Modulation of Aortic Smooth Muscle Cell Membrane Potential by Extracellular Calcium

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Removal of extracellular calcium may result in depolarization of the resting cell membrane potential. This has been attributed to the stabilizing action of calcium on the ionic permeability of the cell membrane. It is unknown whether this phenomenon is exclusively mediated by extracellular calcium or through associated changes in intracellular calcium. To examine this, we exposed rat aortic smooth muscle cells in culture to different calcium concentrations and studied their effects on the resting membrane potential and intracellular calcium activity. The resting membrane potential was dependent on the extracellular potassium concentration. Exposure to reduced extracellular calcium concentrations (0.25 and 0.5 mM) caused a steep and reversible depolarization of the membrane potential, but intracellular calcium, measured with fura 2-AM, was not reduced below that measured in control conditions (1.8 mM). Atomic absorption spectrophotometric measurements did not indicate a measurable gain in cell sodium after reduction of extracellular calcium levels. We conclude that extracellular calcium controls the resting cell membrane potential of vascular smooth muscle through a mechanism that is independent of cytosolic Ca\textsuperscript{2+} activity. (Hypertension 1989;14:549–555)

There is evidence that extracellular calcium ([Ca\textsuperscript{2+}]) is an important regulator of arterial blood pressure,\textsuperscript{1-2} possibly acting through changes in circulating hormones and local vasoactive agents\textsuperscript{3-4} or by directly affecting vascular smooth muscle properties.\textsuperscript{5} In addition, Ca\textsuperscript{2+} regulates a major determinant of vascular tone, the resting membrane potential of the vascular smooth muscle cell membrane (Vcm).\textsuperscript{7} The magnitude and polarity of Vcm depend primarily on the electrochemical gradients and permeability for K\textsuperscript{+} and Na\textsuperscript{+} across the cell membrane. In the resting state, the higher permeability of the membrane for K\textsuperscript{+} drives Vcm closer to the K\textsuperscript{+} equilibrium potential or E\textsubscript{K}. Although transmembrane Ca\textsuperscript{2+} gradients do not contribute significantly to Vcm, Ca\textsuperscript{2+} plays a critical role in the generation of this electrophysiological parameter through its effect on the membrane permeability to K\textsuperscript{+} and Na\textsuperscript{+}.

In 1940, Guttman\textsuperscript{8} observed that Ca\textsuperscript{2+} antagonized the depressing action of extracellular K\textsuperscript{+} on the membrane potential of spider crab nerve and “stabilized” cell membrane ionic permeability. Frankenhaeuser and Hodgkin\textsuperscript{9} demonstrated an increase in inward, depolarizing Na\textsuperscript{+} currents on removal of Ca\textsuperscript{2+} from the electrolyte solution bathing the squid axon, thus suggesting that Ca\textsuperscript{2+} effects on membrane ionic permeability were complex, involving Na\textsuperscript{+} as well as K\textsuperscript{+}. Biochemical studies later provided additional information on the role of Ca\textsuperscript{2+} in ion transport across the cell membrane. Experiments by Gárdos\textsuperscript{10} first determined that Ca\textsuperscript{2+} regulated the K\textsuperscript{+} permeability of the red blood cell membrane. The study of Rb\textsuperscript{+} efflux in inside out vesicles from human red blood cells highlighted the importance of the intracellular Ca\textsuperscript{2+} compartment in transmembrane K\textsuperscript{+} transport.\textsuperscript{11,12}

In recent years additional insight into the electrophysiological properties of the cell membrane has been provided by the patch-clamp technique.\textsuperscript{13,14} Since its initial description, this method has facilitated the individualization of single ion currents in smooth muscle and has clearly demonstrated the existence of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels.\textsuperscript{15} Even though the intracellular Ca\textsuperscript{2+} compartment has been primarily implicated in the regulation of K\textsuperscript{+} channels in smooth muscle, recent evidence supports a role for the extracellular Ca\textsuperscript{2+} compartment as well,\textsuperscript{16} thus raising the possibility that the effects of [Ca\textsuperscript{2+}]

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may result from its action on cell membrane K+ channels responsible for the generation of Vcm.  

