Regulation of $K_{\text{Ca}}$ current by store-operated Ca\textsuperscript{2+} influx depends on internal Ca\textsuperscript{2+} release in HSG cells

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Regulation of $K_{\text{Ca}}$ current by store-operated Ca\textsuperscript{2+} influx depends on internal Ca\textsuperscript{2+} release in HSG cells. Am. J. Physiol. 275 (Cell Physiol. 44): C571–C580, 1998.—This study examines the Ca\textsuperscript{2+} influx-dependent regulation of the Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel (KCa) in human submandibular gland (HSG) cells. Carbachol (CCh) induced sustained increases in the KCa current and cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}], which were prevented by loading cells with 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA). Removal of extracellular Ca\textsuperscript{2+} and addition of La\textsuperscript{3+} or Gd\textsuperscript{3+}, but not Zn\textsuperscript{2+}, inhibited the increases in KCa current and [Ca\textsuperscript{2+}]. Ca\textsuperscript{2+} influx during refill (i.e., addition of Ca\textsuperscript{2+} to cells treated with CCh and then atropine in Ca\textsuperscript{2+}-free medium) failed to evoke increases in the KCa current but achieved internal Ca\textsuperscript{2+} store refill. When refill was prevented by thapsigargin, Ca\textsuperscript{2+} readdition induced rapid activation of KCa.

These data provide further evidence that intracellular Ca\textsuperscript{2+} accumulation provides tight buffering of [Ca\textsuperscript{2+}] at the site of Ca\textsuperscript{2+} influx (H. Mogami, K. Nakano, A. V. Tepikin, and O. H. Petersen. Cell 88: 49–55, 1997). We suggest that the Ca\textsuperscript{2+} influx-dependent regulation of the sustained KCa current in CCh-stimulated HSG cells is mediated by the uptake of Ca\textsuperscript{2+} into the internal Ca\textsuperscript{2+} store and release via the inositol 1,4,5-trisphosphate-sensitive channel.

calcium-activated potassium channel; store-operated calcium influx; salivary gland cells; muscarinic receptor
In this study, we have examined the role of intracellular Ca\(^{2+}\) release and store-operated Ca\(^{2+}\) influx in the regulation of the K\(^+\) channel in HSG cells by CCh. By using the standard patch-clamp whole cell technique, we show that the channel activation is dependent on CCh-stimulated intracellular Ca\(^{2+}\) release, via IP\(_3\) sensitive channels, and that its sustained activation is determined by Ca\(^{2+}\) influx, via the store-operated Ca\(^{2+}\) influx pathway. Importantly, we have examined K\(_{\text{Ca}}\) activity during the Ca\(^{2+}\) influx that occurs during reloading of internal Ca\(^{2+}\) stores, i.e., in the absence of internal Ca\(^{2+}\) release. The results show that Ca\(^{2+}\) influx alone cannot support activation of the K\(_{\text{Ca}}\) because of the rapid buffering of [Ca\(^{2+}\)] in the subplasma membrane region by the activity of the intracellular Ca\(^{2+}\) pump. Thus we suggest that the Ca\(^{2+}\) influx-dependent modulation of K\(_{\text{Ca}}\) activity in CCh-stimulated HSG cells is not directly due to an elevation of [Ca\(^{2+}\)] in the site of Ca\(^{2+}\) influx but rather is mediated via uptake of Ca\(^{2+}\) into the intracellular Ca\(^{2+}\) store and IP\(_3\)-dependent release.

**METHODS**

Cell culture. HSG cells were a gift from Dr. Mitsunobu Sato of the Second Department of Oral and Maxillofacial Surgery, Tokushima University, Tokushima, Japan. Cells were grown in Eagle’s minimum essential medium with Earle’s balanced salt solution (Biofluids, Rockville, MD) with 5% CO\(_2\) in air at 37°C in the presence of 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Biofluids). Cells were fed three times a week and passed when confluent. Cells were passaged by detaching them from the tissue culture dish with 0.25% trypsin-1.0 mM EDTA (Biofluids). A single cell suspension was reseeded on coverslips, kept in a 35-mm culture dish (Corning), and cultured for 24 h before use.

Patch-clamp experiments. The coverslips were cut to \(0.5 \times 0.5 \text{ mm}\) and placed in a perfusion chamber (Warner Instrument, Hamden, CT). The perfusion rate, \(\sim 5 \text{ ml/min}\), was achieved by gravity-fed plastic tubes in a bath solution that was continuously and simultaneously removed through a vacuum line. Complete solution changes were obtained within 15 s. The standard extracellular solution contained (in mM) 145 NaCl, 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 10 glucose, 0.1 EGTA, and 5 HEPES, pH 7.4. The pipette was filled with (in mM) 150 KCl, 2 MgCl\(_2\), 1 ATP, and 5 HEPES, pH 7.2. In some experiments, 150 mM KCl was replaced with 150 mM CsCl, and, in others, either 10 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA) or 10–100 µM IP\(_3\) was included in the pipette solution.

