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Mechanism of Na^+ Deprivation-Induced Catecholamine Secretion From Freshly Isolated Bovine Adrenal Chromaffin Cells

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Summary

We have studied the mechanism of Na^+ deprivation-induced catecholamine secretion from freshly isolated bovine adrenal chromaffin cells. Na^+ deprivation-induced catecholamine secretion depended on free extracellular Ca^{2+} concentrations and was almost parallel to $^{45}Ca^{2+}$ influx into the cells under various experimental conditions. Furthermore, Na^+ deprivation-induced $^{45}Ca^{2+}$ influx and catecholamine secretion were actually induced by a relative Na^+ concentration gradient across the plasma membrane, but not by simple omission of Na^+ from the medium. These results indicate that the deprivation of Na^+ from the medium changes the relative Na^+ gradient across the plasma membrane and results in Ca^{2+} influx *via* a reverse mode of Na^+-Ca^{2+} exchange rather than by inducing Ca^{2+} entry through Ca^{2+} channels by eliminating the competition between extracellular Na^+ and Ca^{2+}.

Key words
Na^+-Ca^{2+} exchange • Na^+ deprivation • Catecholamine secretion • Adrenal medulla • Chromaffin

Introduction

The cytoplasmic free Ca^{2+} concentrations in most cells are maintained below the micromolar range against a large electrochemical gradient. It is well known that Na^+-Ca^{2+} exchange together with Ca^{2+}-ATPase is the major route by which Ca^{2+} exits from cells (Blaustein 1988, DiPolo and Beauge 1988). Na^+-Ca^{2+} exchange is a carrier-mediated transport system and the transmembrane Na^+ electrochemical gradient, which is generated by Na^+, K^+-ATPase, is used for extruding intracellular Ca^{2+} (Philipson 1985, Shigekawa and Iwamoto 2001). The exchange reaction can operate in both directions and it can also promote the net entry of Ca^{2+} into cells under certain circumstances such as membrane depolarization. On the other hand, it is widely known that the Na^+-Ca^{2+} exchange system is present in adrenal medullary chromaffin cells (Pan *et al.* 1998) as well as in other cells and plays a physiologically important role in regulating the intracellular Ca^{2+} concentration (Powis *et al.* 1991, Pan and Kao 1997, Yang and Kao 2001).

Douglas and Rubin (1961) found that the deprivation of Na^+ from the extracellular fluid resulted in exocytotic catecholamine secretion from the perfused cat adrenal gland. Since then, similar results have been reported in perfused cat and bovine adrenal glands, and in isolated bovine adrenal chromaffin cells (Banks *et al.* 1969, Sorimachi and Nishimura 1981, Liu and Kao 1990). The mechanism by which Na^+ deprivation causes catecholamine secretion has been controversial for long

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time. It has been reported that no Ca^{2+} influx coupled with the outward movement of Na^+, the reverse mode of Na^+-Ca^{2+} exchange, was observed in the adrenal medulla (Rink 1977). Moreover, Na^+ deprivation-induced catecholamine secretion might be due to the redistribution of intracellular Ca^{2+}, because it can be observed even in the absence of extracellular Ca^{2+} (Lastowecka and Trifaro 1974). In contrast, it has been shown that Ca^{2+} influx is essential for catecholamine secretion by Na^+ deprivation (Nishimura *et al.* 1981). Furthermore, various kinds of treatments of cells to be expected to raise the concentration of internal Na^+ led to an increase in subsequent Na^+ deprivation-induced Ca^{2+} influx into the cells (Sorimachi *et al.* 1981, Sorimachi and Nishimura 1984, Liu and Kao 1990). In addition, the ratio of noradrenaline to adrenaline in catecholamines released by membrane depolarization has been reported to be a little different from the case in Na^+ deprivation (Duarte *et al.* 1994). These reports are consistent with the view that Na^+ deprivation induces Ca^{2+} influx *via* Na^+-Ca^{2+} exchange, which results in catecholamine secretion. However, the experiments reported until now do not exclude the possibility that the deprivation of Na^+ from the medium induces Ca^{2+} entry by eliminating the competition between extracellular Na^+ and Ca^{2+}. Thus, in the present study, we reexamined the mechanism of Na^+ deprivation-induced catecholamine secretion from freshly isolated bovine adrenal chromaffin cells to test this possibility.

