Sequestration of Depolarization-Induced Ca\textsuperscript{2+} Loads by Mitochondria and Ca\textsuperscript{2+} Efflux via Mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} Exchanger in Bovine Adrenal Chromaffin Cells

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Abstract: We used fura-2 microfluorometry to investigate the role of mitochondria in regulating the increase in the cytosolic Ca\textsuperscript{2+} concentration ([Ca]_i) and the mechanism(s) underlying the subsequent Ca\textsuperscript{2+} efflux from mitochondria in bovine adrenal chromaffin cells. The rate of [Ca]_i decay during and following stimulation with 100 mM KCl depolarization was markedly increased when the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger was inhibited by clonazepam or CGP-37157 (CGP). In contrast, the addition of gramicidin, which increased the cytosolic Na\textsuperscript{+} concentration, following KCl depolarization caused a secondary increase in [Ca]_i. This secondary increase in [Ca]_i was prevented by the addition of clonazepam or CGP, and by the removal of external Na\textsuperscript{+}. The subsequent removal of clonazepam or CGP, or the delayed addition of Na\textsuperscript{+} caused a slow increase in [Ca]_i. A protonophore (FCCP) applied following KCl depolarization also caused a robust, secondary increase in [Ca]_i, which was insensitive to blocking by clonazepam or CGP. Neither gramicidin nor FCCP altered the [Ca]_i decay when applied following stimulation with histamine or caffeine, which mobilized Ca\textsuperscript{2+} from intracellular stores. These results suggest that the large [Ca]_i increase induced by Ca\textsuperscript{2+} influx, but not by intracellular Ca\textsuperscript{2+} release, is buffered by mitochondria, and that the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger makes a major contribution to the subsequent Ca\textsuperscript{2+} efflux from mitochondria. [Japanese Journal of Physiology, 49, 35–46, 1999]

Key words: bovine adrenal chromaffin cells, fura-2 microfluorometry, cytosolic Ca\textsuperscript{2+} concentration, mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, CGP-37157.

Recent studies using the Mn\textsuperscript{2+} quenching of cytosolic indo-1 [1] or mitochondrially targeted aequorin [2, 3] have indicated that mitochondrial Ca\textsuperscript{2+} uptake plays an important role in the regulation of elevations in cytosolic Ca\textsuperscript{2+} concentration ([Ca]_i) in the physiological range. Mitochondria accumulate Ca\textsuperscript{2+} through a membrane potential–dependent Ca\textsuperscript{2+} uniporter, which is inhibited by Ruthenium Red [4]. Once sequestered, Ca\textsuperscript{2+} is effluxed from mitochondria via Na\textsuperscript{+}-independent and Na\textsuperscript{+}-dependent mechanisms, the primary importance of which is dependent on tissues (see reviews of Gunter and Pfeiffer [5] and Gunter et al. [6]). It has been shown that, in rat adrenal chromaffin cells, mitochondrial accumulation is a major route for the rapid clearance of large Ca\textsuperscript{2+} loads, in comparison with Ca\textsuperscript{2+} efflux via the plasma membrane Ca\textsuperscript{2+} pump and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange [7–9]. These studies also indicated that the subsequent Ca\textsuperscript{2+} efflux from mitochondria is reflected by the secondary plateau and slow return to the basal level of [Ca]_i during recovery from stimulation, but the primary mechanism of Ca\textsuperscript{2+} efflux has not been exclusively studied.