Patch clamp in a whole cell configuration was performed at room temperature on single HSG cells attached to coverslips using the standard patch-clamp technique (13). Patch electrodes were made from 1.0-mm borosilicate glass tubing with filament (BF-100–50–10, Sutter Instrument, Novato, CA). The resistance of the pipette was typically between 3 and 6 M\(\Omega\) when filled. The chamber was grounded with an Ag-AgCl pellet through a 150 mM NaCl-containing agar bridge. Cell membrane and pipette capacitative transients were subtracted from the records by the amplifier circuitry before sampling. Voltages were not compensated for liquid junction potentials. Membrane currents were measured with an Axopatch 200A amplifier in conjunction with pCLAMP 6.1 software and a Digidata 1200 analog-to-digital converter (Axon Instruments, Foster City, CA). Whole cell K\(^+\) currents were filtered at 2 kHz (low-pass Bessel filter), sampled with an interval of 10 ms in a gap-free mode, and recorded directly onto the hard drive of a Dell Pentium computer from a holding potential of 0 mV, the Cl\(^-\) equilibrium potential, for analysis. Digitized data were analyzed with the use of using pCLAMP 6.1 and Origin 4.1 (Microcal Software, Northampton, MA). In some experiments, a holding potential of \(-85\) mV, the K\(^+\) equilibrium potential, was used to test whether there was a CCh-induced inward current. In the current-voltage (I-V) relationship experiments, the membrane potential was changed from \(-120\) to \(+80\) mV in a 20-mV step by generating square pulses of 2.56-s duration from a holding potential of \(-35\) mV in a Clampex module. I-V relationships were obtained from 10 µM CCh-induced peak currents. The mean K\(^+\) current (total integrated current induced by agonist application/total time of application) and the amplitude of the current were measured using the Fetchan module. The I-V relationship was calculated using the Clampfit module and exported to the Origin 4.1 for further analysis.

**RESULTS**

CCh stimulation of K\(_{\text{Ca}}\) in HSG cells. Stimulation of HSG cells with CCh induced an increase in the outward current at a holding potential of 0 mV, the Cl\(^-\) equilibrium potential (Fig. 1A), in 94% of the cells. Oscillatory increases were observed at lower concentrations of CCh (1–10 µM), whereas steady-state increases in the current were obtained with higher concentrations (>100 µM). Although the initial amplitude of the current in the same cell was similar at all agonist concentrations (see Fig. 1A), the mean current increased significantly with increasing CCh concentration, from 322 ± 13 pA at 1 µM and 663 ± 87 pA at 100 µM CCh (P < 0.05, n = 5). A concentration of 1 µM CCh typically evoked baseline-separated oscillations with a mean frequency of 3.7 ± 1.7 per minute (n = 5), whereas 10 µM CCh induced either similar, fast baseline-separated oscillations (mean frequency of 6.6 ± 2.1 per minute, n = 6; seen in one-half of the cells tested) or slower oscillations, which were superimposed on a sustained elevation of the current (as shown in Fig. 1A). Higher concentrations of CCh (100 µM to 1.0 mM) consistently induced a fast transient increase in the
outward current that was followed by lower steady-state current (Fig. 1A). Figure 1B shows outward currents in a cell that was stimulated repeatedly by 100 µM CCh; the interval between stimulations was 3 min. The differences in the mean amplitudes of the first three stimulations are not significant (P > 0.05, n = 4). The amplitudes of second and third responses are 88.7 ± 8.1% and 71.6 ± 14.5% (n = 4), respectively, of the first response. These data demonstrate that no rapid desensitization or inactivation of the K⁺ channel response occurs with repeated short exposure to CCh.

Previous reports demonstrated the presence of KCa in HSG cells (18). We carried out some initial studies to confirm these previous findings under our experimental conditions, and the data are summarized here. The effects of Ba²⁺ and Cs⁺, potent inhibitors of K⁺ channels, were examined. Replacement of intracellular K⁺ by Cs⁺ or internal administration of Ba²⁺ (2 mM) (n = 5, data not shown) completely eliminated the CCh-induced responses. Furthermore, BAPTA (10 mM), a Ca²⁺ chelator, was directly introduced into the cytosol through the patch pipette. This treatment completely abolished CCh-induced KCa at all concentrations of CCh tested, from 1 to 100 µM (Fig. 1C, compare with data in Fig. 1A). Loading HSG cells with BAPTA also inhibited CCh-induced [Ca²⁺]ᵢ elevation (data not shown).

