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The effects of hydralazine on lipolysis in subcutaneous adipose tissue in humans

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Abstract

Recent evidence from animal research and in vitro experiments indicates that changes in dietary calcium intake could cause changes in lipolysis through alterations of the intracellular calcium concentration in adipocytes. The objective of the study was to examine whether the calcium antagonist hydralazine affects blood flow and lipolysis in subcutaneous abdominal adipose tissue in vivo in humans. Three different concentrations of hydralazine (12.2, 24.4, and 48.8 μmol/L) were locally administered in adipose tissue using the microdialysis technique to assess effects on lipolysis and blood flow in subcutaneous adipose tissue in the abdominal region. Subjects from the general community were studied ambulatorily at a university hospital. Eight healthy men (age, 33.1 ± 3.3 years; body mass index, 24.2 ± 0.2 kg/m^2) were recruited by local announcement. Subcutaneous adipose tissue in the abdominal region was perfused with increasing concentrations of hydralazine. The main outcome measures were adipose tissue lipolysis and blood flow. Hydralazine had no effect on ethanol outflow-inflow ratios, but significantly increased interstitial glycerol concentration at the highest concentration ($P < .05$). The present results indicate that hydralazine increases lipolysis in abdominal subcutaneous adipose tissue in healthy lean subjects, but hydralazine had no significant effects on local blood flow in adipose tissue.

1. Introduction

The prevalence of obesity has increased markedly during the past 2 decades; and it is an important risk factor for the development of type 2 diabetes mellitus, various types of cancer, and cardiovascular complications. In recent years, an inverse relation between dietary calcium and body mass index (BMI) was repeatedly observed [1-8]. Some intervention studies also show that dietary calcium may have weight-lowering effects [9-12].

A possible mechanism to explain this relation could be as follows: decreasing the concentration of 1,25-OH_2D_3 in cultures of human adipocytes and in transgenic mice caused immediate decreases of the intracellular calcium concentration in adipocytes [13,14]. Interestingly, this reduction in intracellular calcium concentration could decrease lipogenesis through a Ca^{2+}-dependent inhibition of fatty acid synthase expression and increase lipolysis through an inhibition of phosphodiesterase 3B and an increase in the cyclic adenosine monophosphate concentration. The latter increases the phosphorylation of hormone-sensitive lipase, thus increasing the rate of lipolysis [15,16]. In this way, a high dietary calcium intake may lead to a lower body weight by changing the rate of lipolysis and lipogenesis in adipose tissue through a decrease in serum 1,25-OH_2D_3 [13-16].

To be able to study the in situ effects of modifying the intracellular calcium concentration in human adipocytes, we used hydralazine in the present microdialysis study. Hydralazine is a vasodilator clinically used to treat hypertension. Furthermore, it has previously been shown in microdialysis experiments to cause vasodilatation [17,18]. The effects of hydralazine on vasodilatation are mediated through antagonistic effects on the intracellular calcium concentration in smooth muscle tissue of blood vessels. Hydralazine has been shown to cause a reduction in the intracellular calcium concentration of rabbit vessels via a decreased 1,4,5 *myo*-inositol phosphate (IP_3) activity, which in turn caused a decrease in the release of calcium from the sarcoplasmatic reticulum [19]. Furthermore, decreases in body weight in a rabbit model of obesity were also observed as a response to treatment with hydralazine [20]. Moreover, in a previous investigation from our laboratory, lipolytic effects of

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hydralazine were observed in skeletal muscle tissue using the microdialysis technique [18]. Based on these findings, we hypothesized that changes in the intracellular calcium concentration induced by the calcium antagonist hydralazine could induce an increase in lipolysis in human adipose tissue.

In this investigation, we studied the effects on adipose tissue blood flow and lipolysis of local perfusion of subcutaneous adipose tissue in the abdominal region with 3 different concentrations of the calcium antagonist hydralazine.

2. Materials and methods

2.1. Subjects

Eight healthy male subjects were studied in this investigation. All subjects were free from overt disease and not participating in any regular physical activity for >3 h/wk; and the habitual macronutrient, total energy, calcium, and fiber intake of the subjects was determined with a 3-day food intake questionnaire. The Ethical Committee of the Academic Hospital Maastricht and the University of Maastricht approved of the protocol, the nature and risks of the experimental procedures were explained to the subjects, and their informed consent was obtained.

