Pharmacological Study on Ba$^{2+}$-Stimulated Catecholamine Secretion from Cultured Bovine Adrenal Chromaffin Cells: Possible Relation of Ba$^{2+}$ Action to Ca$^{2+}$-Activated Secretory Mechanism

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ABSTRACT—The stimulatory action of Ba$^{2+}$ on catecholamine secretion from cultured bovine adrenal chromaffin cells was studied to elucidate a possible relationship between Ba$^{2+}$ action and the Ca$^{2+}$-mediated secretory mechanism. Catecholamine secretion was dramatically stimulated by Ba$^{2+}$ in the absence of external Ca$^{2+}$, and this stimulatory action was observed in a concentration-dependent manner. Ba$^{2+}$ evoked the concomitant release of dopamine $\beta$-hydroxylase in a similar manner to the Ca$^{2+}$-dependent secretion. The stimulation of catecholamine secretion by low concentrations of Ba$^{2+}$ was markedly inhibited by protein kinase inhibitors, polymyxin B and trifluoperazine (TFP). The inhibitory action of polymyxin B, but not that of TFP, on the Ba$^{2+}$ action was attenuated by elevating the concentration of Ba$^{2+}$ in the incubation mixture. The stimulatory action of Ba$^{2+}$ was enhanced by a protein kinase C activator, 12-O-tetradecanoylphorbol 13-acetate (TPA). In contrast to the acute effect of TPA, chronic exposure of chromaffin cells to high concentration of TPA reduced catecholamine secretion stimulated by Ba$^{2+}$ as well as high K$^+$ and carbamylcholine. These findings suggest the possibility that Ba$^{2+}$ may activate Ca$^{2+}$-mediated secretory processes presumably through its action on protein kinase C, thus resulting in the stimulation of catecholamine secretion from bovine adrenal chromaffin cells.

The properties of catecholamine secretion from the adrenal medulla have already been exhaustively studied to elucidate the mechanism of exocytosis, and considerable evidence for an essential role of Ca$^{2+}$ in the exocytotic secretory process has been obtained (1, 2). Recently, as an approach to the intracellular processes, selective permeabilization of the plasma membranes by either electrical or chemical manipulations has been introduced to gain direct access to the cell interior, and a crucial role of Ca$^{2+}$ as an activator for the intracellular process of exocytosis has been established (3-8). Furthermore, the detailed mechanism of intracellular Ca$^{2+}$ signal transduction has been investigated using intact cells and these permeabilized chromaffin cells, and workers have proposed the possibility that the stimulatory action of Ca$^{2+}$ on the intracellular secretory mechanism may be mediated by phosphorylation of several proteins catalyzed by a Ca$^{2+}$/phospholipid-activated protein kinase, protein kinase C (9-14).

In contrast to the secretory actions of depolarizing agents or cholinergic agonists, the stimulatory action of Ba$^{2+}$ on catecholamine secretion...
secretion has been reported to be independent of the influx of extracellular Ca\(^{2+}\) into the cells, and therefore considered to be mediated by an elevation of the free Ca\(^{2+}\) concentration within the cells, resulting from the displacement of Ca\(^{2+}\) with Ba\(^{2+}\) from the intracellular store sites (15). On the other hand, the stimulation of catecholamine secretion by Ba\(^{2+}\) has also been shown to be similar to that stimulated by nicotine, veratridine, or ionomycin (16). In view of these observations, Ba\(^{2+}\) has therefore been considered to stimulate the phosphorylation of cell proteins presumably through the mechanism activated by intracellular Ca\(^{2+}\), thus resulting in the stimulation of catecholamine secretion from adrenal chromaffin cells.

Recently, Ba\(^{2+}\) has been reported to induce the release of ATP, which is accumulated within chromaffin granules and secreted concomitantly with catecholamines in an exocytotic manner from adrenal chromaffin cells. Furthermore, this ATP release has been shown to be mediated by the Ba\(^{2+}\) entry into these cells, presumably through both voltage- and receptor-operated Ca\(^{2+}\) channels. These findings have therefore been considered to support the hypothesis that the intracellular events leading to the Ba\(^{2+}\)-stimulated secretion may coincide at least in part with those leading to the Ca\(^{2+}\)-mediated exocytotic secretion (17). More recently, Ba\(^{2+}\) has been reported to enter chromaffin cells via voltage-operated Ca\(^{2+}\) channels, and furthermore shown to stimulate catecholamine secretion, presumably through acting on intracellular sites different from those of Ca\(^{2+}\) action (18). Thus, it has not yet been completely elucidated whether the intracellular mechanisms and sites of Ba\(^{2+}\) action may be the same as or distinct from those of Ca\(^{2+}\) action. To approach this issue, we examined the effects of various agents modifying the activities of Ca\(^{2+}\)-activated protein kinases on the Ba\(^{2+}\) action in cultured bovine adrenal chromaffin cells.

