Ca\(^{2+}\) Inactivation of Voltage-Dependent Na\(^{+}\) Channels in Cultured Bovine Adrenal Chromaffin Cells: Further Studies on Inhibition of Veratridine-Induced Catecholamine Secretion by External Ca\(^{2+}\)

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ABSTRACT—The stimulatory actions of veratridine (VTD) on catecholamine secretion and Na\(^{+}\) influx in cultured bovine adrenal chromaffin cells were studied in the presence of high concentrations of Ca\(^{2+}\) in the incubation mixture. Catecholamine secretion evoked by VTD was reduced by elevating the external Ca\(^{2+}\) concentration to higher than 2 mM. Under the same conditions, VTD-stimulated \(^{22}\)Na\(^{+}\) uptake into the cells was also reduced by elevating the extracellular Ca\(^{2+}\) concentration. In contrast, the secretory action of VTD was not significantly suppressed by preloading Ca\(^{2+}\) to the cells. Furthermore, the effects of extracellular Ca\(^{2+}\) on the sensitivities of the cells to VTD and tetrodotoxin (TTX) were examined, and neither their sensitivities to VTD nor those to TTX were shown to be significantly altered by elevating the Ca\(^{2+}\) concentration in the incubation mixture. These results seem to indicate that the elevation of extracellular Ca\(^{2+}\) concentration may cause the inhibition of VTD-induced catecholamine secretion as a consequence of the inactivation of voltage-dependent Na\(^{+}\) channels, and suggest that Ca\(^{2+}\) may directly act on the cell surface, and the site of Ca\(^{2+}\) action is presumably distinct from the sites of both VTD and TTX actions in the plasma membranes of adrenal chromaffin cells.

Keywords: Veratridine, Extracellular Ca\(^{2+}\), Na\(^{+}\) channel (voltage-dependent), Catecholamine secretion, Adrenal chromaffin cell

Exocytosis is commonly appreciated as the mechanism for the release of neurotransmitters and hormones in various cells and tissues, and it has been established that a series of intracellular events in exocytosis is initiated by a rise in the intracellular concentration of free Ca\(^{2+}\), resulting from an increase in the influx of extracellular Ca\(^{2+}\) into the cell. In the adrenal chromaffin cell, the influx of extracellular Ca\(^{2+}\) has been reported to be mediated by two different-types of Ca\(^{2+}\) channels, such as receptor-operated and voltage-dependent Ca\(^{2+}\) channels (1–3). The stimulation of acetylcholine receptors on the cell surface has been well established to stimulate the influx of both Na\(^{+}\) and Ca\(^{2+}\) through receptor-linked cation channels. On the other hand, various neurotoxins, such as veratridine (VTD), aconitine, batrachotoxin and scorpion venom, have been shown to stimulate Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels as a result of the stimulation of tetrodotoxin (TTX)-sensitive Na\(^{+}\) channels (2, 4). Thus, it seems reasonable to consider that Na\(^{+}\) channels may be functionally coupled with Ca\(^{2+}\) channels in the plasma membranes and probably play an important role in the regulation of stimulus-secretion coupling.

According to the general concept that an increase in the intracellular Ca\(^{2+}\) concentration may be responsible for the activation of the intracellular exocytotic process, it seems quite reasonable to consider that the secretory response of the adrenal chromaffin cell may directly be influenced by the mechanism(s) modulating the Ca\(^{2+}\) influx into the cell. In fact, the rate of catecholamine secretion has previously been reported to be declining during prolonged stimulation of the cell with cholinergic agonists and depolarizing agents, and this decline has also been proposed to be attributable to a decrease in the influx of Ca\(^{2+}\) into the cell (2, 3, 5–7), resulting from the inactivation of Ca\(^{2+}\) channels by Ca\(^{2+}\) (3, 8, 9). Further-
more, since Na⁺ channels have been well known to be coupled with Ca²⁺ channels, it also seems possible to assume that Na⁺ channels as well as Ca²⁺ channels may be inactivated by Ca²⁺ itself. The earlier studies have shown that the electrical properties of excitable membranes can be modulated by elevating the external Ca²⁺ concentration, suggesting the possibility that the permeability of Na⁺ through the plasma membranes is presumably regulated by Ca²⁺ in squid axons (10) and mammalian skeletal muscles (11). On the other hand, the stimulatory action of VTD on catecholamine secretion has previously been reported to be suppressed by elevating the external Ca²⁺ concentration in cultured bovine adrenal chromaffin cells (12). These findings suggest that Na⁺ channels as well as Ca²⁺ channels may be inactivated by Ca²⁺ itself in excitable cells and tissues, but little is known about the detailed mechanism underlying the inactivation of Na⁺ channels by external Ca²⁺ in the plasma membranes of excitable cells.

