Cyclic GMP Modulates Depletion-activated Ca\(^{2+}\) Entry in Pancreatic Acinar Cells*

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In the pancreatic acinar cell, hormonal stimulation causes a rise in the intracellular free Ca\(^{2+}\) concentration by activating the inositol 1,4,5-trisphosphate-mediated release of Ca\(^{2+}\) from intracellular stores (Berridge, M. J., and Irvine, R. F. (1989) Nature 341, 197–205). The released Ca\(^{2+}\) is, for the most part, extruded from the cell, necessitating a mechanism for Ca\(^{2+}\) entry and reloading of intracellular Ca\(^{2+}\) stores (Putney, J. W., Jr. (1990) Cell Calcium 11, 611–624; Rink, T. J. (1990) FEBS Lett. 268, 381–385). However, neither the mechanism of depletion-activated Ca\(^{2+}\) entry nor the signal that activates it is known. We report here that a sustained inward current of depletion-activated Ca\(^{2+}\) entry can be measured in pancreatic acinar cells using patch-clamp recording methods. Furthermore, the current can be blocked by an inhibitor of guanylyl cyclase, can be reactivated by 8-bromo-cGMP after inhibition, and can be activated in the absence of Ca\(^{2+}\) depletion by perfusing the cell with cGMP, but not cAMP. Inward perfusion with 1,3,4,5-inositol tetrakisphosphate did not activate an inward current, whereas perfusion with 2,4,5-inositol trisphosphate did activate an inward current. We conclude that cGMP may be an intracellular messenger that regulates depletion-activated Ca\(^{2+}\) entry.

Pancreatic acinar cells were important in the discovery of inositol lipids and inositol 1,4,5-trisphosphate (IP3) signaling (1–5). Ca\(^{2+}\) entry that occurs when intracellular Ca\(^{2+}\) stores become depleted is essential to maintain [Ca\(^{2+}\)]\(_{i}\), during tonic stimulation (6) and to maintain cellular responsiveness to subsequent stimulation in this (7) and other cell types (8). Depletion-activated Ca\(^{2+}\) entry in acinar cells has previously been described using indirect measurements (6, 9, 10); however, direct measurement of a Ca\(^{2+}\) current in these cells corresponding to depletion-activated Ca\(^{2+}\) entry has not been reported. Hoth and Penner (11) described an inward current in mast cells carried by Ca\(^{2+}\), termed I\(_{MAC}\) for “calcium release-activated calcium,” which could be measured when the intracellular stores were depleted. To determine whether depletion-activated Ca\(^{2+}\) entry could similarly be resolved in acinar cells and to investigate the intracellular regulation of Ca\(^{2+}\) entry, whole-cell recordings were made under various conditions of stored Ca\(^{2+}\) depletion. Here, we show that intracellular application of cGMP can mimic Ca\(^{2+}\) depletion as an activator of the Ca\(^{2+}\) entry current, that inhibition of cGMP formation can inhibit the Ca\(^{2+}\) entry current seen after Ca\(^{2+}\) depletion, and that inositol tetrakisphosphate is neither necessary nor sufficient to activate the Ca\(^{2+}\) entry current in this cell type.

MATERIALS AND METHODS

Pancreatic acinar cells were isolated from adult Sprague-Dawley rats as previously described (12), and the tight-seal whole-cell recording configuration of the patch-clamp technique (13) was used to make current measurements. The intracellular recording solution contained 125 mM potassium acetate, 10 mM KCl, 2 mM MgCl\(_2\), 10 mM K, BAPTA, and 10 mM HEPES (pH 7.4 with NaOH). CaCl\(_2\) was added to this solution to give the indicated free [Ca\(^{2+}\)]. The standard bathing solution contained 135 mM NaCl, 4 mM KCl, 2 mM MgCl\(_2\), 12 mM CaCl\(_2\), 2 mM EGTA, 10 mM HEPES (pH 7.4 with NaOH), and 10 mM glucose. Experiments were performed at 22–25°C. The occurrence of depletion-activated Ca\(^{2+}\) entry in acinar cells incubated at room temperature has been verified by spectrofluorometric fura-2 measurements (data not shown). Bath exchanges were accomplished using gravity perfusion. Microperfusion was accomplished using pipettes (tip diameter of 10–20 μm) placed near the test cell. A computer-controlled pressure pulse was applied to the microperfusion pipette to cause discharge. Patch pipettes were pulled from Corning 8161 capillary tubing. Pipette resistance was 2–3 megohms using these solutions. All traces in the figures are taken from single cells and are representative of totals described in the text. Unless otherwise noted, all current axes are marked relative to the initial holding current. Cells were held at either 0 mV (n = 141) or at V\(_{h}\) (n = 28), where the initial holding current was zero. Recordings were made using an Axopatch amplifier (Axon Instruments, Inc., Foster City, CA). Currents were sampled at 5-ms intervals and filtered at 20 Hz unless otherwise noted. Data were digitized on-line using a laboratory computer (Indec Systems, Inc., Sunnyvale, CA) running acquisition and analysis software written in this laboratory. LY83853 (6-amino-5,8-guanosinedione), 1,2,4,5-inositol tetakisphosphate (IP\(_5\)), and 2,4,5-inositol trisphosphate (2,4,5-IP\(_3\)) were purchased from Calbiochem. Other reagents were obtained from Sigma.

