Modulation of Pancreatic Acinar Cell to Cell Coupling during ACh-evoked Changes in Cytosolic Ca\(^{2+}\)**

(Received for publication, June 15, 1998, and in revised form October 8, 1998)

Marc Chanson‡‡‡, Patrice Mollard**, Paolo Meda‡, Susanne Suter§, and Habo J. Jongsm‡

From the ‡Department of Medical Physiology and Sport Medicine, Utrecht University, 3508TA Utrecht, The Netherlands, **INSERM Unité 469, CCIFPE, 34094 Montpellier, France, and the ‡Department of Pediatrics and Department of Morphology, University of Geneva, 1211 Geneva 14, Switzerland

The temporal changes in cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)), Ca\(^{2+}\)-dependent membrane currents (\(I_m\)), and gap junctional current (\(I_j\)) elicited by acetylcholine (ACh) were measured in rat pancreatic acinar cells using digital imaging and dual perforated patch-clamp recording. ACh (50 nm-5 μm) increased [Ca\(^{2+}\)]\(_i\), and evoked \(I_m\) currents without altering \(I_j\) in 19 of 37 acinar cell pairs. Although [Ca\(^{2+}\)]\(_i\), rose asynchronously in cells comprising a cluster, the delay of the [Ca\(^{2+}\)]\(_i\) responses in neighboring cells that were not necessarily in direct contact with the stimulated one. This suggests that extensive coupling between acinar cells provides a pathway for cell-to-cell diffusion of Ca\(^{2+}\)-releasing signals. Strikingly, maximal (1–5 μm) ACh concentrations reduced \(I_j\) by 69 ± 15% (n = 9) in 25% of the cell pairs subjected to dual patch-clamp recording. This decrease occurred shortly after the \(I_m\) peak and was prevented by incubating acinar cells in a Ca\(^{2+}\)-free medium, suggesting that uncoupling was subsequent to the initiation of the Ca\(^{2+}\)-mobilizing responses. Depletion of Ca\(^{2+}\)-sequestering stores by thapsigargin resulted in a reduction of intercellular communication similar to that observed with ACh. In addition, ACh-induced uncoupling was prevented by blocking nitric oxide production with l-nitro-arginine and restored by exposing acinar cells to dibutyryl cGMP. The results suggest that ACh-induced uncoupling and capacitative Ca\(^{2+}\) entry are regulated concurrently. Closure of gap junction channels may occur to functionally isolate nearby cells differing in their intrinsic sensitivity to ACh and thereby to allow for sustained activity of groups of secreting cells.

Gap junctions are intercellular channels formed by twelve subunits of membrane proteins called connexins (Cx). Six subunits are contributed by each cell to form hemichannels, the docking of which provides a low resistance pathway for exchange of ions and small molecules between cells in contact. Gap junctional coupling was shown to be involved in the control of embryonic development, cell proliferation, electrical conduction, and metabolic cooperation (1–3). Because gap junction channels allow for the potential passage of molecules of a molecular mass up to 1000 Da, it is conceivable that second messengers produced in one cell can diffuse between neighboring cells to coordinate their individual response. In support of this hypothesis, the passage of Ca\(^{2+}\) waves has been reported in epithelial cells, glial cells, and various cultured cells (4).

The exocrine pancreas represents a valuable model to search for the role of gap junctional coupling in signal transduction of nonexcitable tissues. Acinar cells are extensively electrically and chemically coupled by Cx32- and Cx26-built gap junction channels (5). A major group of secretagogues in these cells are the Ca\(^{2+}\)-mobilizing agonists, including cholecystokinin and acetylcholine (ACh). In the highly polarized acinar cells, cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) initially rises within the apical secretory region and, when stimulation is sufficient, subsequently spreads as a wave toward the basal pole of the cell (6–9). Intercellular propagation of Ca\(^{2+}\) oscillations and/or Ca\(^{2+}\) waves elicited by Ca\(^{2+}\)-mobilizing secretagogues has been reported to correlate with gap junctional activity (7, 10–13). Increasing evidence indicates that open gap junctions coordinate the frequency of Ca\(^{2+}\) oscillations within individual cells of a same acinus which, in turn, regulates enzyme secretion. This hypothesis, however, is in apparent contradiction with the observation that these secretagogues also evoke acinar cell uncoupling, both in vitro and in vivo, at concentrations that maximally stimulate enzyme secretion (14–17). Thus, the role of gap junctional coupling during acute stimulation of pancreatic acinar cells remains tantalizing.

