Evidence for Contribution by Increased Cytoplasmic Na\textsuperscript{+} to the Insulinotropic Action of PACAP38 in HIT-T15 Cells*

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is localized to pancreatic nerve terminals and stimulates insulin secretion. The insulinotropic effect of PACAP38 in insulin-producing HIT-T15 cells is accompanied by increases in cellular cAMP and cytoplasmic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{cyt}). As also intracellular Na\textsuperscript{+} is important for insulin secretion after glucose and other cAMP forming peptides, we examined the Na\textsuperscript{+} dependence of the insulinotropic effect of PACAP38 in HIT-T15 cells. We found that PACAP38 (100 nM)-induced insulin secretion was diminished by approximately 50% by removal of extracellular Na\textsuperscript{+} (replaced by equimolar N-methyl-D-glucamine). In contrast, removal of Na\textsuperscript{+} did not diminish the formation of cellular cAMP (measured by radioimmunoassay) or the increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} (measured in FURA-2AM-loaded cell suspensions) induced by PACAP38. Furthermore, PACAP-38 increased the cytoplasmic Na\textsuperscript{+} ([Na\textsuperscript{+}]\textsubscript{cyt}) in single HIT-T15 cells as measured by the fluorophore sodium-binding benzofuran isophthalate. This increase was reduced by removal of extracellular Na\textsuperscript{+} and by inhibition of protein kinase A by H-89. We conclude that the insulinotropic action of PACAP38 is Na\textsuperscript{+}-dependent. We propose that PACAP38 opens plasma membrane Na\textsuperscript{+} channels by an action partially mediated by cAMP and protein kinase A, and the subsequent raise in [Na\textsuperscript{+}]\textsubscript{cyt} elicits insulin secretion by an as yet unsolved mechanism.

PACAP has been demonstrated to be a ubiquitously distributed neuropeptide throughout the body (3). In the pancreas, PACAP is localized to nerves innervating the exocrine parenchyma, blood vessels, islets of Langerhans as well as to intrapancreatic ganglia (4, 5), which suggests that the neuropeptide is involved in the neural regulation of pancreatic function. We have shown recently that two types of the presently three known PACAP receptor subtypes are expressed in insulin-producing tissues, the PACAP type 1 and the VIP2/PACAP receptors, which further supports a role for PACAP in regulating islet function (5). It is well established that PACAP potently stimulates insulin secretion, as has been demonstrated in vitro in insulin-producing clonal cells (6, 7), in isolated mouse and rat islets (5, 8), and in perfused rat pancreas (9, 10), as well as in vivo in mice (11) and humans (12). The potent insulinotropic action of PACAP has been thought to be mediated by raised formation of cellular cAMP, since PACAP stimulates cAMP formation in insulin-producing tissues (13, 14) and since cAMP through activation of protein kinase A (PKA) is known to stimulate the exocytosis of insulin containing granules (15). However, we showed previously that PACAP38 (100 nm) induces insulin secretion to a greater extent than the adenylate cyclase-activating agent forskolin (0.25 μM), even though at these doses PACAP38 and forskolin induce formation of cAMP to the same extent (14). This implies that formation of cAMP cannot fully explain the insulinotropic effect of PACAP, which led us to speculate that also one or several other signaling mechanisms contribute to its insulinotropic action. Since PACAP38 also increases cytoplasmic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{cyt}) in insulin-producing HIT-T15 cells (14), such an action might contribute to the action of PACAP on insulin secretion, since Ca\textsuperscript{2+} accentuates the exocytosis of granules in insulin producing cells (16). However, in addition whether other signaling mediators for PACAP exist in insulin-producing cells remains to be established.

Earlier studies in human pituitary adenoma cells have shown that PACAP induces growth hormone secretion with a mechanism that is inhibited by tetrodotoxin, a voltage-gated Na\textsuperscript{+} channel blocker, and that PACAP increases tetrodotoxin-sensitive Na\textsuperscript{+} channel currents in such cells (17). This would suggest that also increased uptake of Na\textsuperscript{+} is a mechanism for actions induced by PACAP. Extracellular Na\textsuperscript{+} has been shown previously to be required for glucose-induced insulin secretion in β-cells (18, 19), and, furthermore, the muscarinic agonist, acetylcholine, has been shown to stimulate insulin secretion in a Na\textsuperscript{+}-dependent manner (20). Moreover, earlier studies from our laboratory have shown that glucagon-like peptide-1 (GLP-1), which, like PACAP, activates adenylate cyclase (21), stimulates insulin secretion in a Na\textsuperscript{+}-dependent manner (22).

