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Muscarinic Stimulation Increases Na^+ Entry in Pancreatic B-Cells by a Mechanism Other than the Emptying of Intracellular Ca^{2+} Pools

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Stimulation of muscarinic (M_3) receptors depolarizes pancreatic B-cells by increasing Na^+ influx. Here, we measured $[Na^+]_i$ and $[Ca^{2+}]_i$ in B-cell clusters to investigate whether depletion of intracellular Ca^{2+} pools triggers this unusual transduction pathway for muscarinic receptors. Acetylcholine emptied Ca^{2+} pools less completely than did the SERCA pump inhibitors, thapsigargin, and cyclopiazonic acid. However, the rise in $[Na^+]^i$ produced by acetylcholine was not mimicked by thapsigargin or cyclopiazonic acid and was not prevented by previous depletion of Ca^{2+} pools. Depolarization of B-cells by acetylcholine stimulates Ca^{2+} influx and steadily increases $[Ca^{2+}]_i$. In the presence of glucose and extracellular Ca^{2+}, B-cells treated with thapsigargin or cyclopiazonic acid displayed large $[Ca^{2+}]_i$ oscillations. Subsequent application of acetylcholine was followed by a sustained rise in $[Ca^{2+}]_i$ as in untreated cells. In conclusion, intracellular Ca^{2+} pool depletion does not mediate acetylcholine stimulation of Na^+ entry and of subsequent events. We propose that the muscarinic receptors are coupled to Na^+ channels in B-cells.

Nutrient stimulation of insulin secretion is markedly amplified by acetylcholine (ACh) released from parasympathetic nerve endings during meals. The neurotransmitter acts directly on pancreatic B-cells where, by activating muscarinic receptors of the M_3 type (1-3), it triggers changes in phospholipid metabolism leading to formation of (1,4,5)$InsP_3$ which mobilizes Ca^{2+} from intracellular stores, and diacylglycerol which activates protein kinase C (4-7). In addition, ACh depolarizes B-cells by increasing the membrane permeability for Na^+ (8). As B-cells are electrically excitable, this depolarization activates voltage-dependent Ca^{2+} channels, resulting in an acceleration of Ca^{2+} influx that plays an important role in the amplification of insulin secretion (8, 9). This transduction pathway is unexpected and still unexplained because, unlike nicotinic receptors (10, 11), muscarinic receptors are not classically causing changes in membrane Na^+ conductance. A small current dependent on extracellular Na^+ and prevented by atropine has been observed in ACh-stimulated B-cells, but it has not been characterized (12).

Recently, the emptying of intracellular Ca^{2+} pools by thapsigargin or cyclopiazonic acid (CPA), two inhibitors of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA pump) (13), has been reported to activate Na^+ influx in platelets and lymphocytes (14, 15). Similarly, a depolarizing current elicited by Ca^{2+} store depletion with thapsigargin, and thought to be caused mainly by Na^+, has been described in pancreatic B-cells (16). Since ACh is a powerful Ca^{2+}-mobilizing agent, it has been suggested that the increase in Na^+ influx brought about by muscarinic stimulation and, hence, the depolarization and secondary influx of Ca^{2+} through voltage-dependent Ca^{2+} channels are secondary to the emptying of intracellular Ca^{2+} pools

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Abbreviations: ACh, acetylcholine; $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; CPA, cyclopiazonic acid; $[Na^+]_i$, free cytosolic Na^+ concentration; PACAP, pituitary adenylate cyclase-activating polypeptide; SERCA pump, sarco-endoplasmic reticulum Ca^{2+}-ATPase.

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(17). This hypothesis was tested in the present study, with mouse pancreatic B-cells loaded with the Na^+ or Ca^{2+} fluorescence indicators SBFI or fura-2. The effects of ACh on the free cytoplasmic concentrations of Na^+ ($[Na^+]_i$) and Ca^{2+} ($[Ca^{2+}]_i$) were compared to those of thapsigargin and CPA.