**MATERIALS AND METHODS**

**Cell preparation and culture**

Chromaffin cells were enzymatically prepared from fresh bovine adrenal medulla according to the method reported previously (19). Isolated cells were plated on 24-well plastic cluster plates at a density of \(5 \times 10^5\) cells/well; and they were maintained for 3 or 4 days as monolayer cultures at 37°C in a humidified atmosphere containing 5% CO\(_2\) in 1.5 ml of Eagle's minimum essential medium containing 5% heat-inactivated newborn calf serum, 2 mM glutamine, 100 U/ml of penicillin, 100 \(\mu\)g/ml of streptomycin, 50 \(\mu\)g/ml of gentamicin, 2 \(\mu\)g/ml of fungizone, and 10 \(\mu\)M cytosine arabinoside.

**Catecholamine secretion from cultured chromaffin cells**

Cells were washed with 1 ml of balanced salt solution [135 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl\(_2\), 2.2 mM CaCl\(_2\), 10 mM glucose, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), adjusted to pH 7.35] and incubated with or without various drugs at 37°C for 30 min in 250 \(\mu\)l of Ba\(^{2+}\)-containing salt solution, in which CaCl\(_2\) was substituted by various concentrations of BaCl\(_2\). At the end of incubation, the medium was withdrawn, and the cells were lysed by adding 250 \(\mu\)l of 10% acetic acid and by subjecting them to a freeze-thaw cycle. Medium and cell lysates were centrifuged at approx. 9000 \(\times\) g for 2 min, and the amounts of catecholamines in the supernatant fractions were determined as previously reported (20, 21).

Catecholamine secretion was calculated as a percentage of the total cellular catecholamine content secreted during the incubation period, and the Ba\(^{2+}\)-stimulated secretion was calculated by subtracting the basal secretion (2.0–3.8%), which was obtained in the absence of Ba\(^{2+}\) in each experiment.
Dopamine 6-hydroxylase release from cultured chromaffin cells

Cells were incubated at 37°C for different time periods in the presence of Ba2+. At the end of the incubation period, the medium was collected, and the cells were lysed by adding 250 μl of distilled water. Both medium and cell lysates were centrifuged as described above, and the supernatant fractions were saved for determination of the release of dopamine 6-hydroxylase. The enzyme activity was measured by spectrophotometric assay as reported previously (22), and the enzyme release was then calculated as a percentage of the total cellular activity released into the medium during the incubation period.

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 used to determine statistical significance.

Chemicals

Polymyxin B, TFP, TPA, and carbamylcholine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals used were of the commercially available reagent grade.

RESULTS

Effect of Ba2+ on catecholamine secretion

Cultured adrenal chromaffin cells were incubated with various concentrations of Ba2+ in the absence of Ca2+, and catecholamine secretion was then determined to confirm further the stimulatory action of Ba2+ on the exocytotic secretory response. As shown in Fig. 1, catecholamine secretion was markedly stimulated by Ba2+, and this stimulatory action was observed in a manner dependent on the Ba2+ concentration. The notable effect of Ba2+ on the secretion was already observed at 0.1 mM, and the maximum stimulation was obtained at 2 mM Ba2+. Catecholamine secretion stimulated by Ba2+ increased according to the incubation time. Under these conditions, dopamine 6-hydroxylase release was also stimulated by Ba2+, and this enzyme was released in parallel with catecholamine secretion (Fig. 2). These results indicated that Ba2+ stimu-
lated the concomitant release of dopamine \(\beta\)-hydroxylase with catecholamines, thus suggesting that \(\text{Ba}^{2+}\) caused the discharge of intragranular components as a consequence of activating the exocytotic secretory mechanism in the adrenal chromaffin cell.