In spite of the usual concentration gradient of approximately 105 between [Ca2+]o and intracellular calcium ([Ca2+]i), it is important to recognize that stabilization of the ionic permeability of the cell membrane by [Ca2+]i could ultimately be mediated or perhaps enhanced by a simultaneous effect on [Ca2+]o. It is also worth pointing out that an increase in [Ca2+]o, by stabilizing the cell membrane, could decrease membrane permeability to Ca2+ and thereby reduce [Ca2+]i.

To better define the site of action of Ca2+ on the cell membrane potential (intracellular vs. extracellular compartment), we exposed cells derived from a rat aortic smooth muscle (RASM) cell culture to solutions with different Ca2+ activities, and their effects on the Vcm and [Ca2+]i were studied with intracellular glass microelectrodes and the cell permeant fluorescent Ca2+ indicator fura 2-AM, respectively.

Materials and Methods

RASM Aortic Smooth Muscle Cell Preparation

RASM cells were prepared by a method modified from Brock et al.  

The thoracic aortas of 10-week-old Wistar rats were removed, trimmed of fat, and rinsed with a physiological salt solution (PSS 1) of the following composition (mM): NaCl 110, KCl 5.4, MgSO4 0.4, Na2HPO4 1.04, NaHCO3 22, CaCl2 1.0, Glucose 5, N-2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) 25, adjusted to pH 7.4 with NaOH. CaCl2 was then omitted or added in a 0.25 or 0.5 mM concentration to the experimental solution.

Measurement of Intracellular Sodium in Rat Aortic Smooth Muscle

RASM cells were grown to confluence on Falcon 3046 plates with DMEM (high glucose and 200 mM glutamine) and 10% fetal calf serum. The plates were divided into four rows of three wells. The media were removed and the wells were rinsed three times with 2.0 ml warm (37°C), nominally Ca2+-free solution. Experimental concentrations of Ca2+ (see above) were then added, and the cells returned to the incubator. After 5 minutes, the plates were removed and placed in an ice bath, the solutions suctioned, and the cells rapidly washed three times with 2 ml ice cold 110 mM MgCl2. Two milliliters of 0.1N HNO3 were then added, and the cells allowed to lyse for 4 hours at room temperature. The well contents were then transferred to plastic 12×75 mm tubes and the Na+ concentration determined by atomic absorption spectrophotometry. The Na+ concentration of the lysing solution was also measured and subtracted from all values as background. Protein concentration was determined by the Lowry method. Results are expressed as nanomoles Na+ per microgram protein.

Intracellular Electrophysiological Measurements

Plastic Petri dishes containing cultured RASM cells were mounted on the stage of an inverted microscope (IMT 2, Olympus Corp., Lake Success, New York), visualized under Hoffman Modulation Contrast (Modulation Optics, Inc, Greenvale, New York), and projected on a video monitor via a color video camera. During the experiment, the cells were continuously gassed with a mixture of 95% O2 and 5% CO2.

Intracellular microelectrodes were fabricated from borosilicate glass pipettes (WP Instr., New Haven, Connecticut) with an outer diameter of 1.8 mm and an inner diameter of 0.8 mm. Microelectrodes were horizontally pulled on a programmable Model P80/PE puller (Sutter Instr. Co., San Rafael, California) and then backfilled with 0.5 M KCl. Microelectrode tip resistance was measured on a Series 750 Dual microprobe amplifier (WP Instr.) where a 1 mV deflection of the baseline current output equals a 1 MΩ resistance across the microelectrode tip. Tip resistances were between 120 and 200 MΩ when tested in 1 M KCl. A microelectrode manipulator with hydraulic microdrive (MO-11N, Narishige, Tokyo, Japan) was mounted on the microscope stage and used to advance the microelectrode into the cell interior. An additional large tip (approxi-
mately 1 mm) micropipette was filled with agar-1 M KCl, immersed in the solution bathing the cells, connected to the ground terminal of the amplifier, and used as a reference electrode. Cell membrane potentials were measured on a FD-223 amplifier (WP Instr.) and recorded on a SC 284 pen recorder (Gould Instr., Cerritos, California) or a Fisher Recordall (Fisher Scientific, Tustin, California).

