Ca^{2+} uptake in GH_3 cells during hypotonic swelling: the sensory role of stretch-activated ion channels

YE CHEN, STEVEN M. SIMASKO, JEFFREY NIGGEL,
WADE J. SIGURDSON, AND FREDERICK SACHS
Department of Biophysical Sciences, State University of New York at Buffalo, Buffalo, New York 14214

Chen, Ye, Steven M. Simasko, Jeffrey Niggel, Wade J. Sigurdson, and Frederick Sachs. Ca^{2+} uptake in GH_3 cells during hypotonic swelling: the sensory role of stretch-activated ion channels. *Am. J. Physiol.* 270 (*Cell Physiol.* 39): C1790–C1798, 1996.—Hypotonic cell swelling triggers an increase in intracellular Ca^{2+} concentration that is deemed responsible for the subsequent regulated volume decrease in many cells. To understand the mechanisms underlying this increase, we have studied the Ca^{2+} sources that contribute to hypotonic cell swelling-induced Ca^{2+} increase (HICI) in GH_3 cells. Fura 2 fluorescence of cell populations revealed that extracellular, but not intracellular, stores of Ca^{2+} were required. HICI was abolished by nifedipine, a blocker of L-type Ca^{2+} channels, and Gd^{3+}, a nonspecific blocker of stretch-activated channels (SACs), suggesting two components for the Ca^{2+} membrane pathway: L-type Ca^{2+} channels and SACs. Using HICI as an assay, we found that venom from the spider *Grammostola spatulata* could block HICI without blocking L-type Ca^{2+} channels. The venom did, however, block SAC activity. This suggests that Ca^{2+}-permeable SACs, rather than L-type Ca^{2+} channels, are the sensing elements for HICI. These results support the model for volume regulation in which SACs, activated by an increase of the membrane tension during hypotonic cell swelling, trigger HICI, leading to a volume decrease.

stretch-activated channels; L-type calcium channels; nifedipine; gadolinium; spider venom; blocker; thyrotropin-releasing hormone; inositol 1,4,5-trisphosphate-sensitive stores; volume regulation; regulated volume decrease; *Grammostola spatulata*

CELL VOLUME REGULATION is essential for cells exposed to an environment with changing osmolarity. Numerous studies have elucidated that regulated volume decrease is caused by extrusion of water accompanying the extrusion of osmolytes, including ions, amino acids, and sugars, through a variety of transport systems (9, 25). However, the signaling mechanisms that relay hypotonic swelling to the activation of transporters remain to be elucidated. In many cells, hypotonic swelling produces an increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$; Ref. 19). The elevation of $[Ca^{2+}]_i$ may activate Ca^{2+}-activated K^+ channels (32) and Ca^{2+}-activated Cl^- channels (13), allowing an outflow of K^+ and Cl^-, thus reducing the cytoplasmic osmolarity and resulting in a volume decrease. We present here data that support the hypothesis that stretch-activated ion channels are the sensory elements.

Previous studies have suggested three Ca^{2+} sources that may contribute to hypotonic cell swelling-induced Ca^{2+} increase (HICI): extracellular Ca^{2+} (19), inositol 1,4,5-trisphosphate (IP_3)-sensitive stores (3), and ryanodine-sensitive stores (12). For example, extracellular Ca^{2+} is necessary for HICI in many cells such as osteosarcoma, intestinal epithelial cells, and gall bladder and urinary bladder cells (19). IP_3-sensitive stores may contribute to HICI in Ehrlich ascites tumor cells (32a), astrocytes (2), and proximal tubule cells (30). Ca^{2+}-induced Ca^{2+} release from ryanodine-sensitive stores may participate in the HICI of human intestinal 407 cells (12). Considering the diversity of available machinery in different cells, it is likely that the involvement of Ca^{2+} sources in HICI is cell specific. In the cells that require extracellular Ca^{2+} for HICI, Ca^{2+}-permeable channels in the plasma membrane may hold important clues about the sensory mechanism of HICI. Previous studies have suggested two Ca^{2+} channels: L-type Ca^{2+} channels and Ca^{2+}-permeable stretch-activated channels (SACs). The evidence that supports the involvement of L-type Ca^{2+} channels comes from the finding that HICI is inhibited by some blockers of L-type Ca^{2+} channels. For example, nicardipine, diltiazem, and verapamil inhibit HICI in osteosarcoma UMR-106-01 cells (33) and in renal proximal tubule cells (17, 18). In astrocytes, nimodipine partially blocks hypotonic swelling-induced $^{45}Ca^{2+}$ entry (22). The hypothesis that SACs play a role in HICI stems from two indirect lines of evidence: *1*) Gd^{3+}, a nonspecific blocker of SACs, inhibits HICI (23), and *2*) disruption of microfilaments inhibits regulatory volume decrease (5, 7). A reasonable hypothesis is that hypotonic swelling exerts a hydrostatic pressure on the cell membrane that increases the average membrane tension. This membrane tension is transmitted by the cytoskeleton to SACs, causing activation (11). However, because of the lack of a specific blocker of SACs, their involvement could not be rigorously tested.

The goal of our study is to dissect the Ca^{2+} pathways responsible for HICI, specifically to distinguish the role of L-type Ca^{2+} channels and Ca^{2+}-permeable SACs. We used the GH_3 cell line because these cells exhibited a significant and sustained HICI response, possessed SACs, and exhibited volume regulation. We first identified the Ca^{2+} sources contributing to HICI and found that extracellular Ca^{2+} was necessary for HICI and intracellular Ca^{2+} stores were not. Then we studied the role of L-type Ca^{2+} channels and SACs and found that blockers of L-type Ca^{2+} channels and SACs inhibited HICI. This suggested the possible involvement of both channels, although the specificity of the blockers remained an issue. To distinguish the roles of the two channels, we sought a blocker with specificity for one channel. Using HICI viewed with fura 2 as an assay, we discovered a spider venom that was able to inhibit HICI

without blocking L-type Ca^{2+} channels. This activity suggested that L-type Ca^{2+} channels were not sufficient for HICI, leaving the suggestion that SACs were the trigger for HICI, either acting directly or through depolarization-induced activation of Ca^{2+} channels.

METHODS

GH_3 cell culture. GH_3 cells, a rat pituitary cell line, were cultured in standard GH_3 culture medium containing 82.5% Ham's F-10 nutrients (GIBCO, no. 430–1200EB), 15% heat-inhibited horse serum (GIBCO, no. 26050–013), and 2.5% fetal bovine serum (GIBCO, no. 26140–012) at 37°C in 10% CO_2. Cells were fed twice per week and subcultured once per week. For fura 2 fluorescence measurements, cells were plated on poly-L-lysine-coated glass coverslips at 95% confluence, cultured under normal conditions, and used between *day 3* and *6* after plating.

Fura 2 fluorescence ratio measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured with the fura 2 fluorescence ratio method on an SLM Aminco-Bowman 2 fluorescence spectrometer. Cells were loaded with fura 2 in normal saline solution containing 2 μM fura 2-acetoxymethyl ester and 0.05% Pluronic-127 detergent with the use of the following procedure: *1*) rinse cells twice in phosphate-buffered saline (PBS), *2*) incubate cells in the loading solution for 30 min at 25°C, *3*) rinse cells twice with PBS, and *4*) incubate cells in the culture medium for 30 min at 25°C. Experiments were performed within 1 h after loading.

The coverslip was mounted in a custom-made holder and placed in a 5-ml quartz cuvette at an angle of 20° to the excitation beam. (In a few later experiments, a 20-μl chamber was used as noted.) Fluorescence emission was collected from a group of ~10^5 cells located in the excitation path. The excitation beam at wavelength 340 nm (Ex340) and 380 nm (Ex380) were used, and the fluorescence intensities at emission wavelength 510 nm (Em510) were monitored. The maximum data acquisition rate was two data points per second. An increase in the Ca^{2+} concentration caused an increase in the fluorescence intensity at Em510/Ex340 and, at same time, a decrease at Em510/Ex380. The ratio of the two (R) was used to calculate the Ca^{2+} concentration, using the formula (9)

$$[Ca^{2+}] = K_d \cdot \frac{R - R_{min}}{R_{max} - R} \cdot \frac{F_f}{F_b} \quad (1)$$

where K_d is the equilibrium constant, R_{max} is the fluorescence ratio, and F_b is the fluorescence intensity at excitation wavelength 380 nm, when fura 2 is saturated with Ca^{2+} in R_{max} solution containing 5 mM Ca^{2+} (Table 1). R_{min} is the fluorescence ratio and F_f is the fluorescence intensity at excitation wavelength 380 nm, when fura 2 is not bound by Ca^{2+} in R_{min} solution containing 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (Table 1). A K_d of 224 nM was used as given by Grynkiewicz et al. (10) for the high-K^+ condition.

