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Cytoplasmic Ca^{2+} in Glucagon-Producing Pancreatic α-Cells Exposed to Carbachol and Agents Affecting Na^+ Fluxes

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The cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured with dual wavelength fluorometry in glucagon-producing mouse pancreatic α-cells loaded with the indicator fura-2. Spontaneous rhythmic activity in terms of slow oscillations from a basal level was observed at 3 m*M* glucose. Like in the insulin-secreting β-cells the generation of $[Ca^{2+}]_i$ oscillations in the α-cells was affected by the activity of the Na/K pump. Blocking the pump with ouabain resulted in an initial rise of $[Ca^{2+}]_i$ followed by gradual return to the basal level. The oscillations were transformed into sustained elevation of $[Ca^{2+}]_i$ by 10 m*M* L-glycine, which is cotransported with Na^+. A similar but less pronounced effect was obtained when Na^+ was cotransported with 10 m*M* of the nonmetabolizable amino acid α-aminoisobutyric acid. L-glycine induced sustained increase of $[Ca^{2+}]_i$ also when the oscillatory activity was suppressed by exposing the α-cells to 20 m*M* glucose in the presence of insulin. The observation that carbachol induces a $[Ca^{2+}]_i$ response in isolated α-cells calls for reconsideration of current ideas that muscarinic stimulation of glucagon release is an indirect effect mediated by adjacent β-cells.

Key Words: α-Cell; glucagon; Ca^{2+}; oscillations; Na^+; carbachol.

Introduction

Glucagon release from the pancreatic α-cells is related in a feedback fashion to the function of the hormone to maintain normal levels of blood glucose *(1–3)*. Accordingly, hypoglycemia evokes a prompt rise of the circulating glucagon concentration, whereas increase of blood glucose inhibits the secretory activity of the α-cells. Like the other islet hormones glucagon is released in pulses *(4–6)*. Recent studies have contributed to the understanding of this pulsatility in revealing the existence of cytoplasmic Ca^{2+} oscillations *(7)* suppressible by raising the glucose concentration in the presence of insulin *(8)*.

The significance of the energy metabolism for the secretory activity of the α-cells points to a central role for ATP in regulating the oscillations of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) responsible for the pulsatile release of glucagon *(8)*. The α-cells differ from the insulin-secreting β-cells both in lacking functionally active K^+ channels regulated by ATP and exhibiting electrical activity at 3 m*M* glucose *(9,10)*. These characteristics of the α-cell have resulted in the proposal that glucose inhibition of glucagon release is mediated by ATP-induced lowering of the cytoplasmic concentration of Ca^{2+} *(8,11)*.

In the present study measurements of $[Ca^{2+}]_i$ were utilized for analyzing the importance of the Na/K pump for oscillatory Ca^{2+} signaling in glucagon release. It will also be shown that isolated α-cells respond both to adrenergic and cholinergic stimuli.

Results

Spontaneous slow oscillations of $[Ca^{2+}]_i$ originating from a basal level were seen in a majority of α-cells exposed to 3 m*M* glucose. After blocking the Na/K pump with ouabain there was an initial increase of $[Ca^{2+}]_i$ to levels exceeding the peaks of the oscillations, followed by a return to basal values during a 10–20 min period (Fig. 1). The latter phase showed considerable variation with regard to interfering fluctuations.

There was a prompt increase of $[Ca^{2+}]_i$ also when the α-cells were exposed to amino acids cotransported with Na^+. When 10 m*M* L-glycine was added to a medium containing 3 m*M* glucose the initial rise was followed by a sustained elevation (Fig. 2A). Glycine had a similar effect on $[Ca^{2+}]_i$ when the spontaneous oscillatory activity was suppressed by exposing the α-cells to 20 m*M* glucose in the presence of insulin (Fig. 2B). Addition of 10 m*M* of the nonmetabolizable α-aminoisobutyric acid (AIB) to a medium containing 3 m*M* glucose resulted in more prominent oscillations (Fig. 3A) or in periods with sustained elevation (Fig. 3B). Exposure to the Ca^{2+} channel blocker methoxyverapamil promptly restored basal levels of $[Ca^{2+}]_i$ both in the presence of L-glycine and AIB (Figs. 2 and 3).

