Activation of muscarinic receptors increases the concentration of free Na^+ in mouse pancreatic B-cells

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The fluorescent probe SBFI was used to monitor the influence of acetylcholine (ACh) on the cytosolic concentration of free Na^+ (Na^+_i) in single mouse pancreatic B-cells. In the presence of 3 mM glucose and 135 mM extracellular Na^+, Na^+_i averaged 16.6 mM. ACh (100 μM) increased Na^+_i by ~ 80%. This rise was prevented by atropine, a blocker of muscarinic receptors, and by omission of extracellular Na^+, but still occurred if the sodium pump was blocked by ouabain. It was unaffected by tetrodotoxin, a blocker of voltage-sensitive Na^+ channels, and was not mimicked by depolarization of the cells with high K^+. It is concluded that activation of muscarinic receptors increases the membrane permeability to Na^+ in pancreatic B-cells.

Acetylcholine; Cytosolic sodium; Insulin release; Muscarinic receptor; Ouabain; Pancreatic B-cell

1. INTRODUCTION

The broad spectrum of biological responses brought about by the activation of muscarinic receptors is due to the existence of several subtypes of receptors linked to multiple transduction pathways.

Parasympathetic signals exert an important influence on glucose homeostasis through their modulation of hormone release by the endocrine pancreas. The major effect of acetylcholine (ACh) is a stimulation of insulin release that results from the activation of M_3 receptors in B-cells [1,2]. Acceleration of phosphoinositide turnover with subsequent mobilization of intracellular Ca^{2+} and activation of protein kinase C certainly contribute to this stimulation [3,4]. However, ACh also depolarizes the B-cell membrane [5,6], and this depolarization has tentatively been attributed to an increase in Na^+ permeability [7]. This conclusion was based on the observations that the depolarizing effect of ACh was accompanied by an increase in $^{22}Na^+$ uptake and was abolished by omission of extracellular Na^+ [7]. These findings were unexpected since the effects of ACh were clearly mediated by muscarinic receptors which, in contrast to nicotinic receptors [8], do not classically use changes in membrane permeability to Na^+ as a transduction pathway. The depolarizing current produced by ACh in B-cells has not yet been identified, probably because of its small magnitude [9]. We therefore used a recently developed technique based on the Na^+-sensitive fluorescent probe SBFI [10] to test more directly the possible effect of ACh on the cytosolic concentration of free Na^+ (Na^+_i) in pancreatic islet cells from normal mice.

2. MATERIALS AND METHODS

Islets were isolated after collagenase digestion of the pancreas of fed female NMRI mice. The cells were then dispersed in a Ca^{2+}- and Mg^{2+}-free solution and cultured for 1–2 days on glass coverslips in RPMI 1640 medium containing 11 mM glucose, 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. They were loaded with the Na^+ indicator SBFI during 1.5–2 h of incubation at 37°C in culture medium supplemented with 7 μM of SBFI acetoxymethyl ester (Molecular Probes, Eugene, OR, USA) and 0.02% of the non-ionic dispersing agent Pluronic F-127. The cover glasses with the SBFI-loaded cells were then used as the bottom of a perifusion chamber mounted on the microspectrofluorimetric set-up described for measurements of cytoplasmic Ca^{2+} [11]. The preparation was then perifused with HEPES-buffered solutions containing 3 mM glucose and 1 mg/ml BSA. They were gassed with O_2 and had a pH of 7.4 at 37°C. The control solutions contained (mM) NaCl 135, KOH 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2 and HEPES 10. Na^+-free solutions were prepared by substituting N-methyl-D-glucamine chloride for NaCl [7]. Among the test agents used, acetylcholine chloride, tetrodotoxin and gramicidin D were obtained from Sigma Chemical Co. (St Louis, MO, USA), and atropine sulfate, ouabain and L-alanine from Merck AG (Darmstadt, Germany).

