the secretion of [^{3}H]norepinephrine from PC12 cells, of neuroendocrine origin. Treatment with ethaverine reduced catecholamine secretion in a concentration-dependent manner. The maximal inhibitory effect (90%) was achieved with 10 μM ethaverine, and the IC_{50} for secretion was ~2 μM. Ethaverine pretreatment for 1 min prior to stimulation by 70 mM K^+ also decreased the level of intracellular Ca^{2+} in a concentration-dependent manner, as measured by fura-2 fluorescence. The IC_{50} for the inhibition of the increase in intracellular Ca^{2+} was ~2 μM. Nifedipine, a dihydropyridine L-type Ca^{2+} channel blocker, did not enhance the inhibitory effect of ethaverine on the 70 mM K^+-induced increase in $[Ca^{2+}]_i$ or catecholamine secretion. In contrast, the addition of the N-type voltage-sensitive Ca^{2+} channel antagonist ω-conotoxin with ethaverine resulted in further reductions in the increase in $[Ca^{2+}]_i$ and catecholamine release. Maximally effective concentrations of ethaverine and nifedipine showed the same inhibitory effect on the 70 mM K^+-evoked responses. However, ethaverine pretreatment did not inhibit the bradykinin-induced secretion and $[Ca^{2+}]_i$ rise, which are known to be produced through the receptor-operated Ca^{2+} channels. We conclude that ethaverine reduces catecholamine secretion by blocking the L-type voltage-sensitive Ca^{2+} channel.

Key words: ethaverine; catecholamine; exocytosis; intracellular calcium; calcium channel; PC12 cell

Ethaverine, a tetraethoxyl homolog of papaverine, which is a substituted isoquinoline alkaloid from *Papaver somniferum*, is used therapeutically as a potent vasodilator in the treatment of peripheral vascular diseases [1]. This action appears to result from its ability to inhibit the L-type Ca^{2+} channels responsible for Ca^{2+} influx in arterial and cardiac smooth muscle cells [2, 3]. Wang and Rosenberg [4] have shown that ethaverine exerts its effects by binding to the verapamil binding sites on the L-type calcium channels in porcine cardiac sarcolemma. This compound is also known to inhibit phosphodiesterase activity in smooth muscle cells [2]. However, thus far, no study has addressed the effects of ethaverine on neurotransmitter secretion.

To study the effects of ethaverine on the secretory mechanism of neuronal cells, we used the undifferentiated clonal rat pheochromocytoma PC12 cell line, which has been shown to be a useful model system for neurosecretion [5]. PC12 cells possess two types of VSCCs*, L- and N-types, which are opened by membrane depolarization with elevated extracellular K^+ [6], as well as ROCCs, which are activated by bradykinin and ATP [7]. Ca^{2+} influx through VSCCs or ROCCs results in dopamine or norepinephrine release from these cells [8]. The two different types of VSCCs can be distinguished by their nature and pharmacological properties [9].

Because the regulation of Ca^{2+} channel activity can affect catecholamine secretion, we examined the function of ethaverine in controlling $[Ca^{2+}]_i$ in PC12 cells. In this report we present evidence showing that depolarization-induced $[Ca^{2+}]_i$ increase and catecholamine secretion are inhibited by ethaverine. Ethaverine blocked the L-type voltage-sensitive channel without affecting the N-type channel or the receptor-operated channel in PC12 cells.

* Abbreviations: VSCCs, voltage-sensitive Ca^{2+} channels; ROCCs, receptor-operated Ca^{2+} channels; ω-CTx, ω-conotoxin GVIA; [^{3}H]NE, [^{3}H]norepinephrine; and $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration.

Materials and Methods

Cell culture. PC12 cells were grown on Primaria culture dishes (Falcon, Lincoln Park, NJ, U.S.A.) in RPMI 1640 (GIBCO, Gaithersburg, MD, U.S.A.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 5% heat-inactivated horse serum (Hyclone, Logan, UT, U.S.A.) and 1% antibiotics (GIBCO) in a humidified atmosphere of 5% CO_2–95% O_2 at 37°. Medium was changed every 3 days, and cells were subcultured about once a week.

