Stimulatory Action of Ba^{2+} on Catecholamine Biosynthesis in Cultured Bovine Adrenal Chromaffin Cells: Possible Relation to Protein Kinase C

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Abstract—The effect of Ba^{2+} on the catecholamine biosynthetic activity was studied by measuring the formation of [^{14}C]catecholamines from L-[^{14}C]tyrosine in cultured adrenal chromaffin cells. In the absence of Ca^{2+} , [^{14}C]catecholamine formation was markedly stimulated by Ba^{2+} , and this stimulation was observed in a manner dependent on its concentration. The stimulation of [^{14}C]catecholamine formation by relatively low concentrations of Ba^{2+} was suppressed by polymyxin B, a typical inhibitor of Ca^{2+} /phospholipid-dependent protein kinase (protein kinase C); and this inhibitory action of polymyxin B was attenuated by increasing the Ba^{2+} concentration. On the other hand, a tendency toward the enhancement of Ba^{2+} -stimulated [^{14}C]catecholamine formation was observed by a protein kinase C activator, 12-O-tetradecanoylphorbol 13-acetate (TPA). In contrast to the acute effect of TPA, [^{14}C]catecholamine formation stimulated by Ba^{2+} was reduced by long-term exposure of chromaffin cells to a high concentration of TPA, which has already been reported to cause the reduction of protein kinase C activity as a result of the down-regulation of this enzyme. These findings suggest that Ba^{2+} stimulates catecholamine biosynthesis, probably through its direct action on protein kinase C in adrenal chromaffin cells.

Catecholamine secretion evoked by cholinergic drugs or the depolarizing agents has already been established to be supported by the presence of extracellular Ca^{2+} (1, 2). Recently, the essential role of Ca^{2+} in the intracellular secretory process has been confirmed using permeabilized chromaffin cells, which allows us to gain direct access to the cell interior (3–8). In contrast, Ba^{2+} has previously been reported to evoke catecholamine secretion without stimulating the plasma membranes, and the secretory action of Ba^{2+} has been shown to be independent of extracellular Ca^{2+} (9–12). The secretory action of Ba^{2+} was therefore considered to be mediated by a rise in the free Ca^{2+} concentration within the cells, resulting from displacing Ca^{2+} with Ba^{2+} from the intracellular store sites. On the other hand, Ba^{2+} has recently been found to stimulate the release of ATP concomitantly with that of catechol-

amines from the adrenal chromaffin cell, and this stimulatory action has furthermore been shown to be mediated by the entry of Ba^{2+} into the cells, probably through voltage- and/or receptor-gated Ca^{2+} channels. Thus, these results were considered to support the hypothesis that the intracellular events induced by stimulation of the cells with Ba^{2+} may in part coincide with those leading to the Ca^{2+} -mediated exocytotic secretion (13). More recently, it has been proposed that Ba^{2+} can enter chromaffin cells through voltage-gated Ca^{2+} channels, and induces the stimulation of catecholamine secretion probably through its action on intracellular sites different from those affected by Ca^{2+} actions (14). Thus, the question of whether the intracellular sites of Ba^{2+} actions may be the same or different from those of Ca^{2+} actions has not yet been completely elucidated.

An earlier study demonstrated that Ba^{2+} -

stimulated catecholamine secretion is accompanied by stimulation of the phosphorylation of several proteins of the chromaffin cell. In particular, the Ba^{2+} -stimulated phosphorylation of one of these proteins (95K protein) has been found to be almost similar to that observed in the cells stimulated by nicotine (15), while previous studies on the direct actions of various divalent cations on exocytotic secretion and protein kinase C activity have shown that Ba^{2+} may be able to substitute for Ca^{2+} in the activation of protein kinase C as well as the stimulation of catecholamine release from electrically permeabilized chromaffin cells (16). It thus seems reasonable to conclude that Ba^{2+} may induce catecholamine secretion as a consequence of stimulating the Ca^{2+} -activated secretory mechanism, probably through its direct action on protein kinase C in adrenal chromaffin cells. Generally, the stimulation of catecholamine secretion by various secretagogues is known to be accompanied by an elevation in the ability of catecholamine biosynthesis in various adrenergic tissues (17). On the other hand, it has been established that tyrosine hydroxylase, which is the rate-limiting enzyme in the catecholamine biosynthetic pathway, is activated by phosphorylation of the enzyme protein catalyzed by various protein kinases including protein kinase C (18–26). Furthermore, Ba^{2+} has been reported to stimulate the phosphorylation of tyrosine hydroxylase accompanied by the activation of this enzyme (27). In view of these findings, it therefore seems reasonable to presume that Ba^{2+} may be able to stimulate catecholamine biosynthesis through the activation of protein kinases within the cell. In the present study, we then examined the effect of Ba^{2+} on catecholamine biosynthesis by measuring the formation of [^{14}C]catecholamines from [^{14}C]tyrosine in cultured bovine adrenal chromaffin cells. The possibility that the effect of Ba^{2+} on catecholamine biosynthesis may be mediated by protein kinase C was also verified using agents that modulate the activity of this enzyme.