SBFI was successively excited at 340 and 380 nm and the emitted fluorescence was filtered at 510 nm and recorded with a camera (CCD Video camera from Photonic Sciences Ltd., Tunbridge Wells, UK). The images were digitized with the system Magical of Applied Imaging (Sunderland, UK). The time interval between the ratioed images (340/380) was 8.5 s. No interference by autofluorescence was detectable at either excitation wavelength under the present conditions. The concentration of Na^+_i in test cells was calculated by comparing the ratio of the fluorescence at 340 and 380 nm in each cell to a calibration curve. This curve was established by exposing reference cells to solutions containing different Na^+ and K^+ concentrations ($Na^+ + K^+ = 150$ mM, 120 mM gluconate, 30 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES and 3 mM glucose) in the presence of 10 μg/ml gramicidin D, an ionophore that equilibrates transmembrane Na^+ and K^+ concen-

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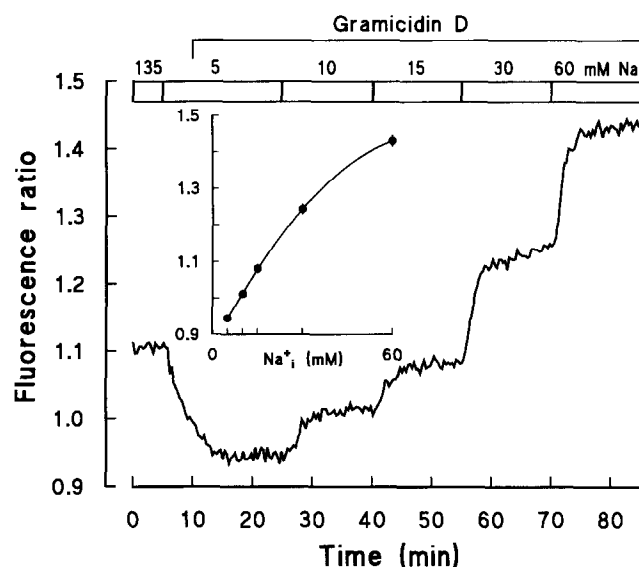
Fig. 1. Calibration curves relating the fluorescence excitation ratio of intracellular SBFI to the concentration of Na^+. Islet cells loaded with SBFI were perifused with a medium containing 3 mM glucose and the indicated concentration of Na^+. To equilibrate Na^+_i and Na^+_o, the cells were exposed to gramicidin D (10 μg/ml) from 10 min. The trace corresponds to the mean response obtained in 33 cells. The inset shows the steady-state fluorescence ratio (R) as a function of Na^+_i. It was fitted by a polynomial equation of the third degree: $[Na^+] = 272R^3 - 828R^2 + 913R - 348$. Values are means ± S.E.M. (which hardly exceed the size of the symbols).

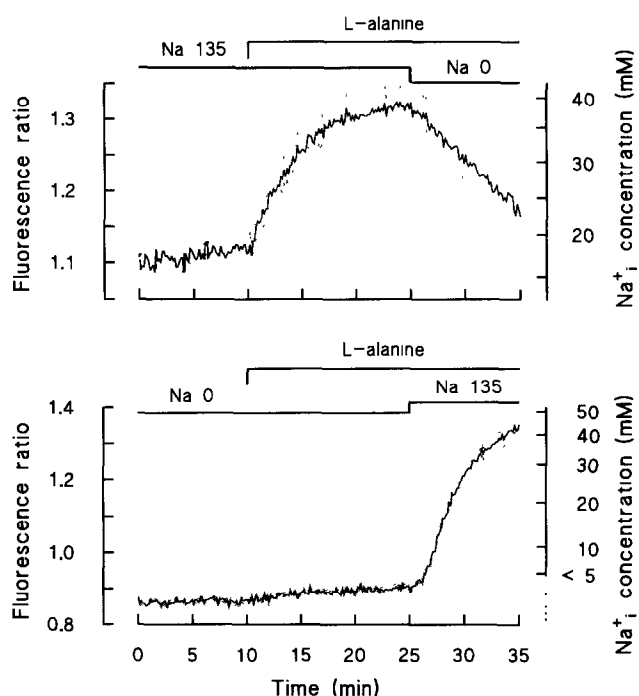Fig. 2. Effect of 20 mM L-alanine on Na^+_i in single mouse islet cells. The perifusion medium contained 3 mM glucose throughout and either 135 mM Na^+ or no Na^+ (Na 0, with N-methyl-D-glucamine as substitute). The traces correspond to the mean responses (±S.E.M.) obtained in 24 (upper panel) and 26 cells (lower panel).