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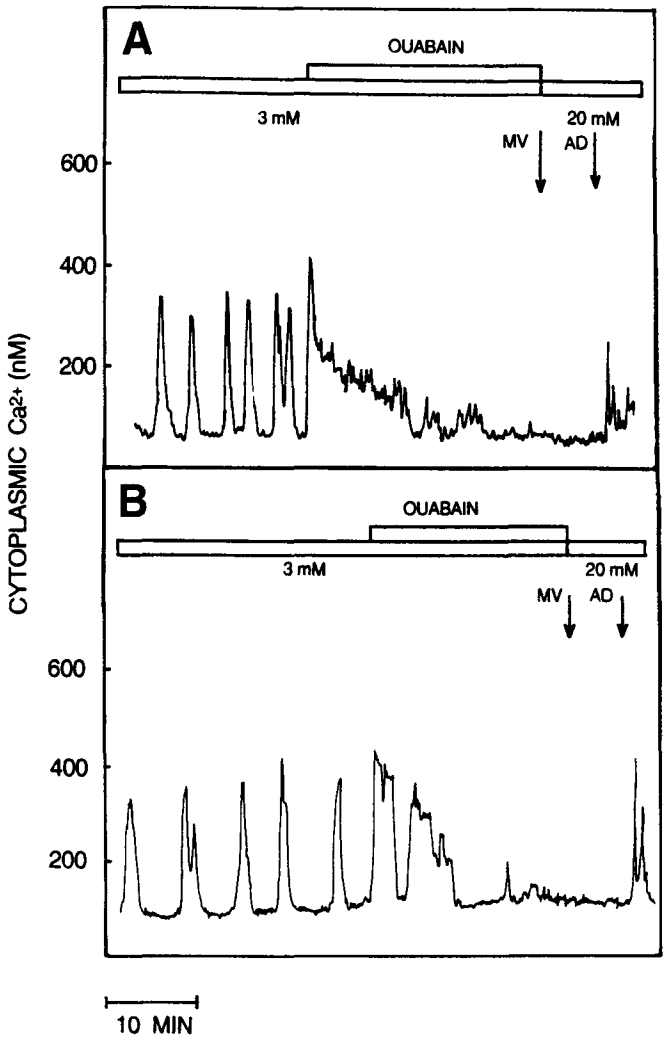

Fig. 1. Alterations of the cytoplasmic Ca^{2+} concentration in α-cells after ouabain inhibition of the Na/K pump. The bars denote the presence of 3 or 20 m*M* glucose and 1 m*M* ouabain and the arrows indicate the additions of 50 μ*M* methoxyverapamil (MV) and 3 μ*M* L-adrenaline (AD). Representative responses of five cells.

