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# $\alpha_1$- AND β-ADRENERGIC REGULATION OF INTRACELLULAR $Ca^{2+}$ LEVELS IN BROWN ADIPOCYTES

Mona Wilcke and Jan Nedergaard

The Wenner-Gren Institute, The Arrhenius Laboratories F3,
University of Stockholm, S-106 91 Stockholm, Sweden

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In order to monitor changes in cytosolic $Ca^{2+}$ levels, brown-fat cells were incubated with the fluorescent $Ca^{2+}$-indicator fura-2 and the fluorescence intensity ratio followed. The addition of norepinephrine led to a rapid and persistent increase in the cytosolic $Ca^{2+}$ level, which was dose-dependent with a maximal effect at about 1 μM. The response was diminished in the absence of extracellular $Ca^{2+}$ and was inhibited more efficiently by phentolamine and prazosin than by propranolol or yohimbine, indicating $\alpha_1$-adrenergic mediation. Accordingly, selective $\alpha_1$-adrenergic stimulation also increased the cytosolic $Ca^{2+}$ level. However, selective β-adrenergic stimulation, as well as the adenylate cyclase activator forskolin, were also able to increase the cytosolic $Ca^{2+}$ level in these cells to a certain extent. It was concluded that the major part of the increase in cytosolic $Ca^{2+}$ was mediated, as in other cell types, via $\alpha_1$-adrenergic receptors, but that $Ca^{2+}$ levels were also positively modulated by a cAMP-mediated process. These observations are discussed in relation to known $\alpha_1/\beta$ synergisms in brown adipose tissue. 

A series of events considered to bring about $Ca^{2+}$ mobilization in brown-fat cells have been reported. Such events include an adrenergically induced increased phosphatidyl inositol turnover (1), $PIP_2$ degradation (2, 3), $IP_3$ production (4, 5) and activation of protein kinase C (6).

Furthermore, several reports indicating regulatory effects of cytosolic $Ca^{2+}$ on different cellular parameters have appeared. Such effects include activation of $K^+$ channels (7, 8) and activation of mitochondrial glycerol-3--phosphate dehydrogenase (9-11) as well as of intramitochondrial enzymes (12, 13). Even the affinity of the uncoupling protein thermogenin for GDP may be influenced by $Ca^{2+}$ (14), and $Ca^{2+}$ may perhaps be involved in the so-called unmasking process (15). Also the presence of calmodulin in the tissue (16) and therefore of possible calmodulin-related mechanisms would indicate significant regulatory effects of changes in cytosolic $Ca^{2+}$ in these cells.

However, to date all information about adrenergically induced changes in cytosolic $Ca^{2+}$ levels in brown-fat cells have had to be deduced indirectly, e.g. from studies on $Ca^{2+}$ mobilization (17) or uptake (18), Thus, despite the persistent interest for the cytosolic $Ca^{2+}$ levels in these cells, no direct ob-

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servations of adrenergically evoked changes in the cytosolic $Ca^{2+}$ level in brown-fat cells have so far been published. This is probably partly a consequence of the technical problems caused by the very low cytosolic volume in these cells and by their high mitochondrial content. In earlier attempts to monitor the $Ca^{2+}$ levels with the fluorescent $Ca^{2+}$-indicator quin-2 and normal spectroscopic equipment we had failed to obtain clear indications of changes in cytosolic $Ca^{2+}$ (unpublished). In the present investigation we have used the fluorescent $Ca^{2+}$-indicator fura-2 (19), which has a much higher fluorescence intensity, and we have used an advanced instrumentation for the analysis of the signals.

We here present the first direct measurements of increased $Ca^{2+}$ levels in adrenergically stimulated brown-fat cells. We have analysed these results for adrenergic specificity and conclude that the major part of the increase is mediated via $\alpha_1$-adrenergic receptors, similar to the case in several other cells types. However, not unexpectedly (based on our earlier studies with $^{45}Ca^{2+}$ (18)), also β-adrenergic stimulation seems to be able to increase cytosolic $Ca^{2+}$ levels in these cells.