One of the first questions to address is whether rises in [Ca\(^{2+}\)]\(_i\), and changes in junctional coupling evoked by acinar cell stimulation are parallel events. The application of the patch-clamp technique to dissociated acinar cells has revealed that the kinetic of Ca\(^{2+}\)-dependent membrane current activation reflects that of the [Ca\(^{2+}\)]\(_i\), changes (18–20). However, accurate monitoring of junctional conductance under dual whole-cell recording conditions has been limited because spontaneous uncoupling occurs within seconds, presumably as a result of cytoplasmic dialysis (21–23). To bypass this problem, we applied here a dual perforated patch-clamp approach, which preserves the integrity of the internal milieu (24, 25), to mon-
itor pairs of acinar cells stimulated with increasing ACh concentrations for both Ca\(^{2+}\)-dependent membrane and gap junctional currents. In parallel experiments, changes in [Ca\(^{2+}\)]\(_i\), were monitored by digital imaging of fluo-3-loaded acinar cells. Under these conditions, we observed that ACh-induced closure of gap junction channels parallels the phase plateau of the [Ca\(^{2+}\)]\(_i\) response, but not the initial peak. We further show that acinar cell uncoupling requires the presence of extracellular Ca\(^{2+}\) and parallels capacitative Ca\(^{2+}\) entry.

**EXPERIMENTAL PROCEDURES**

**Preparation of Acinar Cells—**Acinar cells were prepared as described previously (26). Briefly, acini were first isolated by collagenase (CLS 3, Worthington Biochemical Corp.) digestion from the pancreas of male Wistar or Sprague-Dawley rats (about 200 g), which were killed by decapitation. Single and paired acinar cells were prepared by resuspending the intact acini in a Ca\(^{2+}\)- and Mg\(^{2+}\)-free Krebs-Ringer-bicarbonate medium buffered to pH 7.4 with 12.5 mM Hepes-NaOH and containing 3 mM EGTA. The resulting cell suspension was repeatedly passed through an 18-gauge needle, centrifuged for 3 min at 100 × g in Krebs-Ringer bicarbonate medium (KRB) supplemented with 4% bovine serum albumin. Cells were then resuspended in RPMI 1640 culture medium (Life Technologies, Inc.) supplemented with 0.1% bovine serum albumin and 0.01% trypsin inhibitor (Sigma), plated on bacterial Petri dishes (60 × 15 mm), and kept at 4 °C up to 6 h.