For simplicity, in many experiments, we simply used R as an indicator of Ca^{2+} activity rather than converting it to an estimated Ca^{2+} level. In a population of spontaneously active cells, the calculated Ca^{2+} may in fact be misleading.

Video imaging. The volume of GH_3 cells was measured by video imaging, using dark field and differential interference contrast optics. The images were obtained under a Zeiss Axioskop microscope with a cardioid condenser (40 × 1.0 numerical aperture, iris diaphragm) and a DAGE MTI Series 67 Nuvicon camera, recorded on a JVC-HRS10000V editing recorder and a Panasonic-AG6720A time-lapse recorder, acquired by using a NOVA-810 time-base corrector and a Mac Quadra 950 computer with a Perceptics Pixel Pipeline board. Software BDS-Image (Biological Detection Systems) was used for image acquisition and processing.

GH_3 cells were trypsinized and suspended in the isotonic solution for at least 20 min to allow an equilibration of the initial volume and to become spherical. A glass pipette was then used to hold a cell with low suction to avoid deforming the cell. The cell chamber was then perfused. The mean cell diameter was measured from the images and, assuming a sphere, used to calculate the cell volume that was then normalized to the initial volume in isotonic solution.

Solutions and chemicals. Solutions and chemicals are listed in Table 1. *Grammostola spatulata* venom was obtained from SpiderPharm (Seasterville, PA). Notice that the only difference between the isotonic and hypotonic solutions was the mannitol concentration. The concentration of all the ions was kept constant. To avoid mechanical disturbances, solution flow was generally kept constant. A complete exchange of solutions in the 5-ml perfusion chamber took ~1 min and a few seconds in the 20-μl chamber.

RESULTS

Hypotonic swelling caused a significant increase of $[Ca^{2+}]_i$. Immediately after a hypotonic exposure, GH_3 cells first undergo swelling and then a regulated volume decrease. As shown in a sample trace in Fig. 1*A*, hypotonic exposure caused the cell to swell immediately and, within 1 min, to reach a peak ~30% larger than its initial volume. The cell then regulated its volume back to a steady state that was ~10% larger than its initial volume. Switching to the isotonic solution caused the cell to shrink immediately below control values and then return to the initial volume.

Exposing GH_3 cells to hypotonic solution caused a significant increase of $[Ca^{2+}]_i$. Figure 1*B* demonstrates a typical HICI response. In this case, cells had a basal

Table 1. *Solutions*

Full Name	NaCl	KCl	$CaCl_2$	$MgCl_2$	HEPES	Mannitol	Glucose	EGTA	Triton	Osmolarity
Normal saline	135	5	1	2	10	10	10			319
Isotonic solution	65	5	1	2	10	160				319
Isotonic Ca^{2+} free	65	5	0	3	10	160				319
Hypotonic solution	65	5	1	2	10	20				179
Hypotonic Ca^{2+} free	65	5	0	3	10	20				179
High-K^+ 65 mM isotonic	65	65	1	2	10	40				319
High-K^+ 65 mM Ca^{2+} free	65	65	0	3	10	40				319
R_{max} solution	20	115	5	1	10	20		0	0.10%	
R_{min} solution	20	115	0	1	10	30		5	0.10%	

Values are in mM.

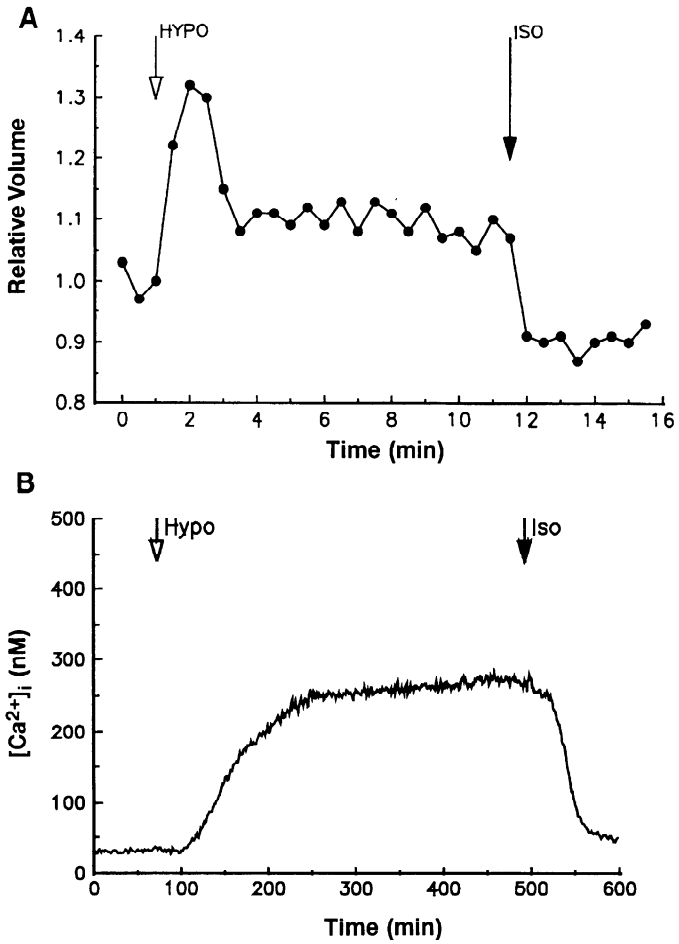

Fig. 1. Hypotonic cell swelling, regulatory volume decrease (RVD), and hypotonic cell swelling-induced Ca^{2+} increase (HICI). *A*: relative cell volume plotted against time course of solution exchange. Hypotonic (Hypo) exposure caused cell to swell by 30% in ~1 min. It then underwent a RVD that brought volume back to a steady state of ~10%. Switching to isotonic (Iso) solution caused cell to shrink rapidly below its initial volume, consistent with a loss of osmolytes during RVD. Cell then underwent a volume increase to recover gradually toward initial volume. *B*: HICI response. Basal level intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in isotonic solution was ~30 nM. Switching to hypotonic solution caused $[Ca^{2+}]_i$ to increase more than 8-fold and reach a plateau level of ~250 nM within 2 min. $[Ca^{2+}]_i$ remained at plateau level throughout hypotonic exposure. Switching back to isotonic solution caused $[Ca^{2+}]_i$ to return to basal level.

level $[Ca^{2+}]_i$ of ~30 nM in the isotonic solution. Switching to hypotonic solution caused $[Ca^{2+}]_i$ to increase to ~250 nM, and restoring the isotonic solution caused $[Ca^{2+}]_i$ to rapidly return to the basal level.

To summarize the results from >80 experiments, the HICI response in GH_3 cells consistently showed four characteristics. *1*) Exposure to hypotonic solution caused a significant increase of $[Ca^{2+}]_i$. The magnitude of the $[Ca^{2+}]_i$ increase varied somewhat among cell preparations, perhaps because of the variations in cell culture conditions such as growth stage and density and possible variations in dye loading. The peak $[Ca^{2+}]_i$ increase ranged from 4 to 11 times the basal level. *2*) The time course of the $[Ca^{2+}]_i$ increase was sigmoidal, with a time to half-maximum ranging from 20 s to 4 min, averaging 1.5 min for the 5-ml chamber. *3*) $[Ca^{2+}]_i$ remained elevated during the entire period of hypotonic exposure. The longest time tested was 12 min. *4*) Returning to isotonic solution caused $[Ca^{2+}]_i$ to return to the basal levels within ~30 s.