To determine the extent of contribution of the Na\textsuperscript{+}-dependent pathway in mitochondrial Ca\textsuperscript{2+} efflux, we investigated whether the increase in cytosolic Na\textsuperscript{+} concentration ([Na]_i) caused by a Na\textsuperscript{+}, K\textsuperscript{+} ionophore gramicidin alters the rate of [Ca]_i decay after KCl depolarization. We found that gramicidin caused a sec-
ondary increase in \([\text{Ca}^{2+}]_{\text{in}}\), which was blocked by an inhibitor of the Na\(^+\)/Ca\(^{2+}\) exchanger in mitochondria, CGP-37157 (CGP [10, 11]) or clonazepam [12–14], and furthermore, that the removal of an inhibitor was followed by a transient increase in \([\text{Ca}^{2+}]_{\text{in}}\). Our results support the notion that the Na\(^+\)/Ca\(^{2+}\) exchanger is the predominant route for mitochondrial Ca\(^{2+}\) eflux in bovine adrenal chromaffin cells.

**MATERIALS AND METHODS**

**Isolation of bovine adrenal chromaffin cells.** Bovine adrenals were retrogradely perfused with a Ca\(^{2+}\)-deficient solution (no Ca\(^{2+}\) added) containing 0.025% collagenase and 0.01% DNase, followed by washing and trituration. The isolated cells were maintained for 1–4 d in Dulbecco’s modified eagle medium (DMEM; Nissui, Japan) containing 10% fetal calf serum and antibiotics as described previously [15].

**Measurement of \([\text{Ca}^{2+}]_{\text{in}}\).** Cells were allowed to adhere to poly-L-lysine- or collagen-coated glass coverslips and were then loaded with 1 \(\mu\text{M}\) fura-2 ace-toxymethyl ester (fura-2/AM), 0.1% dimethyl sulfoxide (DMSO), and 0.2% bovine serum albumin (BSA) for 30–60 min at room temperature (25–27°C). The coverslips were then mounted in a superfusion chamber and placed on the stage of an inverted microscope (Nikon, Diaphot-TMD). Cells on a glass coverslip were continuously superfused at a rate of 1 ml/min with standard saline at room temperature through a polyethylene tube, the tip of which was placed 1–2 mm away from the cells. The standard saline contained 150 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM HEPES, and 5.5 mM glucose, the pH of which was adjusted to 7.4 by adding Tris. In a Ca\(^{2+}\)-free solution, Mg\(^{2+}\) was increased to 2 mM, and 0.2 mM EGTA was added. In a divalent cation-free solution, Ca\(^{2+}\) and Mg\(^{2+}\) were omitted and 0.2 mM EDTA was added. In a Na\(^+\)-free solution, Na\(^+\) was isotonically replaced by 150 mM N-methyl-D-glucamine (NMDG\(^+\)).

Cells were viewed with a \(\times40\) Fluor objective lens (Nikon), and a single cell, to be measured, was fixed in the window positioned between the photomultiplier and the microscope. The changes in fluorescence ratios at 340 and 380 nm excitation wavelengths \((F_{340}/F_{380})\) were measured using a CAM-200 spectrometer (Jasco, Japan). The absolute value of \([\text{Ca}^{2+}]_{\text{in}}\) was calculated using the formula described by Grynkiewicz et al. [16]: \([\text{Ca}^{2+}]_{\text{in}} = b \times K_d (R - R_{\text{ref}})/(R_{\text{max}} - R)\). Calibration constants were determined in separate experiments with the same experimental set-up. The \(R\) value in the presence of 50 \(\mu\text{M}\) BAPTA/AM (a cell-permeant Ca\(^{2+}\) chelating agent) and 0.5 mM EGTA is referred to as \(R_{\text{ref}}\). \(R_{\text{max}}\) was obtained after adding ionomycin (5 \(\mu\text{M}\)) and CaCl\(_2\) (5 mM). Several points on the \(F_{340}/F_{380}\) ratio tracing were selected, and the values of \([\text{Ca}^{2+}]_{\text{in}}\) are shown on the ordinates in the figures after the values of \(F_{340}\) and \(F_{380}\) at each point were corrected by subtracting the background fluorescence at each wavelength.