In addition, the I-V relationship was measured in unstimulated control HSG cells (Fig. 2A) and CCh (10 µM)-stimulated cells (Fig. 2B). As shown in Fig. 2C, the current increased almost linearly between −120 and 0 mV and reached a maximum between 0 and 20 mV. The reversal potential of the current was about −80 mV, which is close to the K⁺ equilibrium potential (−85 mV). When the holding potential was greater than +20 mV, the K⁺ currents became smaller, which is likely due to a decrease in the driving force for Ca²⁺ influx across the plasma membrane. Furthermore, consistent with the previous report by Izutsu et al. (18), ChTX (50 nM), a large-conductance Ca²⁺-dependent K⁺ channel inhibitor, significantly reduced CCh-induced K⁺ current to 12.9 ± 8.4% (P < 0.05, n = 5), which was partially restored to 45.7 ± 13.9% of the control when ChTX was removed (data not shown). On the other hand, apamin, a small-conductance Ca²⁺-dependent K⁺ channel inhibitor, completely abolished CCh-induced K⁺ current (data not shown).

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**Fig. 1. Carbachol (CCh)-induced outward currents.** A: concentration-dependent response of CCh-induced Ca²⁺-activated K⁺ channel (KCa) current in human submandibular gland (HSG) cells at a holding potential of 0 mV. CCh and all other agents were continuously applied to the bath (indicated by bars) at a rate of ~5 ml/min. This is a representative trace of results obtained with >20 cells. B: sequential stimulation of HSG cell. CCh (100 µM) was applied repeatedly for 30 s (shown by bars), with an interval of ~3 min between applications in which the cell was washed by bath solution. This is a representative trace of data from 7 different cells. C: 1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA), directly introduced into the cell via patch pipette, and CCh (1–100 µM) were applied to the cell (indicated by bars).

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**Fig. 2. Current-voltage (I-V) relationship of CCh-induced peak KCa.** A: control currents were recorded at voltages of −120 to +80 mV with 20-mV steps from a holding potential of −35 mV before application of CCh. B: 10 µM CCh-induced peak current was recorded under same conditions as control recording. C: I-V relationship (in pA and mV, respectively) of peak current induced by 10 µM CCh (n = 6).
channel inhibitor, did not have any significant effect on CCh-induced outward current. These data demonstrate that the CCh-induced outward current in HSG cells maintained at 0 mV is mainly carried by K⁺ via a ChTX-sensitive Ca²⁺-dependent K⁺ channel.

IP₃- and TG-dependent stimulation of KCa in HSG cells. CCh-stimulated intracellular Ca²⁺ mobilization is mediated via increases in intracellular IP₃ and IP₃-induced release of Ca²⁺ from internal Ca²⁺ stores (1, 2, 15, 32). Thus further experiments were carried out to test whether the IP₃-induced Ca²⁺ release pathway is involved in the CCh stimulation of KCa. IP₃, directly applied to HSG cells via the patch pipette, typically caused oscillatory increases in KCa at low concentrations of IP₃ (e.g., 10 µM, Fig. 3A) with a frequency of 4.8 ± 1.6 oscillations/min (n = 5). At higher IP₃ concentrations (e.g., 100 µM), a relatively sustained increase in the current was induced that appeared to be superimposed on an oscillatory current and ran down within 2–3 min (Fig. 3B). The mean current increased significantly with increasing IP₃ concentrations [from 662 ± 258 pA at 10 µM (n = 5) to 1,379 ± 424 pA at 100 µM (P < 0.01, n = 9)]. It must be noted that the responses induced by the dialysis of IP₃ were not as stable as that induced by CCh, and 2 of 18 cells tested did not respond to IP₃ stimulation. However, the pattern of currents induced by increasing concentrations of IP₃ was similar to that induced by increasing concentrations of CCh, i.e., oscillations at relatively lower concentrations and relatively sustained increases in the current at higher concentrations. Importantly, addition of CCh during the IP₃-mediated response did not alter the IP₃-induced current (data not shown) and addition of CCh after rundown of the IP₃-stimulated KCa oscillations induced either a very attenuated response (Fig. 3B) or no response at all.

We have previously reported that CCh stimulation of HSG cells induces a Ca²⁺ influx that is dependent on the depletion of internal Ca²⁺ stores, i.e., store-operated Ca²⁺ influx (20). To determine whether the store-dependent Ca²⁺ influx regulates KCa in HSG cells, cells were treated with TG, an irreversible Ca²⁺-ATPase inhibitor that depletes intracellular Ca²⁺ stores by inhibiting the Ca²⁺ pump, thus activating the store-operated Ca²⁺ influx pathway (31). TG (1–10 µM) induced a biphasic increase in K⁺ currents and attenuated a subsequent response to CCh (Fig. 4A). BHQ, a reversible Ca²⁺-ATPase inhibitor, produced effects simi-
lar to TG; however, in this case, the response could be recovered by washing off BHQ from the cells (n = 7, data not shown). These data suggest that CCh, IP₃, and TG (or BHQ) stimulate Kᵥ via internal Ca²⁺ store depletion. Figure 4B shows that TG treatment induced a transient increase in the Kᵥ current in cells perfused with a Ca²⁺-free medium. However, the current was rapidly increased when Ca²⁺ was reintroduced into the medium. These data clearly show that the store-operated Ca²⁺ influx pathway can regulate Kᵥ.