Extracellular Ca^{2+} was necessary for HICI. The dependence of HICI on the extracellular Ca^{2+} was studied by removing Ca^{2+} from the solution at three different stages of hypotonic exposure. In the first experiment, Ca^{2+} was simply removed from the hypotonic solution (Fig. 2*A*). $[Ca^{2+}]_i$ exhibited a monotonic decrease from the basal level: HICI was abolished. Returning to isotonic solution (containing 1 mM Ca^{2+}) caused a transient increase of $[Ca^{2+}]_i$, which declined to the

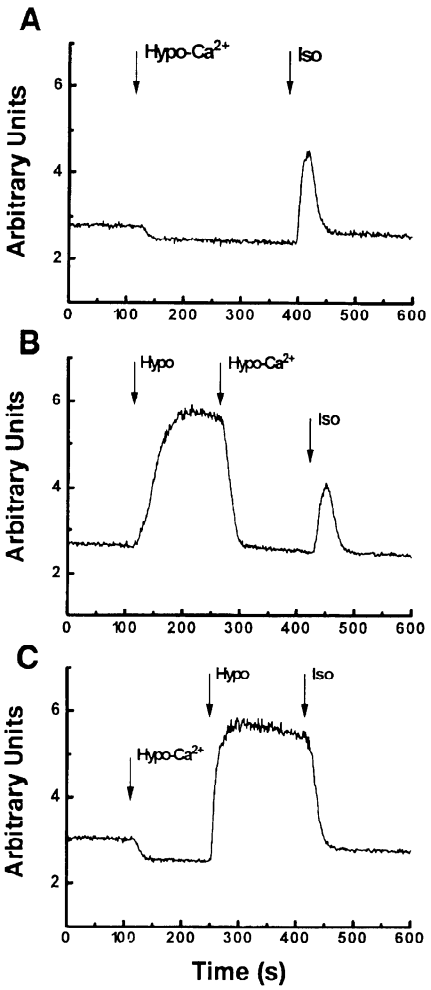

Fig. 2. Dependence of HICI on extracellular Ca^{2+}. *A*: removing Ca^{2+} from hypotonic solution (Hypo − Ca^{2+}) lowered $[Ca^{2+}]_i$ and abolished HICI. Switching back to isotonic solution evoked a transient increase of $[Ca^{2+}]_i$. This increase was probably caused by an influx of $[Ca^{2+}]_i$ through channels previously opened by hypotonic stress and somewhat to increase in concentration caused by cell shrinkage (cf. Fig. 1). (Experiments in *A–C* were performed with a 20-μl chamber.) *B*: extracellular Ca^{2+} is required to maintain HICI. Exposure to hypotonic solution caused $[Ca^{2+}]_i$ to increase in a normal HICI response. Removing Ca^{2+} from hypotonic solution (Hypo − Ca^{2+}) rapidly brought $[Ca^{2+}]_i$ back to basal level. Return to Ca^{2+} containing isotonic solution caused a transient increase in $[Ca^{2+}]_i$ as discussed above. *C*: extracellular Ca^{2+} is required to initiate HICI. Exposing cells to Ca^{2+}-free hypotonic solution (Hypo − Ca^{2+}) lowers cell Ca^{2+}. Returning $[Ca^{2+}]_i$ to hypotonic solution rescued HICI. Return to Ca^{2+} containing isotonic solution caused a transient increase in $[Ca^{2+}]_i$ as discussed above.

basal level within a minute. The $[Ca^{2+}]_i$ transient had a much shorter delay than normal HICI, suggesting that it resulted from an influx through the pathways previously activated by hypotonic exposure. In the second experiment (Fig. 2*B*), a normal hypotonic exposure caused a large increase in $[Ca^{2+}]_i$. When Ca^{2+} was removed from the hypotonic bathing solution, $[Ca^{2+}]_i$ rapidly decreased. Thus a continued presence of extracellular Ca^{2+} was necessary for HICI. In the third experiment (Fig. 2*C*), cells were exposed to Ca^{2+}-free hypotonic solution, causing $[Ca^{2+}]_i$ to fall again below the basal level. Switching to hypotonic solution with normal Ca^{2+} (1 mM) caused $[Ca^{2+}]_i$ to increase much more rapidly than normal HICI, suggesting that the Ca^{2+} pathways are activated by hypotonic exposure in the absence of Ca^{2+}. HICI was rescued simply by the addition of extracellular Ca^{2+}. In summary, our results ($n > 18$ experiments) consistently showed that extracellular Ca^{2+} was necessary for inducing and maintaining an elevated $[Ca^{2+}]_i$ level throughout HICI.

IP_3-sensitive stores were not essential for HICI. IP_3-sensitive stores in GH_3 cells can be stimulated by thyrotropin-releasing hormone (TRH; Ref. 24). To examine the involvement of these stores in HICI, we used TRH to release Ca^{2+} (Fig. 3*A*). Cells had a basal level $[Ca^{2+}]_i$ of 75 nM. TRH (1 μM) caused a transient $[Ca^{2+}]_i$ increase, peaking at 200 nM, followed by a small sustained elevation to 100 nM in agreement with the results reported by Albert and Tashjian (1). Subsequent hypotonic exposure caused a further increase of $[Ca^{2+}]_i$, similar to normal HICI. The fact that HICI occurred despite the TRH pretreatment strongly suggests that HICI was not dependent on these IP_3-sensitive stores ($n = 5$ experiments). We then examined whether IP_3-sensitive stores contribute to HICI (Fig. 3*B*). A normal HICI response increased $[Ca^{2+}]_i$ from a basal level of 75 nM to a plateau level of 300 nM. TRH (1 μM) was then added to the hypotonic solution, which caused a further increase of $[Ca^{2+}]_i$, from 300 nM to a peak of 375 nM. The transient characteristics of this $[Ca^{2+}]_i$ increase resembled a standard TRH response ($n = 5$ experiments). HICI did not deplete Ca^{2+} from these stores. The results clearly suggest that TRH-sensitive stores were not essential for HICI.

Nifedipine, a blocker of L-type Ca^{2+} channels, inhibited HICI. Because extracellular Ca^{2+} is required for HICI, it was likely that membrane Ca^{2+} channels played a critical role. We studied the role of L-type Ca^{2+} channels in HICI by using nifedipine to block these channels (28) at various stages of hypotonic exposure. Cells were exposed to hypotonic solution containing 10 μM nifedipine (Fig. 4*A*), and $[Ca^{2+}]_i$ failed to increase, except during the mixing transient. HICI was basically abolished. Nifedipine (10 μM) was then added to the hypotonic solution after $[Ca^{2+}]_i$ had been elevated from a basal level of ~30 nM to a plateau level of ~210 nM (Fig. 4*B*). Nifedipine caused $[Ca^{2+}]_i$ to decrease rapidly toward the basal level. Thus nifedipine inhibited HICI ($n = 10$ experiments), presumably by blocking L-type Ca^{2+} channels. To study the reversibility of nifedipine, cells were pretreated and then exposed to the hypotonic solution (Fig. 4*C*). In isotonic solution, nifedipine (5 μM) decreased basal level $[Ca^{2+}]_i$ from ~50 to ~30 nM, in agreement with Scherubl's and Hescheler's (26) earlier findings. A subsequent hypotonic exposure (without nifedipine) caused $[Ca^{2+}]_i$ to rise from ~30 to ~70 nM, slightly above the initial basal level. The pattern of this $[Ca^{2+}]_i$ elevation resembled a greatly attenuated HICI response, but the inhibition of HICI was not reversible within 5 min.