Criteria for acceptance of a valid cell membrane potential measurement were: 1) an abrupt negative deflection of the baseline potential indicative of microelectrode penetration into the cell interior, followed by a progressive stabilization of the cell membrane potential over a period not shorter than 30 seconds and 2) a rapid return to baseline when the microelectrode was withdrawn from the cell interior.

**Measurement of Intracellular Calcium**

\([\text{Ca}^{2+}]_i\) in RASM cells cultured on glass cover slips with the cell permeant fluorescent Ca\(^{2+}\) indicator fura 2-AM. 17 RASM cells grown to confluence were washed with PSS 2 and loaded with fura 2-AM (2 \(\mu\)M) during a 30-minute incubation at room temperature. The remaining fura 2-AM was then washed out, and the cover slip with cells attached was placed in a tissue culture bath chamber (Medical Systems Corp., Greeneville, New York) and mounted on an inverted stage fluorescence microscope (MT-2, Olympus Corp.). Fluorescence measurements were made at room temperature with a dual wavelength excitation light source (Photon Technology Intl., Princeton, New Jersey) and a photon-counting photomultiplier tube mounted to the side camera port of the microscope. A computer synchronized the photomultiplier signal collection with alternation of the excitation wavelength to separate the two emission wavelengths. The fluorescence ratio value \(R\) from the fluorescence measurements made at respective wavelengths. The fluorescence ratio value \(R\) between the background-subtracted emission fluorescence with 350 and 380 nm excitation, was used to estimate \([\text{Ca}^{2+}]_i\) according to the equation 17:

\[
[\text{Ca}^{2+}]_i = K_D \times \frac{(R - R_{\text{min}})/(R_{\text{max}} - R)}{(S_{380}/S_{350})}
\]

\(R_{\text{max}}\) and \(R_{\text{min}}\) were obtained by addition of the Ca\(^{2+}\) ionophore ionomycin (1 \(\mu\)M) followed by addition of EGTA (2 mM) and NaOH (10 mM) at the end of each experiment. \(S_{380}/S_{350}\) represents the ratio of fluorescence measurements at 380 nm excitation for \(R\) to that for \(R_{\text{max}}\) values. A \(K_D\) of 224 nM was used.

Results are presented as mean±SEM. Statistical analysis was performed with Student's t test for paired and unpaired observations.

**Results**

Microelectrode impalements were facilitated in dishes containing dispersed cells plated 1 or 2 days before the experiments. These cells displayed a more prominent cytoplasm whereas older, confluent subcultures appeared flattened, a characteristic that hampered the recording of stable intracellular potentials. There were no significant differences among Vcm measurements obtained from cells in DMEM supplemented with fetal calf serum (–46±1.6 mV, \(n=24\)), cells that were serum deprived overnight (–46±2.7 mV, \(n=16\)), or cells that were transferred to PSS 2 before the experiment (–44±2 mV, \(n=30\)). A time-dependent fall in Vcm values was not observed during our electrophysiological experiments, which had an approximate average duration of 2 hours. The occurrence of spontaneous fluctuations in Vcm (action potentials) or visible contractions in cultured RASM was not documented.

Isosmotic, partial replacement of the extracellular NaCl with increasing concentrations of KCl led to progressive depolarization of Vcm. An increase in the extracellular Ca\(^{2+}\) concentration from 5 to 50 mM decreased Vcm from –54±1.5 mV (\(n=3\)) to –11±1 mV (\(n=4\)) (Figure 1). Stepwise decrements in \([\text{Ca}^{2+}]_o\) concentration led to a similar depolarization of Vcm. Figure 2 depicts a representative experiment and demonstrates that the depolarization induced by the reduction in \([\text{Ca}^{2+}]_o\) is reversible. Vcm fell from a control value of –42±1 mV (\(n=11\)) in 1.8 mM Ca\(^{2+}\) to –28±4 mV (\(n=4\)) in 0.5 mM Ca\(^{2+}\) and to –13±2 mV (\(n=6\)) in 0.25 mM Ca\(^{2+}\) (Figure 3) and recovered to an average of –56±2 mV (\(n=4\)) on reintroduction of 1.8 mM Ca\(^{2+}\).