**Measurement of Na\(^+\)-equilibration rate across the membrane.** Cells were incubated for 1.5–2 h at 37°C in standard saline containing 5 \(\mu\text{M}\) sodium binding benzofuranisophthalate (SBFI) tetra(ace-toxymethyl)ester, 0.01% cremophore (surfactant), 0.1% DMSO, and 0.2% BSA [17]. SBFI was loaded more rapidly at 37°C than at room temperature. The microfluorometric procedure used was the same as that for \([\text{Ca}^{2+}]_{\text{in}}\) measurement.

**Reagents.** The following reagents were used: Fura-2/AM (1 mM in DMSO; Dojindo, Japan), SBFI/AM (dissolved in DMSO; Molecular Probes, USA), CGP-37157 (dissolved in DMSO), and collagenase (Worthington, USA). Clonazepam (dissolved in methanol), DNase, gamicidin D (dissolved in methanol), FCCP (dissolved in DMSO), and veratridine (dissolved in DMSO) were obtained from Sigma Chemicals, USA. The final concentration of solvent (DMSO and methanol) used to dissolve reagents was equal to or lower than 0.1%, which did not affect the \([\text{Ca}^{2+}]_{\text{in}}\) decay after KCl stimulation.

**RESULTS**

**[Ca\(^{2+}\)]\(_{\text{in}}\) decay following 100 mM KCl stimulation and the effects of inhibitors of mitochondrial Na\(^+\)/Ca\(^{2+}\) exchanger**

When the 15 s stimulation with 100 mM KCl was followed by washing with a Ca\(^{2+}\)-free solution to avoid the possible occurrence of spontaneous fluctuations in \([\text{Ca}^{2+}]_{\text{in}}\) [15], \([\text{Ca}^{2+}]_{\text{in}}\) decayed rapidly and tended to remain at a plateau for 1–2 min before returning to the resting level in most cells. In some cells, we observed an obvious plateau phase of \([\text{Ca}^{2+}]_{\text{in}}\). Hoyt et al. [18] demonstrated that the variation in the recovery of \([\text{Ca}^{2+}]_{\text{in}}\) following stimulation is due to differences in the extent of mitochondrial loading. The plateau phase of \([\text{Ca}^{2+}]_{\text{in}}\) was never observed when the Ca\(^{2+}\)-free solution contained 10 \(\mu\text{M}\) CGP, and \([\text{Ca}^{2+}]_{\text{in}}\) returned to the basal level within 95 ± 15 s (mean ± SEM; \(N = 7\)) after stimulation, compared with 185 ± 17 s in paired controls (Fig. 1A; \(p < 0.01\) by paired Student’s \(t\)-test). The removal of CGP was followed by a slow increase (87 ± 22 nM increases above the lowest levels, \(N = 4\)) and subsequent fall in \([\text{Ca}^{2+}]_{\text{in}}\) (Fig. 1A, right). The ad-
Mitochondrial Na\(^+\)/Ca\(^{2+}\) Exchanger

![Diagram](image)

**Fig. 1.** [Ca\(_{in}\)] decay during washing with a Ca\(^{2+}\)-free solution following 15 s 100 mM KCl depolarization and the effects of CGP and clonazepam on [Ca\(_{in}\)] decay. Representative traces are shown. A: KCl stimulation was immediately followed by washing with a Ca\(^{2+}\)-free solution (Ca\(^{2+}\) 0, Mg\(^{2+}\) 2) lacking or containing 10 \(\mu M\) CGP-37157 (CGP10; \(N=7\)). Washing with CGP (right) nullified the plateau phase of [Ca\(_{in}\)], observed under the control conditions following stimulation (left), and the removal of CGP caused a small increase in [Ca\(_{in}\)] in the continued absence of external Ca\(^{2+}\) (right). B: The addition of 10 \(\mu M\) CGP (CGP10) during a long plateau phase of [Ca\(_{in}\)] lowered the [Ca\(_{in}\)] toward the basal level, and its removal tended to increase [Ca\(_{in}\)], which was again lowered by CGP (\(N=5\)). C: The addition of 80 \(\mu M\) clonazepam (clonaz-80) during the plateau phase of [Ca\(_{in}\)] reversibly lowered [Ca\(_{in}\)] toward the basal level (\(N=7\)).