**Perforated Patch-Clamp—**Acinar cells were rinsed by centrifugation and allowed to attach for 15 min at room temperature onto glass coverslips, previously coated with 0.5% gelatin (M, 150,000–300,000, Sigma) in distilled water. Coverslips with attached acinar cells were transferred to a chamber, mounted on a Nikon Optiphot microscope, and placed in an inverted stage of an inverted microscope (TMD-300, Nikon AG). Throughout the experiments, cells were continuously superfused with a solution containing 136 mM NaCl, 4 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), and 25 mM glucose, which was buffered to pH 7.4 with 10 mM Hepes-NaOH (control solution). In some experiments, CaCl\(_2\) was omitted from the control solution and 1 mM EGTA was added (Ca\(^{2+}\)-free solution). To allow long-term recording with minimal cell damage, we chose a dual perforated configuration of the patch-clamp technique (25). To increase the success of seal formation, the tip of electrodes was first filled with a solution containing 139 mM KCl, 1 mM CaCl\(_2\), 2 mM MgCl\(_2\), 0.5 mM EGTA, 10 mM Hepes-KOH (pH 7.2) and then back-filled with the same solution supplemented with 120 mM amphotericin B (24). This antibiotic concentration was pre-exposed to acinar cells in a solution of dimethyl sulfoxide (Me2SO) and thoroughly sonicated before use. Under these conditions, the electrode resistance averaged 2.9 ± 0.08 MΩ (mean ± S.E., n = 56) as measured in the bathing control solution. The use of amphotericin B has been reported to yield cell capacitance values that are lower than those of untreated cells, which we correlated to the observed decreased junctional conductance (56). However, ACh induced a marked decrease in junctional conductance (56). For dye coupling studies, intact acini attached to plastic dishes coated with poly-L-lysine were used. Acini were incubated in KRB supplemented with either 1 μM ACh, 0.5 μM thapsigargin, or 1 mM dibutyryl cGMP (Sigma) for up to 30 min. Specificity of these agents was tested by depolarizing acinar cells for their internal Ca\(^{2+}\). To deplete Ylabeled cells, cell coupling was then expressed as percentage of the acinar area. All data are expressed as mean ± S.E. and compared with controls using an unpaired t test. Values were not corrected for the slight overestimation of the Lucifer Yellow stained areas which resulted from their fluorescent labeling (30).

**RESULTS**

**Temporal Relationship between ACh-induced Uncoupling and [Ca\(^{2+}\)]\(_i\)—**The dual perforated patch-clamp approach was applied to pairs of acinar cells to simultaneously monitor Ca\(^{2+}\)-dependent membrane (\(I_{\text{mem}}\)) and gap junctional currents (\(I_{\text{gj}}\)). All cell pairs exhibited a large and stable initial junctional conductance, which averaged 42 ± 4 nS (mean ± S.E., n = 37) over a 30–90 min period of recording. Nine of the 37 cell pairs studied did not respond to ACh at concentrations ranging from 100 nM to 5 μM. In 19 cell pairs, ACh triggered \(I_{\text{gj}}\) currents without affecting the extensive electrical coupling (\(I_{\text{m}}\) traces) of the cell pairs (Fig. 1A). In the remaining nine pairs, a similar \(I_{\text{m}}\) was observed with ACh concentrations ≤ 1 μM. However, ACh induced a marked decrease in junctional coupling, averaging 69 ± 15% (n = 9) of the initial conductance, at concentrations of 1–5 μM. This uncoupling was reversible when the secretagogue was washed out and was inducible again during a second exposure to ACh (Fig. 1B). The decrease
in junctional conductance was low, if any, at the $I_m$ peaks. In contrast, uncoupling was maximal 1–2 min after the $I_m$ peaks, taking place during the sustained phase of the $I_m$ response (Figs. 1B and 3). Close inspection of the recordings showed that $Ca^{2+}$-dependent currents ($I_{m1}$ and $I_{m2}$) were synchronized in both cells of a pair when junctional coupling was high. In contrast, the pattern of $I_{m1}$ and $I_{m2}$ currents was different when junctional coupling was low (Fig. 1B, arrows). In the example shown in Fig. 1B, while $Ca^{2+}$-dependent membrane currents were still observed in one cell ($I_{m1}$, trace), they were readily discontinued in the other ($I_{m2}$, trace). Similar observations were made in stimulated pairs that spontaneously uncoupled during recording ($n = 4$) or which were experimentally uncoupled by superfusing 3.5 mM heptanol ($n = 2$).