MATERIALS AND METHODS

Preparation and solutions. All experiments were performed at 37°C with cells obtained by dispersion (18) of pancreatic islets isolated after collagenase digestion of the pancreas of fed female NMRI mice (25-30g). Cells were then allowed to attach to 22 mm circular coverslips, and were cultured for 1-3 days in RMPI 1640 medium (Gibco BRL, Life Technologies Ltd, Paisley, UK) containing 10 mM glucose and supplemented with 10% heat inactivated fetal calf serum (Gibco BRL), 100 IU/ml penicillin and 100 μg/ml streptomycin. The solution used for islet isolation and for most experiments was a bicarbonate-buffered medium which contained (in mM) 120 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, and 24 $NaHCO_3$, was gassed with O_2/CO_2 (94:6), had a pH of 7.4 and was supplemented with 1 mg/ml BSA. Ca^{2+}-free solutions were prepared by susbstituting $CaCl_2$ for $MgCl_2$, and adding 50 μM EGTA. Na^+-free solutions are usually prepared by substituting choline chloride for NaCl and choline bicarbonate for $NaHCO_3$. However, because choline exerts muscarinic effects, atropine has to be added to these solutions, which precludes their use in experiments where ACh is tested. Therefore, the experiments aiming at compairing the effects of ACh to those of thapsigargin or CPA on $[Na^+]_i$ were performed in a Hepes-buffered medium containing (in mM): 135 NaCl, 4.8 KOH, 2.5 $CaCl_2$, 1.2 $MgCl_2$ and 10 Hepes. Hepes-buffered Na^+-free solutions were prepared by substituting *N*-methyl-D-glucamine chloride for NaCl (8). Whenever possible, control experiments were performed with both types of solutions and no differences were observed. ACh chloride, cyclopiazonic acid and thapsigargin were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Measurements of $[Ca^{2+}]_i$ and of $[Na^+]_i$. Because of the heterogeneity of $[Ca^{2+}]_i$ and $[Na^+]_i$ responses in single cells, all experiments were carried out with clusters of 3-8 islet cells. For $[Ca^{2+}]_i$ measurements, the cells were loaded with fura-2 for 40 min at 37°C in a bicarbonate-buffered solution containing 10 mM glucose and 1 μM fura-2-acetoxymethyl ester (Molecular Probes, Eugene, OR, USA). For $[Na^+]_i$ measurements, the cells were loaded with SBFI during 2 h of incubation at 37°C in culture medium supplemented with 5 μM SBFI acetoxymethyl ester (Molecular Probes) and 0.0125% of Pluronic acid F-127. Thereafter, the cells were equilibrated for 20 min in a Hepes medium similar to that with which the subsequent experiment was started. The coverslips with the fura-2- or SBFI-loaded cells were used as the bottom of a perifusion chamber placed on the stage of a microscope. The loaded cells were excited successively at 340 and 380 nm, and the fluorescence emitted at 510 nm was captured and analyzed by a photomultiplier-based system (Photon Technologies International Ltd, Princeton, NJ, USA). In some $[Ca^{2+}]_i$ experiments, the emitted fluorescence was captured by a CCD camera (Photonic Science Ltd., Turnbridge Wells, UK), and the images were analyzed by the MagiCal system (Applied Imaging, Sunderland, UK). $[Ca^{2+}]_i$ and $[Na^+]_i$ were calculated by comparing the ratio of the signals successively acquired at 340-380 nm to calibration curves. For $[Ca^{2+}]_i$ determinations, the calibration curve was obtained as described previously (19, 20). For $[Na^+]_i$ measurements, the calibration curve was established by exposing reference cells loaded with SBFI to 10 μg/ml gramicidin D, an ionophore that equilibrates transmembrane Na^+ and K^+ concentrations, and perifusing them stepwise with solutions containing different Na^+ (from 5 to 100 mM) and K^+ concentrations ($Na^+ + K^+ = 135$ mM, 105 mM gluconate, 30 mM NaCl or KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM Hepes and 3 mM glucose). The 340/380 ratio (R) plotted as a function of the Na^+ concentration was fitted by a polynomial equation of the third degree ($[Na^+] = 109R^3 - 344R^2 + 418R - 176$) which was used to calculate $[Na^+]_i$.

Presentation of results. The results are given either as means ± SEM for a certain number of cell clusters, or illustrated by recordings which are representative of those obtained with the indicated number of cell clusters. Each protocol was repeated with cells from at least three different cultures. The statistical significance of observed differences was assessed by an analysis of variance followed by a Dunnett test for multiple comparisons.