In this study, we have examined the possible contribution by Na\textsuperscript{+} on influences of PACAP in insulin secretory cells, by studying the Na\textsuperscript{+} dependence of the effect of PACAP38 on insulin secretion, on cAMP formation, and on [Ca\textsuperscript{2+}]\textsubscript{cyt} in insulin-producing clonal hamster insulinoma HIT-T15 cells.

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Since pronounced Na\(^+\)-dependent effects were found on insulin secretion, suggesting that Na\(^+\) indeed is of importance for the insulinotropic action of PACAP, we proceeded and used the fluorophore Na\(^+\)-binding benzofuran isothiophosphate (SBFI) to study whether PACAP38 also affects the cytoplasmic concentration of Na\(^+\) ([Na\(^+\)]\text{cyt}) in these cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—PACAP38 was from Peninsula Europe Laboratories, Merseyside, United Kingdom (UK). RPMI 1640 medium and amphotericin were from Life Technologies AB, Taby, Sweden. Fetal calf serum (FCS), penicillin G, streptomycin, and fungizone were from Roche, Stockholm, Sweden, and Spånga, Sweden. H89 was from Seikagaku Corp., Tokyo, Japan. Flasks, 24-well plates, and 4-well plates were from Nunc, Roskilde, Denmark. Guinea pig anti-porcine insulin, mono-Z\(\text{Z}\)-insulin, and rat insulin were from Linco Research, St. Charles, MO. Radioimmunoassay kit for cAMP with rabbit anti-succinyl AMP serum, cyclic 2-succinyl-3-\(^{125}\)I-methyl ester, and cyclic AMP were from Amersham Pharmacia Biotech, Amersham, UK. All other chemicals, including FURA-2, SBFI-AM, and 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) were from Sigma.

**Cell Culture**—HIT-T15 cells, i.e. the clonal hamster \(\beta\)-cell line, were cultured at +37 °C in 5% CO\(_2\), 95% air in RPMI 1640 medium supplemented with 10% FCS, 100 units/ml penicillin G, 0.1 mg/ml streptomycin, and 2.5 \(\mu\)g/ml of amphotericin B. Passages were performed every 7 days, and the medium was changed every 3–4 days. Cells of the passages 72–82 were used.

**Insulin Secretion**—Cells were seeded on 24-well plates (0.5 million cells/well) and cultured for 48 h (about 80% confluence). They were then washed twice in a Hepes medium (125 mM NaCl, 5.9 mM KCl, 1.28 mM MgCl\(_2\), 25 mM Hepes, 0.1% human serum albumin, pH 7.36) and incubated at +37 °C in the Hepes medium in a volume of 200 \(\mu\)l at 10 mM glucose with or without PACAP38. In the Na\(^+\)-free medium, NaCl was replaced with an equimolar concentration of N-methyl-D-glucamine (NMDG). After the end of incubation, 150 \(\mu\)l of the medium were collected and centrifuged at 350 \(\times\) g for 5 min. Aliquots of 50 \(\mu\)l in duplicate were then stored at -20 °C until analysis of insulin by radioimmunoassay, using guinea pig anti-porcine insulin, mono-Z\(\text{Z}\)-insulin, and, as standard, rat insulin. Free and bound radioactivity were separated by the double antibody technique.