Methods

Isolation of adrenal medullary cells

Fresh bovine adrenal glands were kindly provided by the Nara Livestock Center (Yamato-Koriyama, Nara, Japan). The isolated cells were prepared by sequential digestion of adrenal medullary slices with collagenase and then purified on a Percoll gradient (Amersham Biosci.) as described previously (Isosaki *et al.* 1994). Unless otherwise stated, the freshly isolated cells were preincubated at 37 °C for 10 min in a NaCl medium consisting of 150 mM NaCl, 10 mM Tris.HEPES (pH 7.4), 10 mM glucose and 0.2 % bovine serum albumin to allow the cells to accumulate Na^+ as a consequence of blocking Na^+, K^+-ATPase by K^+ removal, and kept on ice until the experiments were started. For the removal of extracellular Na^+, 150 mM NaCl in the medium was replaced by equimolar concentrations of various salts shown in the figures or 185 mM Tris Cl or 300 mM sucrose.

$^{45}Ca^{2+}$ influx

$^{45}Ca^{2+}$ influx was determined as described previously (Isosaki *et al.* 1994). Briefly, the isolated cells (10^6 cells/tube) were incubated at 37 °C with the various media containing $^{45}Ca^{2+}$ (0.5-1 μCi) as noted in the legends to the figures. The cells were washed three times with 3 ml of an ice-cold NaCl medium containing 2 mM $LaCl_3$ and solubilized in 1 % Triton X-100, and the radioactivity was assessed.

Catecholamine secretion

Catecholamines (adrenaline and noradrenaline) released into the medium and in the cells were estimated by the ethylenediamine condensation method (Weil-Malherbe and Bone 1952) using a fluorescence spectrophotometer with an excitation wavelength of 420 nm and an emission of 540 nm. Catecholamine secretion was expressed as a percentage of the total catecholamine content in the cells.

Na^+ in cells

The cells were incubated in plastic tubes as described in the legends to Figures 3A and 4A, and then washed five times with ice-cold Na^+-free medium consisting of 150 mM choline chloride and 10 mM Tris.HEPES (pH 7.4). The cells were punctured with 10 ml of 0.1 mM CsCl and then subjected to a freeze-thaw cycle. After 20 min centrifugation at 15 000 x *g*, the Na^+ concentrations in the supernatant were measured by flame photometry with a wavelength of about 589 nm.

Statistics

The statistical significance of differences were assessed by the one-way analysis of variance (ANOVA) with the Newman-Keuls multiple comparison test.

Materials

$^{45}Ca^{2+}$ (specific activity 10-40 mCi/mg Ca^{2+}) was purchased from Amersham Biosci. Other chemicals and materials were obtained from commercial sources.

Results

The replacement of NaCl in the incubation medium by Tris Cl, choline Cl, KCl, K glutamate, CsCl, RbCl and sucrose resulted in significant catecholamine secretion and $^{45}Ca^{2+}$ influx into freshly isolated bovine adrenal chromaffin cells (Fig. 1). However, various substitutes showed quantitatively different effects as reported previously (Sorimachi and Nishimura 1984).

Replacing Na^+ by Li^+ showed only a small increase in secretion and the $^{45}Ca^{2+}$ influx was consistent with the report that Li^+ is a weak substitute for Na^+ in Na^+-Ca^{2+} exchange (Ledvora and Hegyvary 1983). On the other hand, the replacement of NaCl by Na isethionate and Na glutamate did not cause large changes in the responses. These results indicate that the deprivation of Na^+ from the extracellular fluid causes $^{45}Ca^{2+}$ influx and catecholamine secretion from the cells.