One possible explanation for the synchronized $I_m$ responses observed in highly coupled pairs is that cells mobilized $[Ca^{2+}]_i$, simultaneously. Alternatively, junctional coupling could be so high that the patch pipettes only recorded the average current of the whole cell pair. To address this question, $[Ca^{2+}]_i$ changes were monitored in multiple acinar cells by digital imaging. As shown in Fig. 2A, superfusion of an acinar cell pair with 100 nM ACh induced first a rise in $[Ca^{2+}]_i$ in one cell followed by a delayed $Ca^{2+}$ response in the second cell. An increase in the concentration of ACh was associated with shortening of the delay between the onset of responses, an event which was observed in all 13 cell pairs tested. At higher ACh concentrations, the $Ca^{2+}$ responses exhibited a typical biphasic “peak-and-plateau” profile (Fig. 2A). Two mechanisms, not mutually exclusive, could be involved in the lack of synchronization of the $Ca^{2+}$ response between coupled acinar cells. The long lasting intervals between individual responses at low ACh concentrations could be because of heterogeneity in ACh responsiveness (11, 13, 31). Furthermore, distinct IP$_3$ receptor sensitivity may exist in acinar cells as illustrated in Fig. 2B. Dialysis of a cell using a patch pipette containing 10–40 $\mu$M IP$_3$ triggered transient rises in $[Ca^{2+}]_i$, which was followed by delayed $Ca^{2+}$ responses in other neighboring or distant cells ($n = 5$).

Together, these data indicate that acinar cells can exhibit asynchronous rises in cytosolic $Ca^{2+}$ in response to $Ca^{2+}$-mobilizing mediators. In dual-patched acinar cells, the asynchrony of $Ca^{2+}$-dependent membrane currents was only unmasked when junctional coupling was strongly reduced (Figs. 1B and 3).

**ACH-induced Uncoupling Is Dependent on Extracellular $Ca^{2+}$**—In acinar and other cell types, it has been shown that the rapid phase of $[Ca^{2+}]_i$ changes reflects $Ca^{2+}$ release from internal stores, whereas the later sustained phase depends on capacitative $Ca^{2+}$ entry into the cells (32–34). We therefore investigated whether removal of external $Ca^{2+}$ could affect ACh-induced changes in junctional coupling. As shown in Fig. 3, a first exposure of acinar cells to ACh induced $I_m$ currents and a delayed reduction of $I_j$. During a second stimulation, the amplitude of the $I_m$ currents was markedly decreased and junctional coupling was not affected. Larger ACh-induced $I_m$ and $I_j$ responses were readily restored after reintroduction of $Ca^{2+}$ in the superfusing solution. The ACh-induced uncoupling of acinar cells observed in cells incubated in the absence of external $Ca^{2+}$ was typically reduced by 49 ± 8% ($n = 4$) as compared with that measured in pairs exposed to the control solution.

To investigate a possible relationship between $Ca^{2+}$ entry and cell uncoupling, the extent of intercellular communication was studied by injecting Lucifer Yellow in isolated acini exposed to various conditions known to deplete internal $Ca^{2+}$ stores and/or to activate capacitative $Ca^{2+}$ entry. Thapsigargin is a specific inhibitor of the endoplasmic reticulum $Ca^{2+}$-ATPase (35), which induces a slow depletion of $Ca^{2+}$ stores and, hence, capacitative entry of $Ca^{2+}$. As shown in Figs. 4 and 5, a 15–20-min exposure to 0.5 $\mu$M thapsigargin resulted in a marked reduction of intercellular communication. Although Lucifer yellow rapidly spread from the injected cell into all its neighbors in control acini (Fig. 4A), the diffusion of the tracer was restricted to the site of injection in the presence of thapsigargin (Fig. 4B). Quantitative analysis revealed that the surface labeled by Lucifer yellow represented 51 ± 6% ($n = 17$) of the acinus profile, a value that is markedly reduced ($p < 0.001$) as compared with that measured under control conditions (Fig. 5A). A similar blockade of acinar cell coupling ($p < 0.001$) was observed with 1 mM dibutyryl cGMP (Figs. 4C and 5A), an agent known to activate $Ca^{2+}$ entry (36). The effects of both agents were inhibited when internal $Ca^{2+}$ stores were previously depleted and acinar cells incubated in the absence of extracellular $Ca^{2+}$ (Fig. 5A). We therefore studied whether $Ca^{2+}$-mobilizing secretagogues could cause parallel activation of capacitative $Ca^{2+}$ entry and cell uncoupling. ACh has been shown previously to stimulate capacitative $Ca^{2+}$ entry by acti-
vation of nitric-oxide synthase, leading to generation of nitric oxide (NO) which, in turn, increases intracellular cGMP concentration (29, 37). When cells were incubated in the presence of 1 mM L-nitro-arginine, an inhibitor of nitric-oxide synthase, the ACh-induced uncoupling was fully prevented (Fig. 5B). In contrast, application of dibutyryl cGMP to acini pre-treated with L-nitro-arginine fully reduced ($p$, 0.001) acinar cell coupling by an extent similar to that observed in the presence of ACh alone (Figs. 4D and 5B). These results suggest that closure of gap junctions by ACh is closely associated with the activation of capacitative Ca$^{2+}$ entry.