dition of 10 \(\mu M\) CGP or 80 \(\mu M\) clonazepam during the plateau phase also quickly lowered the [Ca\(_{in}\)] to the basal level (Fig. 1, B and C; \(N=5\) and 7, respectively). These findings are in good accordance with the view that the slow return of [Ca\(_{in}\)] to the basal level is a consequence of an efflux of sequestered Ca\(^{2+}\) from mitochondria [8, 9], and strongly suggest that the mitochondrial Ca\(^{2+}\) efflux is mediated mainly by the Na\(^+/Ca^{2+}\) exchanger. These results prompted us to investigate whether manipulations leading to an increase in the cytosolic Na\(^+\) concentration ([Na\(_i\)]) increased the Ca\(^{2+}\) efflux from mitochondria.

**Effects of gramicidin and veratridine on [Ca\(_{in}\)] decay after 100 mM KCl stimulation**

We previously demonstrated, in cat chromaffin cells, that the removal of divalent cations from external solution increased [Na\(_i\)] ([19]). However, the peak [Na\(_i\)] was attained only after a few minutes under these conditions, and we therefore added gramicidin D (5–10 \(\mu g/ml\)), a Na\(^+\), K\(^+\)-ionophore, to a divalent cation-free washing solution as an attempt to increase [Na\(_i\)] more rapidly and consistently. The application of this washing solution alone did not affect the basal [Ca\(_{in}\)] (\(N=5\)). When KCl (100 mM) stimulation was terminated by the washing solution containing gramicidin, the secondary increases in [Ca\(_{in}\)] were observed in 30 of 40 cells tested (Fig. 2A), as if substantial amounts of Ca\(^{2+}\) were suddenly released from intracellular stores. The secondary increases in [Ca\(_{in}\)] were still observed in cells treated for 5 min with 1 \(\mu M\) thapsigargin (\(N=4\); data not shown), which abolished the caffeine- and methacholine-induced intracellular Ca\(^{2+}\) release (data not shown) by blocking Ca\(^{2+}\) pumps in the endoplasmic reticulum (ER [20]), thus ruling out the involvement of Ca\(^{2+}\) release from ER in the secondary increase in [Ca\(_{in}\)]. The addition of CGP (\(N=3\); data not shown) or clonazepam (\(N=6\); Fig. 2B) during the secondary increases in [Ca\(_{in}\)] increased the [Ca\(_{in}\)] decay. In fact, the secondary increase in [Ca\(_{in}\)] was never observed when the gramicidin solution contained these agents. In the presence of CGP and clon-
azepam, the [Ca]ₐ dropped to the basal level within 82±7 s (N=8) and 80±8 s (N=19), respectively, after stimulation, as compared with 227±11 s (N=20; p<0.01 by Student's t-test) in controls. As shown in Fig. 2, C and D, [Ca]ₐ slowly rose after the removal of either agent (the increases above the lowest levels were 106±32 nM, N=8, and 65±7 nM, N=8, respectively). Such a late increase in [Ca]ₐ was never observed in the continued presence of CGP or clonazepam (N=6 and 3, respectively).

In ten of the 40 cells tested, there was no obvious secondary increase in [Ca]ₐ in the presence of gramicidin, and only the plateau phase of the [Ca]ₐ decay was observed. The addition of CGP or clonazepam during the plateau phase quickly lowered the [Ca]ₐ (N=8 and 4, respectively; data not shown), as observed under the control conditions (Fig. 1, B and C).