RESULTS AND DISCUSSION

Whole-cell current recordings were made with 10 mM free Ca\(^{2+}\) in the bathing medium and BAPTA in the intracellular solution to buffer [Ca\(^{2+}\)], to the indicated levels (1–75 nM). Under these conditions, carbachol, which stimulates muscarinic receptors on the cell surface, would be expected to release stored Ca\(^{2+}\) and, in the presence of intracellular BAPTA, cause long-lasting Ca\(^{2+}\) depletion (6). The application of carbachol during whole-cell recording activated an inward current (two/four cells) with an amplitude of −6.3 ± 2.4 pA (values are expressed as mean ± S.D.). This current had a
positive reversal potential and was sustained due to the chelation of Ca²⁺ by BAPTA (Fig. 1A). No currents developed in the absence of the carbachol puff (Fig. 1B). To study the regulation of this depletion-activated Ca²⁺ current, we activated it directly by buffering [Ca²⁺], to low levels and including IP₃ in the patch pipette to stimulate Ca²⁺ release and to cause Ca²⁺ depletion. Under these whole-cell recording conditions, a slowly developing, inward current was detected in 50/52 cells (Fig. 1C) that persisted for the duration of the recording (up to 11.5 min) (data not shown). In no cell was the current seen to diminish spontaneously. The mean amplitude of this inward current was -2.9 ± 1.9 pA, and the mean time to half-maximal current (t₁/₂) was 51 ± 27 s (36 cells). This current could also be detected when the extracellular calcium concentration was 2 mM (−3.7 pA, t₁/₂ = 48 s, one cell).

Ramp current voltage curves indicated that the inward current corresponded to an increase in membrane conductance and that its reversal potential was approximately +45 mV (Fig. 2A). In instances where ramp currents were not measured, the positive reversal potential of the inward current was verified by noting that the current was inward when the cell was held at 0 mV. Under our recording conditions, the reversal potentials for Cl⁻, K⁺, and a monovalent-selective nonspecific cation conductance would be negative, causing an outward current for these ions at V_m = 0 mV, opposite to what was observed. After onset of the inward current, microperfusion with Ca²⁺-free solutions containing various divalent cations substituted for Ca²⁺ diminished the current. To replace Ca²⁺ in the perfusate, we made equimolar substitution with Mn²⁺ (current diminished in six/six cells) (Fig. 2B), N-methyl-D-glucamine⁺ (three/three cells) (data not shown), Ni²⁺ (two/two cells) (data not shown), or Cd²⁺ (one/two cells) (data not shown). The current was only partially blocked in these experiments, possibly because of incomplete replacement of the Ca²⁺-containing bath by the perfusate, because Na⁺ or the substituted cations may be somewhat permeant through the Ca²⁺ entry mechanism, or because more than one conductance is activated. Nonetheless, these findings suggest that the inward current was, at least in part, carried by Ca²⁺. A lack of Ca²⁺ selectivity for the entry mechanism would not be unexpected; previously reported ligand-gated Ca²⁺-permeable ion channels in smooth muscle cells (14), lymphocytes

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![Figure 1](image1.png)