**Cellular Cyclic AMP Content**—HIT-T15 cells were seeded on four-well plates (0.5 million cells/well) and cultured for 48 h as above. The cells were then washed twice in the Hepes medium and incubated at +37 °C in a volume of 200 \(\mu\)l in presence of 10 mM glucose and 0.1 mM isobutylmethylxanthine with or without addition of PACAP38 (100 nM) or forskolin (0.25 \(\mu\)M). In the Na\(^+\)-free medium, NaCl was replaced by NMDG. The incubation was stopped after 2 min with addition of ice-cold ethanol (final concentration: 65%), and the cells were scraped off with a rubber policeman. After being washed twice in 65% ice-cold ethanol, the extracts were centrifuged at 2000 \(\times\) g at +4 °C for 15 min, transferred to fresh test-tubes, evaporated at +60 °C under a stream of nitrogen, and then stored at -20 °C until analysis for protein content by the Lowry method (23) and for cAMP by radioimmunoassay, using a rabbit anti-succinyl-AMP serum, cyclic 2-succinyl-3,125I-methyl ester as tracer, and cAMP as standard. Free and bound radioactivity were separated by the double antibody technique.

**Cytosplasmic Ca\(^{2+}\) —[Ca\(^{2+}\)]\text{cyt} was determined in FURA-2-AM-loaded HIT-T15 cells as described previously (14). In brief, cells were grown for 4–7 days in RPMI medium supplemented as above. After trypsinization, cells recovered for 2 h in 10 ml of RPMI medium supplemented with 10% FCS at +37 °C in 5% CO\(_2\) and were thereafter loaded with FURA-2-AM (1 \(\mu\)M) for 45 min. The cells were then washed either in a Hepes buffer or in a Hepes medium in which NaCl was replaced with NMDG. The incubation was stopped after 2 min with addition of ice-cold buffer or NMDG. 

**RESULTS AND DISCUSSION**

**Insulin Secretion**—In the presence of extracellular Na\(^+\), PACAP38 increased medium insulin from 1230 ± 131 pmol/liter to 6410 ± 687 pmol/liter \((p < 0.001)\), whereas in the absence of extracellular Na\(^+\), medium insulin was increased from 1840 ± 107 pmol/liter to only 4640 ± 431 pmol/liter \((p < 0.001)\) by the peptide, representing a reduction of the insulinotropic effect of PACAP38 by 45 ± 0.0% after removal of extracellular Na\(^+\) (Fig. 1). Hence, the results show that the Na\(^+\)-dependence of the action of PACAP is not restricted to pituitary cells (17), and, furthermore, that PACAP resembles glucose, acetycholine, and the cAMP-forming peptide, GLP-1, exhibiting partial Na\(^+\)-dependence for insulinotropic action (18–20, 22). The Na\(^+\)-dependence of the action of PACAP38 might be executed by Na\(^+\) being important for the generation of cAMP or for the increase in [Ca\(^{2+}\)]\text{cyt} since PACAP38 increases cAMP formation and [Ca\(^{2+}\)]\text{cyt} in HIT-T15 cells (14). The potential site of the Na\(^+\)-dependence was therefore further examined by
determining cAMP and \([\text{Ca}^{2+}]_{\text{c}}\) in HIT-T15 cells after PACAP38 activation.

_Cyclic AMP_—To investigate whether removal of extracellular \(\text{Na}^{+}\) affects the PACAP38-induced formation of cellular cAMP, the cells were incubated at 10 mM glucose with the addition of 0.1 mM isobutylmethylxanthine for 2 min in the presence or absence of extracellular \(\text{Na}^{+}\). Fig. 2 shows that PACAP increased cellular cAMP from 10.9 ± 0.9 fmol/\(\mu\)g protein to 41.4 ± 2.6 fmol/\(\mu\)g protein in the presence of \(\text{Na}^{+}\) \((p < 0.001)\). This effect was not reduced by removing \(\text{Na}^{+}\) from the medium \((13.3 ± 0.4 \text{ fmol/} \mu\text{g protein without PACAP38 versus } 40.2 ± 3.4 \text{ fmol/} \mu\text{g protein with PACAP38}; p < 0.001)\). Similarly, the effect of forskolin \((0.25 \mu\text{M})\) on cellular cAMP formation was not affected by removal of extracellular \(\text{Na}^{+}\), since cellular cAMP content was 50.1 ± 5.7 fmol/\(\mu\)g protein in the presence of extracellular \(\text{Na}^{+}\) and 43.5 ± 1.7 fmol/\(\mu\)g protein in a \(\text{Na}^{+}\)-free medium after stimulation by forskolin (not significant, Fig. 2). These results thus show that the activation of adenylate cyclase by PACAP38 or forskolin in HIT-T15 cells is a process not dependent on \(\text{Na}^{+}\). This is in contrast to previous studies in parotid glands showing that the binding of \(\text{G}_{\alpha}\)-protein to the catalytic unit of adenylate cyclase is Na\(^{+}\)-dependent \((29)\), which suggests that the \(\text{Na}^{+}\) dependence of \(\text{G}_{\alpha}\)-protein binding to adenylate cyclase is different in different cell systems or that the PACAP-activated \(\text{G}_{\alpha}\) is different from other \(\text{G}_{\alpha}\)-proteins in this respect. A third possibility is that a minimal amount of \(\text{Na}^{+}\) remains intracellularly despite incubating the cells in a medium devoid of \(\text{Na}^{+}\) during experiments, and this is sufficient to facilitate the binding of the \(\text{G}_{\alpha}\)-protein to adenylate cyclase. In any case, our results show that the \(\text{Na}^{+}\) dependence of the insulinotropic effect of PACAP38 is located further downstream from adenylate cyclase of the intracellular pathway in HIT-T15 cells or executed by signaling mechanisms of PACAP not involving cAMP.