The Na⁺-free (replaced by NMDG⁺) washing solution containing gramicidin never caused a secondary increase in [Ca]ₐ (Fig. 3A, right), and the [Ca]ₐ rapidly returned to the basal level within 87±9 s (N=13). The reduction in [Na] during Na⁺ removal would diminish mitochondrial Na⁺/Ca²⁺ exchange, and Ca²⁺ would be efficiently extruded by the plasma membrane Ca²⁺ pump, although the plasma membrane Na⁺/Ca²⁺ exchange would be inhibited during Na⁺ removal [18]. The delayed restoration of Na⁺ to the washing solution caused a slow increase in [Ca]ₐ in all cells tested (Fig. 3, A and B; 110±25 nM increase, N=13), which was reversibly inhibited by the addition of clonazepam (Fig. 3B; N=4). Pretreatment of the cell with clonazepam also strongly inhibited the [Ca]ₐ increase induced by the delayed addition of Na⁺, and the removal of this agent caused a slow increase in [Ca]ₐ (Fig. 3C; 80±7 nM increase, N=4). It is to be stressed that the delayed addition of Na⁺ during the treatment with gramicidin consistently caused a slow increase in [Ca]ₐ, thus providing a reliable experimental means of verifying the occurrence of Ca²⁺ efflux from mitochondria.
The addition of veratridine (50 μM), a Na⁺ channel activator [21], to a divalent cation-free washing solution also caused a secondary increase in [Ca]ᵢ in 11 of 30 cells and only prolonged the [Ca]ᵢ decay in the other 19 cells (data not shown). The inclusion of CGP in the washing solution quickly lowered the [Ca]ᵢ to the basal level, within 96 ± 9 s (N=9), and the removal of CGP caused a late increase in [Ca]ᵢ (64 ± 8 nM increase).

These results support the notion that the abrupt increase in [Na]ᵢ, when observed, strongly activated the mitochondrial Na⁺/Ca²⁺ exchanger, resulting in a secondary increase in [Ca]ᵢ during the washing period. To verify this, we investigated the effects of these agents on the ratio of 340 nm/380 nm fluorescent intensity for SFBI [17]. The gramicidin (10 μg/ml)- and veratridine (50 μM)-induced increases in the F₃₄₀/F₃₈₀ were preceded by time lags, which ranged from 18 to 46 s (29 ± 2 s, N=20) and 25 to 67 s (38 ± 3 s, N=16), respectively. A lag time shorter than 30 s was observed more often with gramicidin (13 of 20 cells) than with veratridine (4 of 16 cells). When the cells were treated for 1 min with a Na⁺-free (NMDG⁺) solution containing gramicidin, the addition of 150 mM Na⁺ caused an immediate increase in the ratio without a time lag (N=6; data not shown).

**Effect of gramicidin on [Ca]ᵢ decay following stimulation with weaker KCl depolarization**

When cells were stimulated with 25–30 mM KCl (600±95 nM [Ca]ᵢ increase, N=8), the immediate application of washing solution containing gramicidin did not cause a secondary increase in [Ca]ᵢ. In addition, when the Na⁺-free (NMDG⁺) washing solution containing gramicidin was applied after stimulation with 30–40 mM KCl (999±108 nM [Ca]ᵢ increase, N=7), the delayed addition of Na⁺ caused a slow increase in [Ca]ᵢ in only one of nine cells tested, in which the largest [Ca]ᵢ increase (1,330 nM) was elicited with 40 mM KCl. These results support the notion that Ca²⁺ influx through the Ca²⁺ channels was buffered by mitochondria only when [Ca]ᵢ increased above the critical level [8, 9].