RESULTS AND DISCUSSION

Effects of ACh, thapsigargin, and CPA on intracellular Ca^{2+} pools. In the presence of 15 mM glucose and 2.5 mM Ca^{2+} in the medium (start of all traces in Fig. 1), slow and large amplitude oscillations in $[Ca^{2+}]_i$ were observed in clusters of B-cells. Switching to a Ca^{2+}-free medium abolished the oscillations and decreased $[Ca^{2+}]_i$ to low levels. Addition of 100 μM ACh, 8 min after Ca^{2+} removal, elicited a rapid and large $[Ca^{2+}]_i$ peak which averaged 307 ± 10 nM (n=91). Thereafter, $[Ca^{2+}]_i$ returned to basel levels in most clusters (72/91) (Fig. 1A), but started to display oscillations of variable amplitude in ~20 % of the clusters

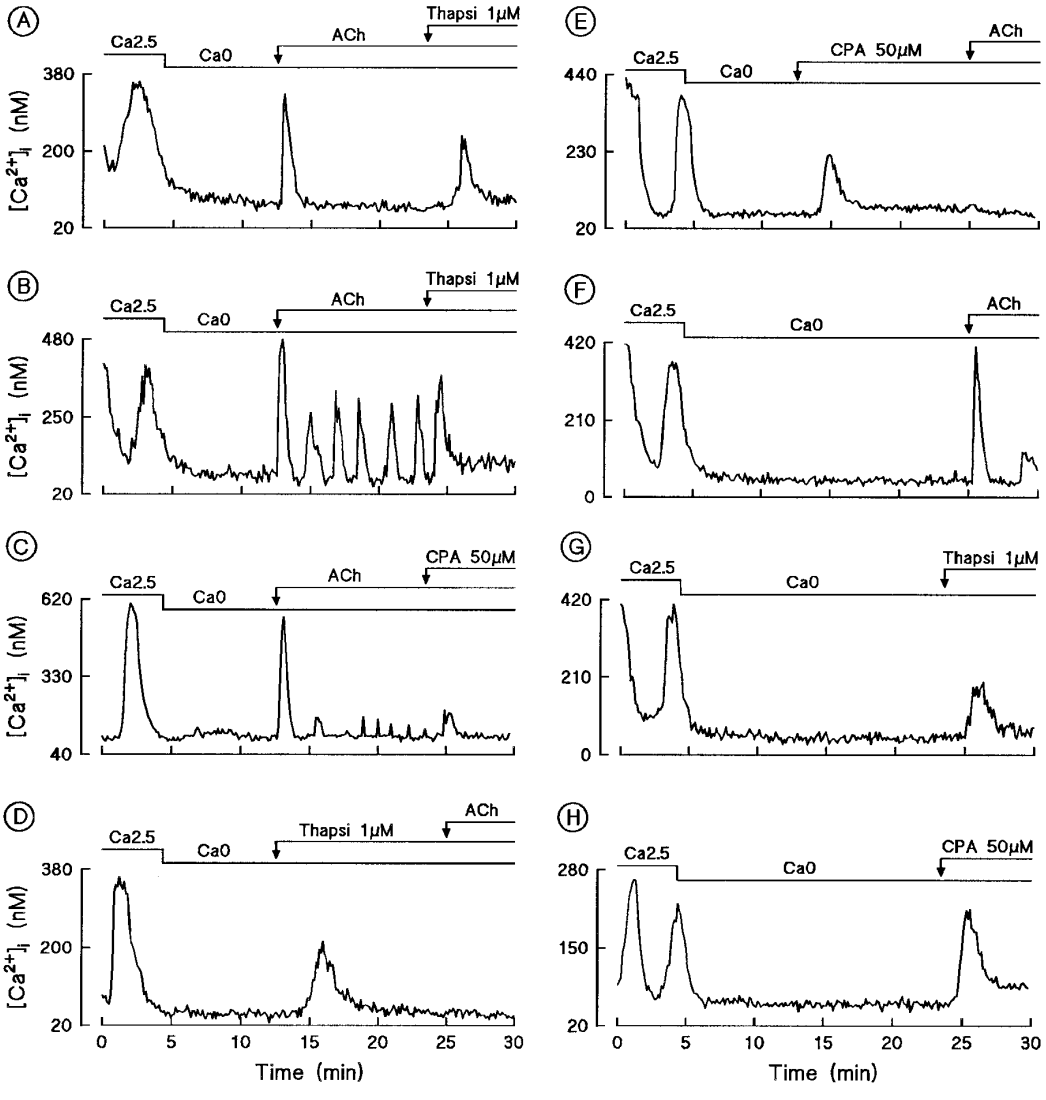

FIG. 1. Emptying of intracellular Ca^{2+} pools of mouse pancreatic B-cells by ACh, thapsigargin, and CPA. The cells were perifused with a bicarbonate-buffered medium containing 15 mM glucose. Extracellular Ca^{2+} was omitted (Ca 0) as indicated on top of each panel, while test agents (100 μM ACh, 1 μM thapsigargin, or 50 μM CPA) were added as indicated by the arrows. The traces are representative of results obtained in 46 (A), 14 (B), 31 (C), 62 (D), 20 (E), 56 (F), 53 (G), and 26 (H) clusters of cells.