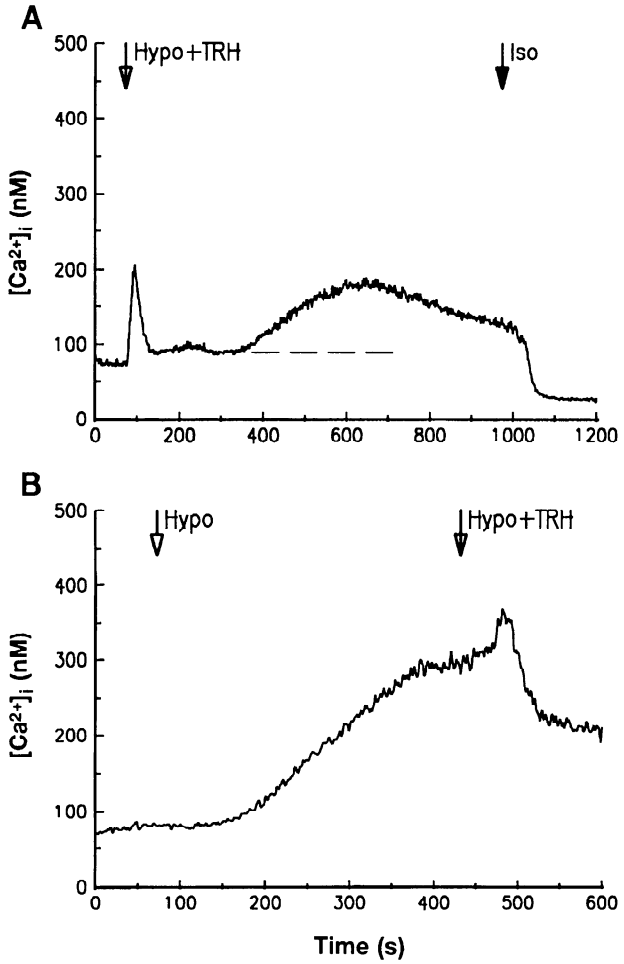

Fig. 3. Relation between HICI and Ca^{2+} release from inositol 1,4,5-trisphosphate (IP_3)-sensitive stores. *A*: HICI acts in presence of thyrotropin-releasing hormone (TRH). Cells were exposed to TRH and hypotonic solution at same time. TRH (1 μM) caused a rapid transient increase of $[Ca^{2+}]_i$ from basal level of 75 to 210 nM, followed by a slight elevation of $[Ca^{2+}]_i$ of 100 nM. This $[Ca^{2+}]_i$ response resembled a standard TRH response as shown by other investigators (shown by dashed line). $[Ca^{2+}]_i$ elevation above dashed line was because of HICI. Peak $[Ca^{2+}]_i$ during HICI was 190 nM, ~2.5 times basal level. *B*: TRH acts in presence of HICI. Cells were first exposed to hypotonic solution. $[Ca^{2+}]_i$ increased to 300 nM, ~4 times basal level of 75 nM. A subsequent application of TRH (1 μM) caused a further transient $[Ca^{2+}]_i$ increase to 375 nM, followed by a rapid decline to 200 nM.

Gd^{3+}, a generic blocker of SACs, inhibited HICI. The involvement of SACs was examined by using Gd^{3+}, as we did with nifedipine for Ca^{2+} channels (34). First, Gd^{3+} (20 μM) was added to the hypotonic solution (Fig. 5*A*). $[Ca^{2+}]_i$ failed to rise: HICI was inhibited. Then Gd^{3+} was added after $[Ca^{2+}]_i$ had reached its peak during HICI (Fig. 5*B*). It caused $[Ca^{2+}]_i$ to rapidly

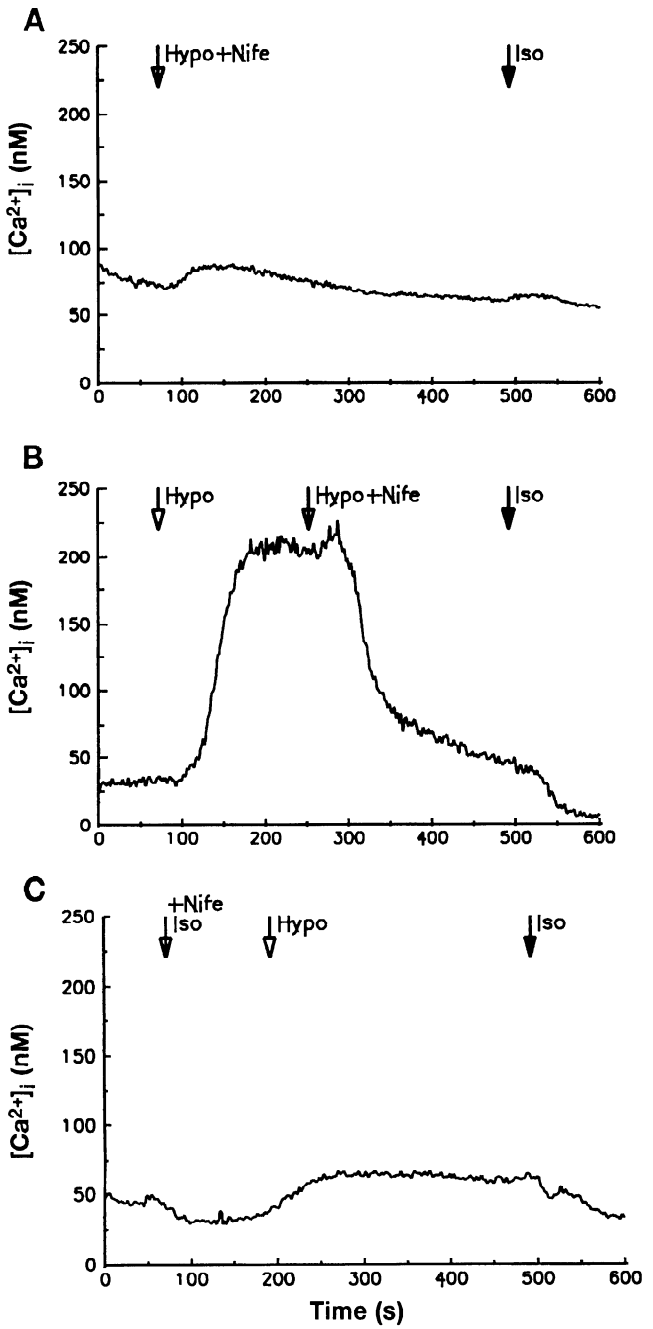

Fig. 4. Nifedipine blocks HICI. *A*: nifedipine (10 μM) was added to hypotonic solution (Hypo + Nife) from beginning of hypotonic exposure. *B*: cells were first exposed to hypotonic solution. HICI response showed a 7-fold increase of $[Ca^{2+}]_i$ from a basal level of 30 to 210 nM. Nifedipine (10 μM) was then added to hypotonic solution (Hypo + Nife), causing a rapid decrease of $[Ca^{2+}]_i$ toward basal level. *C*: effect of nifedipine was not reversed in 5 min. Cells were first treated with 5 mM nifedipine (Nife + Iso), causing $[Ca^{2+}]_i$ to decrease to 30 nM, below basal level of 50 nM. A subsequent hypotonic exposure caused a small $[Ca^{2+}]_i$ increase to ~60 nM. HICI response was greatly attenuated.

decrease toward the basal level (n = 12 experiments). To investigate reversibility, cells were treated with Gd^{3+} before hypotonic exposure (Fig. 5*C*). A subsequent hypotonic exposure (without Gd^{3+}) failed to elevate $[Ca^{2+}]_i$ within 5 min (n = 6 experiments).

Gd^{3+} also blocked L-type Ca^{2+} channels. The activity of L-type Ca^{2+} channels can be assessed by measuring the depolarization-induced Ca^{2+} increase with high K^+ (8). Figure 6*A* shows the $[Ca^{2+}]_i$ elevation induced by three consecutive high-K^+ exposures of 2 min applied at 3-min intervals. Each stimulation caused a similar elevation of $[Ca^{2+}]_i$.