[Ca]ᵢ decay during KCl stimulation with and without clonazepam

It has been shown that the Ca²⁺ concentration in the mitochondrial matrix ([Ca]ₘₐₙ) increased only tran-
siently, even when the agonist-induced [Ca]_in increase was sustained, indicating that once sequestered, mitochondrial Ca^{2+} was effluxed rather rapidly [2, 3, 22, 23]. We therefore attempted to determine the extent of the contribution of mitochondrial Ca^{2+} efflux to [Ca]_in decay during KCl stimulation. Preliminary experiments revealed that treatment of the cells with 10 μM CGP substantially inhibited the [Ca]_in increase induced by 20s KCl (60mM) depolarization (62±5% of the mean of the preceding and following control responses; N=6), though CGP at this concentration has been shown not to affect the Ca^{2+} current in neonatal ventricular myocytes [11]. On the other hand, 80 μM clonazepam did not affect the KCl-induced [Ca]_in increase (N=5; 97±2% of the mean) at all, which is in agreement with the finding indicating no inhibitory effect of this agent on the voltage-dependent Ca^{2+} channels in cardiac muscles [14]. We therefore compared [Ca]_in decay during KCl stimulation in the presence and absence of clonazepam (Fig. 4A). The rate of [Ca]_in decrease during 1-min stimulation in the presence of clonazepam was 2,068±332 nM/min (N=9), which is significantly different from 1,041±134 nM/min in paired controls (p<0.01 by Student's paired t-test). In contrast, when the cells were stimulated with 20–30 mM KCl, the time courses of [Ca]_in decay during stimulation in the presence and absence of clonazepam were very similar (Fig. 4B; N=12).

Effect of FCCP on [Ca]_in decay following stimulation with KCl

The protonophores, CCCP and FCCP, reversibly remove the driving force for mitochondrial Ca^{2+} uptake by dissipating the membrane potential (see Gunter and Pfeiffer [5]), resulting in Ca^{2+} release, and they have thus successfully been employed to verify the role of mitochondria in cellular Ca^{2+} homeostasis [8, 9, 24–31]. FCCP (2 μM) added to a Ca^{2+}-free washing solution caused a robust, secondary increase in [Ca]_in when applied immediately after 15s KCl (100 mM)
stimulation (Figs. 5–7; N=50). FCCP also caused a secondary increase in [Ca]_in following stimulation with 100 μM nicotine (N=5; data not shown). Neither the simultaneous addition of CGP nor pretreatment with 80 μM clonazepam affected [Ca]_in decay during the application of FCCP following KCl stimulation (Fig. 5, A and B; N=6 in each case). These results indicate that clonazepam and CGP have no inhibitory effect on Ca2+ release from mitochondria, which occurred after dissipation of the mitochondrial membrane potential with FCCP.

When the Ca2+ concentration in 100 mM KCl was lowered to 0.5, 0.2, and 0.1 mM, FCCP caused a secondary increase in [Ca]_in in 8 of 9, 5 of 14, and none of 6 cells tested, respectively (the results at 0.5 and 0.1 mM Ca2+ are shown in Fig. 6A). In addition, FCCP only prolonged [Ca]_in decay when applied after 15 s stimulation with 30 mM KCl containing 2 mM Ca2+, which caused 553±129 nM increases (N=10; Fig. 6B).

**Effects of FCCP and gramicidin on [Ca]_in decay following stimulation with histamine or caffeine**

It has been proposed that Ca2+ released to the cytosol in response to an IP3-generating agonist is transferred into the mitochondrial matrix much more effectively than the apparently equivalent [Ca]_in increases induced by Ca2+ influx in Hela cells [2, 3]. It is therefore of interest to determine whether the application of FCCP or the rapid increase in [Na]i results in a secondary increase in [Ca]_in following stimulation with histamine, which mobilizes Ca2+ from internal stores by the generation of IP3 in chromaffin cells [32].

