Effects of Ca^{2+} Blockers on the Various Types of Stimuli-Induced Acetylcholine Release from Guinea Pig Ileum Myenteric Plexus

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Abstract—In the present study, we investigated the effects of various Ca^{2+} blockers on the release of acetylcholine (ACh) induced by nicotine, electrical field stimulation (EFS) and high- K^+ . Cd^{2+} markedly depressed the ACh release due to these stimuli. Verapamil inhibited the nicotine-induced ACh release remarkably and the EFS- or the high- K^+ -induced ACh release to a lesser extent. Since the nicotine- and the EFS-induced ACh releases were inhibited by procaine, the local anesthetic property of verapamil likely contributes in part to the inhibition. Diltiazem abolished the nicotine-induced ACh release completely but did not affect the EFS-induced release and significantly increased the high- K^+ -induced ACh release. These results suggest the absolute requirement for extracellular Ca^{2+} in the release of ACh induced by nicotine as well as EFS and high- K^+ . In addition, these stimuli may open the same Ca^{2+} channel to evoke ACh release.

The importance of Ca^{2+} in transmitter release from central and peripheral nerve terminals has been established. This Ca^{2+} -dependency as well as tetrodotoxin sensitivity is thought to be one of the criteria for neuronal release of transmitters (1). As transmitter release is greatly attenuated or abolished in Ca^{2+} -free media, Ca^{2+} entry from the external media is essential for this process. However, the contribution of Ca^{2+} derived from intracellular store sites cannot be excluded completely since it has been reported that transmitters can be released in the absence of external Ca^{2+} under certain circumstances. For example, veratridine or ouabain caused acetylcholine (ACh) release from brain synaptosomes (2) and motor nerve terminals (3) and noradrenaline release from vas deferens (4) in such a condition. In these cases, an increase of intracellular Na^+ concentration evoked by these drugs may promote the release of Ca^{2+} from intracellular store sites, thereby causing transmitter release.

In the previous report, we showed that the relationship between ACh release and external Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) varied with the kind of stimuli applied to guinea pig ileum

myenteric plexus; electrical field stimulation (EFS)- or high- K^+ -induced ACh release increased with the increment of $[\text{Ca}^{2+}]_o$ but nicotine-induced release increased up to 0.45 mM $[\text{Ca}^{2+}]_o$ and then declined progressively as $[\text{Ca}^{2+}]_o$ was further raised (5), suggesting that the stabilizing effect of external Ca^{2+} might be responsible for the biphasic characteristics of the nicotine-induced ACh release. However, we can not exclude the possibility that the Ca^{2+} source necessary for the nicotine-induced ACh release might differ from that necessary for the EFS- or high- K^+ -induced one.

In the present study, therefore, the source of Ca^{2+} available for the various-stimuli-induced ACh release was investigated using several Ca^{2+} blockers from different chemical classes and with different target sites.

Materials and Methods

Male guinea pigs (350–600 g) were stunned and killed by exsanguination. A length of small intestine was isolated proximal to the last 10 cm, and longitudinal muscle strips were prepared as previously described (6). Each of three strips were tied with cotton

threads and mounted in an organ bath containing 3 ml Tyrode's solution of the following composition: 136.9 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl_2 , 1.05 mM MgCl_2 , 0.4 mM NaHCO_3 and 5.6 mM glucose. Physostigmine salicylate (5 μM) and choline chloride (1 μM) were added to the Tyrode's solution. The bath fluid was maintained at 37°C and bubbled with 5% CO_2 in O_2 .

Effects of drugs on ACh release: Experiments on ACh release were carried out by the previously described procedure (7). In brief, after the strips were equilibrated for 15 min, during which the bath fluid was perfused at a rate of 1–2 ml/min, the perfusion was stopped and the samples for estimating the released ACh were collected by replacing the bath fluid with fresh Tyrode's solution at 37°C every 1 min. After two samples for the spontaneous ACh release were collected, the strips were stimulated by nicotine, EFS or high- K^+ . Each type stimulation was performed twice with an interval of 19 min. When the effect of given drug on ACh release was studied, the first stimulation was carried out in the absence (S_1) and second stimulation in the presence (S_2) of the drug which was administered 17 min before S_2 . For the EFS-induced ACh release, the strips were stimulated with supramaximal voltage (100 V) and 0.5 msec duration at 10 Hz. Stimulation was performed in trains of 20 sec, and bath fluid was collected after a further 10 sec of rest. The high- K^+ (50 mM) Tyrode's solution was kept iso-osmotic by appropriate changes in the Na^+ concentration. The ACh release due to each stimulation was calculated by subtracting the output collected during the immediately preceding 1 min resting period from the total output during the stimulation period. Effects of drugs were quantified by the standard method of comparing the output of ACh induced by the first stimulation with that induced by the second stimulation ($S_2/S_1 \times 100$ (%)).