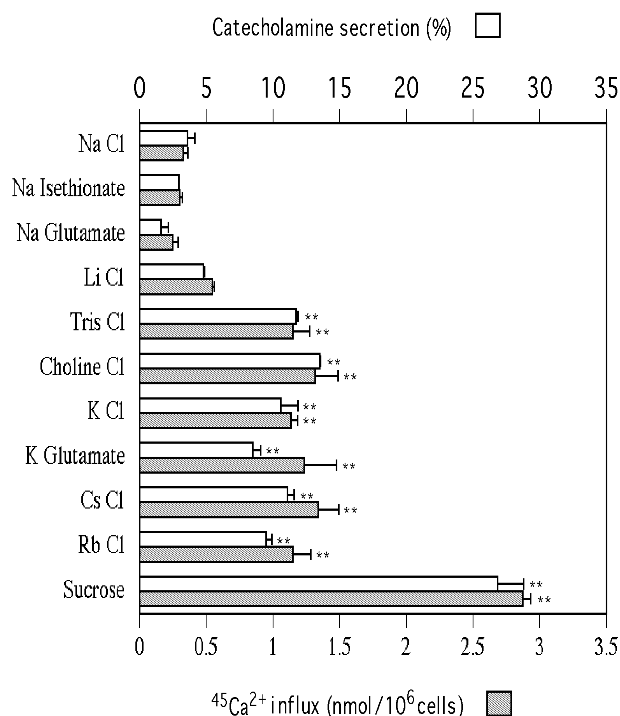

Fig. 1. *Effect of replacement of NaCl in extracellular medium by various substitutes on catecholamine secretion and $^{45}Ca^{2+}$ influx into freshly isolated bovine adrenal chromaffin cells. The cells (10^6 cells/0.1 ml) suspended in ice-cold 0.3M sucrose medium were added to 0.9 ml of the various substitute media (shown in the figure) containing Ca^{2+} and ouabain to yield the compositions as noted below. After the incubation for 10 min at 37 °C, $^{45}Ca^{2+}$ influx or catecholamine secretion was measured as described under "Materials and Methods". The final concentrations in the incubation medium are the following: 300 mM sucrose or 166.5 mM Tris Cl + 30 mM sucrose or 135 mM other various substitutes + 30 mM sucrose, 0.22 mM Ca^{2+} or $^{45}Ca^{2+}$ and 10 μM ouabain. All the media noted in the legends of figures contains 10 mM Tris.HEPES (pH 7.4), 10 mM glucose and 0.2 % bovine serum albumin, although the descriptions are omitted. The data shown are means ± S.E.M. from three separate experiments. Significant differences from the control (NaCl) are given as **P<0.01.*

Catecholamine secretion and $^{45}Ca^{2+}$ influx in response to a Na^+-free sucrose medium almost linearly continued for 10-15 min, and then declined gradually. The maximal responses were observed at 20-30 min (Isosaki *et al.* 1994). As shown in Figure 2A, an increase in Na^+ concentration in the extracellular medium resulted in a decrease of catecholamine secretion from the cells with a parallel decrease in $^{45}Ca^{2+}$ influx into the cells. Inhibition of catecholamine secretion and $^{45}Ca^{2+}$ influx was half maximal with about 15 mM Na^+, and almost complete inhibition was observed with 50-60 mM Na^+ (Fig. 2A).