Some of these results have been presented in abstract form (20).

## MATERIALS AND METHODS

### Brown adipocytes

Brown adipocytes were prepared as earlier described (17) by collagenase digestion in a Krebs-Ringer phosphate buffer (with added glucose, fructose and albumin) from two adult Syrian hamsters of both sexes. The cells were routinely used on the day of preparation but were occasionally stored refrigerated until the next day; no principal differences were evident.

### Incorporation of fura-2

The cell suspension was added to 10 ml Krebs-Ringer phosphate buffer, and 3 µl of a 10 mM fura-2/AM (Calbiochem) solution dissolved in DMSO (dimethylsulfoxide) was added. The cells were incubated in a slowly shaking water bath at 37°C for 1 h. The infranatant was discarded, 10 ml of phosphate buffer added and the cells allowed to floatate for 30 min on ice. The infranatant was discarded and 10 ml of a Krebs-Ringer bicarbonate buffer (for exact composition, see (18)) was added and the cells were left for 30 min on ice to float up. A sample of the infranatant was collected and the rest was discarded. The concentrated cell suspension was counted in a Bürker chamber (about 3 million cells in about 1 ml buffer), and the cell suspension was stored on ice.

Parallel incubations of cells, without the fura-2/AM addition, were run as controls as described.

### Determination of intracellular $Ca^{2+}$ levels

A sample of cells (normally 30 µl), corresponding to about 100 000 cells, was added to a cuvette containing 1.5 ml bicarbonate buffer. The absorbance was followed in a Sigma ZWS II dual wavelength spectrofluorometer in a thermostated cuvette (37°C). The emission caused by excitation at alternating wavelengths of 355 nm and 395 nm was monitored and the ratio between these emissions was continuously recorded. A yellow emission cut-off filter (KV 470),

a time filter of 1 sec and a recording speed of 50 sec/cm was used. With this concentration of cells, oxygen supply is sufficient for several min of norepinephrine-stimulated respiration.

Routinely, each series of experiments was started and finished with controls of the effect of the addition of 1 μM norepinephrine. The results presented here have been observed at least in duplicate in 2-3 different cell preparations.

Materials

Fura-2/AM and fura-2 (free acid) were both obtained from Calbiochem and dissolved in DMSO. Norepinephrine (bitartrate salt) was obtained from Sigma. It was dissolved in distilled water immediately before it was added to the incubations. Propranolol and phentolamine (Sigma) were both dissolved in water to 10 mM stock solution. Prazosin and yohimbine (Sigma) were both dissolved in 95 % ethanol. SZL-49 (1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-bicyclo(2.2.2)-octa-2,5-dienylcarbonyl)piperazine was a kind gift from Dr J. W. Kusiak, NIH, Baltimore, MD, and was dissolved in DMSO. Forskolin (Sigma) was dissolved to 10 mM in DMSO. NP-40 was dissolved in water, and ionomycin (Calbiochem) was dissolved to 10 mM in DMSO.

## RESULTS

### Effect of norepinephrine on $Ca^{2+}$ levels in brown-fat cells

For these experiments, isolated hamster brown-fat cells were preincubated with fura-2/AM, after which aliquots of the preincubated cell suspension were followed in a dual wavelength spectrofluorometer. The ratio between the fluorescence intensity evoked by excitation at 355 nm and 395 nm was followed and is directly depicted here.

As seen in Fig. 1A, the addition of norepinephrine to the fura-loaded cells resulted in a rapid increase in fluorescence intensity, indicating an