2.2. Body composition and anthropometric measurements

Whole-body density was determined by underwater weighing in the morning after an overnight fast. Body weight was measured with a digital balance with an accuracy of 0.01 kg (type E1200; Sauter, New Market, United Kingdom), and height was measured to the nearest 0.01 m. Lung volume was measured simultaneously with the helium dilution technique using a spirometer (Volugraph 2000; Mijnhardt, Bunnik, The Netherlands).

2.3. Microdialysis protocol

Subjects were asked to report to the laboratory for the microdialysis trial in the morning after an overnight fast. They were asked not to consume alcohol and not to perform heavy physical exercise during the 24 hours preceding the experiment. They were studied in the supine position on a hospital bed, with the ambient temperature kept at 23°C to 25°C.

Consequently, 2 microdialysis probes (CMA-60; CMA Microdialysis, Solna, Sweden) were inserted into the subcutaneous abdominal adipose tissue. The skin was anesthetized locally with a lidocaine- and prolocaine-containing crème (25 mg/g) (EMLA; Astra Pharmaceuticals, Zoetermeer, the Netherlands), which was applied 1 hour before the start of the experiment. Probes were placed 5 to 8 cm left and right from the umbilicus.

Probes consisted of dialysis tubing (30 × 0.6 mm^2, 20-kd cutoff) glued to the end of a double lumen polyurethane canula. The perfusion solvent entered the probe through the inner canula, passed down to the tip of the probe, streamed upward in the space between the inner canula and the outer dialysis membrane, and left the probe through the outer canula via a side arm, from which the dialysate was collected.

After insertion, the catheters were flushed with Ringer perfusion fluid (the composition of this fluid was as follows: Na^+, 147 mmol/L; K^+, 4 mmol/L; Ca^{2+}, 2.3 mmol/L; Cl^-, 156 mmol/L [pH = 6]; and an osmolality of 290 mosm/kg) to clear the microdialysis membranes from air bubbles. Thereafter, Ringer perfusion fluid supplemented with 50 mmol/L ethanol was perfused at a flow rate of 0.5 μL/min for 90 minutes before the start of the experiment to allow for recovery of the adipose tissue at the site where the probes were inserted. Consequently, the real interstitial glycerol concentration was determined with the zero flow method [21]. For this determination, dialysate was collected in two 20-minute fractions at a flow rate of 0.5 μL/min and in three 10-minute fractions at flow rates of 1.0, 2.5, and 5.0 μL/min. The perfusion at 5.0 μL/min was used as a baseline measurement for the second part of the experiment. Dialysate glycerol concentrations were log-transformed and plotted against perfusion rates. Linear regression analysis was used to calculate the glycerol concentration at the zero flow rate, corresponding to the real interstitial glycerol concentration. The in vivo recovery rate of the probe was calculated by dividing the dialysate glycerol concentration at 5.0 μL/min by the calculated interstitial glycerol concentration. Furthermore, the ethanol concentration was determined both in the ingoing and outgoing perfusion solvent to assess the ethanol outflow-inflow (out-in) ratio as indicator for local nutritive blood flow (ethanol dilution technique) [22,23].

During the second part of the experiment, one probe was perfused with increasing concentrations of hydralazine: 12.2, 24.4, and 48.8 μmol/L (Sovereign Medical, Basildon, United Kingdom) at a flow rate of 5.0 μL/min. The other probe served as a control probe and was perfused with Ringer fluid at a flow rate of 5.0 μL/min throughout the experiment.

During each 60-minute hydralazine perfusion period, dialysate was collected in six 10-minute fractions. Each perfusion period was followed by a 30-minute washout, during which Ringer solution supplemented with 50 mmol/L ethanol was perfused. These washout periods were included in the protocol to ensure that the effects of the hydralazine perfusion on blood flow and lipolysis could return toward their basal level before the start of the following perfusion period. During these washout periods, dialysate was collected in 10-minute fractions as well.

The glycerol concentrations and ethanol ratios presented here are the averages of the last 3 fractions during the experimental periods and the averages of the last 2 fractions during the washout periods.