To explore the possibility that reduction of \([\text{Ca}^{2+}]_o\) resulted in intracellular sodium ([Na\(^{+}\)]\(_i\)) increments, measurements of [Na\(^{+}\)]\(_i\) were carried out in control conditions (1.8 mM Ca\(^{2+}\)) and during exposure to PSS 2 with a reduced Ca\(^{2+}\) concentration (0.5 or 0.25 mM) for 5 minutes, a time period when alterations in Vcm were already evident. Treatment with these low Ca\(^{2+}\) solutions was not associated with a measurable gain in [Na\(^{+}\)]\(_i\) by RASM cells. A summary of these results is presented in Table 1. In separate control experiments RASM cells were exposed to the Na\(^{+}\) permeabilizing agent monensin (10 \(\mu\)g/ml) for 5 minutes. This resulted in an increase in [Na\(^{+}\)]\(_i\) from 0.144±0.017 (\(n=5\)) to 0.341±0.071 nmol/\(\mu\)g protein (\(n=3\)).

To determine whether an alteration in \([\text{Ca}^{2+}]_o\) resulted from the reduction of \([\text{Ca}^{2+}]_o\), RASM cells were loaded with the Ca\(^{2+}\)-sensitive fluorescent dye fura 2-AM for \([\text{Ca}^{2+}]_i\) measurements, and the effect of lowering \([\text{Ca}^{2+}]_o\) was determined by sequential exchange of the extracellular solution with a Ca\(^{2+}\) concentration of 1.8 mM to one containing 0.5 mM Ca\(^{2+}\), 0.25 mM Ca\(^{2+}\), or a nominally Ca\(^{2+}\)-free solution. At each \([\text{Ca}^{2+}]_o\) decrement, \([\text{Ca}^{2+}]_i\) was monitored for 5 minutes continuously, and the mean of three \([\text{Ca}^{2+}]_i\) measurements (initial, middle,
FIGURE 1. Graph showing relation between cell membrane potential (Vcm) and extracellular potassium ([K+]o). In both 25 mM KCl and 50 mM KCl electrolyte solution, NaCl in physiological salt solution 2 (PSS 2) was reduced to 55 mM and partially replaced by 35 mM and 10 mM Choline Cl, respectively. Thus, NaCl concentration was similar in both high K+ solutions. Numbers in parentheses indicate number of successful microelectrode impalements. Note that Vcm values at a [K+]o of 5 mM (−54±1.5 mV, n=7) represent only measurements made in same cell culture dishes subsequently exposed to increasing [K+]o. Average Vcm obtained for all punctures in control PSS 2 was −44±2 mV, n=30.

Figure 2. Representative tracing showing reduction in extracellular calcium ([Ca2+]o) leads to a reversible depolarization of cell membrane potential (Vcm) in rat aortic smooth muscle (RASM) cells. Recording is read from left to right and arrows indicate penetration of cell interior with a glass microelectrode. Vcm is first measured when [Ca2+]o is 1.8 mM (tracing on extreme left) and after exposure to 0.5 mM Ca2+ solution (central tracing). Note slight overshoot in Vcm as [Ca2+]o is restored to control values (recovery, extreme right). Brackets indicate time intervals of 5 to 15 minutes between different experimental conditions.

Discussion

The measurements represented in Figure 1 confirm the known sensitivity of the resting Vcm in vascular smooth muscle to the extracellular potassium ([K+]o), resulting from the predominantly K+ permselective behavior of its cell membrane. Our slope of 42 mV per decade change in [K+]o is consistent with values previously published but is significantly less than the EK+ estimated to be in the vicinity of −80 mV (for a review on this subject see Reference 20). In our case, the discrepancy of Vcm with EK+ could be explained in part by experimental conditions. Our microelectrode experiments were conducted at room temperature, which may have diminished the contribution of the electrogenic, temperature-dependent Na+-K+ pump of vascular smooth muscle, thus accentuating the deviation of Vcm from the estimated EK+. It must be noted, however, that in one experiment we were able to determine Vcm while RASM cells were incubated...
TABLE 1. Measurement of Intracellular Sodium ([Na+]i, in Rat Aortic Smooth Muscle: Effect of Extracellular Calcium