To characterize the pharmacological effect of Ca²⁺ on voltage-dependent Na⁺ channels in the adrenal chromaffin cell, the stimulatory actions of VTD on both catecholamine secretion and Na⁺ influx were examined in the presence of relatively high concentrations of extracellular Ca²⁺, and confirmed that catecholamine secretion evoked by VTD was reduced by elevating the Ca²⁺ concentration in the incubation mixture. This reduction was also shown to be accompanied by the inhibition of Na⁺ influx into the cells. Further studies on the mechanism of this Ca²⁺ inhibition suggested the possibility that the site(s) of Ca²⁺ action might be different from the sites of both VTD and TTX actions in the plasma membranes of adrenal chromaffin cells.

MATERIALS AND METHODS

Cell preparation and culture

Chromaffin cells were enzymatically prepared from fresh bovine adrenal medulla according to the previously reported method (13). Cells were plated on a 24-well plastic cluster plate at a density of 5 x 10⁵ cells/well and maintained as a monolayer culture in 1.5 ml of Eagle’s minimum essential medium containing 5% heat-inactivated newborn calf serum, 2 mM glutamine, 100 units/ml of penicillin, 100 μg/ml streptomycin, 50 μg/ml of gentamicin, 2 μg/ml of fungizone and 10 μM cytosine arabinoside. For the experiments, cells cultured for 3 or 4 days were usually employed.

Determination of catecholamine secretion

Cultured cells were washed with 1.0 ml of balanced salt solution consisting of 135 mM NaCl, 5.6 mM KCl, 1.2 mM MgSO₄, 2 mM CaCl₂, 10 mM glucose and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, and then stimulated by secretagogues at 37°C for 10 min in 250 μl of balanced salt solution containing different concentrations of CaCl₂. At the end of the incubation period, the medium covering the cells was withdrawn, and the cells were lysed by adding 250 μl of 10% acetic acid followed by a freeze-thaw cycle. Both the medium and the lysates were centrifuged at the maximum speed for 2 min in an Eppendorf microcentrifuge, and the amounts of catecholamines in the supernatants were then determined by the previously reported method (14). Catecholamine secretion was expressed as the percentage of total cellular content secreted into the medium during the incubation period.

Measurement of ²²Na⁺ uptake

Cells were washed with balanced salt solution as described above and then incubated with VTD at 37°C for 10 min in 250 μl of balanced salt solution containing various concentrations of CaCl₂ with ²²NaCl (0.5 μCi/well). At the end of the incubation period, the medium was removed by aspiration, and the cells were washed 4 times with 1.0 ml of ice-cold balanced salt solution. The cells were solubilized by adding 200 μl of 1% Triton X-100, and the wells were washed twice with 200 μl of distilled water. The lysates and the washings were combined together, and the radioactivities in these solutions were determined by a liquid scintillation spectrometer. The uptake of Na⁺ into the cells was calculated on the basis of the initial specific activity of ²²NaCl in the incubation mixture and expressed as nmoles Na⁺/well.

The concentration of NaCl in high K⁺ medium (56 mM KCl) was reduced from 135 mM to 84.6 mM to keep the solution isotonic. Standard deviation (S.D.) of the difference between two groups was calculated as 
\[(\text{S.D.}_1^2 + \text{S.D.}_2^2)^{1/2}\], and Student’s t-test was employed to determine the statistical significance.

Chemicals

²²NaCl was purchased from New England Nuclear Corp (Boston, MA, USA). Carbamylcholine, VTD and A23187 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). TTX was from Sankyo Co., Ltd. (Tokyo). Other chemicals were of commercially available reagent grades.

RESULTS

Effects of external Ca²⁺ concentration on catecholamine secretion

To elucidate the influence of external Ca²⁺ concentration on the secretory responses of adrenal chromaffin cells, the stimulatory actions of secretagogues on catechol-
amine secretion from these cells were examined in the presence of relatively high concentrations of Ca\(^{2+}\) in the incubation mixture. As shown in Fig. 1, catecholamine secretion evoked by VTD was markedly reduced by elevating the external Ca\(^{2+}\) concentration from 2 mM to 20 mM. In comparison to the maximum secretion observed at 2 mM Ca\(^{2+}\), VTD-induced catecholamine secretion was significantly reduced by the presence of 4 mM Ca\(^{2+}\), and an approximately 50% inhibition of the secretory response was obtained at 8 mM. In contrast, catecholamine secretion evoked by either carbamylcholine or high K\(^+\) was substantially enhanced by elevating the external Ca\(^{2+}\) concentration to a level as high as that producing the marked reduction of VTD-induced secretion (Fig. 2). These results seemed to indicate that the VTD-stimulated step in the secretory process might be specifically influenced by elevating the extracellular Ca\(^{2+}\) concentration, thus suggesting the possibility that VTD-stimulated, voltage-dependent Na\(^+\) influx into the cells might be tightly connected with the reduction of VTD-induced catecholamine secretion caused by high concentrations of extracellular Ca\(^{2+}\).