_Cytoplasmic \(\text{Ca}^{2+}\)—_It is known that PACAP38 increases \([\text{Ca}^{2+}]_{\text{c}}\) in HIT-T15 cells \((14)\). In isolated rat islets, uptake of extracellular \(\text{Ca}^{2+}\) induced by GLP-1 is abolished by removal of extracellular \(\text{Na}^{+}\) \((22)\). This would infer that a site of the \(\text{Na}^{+}\) dependence for GLP-1, and therefore perhaps also for PACAP38, resides in the mechanism of increased \([\text{Ca}^{2+}]_{\text{c}}\). We therefore examined the \(\text{Na}^{+}\) dependence of the increase in \([\text{Ca}^{2+}]_{\text{c}}\) induced by PACAP38 in FURA-2-AM-loaded cell suspensions. We found, as seen in Fig. 3, that PACAP38 increased the cytoplasmic concentration of \(\text{Ca}^{2+}\) both in the presence and in the absence of extracellular \(\text{Na}^{+}\). Furthermore, in the absence of extracellular \(\text{Na}^{+}\), the increase in \([\text{Ca}^{2+}]_{\text{c}}\) in response to PACAP38 was greater than in the control cells incubated in the presence of extracellular \(\text{Na}^{+}\). Thus, at 300 s after addition of PACAP38, \([\text{Ca}^{2+}]_{\text{c}}\) had increased by 200 ± 8 nmol/liter in the absence of extracellular \(\text{Na}^{+}\) versus 52.0 ± 7 nmol/liter in the presence of extracellular \(\text{Na}^{+}\) \((p < 0.001)\). Therefore, in contrast to previous results that the \(\text{Ca}^{2+}\) uptake in isolated rat islets in response to GLP-1 was reduced by...
removal of extracellular Na\(^+\) (22), PACAP38 induced an exaggerated increase in [Ca\(^{2+}\)]\(_{cyt}\) after omission of Na\(^+\) from the medium. Hence, the Na\(^+\) dependence of the insulinotropic action of PACAP38 does not reside in impaired increase in [Ca\(^{2+}\)]\(_{cyt}\). The finding that the cytoplasmic concentration of Ca\(^{2+}\) increased after activation by PACAP38 also in the absence of extracellular Na\(^+\) suggests that the opening of Ca\(^{2+}\) channels is not dependent on depolarization of the cell by the influx of Na\(^+\), but is instead dependent on other mechanisms. One such mechanism could involve opening of PKA-dependent Ca\(^{2+}\) channels (15). We have shown previously that in HIT-T15 cells, the PACAP38-induced increase in [Ca\(^{2+}\)]\(_{cyt}\) is abolished when adenylate cyclase is activated by forskolin prior to introduction of PACAP38, suggesting that cAMP in fact mediates the opening of membranous Ca\(^{2+}\) channels (14). Another possibility involves opening of Ca\(^{2+}\) channels directly coupled to a G-protein activated by PACAP38. Such a Ca\(^{2+}\) channel, which is directly opened after activation of a G-protein, has been described previously to be coupled to at least one of the PACAP receptors expressed in the pancreatic endocrine tissue (30).