Effects of protein kinase inhibitors on \(\text{Ba}^{2+}\) action

To solve the question of whether \(\text{Ba}^{2+}\)-stimulated catecholamine secretion through its action on the \(\text{Ca}^{2+}\)-mediated secretory mechanism or some other mechanism, the effects of inhibitors of \(\text{Ca}^{2+}\)-activated protein kinases on \(\text{Ba}^{2+}\)-stimulated catecholamine secretion were first studied. As shown in Fig. 3, catecholamine secretion evoked by relatively low concentrations of \(\text{Ba}^{2+}\) was markedly inhibited by the protein kinase C inhibitor polymyxin B. This inhibitory action was attenuated by elevating the \(\text{Ba}^{2+}\) concentration, and it was no longer observed at 2 mM \(\text{Ba}^{2+}\), while the secretory action of \(\text{Ba}^{2+}\) was inhibited by the calmodulin-dependent protein kinase inhibitor TFP. In contrast to the effect of polymyxin B, this inhibitory action was not affected by increasing the \(\text{Ba}^{2+}\) concentration (Fig. 4). These results seemed to indicate that the \(\text{Ba}^{2+}\)-stimulated secretion is related to the \(\text{Ca}^{2+}\)-mediated secretory process, in which \(\text{Ca}^{2+}\)-activated protein kinases might be involved.

Effect of phorbol ester TPA on \(\text{Ba}^{2+}\) action

To test the possibility that protein kinase C may be involved in the mechanism of \(\text{Ba}^{2+}\)-stimulated catecholamine secretion, the effect of the protein kinase C activator TPA on the secretory action of \(\text{Ba}^{2+}\) was examined. As shown in Fig. 5, the stimulatory action of \(\text{Ba}^{2+}\) on catecholamine secretion was enhanced by TPA, although TPA itself had no influence on the secretion (data not shown). Furthermore, this effect of TPA on \(\text{Ba}^{2+}\)
stimulated catecholamine secretion was no longer observed when the cells were stimulated by \( \text{Ba}^{2+} \) at concentrations higher than 1 mM (data not included). In contrast to the acute effect of TPA, the prolonged exposure of the cells to a relatively high concentration of TPA resulted in the suppression of catecholamine secretion stimulated by \( \text{Ba}^{2+} \) without any notable change in the basal secretion. Under these experimental conditions, the secretory actions of carbamylcholine and high K\(^{+}\) were also suppressed by TPA treatment (Fig. 6). These results seemed to provide further evidence for a possible role of protein kinase C in the mechanism of \( \text{Ba}^{2+} \)-stimulated catecholamine secretion from adrenal chromaffin cells.

**DISCUSSION**

Catecholamine secretion from cultured adrenal chromaffin cells was shown to be dramatically stimulated by \( \text{Ba}^{2+} \) in the absence of extracellular Ca\(^{2+}\). This \( \text{Ba}^{2+} \)-stimulated secretion was also shown to be saturable, and more than half of the cellular catecholamine content was secreted at the maximum level (Fig. 1). Catecholamine secretion stimulated by \( \text{Ba}^{2+} \) was furthermore shown to be accompanied by the concomitant release of an intragranular enzyme, dopamine \( \beta \)-hydroxylase (Fig. 2). Thus, these results seem to indicate that \( \text{Ba}^{2+} \) causes the secretion of massive amounts of intragranular materials from adrenal chromaffin cells, and they provide further evidence confirming the earlier findings that \( \text{Ba}^{2+} \) can stimulate the exocytotic secretory process even in the absence of extracellular Ca\(^{2+}\) (17, 18, 23, 24).

The secretory actions of relatively low concentrations of \( \text{Ba}^{2+} \) was shown to be markedly inhibited by polymyxin B, and this inhibitory action was overcome by elevating the \( \text{Ba}^{2+} \) concentration in the incubation mixture (Fig. 3). Polymyxin B has been reported to inhibit more specifically protein kinase C competitive-
ly with respect to phosphatidylserine (25). On the other hand, phospholipid cofactors are well-known to activate protein kinase C as a result of increasing the affinity of the enzyme for Ca\(^{2+}\). Polymyxin B is therefore considered to decrease the affinity of protein kinase C for Ca\(^{2+}\), presumably through its blocking action on the effects of phospholipid cofactors. In view of these facts, it seems reasonable to assume that the inhibition of the secretory action of Ba\(^{2+}\) by polymyxin B is attributed to a decrease in the affinity of the secretory mechanism for Ba\(^{2+}\). In fact, the finding that the inhibitory action of polymyxin B on Ba\(^{2+}\)-stimulated catecholamine secretion was overcome by elevating the Ba\(^{2+}\) concentration seems to support this assumption. Furthermore, it seems possible to assume that the site related to the inhibition of Ba\(^{2+}\) action is the same as that related to the inhibition of Ca\(^{2+}\) action, and this site that is commonly related to both the Ba\(^{2+}\) and Ca\(^{2+}\) actions is supposed to be protein kinase C.