trations [12]. Fig. 1 shows the mean 340/380 nm excitation ratio obtained in 33 cells exposed to Na^+ concentrations ranging from 5 to 60 mM. The ratio plotted as a function of the Na^+ concentration (Fig. 1, inset) was fitted by a polynomial equation of the third degree rather than by a linear relationship ($r = 0.972$). It shows that the fluorescence of SBFI tends to saturate at high Na^+ concentration as also indicated by the equation of Grynkiewicz [13]. This calibration curve was used to calculate Na^+_i from the observed ratios in the following experiments.

All experiments are presented as means ± S.E.M. for the indicated number of cells from at least three different cell cultures. The statistical significance of differences between observations was assessed by analysis of variance followed by a Neuman-Keuls test.

3. RESULTS

When the perifusion medium contained 3 mM glucose, 135 mM Na^+ and no test agent, Na^+_i averaged 16.6 ± 0.9 mM ($n = 256$) in islet cells.

The validity of our methodology was first assessed in a series of control experiments. L-alanine (20 mM), which enters B-cells through a Na^+-dependent transport system [14], rapidly increased Na^+_i in all cells studied (Fig. 2, upper panel). This increase was reversed by omitting Na^+ from the medium containing alanine, and did not occur when alanine was added to a Na^+-free medium (Fig. 2, lower panel). Blocking the sodium pump with 100 μM ouabain caused a progressive rise in Na^+_i in 86% of the cells studied (Fig. 3, upper panel). This increase was also reversed by omitting Na^+ from

the medium and did not occur when ouabain was added to a Na^+-free medium (Fig. 3, lower panel). The increase in Na^+_i brought about by 100 μM ouabain was smaller ($P < 0.01$) than that produced by 20 mM alanine (Table I), and was not augmented if a higher concentration of ouabain was used (not shown).

Stimulation with 100 μM ACh increased Na^+_i in 79% of the cells studied (Table I). The increase was rapid and reversed by Na^+ omission from the perifusion medium (Fig. 4, upper panel). It did not occur in a Na^+-free solution (Fig. 4, middle panel) or when the muscarinic receptors were blocked by atropine (Fig. 4, lower panel). By contrast, 2 μM tetrodotoxin, a blocker of voltage-dependent Na^+ channels, did not significantly affect the rise in Na^+_i induced by ACh (Table I). Stimulation by ouabain and ACh together increased Na^+_i to a larger extent than did ouabain or ACh alone ($P < 0.01$), indicating that the effects of both agents are additive (Table I). Perifusion with 8.4 mM K^+, which depolarizes B-cells by 3–4 mV more than ACh, slightly decreased Na^+_i ($P < 0.05$ by paired t-test) (Table I).