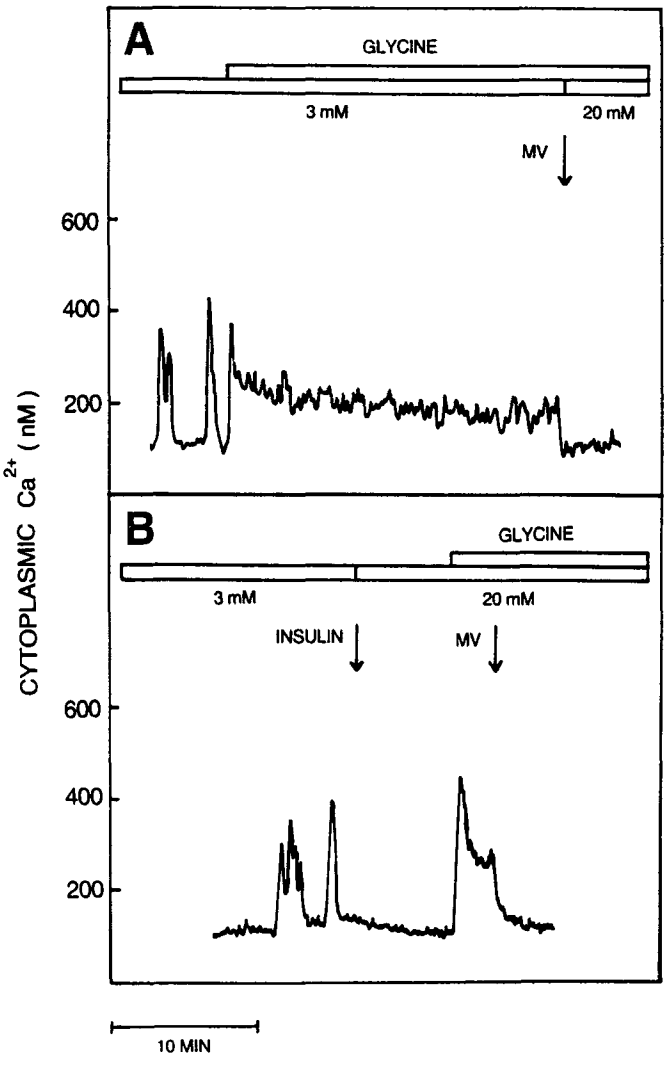

Fig. 2. Effects of L-glycine on the cytoplasmic Ca^{2+} concentration in α-cells. The bars denote the presence of 3 or 20 m*M* glucose and 10 m*M* L-glycine and the arrows indicate the additions of 50 μ*M* methoxyverapamil (MV) and 2 μg/mL insulin. Additions of L-glycine were made both in the absence **(A)** and presence **(B)** of stimulated glucose metabolism. Representative responses of five cells for each experimental situation.

It was tested whether the α-cells react to carbachol with mobilization of intracellular calcium. To promote the loading of internal calcium stores and avoid interference with spontaneous oscillations, these experiments were performed in the presence of 20 m*M* glucose, insulin, and methoxyverapamil. Most cells responded to 10 μ*M* carbachol with $[Ca^{2+}]_i$ peaks often followed by a slight elevation (Fig. 4A). This effect was blocked by 10 μ*M* atropine (not shown). Also cells not responding to carbachol reacted to L-adrenaline with a transient increase of $[Ca^{2+}]_i$ (Fig. 4B).

Discussion

The slow $[Ca^{2+}]_i$ oscillations in the α-cells mimic those in the β-cells in reflecting periodic entry of the ion via voltage-dependent channels *(8)*. Nevertheless, glucose has opposite effects on the secretory activity in the two types of cells. The stimulatory action on the β-cell release of insulin can be attributed to the metabolism of the sugar with resulting closure of ATP-regulated K^+ channels *(12)*. Since the α-cells lack functionally active K^+ channels of this type, it has been proposed that glucose inhibition of glucagon release reflects ATP-induced lowering of $[Ca^{2+}]_i$ *(13)*. This lowering can be attributed both to stimulated removal of Ca^{2+} from the cytoplasm *(11)* and to reduced entry of the ion mediated by a hyperpolarizing action of electrogenic membrane transporters *(8)*. The potential importance of the latter alternative is evident from the observations of Ca^{2+}-dependent action potentials under low glucose conditions, the frequency of which is markedly affected by minor alterations of the membrane potential *(9,10)*.

In pancreatic β-cells the slow oscillations of $[Ca^{2+}]_i$ are transformed into sustained elevation during expo-