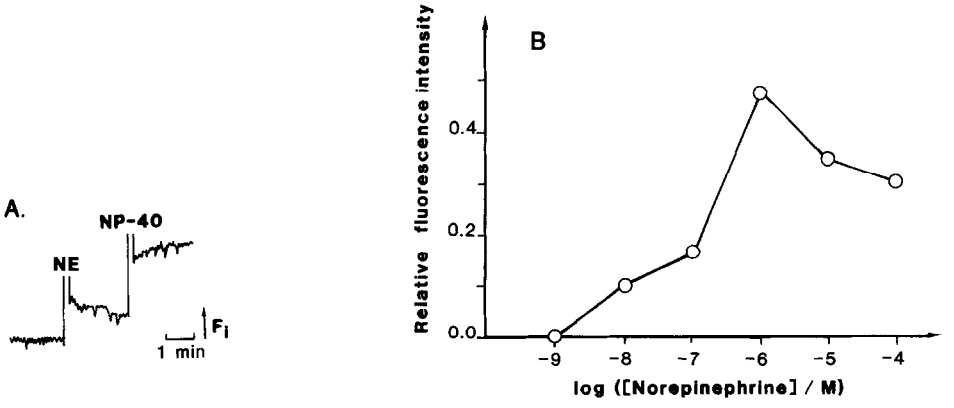


Fig. 1. Effect of norepinephrine on the level of cytosolic $Ca^{2+}$ in isolated brown adipocytes. (A) About 100 000 fura-2-loaded brown-fat cells were incubated as described in Methods. Norepinephrine (NE) was added to 10 μM and NP-40 to 10 μM. The $F_i$ (fluorescence intensity ratio) monitored is the ratio of the emission intensities evoked by excitation at 355 and 395 nm. (B) Dose-response curve for the effect of norepinephrine on $F_i$. The experiments were performed as depicted in Fig. 1A. The points are means of at least 2 determinations in one cell preparation. Each trace was internally calibrated by the subsequent addition of NP-40, and the values given are the ratios between the effect of norepinephrine and the total increase observed after the subsequent addition of NP-40.

increase in the intracellular level of $Ca^{2+}$. The subsequent addition of the non-fluorescent detergent NP-40 resulted in a further increase in fluorescence intensity, indicating that the $Ca^{2+}$ level observed after norepinephrine addition was below the saturation level for the fura-2 in the cells.

This increase in fluorescence intensity was dose-dependent, and maximal effects of norepinephrine were observed at 1 μM; with higher norepinephrine concentrations there was a tendency to a lower response (Fig. 1B).

Control experiments

As effects of norepinephrine on intracellular $Ca^{2+}$ levels in brown-fat cells, monitored with the fura-2 technique, have not earlier been described, it was of importance to perform a series of control experiments to validate that the effects observed represented true increases in intracellular $Ca^{2+}$ levels.

In the Krebs-Ringer bicarbonate buffer alone, there was no effect on fluorescence intensity ratio of the addition of norepinephrine, NP-40 or the $Ca^{2+}$ chelator EGTA (not shown). Similarly, in cells treated in parallel with those investigated here, but preincubated without fura-2/AM, there was practically no effect of norepinephrine, NP-40 or EGTA on the fluorescence intensity ratio. In incubations with the (cell-free) infranatant saved after the final wash after the fura-2 AM preincubation, norepinephrine and NP-40 were practically without effect, whereas EGTA led to a decrease in fluorescence intensity ratio (not shown). This indicates that an appreciable amount of hydrolyzed fura-2/AM had leaked from the cells and that EGTA could not be used for zero-point titrations in the cells. This caused, however, no problem for the interpretation of the changes in the cell-associated signals.

In further control experiments, full excitation/emission spectra were taken (300 - 435 nm excitation wavelength). It was clear from these experiments that the cells had a very high autofluorescence (probably due to their high content of mitochondria) which always dominated the spectra. Despite the high autofluorescence, qualitative effects on the spectra of the addition of norepinephrine, NP-40 and EGTA could be observed which were in accordance with the results obtained with the fluorescence intensity ratio method (not shown). As pointed out by Grynkiewicz et al. (19), a prerequisite for using the fluorescence intensity ratio method to directly calculate absolute values of intracellular $Ca^{2+}$ concentration, is that the autofluorescence is negligible. Thus, it was concluded that it was not possible to use this method to directly obtain absolute values for $Ca^{2+}$ concentration in these cells, and the changes observed here should be considered to be only semiquantitative. However, we concluded in preliminary experiments, comparing the fluorescence intensity obtained after excitation at 355 nm alone, as the difference 355-395 nm, or as the ratio 355/395, that the ratio parameter was the most stable. It should be