FCCP did not affect [Ca]_in decay at all when applied after 20 s stimulation with 100 μM histamine in the absence of Ca2+, which increased [Ca]_in to 420±53 nM (N=15), although a second application of FCCP caused a secondary increase in [Ca]_in in all cells following 100 mM KCl stimulation (Fig. 7A). Also, the application of gramicidin following histamine stimulation (541±77 nM [Ca]_in increase, N=9)
did not alter [Ca]_i decay at all (time course of [Ca]_i decay was very similar to that found with the application of FCCP, shown in Fig. 7A). In addition, the delayed addition of Na⁺ failed to increase the [Ca]_i amount when histamine stimulation (463±55 nM increase, N=14) was followed by the Na⁺-free (NMDG⁺) washing solution containing gramicidin. The same results were obtained even when the cells were pretreated with clonazepam to reduce the Ca²⁺ efflux from mitochondria before the re-addition of Na⁺ (N=3). In contrast, the delayed addition of Na⁺ consistently caused a slow increase in [Ca]_i (120±39 nM, N=7) following 100 mM KCl stimulation in other cells plated on the same coverslips, but not previously exposed to histamine and gramicidin.

Essentially the same results were obtained when the cells were stimulated with caffeine, which mobilizes Ca²⁺ from intracellular stores partially shared with IP₃-sensitive channels [33, 34]. The immediate application of FCCP (Fig. 7B) or gramicidin (data not shown) did not alter [Ca]_i decay at all following stimulation with 20 mM caffeine (668±182 nM [Ca]_i increase in the former case, N=10, or 656±66 nM in the latter, N=11).

**DISCUSSION**

Previous studies on rat chromaffin cells have clearly indicated that mitochondrial Ca²⁺ uptake is the major mechanism responsible for rapid clearance following relatively large Ca²⁺ loads [7–9]. In the present study, we found that [Ca]_i decay during strong KCl depolarization was markedly increased when the mitochondrial Na⁺/Ca²⁺ exchanger was blocked by clonazepam (Fig. 4), indicating that the mitochondrial Ca²⁺ uptake is followed by a Na⁺-dependent efflux when a Ca²⁺ influx causes a large increase in [Ca]_i. In fact, the plateau phase of [Ca]_i decay following 100 mM KCl stimulation, when observed, was abolished with CGP or clonazepam (Fig. 1), suggesting that Ca²⁺ efflux via the Na⁺/Ca²⁺ exchanger makes a major contribution to the generation of the plateau phase. This notion is further supported by the following findings: 1) The application of gramicidin, which caused a rapid increase in [Na⁺], with a relatively shorter lag time, following KCl stimulation often caused a secondary increase in [Ca]_i, and this increase was nullified by the inhibition of the Na⁺/Ca²⁺ exchanger (Fig. 2) and removal of external Na⁺ (Fig. 3); and 2) the removal of
an inhibitor of the Na⁺/Ca²⁺ exchanger (Fig. 2, C and D) or the restoration of external Na⁺ (Fig. 3) caused a slow increase and subsequent decay in [Ca]ᵢ, as if the stored Ca²⁺ was slowly released. Although these results are consistent with the production of a reversible inhibition of the mitochondrial Na⁺/Ca²⁺ exchanger with an inhibitor or the lowered [Na]ᵢ, a question may arise as to the specificity of an inhibitor. For example, the rapid [Ca]ᵢ decay in the presence of clonazepam or CGP following KCl stimulation could be explained if one assumes its stimulatory effect on the plasma membrane Na⁺/Ca²⁺ exchange and Ca²⁺ pump. However, neither of these agents increased [Ca]ᵢ decay when mitochondrial Ca²⁺ was released by the dissipation of the mitochondrial membrane potential with FCCP (Fig. 5, A and B), and clonazepam did not increase [Ca]ᵢ decay during mild depolarization (Fig. 4). In addition, the removal of these agents caused a slow increase in [Ca]ᵢ even in the absence of Ca²⁺ influx, an observation which is difficult to explain on the basis of the agents’ stimulatory effects on Ca²⁺ efflux at the plasma membrane (Figs. 1–3). White and Reynolds [31] have also pointed out the important role of the Na⁺/Ca²⁺ exchanger in the regulation of [Ca]ᵢ in central neurons, using CGP as an experimental tool.