Next, the effect of extracellular free Ca^{2+} on Na^+ deprivation-induced catecholamine secretion was examined. The replacement of the NaCl medium by a Na^+-free sucrose medium containing only contaminant (not added) Ca^{2+} resulted in significant (P<0.05) catecholamine secretion from the cells (Fig. 2B). This was in good agreement with a previous report (Lastowecka and Trifaro 1974). An increase in Ca^{2+} concentration in the medium enhanced catecholamine secretion, and maximal secretion was observed with 0.22 mM Ca^{2+}. On the other hand, the addition of 1 mM EGTA to the medium strongly inhibited Na^+ deprivation-induced catecholamine secretion. This inhibitory effect of EGTA was completely reversed by 1.2 mM Ca^{2+} (Fig. 2B). Furthermore, the Ca^{2+} antagonistic divalent cations Ni^{2+} and Co^{2+} (0.03-1 mM) inhibited Na^+ deprivation-induced $^{45}Ca^{2+}$ influx and catecholamine secretion in a concentration-dependent manner, and almost complete inhibitions were observed at 0.3 mM and 1 mM, respectively (data not shown). Taken together, these results indicate that Na^+ deprivation from the extracellular fluid induces Ca^{2+} influx into the cells, which results in catecholamine secretion.

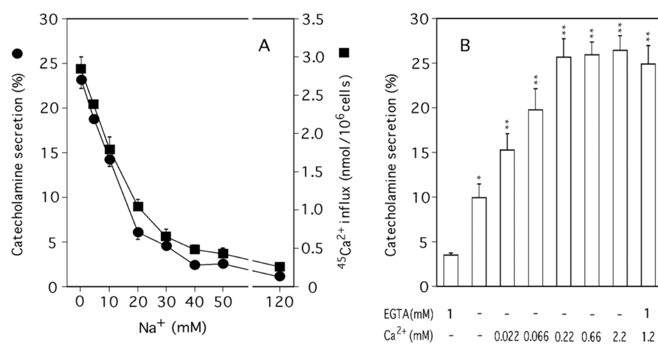

Fig. 2. *Panel A. Effects of different concentrations of extracellular Na^+ on catecholamine secretion and $^{45}Ca^{2+}$ influx. The cells (10^6 cells/tube) were incubated for 10 min with 1 ml of the medium consisting of various concentrations (0-120 mM) of NaCl, 0.22 mM Ca^{2+} or $^{45}Ca^{2+}$ (0.5-1.0 μCi) and 10 μM ouabain. NaCl was replaced by osmotically equivalent amounts of sucrose. Panel B. Effects of different concentrations of extracellular Ca^{2+} on Na^+ deprivation-induced catecholamine secretion. The cells were incubated for 10 min with 1 ml of sucrose medium containing 1 mM EGTA and/or different concentrations of Ca^{2+}. The pH of EGTA solution was adjusted to 7.4 with Tris base. The data shown are means ± S.E.M. from three separate experiments. Significant differences from the control (1 mM EGTA) are given as *P<0.05 and **P<0.01.*

Figure 3 shows the effects of modulations of intracellular Na^+ on Na^+ deprivation-induced $^{45}Ca^{2+}$ influx and catecholamine secretion. Incubation (20 min) of the cells with NaCl medium containing 5.6 mM K^+, which activated the Na^+, K^+-ATPase, led to a significant decrease in cellular Na^+ (Fig. 3A). This decrease in cellular Na^+ was strongly prevented by 10 μM ouabain, an inhibitor of Na^+, K^+-ATPase. On the other hand, as shown in Figure 3B, preincubation of the cells with 5.6 mM K^+ markedly inhibited subsequent Na^+ deprivation-induced $^{45}Ca^{2+}$ influx and catecholamine secretion, and this inhibition was also prevented by 10 μM ouabain. Taken together, these results indicate that Na^+ deprivation-induced $^{45}Ca^{2+}$ influx and catecholamine secretion depends on intracellular Na^+.