(19/91) (Figs. 1B,C). Addition of thapsigargin or CPA, 8 min after removal of Ca^{2+} from the medium, also elicited peaks of $[Ca^{2+}]_i$ which were slower, smaller but longer than those produced by ACh (Figs. 1D,E). Perifusion of the cells with a Ca^{2+}-free medium for a longer period of time (~20 min) did not prevent ACh, thapsigargin or CPA from inducing large $[Ca^{2+}]_i$ peaks (Figs. 1F-H). This indicates that the intracellular Ca^{2+} pools do not spontaneously empty under these conditions. When thapsigargin or CPA was added 11 min after ACh, a peak of $[Ca^{2+}]_i$ still occurred, and the oscillations sometimes induced by ACh were abolished (Figs. 1A-C). By contrast, ACh consistently failed to increase $[Ca^{2+}]_i$ in clusters treated with

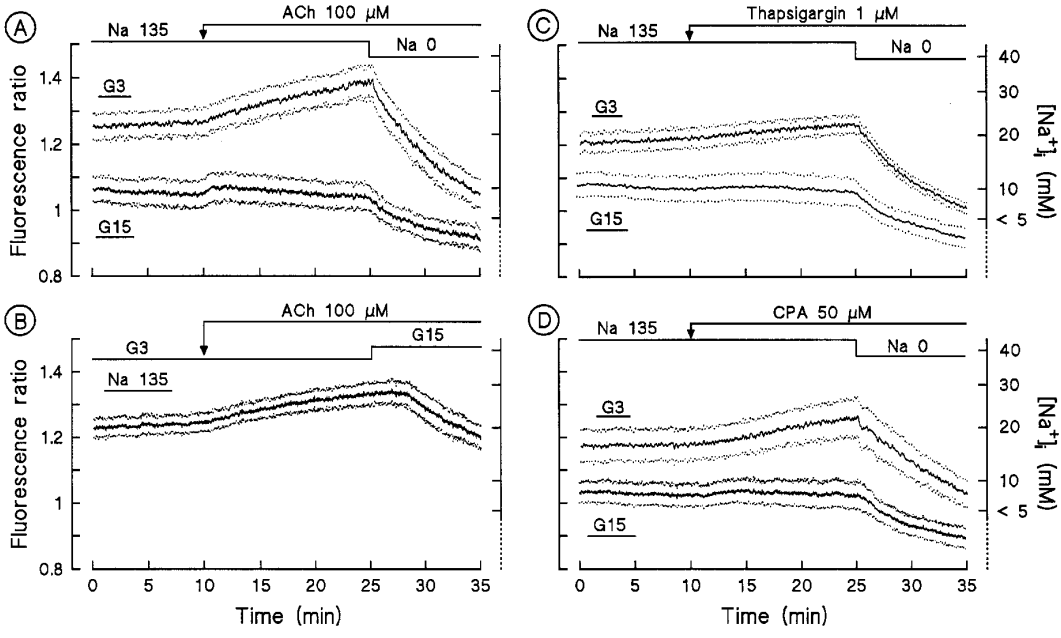

FIG. 2. Effects of ACh, thapsigargin, and CPA on $[Na^+]_i$ in mouse pancreatic B-cells. The cells were perifused with a Hepes-buffered medium containing either 3 or 15 mM glucose (G), and 135 mM Na^+ or no Na^+ (Na 0) as indicated. In (B), the Na^+ concentration of the medium was 135 mM throughout and the glucose concentration was increased from 3 to 15 mM as indicated. The traces correspond to the mean responses (± SEM) obtained in 6–14 clusters of cells.