The mitochondrial accumulation of Ca²⁺ following strong KCl depolarization was also demonstrated by the robust, secondary increase in [Ca]ᵢ caused by the application of FCCP (Figs. 5–7), as frequently observed in other cells [8, 9, 24–31]. The FCCP-induced Ca²⁺ release from mitochondria was much larger than that caused by gramicidin (compare Fig. 2 with Figs. 5–7). This could be explained if one assumes that FCCP diffuses rapidly into the cytoplasm causing the massive release of Ca²⁺ from the mitochondria. In comparison, a rapid increase in [Na]ᵢ induced by gramicidin occurred after a time lag, during which substantial amounts of Ca²⁺ may have been released from mitochondria. In fact, the occurrence of a secondary increase in [Ca]ᵢ was less frequent when the washing solution contained veratridine, which increased [Na]ᵢ with a time lag longer than that with gramicidin. However, FCCP only prolonged [Ca]ᵢ
decay without causing a secondary increase in [Ca]m when applied following mild KCl depolarization, which moderately increased [Ca]m (Fig. 6, A and B), suggesting that only a small proportion of mitochondria near the plasma membrane is exposed to domains of high [Ca]m generated by the weak stimulus. It is of note that the selective increase in Ca2+ influx evoked only a small increase in [Ca]m, despite causing a large increase in [Ca]m in a HeLa cell line, where a small proportion of mitochondria is located close to the plasma membrane [35].

In the present study, neither gramicidin nor FCCP released Ca2+ from mitochondria when applied following stimulation with histamine or caffeine, both of which mobilize Ca2+ from intracellular stores. These results suggest that the Ca2+ released from the ER was not efficiently accumulated into mitochondria. It has been shown that the close apposition of IP3-sensitive channels to the mitochondria results in microdomains of high [Ca]m sufficient to facilitate rapid Ca2+ uptake into the mitochondria when stimulated with an IP3-generating agonist in HeLa cells [3, 23] and hepatocytes [22]. In contrast, the release of Ca2+ from the ER was less efficient in increasing [Ca]m than the Ca2+ influx in an endothelial cell line, where less than 4% of mitochondria are close to the ER [35]. These findings led us to speculate that a low degree of association exists between the mitochondria and the ER in bovine chromaffin cells, although there is no available morphological information. In contrast, it has been reported, in rat chromaffin cells, that the muscarine (MUS)- and bradykinin (BK)-induced Ca2+ release from the ER was taken up by mitochondria to increase [Ca]m, which was measured using rhod-2 [7]. Although the authors assumed that both Mus and BK mobilized intracellular Ca2+ alone in the presence of external Ca2+, the available information indicates that both agonists evoked Ca2+ influx associated with catecholamine (CA) secretion [36], and Mus induced an elevation of action potential frequency [37] by suppression of the K+ channel [38–40]. Thus, the possibility cannot be neglected that both agonists increase [Ca]m by increasing the Ca2+ influx, though further studies using rhod-2 are necessary to resolve this discrepancy between rat and bovine cells.

The present results indicate that the relatively large Ca2+ loads caused by Ca2+ influx through Ca2+ channels, but not by Ca2+ release from stores, are taken up by mitochondria possibly located near the plasma membrane in bovine chromaffin cells. In light of the marked dependency of CA secretion from the chromaffin cells on Ca2+ influx, but not on Ca2+ release from stores [19, 36, 41–45], mitochondrial Ca2+ uptake may play a physiologically important role in the secretory process in two ways. One is that Ca2+ uptake and the subsequent Ca2+ efflux via the Na+/Ca2+ exchanger determine the time course of secretion by affecting [Ca]m changes in domains close to the exocytotic sites following stimulation. The second is that the increase in [Ca]m activates Ca2+-sensitive intramitochondrial enzymes such as several dehydrogenases [46, 47] and ATP synthase [48]. The resultant increase of ATP synthesis may meet the demands for the subsequent energy-dependent exocytotic process.

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