Figure 6*D* shows that $[Ca^{2+}]_i$ elevation during depolarization requires extracellular Ca^{2+}. The protocol of three consecutive stimuli was used again, except that

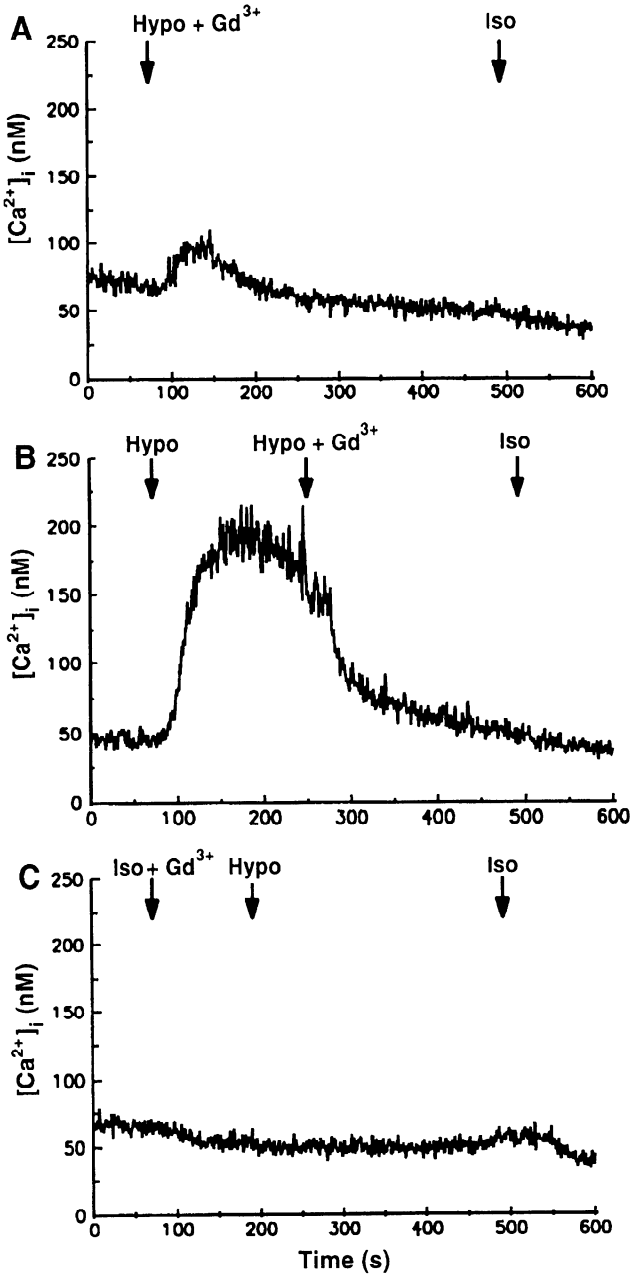

Fig. 5. Gd^{3+} blocks HICI. *A*: when added to hypotonic solution (Hypo + Gd^{3+}) from beginning of hypotonic exposure, Gd^{3+} (20 μM) inhibited HICI. *B*: cells were first exposed to hypotonic solution. $[Ca^{2+}]_i$ increased from a basal level of 50 to 200 nM. Gd^{3+} (20 μM) was then added to hypotonic solution (Hypo + Gd^{3+}), which caused a rapid decrease of $[Ca^{2+}]_i$ toward basal level. *C*: effect of Gd^{3+} was irreversible in 5 min. Cells were first treated by 20 μM Gd^{3+} (Iso + Gd^{3+}). A subsequent hypotonic exposure failed to induce HICI.

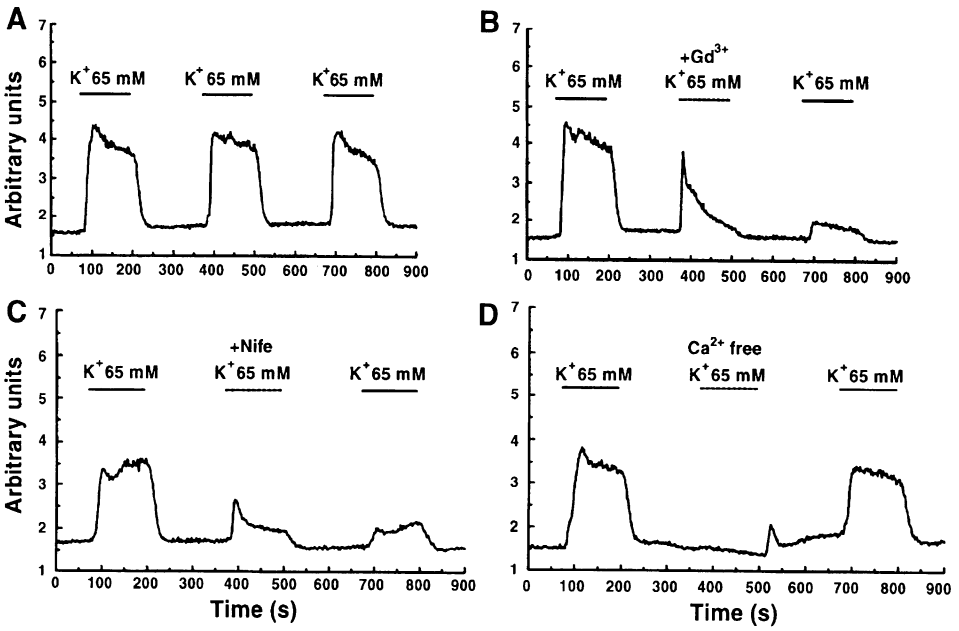

Fig. 6. Depolarization-induced Ca^{2+} increase. *A*: an increase in $[Ca^{2+}]_i$ was evoked by using a high K^+ (65 mM) isosmotic solution (K^+ 65 mM) to depolarize cell. Peak and plateau levels of $[Ca^{2+}]_i$ were similar in 3 repetitive responses. $[Ca^{2+}]_i$ always returned to basal level on switching back to isotonic solution containing normal K^+ concentration (5 mM). *B*: Gd^{3+} (10 μM) inhibited Ca^{2+} increase caused by depolarization following same protocol used in *A*. *C*: adding nifedipine to solution (K^+ 65 mM + Nife) during 2nd stimulation significantly inhibited $[Ca^{2+}]_i$ elevation. Inhibitory effect of nifedipine was not reversible within experimental duration as shown in 3rd stimulation. *D*: same protocol was used except that, during 2nd stimulation, Ca^{2+} was removed from high-K^+ isotonic solution (Ca^{2+}-free K^+ 65 mM). $[Ca^{2+}]_i$ failed to rise in absence of extracellular Ca^{2+}. Returning Ca^{2+} to solution rescued $[Ca^{2+}]_i$ elevation in 3rd stimulation.

Ca^{2+} was removed from the extracellular solution during the second stimulation. The first stimulation caused a normal $[Ca^{2+}]_i$ elevation. During the second stimulation, without extracellular Ca^{2+}, $[Ca^{2+}]_i$ failed to increase. Restoring Ca^{2+} to the extracellular solution during the third stimulation rescued the $[Ca^{2+}]_i$ elevation. The experiment shown in Fig. 6*C* suggests that L-type Ca^{2+} channels were responsible for the depolarization-induced Ca^{2+} increase. With the use of three consecutive stimuli, nifedipine (10 μM) was added during the second. The first stimulation caused a normal elevation of $[Ca^{2+}]_i$. Blocking Ca^{2+} channels during the second inhibited $[Ca^{2+}]_i$ elevation. The nifedipine inhibition was only slightly reversible within 5 min.

The effect of Gd^{3+} on L-type Ca^{2+} channels was unknown in this preparation. To investigate the Gd^{3+} effect, the protocol of three consecutive depolarizations was again used with Gd^{3+} (10 μM) added during the second stimulation (Fig. 6*B*). The first stimulation induced a normal $[Ca^{2+}]_i$ increase. The second failed to evoke a normal $[Ca^{2+}]_i$ increase. The inhibitory effect of Gd^{3+} was not reversible within 5 min, as seen in the third stimulation. These results suggested that Gd^{3+} blocked L-type Ca^{2+} channels in GH_3 cells.

Spider venom inhibited HICI without blocking L-type Ca^{2+}. Because Gd^{3+} appears to block both L-type Ca^{2+} channels and SACs, and the effect of nifedipine on SACs was unknown in GH_3 cells, neither Gd^{3+} nor nifedipine could be used to differentiate the role of the two channels. Prompted by patch-clamp studies on *Xenopus* oocytes (C. Bowman and F. Sachs, unpublished observations) in which venom from the spider *Grammostola* was found to block cationic SACs, we studied its effect on GH_3 cells.

