Effects of Low Extracellular Sodium on Cytosolic Ionized Calcium

Na\(^{+}\)-Ca\(^{2+}\) EXCHANGE AS A MAJOR CALCIUM INFUX PATHWAY IN KIDNEY CELLS

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The effects of extracellular Na\(^{+}\) (Na\(^{+}\)) on cytosolic ionized calcium (Ca\(^{2+}\)) and on calcium and sodium fluxes were measured in monkey kidney cells (LLC-MK\(\lambda\)). Ca\(^{2+}\) was measured with aequorin and the ion fluxes with \(^{45}\)Ca and \(^{22}\)Na. Na\(^{-}\)-free media rapidly increased Ca\(^{2+}\) from 60 to a maximum of about 700 nM in 2–3 min. After the peak, Ca\(^{2+}\) declined and reached a plateau of about twice the resting Ca\(^{2+}\). The peak Ca\(^{2+}\) was inversely proportional to the Na\(^{-}\) and directly proportional to the extracellular calcium concentration (Ca\(^{2+}\)). On the other hand, a pH of 6.8 reduced and Ca\(^{2+}\) substitution with Sr\(^{2+}\) completely blocked the Ca\(^{2+}\) response to low Na\(^{-}\). A Na\(^{-}\)-free medium stimulated calcium influx from the cells 4–5-fold, a response which was abolished in the absence of extracellular Ca\(^{2+}\). Na\(^{-}\)-free media also stimulated calcium influx and sodium efflux. The cell calcium content, however, was not increased. These results indicate that removal of extracellular Na\(^{+}\) increases Ca\(^{2+}\) by stimulating calcium influx and not by inhibiting calcium efflux; the increased calcium influx takes place on the Na\(+\)-Ca\(^{2+}\) antiporter operating in the reverse mode in exchange for sodium efflux. The increased calcium efflux occurs as a consequence of the rise in Ca\(^{2+}\) and presumably takes place on the (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase-dependent calcium pump.

After its discovery in heart and nerves (1, 2), Na\(+\)-Ca\(^{2+}\) exchange has been generally regarded as a mechanism for the uphill extrusion of calcium from the cell, energized by the Na\(^{+}\) electrochemical potential gradient \(\Delta\mu\)Na\(^{+}\). Since then, Na\(+\)-Ca\(^{2+}\) exchange has been reported in many other tissues (3), in renal and intestinal epithelia (4, 5), and in vesicles isolated from their basolateral plasmalemma (6, 7). Although it was recognized early that Na\(+\)-Ca\(^{2+}\) exchange could operate in the reverse mode, i.e. Na\(^{+}\) efflux and Ca\(^{2+}\) influx energized by the Ca\(^{2+}\) electrochemical potential gradient \(\Delta\mu\)Ca\(^{2+}\) (2, 8–11), it has never been considered to be a major calcium influx pathway. Indeed, the rise in cytosolic free calcium (Ca\(^{2+}\))

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The abbreviations used are: Ca\(^{2+}\), cytosolic ionized calcium; Ca\(^{2+}\), extracellular ionized calcium; KHB, Krebs-Henseleit bicarbonate buffer; EDTA, ethylenediaminetetraacetic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Na\(^{+}\), intracellular sodium concentration; Na\(^{-}\), extracellular sodium concentration; TMA, tetramethylammonium ion.
sers were purchased from Microbiological Associates (Walkersville, MD). MgATP, EGTA, EDTA, HEPES, and Triton X-100 were from Sigma; 4CaCl₂, 4NaCl, and scintillator Formula 963 were from New England Nuclear. Ultrapure grades of MgCl₂ and CaCl₂ were purchased from Johnson Matthey Ltd., United Kingdom and BDH Chemicals, Ltd., United Kingdom, respectively.

Measurement of Cytosolic Ionized Calcium—The cells were loaded with the calcium-sensitive photoprotein aequorin by a modification of the scrape-loading method of McNeiil and Taylor (21, 22). They were held and perfused in a matrix of fiberglass in a cuvette placed in an aequorin photometer (22). The perfusate was a Krebs-Henseleit bicarbonate buffer (KHB) comprising (in mM) NaCl 120, KCl 5, CaCl₂ 1.5, MgSO₄ 1.0, KH₂PO₄ 1.0, NaHCO₃ 24, and glucose 5, equilibrated with a gas phase of 95% O₂, 5% CO₂. The light emitted by the Ca²⁺-aequorin reaction was collected by a photomultiplier tube (EMI 9635A), and the current was recorded on a strip chart recorder. All experiments were performed at 25°C. The conversion of the aequorin light signal measured in nA into ionized calcium concentration was done according to Allen and Blinks (23). In the end of each experiment, the amount of aequorin incorporated by the cells was assessed by exposing the cells to 2% Triton X-100, in the presence of 140 mM KCl, 1 mM MgCl₂, 13 mM HEPES (pH 7.4), and 10 mM CaCl₂ to ignite all the intracellular aequorin. The light signal obtained after Triton X (Lₓ) was interpolated on a standard curve relating the fractional luminescence of a constant amount of the same lot of aequorin reacting with standard calcium concentrations at the same temperature (25°C) and in a solution whose ionic composition was assumed to be that of the intracellular milieu (140 mM KCl, 1 mM MgCl₂, pH 7.4).