Effect of extracellular Ca²⁺ on the regulation of the Kᵥ current in CCh-stimulated HSG cells. As discussed above, activation of the muscarinic receptor in HSG cells induces a biphasic increase in [Ca²⁺]: an initial rapid transient increase and a subsequent lower sustained elevation (15, 20, 35). The initial elevation of Ca²⁺ is due to intracellular Ca²⁺ release from IP₃-sensitive Ca²⁺ stores, whereas the sustained elevation is dependent on Ca²⁺ influx from extracellular medium. Consistent with these previous discoveries, Fig. 5A shows the CCh-stimulated biphasic [Ca²⁺] increase in a single HSG cell loaded with indo 1. A concentration of 100 µM CCh induced a rapid transient increase followed by a sustained elevation of Ca²⁺. The resting and peak levels of [Ca²⁺], following addition of 100 µM CCh were 138 ± 8.5 nM (n = 8) and 375 ± 59 nM (n = 8). The sustained elevation of [Ca²⁺] was dependent on Ca²⁺ influx, since removal of extracellular Ca²⁺ reduced [Ca²⁺] to the resting level and reintroduction of extracellular Ca²⁺ restored sustained [Ca²⁺]. The pattern of CCh-induced increases in the Kᵥ current was similar to that of [Ca²⁺] (Fig. 5B). The sustained Kᵥ current was decreased to resting levels when external Ca²⁺ was removed and recovered when Ca²⁺ was reintroduced into the medium. These data, together with the effect of BAPTA (Fig. 1C), demonstrate that the Kᵥ current reflects, and is dependent on, the underlying changes in [Ca²⁺] induced following CCh stimulation of the cells.

Consistent with the above discussion, the initial activation of the Kᵥ channel by CCh was not affected by the removal of external Ca²⁺ (Fig. 6A). The amplitude of the transient increase in the CCh-induced Kᵥ current in a Ca²⁺-free medium (second and third responses) was not significantly different from that induced in the presence of extracellular Ca²⁺ (82.1.8 ± 8.1% and 70.8 ± 13.7%, respectively, of the first response, P > 0.05, n = 4). The differences in amplitudes induced by repeated CCh stimulation in these experiments and in control experiments shown in Fig. 1B were not significant. Note that in these experiments...
Ca\(^{2+}\) was removed from the medium after removal of CCh, which allows refill of internal Ca\(^{2+}\) stores. When Ca\(^{2+}\) was removed before CCh (Fig. 6B), a condition in which refill of internal Ca\(^{2+}\) stores does not occur, the current induced by subsequent simulation of the cells with CCh was greatly reduced to 20.7 ± 6.8% of the control response (P < 0.05, n = 5). However, when Ca\(^{2+}\) was reintroduced, the stores refilled and the CCh-induced response was restored to 65.1 ± 5.1% (Fig. 5C). These data suggest that CCh stimulation of the K\(^{+}\) current is dependent on internal Ca\(^{2+}\) release. However, because the refill status of the internal Ca\(^{2+}\) store is dependent on Ca\(^{2+}\) influx, the initial activation of K\(_{Ca}\) is also dependent, indirectly, on store-operated Ca\(^{2+}\) influx.

Ca\(^{2+}\) influx-dependent regulation of K\(_{Ca}\) in HSG cells. The role of Ca\(^{2+}\) influx in CCh-stimulated oscillations of the K\(_{Ca}\) current was next examined. Removal of external Ca\(^{2+}\) abolished CCh-induced sustained oscillations of K\(_{Ca}\) (Fig. 7A), which were recovered when Ca\(^{2+}\) was reintroduced to the medium. Similarly, the sustained oscillations and steady-state increases in K\(_{Ca}\) induced by introducing IP\(_{3}\) in the patch pipettes were also inhibited by removal of extracellular Ca\(^{2+}\) (n = 6, data not shown). To more directly demonstrate the involvement of Ca\(^{2+}\) influx, La\(^{3+}\) (1 mM), which is an effective Ca\(^{2+}\) channel antagonist and blocker of Ca\(^{2+}\) influx in a wide variety of nonexcitable cells including salivary gland cells (26, 30), was introduced into the cell medium. CCh-induced sustained oscillations in K\(_{Ca}\) were first decreased and then abolished in the continued presence of La\(^{3+}\). The current recovered once La\(^{3+}\) was removed from the medium (Fig. 7B). Sustained elevation of [Ca\(^{2+}\)]\(_i\) in CCh-stimulated HSG cells was also blocked by addition of La\(^{3+}\) to the cell medium (data not shown). These data suggest that Ca\(^{2+}\) influx regulates the sustained activation of K\(_{Ca}\) in CCh-treated HSG cells. As mentioned above, internal Ca\(^{2+}\) store refill is achieved by Ca\(^{2+}\) influx via the store-dependent pathway. Thus, to further demonstrate that La\(^{3+}\) blocks K\(_{Ca}\) by inhibiting Ca\(^{2+}\) influx, the effect of La\(^{3+}\) on the refill of internal Ca\(^{2+}\) stores was examined (Fig. 8A). The cells were first stimulated with CCh, and then La\(^{3+}\) was added before removal of CCh. Cells were then restimulated with CCh in the continued presence of La\(^{3+}\). The amplitude of the second response to CCh was significantly reduced to 15.1 ± 5.1% of that in the control response (P < 0.01, n = 6). The inhibition was partially recovered to 37.6 ± 9.6% when La\(^{3+}\) was washed out.