The venom exerted at least two effects on $[Ca^{2+}]_i$ (Fig. 7*A*). The main effect was a block of HICI. At 15,000× dilution (vol/vol), it inhibited significantly; at 7,500× dilution, it almost completely abolished HICI (n = 35 experiments). Addition of venom usually caused a transient increase in $[Ca^{2+}]_i$, much like the TRH response. The response was inhibited during HICI, suggesting that it may be inhibited by elevated levels of intracellular Ca^{2+} (Fig. 7*B*, *top trace*). When venom was added to cells in normal saline, the transient increase in activity was followed by a drop in cell Ca^{2+} (Fig. 7*B*, *middle trace*). This suggests, surprisingly perhaps, that in the resting state $[Ca^{2+}]_i$ levels are set by the activity of venom-sensitive channels, probably SACs. When Ca^{2+} was removed from the isotonic saline, there was a drop in intracellular Ca^{2+}, and the subsequent addition of venom had no further effect on Ca^{2+} (Fig. 7*B*, thin *bottom trace*). This result is consistent with the interpretation that most of the resting Ca^{2+} influx is sensitive to the venom, probably arising from finite levels of resting SAC activity.

To investigate whether venom blocks L-type Ca^{2+} channels, we tested it on the depolarization-induced Ca^{2+} increase (Fig. 7*C*). With the use of the protocol of three consecutive K^+ depolarizations, venom (5,000× dilution) was applied during the second stimulus. Unlike Gd^{3+} or nifedipine, the venom did not block depolarization-induced Ca^{2+} increase, suggesting that the venom did not block L-type Ca^{2+} channels (n = 4 experiments). Thus the venom was capable of inhibiting HICI without blocking L-type Ca^{2+} channels.

We suspected that the venom blocks HICI by blocking stretch-activated ion channels. We tested this using back diffusion of the venom (~1:1,000 dilution) onto cell-attached patches and superfusion of the venom onto outside-out patches. The venom blocked in both cases, but the outside-out patches permitted washout. A typical result is shown in Fig. 8. Without an attempt to work at high time resolution, the venom effect washed in within 30 s and washed out within 2 min. Detailed dose-response data with whole venom have not been obtained, but experiments on the active component are in progress.

DISCUSSION

Ca^{2+} sources involved in HICI. GH_3 cells respond to a hypotonic exposure with an increase of $[Ca^{2+}]_i$ that is comparable with that seen in proximal tubule cells (17) but much larger than that seen in epithelial cells (12). The delay time for developing HICI is well beyond that required for exchange, as can be seen in Fig. 2*B* in which the response due to the removal and addition of Ca^{2+} is much faster than that for HICI. The delay time is comparable with the rate of swelling, suggesting that they are correlated. Presumably, swelling precedes HICI, although we have no kinetic data to resolve causality. The delay time for swelling reflects the integration of water flux to produce a change in volume.

We examined three Ca^{2+} sources for their involvement in HICI, and the data are clear in illustrating that extracellular Ca^{2+} was required for both initiating and

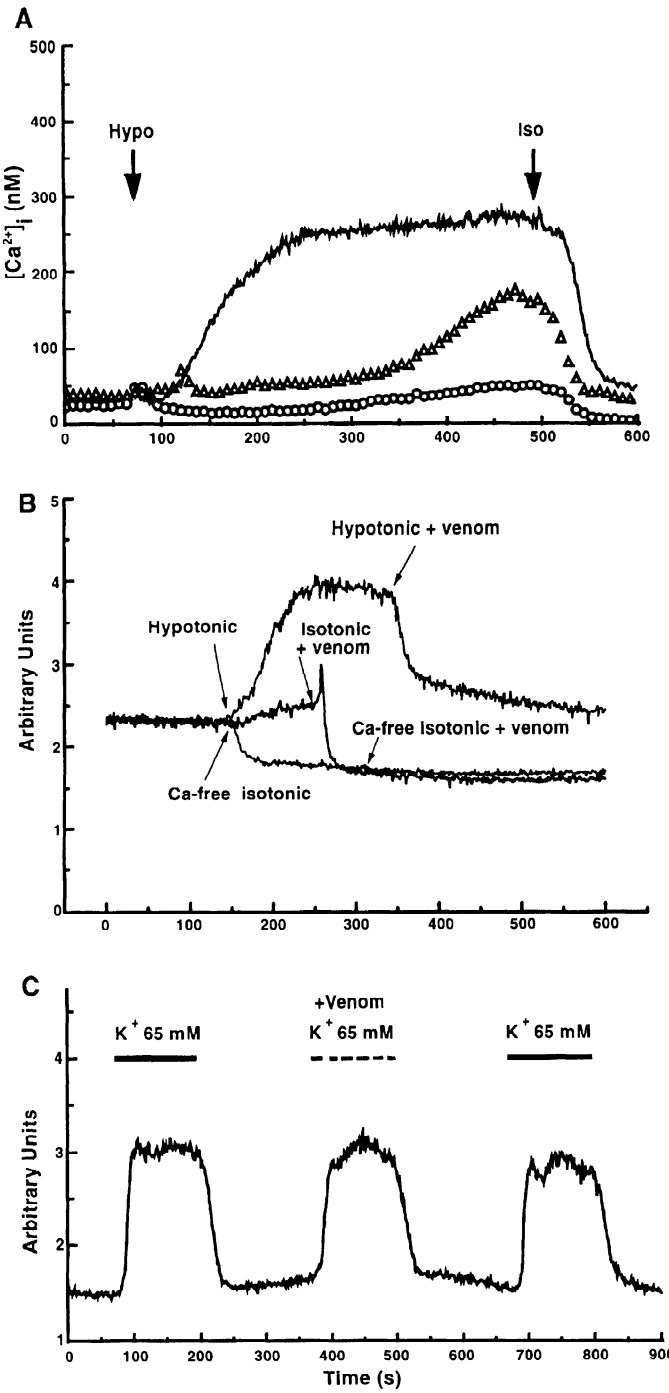

Fig. 7. Effects of *Grammostola spatulata* venom on HICI and depolarization-induced Ca^{2+} increase. *A*: normal HICI is shown in solid line. Venom was added to hypotonic solution at beginning of hypotonic exposure. Compared with normal HICI response, venom of 7,500× dilution (vol/vol) almost completely blocked HICI (○). Venom of 15,000× dilution significantly inhibited HICI (△). *B*: effects of *Grammostola* venom on cells in hypertonic, normal isotonic, and Ca^{2+}-free isotonic solutions. Cells (3 separate coverslips) were initially perfused (~1 ml/min) with isotonic saline in 20-μl chamber, and raw venom was injected (without perfusion to reduce requisite volume) at 1:1,000 dilution. *Top trace*: quenching of HICI by venom. Perfusate was switched to hypotonic saline at ~150 s. At 345 s, 95 μl of hypotonic saline containing venom were injected, immediately suppressing HICI. *Middle trace* (thick line): effect of venom on resting cells. At 100 s, normal perfusion was stopped (producing a slight rise in Ca^{2+}). At ~250 s, we made two 95-μl venom injections, 30 s apart. This produced a transient rise in Ca^{2+}, followed by a maintained suppression of resting $[Ca^{2+}]_i$. (Note that venom-induced transient seen in *top trace* is not seen when cell is previously swollen.) *Bottom trace* (thin line): effect of venom in Ca^{2+}-free isotonic solution. At 150 s, cells were perfused with Ca^{2+}-free saline, producing an immediate drop in $[Ca^{2+}]_i$. As described above, we made 2 injections of venom at ~250 s, producing no further change in $[Ca^{2+}]_i$. *C*: depolarization-induced Ca^{2+} increase was not blocked by venom. Cells were depolarized by using a high-K^+ isotonic solution (K^+ 65 mM). Venom (5,000× dilution) was added to solution (K^+ 65 mM + Venom) during 2nd stimulation. Venom did not block depolarization-induced Ca^{2+} increase, and 3rd stimulation also showed a normal depolarization-induced Ca^{2+} increase.