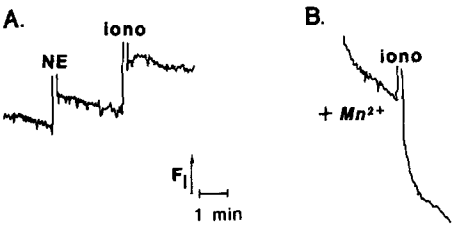


Fig. 2. Effects of ionomycin and $Mn^{2+}$ on fura-2 fluorescence intensity ratio in brown-fat cells. Cells were incubated as in Fig. 1, and 30 μM norepinephrine (NE) and 30 μM ionomycin (iono) were added where indicated. In (B), 2 mM $MnCl_2$ was added to the buffer with the cells, 2 min before the addition of ionomycin.

noted that this parameter is not directly proportional to cytosolic $Ca^{2+}$ levels but rather is logarithmic in nature.

In other control experiments, we also tested the effect of the $Ca^{2+}$ ionophore ionomycin. As seen, when the intracellular level of $Ca^{2+}$ was increased due to the addition of this ionophore, there was a clear increase in fluorescence intensity ratio, equivalent to that observed after the unspecific permeabilization of the cells with the detergent NP-40 (Fig. 2A).

$Mn^{2+}$ quenches fura-2 fluorescence (19), and $Mn^{2+}$ is also carried by ionomycin over the cell membrane. As seen in Fig. 2B, addition of ionomycin in the presence of $Mn^{2+}$ led to a decrease in fluorescence, indicating that the $Mn^{2+}$, when it entered the cytosol, quenched the basal fluorescence caused by intracellular $Ca^{2+}$ interacting with intracellular fura-2.

Thus, all control experiments indicated that the signal monitored represented a reliable estimate of changes in intracellular $Ca^{2+}$ levels.

## The source of $Ca^{2+}$

In order to investigate the source of the $Ca^{2+}$ for the increased cytosolic $Ca^{2+}$ level, we investigated the effects of elimination of extracellular $Ca^{2+}$ on the norepinephrine-stimulated increase in cytosolic $Ca^{2+}$. This was done either by addition of an excess of the $Ca^{2+}$ chelator EGTA or by the use of a bicarbonate buffer mixed without the addition of $CaCl_2$.

In both these low-$Ca^{2+}$ conditions, a response to norepinephrine was still obtained, but it was both smaller in magnitude and shorter in duration than that observed with normal extracellular $Ca^{2+}$ (Fig. 3). No consistent difference between the two conditions of low $Ca^{2+}$ was observed.

It was concluded that a persistent and full norepinephrine response demands the presence of extracellular $Ca^{2+}$ but that a small transitory increase in cytosolic $Ca^{2+}$ may still be observed in its absence, demonstrating $Ca^{2+}$ release from intracellular stores. These experiments may indicate that adrenergic stimulation of the cells leads to the opening of $Ca^{2+}$ channels in the cell membrane and that a putative ensuing influx of $Ca^{2+}$ is of significance for the

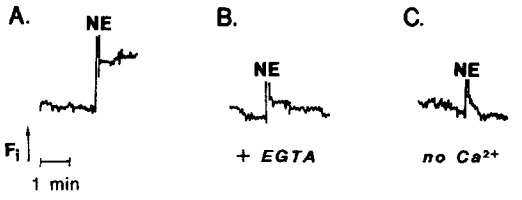


Fig. 3. Effect of extracellular $Ca^{2+}$ depletion on the norepinephrine-stimulated increase in the level of cytosolic $Ca^{2+}$. Cells were incubated as in Fig. 1 (trace A), or in the same buffer to which 5 mM EGTA was added 2 min before the addition of 1 μM norepinephrine (NE) (trace B), or for 2 min in a Krebs-Ringer bicarbonate buffer as described above into which no $CaCl_2$ was added (trace C). (Normal $Ca^{2+}$ concentration in the Krebs-Ringer buffer is 2.5 mM and the $Mg^{2+}$ concentration is 1.2 mM).