Measurements of $[Ca^{2+}]_i$. Fura-2 fluorescence was used to measure cytosolic calcium. PC12 cells were loaded with fura-2 acetoxymethylester (fura-2/AM) (Molecular Probes, Eugene, OR, U.S.A.) to a final concentration of 2–5 μM in complete medium at 37° for 40 min. The final concentration of DMSO in the incubation medium was less than 0.3%. After loading, the cells were washed twice with Locke's solution (NaCl, 154 mM; KCl, 5.6 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 2.2 mM; HEPES, 5.0 mM; glucose, 10 mM, pH 7.4) to remove the extracellular dye. Sulfinpyrazone (Sigma, St. Louis, MO, U.S.A.) was added to both the loading medium and the washing solution to a final concentration of 250 μM to prevent dye leakage. For the fluorimetric measurement of $[Ca^{2+}]_i$, 1×10^6 cells in 1 mL Locke's solution were placed in a quartz cuvette in a thermostatically controlled cell holder at 37°, and the cell suspension was stirred continuously. Fluorescence ratios were taken by dual excitation at 337 nm and 380 nm and emission at 500 nm by an alternative wavelength time scanning method, using a Shimadzu RF-5000 spectrofluorometer. Calibration of the fluorescence signal in terms of $[Ca^{2+}]_i$ was performed according to Grynkiewicz *et al.* [10].

Measurement of catecholamine release. The release of

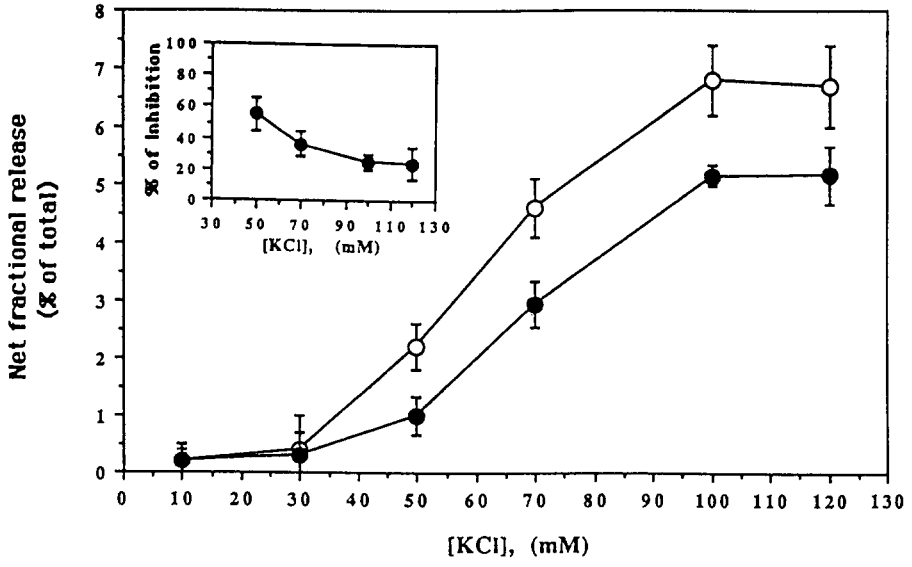

Fig. 1. Inhibitory effect of ethaverine on catecholamine secretion evoked by variable extracellular K^+ concentrations. Cells were treated with variable concentrations of extracellular K^+ in the absence (○) or presence (●) of 1 μM ethaverine. Inset: percent inhibition of K^+-stimulated secretion by 1 μM ethaverine. Each data point was obtained from triplicate experiments and is shown as the mean ± SEM.

[^{3}H]NE from the PC12 cells was measured by a method reported previously [11]. Briefly, cells were grown on 100-mm dishes and incubated with 1 μCi/mL of [^{3}H]NE for 90 min. Cells were harvested by gently pipetting the culture medium onto the surface of the monolayers, and then were divided into 1×10^6 cells per Eppendorf tube. The pellets were washed three times with Ca^{2+}-free Locke's solution, and the final fourth washing was done by incubating cells with Ca^{2+}-containing Locke's solution for 10 min. The experiment consisted of five consecutive 5-min incubation periods. The measurement of secretion was carried out with two 5-min incubation periods in buffer containing 2.2 mM $CaCl_2$ with variable concentrations of ethaverine (Sigma). This provided a measure of the basal release. The above buffer was removed, and then cells were incubated with Locke's solutions containing elevated concentrations of K^+ and variable concentrations of ethaverine. Finally, the decline of the K^+-induced release back to the basal level was measured in two 5-min incubation periods following the stimulation period. At the end of the five consecutive 5-min incubations, residual catecholamine was extracted from the cells with 0.1 N HCl. Radioactivity was measured by scintillation counting, and secretion was calculated as the percentage of total catecholamine.