2.4. Analytical methods

Glycerol and ethanol concentrations in the dialysate were determined on a Cobas Fara centrifugal analyser (Roche Diagnostics, Basel, Switzerland); glycerol concentrations were measured fluorometrically using a standard glycerol kit

(Boehringer, Mannheim, Germany), but with adapted concentrations of nicotinamide adenine-dinucleotide hydride (NADH), enzymes, and buffer to achieve accurate fluorometric detection; and ethanol concentrations were measured spectrophotometrically at 340 nm using a standard ethanol assay kit (176290, Boehringer).

2.5. Statistical analysis

To compare the effects of the different concentrations of hydralazine, a repeated-measures analysis of variance (ANOVA) was performed and a Scheffé test was used for a nonpaired post hoc analysis. To compare the effects of the hydralazine perfusion on the Δs of the glycerol and ethanol out-in ratios, a 2 × 6 repeated-measures ANOVA was performed and a Scheffé test was used for a nonpaired post hoc analysis. A paired-samples *t* test was used to test for differences between the experimental and control probes. The SPSS 11.0.4 for Mac OS X (Chicago, IL) was used for statistical analysis. All values are means ± SEM. Statistical difference was set at $P < .05$.

3. Results

3.1. Subject characteristics

The subjects had an average age (in years) and BMI (in kilograms per square meters) of 33.1 ± 3.3 and 24.2 ± 0.2, respectively. The average body fat percentage of our subjects was 21.3 ± 1.7 as determined with the hydrodensitometry measurements (Table 1). In general, the habitual nutrient intake was within the reference range for this age and sex group. However, the habitual calcium intake was around 200 mg/d higher than the average for this age and sex group [24].

3.2. Ethanol out-in ratio

The baseline ethanol out-in ratio was 0.80 ± 0.04 in the experimental probe and 0.86 ± 0.05 in the control probe ($P = .18$). These values decreased to 0.68 ± 0.05 and 0.78 ± 0.06 at the end of the experiment (ie, after perfusion with the highest concentration of hydralazine) in the experimental and control probes, respectively; and they were significantly different from baseline ($P = .001$ for the experimental and $P < .05$ for the control probe). There were no between-treatment differences in the ethanol ratios at any of the doses used in this study. The ethanol Δ, calculated by subtracting the concentration of ethanol in the control probe from the concentration in the experimental probe at corresponding time points, did not change significantly throughout the experiment (Fig. 1A, B).

Table 1
Physical characteristics and habitual nutrient intake of the male subjects (N = 8)

Age (y)	33 ± 3
Height (m)	1.84 ± 0.03
Weight (kg)	84.3 ± 2.8
BMI (kg/m^2)	24.3 ± 0.4
Body fat (%)	21.3 ± 1.7
Energy (MJ)	12.8 ± 2.1
EN%PRO	15.8 ± 0.4
EN%CHO	48.8 ± 0.9
EN%FAT	33.9 ± 1.6
Calcium (mg)	1471 ± 158
Fiber (g)	23.0 ± 4.4

All values are mean ± SEM. EN%PRO indicates energy percentage as protein intake; EN%CHO, energy percentage as carbohydrate intake; EN% FAT, energy percentage as fat intake.

3.3. Interstitial glycerol concentration

The baseline interstitial glycerol concentration was 218 ± 34 μmol/L in the experimental probe and 207 ± 29 μmol/L in the control probe, but this difference was not statistically significant ($P = .715$). These values decreased to 204 ± 30 and 140 ± 30 μmol/L at the end of the experiment in the experimental and control probes, respectively; and this decrease was significantly different from baseline in the control condition ($P < .01$). The glycerol Δ, calculated by subtracting the concentration of glycerol in the control probe from the concentration in the experimental probe at corresponding time points, was 63 ± 27 μmol/L at the end of the experiment, which meant a significant increase compared with baseline ($P = .05$) (Fig. 2A, B).

4. Discussion

We observed that perfusing the abdominal subcutaneous adipose tissue of healthy men with hydralazine increased the interstitial glycerol concentration despite a nonsignificant increase in blood flow in the experimental probe. This could indicate a lipolytic effect of hydralazine, which was only observed at the highest concentration of 48.8 μmol/L.