Further studies are required to examine this possibility. The potentiated PACAP38-induced increase in [Ca\(^{2+}\)]\(_{cyt}\) by PACAP38 is accentuated.

**Cytoplasmic Na\(^+\)**—The results above imply that Na\(^+\) is involved in the insulinotropic effect of PACAP38 in HIT-T15 cells, although the exact site of this involvement is still an open question. Indirect actions through cAMP or Ca\(^{2+}\) seem less likely, since neither the formation of cAMP nor the increase in [Ca\(^{2+}\)]\(_{cyt}\) after PACAP38 was reduced by removal of Na\(^+\), although the insulin secretory response to PACAP38 was impaired. This pattern of effects could be executed by activation of a channel increasing the uptake of Na\(^+\), with a subsequent increase in cytoplasmic concentration of Na\(^+\)\(_{cyt}\) yielding a direct secretory action of intracellular Na\(^+\). To examine this possibility, we measured [Na\(^+\)]\(_{cyt}\) by using the fluoroephore SBF1 in HIT-T15 cells. Measurement of [Na\(^+\)]\(_{cyt}\) in insulin-producing cells by using SBF1 has been performed previously by several groups showing that glucose, glycerol, and acetyicholine increase the [Na\(^+\)]\(_{cyt}\), which might contribute to the insulinotropic action of these secretagogues (25, 26, 32, 33). In our hands, measurement of [Na\(^+\)]\(_{cyt}\) in suspensions of HIT-T15 cells by using SBF1 did not yield reliable results, despite extensive trials in our laboratory (data not shown). We therefore proceeded to measure [Na\(^+\)]\(_{cyt}\) in single cells, which proved successful. As is seen in Fig. 4A, PACAP38 increased [Na\(^+\)]\(_{cyt}\) in a medium containing both extracellular Na\(^+\) and Ca\(^{2+}\). At 400 s after introduction of PACAP38, the ratio of [Na\(^+\)]\(_{cyt}\) fluorescence had increased from 0.68 \(\pm\) 0.02 in controls to 0.80 \(\pm\) 0.03 (p = 0.018) after stimulation by PACAP38. Fig. 4B shows a typical trace of such an experiment. In contrast, when extracellular Na\(^+\) was replaced with NMDG (125 mM), the effect of PACAP38 was abolished (Fig. 4C). This implies that PACAP38 activates a Na\(^+\) channel mediating the uptake of extracellular Na\(^+\), thereby increasing the intracellular Na\(^+\) concentration. The PACAP38-induced increase in [Na\(^+\)]\(_{cyt}\) was abolished also when the cells were treated with 20 \(\mu\)M H89 (Fig. 4C), which is a specific PKA inhibitor (34). These results imply that PACAP38 causes an uptake of Na\(^+\) into the cells and that this uptake is mediated by PKA and therefore probably executed through a PKA-sensitive Na\(^+\) channel. These findings support the important notion by Leech et al. (35) that PACAP stimulates an inward current in HIT-T15 cells, which is mainly caused by an influx of Na\(^+\) into the cells and which is mediated by cAMP. This is also consistent with a previous study showing that cAMP activates a cation channel in the rat isletoma cell line CRI-G1 (36).