Measurement of Cell Calcium—To determine the effects of extracellular Na⁺ on cell calcium, the cells were first incubated in KHB as a suspension gently stirred with a magnetic stirrer at 25°C for 60 min. The cells were then centrifuged at 200 × g and resuspended in a Na⁺-free KHB with TMA as the substituting ion. After 60 min in Na⁺-free media, the cell samples were added to 40 ml of GKN (a phosphate-buffered saline solution containing in mM: K₂HPO₄ 0.15, KH₂PO₄ 0.51, NaCl 135, KCl 4, glucose 11), centrifuged, and resuspended in 3 ml of deionized water. The cells were homogenized with an ultrasonic probe. Calcium was assayed by the method of Borle and Briggs (24) and cell protein by the method of Lowry et al. (25).

Measurement of Fractional Calcium Efflux—Kidney cells were first labeled for 60 min with ⁴⁰Ca, and the isotope desaturation was performed by perfusion at 25°C according to the method published by Borle et al. (26). The cells were placed into a loosely packed matrix of fiberglass over a 10-μm mesh nylon net placed at the outlet of the chamber and were perfused at a rate of 0.6 ml/min. The dead space of the system from medium reservoir to collection vials was 1.5 ml. The effluent was collected in scintillation minivials and analyzed for radioactivity and protein. Data are expressed as the ⁴⁰Ca fractional efflux ratio. The fractional efflux is the radioactivity released per unit of time expressed as the percentage of the mean cell radioactivity during the collection period. The fractional efflux ratio is the fractional efflux of the experimental group divided by the fractional efflux of controls perfused concurrently.

Measurement of Fractional Sodium Efflux—The same method and calculations as those just described for calcium were used to determine Na⁺ fractional efflux.

Na⁺ Uptake—Na⁺ uptake studies were performed according to Uchikawa and Borle (27). Kidney cells were harvested from the culture flasks with a rubber policeman and placed in suspension in KHB at 25°C. After an equilibration period of 25 min, Na⁺ was added, and 1-ml aliquots were taken from 2-60 min, centrifuged through a 40-ml column of ice-cold GKN, and the cell pellet was homogenized with an ultrasonic probe in 3 ml of deionized water. ⁴⁰Ca, ⁴⁰Ca, and protein were determined as described above. To study the effects of Na⁺ on media, ¹⁴Na⁺ was suspended in KHB in which Na⁺ was substituted by TMA.

Extracellular Na⁺ Substitution—Table I shows the composition of the various incubation media used in these experiments. Na⁺ was either reduced to 24 mM and replaced with TMA, lithium, and choline or totally replaced by the same substituting ions. In Na⁺-free media, the pH of 7.4 was always maintained by 24 mM choline bicarbonate in equilibrium with a gas phase of 5% CO₂, 95% O₂. In ⁴⁰Ca and ¹⁴Na fractional efflux experiments the pH of 7.4 was maintained by 20 mM Hepes and Na⁺ was totally replaced by 144 mM TMA.

RESULTS

Effects of Lowering Extracellular Sodium on Cytosolic Ionized Calcium—The lowering or the removal of extracellular sodium and its substitution by TMA, lithium, or choline resulted in an immediate rise in the cytosolic ionized calcium of cultured monkey kidney cells. This confirms the results obtained in perfused proximal tubules of Necturus kidney by Lorenzen et al. and Windhager (13, 15) who measured Ca²⁺ with Ca²⁺-selective microelectrodes. Fig. 1 shows a composite drawing derived from 5 separate experiments in Na⁺-free media in which Na⁺ was replaced by TMA. The inset presents a typical recording of such an experiment. Ca²⁺ rose from a basal concentration of 65 ± 19 to 696 ± 78 nM in 90 s. For the next 10 min, Ca²⁺ declined to a quasi-plateau at approximately 171 ± 59 nM. When Na⁺ was restored to its normal concentration of 144 mM, Ca²⁺ returned to the basal levels within 5 min. In spite of this rise in Ca²⁺, the total cell calcium measured throughout the period of incubation in Na⁺-free media up to 60 min did not increase. In fact cell calcium decreased slightly from 8.35 ± 1.21 nmol/mg of protein (n = 8) to 7.15 ± 0.43 nmol/mg of protein (n = 16). The peak level of Ca²⁺ depended both on the substituting ions and on the Na⁺ concentration. Fig. 2 shows that total Na⁺ substitution with choline and lithium also increased Ca²⁺ but less than with TMA. Table II presents the results of 30 separate experiments with the 3 different substituting ions and with low Na⁺ (24 mM) or Na⁺-free media. When Na⁺ was lowered from 140 to 24 mM, lithium produced the greater rise in Ca²⁺ to 479 ± 56 nM, choline increased it to 298 ± 48 and TMA to 170 ± 14 nM. The effects of choline are comparable to those reported by Lorenzen et al. who found that intracellular calcium activity rose from 73 ± 14 to 382 ± 69 nM when Na⁺ was decreased to 10 mM. In our experiments, the substitution of Na⁺ with any of the three substitutes caused a very rapid rise in Ca²⁺ at a rate approaching 300 nM/min. In all cases, the peak Ca²⁺ was reached within 1–3 min. The peak Ca²⁺ was maintained only briefly, and the rate of fall in Ca²⁺ was approximately 10% of