Although modest increases in blood flow in the control condition have been observed previously in microdialysis experiments [18], the increase in blood flow in the control condition we observed here is larger than that in previous investigations. The subjects in this investigation were relatively lean and young, and it could be possible that they were more sensitive to changes in blood flow due to the long fasting period. However, the increase in blood flow was slightly more pronounced in the experimental condition, although this was not significant. It could be speculated that an increased local spillover of noradrenalin due to an increased sympathetic stimulation, which has been observed previously after prolonged fasting [25], caused an increase in the local blood flow throughout the experiment. Sympathetic stimulation has previously been shown to increase local blood flow during microdialysis experiments [26].

Furthermore, we included a washout period of 30 minutes between the perfusion with the 3 different concentrations of hydralazine to allow blood flow and lipolysis to return to baseline. However, blood flow and lipolysis did not return to baseline during these periods; and it cannot be excluded that

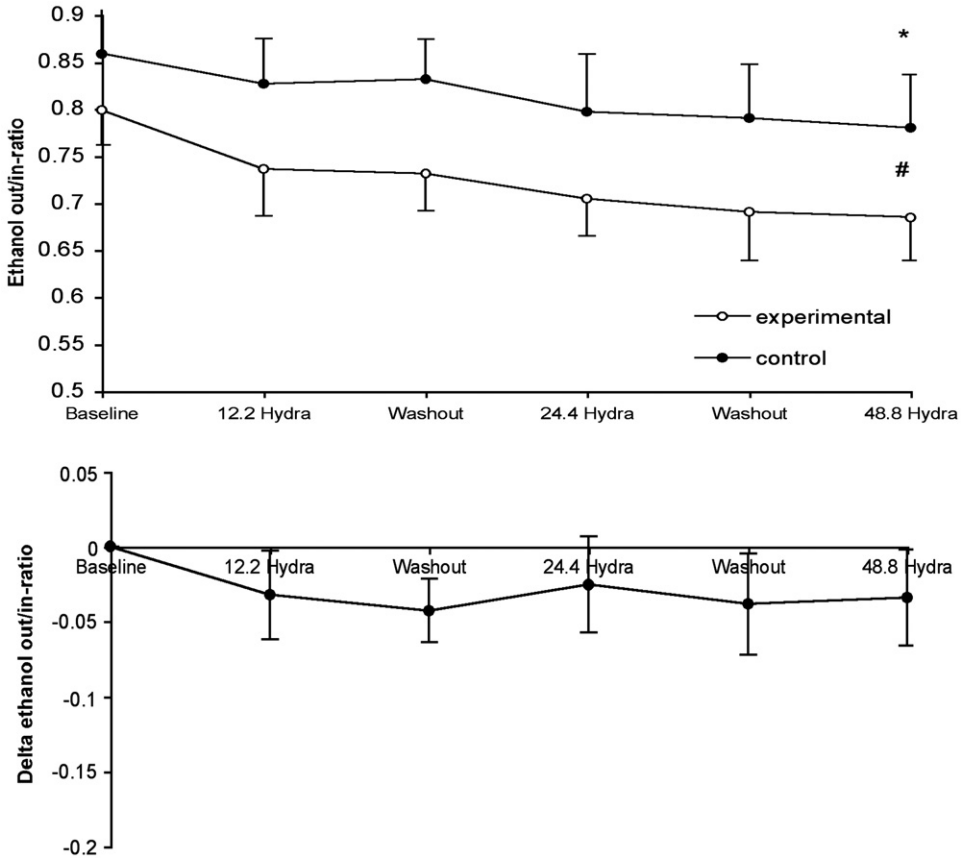

Fig. 1. Effects of hydralazine on the ethanol out-in ratios in abdominal subcutaneous tissue. Data in bottom figure are adjusted for corresponding changes in the control probes and presented as changes from baseline. All values are mean ± SEM (N = 8). Differences induced by hydralazine were tested with repeated-measures ANOVA; a Scheffé test was used for post hoc analysis. *$P < .05$ compared with baseline; #$P = .001$ compared with baseline.

there was a residual effect on blood flow and lipolysis of the previous perfusions at the start of the last perfusion with hydralazine. Therefore, it could be that the hydralazine effects that were observed at the beginning of the last perfusion period are slightly increased because of this carryover effect. However, it is important to realize that at each dose of hydralazine (60-minute infusion), a steady-state condition in both lipolysis and blood flow was reached at the end of the infusion period; and it is very unlikely that a small carryover effect has affected the steady-state level.