We used the K\(_{Ca}\) current to further characterize the Ca\(^{2+}\) influx pathway. Gd\(^{3+}\) has been reported to block stretch-activated and nonspecific cation channels (5, 33), and Zn\(^{2+}\) has been shown to inhibit the internal Ca\(^{2+}\) release-activated Ca\(^{2+}\) current (I\(_{CRAC}\)) in mast cells and T lymphocytes (2, 6, 8). Zn\(^{2+}\) also inhibits store-operated Ca\(^{2+}\) influx in salivary gland cells (4, 11, 22). The effects of these cations on K\(_{Ca}\) are shown in Fig. 8. The amplitude of CCh-induced K\(_{Ca}\) in the presence of Gd\(^{3+}\) was significantly decreased to 25.9 ± 7.4% (P < 0.01, n = 5) of the control current, and this reduction was also partially restored when Gd\(^{3+}\) was removed (to 46.7 ± 10.2%, Fig. 8B). In general, La\(^{3+}\) and Gd\(^{3+}\) mimicked the effects of extracellular Ca\(^{2+}\) removal on the CCh-induced activation and sustained increases in K\(_{Ca}\). However, Zn\(^{2+}\) did not inhibit CCh-induced K\(_{Ca}\) in HSG cells (Fig. 8C). The mean amplitude of CCh-induced current in the presence of Zn\(^{2+}\) was 88.6 ± 6.9% of that in the control (P > 0.05, n = 5). In aggregate, these results indicate that 1) Ca\(^{2+}\) influx is necessary for maintaining CCh-induced sustained oscillations and steady-state increases in K\(_{Ca}\), 2) Ca\(^{2+}\) influx, via internal Ca\(^{2+}\) store refill, also determines the initial activation of K\(_{Ca}\) by CCh, and 3) Ca\(^{2+}\) influx is mediated via a La\(^{3+}\)- and Gd\(^{3+}\)-sensitive, but Zn\(^{2+}\)-insensitive, pathway. We have also measured the effect of Gd\(^{3+}\) and Zn\(^{2+}\) on CCh-induced elevation in [Ca\(^{2+}\)]\(_i\), and have observed that Gd\(^{3+}\), but not Zn\(^{2+}\), is similar to La\(^{3+}\) in blocking the sustained elevation of [Ca\(^{2+}\)]\(_i\) (data not shown).

Fig. 7. Effect of Ca\(^{2+}\) influx on CCh-induced sustained oscillations of K\(_{Ca}\). A: current was measured in continued presence of 10 µM CCh (dashed line). Perfusion with Ca\(^{2+}\)-free medium and EGTA is shown by the bar. B: experimental conditions were similar to those in A. Cells were continuously perfused with medium containing CCh (10 µM), indicated by dashed line. Addition of 1 mM La\(^{3+}\) to the bath is also indicated (solid bar).

Fig. 8. Effects of Gd\(^{3+}\) and Zn\(^{2+}\) on CCh-induced sustained oscillations of K\(_{Ca}\) in HSG cells. A: current was measured in continued presence of 10 µM CCh (dashed line). Perfusion with Ca\(^{2+}\)-free medium and EGTA is shown by the bar. B: experimental conditions were similar to those in A. Cells were continuously perfused with medium containing CCh (10 µM), indicated by dashed line. Addition of 1 mM La\(^{3+}\) to the bath is also indicated (solid bar).
Regulation of K\textsubscript{Ca} by Ca\textsuperscript{2+} influx is dependent on internal Ca\textsuperscript{2+} release in HSG cells. The data presented above demonstrate that the sustained K\textsubscript{Ca} current in CCh-stimulated HSG cells is primarily regulated by Ca\textsuperscript{2+} influx. To examine the effect of Ca\textsuperscript{2+} influx in the absence of internal Ca\textsuperscript{2+} release, the K\textsubscript{Ca} current was measured during refill of internal Ca\textsuperscript{2+} stores. Cells were first stimulated with CCh in a Ca\textsuperscript{2+}-free medium, and atropine was then added to terminate the muscarinic receptor-mediated signaling (i.e., IP\textsubscript{3}-dependent intracellular release was inactivated). Reintroduction of Ca\textsuperscript{2+} into the cell medium induced rapid activation of K\textsubscript{Ca} (Fig. 9C). Thus the activation of K\textsubscript{Ca} by Ca\textsuperscript{2+} influx alone is achieved only when internal Ca\textsuperscript{2+} release is activated (CCh or TG treated) or internal Ca\textsuperscript{2+} accumulation is inhibited (TG treated, also see Fig. 10).

