α_1-Adrenergic stimulation of Cl^- efflux in isolated brown adipocytes

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Unidirectional $^{36}Cl^-$ efflux from preloaded isolated brown adipocytes was studied. A norepinephrine-stimulated $^{36}Cl^-$ efflux pathway was found which approximately doubled the rate of $^{36}Cl^-$ efflux from the cells. The response to norepinephrine was fully inhibited by the α_1-adrenergic antagonist prazosin, but was unaffected by the β-adrenergic antagonist propranolol, showing that norepinephrine stimulated the $^{36}Cl^-$ efflux pathway via the α_1-adrenoceptor. The stimulation of $^{36}Cl^-$ efflux could not be mimicked by the Ca^{2+} ionophore A23187, indicating that the effect was not mediated by elevation of the intracellular Ca^{2+} level. It is concluded that brown fat cells possess a specific mechanism for α_1-adrenergic stimulation of Cl^- efflux. The possibility is discussed that this Cl^- efflux pathway could be the basis for the early α-adrenergic depolarization seen in brown fat cells.

Brown adipose tissue; Cl^- flux; Membrane depolarization; Nonshivering thermogenesis; Prazosin; (Hamster)

1. INTRODUCTION

Physiological stimulation of brown adipose tissue occurs via the release of norepinephrine from the sympathetic nervous system. Norepinephrine binds to both α_1- and β-adrenergic receptors present in the brown adipocyte plasma membrane [1,2]. Stimulation of each of these receptors finally elicits an increased rate of oxygen consumption (= thermogenesis) but the biochemical processes behind this increase are functionally distinct for the responses mediated via the β- and the α_1-receptors [3–6].

Our laboratory has been investigating the adrenergic regulation of ionic movements across the plasma membrane of the isolated brown adipocyte. We have found that α_1-adrenergic stimulation leads to Ca^{2+} mobilization from intracellular stores and, via an elevation of cytosolic Ca^{2+} levels, to an activation of an apamin-sensitive Ca^{2+}-dependent K^+ channel which mediates K^+ efflux [7–10]. Ca^{2+} entry is not stimulated α_1-adrenergically but is inhibited by β-adrenergic stimulation [11]. Norepinephrine also induces an increased permeability of the brown adipocyte plasma membrane to Na^+ ions; this response is β-adrenergic and is mediated via an elevated cAMP level within the cells [12].

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Cl^-, which was earlier believed to move only passively across the plasma membrane and follow a Nernst distribution, has now been found to deviate from this behaviour in many cell types [13]. A possible interaction of intracellular Cl^- with the hormone-receptor coupled G-proteins [14] implicates regulatory effects of changes in intracellular Cl^- levels.

We have therefore investigated the possible presence of adrenergically regulated Cl^- fluxes in isolated brown fat cells. We here demonstrate the presence of an α_1-stimulated Cl^- transport pathway which mediates Cl^- efflux from the isolated brown adipocyte. The possibility that this Cl^- efflux could be responsible for the initial rapid α-adrenergic depolarization, earlier observed in brown fat cells, is discussed.

2. MATERIALS AND METHODS

2.1. *Cell preparation*

Brown adipocytes were isolated by collagenase digestion of brown adipose tissue of adult Syrian hamsters (*Mesocricetus auratus*) as described previously [8]. The cells were finally washed and suspended in a Krebs-Ringer bicarbonate-Hepes buffer (KRB-Hepes buffer) of the following composition (in mM): Na^+ 145.5; K^+ 6.0; Mg^{2+} 1.2; Ca^{2+} 2.5; Cl^- 130.0; HCO_3^- 25.5; $HPO_4^{2-}/H_2PO_4^-$ 1.2; SO_4^{2-} 1.2; Hepes 15; glucose 10; fructose 10; containing 2% fatty acid-free bovine serum albumin. The buffer was equilibrated with O_2/CO_2 (95%/5%); final pH was 7.4 at 37°C. Freshly isolated cells were used throughout. Some seasonal variation was noted in the adrenergic ionic response of the hamster cells but this did not seem to occur in some control rat cell preparations. Cell viability was typically more than 95% as assessed by permeability to Alcian blue.

The viability of the cells was determined by measuring their respiratory response to 1 μM norepinephrine at 37°C in an oxygen chamber fitted with a Yellow Springs Instruments 4004 Clark-type oxygen probe, under conditions which were identical to those in the $^{36}Cl^-$ efflux experiments, except that the buffer was equilibrated with

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air/CO_2 (95%/5%). The basal respiration of the cells was approximately 45 fmol $O \cdot min^{-1} \cdot cell^{-1}$ and the increase in oxygen consumption evoked by norepinephrine was routinely 10–15-fold, leading to a final respiratory rate of about 650 fmol $O \cdot min^{-1} \cdot cell^{-1}$. The specific α_1-adrenergic respiratory response was measured with phenylephrine (50 μM) in the presence of propranolol (10 μM) [6], giving typically a 2-fold prazosin-sensitive increase of the basal respiration.

2.2. *$^{36}Cl^-$ efflux*

The brown adipocytes (3.5×10^6/ml) were loaded with $^{36}Cl^-$ for 1 h at 37°C in 2 ml KRB-Hepes buffer containing 7 μCi/ml (54 μCi/mmol) of $^{36}Cl^-$, under a water-saturated gas stream of O_2/CO_2 (95%/5%). The KRB-Hepes buffer was adjusted so that the ionic concentration after the addition of $Na^{36}Cl$ remained unchanged.

The assay of efflux was started by diluting 50 μl aliquots of the cells 20 times into $^{36}Cl^-$-free KRB-Hepes buffer (950 μl). The $^{36}Cl^-$ remaining in the cells was then measured after recovery, as described below. Additions of adrenergic agonists, antagonists and other agents investigated were made to the efflux buffer, prior to the addition of the cells.