To explore whether clinical treatment with hydralazine could affect adipose tissue lipolysis through changes in the interstitial hydralazine concentration, we have made a rough calculation. The maximal bolus of hydralazine that is normally administered is around 5 mg or 0.0138 mmol [27]. Assuming a total blood volume of 6 L, this could maximally yield a plasma concentration of 2.3 μmol/L. It can be estimated that a clinical dose of hydralazine results in a plasma concentration that is approximately 100-fold higher than the interstitial hydralazine dose that was achieved in the present study during perfusion with the highest hydralazine concentration. Therefore, clinical doses of hydralazine may lead to modest increases in adipose tissue lipolysis.

In previous investigations from our laboratory, we have looked at possible effects of calcium intake on body weight because a possible obesity-inducing effect of a decreased dietary calcium intake was proposed by Zemel et al [28]. They observed immediate increases of the intracellular calcium concentration in adipocytes when they increased the concentration of 1,25-OH_2D_3 in cultures of human adipocytes and in an agouti transgenic mouse model [13,14]. This increased level of Ca^{2+} in the adipocytes lowered lipolysis. Lowering dietary calcium intake leads to an increase in the serum concentration of 1,25-OH_2D_3 within a few days [29]. Putting these observations together, a low dietary calcium intake may lead to a higher body weight by changing the rate of lipolysis and lipogenesis in adipose tissue through an increase in serum 1,25-OH_2D_3 and a consequent increase in intracellular calcium in adipocytes [1]. In a number of intervention studies, it was indeed observed that a high dietary calcium intake leads to larger decreases in body fat and a smaller decrease of lean body mass during energy restriction in both humans and rodents [11,28,30-32]; but data are still controversial [33-38] in both humans and rodents. Furthermore, recent evidence from this laboratory also does not support the hypothesis mentioned above [39,40]. In these experiments, although we did observe that