Materials and Methods

Cell preparation and culture: Chromaffin cells were enzymatically prepared from fresh

bovine adrenal medulla according to the previously reported method (28). Isolated cells were plated on 24-well plastic cluster plates at a density of 5×10^5 cells/well, and 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 $\mu\text{g}/\text{ml}$ of streptomycin, 50 $\mu\text{g}/\text{ml}$ of gentamicin, 2 $\mu\text{g}/\text{ml}$ of fungizone, and 10 μM cytosine arabinoside.

Determination of [^{14}C]catecholamine formation: 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), pH 7.35], and incubated with 20 μM L-[^{14}C]-tyrosine (0.25 μCi) at 37°C for 30 min in 500 μl of Ba^{2+} -containing balanced salt solution in which CaCl_2 was substituted by various concentrations of BaCl_2 . At the end of the incubation period, the medium was discarded by aspiration, and the cells were washed twice with 1 ml of ice-cold balanced salt solution on ice, and then lysed by adding 500 μl of 0.4 M perchloric acid and subjecting them to a freeze-thaw cycle. The cell lysates were centrifuged at $9,000 \times g$ for 5 min, and radioactivity in an aliquot (50 μl) of the supernatant fraction was counted by a liquid scintillation spectrometer to measure the amount of [^{14}C]tyrosine taken up into the cells. Radioactive catecholamines contained in the acid extracts (400 μl) were isolated onto aluminum hydroxide gel, and radioactivity eluted from the gel was then determined by liquid scintillation spectrometry (29). Ba^{2+} -induced [^{14}C]catecholamine formation was calculated by subtracting the values obtained in the absence of Ba^{2+} from those obtained in the presence of Ba^{2+} in the reaction mixture.

Statistical analysis: Standard deviation (S.D.) of the difference between two groups was calculated as $(\text{SD}_1^2 + \text{SD}_2^2)^{1/2}$, and Student's *t*-test was used to determine statistical significance.

Chemicals: L-[U- ^{14}C]tyrosine was purchased from Amersham Japan Corp. (Tokyo, Japan). Polymyxin B, TPA, and carbamylcho-

line were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals used were of commercially available reagent grades.

Results

Effect of Ba²⁺ on catecholamine biosynthesis in cultured adrenal chromaffin cells:

To examine the effect of Ba²⁺ on the activity of catecholamine biosynthesis in cultured adrenal chromaffin cells, [¹⁴C]catecholamine formation from L-[¹⁴C]tyrosine in the cells stimulated with Ba²⁺ was first determined. As shown in Fig. 1, [¹⁴C]catecholamine formation was observed as a function of the incubation time, and the accumulation of [¹⁴C]catecholamines within the cells reached almost the plateau level after a 45-min incubation in the absence of Ba²⁺. Under these conditions, the formation of [¹⁴C]catecholamines was markedly enhanced by Ba²⁺, and this enhancement was observed even at a 60-min incubation. In general, since it is prerequisite for [¹⁴C]catecholamine formation that the precursor pools within the cells are label-

led with L-[¹⁴C]tyrosine, a lag of the increase in [¹⁴C]catecholamine formation is usually observed at the beginning of the incubation, while because newly formed [¹⁴C]catecholamines are thought to be released by stimulation of the cells with Ba²⁺, an increase in the amount of [¹⁴C]catecholamines within the cells tends to level off after a long-time incubation. Thus, to properly evaluate the effect of Ba²⁺ on the catecholamine biosynthetic activity, it is important to choose an appropriate incubation time. Under the conditions used here, the Ba²⁺-stimulated fraction of [¹⁴C]catecholamine formation was shown to be well-correlated to the incubation time from 15 to 60 min (Fig. 2). In our subsequent experiments, a 30 min incubation was therefore employed to determine the stimulatory action of Ba²⁺ on the formation of [¹⁴C]catecholamines in adrenal chromaffin cells.

The stimulatory action of Ba²⁺ on [¹⁴C]-catecholamine formation was also observed as a function of its concentration. As shown in Fig. 3, a substantial increase in Ba²⁺-induced [¹⁴C]catecholamine formation was

Fig. 1. Catecholamine biosynthesis as a function of the incubation time. Cells were incubated with 20 μ M L-[¹⁴C]tyrosine (0.25 μ Ci/well) at 37°C for different time periods in the presence (●) and absence (○) of 2 mM BaCl₂. The amount of [¹⁴C]-catecholamines within the cells was determined as described in the text. Values are the mean $\pm$ S.D. (n=3).