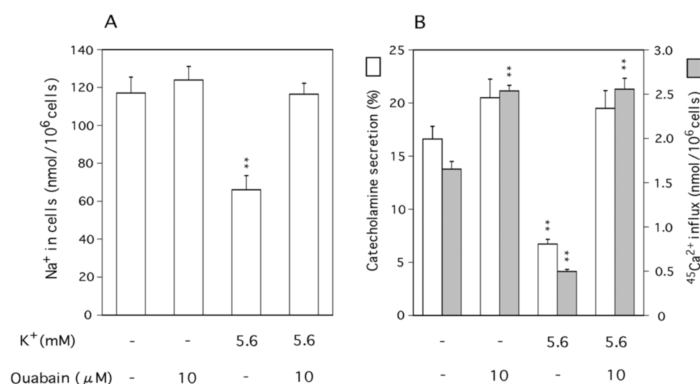

Fig. 3. *Effects of activation (induced by extracellular K^+) and inhibition (by ouabain) of Na^+, K^+-ATPase on cellular Na^+ concentrations (Panel A) and Na^+ deprivation-induced catecholamine secretion and $^{45}Ca^{2+}$ influx (Panel B). Panel A. The cells (10^6 cells/tube) were incubated for 20 min with 0.1 ml NaCl medium containing 0 or 5.6 mM KCl and with or without 10 μM ouabain as shown in the figure. After incubation, the intracellular Na^+ was measured by flame photometry as described under "Materials and Methods". Panel B. The cells preincubated as described above were diluted ten times with 0.9 ml of the 0.3 M sucrose medium containing various reagents to yield the same compositions as noted below and then incubated for 10 min. The final concentrations in all the tubes were adjusted as follows: 270 mM sucrose, 15 mM NaCl, 0.56 mM KCl, 0.22 mM Ca^{2+} or $^{45}Ca^{2+}$ (0.5-1.0 μCi) and 10 μM ouabain. The data shown are means ± S.E.M. from three separate experiments. Significant differences from the corresponding control (no K^+, no ouabain in the preincubation medium) are given as ** P<0.01.*

Furthermore, the possibility that the deprivation of Na^+ from the medium induces Ca^{2+} entry through Ca^{2+} channels by eliminating the competition between extracellular Na^+ and Ca^{2+} was examined. Instead of loading with Na^+ as described under "Methods", the freshly isolated cells were first incubated for 1 h in a

NaCl medium containing 2.2 mM Ca^{2+} and 5.6 mM K^+ to activate Na^+, K^+-ATPase and decrease intracellular Na^+ concentration. These cells incubated for one hour were then loaded for 20 min with the various substitute solutions shown in Figure 4. As expected, the concentrations of Na^+ in the cells incubated with Na^+ salts were significantly higher than those incubated with other substitutes (Fig. 4A). After ten-fold dilution of the cell suspensions with a sucrose medium, the cells were then stimulated for 10 min. Although the concentrations of extracellular Na^+ in the tubes preincubated with Na^+ salts should be much higher than those in the case of other substitutes, interestingly, the cells preincubated with Na^+ salts showed a remarkable $^{45}Ca^{2+}$ influx and catecholamine secretion, whereas the cells preincubated with the other substitutes did not (Fig. 4B).