maintenance of the increase in cytosolic $Ca^{2+}$ levels. However, such an adrenergically-stimulated increase in $Ca^{2+}$ influx has not been observable in experiments monitoring the uptake of $^{45}Ca^{2+}$ (18). Alternatively, as a constant (passive) influx of $Ca^{2+}$ occurs (18), probably due to the high concentration gradient and the negative membrane potential, it may be envisaged that in the absence of this influx the intracellular mobilization mechanisms may not possess the force to counteract resequestration into the cellular $Ca^{2+}$ pools. Finally, at least when the cells are incubated with EGTA, the extracellular $Ca^{2+}$ level may be so low that the medium constitutes a $Ca^{2+}$ sink; the passive $Ca^{2+}$ flux is then outward, thus actively counteracting the increased mobilization of intracellular $Ca^{2+}$ (8, 17).

Determination of adrenergic receptor subtype involved

In order to investigate the adrenergic receptor type involved, the ability of the α- and β-antagonists phentolamine and propranolol to inhibit the norepinephrine-induced increase in intracellular $Ca^{2+}$ level was studied. These agents had in themselves no effect on the signal.

It is seen in Fig. 4A that the α-adrenergic antagonist phentolamine was more efficient than the β-adrenergic antagonist propranolol in inhibiting the norepinephrine-induced increase in cytosolic $Ca^{2+}$. It was therefore concluded that the response was primarily α-adrenergically mediated.

For further subtype differentiation, the antagonists prazosin ($\alpha_1$) and yohimbine ($\alpha_2$) were used. As expected, the $\alpha_1$-antagonist prazosin possessed an appreciable amount of autofluorescence. However, it was still possible to perform these experiments, as the autofluorescence could be backed off before the addition of norepinephrine. As seen in Fig. 4B, prazosin was more efficient than yohimbine in inhibiting the norepinephrine-induced response. This indicated that the response was mainly mediated via $\alpha_1$-receptors. Also the non-fluorescent $\alpha_1$-antagonist SZL-49 (21) was very efficient in abolishing the norepinephrine response (Fig. 4B).

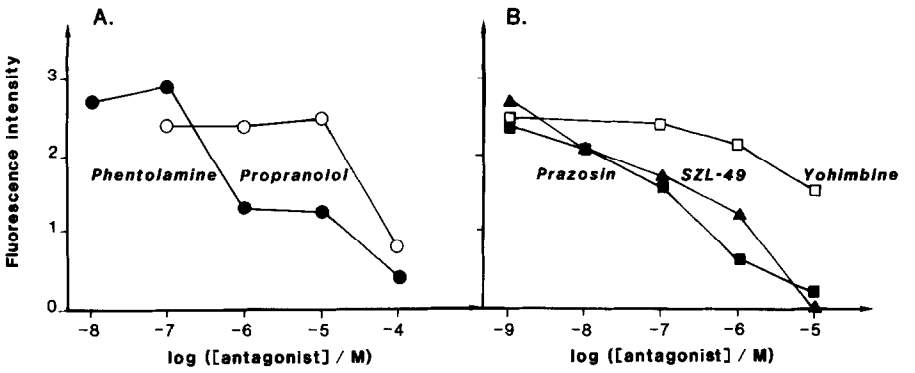


Fig. 4. Effects of adrenergic antagonists on the norepinephrine-induced increase in cytosolic $Ca^{2+}$. Cells were incubated principally as in Fig. 1, but 2 min after the addition of cells, the indicated concentrations of antagonist were added. After a further 1 min, 1 µM norepinephrine was added, and the resulting increase in fluorescence intensity ratio measured and plotted. Note that as the parameter depicted (fluorescence intensity ratio) is not a linear function of $Ca^{2+}$ concentration, the curve shape is not expected to follow Michaelis-Menten-like kinetics. The results in A and B are from different cell preparations.