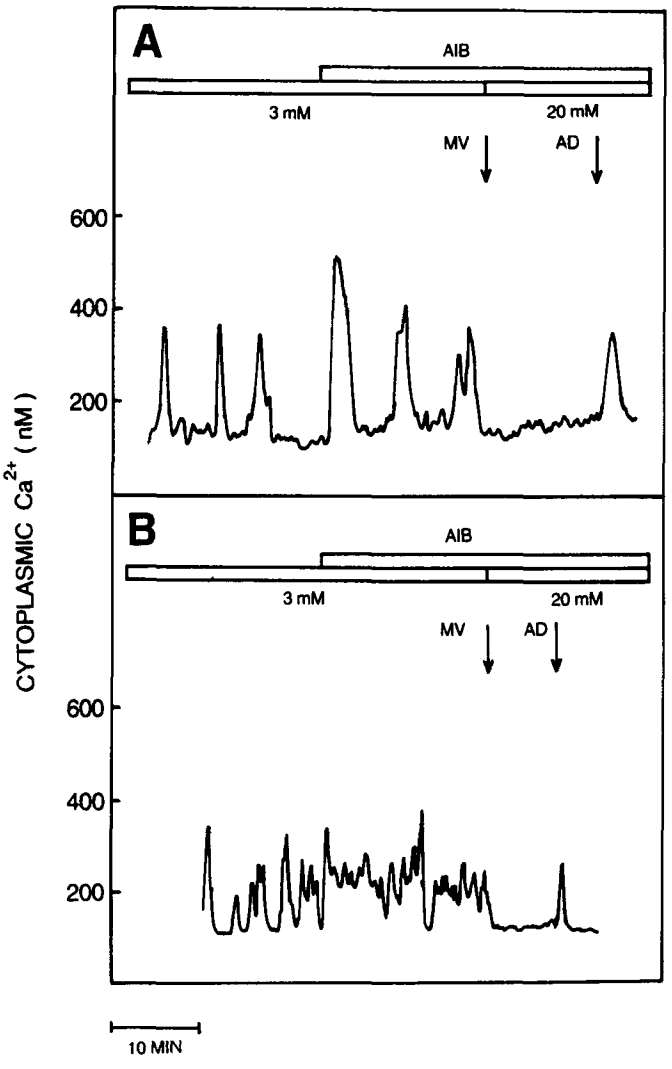

Fig. 3. Effects of α-aminoisobutyric acid (AIB) on the cytoplasmic Ca^{2+} concentration in α-cells. The bars denote the presence of 3 or 20 m*M* glucose and 10 m*M* AIB and the arrows indicate the additions of 50 μ*M* methoxyverapamil (MV) and 3 μ*M* L-adrenaline (AD). In the 18 experiments performed, the exposure to AIB resulted either in more prominent oscillations from the basal level **(A)** or in periods of sustained elevation **(B)**.

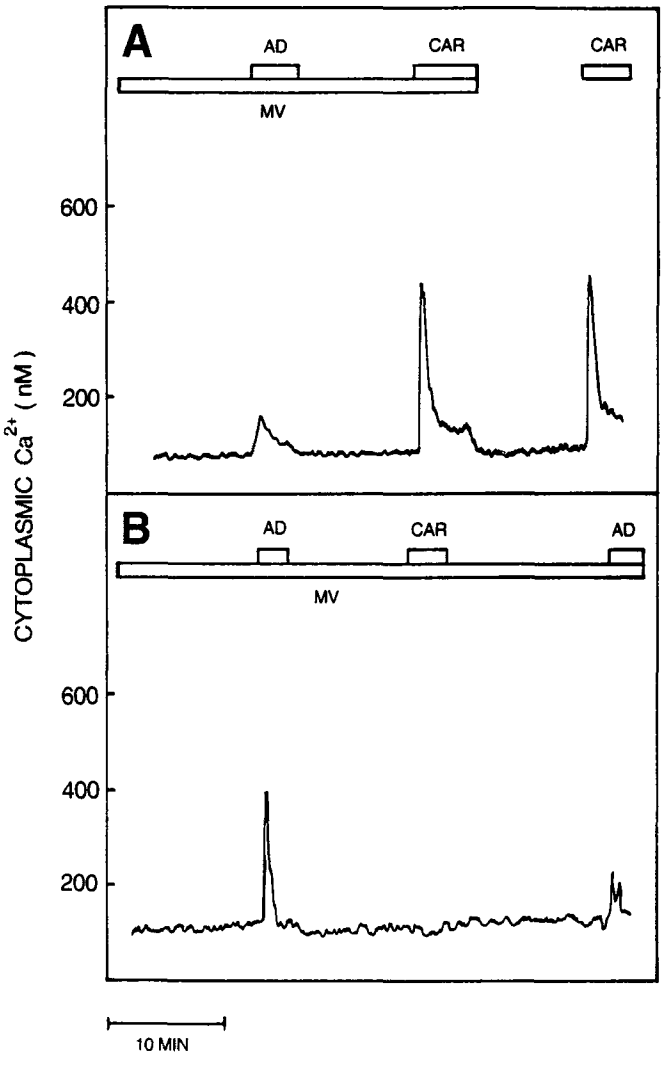

Fig. 4. Effects of carbachol and L-adrenaline on the cytoplasmic Ca^{2+} concentration in α-cells exposed to 20 m*M* glucose. The bars denote the presence of 3 μ*M* L-adrenaline (AD), 10 μ*M* carbachol (CAR), and 50 μ*M* methoxyverapamil (MV). Most cells (six of nine) responded to carbachol with a distinct peak of $[Ca^{2+}]_i$ often followed by suprabasal elevation **(A)**. In the remaining cells carbachol did not affect $[Ca^{2+}]_i$ despite a clear response to L-adrenaline **(B)**.