Fig. 2. Correlation between Ba²⁺-stimulated catecholamine biosynthesis and the incubation time. The Ba²⁺-stimulated fraction of [¹⁴C]catecholamine formation was obtained as the difference between the basal and the Ba²⁺-stimulated values presented in Fig. 1, and then plotted against the incubation time. The line in the figure was obtained by linear-regression analysis. Values are the mean $\pm$ S.D.

observed at 0.1 mM Ba^{2+} , and it reached almost the maximum level at 2 mM. This increase in [^{14}C]catecholamine formation was not enhanced any further by increasing the Ba^{2+} concentration up to 4 mM (data not included in Fig. 3). In contrast, the uptake of [^{14}C]tyrosine into the cells was not affected by Ba^{2+} at any concentrations tested here (data not shown).

Effects of protein kinase C modulators on Ba^{2+} -induced catecholamine biosynthesis in cultured adrenal chromaffin cells: To test the possibility that the stimulatory action of Ba^{2+} on catecholamine biosynthesis is probably mediated by protein kinase C, the effect of the protein kinase C inhibitor polymyxin B on Ba^{2+} -stimulated [^{14}C]catecholamine formation was examined. As shown in Fig. 4, [^{14}C]catecholamine formation stimulated by relatively low concentrations of Ba^{2+} was markedly inhibited by polymyxin B (400 units/ml), and this inhibitory action was attenuated by elevating the Ba^{2+} concentration in the incubation mixture. The significant effect of polymyxin B on [^{14}C]catecholamine formation was no longer observed when the cells were stimulated by 1 mM Ba^{2+} , and the in-

hibitory action of this drug almost completely disappeared in the presence of Ba^{2+} at concentrations higher than 2 mM (data not included in Fig. 4). The effect of TPA, which is known as an activator of protein kinase C, on [^{14}C]catecholamine formation was studied in the presence of lower concentrations of Ba^{2+} . TPA itself caused no significant influence on the basal level of [^{14}C]catecholamine formation, but this compound was shown to have a tendency to enhance the effect of a low concentration of Ba^{2+} on [^{14}C]catecholamine formation under the experimental conditions used here (Fig. 5).

To confirm the possibility that the stimulatory action of Ba^{2+} on catecholamine biosynthesis may be related to the activation of protein kinase C, the cells were cultured for 24 hr in the medium containing 1 μM TPA, and the effect of Ba^{2+} on [^{14}C]catecholamine formation was then determined in these cells. As shown in Fig. 6, the prolonged exposure of the

Fig. 3. Effect of various concentrations of Ba^{2+} on catecholamine biosynthesis. Cells were incubated with various concentrations of BaCl_2 at 37°C for 30 min in the presence of L-[^{14}C]tyrosine, and the amount of [^{14}C]catecholamines was determined as described in the text. Values are the mean $\pm$ S.D. ($n=3$).

Fig. 4. Inhibition of Ba^{2+} -stimulated catecholamine biosynthesis by polymyxin B. Cells were incubated with L-[^{14}C]tyrosine at 37°C for 30 min in the mixture containing different concentrations of BaCl_2 with (hatched column) or without (open column) 400 units/ml of polymyxin B. The amount of [^{14}C]catecholamines was determined as described in the text. Values are the mean $\pm$ S.D. ($n=3$). Statistically significant differences between two groups were observed at 0.2 and 0.5 mM, but not at 1 mM Ba^{2+} ($P < 0.025$).

Fig. 5. Effect of phorbol ester TPA on Ba²⁺-stimulated catecholamine biosynthesis. Cells were incubated with L-[¹⁴C]tyrosine at 37°C for 30 min in the mixture containing low concentrations of BaCl₂ with (hatched column) or without (open column) 100 nM TPA. The amount of [¹⁴C]catecholamines was determined as described in the text. Values are the mean±S.D. (n=3). The difference between two groups was not statistically significant (P<0.025).

cells to TPA resulted in a decrease in [¹⁴C]-catecholamine formation evoked by carbamylcholine or high K⁺. Similarly, the stimulation of [¹⁴C]catecholamine formation by Ba²⁺ was also reduced by the long-term exposure to TPA. Thus, the chronic exposure of the cells to relatively high concentration of TPA was shown to cause the reduction of the stimulatory action of Ba²⁺ on catecholamine biosynthesis in adrenal chromaffin cells.