Assay of ACh: The collected samples were gently shaken with one tenth volume of Amberlite XAD-2 for 15 min and filtered to remove the added nicotine and endogenous prostaglandins, which may be present in the samples (8). Aliquots were then assayed against ACh on a longitudinal muscle strip as

previously described (7). When the samples containing a drug were assayed, the responses to the bath fluids were compared with the standard ACh solution to which the used drug was added to give the same final concentration. The active substance in the samples was identified as ACh by demonstrating that it was destroyed by boiling in alkali and that its action was antagonized by atropine and potentiated by physostigmine.

Drugs: The following materials were obtained from commercial sources: acetylcholine chloride (Sigma), Amberlite XAD-2 (Rohm & Hass), choline chloride (Wako), cadmium chloride (Wako), dantrolene sodium (Yamanouchi), sodium nitroprusside (Wako), morphine hydrochloride (Takeda), physostigmine salicylate (Sigma), procaine hydrochloride (Sigma) and verapamil hydrochloride (Eisai). D600 hydrochloride and diltiazem hydrochloride were the generous gifts of the Department of Veterinary Pharmacology at the University of Tokyo and Tanabe Pharmaceutical Co., respectively. All other drugs were of analytical grade.

Results

In a series of control experiments, no significant difference in ACh release was observed between the first (S_1) and the second (S_2) stimulation. The values of the relative ACh release ($S_2/S_1 \times 100$ (%)) were 104.1 ± 5.1 , 105.7 ± 5.5 and 112.0 ± 3.5 for nicotine-, EFS- and high- K^+ -induced ACh release, respectively. The amounts of ACh released by each stimulus during S_1 were (ng/g tissue/min): 167.2 ± 39.5 (nicotine, $N=6$), 185.5 ± 12.7 (EFS, $N=5$) and 150.8 ± 11.6 (high- K^+ , $N=4$).

Figure 1 illustrates the effect of Cd^{2+} on nicotine-, EFS- and high- K^+ -induced ACh release. As can be seen in the figure, Cd^{2+} (50 μM) markedly reduced all the stimuli-induced ACh releases to the same extent, i.e., by about 90%, irrespective of the types of stimuli.

Figure 2 shows the effects of verapamil and D600 on the ACh release. Nicotine-induced ACh release was markedly inhibited by verapamil (10 μM). On the other hand, the EFS- or the high- K^+ -induced release was suppressed by verapamil at the same concen-

Fig. 1. Effect of Cd²⁺ on various stimuli-induced ACh release from guinea pig ileum myenteric plexus. In each experiment, stimulations were performed twice in the absence (S₁) and the presence (S₂) of 50 μ M Cd²⁺. Cd²⁺ was added to the bath fluid 17 min before S₂. Each column represents the mean $\pm$ S.E. of 4–6 experiments. Significant differences from the control value were determined by Student's *t*-test (****P* < 0.001). EFS: electrical field stimulation.

Fig. 2. Effects of verapamil (10 μ M), D600 (10 μ M) and diltiazem (10 μ M) on various stimuli-induced ACh releases from guinea pig ileum myenteric plexus. EFS: electrical field stimulation, C: control, V: verapamil, D: D600, Dil: diltiazem. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. ND: not determined.

tration, but the extent of the inhibition was less pronounced than that of the nicotine-induced release. A similar inhibition of ACh release was found with the another phenylalkylamine Ca²⁺ blocker, D600 (Fig. 2). Nicotine-induced ACh release was completely

suppressed by D600 (10 μ M).

At concentrations above 10 μ M, phenylalkylamines such as verapamil and D600 have a pronounced membrane stabilizing effect related to the inhibition of Na⁺ channels in neurons (9). In the following experiment,

Fig. 3. Effect of procaine (0.1 mM) on various stimuli-induced ACh release from guinea pig ileum myenteric plexus. EFS: electrical field stimulation. * $P < 0.05$, *** $P < 0.001$.

therefore, the effect of the local anesthetic procaine on the nicotine-, EFS- and high- K^+ -induced ACh release was investigated (Fig. 3). Procaine (0.1 mM) failed to affect the high- K^+ -induced ACh release. In contrast, both the nicotine- and the EFS-induced ACh releases were inhibited by procaine, but the extent of the inhibition in the nicotine-induced release was greater than that in the EFS-induced one (Fig. 3). These results indicate that nicotine- and EFS-induced ACh releases are more susceptible to the local anesthetic as compared with high- K^+ -induced release. Furthermore, the inhibitory effect of phenylalkylamines on the nicotine- and the EFS-induced ACh releases appear to be partly dependent on their local anesthetic properties.