4. DISCUSSION

The present study directly shows that stimulation of muscarinic receptors rapidly increases the concentration of free cytosolic Na^+_i in islet cells. A recent study using integrating flame photometry has shown that car-
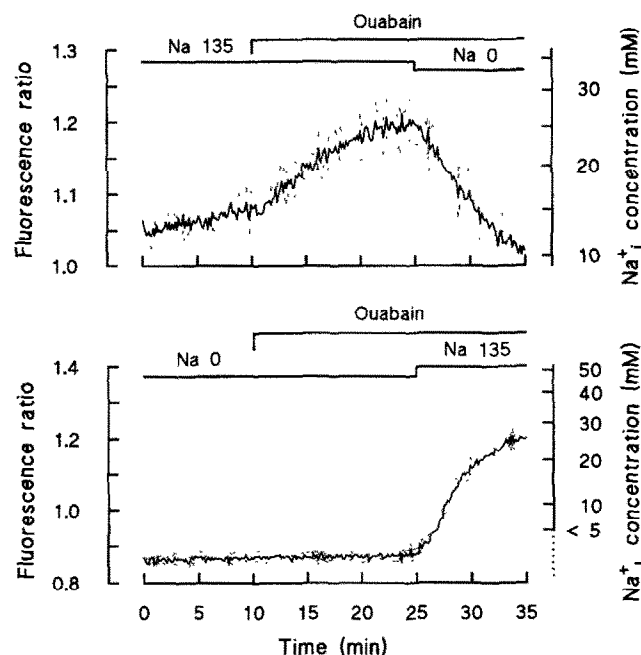
Fig. 3. Effect of 100 μM ouabain on Na^+_i in single mouse islet cells. The perifusion medium contained 3 mM glucose throughout and either 135 mM Na^+ or no Na^+ (Na 0, with N-methyl-D-glucamine as substitute). The traces correspond to the mean responses ($\pm$ S.E.M.) obtained in 24 (upper panel) and 32 cells (lower panel).

bachol causes a steady-state increase in total Na concentration in whole islets of *ob/ob* mice [15]. The 85% rise in cytosolic Na^+_i that we have observed upon stimulation by ACh is much larger than could have been expected from the 25–30% increase in total Na [15]. This is probably due to the fact that 75–80% of Na may be bound in islet cells [16].

We do not know whether the 79% of islet cells showing an increase in Na^+_i in response to ACh only correspond to B-cells, but this proportion is similar to that of B-cells in mouse islets. It has not been established whether the effects of ACh on A and D cells depend on Na^+. On the other hand, it is not clear why ouabain was effective in only 86% of the cells.

The pathway through which Na^+ enters B-cells upon muscarinic stimulation has not been identified. However, several mechanisms can already be excluded. Mouse B-cells possess voltage-dependent, tetrodotoxin-sensitive Na^+ channels, but these are essentially inactivated at the resting membrane potential [17]. The inability of tetrodotoxin to reduce the rise in Na^+_i brought about by ACh is consistent with its inability to affect the depolarization and the uptake of $^{22}Na^+$ also caused by ACh [7]. The conclusion that the stimulation of Na^+ influx is not the consequence (but rather the cause) of the depolarization is also borne out by the lack of effect of high K^+ on Na^+_i. The additivity of the effects of ACh and ouabain on Na^+_i (this study) and on the B-cell membrane potential (J.C. Henquin, unpublished data)
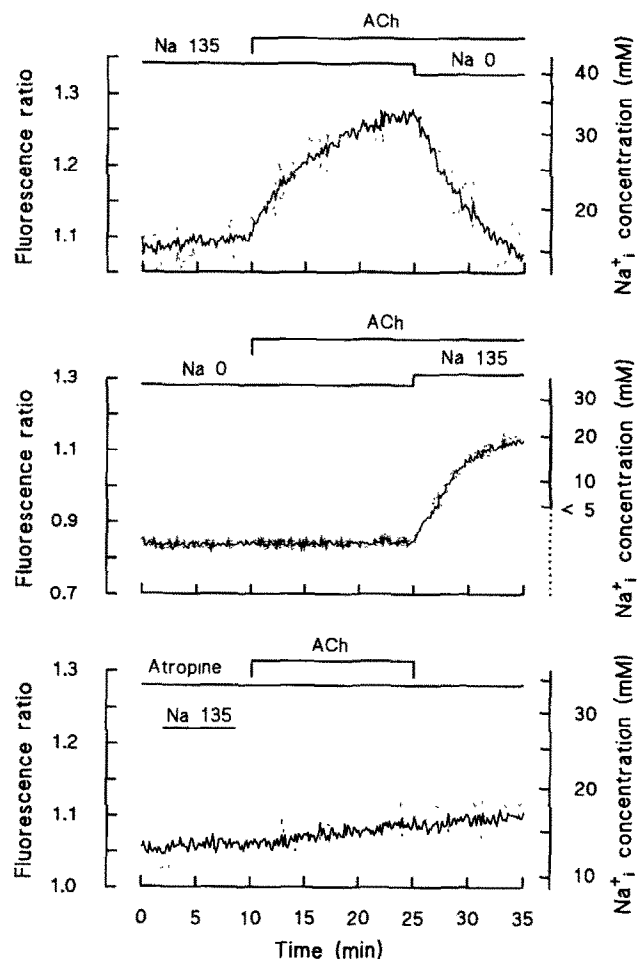