**Effect of external Ca\(^{2+}\) concentration on Na\(^+\) influx**

To solve the question of whether the reduction of VTD-induced catecholamine secretion caused by high concentrations of external Ca\(^{2+}\) might be attributed to an alteration in the activity of VTD-stimulated, voltage-dependent Na\(^+\) channels, the stimulatory action of VTD on \(^{22}\)Na\(^+\) uptake into the cells was examined in the presence of high concentrations of Ca\(^{2+}\) in the incubation mixture. As shown in Fig. 3, VTD-stimulated \(^{22}\)Na\(^+\) uptake into chromaffin cells was markedly reduced by elevating the extracellular Ca\(^{2+}\) concentration, and this reduction of \(^{22}\)Na\(^+\) uptake was more pronouncedly observed in comparison with that of the VTD-induced secretion. These results seemed to provide evidence supporting the possibility that the reduction of VTD-induced catecholamine secretion

![Graph](image1.png)

**Fig. 1.** Effect of extracellular Ca\(^{2+}\) concentration on catecholamine secretion evoked by VTD from cultured bovine adrenal chromaffin cells. Cells were stimulated by 20 \(\mu\)M VTD at 37°C for 10 min in the presence of high concentrations of Ca\(^{2+}\), and catecholamine secretion was then determined as described in the text. Values are the mean±S.D. (n=3).

![Graph](image2.png)

**Fig. 2.** Effect of extracellular Ca\(^{2+}\) concentration on catecholamine secretion evoked by various secretagogues from cultured bovine adrenal chromaffin cells. Cells were incubated with 20 \(\mu\)M VTD, 100 \(\mu\)M carbamylcholine (CCh) or high K\(^+\) at 37°C for 10 min in the presence of high concentrations of Ca\(^{2+}\), and catecholamine secretion was determined as described in the text. Values are the mean±S.D. (n=3).

![Graph](image3.png)

**Fig. 3.** Effect of extracellular Ca\(^{2+}\) concentration on catecholamine secretion and \(^{22}\)Na\(^+\) influx stimulated by VTD in cultured bovine adrenal chromaffin cells. Cells were stimulated by 20 \(\mu\)M VTD at 37°C for 10 min in the mixture containing high concentrations of Ca\(^{2+}\) and \(^{22}\)NaCl, and catecholamine secretion (open column) and \(^{22}\)Na\(^+\) uptake (hatched column) were determined as described in the text. Values are the mean±S.D. (n=3).
caused by elevating the external Ca\(^{2+}\) concentration might be attributed to the reduction of Na\(^+\) influx through voltage-dependent Na\(^+\) channels.

**Properties of Ca\(^{2+}\) action on voltage-dependent Na\(^+\) channels**

To further elucidate the properties of Ca\(^{2+}\) action on VTD-induced catecholamine secretion, the cells were first incubated with the Ca\(^{2+}\)-ionophore A23187 in the medium containing high concentration of Ca\(^{2+}\) (8 mM), and the secretory actions of various secretagogues on the Ca\(^{2+}\)-preloaded cells were examined in the presence of a normal concentration of Ca\(^{2+}\) (2 mM). As shown in Fig. 4, the pretreatment of the cells with A23187 caused the enhancement rather than the reduction of catecholamine secretion evoked by VTD as well as high K\(^+\), while the carbamylcholine-induced secretion was slightly reduced by pretreatment of the cells with A23187 under the same conditions. These results seemed to indicate that the intracellular Ca\(^{2+}\) was probably not related directly to the reduction of VTD-induced catecholamine secretion caused by elevating the Ca\(^{2+}\) concentration in the incubation mixture, thus suggesting strongly the possibility that the elevation of extracellular Ca\(^{2+}\) concentration might be able to cause the suppression of the secretory responses to VTD as a consequence of direct action of Ca\(^{2+}\) on the cell surface.