Discussion

In the present study, Ba²⁺ was found to stimulate dramatically the formation of [¹⁴C]-catecholamines in cultured chromaffin cells without any significant alteration in the uptake of [¹⁴C]tyrosine into the cells, and this effect was also shown to be dependent on the Ba²⁺ concentration in the incubation mixture (Figs. 1 and 3). The dose-response curve of [¹⁴C]-catecholamine formation obtained here was almost similar to that of catecholamine secretion stimulated by Ba²⁺ from cultured adrenal

Fig. 6. Chronic effect of phorbol ester TPA on catecholamine biosynthesis stimulated by different-type secretagogues. Cells were pretreated with (hatched column) or without (open column) 1.0 μM TPA for 24 hr in the culture medium and then stimulated by 56 mM KCl, 300 μM carbamylcholine, or 2 mM BaCl₂ at 37°C for 30 min in the mixture containing L-[¹⁴C]tyrosine. The amount of [¹⁴C]-catecholamines was then determined as described in the text. Values are the mean±S.D. (n=3). The statistically significant difference between two groups was observed in all three cases (P<0.025).

chromaffin cells (K. Morita et al., unpublished data). In addition to the stimulatory action of Ba²⁺ on catecholamine secretion reported previously (9–12), Ba²⁺ is clearly shown here to stimulate catecholamine biosynthesis in adrenal medullary cells.

The stimulation of [¹⁴C]catecholamine formation induced by low concentrations of Ba²⁺ was shown to be significantly inhibited by the protein kinase C inhibitor polymyxin B, and this inhibitory action seemed to be overcome by elevating the Ba²⁺ concentration in the incubation mixture (Fig. 4). It has already been established that phosphatidylserine causes the activation of protein kinase C as a result of increasing the affinity of the enzyme for Ca²⁺. On the other hand, since polymyxin B has already been shown to inhibit more specifically protein kinase C competitively with respect to phosphatidylserine (30), it seems conceivable that polymyxin B may indirectly decrease the affinity of protein

kinase C for Ca^{2+} through its blocking action on the effect of phospholipid cofactor. In view of these findings, the stimulatory action of Ba^{2+} on catecholamine biosynthesis seems to be mediated by the activation of protein kinase C. Furthermore, the finding that the inhibitory action of polymyxin B on Ba^{2+} -stimulated catecholamine biosynthesis was overcome by elevating the Ba^{2+} concentration is therefore considered to indicate that polymyxin B may act on the site(s) of Ba^{2+} action, and then causes a decrease in the affinity for Ba^{2+} , similar to the decrease in the affinity of protein kinase C for Ca^{2+} . It seems possible to presume that the site related to the inhibitory action of polymyxin B on the Ba^{2+} action is probably the same as that involved in its inhibitory action on the Ca^{2+} action, and the site commonly related to both the Ba^{2+} and Ca^{2+} actions is supposed to be protein kinase C. If this idea is correct, it seems reasonable to consider that the stimulation of catecholamine biosynthesis by Ba^{2+} is enhanced by TPA, a protein kinase C activator. In fact, a tendency to enhance Ba^{2+} -stimulated [^{14}C]catecholamine formation was observed by adding TPA to the incubation mixture (Fig. 5).

In view of the earlier findings that the prolonged exposure to relatively high concentrations of TPA causes the reduction of the amount of protein kinase C as a result of down-regulation of this enzyme in several cell types (31–34) including adrenal chromaffin cells (35), the chronic treatment of the cells with TPA therefore seemed to be the most suitable way to confirm the possible involvement of protein kinase C in the cell function. Catecholamine biosynthesis stimulated by carbamylcholine or high K^+ , which is well-established to be mediated by Ca^{2+} -activated protein kinases, was shown to be markedly reduced by the exposure of the cells to TPA. It therefore seems conceivable that the decrease in catecholamine biosynthesis observed here may directly reflect a decrease in the amount of protein kinase C. In addition, the substantial decrease in Ba^{2+} -stimulated catecholamine biosynthesis was observed in the cells pretreated with TPA under the conditions in which the down-regulation of protein kinase C might occur (Fig. 6). It there-

fore seems likely that the stimulatory action of Ba^{2+} on catecholamine biosynthesis may be mediated by the stimulation of protein kinase C activity in the adrenal chromaffin cell.

Although the present study suggests that Ba^{2+} may stimulate the Ca^{2+} -dependent cell functions as a consequence of the stimulation of protein phosphorylation mediated by protein kinase C, it seems still questionable whether Ba^{2+} may be able to stimulate directly the Ca^{2+} -dependent mechanism or stimulate indirectly this process through displacing Ca^{2+} from the intracellular store sites, since a previous study has shown that Ba^{2+} can substitute for Ca^{2+} in the activation of protein kinase C *in vitro* (16). Furthermore, because it has been reported that Ba^{2+} can induce the activation of tyrosine hydroxylase as a result of stimulating the enzyme phosphorylation within the cells (27), it therefore seems likely that Ba^{2+} may directly activate protein kinase C, leading to the activation of tyrosine hydroxylase through the phosphorylation of the enzyme itself, thus resulting in the stimulation of catecholamine biosynthesis in the adrenal chromaffin cell.

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