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**Table I**

| Substituting ion | Control | TMA | Lithium | Choline |
|-----------------|---------|-----|---------|---------|
| Na⁺            | 144     | 24  | 0       | 24      |
| TMA            | 0       | 120 | 0       | 120     |
| Na⁺            | 0       | 120 | 0       | 120     |
| Choline        | 0       | 24  | 0       | 24      |
| HCO₃⁻          | 24      | 24  | 24      | 24      |
| Hepes          | 0       | 0   | 0       | 0       |
| K⁺             | 1.3     | 1.3 | 1.3     | 1.3     |
| Ca²⁺           | 1.3     | 1.3 | 1.3     | 1.3     |
| Mg²⁺           | 1       | 1   | 1       | 1       |
| Cl⁻            | 120     | 120 | 120     | 120     |
| SO₄²⁻          | 1       | 1   | 1       | 1       |
| Glucose        | 5       | 5   | 5       | 5       | 5

*When HCO₃⁻ buffer was used, the media were equilibrated with 95% O₂, 5% CO₂.*
**Na⁺-Ca²⁺ Exchange and Ca²⁺ in Kidney Cells**

### Effects of substitution of extracellular Na⁺ by TMA, choline, and lithium on the cytosolic ionized calcium of kidney cells

| Substituting ion | Basal Ca²⁺ (nM) | Peak Ca²⁺ (nM) | Plateau Ca²⁺ (nM) | Rate of Ca²⁺ rise (nM/min) | Rate of Ca²⁺ fall (nM/min) |
|------------------|-----------------|----------------|------------------|---------------------------|---------------------------|
| **TMA**          |                 |                |                  |                           |                           |
| 24 mM Na⁺        | 65 ± 19         | 170 ± 14       | 91 ± 14          | 74 ± 30                   | 7.1 ± 2.3                 |
| 0 mM Na⁺         | 63 ± 15         | 288 ± 48       | 171 ± 39         | 446 ± 69                  | 50 ± 5.9                  |
| **Choline**      |                 |                |                  |                           |                           |
| 24 mM Na⁺        | 63 ± 15         | 288 ± 48       | 171 ± 39         | 446 ± 69                  | 50 ± 5.9                  |
| 0 mM Na⁺         | 48 ± 11         | 440 ± 42       | 124 ± 13         | 245 ± 17                  | 17 ± 2.6                  |
| **Lithium**      |                 |                |                  |                           |                           |
| 24 mM Na⁺        | 58 ± 14         | 479 ± 66       | 117 ± 9          | 153 ± 26                  | 13 ± 2.6                  |
| 0 mM Na⁺         | 47 ± 11         | 483 ± 85       | 158 ± 35         | 230 ± 55                  | 24 ± 4.4                  |

*Numbers in parentheses indicate number of experiments.

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**Fig. 1.** Composite drawing from 5 recordings showing the effects of the total replacement of extracellular Na⁺ with 120 mM TMA chloride and 24 mM choline bicarbonate on the cytosolic ionized calcium measured with aequorin in monkey kidney cells. The period of Na⁺-free perfusion is indicated by the horizontal line below the tracing. Inset, original recording of one typical experiment.

The rise in cytosolic ionized calcium when Na⁺ is decreased and the inverse relation between Na⁺ and Ca²⁺ strongly suggest that Na⁺-Ca²⁺ exchange may be implicated. The question is whether Ca²⁺ rises because of a decreased calcium efflux or because of an increased calcium influx, that is whether the Na⁺-Ca²⁺ exchanger operates in the forward or in the reverse mode. To distinguish between these two possibilities, calcium efflux was measured by the ⁴⁰Ca fractional efflux ratio technique. Fig. 4A shows that when Na⁺ was totally replaced by 144 mM TMA, calcium efflux immediately increased 4.5-fold.

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**Fig. 2.** Effects of different substituting ions on the rise in Ca²⁺ induced by the total removal of extracellular Na⁺. In the first recording, Na⁺ was replaced by 120 mM TMA and 24 mM choline bicarbonate. In the second recording, Na⁺ was replaced by 120 mM lithium and 24 mM choline bicarbonate. In the third recording, Na⁺ was replaced by 144 mM choline.