**FIG. 1. Inward current of depletion-activated Ca²⁺ entry in pancreatic acinar cells.** The time scale on all current traces begins the moment the whole-cell recording mode was established. The intracellular saline contained 1 mM MgATP and 100 μM GTP; cells were held at 0 mV. A, activation of the inward current following carbachol-stimulated release of Ca²⁺ from intracellular stores. Carbachol (30 μM) was applied by microperfusion for the duration indicated by the horizontal line. Approximately 5 s after the beginning of carbachol application, an inward current developed that was sustained and reached a maximum of −5 pA after −40 s. The intracellular saline contained 74 nM free Ca²⁺ to prevent depletion of the Ca²⁺ stores by low [Ca²⁺] alone. The ragged current deflections on the trace are probably Ca²⁺-activated single channel currents; occasional single channel currents were seen whenever [Ca²⁺] was elevated to the normal physiological range. B, no inward current in the absence of Ca²⁺ depletion. With recording conditions identical to those described above but in the absence of any stimulus for Ca²⁺ release, no inward currents were evident for more than 5 min. C, activation of the inward current in the presence of IP₃ and low [Ca²⁺]. The pipette contained 10 μM IP₃ and 3 nM free Ca²⁺. A sustained inward current developed with a peak amplitude of −3 pA and a t₁/₂ of 43 s.

![Figure 2](image2.png)

**FIG. 2. Characteristics of depletion-activated Ca²⁺ entry.** A, reversal potential of the inward current. Multiple voltage ramps (depicted at the top) lasting 128 ms were applied from −70 to +90 mV to elicit ramp currents prior to development of the inward current and when the current was fully developed. The difference between the averaged ramp currents (response in the presence of the inward current minus control response) was plotted. The positive slope of the current difference indicates that an increased conductance underlies the inward current. Its reversal potential had a value of approximately +45 mV, [Ca²⁺], was buffered to 12 nM; the pipette solution was otherwise identical to that used in Fig. 1C. Currents were sampled at 500-μs intervals and filtered at 1 kHz. B, decrease of the inward current by microperfusion with a Ca²⁺-free solution. The inward current was induced as described for Fig. 1C prior to the start of this record. A Ca²⁺-free solution containing 11 mM MnCl₂, but otherwise identical to the standard bathing solution, was applied by microperfusion for the duration indicated by the horizontal line. This record was filtered at 50 Hz.
pancreatic acinar cells with nitroprusside, which increases the cell interior. In six/seven cells pretreated with LY83583 treatment with LY83583 and of adding exogenous cGMP to the inward current, we used whole-cell recording conditions on [Ca\textsuperscript{2+}] or on the carbachol-stimulated release of Ca\textsuperscript{2+} from the current of depletion-activated Ca\textsuperscript{2+} entry as we observe. Although LY83583 may have multiple actions in this and cGMP restored the inward current (six/seven cells) (Fig. 3C). When LY83583 was added to the bath after directly activating the inward current, the current decreased to near base line (12/15 cells) (Fig. 3B). Furthermore, after LY83583 inhibition of the inward current, microperfusion with 8-Br-cGMP restored the inward current (six/seven cells) (Fig. 3C). Although LY83583 may have multiple actions in this and other cell types, the finding that the LY83583-mediated inhibition of the inward current was reversed by the addition of 8-Br-cGMP suggests that, in this instance, LY83583 was acting by preventing cGMP formation. A recent report (23) proposed that cGMP itself might cause release of Ca\textsuperscript{2+} from IP\textsubscript{3}-sensitive stores in sea urchin eggs. If cGMP were acting exclusively in this way in acinar cells, then blockade of GMP formation with LY83583 after the intracellular stores had already been depleted with IP\textsubscript{3} should not have terminated the current of depletion-activated Ca\textsuperscript{2+} entry as we observe. Furthermore, previous data demonstrated that treatment of pancreatic acinar cells with nitroprusside, which increases intracellular GMP levels several hundredfold, had no effect on [Ca\textsuperscript{2+}], or on the carbachol-stimulated release of Ca\textsuperscript{2+} from intracellular stores as measured by fura-2 fluorescence (24). Thus, cGMP-mediated depletion of intracellular Ca\textsuperscript{2+} stores does not appear to be the means by which GMP stimulates depletion-activated Ca\textsuperscript{2+} entry in these experiments. The observations that LY83583 treatment can inhibit and 8-Br-cGMP can then reactivate the inward current of depletion-activated Ca\textsuperscript{2+} entry suggest that elevation of intracellular GMP levels is important in regulating depletion-activated Ca\textsuperscript{2+} entry.