<table>
<thead>
<tr>
<th>Ca2+-free</th>
<th>0.25 mM Ca2+</th>
<th>0.5 mM Ca2+</th>
<th>1.8 mM Ca2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Na+]i</td>
<td>0.154±0.019</td>
<td>0.140±0.023</td>
<td>0.135±0.021</td>
</tr>
<tr>
<td></td>
<td>(n=18)</td>
<td>(n=16)</td>
<td>(n=18)</td>
</tr>
<tr>
<td></td>
<td>0.142±0.03</td>
<td></td>
<td>(n=17)</td>
</tr>
</tbody>
</table>

Values (mean±SEM) are expressed as nanomoles Na+ per microgram protein. Control intracellular sodium ([Na+]i) measurements with the Na+ permeabilizing agent monensin are presented in the Results section. n, number of measurements.

in a Leiden microincubator system held at 37°C, and the average Vcm was -46.3±4 mV (n=3), a value that was similar to that obtained at room temperature (see Results).

The resting Vcm of vascular smooth muscle appears to be more sensitive to [Ca2+]o, as compared with other preparations.23-25 Casteels et al25 reported findings similar to ours in rabbit pulmonary artery smooth muscle, but the magnitude of the depolarization of Vcm observed by these authors on [Ca2+]o reduction was significantly less pronounced than that observed in the present study. Their work revealed that [Ca2+]o effects on Vcm did not require the presence of Na+, nor did they result from a disruption in cell membrane integrity since treatment with low Ca2+ solutions resulted in an increase and not a decrease in membrane input resistance. [Ca2+]o was not measured in this study.25

Addition of divalent ions to solutions bathing negatively charged artificial lipid membranes containing K+ carriers produces a marked increase in surface potential.26 Through a similar mechanism, binding of divalent cations to the cell membrane could result in substantial modification of the electrical field in the immediate vicinity of ion channels.

Reduction of [Ca2+]o could have depolarized Vcm through an alteration of surface charge or gating properties of ion channels responsible for the generation of Vcm in RASM cells. The 29 mV depolarization observed when [Ca2+]o was reduced approximately 1 decade (Figure 3) is in agreement with that predicted for charged phospholipid membranes26; yet a careful analysis of the "surface potential" theory suggests that it cannot fully explain the electrophysiological effects of a reduction in extracellular divalent ion concentration.27 In a careful patch-clamp study, Inoue et al16 reported a K+ channel in rabbit portal vein smooth muscle with distinctive electrophysiological properties. In contrast to previously reported K+ channels in this preparation,28 this channel exhibited no sensitivity to [Ca2+]o of the magnitude encountered in the cytosol (10-7 to 10-6 M) but was highly sensitive to [Ca2+]o (7.5 μM to 2.8 mM). Current voltage relation studies of this K+ channel suggested that it may contribute to the generation of the resting Vcm of rabbit portal vein smooth muscle. The possibility that similar K+ channels, mainly responsive to [Ca2+]o, are present in the cell membrane of RASM is particularly appealing in view of the effects of [Ca2+]o on Vcm that we describe (Figures 2 and 3).

Our measurements of [Ca2+]o with fura 2-AM reveal that [Ca2+]o in the presence of reduced [Ca2+]o was approximately similar to that obtained when [Ca2+]o was 1.8 mM. We therefore consider it improbable that changes in Vcm that accompanied [Ca2+]o reduction resulted from a decrease in [Ca2+]i. Moreover, our results support the notion that [Ca2+]o, interaction with the cell membrane rather than cytosolic Ca2+ affects the resting Vcm in RASM.