sure to amino acids cotransported with Na^+ *(14)*. Apparently, this is also the case in the α-cells. It has now been confirmed from previous observations that L-glycine transforms existing oscillations in α-cells into sustained elevation of $[Ca^{2+}]_i$ *(8)* and shown that this is often the case for the nonmetabolizable AIB. The influx of Na^+ results in depolarization, which opens voltage-dependent Ca^{2+} channels. Moreover, increase of cytoplasmic Na^+ can be expected to elevate $[Ca^{2+}]_i$ by mobilizing intracellular Ca^{2+} and inhibiting the outward transport by Na/Ca exchange *(15)*. These actions were sufficiently pronounced for glycine to evoke a sustained increase of $[Ca^{2+}]_i$ also when the oscillations were suppressed by raising the glucose concentration in the presence of insulin. Testing another situation when depolarization and rise of intracellular Na^+ is probably associated with increased levels of ATP, also ouabain was found to interfere with the $[Ca^{2+}]_i$ oscillations. Inhibition of the electrogenic Na/K pump by ouabain thus resulted in a prompt increase of $[Ca^{2+}]_i$. However, contrary to β-cells exposed to ouabain *(16)*, the increase was not sustained but slowly returned to basal $[Ca^{2+}]_i$ levels. If the inhibition of the Na/K pump results in a similar increase of ATP as found in β-cells *(16)*, the gradual lowering of $[Ca^{2+}]_i$ in the α-cells may be a reflection of the mechanism by which the stimulation of energy metabolism inhibits glucagon release.

Like other stress hormones, catecholamines serve a vital function by securing glucose delivery to brain. Part of this effect is a result of actions on the pancreatic islets, manifested as suppression of glucose-stimulated insulin secretion and stimulation of glucagon release. Studies of

isolated α-cells have provided evidence for increase of the cAMP content after addition of adrenaline *(17)*, an effect resulting in mobilization of intracellular calcium incorporated in response to glucose *(18)*. Since the islet cells secreting insulin and somatostatin do not respond in such a way, a $[Ca^{2+}]_i$ increase in response to adrenaline can be used for rapid identification of the α-cells *(8)*. It has been suggested that the catecholamine-induced secretion of glucagon is mediated not only by β- *(17)*, but also by α-adrenoceptors *(19,20)*, and depend on circulating adrenaline from the adrenal medulla as well as on noradrenaline released from adrenergic nerves within the pancreas *(21)*.

Although doubts have been expressed whether cholinergic innervation is a physiologically important regulator of α-cells *(21)*, the secretory activity has been reported to be stimulated by vagal nerve stimulation *(22)* or addition of acetylcholine *(23)*, and inhibited by atropine *(24)*. In pancreatic β-cells the activation of muscarinic receptors, or other measures for raising IP_3, results in a mobilization of calcium from the endoplasmic reticulum *(25,26)*. Since previous studies have failed to demonstrate a similar action of carbachol on guinea-pig α-cells, it has been suggested that muscarinic stimulation of glucagon release is an indirect effect requiring the participation of adjacent β-cells *(18)*. The present results call for a reconsideration of this view in demonstrating that a majority of isolated α-cells indeed respond to carbachol with rise of $[Ca^{2+}]_i$. The absence of a $[Ca^{2+}]_i$ response to carbachol may be caused by a heterogeneity in the distribution of muscarinic receptors and a loss of the receptors during the enzymatic isolation of the islets. The fact that no carbachol response was found in some of the studied cells may also be a result of emptying of the IP_3-sensitive store by previous exposure to L-adrenaline.