Fig. 8. La\textsuperscript{3+} and Gd\textsuperscript{3+}, but not Zn\textsuperscript{2+}, block Ca\textsuperscript{2+} influx-dependent regulation of K\textsubscript{Ca}. Same protocol as described in Fig. 1B was used. Perfusion with CCh-containing medium is shown by solid bars. Addition of the test cations (1 mM) is shown by dashed lines, that is, with La\textsuperscript{3+} (A), Gd\textsuperscript{3+} (B), and Zn\textsuperscript{2+} (C). Data are representative of results obtained with 16 cells.

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CCH-INDUCED K\textsubscript{Ca} IN HSG CELLS

Regulation of K\textsubscript{Ca} by Ca\textsuperscript{2+} influx is dependent on internal Ca\textsuperscript{2+} release in HSG cells. The data presented above demonstrate that the sustained K\textsubscript{Ca} current in CCh-stimulated HSG cells is primarily regulated by Ca\textsuperscript{2+} influx. To examine the effect of Ca\textsuperscript{2+} influx in the absence of internal Ca\textsuperscript{2+} release, the K\textsubscript{Ca} current was measured during refill of internal Ca\textsuperscript{2+} stores. Cells were first stimulated with CCh in a Ca\textsuperscript{2+}-free medium, and atropine was then added to terminate the muscarinic receptor-mediated signaling (i.e., IP\textsubscript{3}-dependent intracellular release was inactivated). Reintroduction of Ca\textsuperscript{2+} in the cell medium did not induce any change in K\textsubscript{Ca} (Fig. 9B, also see trace in Fig. 6, A and B). However, under these conditions, Ca\textsuperscript{2+} influx did occur, resulting in the refill of internal Ca\textsuperscript{2+} stores. This is shown by the response to a subsequent addition of TG that was larger than that obtained in cells in which the internal stores were not allowed to fully refill (compare data in Fig. 9B with Fig. 9A; in A, TG was added ~1 min after perfusion with CCh-containing medium was stopped). In the absence of atropine, the IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release pathway remains activated, and in this case readdition of Ca\textsuperscript{2+} to the medium induced rapid activation of K\textsubscript{Ca}. Subsequent removal of Ca\textsuperscript{2+} from the medium and addition of TG induced a small increase in [Ca\textsuperscript{2+}], due to release of Ca\textsuperscript{2+} from partially refilled stores or from stores not mobilized by CCh. Intracellular Ca\textsuperscript{2+} accumulation has been suggested to strongly buffer Ca\textsuperscript{2+} in the subplasma membrane region in exocrine acinar cells (21, 25). Furthermore, previous studies have indicated that refill of internal Ca\textsuperscript{2+} stores is achieved without significant increases in [Ca\textsuperscript{2+}], (24, 26, 31, 34). The data in Fig. 9 are consistent with these previous findings and indicate that the K\textsubscript{Ca} activity monitors [Ca\textsuperscript{2+}] in the region of Ca\textsuperscript{2+} influx. To examine the role of intracellular Ca\textsuperscript{2+} pump activity on the regulation of K\textsubscript{Ca}, cells were treated with CCh, followed by atropine and then TG (i.e., Ca\textsuperscript{2+} influx activated but IP\textsubscript{3} receptor and Ca\textsuperscript{2+} pump inhibited). Reintroduction of Ca\textsuperscript{2+} into the cell medium induced rapid activation of K\textsubscript{Ca} (Fig. 9C). Thus the activation of K\textsubscript{Ca} by Ca\textsuperscript{2+} influx alone is achieved only when internal Ca\textsuperscript{2+} release is activated (CCh or TG treated) or internal Ca\textsuperscript{2+} accumulation is inhibited (TG treated, also see Fig. 10).