Fig. 4. Effect of 100 μM ACh on Na^+_i in single mouse islet cells. The perifusion medium contained 3 mM glucose throughout and either 135 mM Na^+ (upper panel) or no Na^+ (Na 0, middle panel). Lower panel: 10 μM atropine was added to the medium from the beginning of the experiment. The traces correspond to the mean responses ($\pm$S.E.M.) obtained in 32 (upper panel), 24 (middle panel) and 19 cells (lower panel).

indicates that the effects of ACh are not mediated by a blockade of the Na^+ pump. This is in contrast with the mechanism whereby ACh was reported to increase Na^+_i in cardiac Purkinje cells [18]. Muscarinic agonists slightly increase islet cell pH_i [19] probably through an activation of the Na^+/H^+ exchange by protein kinase C. However, this mechanism is unlikely to account for more than a fraction of the increase in Na^+_i because direct stimulation of protein kinase C by phorbol esters has larger effects on pH_i [20] but smaller effects on total islet Na than carbachol [15]. Anyhow, activation of the electroneutral Na^+/H^+ exchange would not explain the depolarizing action of ACh. Arguments against the role of the Na^+/Ca^{2+} exchange and the Na^+-K^+-2Cl$^-$ cotransporter have also been put forward elsewhere [7,21]. Non-specific cationic channels are present in the plasma membrane of many cells, including B-cells [22] and can be activated by various intracellular second

Table I

Changes in Na^+_i induced by different agents in single mouse islet cells

Test agents	Na^+_i (mM)			
	Control (A)	Agent (B)	Effect (B–A)	Number of responding cells
L-Alanine (20 mM)	16.1 ± 1.9	37.8 ± 4.4	21.8 ± 1.3	49/49
Ouabain (100 μM)	15.4 ± 3.0	25.2 ± 5.1	9.7 ± 1.5	24/28
ACh (100 μM)	16.3 ± 2.3	30.1 ± 3.7	13.8 ± 0.9	68/86
ACh (100μM) + Tetrodotoxin (2 μM)	17.0 ± 2.5	29.6 ± 3.3	12.6 ± 1.1	32/40
ACh (100 μM) + Ouabain (100 μM)	18.9 ± 2.5	40.3 ± 4.6	21.3 ± 2.1	31/35
K (8.4 mM)	14.9 ± 3.0	12.3 ± 2.6	−2.6 ± 0.6	25/25

Cells loaded with SBFI were perifused with a control medium containing 3 mM glucose alone before being stimulated for 15 min with the test agents. The effect corresponds to the change in Na^+_i between basal and test conditions in each individual cell. Values are means ± S.E.M. for the changes recorded in the responding cells. Basal Na^+_i in cells that did not show a change in Na^+_i upon stimulation by ouabain or ACh was 18.1 ± 2.4 mM ($n = 34$).

messengers [23]. This, again, is unlikely to explain the effects of ACh because activation of phosphoinositide turnover by vasopressin does not mimic the electrical and ionic effects of the muscarinic agonist [24]. Finally, M_3 muscarinic receptors in pancreatic B-cells could be coupled, perhaps by G proteins, to tetrodotoxin-insensitive Na^+ channels. A similar suggestion has been made recently for muscarinic receptors in cardiac cells [25].

In conclusion, this study provides direct support to the previous proposal [7] that an increase in the membrane permeability to Na^+ is involved in the pancreatic B-cell response to muscarinic stimulation. It further suggests that a rise in cytoplasmic Na^+_i may be one of the several factors which contribute, directly or indirectly, to the amplification of insulin release by parasympathetic signals.

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