To characterize the site of Ca\(^{2+}\) action on the cell surface, the effect of extracellular Ca\(^{2+}\) on the sensitivities of chromaffin cells to VTD were investigated by determining the secretory action of VTD in the presence of normal and high concentrations of Ca\(^{2+}\). As shown in Fig. 5, catecholamine secretion was markedly enhanced by VTD in the presence of 2 mM Ca\(^{2+}\), and this stimulatory action was observed in a concentration-dependent manner. In contrast, the VTD action on the secretion was markedly suppressed by the presence of 8 mM Ca\(^{2+}\), and this suppression was still observed when the cells were stimulated by high concentrations of VTD (50 and 100 \(\mu\)M). If the Lineweaver-Burk analysis was forcibly applied to these data obtained here, the apparent \(K_m\) value of the cells for VTD and the \(V_{\text{max}}\) of catecholamine secretion were estimated as 57.5 \(\mu\)M and 49.8% of the cellular catecholamines released/10 min in the presence of 2 mM Ca\(^{2+}\), and 50.9 \(\mu\)M and 20.1% of the cellular content released/10 min in the presence of 8 mM Ca\(^{2+}\). Thus, the elevation of extracellular Ca\(^{2+}\) concentration was thought to cause a marked decrease in the \(V_{\text{max}}\) without any significant alteration in the apparent \(K_m\) value for VTD. It therefore seemed conceivable that the sensitivities of the cells to VTD might not be altered by elevating the Ca\(^{2+}\) concentration in the incubation mixture.

The inhibitory action of TTX, an inhibitor of voltage-dependent Na\(^+\) channels, on VTD-induced catecholamine secretion was furthermore examined in the incubation mixture containing 2 mM or 8 mM Ca\(^{2+}\). As shown in Fig. 6, the inhibitory action of TTX on the VTD-induced secretion was observed in a concentration-dependent manner. The maximum inhibition of the secretory
action of VTD was observed at $3 \times 10^{-7}$ M TTX, and the 50% inhibition was obtained by approximately $10^{-8}$ M in the presence of 2 mM Ca$^{2+}$. On the other hand, the concentrations of TTX required to produce both the maximum and the 50% inhibition of VTD-induced catecholamine secretion in the presence of 8 mM Ca$^{2+}$ were shown to be nearly identical to those obtained in the presence of 2 mM Ca$^{2+}$. These results were considered to indicate that the inhibitory action of TTX on VTD-induced catecholamine secretion might not be significantly altered by the Ca$^{2+}$ concentration in the incubation mixture.

**DISCUSSION**

The present study indicated that the stimulatory action of VTD on catecholamine secretion from cultured bovine adrenal chromaffin cells was markedly suppressed by elevating the extracellular concentration of Ca$^{2+}$ from 2 mM to 8 mM (Fig. 1), thus confirming the previous finding that VTD-induced catecholamine secretion is reduced by high concentrations of extracellular Ca$^{2+}$ (12). However, the mechanism underlying this Ca$^{2+}$ action on VTD-induced catecholamine secretion has not yet been elucidated. On the other hand, the secretory actions of carbamylcholine and high K$^+$ were shown to be enhanced rather than suppressed by elevating the Ca$^{2+}$ concentration to the level causing the suppression of VTD action (Fig. 2). Both carbamylcholine and high K$^+$ are well known to stimulate the Ca$^{2+}$ influx into the cells, and their stimulatory actions are also known to be mediated by mechanisms different from that underlying the stimulatory action of VTD on the Ca$^{2+}$ influx. Namely, carbamylcholine can stimulate the influx of Ca$^{2+}$ through acetylcholine receptor-mediated Ca$^{2+}$ channels, and high K$^+$ causes the stimulation of voltage-dependent Ca$^{2+}$ influx without accompanying the Na$^+$ influx into the cells. In view of these facts, it seems unlikely that the suppression of VTD action observed here may simply be due to the direct inactivation of Ca$^{2+}$ channels in the plasma membranes of adrenal chromaffin cells.