thapsigargin, and produced a small and short-lived peak of $[Ca^{2+}]_i$ in 25% of the clusters treated with CPA (Figs. 1D,E). These results indicate that the ACh-sensitive Ca^{2+} pool is largely emptied by CPA and completely emptied by thapsigargin, whereas ACh only partially empties the CPA- and thapsigargin-sensitive pools.

Effects of ACh, thapsigargin, and CPA on $[Na^+]_i$. If the increase in Na^+ conductance brought about by ACh results from intracellular Ca^{2+} pool depletion, it should be mimicked by thapsigargin and CPA. This was tested by comparing the effects of ACh on $[Na^+]_i$ with those of the two SERCA pump inhibitors. Addition of ACh to a medium containing 15 mM glucose had no consistent effect on $[Na^+]_i$ (Fig. 2A). Since we had previously observed that ACh increases $[Na^+]_i$ in B-cells in the presence of 3 mM glucose (21), the experiments were also performed under these conditions. $[Na^+]_i$ was twice higher (19.5 ± 1.3 mM, n=39) in the presence of 3 mM than of 15 mM glucose (9.5 ± 1.1 mM, n=19). Stimulation with ACh in low glucose increased $[Na^+]_i$ by 10 mM ($P < 0.01$) (Fig. 2A). This rise of $[Na^+]_i$ was abolished by atropine (21) and reversed by omission of Na^+ from the medium. When the concentration of glucose was increased from 3 to 15 mM after ACh stimulation, $[Na^+]_i$ rapidly decreased (Fig. 2B). These inhibitory effects of glucose on $[Na^+]_i$ are consistent with the report that total Na^+ content of islets (measured with integrating flame photometry) is lowered by 20 mM glucose and increased by ACh in the presence of 3 mM but not 20 mM glucose (22). This does not mean that ACh does not accelerate Na^+ influx in the presence of high glucose. Thus, ACh-induced $^{22}Na^+$ uptake is not affected by the glucose concentration (8). The lack of a rise in $[Na^+]_i$ in the presence of high glucose can be explained by the facilitation of Na^+ extrusion that the sugar produces (23).

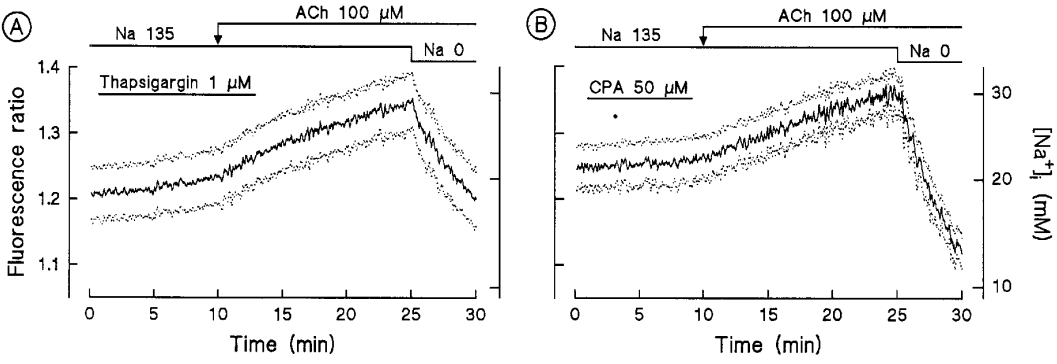

FIG. 3. Effects of ACh on $[Na^+]_i$ in mouse pancreatic B-cells pretreated with thapsigargin or CPA. The experiments were performed with a Hepes-buffered medium containing 3 mM glucose. Thapsigargin and CPA were present during the whole experiments which began about 20 min before the start of the traces. The traces correspond to the mean responses (± SEM) obtained in 10 clusters of cells.