FIGURE 4. Representative tracing showing measurement of intracellular calcium ([Ca2+]i) in rat aortic smooth muscle cells loaded with fluorescent Ca2+ indicator fura 2-AM (see Materials and Methods). Tracing of [Ca2+]i in representative time course study is shown. [Ca2+]i was monitored continuously when extracellular calcium ([Ca2+]o) was reduced from 1.8 mM to 0.5 mM, 0.25 mM, and during exposure to nominally Ca2+-free physiological salt solution 2. Note that [Ca2+]i remained essentially stable during changes in [Ca2+]o. At end of experiment (right), [Ca2+]i was changed to 1.8 mM and angiotensin II (AlI) (10-7 M) was added resulting in a brisk increase in [Ca2+]i.
Similarly, other Ca\(^{2+}\)-dependent cellular functions, such as certain forms of exocytosis, require continuous transmembrane Ca\(^{2+}\) transport rather than a change in cytosolic Ca\(^{2+}\) activity.\(^{29}\) The importance of alterations in [Ca\(^{2+}\)], that occur within various cell compartments and are not measured by techniques that only provide an average [Ca\(^{2+}\)], signal has been stressed\(^{30}\) and may explain our failure to detect a sustained fall in [Ca\(^{2+}\)] during treatment with low Ca\(^{2+}\) solutions. Also our [Ca\(^{2+}\)] measurements reflect average values for 5–10 cells (see Materials and Methods). Accordingly, it was possible to miss a decrease in [Ca\(^{2+}\)], in an individual cell depolarized on reduction of [Ca\(^{2+}\)].

The possibility that inhibition of the Na\(^+\),K\(^+\) adenosine triphosphatase (ATPase) on [Ca\(^{2+}\)], reduction resulted in depolarization of Vcm must be considered. Kino et al\(^{31}\) explored the effects of [Ca\(^{2+}\)], on the Na\(^+-\)K\(^+\)-ATPase in cultured rat carotid artery smooth muscle cells. [Ca\(^{2+}\)], reduction was associated with stimulation and not inhibition of the Na\(^+-\)K\(^+\)-pump. This increased activity was brought about by a rise in [Na\(^+\)] but intracellular potassium ([K\(^+\)]) concentration was not altered significantly. In contrast to these findings, we did not detect an increase in [Na\(^+\)], as a result of [Ca\(^{2+}\)], reduction. The difference may reside in the methodologies employed. We measured [Na\(^+\)], 5 minutes after exposure to low Ca\(^{2+}\) solutions, whereas Kino et al\(^{31}\) used lengthier incubations under similar conditions and presumably induced changes in [Na\(^+\)], that did not become manifest in our relatively short time course experiments. In any case, it is of critical importance to bear in mind that only a relatively small increment in Na\(^+\) influx,\(^{2}3\) coupled with a compensatory rise in Na\(^+\),K\(^+\)-ATPase activity\(^{31}\) and Na\(^+\) efflux, could have depolarized Vcm without a measurable gain in [Na\(^+\)]. In other words, our inability to detect a rise in [Na\(^+\)], on [Ca\(^{2+}\)], reduction, does not rule out a major role for Na\(^+\) in our electrophysiological findings.

In a recent review, Haecusler\(^{21}\) noted that raising the extracellular K\(^+\) from 15 to 30 mM resulted in more than 70% contraction of rabbit main pulmonary artery smooth muscle cell strips and a drop in Vcm from -45 to -33 mV. This suggests that a relatively small cell membrane depolarization is required to induce a significant increase in arterial smooth muscle tension. In addition, depolarization or hyperpolarization of the resting Vcm by only 4 to 6 mV may result in significant changes in tension development on smooth muscle cell interaction with vasoactive agents and neurotransmitters.\(^{20}\) In view of this, variations in [Ca\(^{2+}\)], concentrations could play an important role in the modulation of vascular reactivity to agonists in the absence of a simultaneous perturbation in [Ca\(^{2+}\)].

In summary, we have measured the resting cell membrane potential of RASM in the presence of a varying [Ca\(^{2+}\)], and correlated these measurements with [Ca\(^{2+}\)]. Reduction of [Ca\(^{2+}\)], was not associated with parallel changes in [Ca\(^{2+}\)] despite marked depolarization of Vcm. Our observations support the notion that [Ca\(^{2+}\)], plays a critical modulatory role of the resting ionic permeability of vascular smooth muscle, but its effects do not appear to be mediated through [Ca\(^{2+}\)].

Acknowledgments

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