In contrast to the effect of polymyxin B, the calmodulin-dependent protein kinase inhibitor TFP was shown to inhibit Ba\(^{2+}\)-stimulated catecholamine secretion, and this inhibitory action was not overcome by elevating the Ba\(^{2+}\) concentration (Fig. 4). TFP has previously been reported to inhibit catecholamine secretion stimulated by carbamylcholine or high K\(^+\) as a consequence of inhibiting Ca\(^{2+}\) influx into the cells (26). The secretory action of Ba\(^{2+}\) has been shown to be mediated by the Ba\(^{2+}\) entry into the cells through the Ca\(^{2+}\) channels (17, 18). In addition, the Ba\(^{2+}\)-stimulated secretion was found to be inhibited by neomycin, which can inhibit the Ca\(^{2+}\) influx into the cells, and this inhibitory action as well as that of TFP was not overcome by elevating the Ba\(^{2+}\) concentration (data not shown). In view of these findings, it therefore seems reasonable to consider that the inhibition of Ba\(^{2+}\)-stimulated secretion by TFP may be mainly due to its blocking action on the Ba\(^{2+}\) entry into the cells.

The inhibition by polymyxin B of Ba\(^{2+}\)-stimulated catecholamine secretion observed here seems to provide evidence for the possible involvement of protein kinase C in the Ba\(^{2+}\)-stimulated secretory mechanism. However, because this drug is not strictly specific for protein kinase C, the result obtained using this inhibitor is considered to be insufficient to support the possibility that the secretory action of Ba\(^{2+}\) is mediated by the activation of protein kinase C. To obtain further evidence supporting this possibility, the effect of the protein kinase C activator TPA on the secretory action of Ba\(^{2+}\) was examined, and catecholamine secretion stimulated by Ba\(^{2+}\) was shown to be enhanced by the direct addition of TPA to the incubation mixture (Fig. 5). Furthermore, this effect of TPA on the secretory action of Ba\(^{2+}\) was no longer observed when the cells were stimulated by Ba\(^{2+}\) at the concentration producing the maximum secretion (data not included). These findings seem to provide further evidence that the secretory action of Ba\(^{2+}\) may be related to the activation of protein kinase C. On the other hand, the secretory actions of both carbamylcholine and high K\(^+\), which are established to be mediated by protein kinase C, were shown to be suppressed by long-term treatment of the cells with TPA. Under these conditions, the Ba\(^{2+}\) action was also shown to be suppressed by TPA treatment (Fig. 6). In the previous studies, the prolonged exposure to relatively high concentrations of TPA has been shown to cause a decrease in the activity of protein kinase C as a consequence of down-regulation of the enzyme in several-type cells (27-30) including adrenal chromaffin cells (31). In view of these findings, it seems very likely that Ba\(^{2+}\) may be able to stimulate catecholamine secretion through its stimulatory action on protein kinase C in the adrenal chromaffin cell.

The present study suggests the possibility that Ba\(^{2+}\) may cause the activation of protein kinase C, resulting in the stimulation of catecholamine secretion through the Ca\(^{2+}\)-dependent exocytotic process. While, we have previously shown that Ba\(^{2+}\) can stimulate catecholamine biosynthesis as a consequence
of the activation of tyrosine hydroxylase, which may be attributed to the stimulation of the phosphorylation of this enzyme mediated by protein kinase C in the adrenal chromaffin cell (32), it thus seems more likely that Ba\(^{2+}\) can stimulate the Ca\(^{2+}\)-mediated cell functions probably through the activation of protein kinase C. However, the question of whether Ba\(^{2+}\) can directly stimulate the Ca\(^{2+}\)-activated process or indirectly stimulate this process probably through displacing Ca\(^{2+}\) from the intracellular store sites still remains to be elucidated. Since Ba\(^{2+}\) has already been shown to substitute for Ca\(^{2+}\) in the in vitro activation of protein kinase C (13), it therefore seems possible to consider that the stimulatory action of Ba\(^{2+}\) on catecholamine secretion may reflect the direct action of Ba\(^{2+}\) on protein kinase C, leading to the activation of the Ca\(^{2+}\)-mediated secretory mechanism. Furthermore, the previous studies on the mode of protein kinase C activation have shown that Ba\(^{2+}\) can directly activate type I (\(\gamma\)) and III (\(\alpha\)) enzymes purified from rat brain cytosol (33). It therefore seems reasonable to presume that the stimulatory action of Ba\(^{2+}\) on the exocytotic secretion may be mediated by the activation of both types I and III, but not type II (\(\beta\)), of protein kinase C in the adrenal chromaffin cell.

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