To evaluate whether cGMP alone is sufficient to activate the inward current, we used whole-cell recording conditions that prevented depletion of Ca\textsuperscript{2+} from the intracellular stores and then tested whether the addition of cGMP could stimulate the inward current. Roth and Penner (11) reported that buffering [Ca\textsuperscript{2+}], to very low levels in mast cells can cause Ca\textsuperscript{2+} depletion, leading to activation of Ca\textsuperscript{2+} entry in the absence of IP\textsubscript{3}. We found that buffering [Ca\textsuperscript{2+}] to 12 nM or less in the absence of IP\textsubscript{3} caused the inward current to develop in five/six cells tested (−4.8 ± 2.2 pA, \(t_{1/2} = 58 ± 17\) s, five cells) (data not shown). To prevent Ca\textsuperscript{2+} depletion, IP\textsubscript{3} was omitted from the pipette solution, and [Ca\textsuperscript{2+}], was buffered to 74 nM, near its normal resting level of 70–90 nM in these cells (22) and near the \(K_{d}\) of the Ca-ATPase that reloa...
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Fig. 4. cGMP mimics Ca²⁺ depletion as stimulus for inward current. The cells were held at 0 mV. The pipette solution contained 1 mM MgATP, 100 μM GTP, no IP₃, and 74 nM free Ca²⁺ to prevent depletion of stored Ca²⁺. A, cGMP activates the inward current in the absence of Ca²⁺ depletion. With the addition of 10 μM cGMP to the pipette, the inward current developed. B, the inward current activated by cGMP had a reversal potential near that for Ca²⁺. The voltage ramp method (ramps from −40 to +80 mV) (see Fig. 2A) was used to establish a reversal potential of +50 mV for the inward current activated by cGMP in the absence of Ca²⁺ depletion.

pA and a t₁/₂ of 36 ± 9 s (seven cells), similar to that seen after Ca²⁺ depletion (Fig. 1C).

Ramp currents recorded before and after development of the cGMP-activated inward current showed that its reversal potential was approximately +50 mV (Fig. 4B), nearly identical to that measured for the depletion-activated current stimulated by IP₃ and low [Ca²⁺]. (Fig. 2A). Furthermore, the time course of the inward current after cGMP treatment (t₁/₂ = 36 ± 9 s) was similar to that after Ca²⁺ depletion with IP₃ and low [Ca²⁺]; (t₁/₂ = 51 ± 27 s) and with low [Ca²⁺]; alone (t₁/₂ = 58 ± 17 s). Finally, microperfusion with a Ca²⁺-free bath containing equimolar Mn²⁺ (two cells) or Ni²⁺ (three cells) substituted for Ca²⁺ caused a transient decrease in the cGMP-activated inward current that was similar to that seen with the IP₃ plus low [Ca²⁺]-treated cells (see Fig. 2B). The finding that the depletion-activated Ca²⁺ currents and the cGMP-activated currents had similar peak amplitudes, time courses, reversal potentials, and qualitative divalent ion permeability under whole-cell recording conditions suggest that they are the same current. When CAMP (10 μM) was substituted for cGMP in the cell perfusate, no inward current was detected (three/three cells) (data not shown).

Previous studies (26–28) have suggested that IP₃ may be involved in regulating depletion-activated Ca²⁺ entry. To determine whether this is the case in pancreatic acinar cells, the necessity and sufficiency of IP₃ to stimulate the depletion-activated current was tested in separate experiments. To test whether IP₃ is necessary for the development of depletion-activated Ca²⁺ entry, we used 2,4,5-IP₃, a potent intracellular Ca²⁺-releasing agent (29) and a nonphosphorylatable analog of IP₃, instead of IP₃, to cause Ca²⁺ depletion. Intracellular [Ca²⁺₁ was maintained above 70 nM so that low [Ca²⁺], alone would not deplete intracellular Ca²⁺ stores. In 8/14 cells perfused with 2,4,5-IP₃ (10 μM), an inward current developed (Fig. 5A) with a positive reversal potential, a peak amplitude (−4.5 ± 2.6 pA, eight cells), and time course (t₁/₂ = 43 ± 16 s, eight cells) nearly identical to those seen after IP₃ treatment.

Under these conditions, no substrate for IP₄ formation should have been present, which suggests that IP₄ is not required for activation of the inward current. However, these data do not exclude a positive modulatory influence of IP₄ on Ca²⁺ entry.