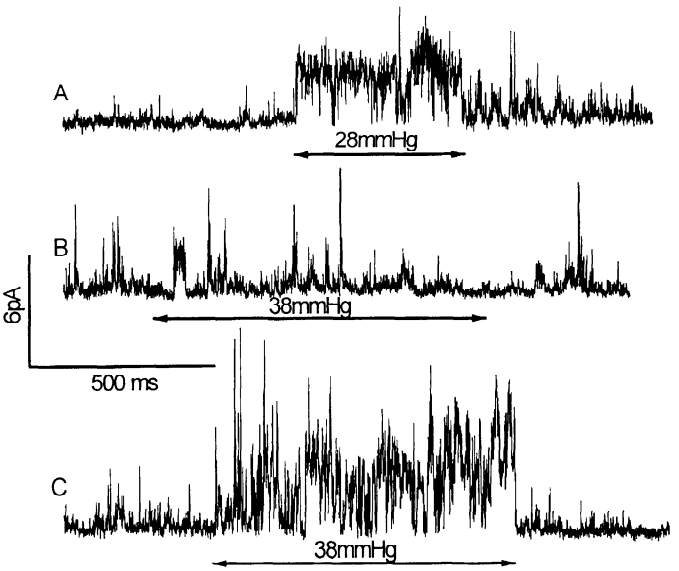

Fig. 8. Stretch-activated single channel currents reversibly blocked by a 5,000:1 dilution of *Grammostola* venom. Single-channel currents were recorded from GH_3 outside-out patches before (*A*), during (*B*), and after (*C*) application of venom. *A*: Stretch-activated channels were observed while 28 mmHg positive pressure (double-headed arrow) was applied to patch pipette. During perfusion with venom (*B*), application of 38 mmHg did not elicit significant channel activation. After ~2-min washout of venom (*C*), 38 mmHg of pressure produced channel activation greater than in *A*. Patch electrode contained (in mM) 120 CsF, 10 CsCl, 10 EGTA, and 10 HEPES (pH 7.3). Bath contained (in mM) 140 sodium gluconate, 5 potassium gluconate, 1 $MgSO_3$, 10 HEPES, and 6 glucose (pH 7.4). Pipette potential was −120 mV. Venom was diluted in bath solution.

maintaining HICI. The involvement of Ca^{2+} stores seemed, at best, contributory to maintaining HICI.

We attempted to use caffeine to study the involvement of Ca^{2+}-induced Ca^{2+} release (30). Our preliminary data showed that caffeine caused an increase of $[Ca^{2+}]_i$ in GH_3 cells (4), perhaps by releasing Ca^{2+} from intracellular stores. But caffeine also inhibited HICI, which might be attributed to the block of L-type Ca^{2+} channels (29). Because of the complexity in caffeine action, it remains unclear whether Ca^{2+}-induced Ca^{2+} release contributed to HICI. Because pretreatment with caffeine did not block HICI, those stores are not essential. We also employed ryanodine to empty ryanodine-sensitive stores and thapsigargin to deplete intracellular Ca^{2+} stores. Neither of these had any obvious effect on $[Ca^{2+}]_i$, although thapsigargin is reported to raise $[Ca^{2+}]_i$ in the related GH_4C_1 cells (15).

We have focused our attention on Ca^{2+} influx to the cytoplasm. However, $[Ca^{2+}]_i$ is set by a dynamic balance between the Ca^{2+} influx and efflux, and HICI could result from a decreased Ca^{2+} efflux. A decreased efflux could be caused by inhibition of the Na^+/Ca^{2+} exchanger and/or Ca^{2+} pumps in the plasmalemma or by decreasing the activity of Ca^{2+}-ATPase of intracellular compartments. For example, in ferret red blood cells, the Na^+/Ca^{2+} exchanger is a primary pathway for Ca^{2+} influx and determines cell volume regulation (20). Although we have not fully investigated whether a decreased Ca^{2+} efflux could play a role in HICI of GH_3 cells, the Na^+/Ca^{2+} exchanger is unlikely to contribute, since we kept the extracellular Na^+ concentration constant.

Role of L-type Ca^{2+} channels and Ca^{2+}-permeable SACs in HICI. Nifedipine inhibited HICI in a dose-dependent manner (data not shown), abolishing HICI at a concentration of 10 μM. With the assumption that nifedipine inhibited HICI by blocking L-type Ca^{2+} channels, our results suggest these channels may play an important role in HICI. Evidence against the requisite role of L-type Ca^{2+} channels in HICI comes from the discovery of a spider venom that inhibited HICI without blocking Ca^{2+} channels. In addition, voltage-clamp experiments showed that hypotonic-swelling activates a voltage-independent Ca^{2+} influx (4). It is likely that some type of Ca^{2+}-permeable channel other than L-type Ca^{2+} channels is required.

SACs are present in GH_3 cells (21) and are commonly Ca^{2+} permeable. Gd^{3+}, a nonspecific blocker of SACs, inhibited HICI, but any conclusions are limited because it can also block voltage-dependent Ca^{2+} channels (3). Our results show that Gd^{3+} effectively blocked L-type Ca^{2+} channels in GH_3 cells. This result is not surprising, especially since other multivalent cations such as La^{3+} and Cd^{2+} also block Ca^{2+} channels in GH_3 cells (28). Interestingly, both nifedipine and Gd^{3+} decreased the basal level $[Ca^{2+}]_i$ (Figs. 4 and 5). Because GH_3 cells are normally spontaneously active (4, 27), it is reasonable that these reagents decreased $[Ca^{2+}]_i$ by inhibiting action potential generation.

Effects of Grammostola spatulata venom. *Grammostola* venom inhibited HICI in a dose-dependent manner, and it apparently did not block L-type Ca^{2+} channels. The raw venom contains many components (16), with at least two kinetically distinguishable components capable of modulating $[Ca^{2+}]_i$. One blocked HICI, whereas the other produced a transient Ca^{2+} elevation that appeared to be inhibited during HICI (Fig. 7*B*, *middle trace*). Purification of the component that is active against HICI revealed that it is different from the stimulatory component.

The patch-clamp experiments have shown that the crude venom (and, although not shown here, the purified fraction) that blocks HICI is capable of blocking SACs in GH_3 cells (Fig. 8). Applied at rest, the venom has the striking effect of reducing the Ca^{2+} level of resting cells. This suggests that SACs are active in resting cells, a conclusion that cannot be tested with patch clamping because of the intrinsic stresses of patch formation. In any case, this HICI assay seems to be a useful method for screening agents that are active on SACs.

Mechanism of HICI and volume regulation in GH_3 cells. The main findings of our studies in GH_3 cells are as follows. *1*) HICI requires extracellular Ca^{2+}, and intracellular Ca^{2+} stores are not essential. *2*) HICI is inhibited by both nifedipine and Gd^{3+}. *3*) Spider venom from *Grammostola* can inhibit HICI without blocking L-type Ca^{2+} channels. *4*) *Grammostola* venom inhibits SAC activity. These results are consistent with the following model. Hypotonic cell swelling exerts a hydrostatic pressure on the cell membrane that increases membrane tension. This activates cationic SACs, allowing Ca^{2+} to enter the cell directly and via depolarization (from Na^+ flux) through L-type Ca^{2+} channels. This Ca^{2+} influx initiates an elevation of $[Ca^{2+}]_i$ that may trigger a further increase of $[Ca^{2+}]_i$ via Ca^{2+}-induced Ca^{2+} release and the activation of Ca^{2+}-sensitive Cl^- channels that would lead to a further depolarization. In the context of this model, the inhibitory effect of nifedipine arises because the Ca^{2+} entry through L-type Ca^{2+} channels contributes heavily to HICI. Gd^{3+} inhibits by blocking both SACs and L-type Ca^{2+} channels, and *Grammostola* venom inhibits by blocking SACs.

We thank Dr. Charles Bowman for sharing data, Drs. Robert Hard and Scott Diamond for their help in experiments, and Mary Teeling and Robert Borschel for their technical support.

This work was supported by the American Heart Association New York Chapter and the United States Army Research Office (Grant DAAL0392G0014).

Present address of Y. Chen: Laboratory of Cardiovascular Sciences, National Institute on Aging, Baltimore, MD 21224.

Address for reprint requests: F. Sachs, 120 Cary Hall, Channels Group, Dept. of Biophysical Sciences, SUNY at Buffalo, Buffalo, NY 14214 (E-mail: sachs@fred.med.buffalo.edu).

Received 20 June 1995; accepted in final form 1 December 1995.