Diltiazem (10 μ M) abolished the nicotine-induced ACh release almost completely (Fig. 2). However, the EFS-induced ACh release was unaffected by this drug. Unexpectedly, diltiazem strongly increased the high- K^+ -induced release to about 180% of the control value.

Then, the effect of dantrolene, an inhibitor of Ca^{2+} release from the intracellular store (10), on the nicotine-induced ACh release was assessed. Nicotine-induced ACh release was unchanged by dantrolene at 20 μ M (data not shown). This result suggests that the ACh

release depends on external Ca^{2+} but not on intracellular Ca^{2+} . Nitroprusside is thought to block predominantly the receptor-operated Ca^{2+} channels but has no effect on the voltage-dependent Ca^{2+} channels (11). Therefore, in order to test whether the receptor-operated Ca^{2+} channels might be involved in the nicotine-induced ACh release, the effect of nitroprusside was examined. The nicotine-induced ACh release was found to be virtually insensitive to nitroprusside at 1 μ M (data not shown).

Discussion

The present results showed that Cd^{2+} , verapamil, D600 and diltiazem, all of which have been shown to inhibit the voltage-dependent Ca^{2+} channels selectively, markedly suppressed the release of ACh induced by nicotine, whereas dantrolene and nitroprusside did not modify the ACh release. These results suggest that nicotine stimulates ACh release by promoting the entry of Ca^{2+} through the voltage-dependent Ca^{2+} channels but not by releasing intracellularly stored Ca^{2+} nor by activating the receptor-operated Ca^{2+} channels.

Verapamil and D600 also caused qualitatively similar inhibitions of the high- K^+ - and the EFS-induced ACh release. Therefore, these results suggest that the voltage-de-

pendent Ca²⁺ channels may also be utilized for the EFS- or high-K⁺-induced ACh release.

On the other hand, the sensitivity to diltiazem varied with the stimuli applied to release ACh. The failure of diltiazem to inhibit the EFS-induced ACh release is consistent with the observation of Kaplita and Trigg (12) that diltiazem did not block the electrically stimulated release of ACh from guinea pig ileum myenteric plexus. The unexpected finding was that the high-K⁺-induced ACh release was not reduced but rather enhanced by diltiazem. Although the reason is unclear at present, a similar increasing effect of diltiazem on transmitter release has been recently demonstrated in the isolated rat kidney, in which diltiazem reduced noradrenaline release induced by electrical stimulation and veratridine, but enhanced that caused by high-K⁺ (13).

The local anesthetic properties of phenylalkylamines are related to the interference with Na⁺ entry into neurons (9). Since the high-K⁺-induced ACh release is unaffected by tetrodotoxin (14) and procaine (present study), it is unlikely that the inhibition of the high-K⁺-induced ACh release by verapamil and D600 is due to the local anesthetic properties of these drugs; rather, it appears to derive from the inhibition of transmembrane Ca²⁺ entry. On the contrary, EFS- and nicotine-induced ACh releases were sensitive to tetrodotoxin (14). Therefore, it seems likely that the inhibitory effects of verapamil and D600 on the ACh release are thought to be partly due to the blockade of Na⁺ channels. This possibility is supported by the results that procaine had an inhibitory effect on the nicotine- and the EFS-induced ACh release. It is well-known that procaine inhibits Ca²⁺-induced Ca²⁺ release from the sarcoplasmic reticulum in muscle cells (15). However, dantrolene, which prevents the release of Ca²⁺ from intracellular store sites (10), had no effect on the nicotine-induced ACh release. Thus, the inhibitory effect of procaine on the release appears to depend not on a blockade of Ca²⁺ release from intracellular sites, but depend on its local anesthetic property.

In summary, it is concluded that extracellular Ca²⁺ is essential for the various stimuli-induced ACh releases from guinea pig ileum

myenteric plexus, and the contribution of Ca²⁺ supplied by intracellular store sites play a minor role in the release. Moreover, Ca²⁺ necessary for the ACh release induced by nicotine as well as EFS- and high-K⁺ appears to be transported via the voltage-dependent Ca²⁺ channels across the neuronal cell membranes.

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