**Fig. 3.** Relation between peak rise in Ca²⁺ and the concentration of Na⁺ during the period of low Na⁺ perfusion. Extracellular Na⁺ was replaced with TMA. Below 24 mM Na⁺, sodium bicarbonate was replaced by choline bicarbonate.
or, less likely, from some intracellular pool of sequestered calcium. Supporting the former idea, Fig. 4B shows that in the perfusate, calcium efflux returned to control levels in 10 min. This rapid and dramatic increase in calcium efflux is an inhibitor of Na+-Ca2+ exchange (28). Fig. 5 shows a typical experiment in which kidney cells were exposed sequentially to 1) a Na+-free medium containing 1.3 mM Ca2+ with TMA as substituting ion (120 mM TMA and 24 mM choline bicarbonate), 2) a Na+-free medium in which all the calcium was replaced by 1.3 mM Sr2+, and 3) a Na+-free medium containing again 1.3 mM Ca2+. First, Na+ substitution with TMA produced the typical rise in Ca2+ in the presence of extracellular calcium. When extracellular calcium was replaced by strontium, the Na+-free medium produced practically no change in luminescence, indicating that the main source of calcium must be extracellular and that intracellular pools of sequestered calcium contribute essentially nothing to the rise in Ca2+ induced by TMA. When Ca2+ was reintroduced for the third stimulation, the peak Ca2+ and the plateau were depressed. A possible explanation is that a significant influx of strontium during the second stimulation has increased the cytosolic concentration of Sr2+ which competes with Ca2+ for binding sites on the aequorin molecules.

Effects of Different Extracellular Calcium Concentrations—If the rise in Ca2+ induced by a Na+-free medium is caused by an influx of calcium and not by a mobilization of intracellular calcium efflux rose for 3 min and then declined to a plateau at about twice the basal level. When Na+ was reintroduced in the perfusate, calcium efflux returned to control levels in 5–10 min. This rapid and dramatic increase in calcium efflux excludes an inhibition of calcium efflux as a cause of the rise in Ca2+ as suggested by many authors. On the contrary, these results suggest that calcium efflux is increased because of the rise in Ca2+. Indeed, the magnitude of the stimulation of calcium efflux (4.5-fold), the duration of its rise (3 min), its decline, and finally its plateau at twice the control levels closely match the magnitude and the time course of the rise in Ca2+. A smaller Na+ reduction to 100 mM was also followed by an increase in fractional calcium efflux from 1.1 ± 0.08 to 1.37 ± 0.13 (n = 6).

Since the rise in Ca2+ induced by low Na+ is not caused by a decreased calcium efflux, the only possible explanation is that calcium enters the cytosol from the extracellular fluids or, less likely, from some intracellular pool of sequestered calcium. Supporting the former idea, Fig. 4B shows that in the absence of extracellular calcium, perfusion with a Na+-free medium did not increase calcium efflux. This suggests that when calcium influx is abolished, the rise in Ca2+ and the secondary increase in calcium efflux do not take place. These results also indicate that mobilization of calcium from intracellular pools does not contribute much, if anything, to the rise in Ca2+ evoked by low Na+.

\[ \text{Effects of Substitution of Extracellular Calcium with Strontium—} \]

To further test the idea that a low Na+ triggers an influx of calcium into the cell, the influence of strontium and of various extracellular concentrations of calcium was investigated. Sr2+ activates aequorin only weakly, so that even if it enters the cell through the various calcium influx pathways, its presence in the cytosol would not enhance the aequorin luminescence. Moreover, Sr2+ is an inhibitor of Na+-Ca2+ exchange (28).
calcium as suggested by the previous experiments, the peak Ca$^{2+}$ should be dependent upon the extracellular calcium concentration. Therefore, we studied the effects of Ca$^{2+}$ from 30 μM to 1.3 mM on the peak Ca$^{2+}$ induced by the total substitution of Na$^+$ by 120 mM TMA and 24 mM choline bicarbonate. In these experiments, the cells were continuously exposed to 2 μg of FCCP/ml to eliminate or minimize the intracellular buffering of Ca$^{2+}$ by mitochondria (see below). Fig. 6 shows a typical recording of such a test, and Fig. 7 the relation between the peak Ca$^{2+}$ and the extracellular calcium concentration in 9 different experiments. The results clearly show that the rise in Ca$^{2+}$ evoked by a Na$^+$-free medium is totally dependent on extracellular calcium and support the postulate that it is caused by a stimulation of calcium influx.

Effect of Lowering Na$^+$ on Calcium Influx—To test whether the substitution of extracellular Na$^+$ with TMA increases calcium influx, the uptake of 45Ca by kidney cells in suspension was studied in control media (KHB) and in media where Na$^+$ was totally replaced by 120 mM TMA and 24 mM choline bicarbonate. Fig. 8 shows that 45Ca uptake was significantly increased in Na+-free media. The early time points of the 45Ca uptake curve represent both calcium binding to the cell surface and unidirectional calcium influx, so that it is impossible to calculate exactly the magnitude of the increase in calcium influx. If one assumes that calcium binding is not increased by TMA substitution, calcium influx can be estimated to be raised 4-6-fold. In addition, 2 mM Mn$^{2+}$ which is an inhibitor of Na$^+$-Ca$^{2+}$ exchange, markedly reduced the increased calcium influx evoked by a Na$^+$-free medium.