Fig. 9. Ca\textsuperscript{2+} influx-dependent regulation of K\textsubscript{Ca} in CCh-stimulated HSG cells is mediated via internal Ca\textsuperscript{2+} release. A: K\textsubscript{Ca} regulation by Ca\textsuperscript{2+} influx in CCh-stimulated cells. Cells were stimulated with CCh in a Ca\textsuperscript{2+}-free medium. Addition and removal of 1 mM Ca\textsuperscript{2+} are indicated. Status of internal Ca\textsuperscript{2+} stores was checked by addition of TG. B: effect of Ca\textsuperscript{2+} influx on K\textsubscript{Ca} during refill of internal Ca\textsuperscript{2+} stores. Additions of CCh, atropine, Ca\textsuperscript{2+}, and TG are indicated. C: Ca\textsuperscript{2+} influx-dependent regulation of K\textsubscript{Ca} following inhibition of intracellular Ca\textsuperscript{2+} pump. Additions of CCh, TG, and Ca\textsuperscript{2+} are indicated. Data represent 3–5 experiments in each condition.
DISCUSSION

The data presented describe the [Ca\textsuperscript{2+}]\textsubscript{i}-dependent regulation of a large-conductance K\textsubscript{Ca} in CCh-stimulated HSG cells. The data demonstrate that there is a strong association between the increase in [Ca\textsuperscript{2+}]\textsubscript{i} and the increase in the K\textsuperscript{+} current in HSG cells stimulated with CCh. We have shown that the initial increase in K\textsubscript{Ca} is dependent on the initial elevation of [Ca\textsuperscript{2+}]\textsubscript{i}. Furthermore, consistent with previous [Ca\textsuperscript{2+}]\textsubscript{i} measurements, the initial amplitude of K\textsubscript{Ca} is not altered by removal of extracellular Ca\textsuperscript{2+}. However, under conditions in which the internal Ca\textsuperscript{2+} store is depleted, CCh activation of K\textsubscript{Ca} is decreased, i.e., in cells treated with IP\textsubscript{3}, TG, or BHQ. Furthermore, several different Ca\textsuperscript{2+} influx pathways have been proposed to be present in nonexcitable cells, including store-operated (capacitative) Ca\textsuperscript{2+} entry, second messenger (i.e., IP\textsubscript{3})-operated Ca\textsuperscript{2+} entry, and receptor-operated Ca\textsuperscript{2+} entry (1, 2, 6, 20, 31). Several different Ca\textsuperscript{2+} influx pathways have been proposed to be present in nonexcitable cells, including store-operated (capacitative) Ca\textsuperscript{2+} entry, second messenger (i.e., IP\textsubscript{3})-operated Ca\textsuperscript{2+} entry, and receptor-operated Ca\textsuperscript{2+} entry (1, 2, 6, 20, 31). We have previously reported the presence of store-operated Ca\textsuperscript{2+} entry in HSG cells. In addition, we had also reported a small Ca\textsuperscript{2+} entry component that appeared to be independent of the store status and was regulated by the muscarinic receptor, either directly or via a G protein (20). In the present study, CCh did not stimulate further increases in K\textsubscript{Ca} in TG- or BHQ-stimulated cells, suggesting that only the store-operated Ca\textsuperscript{2+} influx pathway is primarily involved in sustaining the K\textsuperscript{+} current in HSG cells. This is consistent with our earlier results showing membrane potential changes in CCh-stimulated HSG cell by using membrane potential-sensitive fluorescent dyes (20).

The molecular mechanism involved in mediating Ca\textsuperscript{2+} influx in nonexcitable cells is not yet known. However, it has been reported recently that the store-operated Ca\textsuperscript{2+} entry pathway, where depletion of intracellular Ca\textsuperscript{2+} stores stimulates Ca\textsuperscript{2+} influx across the plasma membrane, is mediated via an I\textsubscript{CRAC} channel (2, 6, 8, 16). Electrophysiological studies with mast cells and T lymphocytes have shown that I\textsubscript{CRAC} has a very low conductance: ~1,000-fold lower than the conductance of classical voltage-sensitive Ca\textsuperscript{2+} channels (6, 8). I\textsubscript{CRAC} is activated by various stimuli, such as the Ca\textsuperscript{2+}-mobilizing agonists (e.g., CCh) or second messengers (e.g., IP\textsubscript{3}) or the inhibitors of the Ca\textsuperscript{2+} pump (e.g., BHQ or TG). It is highly Ca\textsuperscript{2+} selective and is strongly inhibited by La\textsuperscript{3+} or low concentrations of Zn\textsuperscript{2+} and by high [Ca\textsuperscript{2+}]\textsubscript{i}. Although we have not shown direct measurements of the Ca\textsuperscript{2+} influx current in HSG cells, here, we have shown that the sustained activation of K\textsubscript{Ca}, which reflects a sustained elevation of [Ca\textsuperscript{2+}]\textsubscript{i}, is induced by stimulation of the cells with CCh, BHQ, or TG or by introduction of IP\textsubscript{3} into the cells. These results are similar to our previously reported data in which Ca\textsuperscript{2+} entry into fura 2-loaded HSG cells was measured. Furthermore, we have also shown here that 1) the sustained activation of K\textsubscript{Ca} is dependent on extracellular Ca\textsuperscript{2+}, i.e., on Ca\textsuperscript{2+} influx, and is blocked by La\textsuperscript{3+} and Gd\textsuperscript{3+}, but not by Zn\textsuperscript{2+}, and that 2) the inhibition of K\textsubscript{Ca} by the divalent cations is due to the inhibition of Ca\textsuperscript{2+} influx. Zn\textsuperscript{2+} has been reported to effectively block I\textsubscript{CRAC} in RBL mast cells. Thus the Ca\textsuperscript{2+} influx pathway in HSG cells does not appear to show typical characteristics of I\textsubscript{CRAC}. On the other hand, Gd\textsuperscript{3+}, which blocks Ca\textsuperscript{2+} influx into HSG cells (data not shown), has been used extensively to block stretch-activated and voltage-gated cation channels (5, 33). More recently, it has been shown to block cation influx mediated by the Trp gene product, which has been proposed as a candidate protein for the store-operated Ca\textsuperscript{2+} influx activity (3). However, further studies are required to fully describe the electrophysiological characteristics of the Ca\textsuperscript{2+} influx pathway in HSG cells.