**DISCUSSION**

Our results describe the temporal relationship between changes in [Ca$^{2+}$]$_i$ and gap junctional conductance within pairs of pancreatic acinar cells exposed to ACh. Uncoupling of pancreatic acinar cells by concentrations of Ca$^{2+}$-mobilizing secretagogues that maximally stimulate exocrine secretion is well documented (14–17). However, the intracellular mechanism that mediates this effect is not known (38). Using a dual per-
Temporal Changes of Acinar Cell Coupling and \([Ca^{2+}]_i\)

**Fig. 5. Role of capacitative Ca\(^{2+}\) influx on acinar cell uncoupling.** A, the extent of dye coupling between acinar cells incubated in the presence of 0.5 \(\mu M\) thapsigargin (Tg) or 1 \(\mu M\) dibutyryl cGMP (dB-cGMP) was markedly reduced \((p < 0.001)\) as compared with that observed under control conditions \((\text{CONT or Me}_2\text{SO (DMSO)}\text{ see “Experimental Procedures”})\). The uncoupling effect induced by both Tg and dB-cGMP was prevented when Ca\(^{2+}\)-sequestering stores were depleted and acini incubated in a Ca\(^{2+}\)-free medium \((\text{Ca}^{2+}\)-depleted). B, the uncoupling evoked by 1 \(\mu M\) ACh could be prevented by a 10-min preincubation of acini with 1 \(\times\) i-nitro-arginine \((\text{i-NA})\). Addition of dB-cGMP to acini exposed to i-NA and ACh restored the uncoupling effect \((p < 0.001)\). Stars indicate differences at \(p < 0.001\) levels compared with controls.

Forced patch-clamp approach and confocal digital imaging, we report here that ACh-induced uncoupling and \([Ca^{2+}]_i\), changes had distinct kinetics. Thus, decrease in junctional conductance of acinar cells consistently develops after the initial \([Ca^{2+}]_i\) peak and is maximal during the \([Ca^{2+}]_i\) plateau. Furthermore, ACh-induced uncoupling was no longer detected when reloading of internal \(Ca^{2+}\) stores was prevented by incubation of the cells in a \(Ca^{2+}\)-free medium. These results suggest that depletion-activated \(Ca^{2+}\) entry is a key determinant for the ACh-induced uncoupling of acinar cells.