**Cytoplasmic pH**—A possible confounding factor in studying effects of removal of Na\(^+\) from the extracellular medium on insulin secretion is the potential influence through changes in pH\(_{cyt}\). Omission of Na\(^+\) from the medium abolishes the Na\(^+\)-H\(^+\) exchange, thus preventing H\(^+\) to be transported out from the cytoplasm, which has been shown previously to be of importance for the regulation of pH\(_{cyt}\) in the \(\beta\)-cell (37). Therefore, removal of extracellular Na\(^+\) might reduce the pH\(_{cyt}\), which might then affect exocytosis. The inhibitory effect of removal of extracellular Na\(^+\) on PACAP38-induced insulin secretion might thus partially be explained by alteration in pH\(_{cyt}\). To examine whether removal of Na\(^+\) actually affects pH\(_{cyt}\) in HIT-T15 cells, we measured pH\(_{cyt}\) in cell suspensions by using the fluorophore BCECF-AM. We found that removal of extracellular Na\(^+\) expectedly decreased pH\(_{cyt}\) (Fig. 5). Thus, at time
0, \( \text{pH}_{\text{cyt}} \) was 7.41 ± 0.04 in the presence of extracellular \( \text{Na}^+ \) and 7.02 ± 0.11 (\( p = 0.035 \)) when \( \text{Na}^+ \) in the medium was replaced with NMDG (125 mM). \( \text{pH}_{\text{cyt}} \) was stable throughout the study period, and therefore this difference in \( \text{pH}_{\text{cyt}} \) in the presence versus in the absence of extracellular \( \text{Na}^+ \), persisted throughout the study period (Fig. 5). Introduction of PACAP38 did not alter \( \text{pH}_{\text{cyt}} \), neither in the presence nor in the absence of extracellular \( \text{Na}^+ \). To test whether this reduction in \( \text{pH}_{\text{cyt}} \) contributes to the reduced insulin secretion seen after PACAP38 in a \( \text{Na}^- \)-free medium, HIT-T15 cells were incubated with or without PACAP38 in media of different extracellular pH. The cellular buffering of changes in extracellular pH (38) was compensated by decreasing the extracellular pH to 6.8 to examine the influence of \( \text{pH}_{\text{cyt}} \) of 7.0. We found that lowering of extracellular pH from 7.36 to 6.8 did not affect insulin secretion stimulated by 10 mM glucose alone or by 10 mM glucose together with 100 nM PACAP38 (Fig. 6). Thus, the inhibitory effect of removal of \( \text{Na}^+ \) on PACAP38-induced insulin secretion cannot be explained by the accompanying change in \( \text{pH}_{\text{cyt}} \).

Summary and Conclusions—Our results thus show that the insulinotropic effect of PACAP38 is partially (~50%) dependent on extracellular \( \text{Na}^+ \). PACAP38 therefore resembles GLP-1 in this respect (22), and the \( \text{Na}^+ \) dependence of actions of PACAP is not restricted to pituitary cells (17). The failure of removal of extracellular \( \text{Na}^+ \) to completely abolish the insulinotropic action of PACAP38 infers that the signaling pathway mediating the insulinotropic effect of PACAP38 may be partly activated in the absence of extracellular \( \text{Na}^+ \) or, alternatively, that several intracellular pathways might be involved, whereof at least one is strictly \( \text{Na}^- \)-sensitive. Examining these possibilities, we found that the \( \text{Na}^+ \) dependence does not involve the formation of cAMP or the increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\), and that PACAP38 increases [Ca\(^{2+}\)]\(_{\text{cyt}}\) by a mechanism abolished by inhibition of PKA. This shows that neither activation of adenylyl cyclase nor a rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\) can explain the \( \text{Na}^+ \) dependence of PACAP and suggests that cAMP and raised [Ca\(^{2+}\)]\(_{\text{cyt}}\) can not solely explain PACAP38-induced insulin secretion. Instead, our results are integratively interpreted to indicate that PACAP38 

\[ \text{Na}^+ \] independently stimulates formation of cAMP, which activates PKA, which, in turn, opens both Ca\(^{2+}\) and Na\(^+\) channels. The subsequent influx of Na\(^+\) raises the cytoplasmic level of Na\(^+\), which then contributes to the PACAP-induced insulin secretion. This contribution could be mediated by depolarization induced by increased intracellular Na\(^+\), which, in turn, could augment Ca\(^{2+}\) uptake by opening of voltage sensitive Ca\(^{2+}\) channels. A tentative remaining possibility is that Na\(^+\) per se is of importance for the exocytotic mechanism. However, although exocytosis of secretory granules in β-cells has been shown to be mediated by several proteins (39), the Na\(^+\) dependence of the action of these proteins remains to be studied. Finally, it should be emphasized that in several excitable tissues, such as neuronal cells, influx of Na\(^+\) through voltage-gated ion channels plays a major role in cell activation by inducing depolarization (40). Our present results therefore strengthen the similarity between insulin producing cells and other excitable cells also in this respect.

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