Results and Discussion

To determine the inhibitory effect of ethaverine on high K^+-induced catecholamine secretion, PC12 cells were exposed to various concentrations of extracellular K^+ in the presence of 1 μM ethaverine. As shown in Fig. 1, a concentration-dependent sigmoidal increase in catecholamine secretion was observed with increases in extracellular K^+. Secretion was measured by the radioactivity released into the medium after stimulation and shown as the percentage secretion of the total [^{3}H]NE loaded. Maximal secretion was 6.73 ± 0.72% with 100 mM K^+, and half-maximal secretion was obtained with 60 mM K^+. Cells treated with 1 μM ethaverine for 10 min prior to the stimulation showed a 55% inhibition of the 50 mM K^+-induced stimulation and a 25% inhibition of the 100 and 120 mM K^+-induced secretions (Fig. 1, inset). This suggests that the inhibition caused by ethaverine was reduced by increasing the probability of VSCCs being open with strong membrane depolarization.

Table 1. Inhibition of $[Ca^{2+}]_i$ increase and [^{3}H]NE release by Ca^{2+}-channel blockers and ethaverine

	ΔCa^{2+} (nM)	Net secretion (% of total)
70 mM K^+	192 ± 16 (100)	4.65 ± 0.12 (100)
Nifedipine	24 ± 8 (12.5)	0.56 ± 0.15 (12.0)
ω-Conotoxin	160 ± 11 (83.3)	3.45 ± 0.22 (74.2)
Ethaverine	25 ± 5 (13.0)	0.50 ± 0.36 (10.7)
Nifedipine + ethaverine	22 ± 7 (11.4)	0.65 ± 0.21 (14.0)
Nifedipine + ω-conotoxin	7 ± 3 (3.6)	0.35 ± 0.06 (7.5)
Ethaverine + ω-conotoxin	9 ± 2 (4.7)	0.29 ± 0.12 (6.2)

The increment of $[Ca^{2+}]_i$ was obtained by subtracting the basal level from the peak height. The Ca^{2+}-channel antagonists and ethaverine were preincubated for 2 min when determining 70 mM K^+-induced $[Ca^{2+}]_i$. Net secretion is given as a percentage of the total [^{3}H]NE loaded by subtracting the basal level from the secretion caused by 70 mM K^+ in the presence of drugs, as described in Materials and Methods. ω-Conotoxin and ethaverine were tested at 10 μM and nifedipine was tested at 5 μM in all cases. $[Ca^{2+}]_i$ values are means ± SEM from five independent experiments, and net secretions are means ± SEM of two experiments done in triplicate.