Effect of Lowering Na$^+$ on Sodium Efflux—If the observed rise in Ca$^{2+}$ occurs by Na$^+$-Ca$^{2+}$ exchange one would predict that the increased calcium influx on the Na$^+$-Ca$^{2+}$ antiporter should be accompanied by an simultaneous efflux of sodium that would be dependent upon the presence of extracellular calcium. To test this prediction, the effects of sodium substitution with TMA on 22Na efflux were studied in the presence and in the absence of extracellular calcium. Fig. 9A shows that the 22Na fractional efflux was increased 40% in Na+-free media. 22Na efflux reached a peak in 3.5 min and then declined to a plateau about 20% above basal levels and returned to the control rate within 2 min when sodium was reintroduced in the perfusate bathing the cells. The time course of the increased sodium efflux and of its various phases closely matched that of the rise in Ca$^{2+}$ and that of calcium efflux. Fig. 9B shows that in the absence of extracellular calcium, sodium efflux was not stimulated by lowering Na$^+$. These results strongly support the hypothesis that Na$^+$-free media stimulates calcium influx and sodium efflux on the Na$^+$-Ca$^{2+}$ antiporter operating in the reverse mode. The increased sol-
active transport would be expected to fall (13).

**Effect of Ouabain on Ca\(^{2+}\) in Na\(^+-\)free Media**—One can postulate that the fall in Na\(^{+}\) caused by Na\(^{+}\) efflux could be responsible for the transient nature of the rise in Ca\(^{2+}\). Since calcium influx on the antipporter may only occur in exchange for sodium efflux, the resulting rise in Ca\(^{2+}\) would be limited by the availability of intracellular sodium. Once Na\(^{+}\) is depleted, calcium influx would decrease, allowing the plasmalemmal calcium pump and intracellular buffering of Ca\(^{2+}\) by mitochondria to lower Ca\(^{2+}\) to a new steady state plateau. If this assumption is correct, one would predict that the peak Ca\(^{2+}\) and its duration will depend upon Na\(^{+}\) when the cells are exposed to Na\(^+-\)free media. To test this hypothesis the effects of Na\(^{+}\) substitution by TMA on Ca\(^{2+}\) were studied in the presence and in the absence of ouabain.

Fig. 10 shows a typical recording representative of 3 separate experiments in which the effects of Na\(^{+}\) substitution with 120 mM TMA and 24 mM choline bicarbonate on Ca\(^{2+}\) were recorded in the presence and in the absence of 5 mM ouabain. In the first control test with TMA and choline, Ca\(^{2+}\) rose to 421 nM and followed the typical time course of peak, decline, plateau, and return to control levels. In the presence of ouabain, the response became progressively more marked and prolonged. The second and third stimulation of Ca\(^{2+}\) in Na\(^{-}\)-free medium was obtained 10 and 60 min after the cells were exposed to the glycoside. The initial peak Ca\(^{2+}\) was increased by ouabain from 421 nM in the first trace to 471 and 504 nM, 10 and 60 min after ouabain. The rate of decline was also markedly slowed down from 58 nM/min in the control test to 17.1 nM/min and 11.2 nM/min in the second and third test. Finally the Ca\(^{2+}\) plateau was also increased from 145 to 163 and 363 nM in the 3 responses, respectively. Such results can be interpreted as an enhancement of calcium influx on the Na\(^+-\)Ca\(^{2+}\) exchanger by progressively higher concentrations of intracellular Na\(^{+}\) caused by the inhibition of the Na\(^{+}\) pump by ouabain. They support the idea that the decline in Ca\(^{2+}\) after the initial peak may be caused by a depletion of intracellular sodium as sodium efflux on the Na\(^+-\)Ca\(^{2+}\) antiport is stimulated.

**Effects of pH on Ca\(^{2+}\) in Na\(^{-}\)-free Media**—Na\(^+-\)Ca\(^{2+}\) efflux is probably not caused by a stimulation of the (Na\(^+-\)K\(^{-}\))-ATPase-dependent sodium pump because 1) the removal of extracellular potassium which would inhibit the Na\(^{+}\) pump did not prevent the rise of \(^{22}\)Na efflux in Na\(^{-}\)-free media (Fig. 9C), 2) the very high concentration of Ca\(^{2+}\) would if anything inhibit the (Na\(^{-}\)-K\(^{-}\))-ATPase, and 3) when Na\(^{+}\) is lowered from 144 mM to zero, Na\(^{+}\) and consequently Na\(^{-}\)
change is very pH sensitive in cardiac sarcolemmal vesicles and was shown to be a sigmoidal function of pH; it is inhibited at pH 6 and stimatated at pH 9 (28). If the rise in Ca²⁺ induced by Na⁺-free media was caused by calcium influx on the Na⁺-Ca²⁺ antiporter, it should also be influenced by pH. To test this hypothesis, the rise in Ca²⁺ induced by Na⁺ substitution with TMA was studied at pH 6.8, 7.4, and 7.8 by increasing or decreasing the pCO₂ of the gas phase equilibrating the KHB buffer. Fig. 11 shows a typical recording representative of 3 separate experiments. During the first stimulation produced by TMA at pH 7.4, Ca²⁺ rose from 26 to 380 nM in 1.3 min and then fell to a plateau of 150 nM after 10 min. When the pH was reduced to 6.8, TMA substitution increased Ca²⁺ from 36-241 nM in 1.2 min, and the plateau of 37 nM was reached in 12 min. When the pH was raised to 7.8, the rise in Ca²⁺ induced by TMA reached 592 nM in 2.2 min, and a plateau of 145 nM was reached after 36 min. It appears, therefore, that the calcium influx pathway responsible for the rise in Ca²⁺ is markedly pH sensitive. It is inhibited at low pH and enhanced at high pH.