The involvement of store-operated Ca\textsuperscript{2+} influx in CCh-dependent regulation of K\textsubscript{Ca} in HSG cells is demonstrated by the following. 1) TG and BHQ mimic CCh-induced increases in K\textsubscript{Ca} conductance and attenuate the response induced by CCh and vice versa. 2) Inhibition of Ca\textsuperscript{2+} influx prevents initial activation of K\textsubscript{Ca} by preventing refill of internal Ca\textsuperscript{2+} store(s). 3) Inhibition of Ca\textsuperscript{2+} influx prevents sustained activation of K\textsubscript{Ca} due to loss of sustained [Ca\textsuperscript{2+}]\textsubscript{i} elevation. Our model for the regulation of K\textsubscript{Ca} by Ca\textsuperscript{2+} influx in HSG cells is shown in Fig. 10. We have shown that Ca\textsuperscript{2+} influx alone, in the
absence of internal Ca$^{2+}$ release (i.e., during refill of internal Ca$^{2+}$ stores), does not activate K$_{Ca}$ (Fig. 10B, see data in Fig. 9B). When the intracellular Ca$^{2+}$ accumulation is inhibited, K$_{Ca}$ is activated by Ca$^{2+}$ influx (Fig. 10C, see data in Fig. 9C). These data clearly indicate that the intracellular Ca$^{2+}$ store membrane and the plasma membrane are likely to be in close proximity, consistent with previous studies (10, 25). However, presently we cannot rule out the possibility that other Ca$^{2+}$ stores may be present that are not closely situated to the plasma membrane and thus likely not involved in the regulation of K$_{Ca}$ activity.

In aggregate, the data presented above suggest that the [Ca$^{2+}$]$_i$ increase in the region of Ca$^{2+}$ influx appears to be strongly buffered by intracellular Ca$^{2+}$ influx and that there is minimal diffusion of Ca$^{2+}$ from this region under conditions when Ca$^{2+}$ influx is activated. Such buffering has been recently suggested in pancreatic cells, where it was shown that influx of Ca$^{2+}$ induced refill of internal Ca$^{2+}$ stores without giving rise to elevations in [Ca$^{2+}$]$_i$, unless the intracellular Ca$^{2+}$ accumulation was inhibited by TG (Ref. 25, also see footnote1). The present studies provide further evidence for this buffering by using the K$_{Ca}$ activity as a readout for subplasma membrane changes in [Ca$^{2+}$]$_i$. The data (see Fig. 9A) indicate that, when the IP$_3$-dependent Ca$^{2+}$ release pathway in the internal Ca$^{2+}$ store is activated, Ca$^{2+}$ entering the cell via the Ca$^{2+}$ influx pathway in the plasma membrane can reach the K$^+$ channel and activate it. We suggest that, in Ca$^{2+}$-stimulated HSG cells, this is mediated by the uptake of Ca$^{2+}$ into the internal store and release via the IP$_3$-sensitive channel, without significant accumulation in the store (Fig. 10A). An assumption in our model is that IP$_3$-dependent release of Ca$^{2+}$ from the store does not induce a change in the Ca$^{2+}$ pump activity (decrease) or in the diffusion (increase) of Ca$^{2+}$ from the site of influx. An important question that arises from the above model is why the cell would expend considerable energy to pump Ca$^{2+}$ into the store while the IP$_3$-sensitive release channel is activated. A possible explanation is that such a mechanism allows the cell to direct localized release of Ca$^{2+}$ and also prevents significant increase in [Ca$^{2+}$]$_i$ at the site of influx. Localized sites for intracellular Ca$^{2+}$ uptake and release have recently been proposed in salivary and pancreatic acinar cells (21, 25). Further studies will be required to determine whether the subcellular localization of the Ca$^{2+}$ influx protein(s), the K$^+$ channel, the IP$_3$ receptor, and the internal Ca$^{2+}$ pump in the sub-plasma membrane region of the HSG cell determine the regulation of K$_{Ca}$ by [Ca$^{2+}$].

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