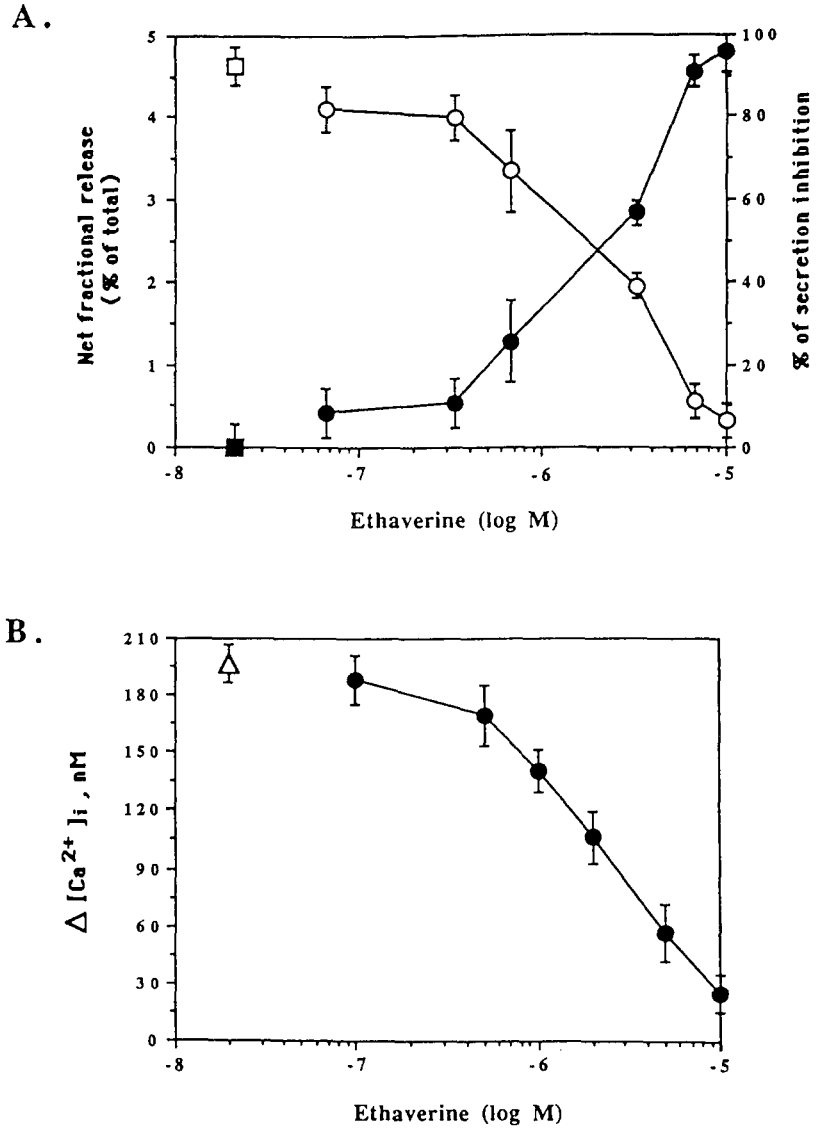

Fig. 2. Concentration-dependent effect of ethaverine on secretion and $[Ca^{2+}]_i$ caused by 70 mM K^+. (A) Cells were preincubated with each concentration of ethaverine for 10 min and subsequently were treated with 70 mM K^+ plus variable concentrations of ethaverine for 5 min. Inhibition of secretion compared with control (○) and percent inhibition (●) are shown. Open (□) and closed (■) squares denote stimulation with 70 mM K^+ alone. (B) Inhibition of the initial calcium peak was measured in cells pretreated with variable concentrations of ethaverine for 1 min. The open triangle (△) is the calcium peak caused by 70 mM K^+ alone. Each value was obtained from triplicate experiments and is shown as mean ± SEM.

The concentration-dependent inhibition by ethaverine of catecholamine secretion caused by 70 mM K^+ stimulation is shown in Fig. 2. The IC_{50} of catecholamine secretion inhibition by ethaverine was ~2 μM, and the maximal inhibition with 10 μM ethaverine showed only ~10% secretion compared with the 70 mM K^+-evoked secretion (Fig. 2A). The concentration-dependent inhibition of the $[Ca^{2+}]_i$ increase by ethaverine pretreatment can be seen in Fig. 2B. The maximal effect was seen with 10 μM ethaverine, which gave 88% inhibition compared with the untreated control; the IC_{50} was ~2 μM.

To demonstrate the effect of ethaverine on bradykinin-induced catecholamine secretion, cells were pretreated with 10 μM ethaverine for 10 min, and then 5 μM bradykinin plus 10 μM ethaverine was added (Fig. 3A). Ethaverine had little effect on the bradykinin-evoked secretion and rise in $[Ca^{2+}]_i$. Nifedipine, a voltage-sensitive Ca^{2+} channel blocker, was used to demonstrate that the bradykinin-induced catecholamine secretion was not caused by activation of the L-type channel.

Bradykinin-induced catecholamine secretion is known to occur by an increase in $[Ca^{2+}]_i$ due to Ca^{2+} release from internal stores and Ca^{2+} influx through the receptor-operated channel [5, 8]. The maximal effective concentration of bradykinin increased the internal Ca^{2+} level and induced secretion. Ethaverine or nifedipine