Effects of Repeated Stimulations and of Mitochondrial Inhibitors—The availability of intracellular sodium may not be the only condition that affects the peak Ca²⁺ and the time course of its fall evoked by Na⁺-free media. Fig. 12 shows that the peak Ca²⁺ response to repeated exposures to Na⁺-free media grew progressively smaller. In some instances the peak disappeared and only the plateau phase of Ca²⁺ elevation was obtained. This could be caused by a depletion of intracellular sodium (13) or alternatively by an increased buffering action of intracellular organelles, particularly by mitochondria. To estimate the contribution of mitochondrial buffering to the decline in peak Ca²⁺ levels, we tested two mitochondrial inhibitors, FCCP and NaCN, with choline as Na⁺ substitute. Fig. 13 presents one representative example of 3 separate experiments performed with 2 μg of FCCP/ml. The first Ca²⁺ elevation of the graph was obtained after a series of Na⁺-free stimulations when the response was markedly depressed and only showed the plateau phase of the Ca²⁺ rise. Ca²⁺ rose from 125 to 210 nM. FCCP alone added to the perfusate caused a transient increase in Ca²⁺ to 186 nM. Later,

when extracellular sodium was totally replaced by choline, Ca²⁺ increased to 460 nM; a second stimulation 9 min later increased it to 350 nM; after 1-h exposure to FCCP a third stimulation increased Ca²⁺ to the same level (data not shown). Fig. 14 shows another recording representative of 2 experi-
ments performed with 2 mM NaCN with choline as the substituting ion. The first stimulation of Fig. 14 was obtained after a series of Na+-free exposures and was markedly blunted. Ca\(^{2+}\) rose from 10 to 75 nM. After NaCN was added to the perfusate, Ca\(^{2+}\) transiently increased to 118 nM. In the presence of cyanide, Na\(^+\) substitution by choline increased Ca\(^{2+}\) to 190 nM. In two subsequent stimulations after 60 and 90 min, Ca\(^{2+}\) rose to 104 and 125 nM, respectively. This series of experiments suggests that mitochondrial buffering of cytosolic calcium contributes to the progressive decrease in the peak Ca\(^{2+}\). When this buffering is eliminated or blunted by FCCP or NaCN the high levels of Ca\(^{2+}\) evoked by Na+-free media are restored.

**DISCUSSION**

The physiological and functional importance of Na+-Ca\(^{2+}\) exchange in transporting epithelia is still controversial. On one hand, the existence of a Na+-Ca\(^{2+}\) antiporter in basolateral membrane vesicles isolated from renal, intestinal, and toad bladder epithelial cells has been conclusively demonstrated (6, 10, 28, 29). On the other hand, the functional importance of Na+-Ca\(^{2+}\) exchange as a major pathway for the active transport of calcium out of intact epithelial cells has been challenged on several grounds by many investigators (5, 7, 16-18): calcium transport on the ATP-dependent calcium pump exceeds that of the Na+-Ca\(^{2+}\) exchanger by a factor of 4-5-fold (7); the effects of Na\(^+\) on calcium transport are claimed to be competitive or allosteric and not due to Na+-Ca\(^{2+}\) countertransport (16, 17); and there is no gain in tissue calcium in the absence of extracellular Na\(^+\) (18, 30). The conflict may arise from the common assumption that Na+-Ca\(^{2+}\) exchange is operating in the forward mode as a calcium efflux pathway. If one assumes that the Na+-Ca\(^{2+}\) antiport is not functioning when \(\Delta_{m}Na^+\) and \(\Delta_{m}Ca^{2+}\) are exactly balanced or that it operates in the reverse mode as a calcium influx pathway, the apparent contradiction may no longer exist.

Our results show that decreasing \(\Delta_{m}Na^+\) by lowering extracellular Na\(^+\) to 100, 24, or 0 mM immediately increased Ca\(^{2+}\). The peak Ca\(^{2+}\) was inversely proportional to the concentration of extracellular Na\(^+\) and directly proportional to the concentration of extracellular Ca\(^{2+}\). Our measurements of calcium and sodium fluxes further showed that decreasing \(\Delta_{m}Na^+\) by totally replacing extracellular Na\(^+\) with TMA stimulated calcium efflux from the cells, a response abolished in Ca\(^{2+}\)-free media; it significantly increased calcium influx. Finally the cell calcium content was not increased in Na+-free media, confirming previous results obtained in rat and rabbit kidney cells (18, 30).