In many nonexcitable cells, depletion of intracellular \(Ca^{2+}\) stores by IP\(_3\) is the primary mechanism by which cell surface receptors activate \(Ca^{2+}\) influx. This phenomenon, which is termed capacitative \(Ca^{2+}\) entry \((33)\), has been involved in the control of \(Ca^{2+}\) oscillations \((32, 39)\), secretion \((40)\), and enzymatic regulation \((41)\). The signal that couples store depletion to \(Ca^{2+}\) entry has not yet been identified \((33)\). In pancreatic acinar cells, however, there is definite evidence that NO produced by nitric-oxide synthase mediates the stimulation of cGMP formation by cholinergic agonists. cGMP, in turn, is known to modulate \(Ca^{2+}\) entry \((29, 36, 37, 42, 43)\). Consistent with these data, we observed that activation of capacitative \(Ca^{2+}\) entry by either depletion of internal \(Ca^{2+}\) stores with thapsigargin or by exposure of acinar cells to dibutyryl cGMP decreased intercellular communication to an extent similar to that observed with ACh alone. Side effects of these agents on intercellular communication appear unlikely because uncoupling was abolished in acini that were depleted for their internal \(Ca^{2+}\) and incubated in a \(Ca^{2+}\)-free medium. In addition, cell uncoupling evoked by ACh was prevented in the presence of a nitric-oxide synthase inhibitor, supporting the view that gap junctional conductance is concurrently regulated. These results, however, do not rule out the possibility that capacitative \(Ca^{2+}\) entry may activate another intracellular pathway leading to modulation of junctional conductance. Support for this idea is provided by the earlier observation that okadaic acid, a phosphatase inhibitor that modulates capacitative \(Ca^{2+}\) entry \((44, 45)\), also prevents ACh-induced uncoupling \((38)\). Although several studies have shown that gap junction channels are blocked by cGMP \((25)\) and nitric oxide \((46)\), our data provide the first observation that ACh-induced uncoupling is linked to capacitative \(Ca^{2+}\) entry.

Previous *in vitro* and *in vivo* studies have documented a relationship between intercellular communication and the secretory activity of pancreatic acinar cells \((17, 30, 47)\). In this context, gap junctional coupling is thought to coordinate the \(Ca^{2+}\) response of individual acinar cells within an acinus and thereby to regulate exocytosis \((11, 48)\). This idea is supported by our present finding that the large and stable junctional conductance observed in most acinar cell pairs was not altered during changes in \([Ca^{2+}]_i\) evoked by ACh. In agreement with a previous study using cholecystokinin \((13)\), we observed that rises in \([Ca^{2+}]_i\), were asynchronous in acinar cells stimulated with low concentrations of ACh. Increasing the agonist concentration was associated with shortening of the delay between the onset of the \(Ca^{2+}\) responses, suggesting that these cells were coupled in terms of \(Ca^{2+}\) mobilization. Also, perfusion of IP\(_3\) into one cell evoked a rise in \([Ca^{2+}]_i\), in neighboring cells even though not necessarily in those that directly contacted the stimulated one. This observation suggests that rat pancreatic acinar cells differ in their ability to mobilize \(Ca^{2+}\) from internal stores, as indicated by previous studies reporting similar heterogeneity in \([Ca^{2+}]_i\), mobilization \((31)\) and amylase secretion \((48, 49)\). This differential responsiveness may be essential to provide a properly modulatory response to agonist-specific stimulation \((11, 50)\).

These results, however, are not immediately reconcilable with the observation that ACh decreases gap junctional coupling while maximally stimulating the secretory activity of acinar cells \((30, 47, 48)\). The blockade of acinar cell-to-cell communication is known to enhance the basal release of amylase *in vitro* and *in vivo* \((16, 26, 30, 47)\). Under conditions of gap junction blockade, the potency of several agonists in stimulating exocytosis has also been found to be reduced when the effect of acinar cell uncoupling on basal secretion was taken into account \((11, 16, 30)\). Therefore, uncoupling may provide acinar cells with a mechanism to sustain enzyme release during acute stimulation by increasing their rate of basal secretion. In this context, the delayed uncoupling evoked by ACh may compartmentalize cells that are highly sensitive to ACh from cells that are less sensitive to the secretagogue, therefore decreasing the effective volume of cytoplasm of interconnected cells. This regulation may be essential to ensure that the intracellular levels of critical factor(s) are maintained to allow for sustained activity of groups of actively secreting cells. Future studies should determine whether uncoupling induced by \(Ca^{2+}\) store depletion
is a common mechanism to control junctional communication in other types of nonexcitable cells.

Acknowledgments—We thank D. Bosco, N. Guérineau, M. B. Rook, and S. Verheule for continuous support during the preparation of this work, and Luc Anibers and Isabelle Duperrut for excellent technical help.

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