From the inhibition results shown in Fig. 4, it would be concluded that the increase in cytosolic $Ca^{2+}$ was fully mediated via $\alpha_1$-adrenergic receptors (as expected from results in other systems) (22, 23). However, we also tested whether stimulation of the cells by selective $\alpha_1$- or β-adrenergic agents could affect the cytosolic $Ca^{2+}$ levels.

Addition of phenylephrine in the presence of propranolol, earlier demonstrated to be a specific $\alpha_1$-adrenergic stimulus (24), led to a marked increase in the cytosolic $Ca^{2+}$ level, in accordance with the results of the inhibitor experiments above (Fig. 5A).

However, selective β-adrenergic stimulation, performed by addition of isoprenaline in the presence of prazosin according to Mohell et al. (24), generally led to a small but clearly observable increase in fluorescence intensity (Fig. 5B). This would indicate that also stimulation of β-adrenergic receptors could increase cytosolic $Ca^{2+}$ levels, and thus this increase should be mediated via an activation of adenylate cyclase. In accordance with this, forskolin, the


Fig. 5. Effects of selective $\alpha_1$- or β-adrenergic stimulation on cytosolic $Ca^{2+}$ levels in brown-fat cells. In trace A, cells were incubated for 1 min with 5 µM propranolol (PRO) and 10 µM phenylephrine (PHE) was then added. In trace B, cells were incubated with 5 µM prazosin (PRA) and 10 µM isoprenaline (ISO) was then added. In trace C, 10 µM forskolin was added directly.

direct stimulator of adenylate cyclase, could in itself also lead to a small but clear increase in cytosolic $Ca^{2+}$ (Fig. 5C).

It was therefore concluded that although the major part of the norepinephrine-stimulated increase in cytosolic $Ca^{2+}$ level was mediated via $\alpha_1$-adrenergic receptors, a pathway involving stimulation of β-receptors and adenylate cyclase was apparently also able to increase the cytosolic $Ca^{2+}$ level.

## DISCUSSION

The experiments described here represent the first direct observations that an increase in cytosolic $Ca^{2+}$ occurs in brown-fat cells as an effect of adrenergic stimulation.

Due to the high autofluorescence of the cells, it was not possible to use the fluorescence intensity ratio method to directly obtain a value for the cytosolic $Ca^{2+}$ concentration (19). However, based on experiments such as those depicted in Fig. 2, on a $K_d$ of fura-2 for $Ca^{2+}$ of 135 nM (19), and on approximate Michaelis-Menten kinetics, a first estimate of basal $Ca^{2+}$ concentration in brown-fat cells would be about 200 nM. As an effect of adrenergic stimulation, the $Ca^{2+}$ concentration would increase to about 600 nM. These values are similar to those which e.g. have been found to be equilibrium values for $Ca^{2+}$ uptake into brown-fat mitochondria (11, 25).

It was clear from inhibition studies that the major part of the $Ca^{2+}$ response was mediated via $\alpha_1$-adrenergic receptors, as expected both from indirect studies in these cells (see Introduction) and from what has been concluded from observations in other tissues (22, 23).

However, we observed that in these cells an increase (albeit small) in cytosolic $Ca^{2+}$ could also be induced via β-adrenergic receptors. Such an effect of β-adrenergic stimulation, although rare, is not unique, and has e.g. been observed in parotid cells (26). The β-induced increase in cytosolic $Ca^{2+}$ level is also in very good agreement with our recent observation that β-adrenergic stimulation can influence the $Ca^{2+}$ metabolism of these cells, probably via a decrease in the mitochondrial membrane potential and an ensuing decrease in mitochondrial $Ca^{2+}$ uptake (18). In a steady-state system, this would be expected to lead to an increase in cytosolic $Ca^{2+}$ levels, as indeed observed here.

Further, a β-mediated inhibition of mitochondrial $Ca^{2+}$ sequestration in connection with an $\alpha_1$-stimulated $Ca^{2+}$ mobilization would together have additive or even synergistic effects on the resulting cytosolic $Ca^{2+}$ level. It is therefore an interesting question whether some of the reported α/β synergisms in this tissue or in these cells (27-32) could have their mechanistic explanation in the phenomena reported here.

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