From these observations, we can eliminate several explanations for the rise in Ca\(^{2+}\) commonly offered by many investigators: the rise in Ca\(^{2+}\) induced by a low extracellular Na\(^+\) is not caused by an inhibition of calcium efflux since calcium efflux increased 4-fold (4, 10-15, 29). The rise in Ca\(^{2+}\) is not caused by a mobilization of calcium from intracellular pools of sequestered calcium as proposed by Mandel and Murphy (30); indeed, when extracellular Ca\(^{2+}\) is substituted by Sr\(^{2+}\), there is no longer any increase in Ca\(^{2+}\), and after the cells are pretreated with mitochondrial inhibitors that release mitochondrial calcium, the effects of low Na\(^+\) are enhanced and not reduced (Figs. 13 and 14). Finally, we can eliminate the possibility that the increased sodium efflux is taking place on the Na+-K\(^+\) pump; it is not abolished by the elimination of extracellular K\(^+\) and, on theoretical ground, one can assume that intracellular Na\(^+\) would be reduced in Na+-free media (13) and that the Na+-K\(^+\) pump would be inhibited by the high cytosolic Ca\(^{2+}\).

All the evidence tends to support the view that the rise in Ca\(^{2+}\) induced by low Na\(^+\) is caused by an increased calcium influx and that this increased calcium influx is taking place on the Na+-Ca\(^{2+}\) antiporter: 1) the increased calcium efflux is accompanied by an increased Na efflux; 2) the increased calcium influx is inhibited by Mn\(^{2+}\) which is a recognized blocker of Na+-Ca\(^{2+}\) exchange (28); 3) the resulting increase in Ca\(^{2+}\) is blocked by Sr\(^{2+}\); and 4) like the Na+-Ca\(^{2+}\) exchange mechanism, it is inhibited by low pH and enhanced by high pH; finally, 5) the increased Na efflux is abolished in Ca\(^{2+}\)-free media.

The following sequence of events could explain the effects of extracellular Na\(^+\) substitution. 1) Lowering extracellular Na\(^+\) suddenly reduces \(\Delta_{m}Na^+\) to the point where the driving force for calcium influx \(\Delta_{m}Ca^{2+}\) greatly exceeds that for sodium influx. 2) A sudden and massive influx of calcium takes place on the Na+-Ca\(^{2+}\) antiporter in exchange for sodium efflux. 3) The increased calcium influx floods the cytosol and increases Ca\(^{2+}\). 4) The rise in Ca\(^{2+}\) immediately stimulates the (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase-dependent calcium pump and calcium efflux is markedly stimulated; the close interrelation among calcium influx, sodium efflux, the rise in Ca\(^{2+}\), and calcium efflux is supported by their similar time course characterized by the 3 phases of peak, decline, and plateau. 5) After reaching a peak, Ca\(^{2+}\) declines to a new plateau, at about twice the control levels of Ca\(^{2+}\); the fall in Ca\(^{2+}\) may be caused by at least 3 processes: (a) a decline in calcium influx limited by the decrease in Na\(^+\) that follows the sudden rise in Na\(^+\) efflux and the absence of Na\(^+\) influx; (b) a rise in calcium efflux on the calcium pump; and (c) intracellular buffering particularly by mitochondria. Indeed, pretreatment with ouabain which presumably increases Na\(^+\) or with mitochondrial inhibitors enhances the peak Ca\(^{2+}\) and delays the decline. 6) The plateau phase of elevated Ca\(^{2+}\) is determined by the combined actions of an increased calcium influx, efflux, and mitochondrial buffering, and it is presumably affected by the intracellular concentration of Na\(^+\), of ATP, and by other unknown factors. Initially, however, mitochondrial calcium buffering may not be an important determinant of Ca\(^{2+}\) in Na+-free media, and calcium efflux on the calcium pump constitutes the main compensation to the elevated Ca\(^{2+}\); indeed the cell calcium content does not increase, and the effects of mitochondrial inhibitors are seen only after several stimulations when the response to low Na\(^+\) is declining.