REFERENCES

1. **Albert, P. R., and A. H. J. Tashjian.** Thyrotropin-releasing hormone-induced spike and plateau in cytosolic free Ca^{2+} concentrations in pituitary cells. Relation to prolactin release. *J. Biol. Chem.* 259: 5827–5832, 1984.
2. **Bender, A. S., J. T. Neary, and M. D. Norenberg.** Role of phosphoinositide hydrolysis in astrocyte volume regulation. *J. Neurochem.* 61: 1506–1514, 1993.

3. **Biagi, B. A., and J. J. Enyeart.** Gadolinium blocks low- and high-threshold calcium currents in pituitary cells. *Am. J. Physiol.* 259 (*Cell Physiol.* 28): C515–C520, 1990.
4. **Chen, Y.** *A Study of the Sensory Mechanism of Cell Volume Regulation* (PhD thesis). Buffalo, NY: State University of New York at Buffalo, 1994.
5. **Cornet, M., I. H. Lambert, and E. K. Hoffmann.** Relation between cytoskeleton, hypo-osmotic treatment and volume regulation in Ehrlich ascites tumor cells. *J. Membr. Biol.* 131: 55–66, 1993.
7. **Cornet, M., J. Ubl, and H. A. Kolb.** Cytoskeleton and ion movements during volume regulation in cultured PC12 cells. *J. Membr. Biol.* 133: 161–170, 1993.
8. **Drummond, A. H.** Bidirectional control of cytosolic free calcium by thyrotropin-releasing hormone in pituitary cells. *Nature Lond.* 315: 752–755, 1985.
9. **Dube, L., L. Parent, and R. Sauve.** Hypotonic shock activates a maxi K^+ channel in primary cultured proximal tubule cells. *Am. J. Physiol.* 259 (*Renal Fluid Electrolyte Physiol.* 28): F348–F356, 1990.
10. **Grynkiewicz, G., M. Poenie, and R. Y. Tsien.** A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260: 3440–3450, 1985.
11. **Guharay, F., and F. Sachs.** Stretch-activated single ion channel currents in tissue-cultured embryonic chick skeletal muscle. *J. Physiol. Lond.* 352: 685–701, 1984.
12. **Hazama, A., and Y. Okada.** Involvement of Ca^{2+}-induced Ca^{2+} release in the volume regulation of human epithelial cells exposed to a hypotonic medium. *Biochem. Biophys. Res. Commun.* 167: 287–293, 1990.
13. **Hoffmann, E. K., L. O. Simonsen, and I. H. Lambert.** Volume-induced increase of K^+ and Cl^- permeabilities in Ehrlich ascites tumor cells. Role of internal Ca^{2+}. *J. Membr. Biol.* 78: 211–222, 1984.
14. **Jessen, F., and E. K. Hoffmann.** Activation of the $Na^+/K^+/Cl^-$ cotransport system by reorganization of the actin filaments in Ehrlich ascites tumor cells. *Biochim. Biophys. Acta* 1110: 199–201, 1992.
15. **Koshiyama, H., and A. H. J. Tashjian.** Evidence for multiple intracellular calcium pools in GH4C1 cells: investigation using thapsigargin. *Biochem. Biophys. Res. Commun.* 177: 551–558, 1991.
16. **Lampe, R. A., P. A. Defeo, M. D. Davison, J. Young, J. L. Herman, R. C. Spreen, M. B. Horn, T. J. Mangano, and R. A. Keith.** Isolation and pharmacological characterization of omega-grammotoxin SIA, a novel peptide inhibitor of neuronal voltage-sensitive calcium channel responses. *Mol. Pharmacol.* 44: 451–460, 1993.
17. **McCarty, N. A., and R. G. O'Neil.** Calcium-dependent control of volume regulation in renal proximal tubule cells: I. swelling activated Ca entry and release. *J. Membr. Biol.* 123: 149–160, 1991.
18. **McCarty, N. A., and R. G. O'Neil.** Calcium-dependent control of volume regulation in renal proximal tubule cells: II. role of dihydropyridine-sensitive and -insensitive Ca entry pathways. *J. Membr. Biol.* 123: 161–170, 1991.
19. **McCarty, N. A., and R. G. O'Neil.** Calcium signaling in cell volume regulation. *Physiol. Rev.* 72: 1037–1061, 1992.
20. **Milanick, M. A.** Ferret red cells: Na/Ca exchange and Na-K-Cl cotransport. *Comp. Biochem. Physiol.* 102: 619–624, 1992.
21. **Morris, C. E., and R. Horn.** Failure to elicit neuronal macroscopic mechanosensitive currents anticipated by single-channel studies. *Science Wash. DC* 251: 1246–1249, 1991.
22. **O'Connor, E. R., and H. K. Kimelberg.** Role of calcium in astrocyte volume regulation and in the release of ions and amino acids. *J. Neurosci.* 13: 2638–2650, 1993.
23. **Okada, Y., A. Hazama, and W. L. Yuan.** Stretch-induced activation of Ca^{2+}-permeable ion channels is involved in the volume regulation of hypotonically swollen epithelial cells. *Neurosci. Res. Suppl.* 12: S5–S13, 1990.
24. **Ronning, S. A., G. A. Heatley, and T. F. Martin.** Thyrotropin-releasing hormone mobilizes Ca from endoplasmic reticulum and mitochondria of GH_3 pituitary cells: characterization of cellular calcium pools by a method based on digitonin permeabilization. *Proc. Natl. Acad. Sci. USA* 79: 6294–6289, 1982.
25. **Rorive, G., and R. Gilles.** Intracellular inorganic osmotic effectors. In: *Mechanisms of Osmoregulation in Animals: Maintenance of Cell Volume*, edited by R. Gilles. Chichester, UK: Wiley, 1989, p. 83–109.
26. **Scherubl, H., and J. Hescheler.** Steady-state currents through voltage-dependent, dihydropyridine-sensitive Ca^{2+} channels in GH_3 pituitary cells. *Proc. R. Soc. Lond. B Biol. Sci.* 245: 127–131, 1991.
27. **Simasko, S. M.** A background sodium conductance is necessary for spontaneous depolarization in rat pituitary cell line GH_3. *Am. J. Physiol.* 266 (*Cell Physiol.* 35): C709–C719, 1994.
28. **Simasko, S. M., G. A. Weiland, and R. E. Oswald.** Pharmacological characterization of two calcium currents in GH_3 cells. *Am. J. Physiol.* 254 (*Endocrinol. Metab.* 17): E328–E336, 1988.
29. **Simasko, S. M., and S. Yan.** 3-Isobutyl-1-methylxanthine inhibits sustained calcium current independently of cyclic AMP in neuronal and endocrine cells. *Mol. Pharmacol.* 44: 622–627, 1993.
30. **Suzuki, M., K. Kawahara, A. Ogawa, T. Morita, Y. Kawaguchi, S. Kurihara, and O. Sakai.** $[Ca^{2+}]_i$ rises via G protein during regulatory volume decrease in rabbit proximal tubule cells. *Am. J. Physiol.* 258 (*Renal Fluid Electrolyte Physiol.* 27): F690–F696, 1990.
31. **Tsien, R. Y.** Intracellular signal transduction in four dimensions: from molecular design to physiology. *Am. J. Physiol.* 263 (*Cell Physiol.* 32): C723–C728, 1992.
32. **Ubl, J., H. Murer, and H. A. Kolb.** Hypotonic shock evokes opening of Ca^{2+}-activated K^+ channels in opossum kidney cells. *Pfluegers Arch.* 412: 551–553, 1988.

32a. **Widdicombe, J. H., M. Kondo, and H. Mochizuki.** Regulation of airway mucosal ion transport. *Int. Arch. Allergy Appl. Immunol.* 94: 56–61, 1991.

33. **Yamaguchi, D. T., J. Green, C. R. Kleeman, and S. Muallem.** Characterization of volume-sensitive, Ca-permeating pathways in the osteosarcoma cell line UMR-106–01. *J. Biol. Chem.* 264: 4383–4390, 1989.
34. **Yang, X. C., and F. Sachs.** Block of stretch-activated ion channels in *Xenopus* oocytes by gadolinium and calcium ions. *Science Wash. DC* 243: 1068–1071, 1989.