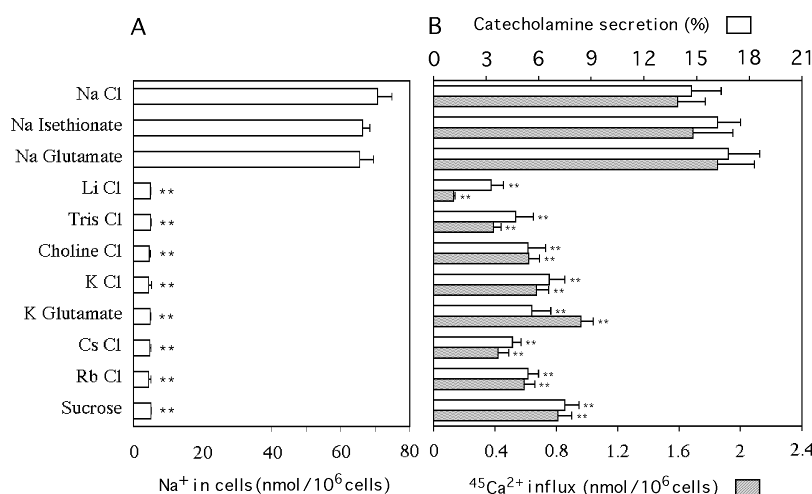

Fig. 4. *Effect of incubation of cells with various substitutes on cellular Na^+ concentrations (Panel A) and Na^+ deprivation-induced catecholamine secretion and $^{45}Ca^{2+}$ influx (Panel B). Panel A. Instead of loading with Na^+ as described under "Methods", the isolated cells (10^6 cells/tube) were first incubated for 1 h with NaCl medium containing 5.6 mM K^+ and 0.22 mM Ca^{2+} to exclude Na^+. The incubated cells were suspended in ice-cold 0.3M sucrose medium, and the aliquots (10^6 cells/50 µl) were added to 50 µl of various substitute media (shown in figure) containing EGTA and ouabain to yield the compositions as noted below. After the incubation for 10 min, the intracellular Na^+ was measured by flame photometry The final concentrations in the second incubation medium (0.1 ml) are the following: 300 mM sucrose or 92.5 mM Tris Cl + 150 mM sucrose or 75 mM other various substitutes + 150 mM sucrose, 0.2 mM EGTA and 10 µM ouabain. Panel B. The cells incubated as above were diluted ten times with 0.9 ml of 0.3M sucrose medium containing various reagents to yield the compositions as noted below and then incubated for 10 min. The final concentrations in the incubation medium (1 ml) are the following: 300 mM sucrose or 9.25 mM Tris Cl + 285 mM sucrose or 7.5 mM other various substitutes + 285 mM sucrose, 0.02 mM EGTA, 0.3 mM Ca^{2+} or $^{45}Ca^{2+}$ (0.5-1.0 µCi), 0.032 mM Tris base and 10 µM ouabain. Tris base was added for pH adjustment. The data shown are means ± S.E.M. from three separate experiments. Significant differences from the control (NaCl medium) are given as **P<0.01.*

Discussion

In this report, we have studied the mechanism of Na^+ deprivation-induced catecholamine secretion from freshly isolated bovine adrenal chromaffin cells. Na^+ deprivation-induced catecholamine secretion depended on extracellular free Ca^{2+} concentrations and went almost in parallel with the $^{45}Ca^{2+}$ influx into the cells under various experimental conditions (see "Results"). Furthermore, it is interesting to compare the results shown in Figure 1 with those in Fig. 4B. Na^+ deprivation-induced $^{45}Ca^{2+}$ influx and catecholamine secretion were actually induced by the relative Na^+ concentration gradient across the plasma membrane rather than the simple omission of Na^+ from the medium.

It is well known that membrane depolarization causes Ca^{2+} influx through voltage-dependent Ca^{2+} channels, which results in catecholamine secretion from adrenal chromaffin cells. It is plausible that the inhibition of Na^+, K^+-ATPase by ouabain leads to membrane depolarization, although we used ouabain as a reagent to prevent the extrusion (*via* Na^+, K^+-ATPase) of Na^+ loaded into the cells. However, ouabain itself did not cause any secretion from the cells in a NaCl medium containing

0.22 mM Ca^{2+} (data not shown), a concentration that induced the maximal secretion in response to Na^+ deprivation (Fig. 2B). Furthermore, high K^+ depolarization induced only a small increase (within 2 %) in catecholamine secretion from the normal Na^+-unloaded cells at 0.22 mM Ca^{2+} (data not shown). In addition, it has been reported that a synaptosomal membrane potential did not show any depolarization, when choline was substituted for extracellular Na^+ (Taglialatela *et al.* 1990). Therefore, it is unlikely that Na^+ deprivation-induced Ca^{2+} influx and catecholamine secretion in the present study is simply due to membrane depolarization, although we cannot exclude a minor contribution.

In the present study, our results indicate that the deprivation of Na^+ from the medium changes the relative Na^+ gradient across the plasma membrane and results in Ca^{2+} influx *via* a reverse mode of Na^+-Ca^{2+} exchange rather than by induced Ca^{2+} entry through Ca^{2+} channels by eliminating the competition between extracellular Na^+ and Ca^{2+}.

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Reprint requests

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