Our data strongly support the idea that, in Na+-free media, the Na+-Ca\(^{2+}\) antiporter operates in the reverse mode as a...
calcium influx pathway. The question remains whether in physiological conditions, the antipporter operates in the reverse or forward mode, i.e. in other words whether it normally behaves as a calcium influx pathway or as a calcium efflux pathway. That will depend of course on the stoichiometry of the exchange mechanism, on the intracellular Na\(^+\) and Ca\(^{2+}\) concentration, and on the cell membrane potential difference \(\Delta \psi\), which influence \(\Delta \mu_{Ca^{2+}}\) and \(\Delta \mu_{Na^{+}}\). Most investigators found that the stoichiometry of the Na\(^+-\)Ca\(^{2+}\) antipporter measured in isolated plasma membrane vesicles or in intact cells is 3 Na\(^+\) for 1 Ca\(^{2+}\) in both forward and reverse modes (9–11, 28, 31–35). The intracellular Na\(^+\) concentration of kidney cells reported in the literature ranges between 12 and 35 mM (13, 15, 36–38) with an average of about 15 mM, and the membrane potential difference of renal proximal tubule cells ranges between −48 and −60 mV (13–15, 36, 38, 39). Accepting a cytosolic ionized calcium of 10\(^{-10}\) M, an extracellular concentration of Na\(^+\) and of Ca\(^{2+}\) of 140 and 1.0 mM, respectively, a Na\(^+\) of 14 mM and \(\Delta \psi\) of −60 mV one can calculate \(\Delta \mu_{Ca^{2+}}\) and \(\Delta \mu_{Na^{+}}\) in normal physiological conditions. \(\Delta \mu_{Ca^{2+}}\) is found to be 34.56 kJ/mol and \(\Delta \mu_{Na^{+}}\) 11.52 kJ/mol. Assuming a stoichiometry of 3 Na\(^+\)/1 Ca\(^{2+}\), the two driving forces are exactly equal: \(\Delta \mu_{Ca^{2+}} = 3 \Delta \mu_{Na^{+}} = 34.56 \text{ kJ/mol}\). We can conclude that, unless Na\(^+\) is significantly less than 14 mM or \(\Delta \psi\) significantly greater than −60 mV the driving force from the sodium electrochemical potential is not great enough to energize calcium efflux in kidney cells. If in physiological conditions the two driving forces were exactly balanced, Na\(^+-\)Ca\(^{2+}\) exchange would not be functioning. On the other hand, one can speculate that if the intracellular Na\(^+\) concentration was greater than 14 mM and \(\Delta \psi\) less than −60 mV, which is more likely according to all the values published in the literature, the driving force for calcium influx would exceed that for sodium influx (\(\Delta \mu_{Ca^{2+}} > 3 \Delta \mu_{Na^{+}}\)) and the Na\(^+-\)Ca\(^{2+}\) antipporter would operate as a calcium influx pathway. The reverse mode of Na\(^+-\)Ca\(^{2+}\) exchange could be the usual and physiological mode. The net driving force for calcium influx on the Na\(^+-\)Ca\(^{2+}\) antipporter would be \(\Delta \mu_{Ca^{2+}} - 3 \Delta \mu_{Na^{+}}\) which would vary with the extracellular Na\(^+\) concentration as shown in Fig. 15. If in physiological conditions the Na\(^+-\)Ca\(^{2+}\) antipporter was working in the forward mode as a calcium efflux pathway, one would predict that a small decrease in \(\Delta \mu_{Na^{+}}\) would reduce calcium efflux while a large decrease in \(\Delta \mu_{Na^{+}}\) would increase calcium influx as the antipporter shifts in the reverse mode. Since a small reduction in Na\(^+\) to 100 mM (which slightly lowers \(\Delta \mu_{Na^{+}}\) by −0.84 kJ/mol results in a 25% stimulation in calcium efflux, it is likely that under physiological conditions the antipporter is not functioning (\(\Delta \mu_{Ca^{2+}} = 3 \Delta \mu_{Na^{+}}\)) or is operating as a calcium influx pathway (\(\Delta \mu_{Ca^{2+}} < 3 \Delta \mu_{Na^{+}}\)). If so, the peak Ca\(^{2+}\) obtained when Na\(^+\) is lowered should be directly proportional to the influx of calcium on the Na\(^+-\)Ca\(^{2+}\) antipporter and, therefore, directly proportional to its driving force. Fig. 16 shows that there is an excellent correlation between the peak Ca\(^{2+}\) measured at various Na\(^+\) in our experiments and the net driving force \(\Delta \mu_{Ca^{2+}} - 3 \Delta \mu_{Na^{+}}\) calculated for the appropriate Na\(^+\). We conclude that in kidney cells, the Na\(^+-\)Ca\(^{2+}\) antipporter can operate as a calcium influx pathway. The magnitude of the calcium influx depends on the relative electrochemical potential gradients for Na\(^+\) and Ca\(^{2+}\). When \(\Delta \mu_{Na^{+}}\) is decreased by lowering Na\(^+\) or by increasing Na\(^+\), calcium influx on the antipporter is markedly stimulated, and it increases Ca\(^{2+}\) to an extent that is directly related to the driving force \(\Delta \mu_{Ca^{2+}} - 3 \Delta \mu_{Na^{+}}\). Calcium efflux on the (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase-dependent calcium pump is secondarily enhanced by the high Ca\(^{2+}\). From these considerations, we can speculate that the only transport mechanism available for calcium efflux in kidney cells is the (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase-dependent calcium pump. Only in exceptional and unlikely conditions, such as hyperpolarization (\(\Delta \psi > -60\) mV) or very low intracellular Na\(^+\) (Na\(^+\) < 10 mM), could the Na\(^+-\)Ca\(^{2+}\) exchange operate as a calcium efflux pathway. These results and their interpretation may help solve some of the conflicts existing between the proponents of Na\(^+-\)Ca\(^{2+}\) exchange as an important transport mechanism.
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