As shown in Figs. 2C and 2D, thapsigargin and CPA were without significant effect on $[Na^+]_i$ in clusters of B-cells, regardless of the ambient concentration of glucose. This means that the SERCA pump inhibitors activate no or only minimal entry of Na^+. Since ACh is less potent than thapsigargin and CPA in emptying intracellular Ca^{2+} pools, the rise in $[Na^+]_i$ that it causes must be ascribed to another mechanism. This conclusion is made inescapable by the demonstration that ACh remained able to increase $[Na^+]_i$ after depletion of Ca^{2+} pools with the SERCA pump inhibitors (Fig. 3).

Effects of ACh on $[Ca^{2+}]_i$ after intracellular Ca^{2+} pool depletion. In B-cells stimulated by glucose in the presence of extracellular Ca^{2+} (physiological conditions), ACh produces a sustained rise in $[Ca^{2+}]_i$ which results from an influx of Ca^{2+} through Ca^{2+} channels activated by a Na^+-dependent membrane depolarization (8, 9). If this depolarization was mediated by emptying of intracellular Ca^{2+} pools, it should not occur when ACh is applied after thapsigargin or CPA, and ACh should not cause a sustained rise in $[Ca^{2+}]_i$ under these conditions. Fig. 4 shows that this is not the case. In the presence of 15 mM glucose and 2.5 mM Ca^{2+} in the medium, B-cells stimulated by thapsigargin or CPA displayed large amplitude oscillations in $[Ca^{2+}]_i$. Subsequent application of ACh was followed by a sustained elevation of $[Ca^{2+}]_i$ that was reversible after ACh removal. This effect was already produced by 1 μM ACh (0.1 μM in other studies with whole islets), i.e. low concentrations that have little effect on intracellular Ca^{2+} mobilization (9).

CONCLUSIONS

Our study provides strong evidence against the hypothesis (17) that depletion of intracellular Ca^{2+} pools mediates the increase in Na^+ entry and, hence, the resulting depolarization and Ca^{2+} influx brought about by ACh in pancreatic B-cells. Several other possible electrogenic mechanisms including activation of tetrodotoxin-sensitive Na^+ channels and blockade of the Na^+ pump have also been ruled out (8, 21). Similar events seem to occur in adrenal chromaffin cells, which are electrically excitable like B-cells, when they are stimulated by pituitary adenylate cyclase-activating polypeptide (PACAP) (24). PACAP was found to cause a rapid release of Ca^{2+} from intracellular stores and to activate a Na^+ dependent depolarization which in turn induced a sustained increase in $[Ca^{2+}]_i$. However, the depolarization and sustained rise of $[Ca^{2+}]_i$ were independent from intracellular Ca^{2+} pool depletion (24). Since neither activation of phospholipase C by vasopressin (25) nor direct stimulation of protein kinase C by a phorbol

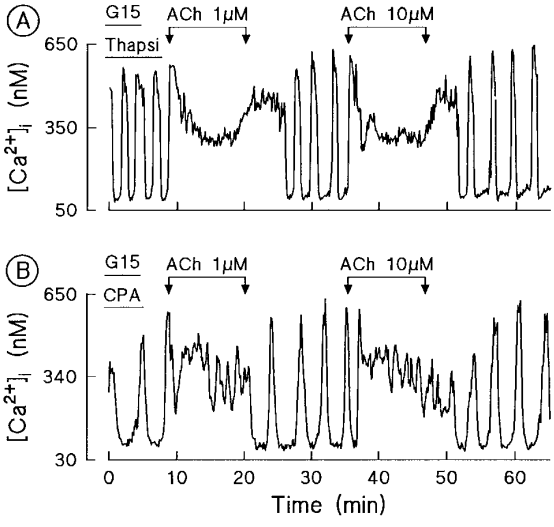

FIG. 4. ACh increases $[Ca^{2+}]_i$ in B-cells with empty Ca^{2+} pools. The cells were perifused with a bicarbonate-buffered medium containing 15 mM glucose (G15). Thapsigargin (1μM) and CPA (50 μM) were added about 20 min before the start of the recordings and remained present throughout. The traces are representative of results obtained in 16 (A) and 23 (B) clusters of cells.

ester (26) mimicks the depolarizing action of ACh, we suggest that M_3 receptors in pancreatic B cells are coupled to Na^+ channels.

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