The second experiment tested whether IP₄ is sufficient to activate the inward Ca²⁺ current in pancreatic acinar cells. Whole-cell recordings were made with IP₄ (10 μM) replacing cGMP, and 74 nM Ca²⁺ in the pipette. The bath and pipette solutions were otherwise identical to those used with the cGMP perfusion experiments. No inward currents were evident under these conditions in eight/nine cells. The ability of the cell to generate an inward current was verified by puffer application of 8-Br-cGMP after intracellular perfusion with IP₄; in five/seven cells, a transient inward current (−2.6 ± 1.1 pA) was evident for the duration of perfusion with 8-Br-cGMP (Fig. 5B). A recent report (30) suggested that IP₄ is sufficient to activate a Ca²⁺-permeable conductance in vascular endothelial cells. However, our data plus other studies show that a nonphosphorylatable analog of IP₃ is sufficient to activate Ca²⁺ entry in acinar cells (29), and other studies found that thapsigargin can activate Ca²⁺ entry in rat lacrimal acinar cells (31) and rat parotid acinar cells (32) in the absence of IP₃ or IP₄ (32, 33). These results suggest that IP₄ is not the final messenger in the pathway in acinar cells, although a modulatory role in the phenomenon cannot be ruled out.

We find that intracellular cGMP stimulates the depletion-activated Ca²⁺ entry mechanism in acinar cells under conditions in which no endogenous activating signal is produced and that an agent that blocks cGMP formation inhibits the current. These results suggest that cGMP is a physiological mediator of depletion-activated Ca²⁺ entry in pancreatic acinar cells.
been demonstrated to elevate cGMP levels in the absence of perfusion from "washing out" the response. Whether guanylyl cyclase (GC) is directly or indirectly activated by Ca$^2+$ store depletion or activation of Caz+ entry under conditions of whole-cell recording is as yet unknown. A pathway signaling Ca$^2+$ entry remains unknown. A possible mechanism for Ca$^2+$ entry, either directly or possibly via a CaM-dependent kinase or phosphodiesterase. As yet unidentified messengers may also modulate the Ca$^2+$ entry mechanism.

The inward currents recorded from acinar cells under conditions of Ca$^2+$ depletion or cGMP perfusion demonstrated outward rectification at positive voltages over the ramp voltage range tested and showed positive reversal potentials (see Figs. 2A and 4B). Hoth and Penner (11) reported inward rectification of the Ca$^2+$ current in mast cells, which was evident at negative voltages and which was somewhat accentuated by the ramp protocol used (11). Our measurements were made over a more limited voltage range because our goal was to identify the reversal potential of the current, and the cells became unstable at extreme negative voltages. Thus, we cannot evaluate this feature of the Ca$^2+$ entry current in acinar cells. The time course, amplitude, reversal potential, and divalent ion permeability of depletion-activated Ca$^2+$ entry in acinar cells are similar to those previously reported by Hoth and Penner (11) for mast cells. This suggests that a similar Ca$^2+$ entry mechanism may exist in both cell types.

While the mechanism of depletion-activated Ca$^2+$ entry remains unknown, the finding that cGMP can regulate the current here suggests that a cGMP-dependent kinase or phosphodiesterase is involved in the signaling pathway or that cGMP is interacting directly with an ion channel (see Fig. 6). If the latter proves to be true, the Ca$^2+$ entry mechanism in acinar cells may be one of a family of calcium and Mg$^2+$ permeable ion channels such as the IP$_3$ channel in cardiac myocytes (39), the L$_v$ channel in neurons (40), and the cyclic nucleotide-gated channels in photoreceptors (16, 41) and olfactory receptor cells (42).

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References

Fig. 6. Proposed mechanism for regulation of depletion-activated Ca$^2+$ entry. Receptor (R) stimulation results in the activation of phospholipase C (PLC) via a membrane-associated guanine nucleotide regulatory protein (G). The soluble intracellular messenger IP$_3$ then activates phospholipase D (PLC) and diffuses to the endoplasmic reticulum, where it interacts with the IP$_3$ receptor to cause Ca$^2+$ release. Released Ca$^2+$ is extruded from the cell (not shown). The lowering of intracellular [Ca$^{2+}$]$_i$ in the store activates guanylyl cyclase (GC), either directly (as in a) or indirectly via an unknown messenger (as in b), causing an elevation of intracellular cGMP levels. cGMP, in turn, activates a plasmalemmal mechanism for Ca$^2+$ entry, either directly or possibly via a CaM-dependent kinase or phosphodiesterase. As yet unidentified messengers may also modulate the Ca$^2+$ entry mechanism.