In view of the previous findings that VTD can stimulate voltage-dependent Na$^+$ channels linked with voltage-dependent Ca$^{2+}$ channels, leading to the stimulation of catecholamine secretion from adrenal medullary cells and tissues (1, 2, 4, 15, 16), it seemed reasonable to assume that the reduction of VTD-induced catecholamine secretion by elevating the external Ca$^{2+}$ concentration is probably mediated by the inhibitory action of Ca$^{2+}$ on VTD-stimulated, voltage-dependent Na$^+$ channels. In fact, the stimulatory action of VTD on Na$^+$ influx into the cells as well as that on catecholamine secretion was shown to be markedly suppressed by elevating the Ca$^{2+}$ concentration in the reaction mixture (Fig. 3). It therefore seems reasonable to conclude that the elevation of external Ca$^{2+}$ concentration may cause the reduction of VTD-induced catecholamine secretion as a consequence of inhibition of Na$^+$ influx through voltage-dependent Na$^+$ channels in cultured bovine adrenal chromaffin cells. Furthermore, the suppression of VTD-induced catecholamine secretion was less pronouncedly observed as compared with that of VTD-stimulated Na$^+$ influx under the experimental conditions employed here. In view of the fact that catecholamine secretion is supported by the intracellular Ca$^{2+}$ concentration which is determined by different mechanisms, such as the influx of extracellular Ca$^{2+}$ through different-type Ca$^{2+}$ channels and the mobilization of intracellular Ca$^{2+}$, it also seems possible to consider that the Ca$^{2+}$ influx coupled with voltage-dependent Na$^+$ influx may partly contribute to the secretory response of the chromaffin cell.

The stimulatory action of VTD on catecholamine secretion was shown to be suppressed by elevating the external Ca$^{2+}$ concentration, and this suppression of the VTD action has also been suggested to be the result of the inactivation of voltage-dependent Na$^+$ channels by Ca$^{2+}$ itself. However, the question of whether the intracellular Ca$^{2+}$ level might be responsible for the inactivation of voltage-dependent Na$^+$ channels still remained to be elucidated.
To address this critical question, the secretory action of VTD was examined using Ca\(^{2+}\)-preloaded cells, which were obtained by preincubating the cells with the Ca\(^{2+}\)-ionophore A23187 in the presence of a high concentration of extracellular Ca\(^{2+}\). Consequently, the enhancement rather than the suppression of the secretory action of VTD was observed in these Ca\(^{2+}\)-preloaded cells, although the Ca\(^{2+}\) level within these cells was presumed to be higher than the physiological level of Ca\(^{2+}\) concentration in the adrenal chromaffin cell (Fig. 4). It therefore seems possible to exclude the possibility that the elevation of extracellular Ca\(^{2+}\) concentration may cause the inactivation of voltage-dependent Na\(^{+}\) channels presumably through an alteration in the intracellular Ca\(^{2+}\) level. Furthermore, the present study indicated that the secretory action of carbamylcholine was not significantly suppressed by preincubation of the cells with the Ca\(^{2+}\)-ionophore A23187 in the presence of a high concentration of Ca\(^{2+}\), thus suggesting that the function of the stimulus-secretion coupling system is still kept intact during the Ca\(^{2+}\) preloading. In contrast, the secretory action of high K\(^{+}\) was shown to be enhanced by pretreatment of the cells with A23187 under the same conditions. In view of the previous finding that catecholamine secretion evoked by high K\(^{+}\) is markedly reduced by pretreatment of the cells with A23187 (9), the unexpected result described here seems to raise another critical question of whether the intracellular Ca\(^{2+}\) level in the A23187-pretreated cells may be maintained as high as that required to cause the inactivation of voltage-dependent cation channels.

Voltage-dependent Na\(^{+}\) channels in the excitable membranes have been well established to be stimulated by VTD and inhibited by TTX in various cells and tissues, and the binding site of VTD has been suggested to be distinct from that of TTX on the Na\(^{+}\) channel (17). Although the present results suggested that voltage-dependent, TTX-sensitive Na\(^{+}\) channels might be inactivated by high concentrations of extracellular Ca\(^{2+}\), the question of whether the site affected by Ca\(^{2+}\) might be the same as or different from the binding site of VTD or that of TTX still remained to be solved. Further studies showed that the elevation of extracellular Ca\(^{2+}\) concentration failed to cause any notable alteration in the potencies of both the secretory action of VTD (Fig. 5) and the inhibitory action of TTX on the VTD-induced secretion (Fig. 6), thus suggesting the possibility that the sensitivities of Na\(^{+}\) channels to both VTD and TTX were probably not affected by elevating the external Ca\(^{2+}\) concentration. It therefore seems possible to consider that the site involved in the inactivation of VTD-stimulated Na\(^{+}\) channels by high concentration of extracellular Ca\(^{2+}\) may be different from both the binding site of VTD and that of TTX in the plasma membranes of adrenal chromaffin cells. As one of possible mechanisms, it seems conceivable that external Ca\(^{2+}\) may cause a non-specific stabilizing action on the plasma membranes, thus resulting in an alteration in the membrane functions. Further studies on the characteristics of Na\(^{+}\) channels are still required to elucidate a possible mechanism(s) for the Ca\(^{2+}\) inactivation of Na\(^{+}\) channels as well as a possible site(s) of Ca\(^{2+}\) action in the adrenal chromaffin cell.

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