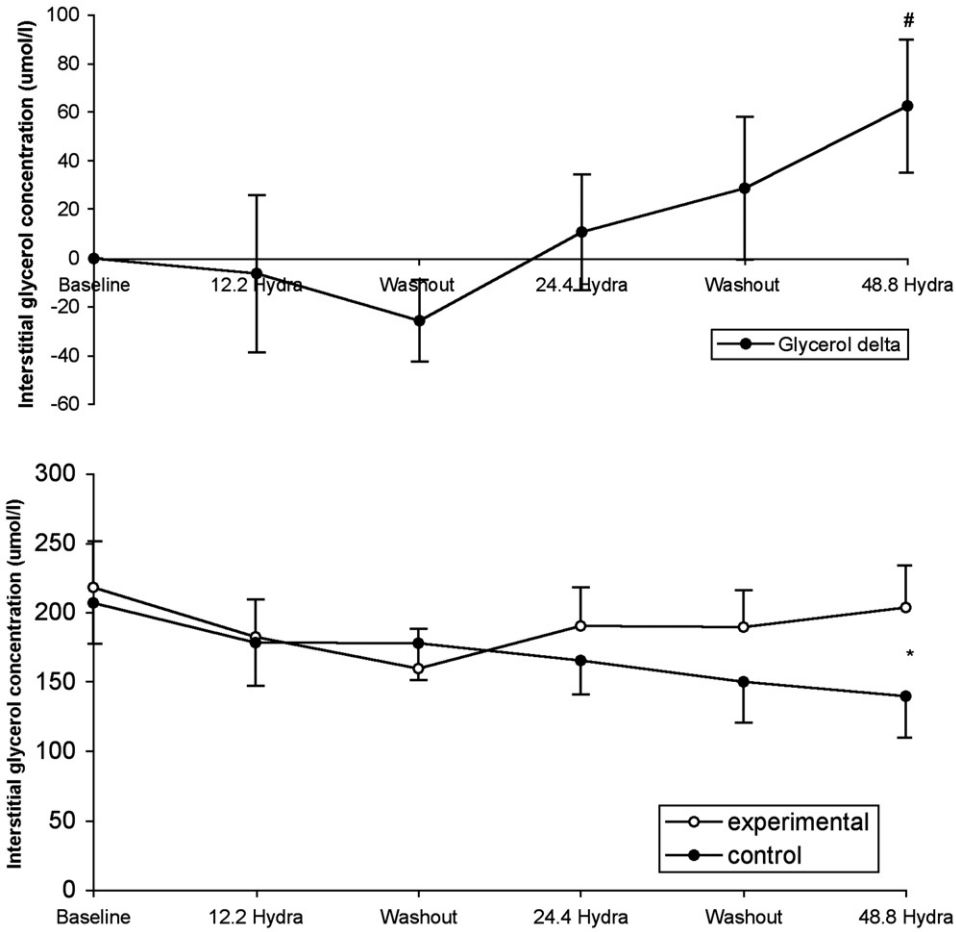

Fig. 2. Effects of hydralazine on the interstitial glycerol concentration in abdominal subcutaneous tissue. Data in bottom figure are adjusted for corresponding changes in the control probes and presented as changes from baseline. All values are mean ± SEM (N = 8). Differences induced by hydralazine were tested with repeated-measures ANOVA; a Scheffé test was used for post hoc analysis. *$P < .01$ compared with baseline; #$P < .05$ compared with baseline.

an increase in dietary calcium intake or an oral supplementation with 1,25-OH_2D_3 increased serum 1,25-OH_2D_3, no effects on fat and energy metabolism or on messenger RNA expression in adipose tissue of various genes involved in fat metabolism were found [39,40]; so we wanted to test the last part of the hypothesis (ie, lowering intracellular calcium increases lipolysis) in more detail.

Interestingly, Goossens et al [18] observed a lipolytic effect of hydralazine on skeletal muscle tissue. Their data suggested that hydralazine might also exert lipolytic effects on adipose tissue because the antilipolytic effects of angiotensin II were completely abolished when coinfused with hydralazine. A possible mechanism to explain the effects observed in this investigation is that hydralazine increases lipolysis by decreasing intracellular calcium in adipocytes. Hydralazine was shown to decrease the activity of IP_3 in rabbit aorta and pulmonary artery [19]. A decreased activity of IP_3 has been shown to reduce the calcium released from isolated rat adipocyte endoplasmatic reticulum. As mentioned above, decreases in intracellular calcium have been shown to increase lipolysis [15]. Taken together, hydralazine could increase lipolysis in adipose tissue via a decrease in intracellular calcium through a decreased activity of IP_3. Furthermore, it has been observed in rat adipocytes that angiotensin II increases intracellular calcium in rat adipocytes [41], which fits in with the findings from Goossens et al because they observed antilipolytic effects of the calcium agonist angiotensin II in adipose tissue. This could point to a possible role for the intracellular calcium concentration in regulating lipolysis in human adipose tissue. However, because in none of these experiments in which lipolytic effects of hydralazine were observed were measurements of intracellular calcium done, it remains unclear if these effects of hydralazine are directly mediated by intracellular calcium or by other mechanisms. Alternatively, there is some evidence that hydralazine may act as an oxidase inhibitor, which may lead to increased adipocyte lipolysis [42].

Data from this experiment may lead to a better understanding of the relevance of the hypothesis postulated by Zemel et al [13,14,43,44]. We have previously shown that the first step of this hypothesis (ie, increasing dietary calcium intake decreases serum 1,25-OH_2D_3) is present in humans [45,46]; and in the present investigation, we have obtained indirect evidence that the last step (ie, decreasing

intracellular calcium in adipocytes increases lipolysis) could also be valid in humans. However, because we observed no effects on whole-body level of increasing dietary calcium on energy expenditure or fat metabolism, it could be postulated that the second step (ie, changes in serum 1,25-OH_2D_3 induce changes in intracellular calcium) is not valid in humans or that increases in lipolysis do not necessarily lead to increases in fat oxidation because the demand for fatty acids hardly ever exceeds the supply, thus making lipolysis not a rate-limiting step in fat oxidation [47].

In conclusion, we have observed that perfusing adipose tissue of healthy subjects with hydralazine increased lipolysis in abdominal adipose tissue in healthy lean subjects. This effect may be mediated by alterations of the intracellular calcium concentration in adipocytes, but the present data do not provide direct evidence for this mechanism. Therefore, this mechanism warrants further investigations using an experimental setup that allows for a direct measurement of intracellular calcium.

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