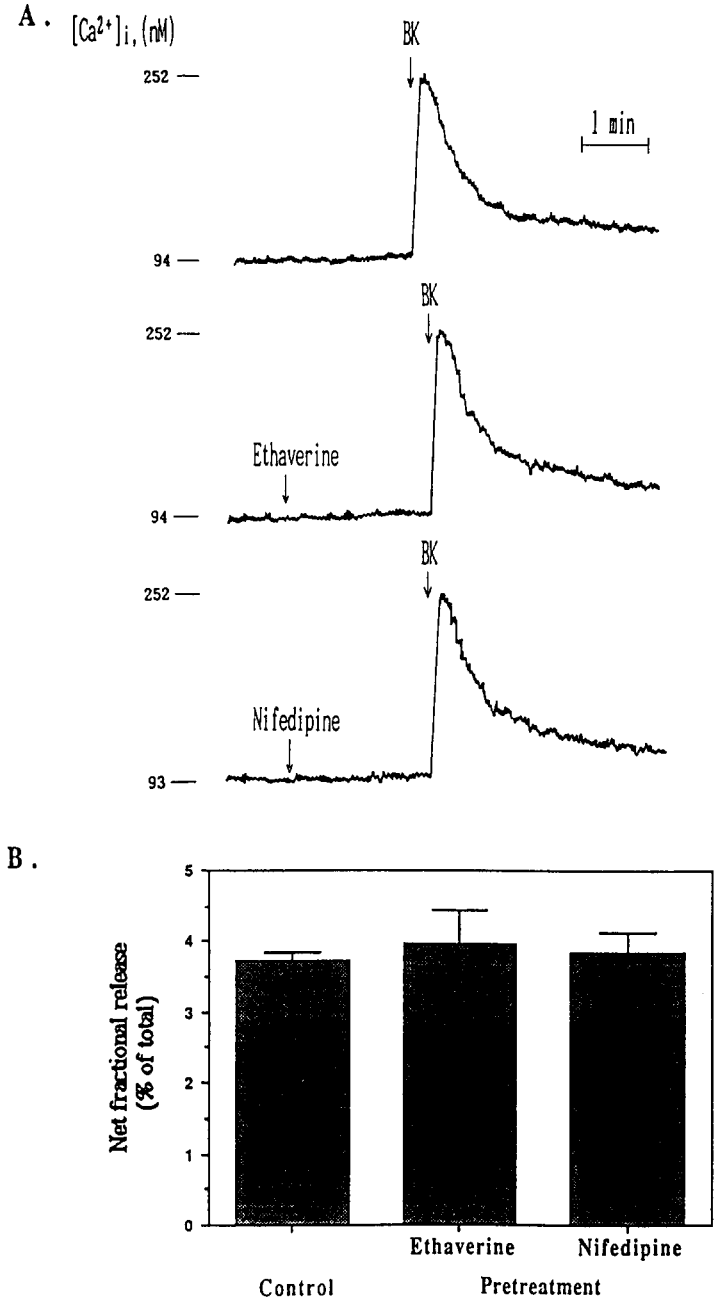

Fig. 3. Effects of ethaverine on bradykinin-induced increase in $[Ca^{2+}]_i$ (A) and catecholamine secretion (B). Ethaverine (10 μM) or nifedipine (5 μM) was added for 2 min followed by bradykinin (BK, 5 μM). Net secretions are means ± SEM of five experiments performed in triplicate.

pretreatment for 2 min did not affect the increase in $[Ca^{2+}]_i$ evoked by bradykinin (Fig. 3B). This implies that ethaverine has no effect on the ROCC opening and phosphoinositide turnover caused by bradykinin treatment.

To study the mechanism of action of ethaverine on depolarization-induced secretion and Ca^{2+} influx, we carried out a series of parallel experiments to determine both $[Ca^{2+}]_i$ and [^{3}H]NE release in the presence of ethaverine and VSCC antagonists when the cells were stimulated with 70 mM K^+. As shown in Table 1, the mean inhibition produced by a maximal concentration of ethaverine was ~90% compared with the untreated control, which is the same extent of inhibition caused by treatment with nifedipine, an L-type VSCC blocker. However, there was no further inhibition when ethaverine and nifedipine were added together. Treatment with a combination of ethaverine and ω-CTx, an N-type VSCC blocker, gave a significantly greater inhibition than ω-CTx or ethaverine alone. This inhibitory effect of ethaverine and ω-CTx mimics the additive effect of nifedipine plus ω-CTx. The above data clearly show that ethaverine blocked the L-type VSCC and also induced inhibition of catecholamine

secretion in PC12 cells. This is the first study to demonstrate that ethaverine, well known for its action as a muscle relaxant, also decreases neurotransmitter secretion in the neuroendocrine system. The above data suggest that L-type VSCCs present in PC12 cells have pharmacological properties similar to those of the cardiovascular system, even though the primary amino-acid sequences of two L-type channels are not the same [12]. This provides the possibility of studying the actions of drugs effective on the cardiovascular system by using PC12 cells. Table 1 also shows that there was detectable inhibition of $[Ca^{2+}]_i$ and secretion by ω-CTx, suggesting that opening of the N-type channels also contributes significantly to exocytosis in PC12 cells.

Ethaverine was reported to have an inhibitory effect on phosphodiesterase in smooth muscle cells [2]. However, it seemed that its inhibition of phosphodiesterase activity was not related to its inhibition of secretion in PC12 cells, as the increase in cyclic AMP level by forskolin resulted in enhancement of depolarization-dependent catecholamine secretion in these cells [13]. It has also been reported that the depolarization-induced increase in intracellular Ca^{2+} was not influenced by forskolin pretreatment of PC12 cells [14].

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