The large amplitude oscillations of cytoplasmic Ca^{2+} in the α-cells mimic those in the insulin-secreting β-cells in reflecting a temporal imbalance between the entry of the ion and its subsequent removal from the cytoplasm *(8)*. Using the ATP-regulated K^+ channels as an indicator of the energy metabolism, it has been suggested that oscillatory $[Ca^{2+}]_i$ in the β-cells reflects periodic depolarization caused by fluctuations in the ATP production *(27)*. Whereas increase of ATP has a depolarizing effect on the β-cells, it is supposed to affect the membrane potential of the α-cells in the opposite way *(8)*. Accordingly, if the metabolism of the β- and α-cells are somehow coupled, the pulsatile release of glucagon should be in opposite phase to that of insulin (cf *4*). However, if the synchronization is based on changes in membrane potential, it is possible to explain why concomitant pulses of glucagon and insulin have also been reported *(5)*. Future studies will have to clarify whether the type of coupling between the islet cells varies under different physiological conditions.

Materials and Methods

Materials

Reagents of analytical grade and deionized water were used. Collagenase, HEPES, and bovine serum albumin (fraction V) were purchased from Boehringer Mannheim GmbH (Mannheim, Germany), and fetal calf serum was obtained from Gibco (Paisley, UK). L-adrenaline, L-glycine, AIB, carbachol, and atropine were products of Sigma (St. Louis, MO) and fura-2 acetoxymethyl ester was provided by Molecular Probes Inc. (Eugene, OR). Methoxyverapamil was donated by Knoll AG (Ludwigshafen am Rhein, Germany) and porcine monocomponent insulin was a gift from Novo Nordisk A/S (Bagsvaerd, Denmark). Material for immunostaining of glucagon was provided by Dako (Carpinteria, CA).

Isolation and fura-2 Loading of α-Cells

Islets of Langerhans were isolated by collagenase digestion from pancreatic glands (avoiding the duodenal portion) of 10–12-wk-old female NMRI mice. Single cells were prepared by shaking the islets in a Ca^{2+}-deficient medium *(28)*. After centrifugation through RPMI-1640 medium the cells were resuspended in such medium and allowed to attach overnight to circular 25 mm cover glasses at 37°C in an atmosphere of 5% CO_2 in humidified air. Further experimental handling was performed with a basal medium containing 125 m*M* NaCl, 5.9 m*M* KCl, 1.2 m*M* $MgCl_2$, 2.56 m*M* $CaCl_2$, 0.5 mg/mL albumin, and 25 m*M* HEPES (adjusted to pH 7.40 with NaOH). After rinsing, the cells were loaded with fura-2 by 30–40 min incubation at 37°C with 0.5 μ*M* of its acetoxymethyl ester in the presence of 3 m*M* glucose. The cover glasses with the attached cells were then washed and used as bottoms of an open chamber *(29)*, which was placed on the stage of an inverted Nikon microscope. The chamber was connected to a peristaltic pump allowing superfusion of the cells at a rate of 0.75 mL/min with medium kept at 37°C.

Measurements of Cytoplasmic Ca^{2+}

The measurements were based on time-sharing dual wavelength fluorometry using a 40 X Fluor oil immersion objective. A 75 W xenon arc lamp and 10–13 nm half-bandwidth interference filters were used for excitation at 340 and 380 nm. Images were collected through a 30 nm half-bandwidth filter at 510 nm with an intensified CCD camera (Extended ISIS-M, Photonic Science, Robertsbridge, UK). The excitation filter changer was part of a Magiscan image analysis system (Applied Imaging, Gateshead, UK). The amplification of the intensified video camera was set to utilize optimally the dynamic range of the analog-to-digital converter at either wavelength or any $[Ca^{2+}]_i$ level encountered. The specimens were illuminated only during image capture. Photodamage of the cells was minimized with neutral density filters. Calibration

was performed according to previously described procedures *(30)*. The Tardis program (Applied Imaging) was used for the image analysis. All 340/380 nm ratio frames were calculated after subtraction of background frames. The calculations were based on capture of an image pair every 3.4–8.4 s. Each image consisted of 16 accumulated video frames divided by 8, the time between the averaged 340 and 380 nm images being 1.1 s.

All measurements were performed on individual α-cells lacking contact with other cells. Paracrine influences between the few cells on the cover glasses are unlikely because of a large chamber volume and a rapid exchange of medium.

Identification of α-Cells

Immunostaining for glucagon was performed at the end of the experiments adhering to a previously described protocol *(8)*.

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