2.3. *Cell recovery*

Cells were recovered with the oil flotation technique described by Gliemann et al. [15] with several modifications.

The cell suspension (1 ml) was quickly mixed into the upper phase of a tube containing 1 ml of ice-cold phthalate oil mixture (dibutylphthalate, density 1.045/bis(2-ethylhexyl)phthalate, density 0.985; 3:5 v/v) overlaid by 1 ml of ice-cold KRB-Hepes buffer containing 1.5 μCi/ml L-[^{3}H]glucose as extracellular marker. (The density of the oil mixture used was intermediate between that of the buffer and the brown adipocytes.) The tube was immediately centrifuged for 50 s at 1000 × *g* on a Hettich Universal centrifuge and the floating cell cake thus obtained was removed for scintillation counting from the oil surface, together with approximately 0.6 ml of the oil mixture. ^{3}H and ^{36}Cl associated with the cells were measured on a Beckman LS 3801 liquid scintillation counter using a dual label quench curve, set up with the phthalate oil mixture as the quenching agent. Results are expressed in terms of the amount of $^{36}Cl^-$ remaining within the cells, after correction for trapped buffer volume (the L-[^{3}H]glucose space) in the cell cake. For the hormone-stimulated effluxes, the assay was always performed for 1 min and the $^{36}Cl^-$ content of the control cells was set to 100%. The effect of the hormone was then calculated as the % reduction in the $^{36}Cl^-$ content (i.e. the increase in $^{36}Cl^-$ efflux), caused by the hormone.

2.4. *Materials*

$Na^{36}Cl$ was from ICN Radiochemicals. L-[^{3}H]Glucose was from New England Nuclear. Collagenase, norepinephrine bitartrate, propranolol hydrochloride, forskolin and calcium ionophore A23187 were from Sigma. Prazosin hydrochloride was a generous gift from Pfizer. All other chemicals were of analytical grade.

Forskolin and A23187 were prepared as 200-fold stock solutions in dimethyl sulphoxide (DMSO). Prazosin was prepared daily as a 200-fold stock solution in 10% DMSO. All controls received equivalent volume additions of the respective solvents. The final concentration of DMSO was never more than 0.5%; this amount had no apparent effect on the transport processes investigated (not shown). Norepinephrine and propranolol were dissolved in double distilled water immediately before use.

3. RESULTS

Preincubation (loading) of brown fat cells with $^{36}Cl^-$ for 1 h led typically to a cell-associated isotope level corresponding to 40–45 fmol $^{36}Cl^-$/cell. After the preincubation, the isotope content of the cells remained constant over the whole period of the experiment (not shown).

Unidirectional efflux of $^{36}Cl^-$ from the preloaded brown adipocytes was evoked by transfer of the cells to a 20-fold greater volume of an identical buffer with unlabelled Cl^-. In order to investigate whether an adrenergically stimulated Cl^- efflux existed in isolated brown adipocytes, we measured this unidirectional efflux of $^{36}Cl^-$ from preloaded cells in the presence and absence of 1 μM norepinephrine. This concentration of norepinephrine has been shown to induce maximal α- and β-adrenergic respiratory responses in brown adipocytes [6,16]. As shown in fig.1, addition of norepinephrine to the efflux medium induced an accelerated rate of $^{36}Cl^-$ efflux from the cells. The rate was practically doubled, with the half-life being reduced from 1.8 min to 1.0 min (k = 0.38 min^{-1} and 0.66 min^{-1}, respectively).

These results demonstrate the existence of an adrenergically stimulated Cl^- transport pathway in isolated brown adipocytes.

The nature of the adrenoceptor regulating the norepinephrine-stimulated Cl^- efflux was studied with adrenergic antagonists. As shown in fig.2A, the presence of the β-adrenergic antagonist propranolol (40 μM) did not influence the norepinephrine-induced increase of $^{36}Cl^-$ efflux. On the other hand, the selec-

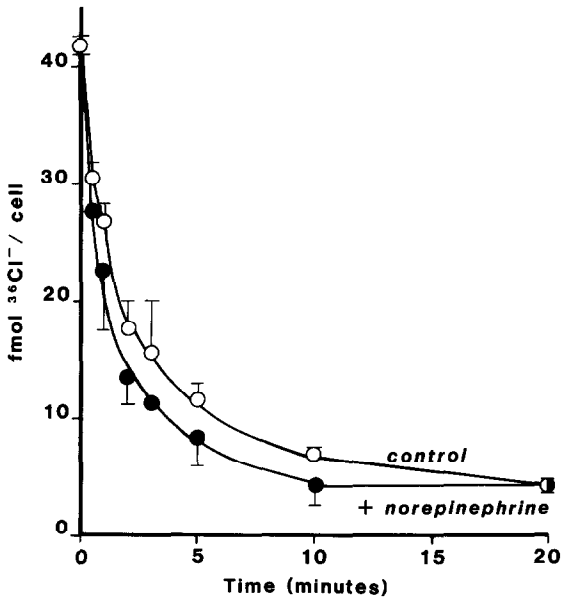

Fig.1. Time course for $^{36}Cl^-$ efflux from control and norepinephrine-stimulated brown adipocytes. Cells were preloaded with $^{36}Cl^-$ for 1 h at 37°C and at zero time incubated in unlabelled efflux medium for the indicated times in the absence (○) or presence (●) of 1 μM norepinephrine. Values are the means ± SE of two experiments; absence of error bar indicates that SE was smaller than the symbol. Two-way analysis of variance with duplicates (all time points included) indicated a significant effect of norepinephrine ($P < 0.01$). The final value is close to the one expected, corresponding to the new isotopic equilibrium.

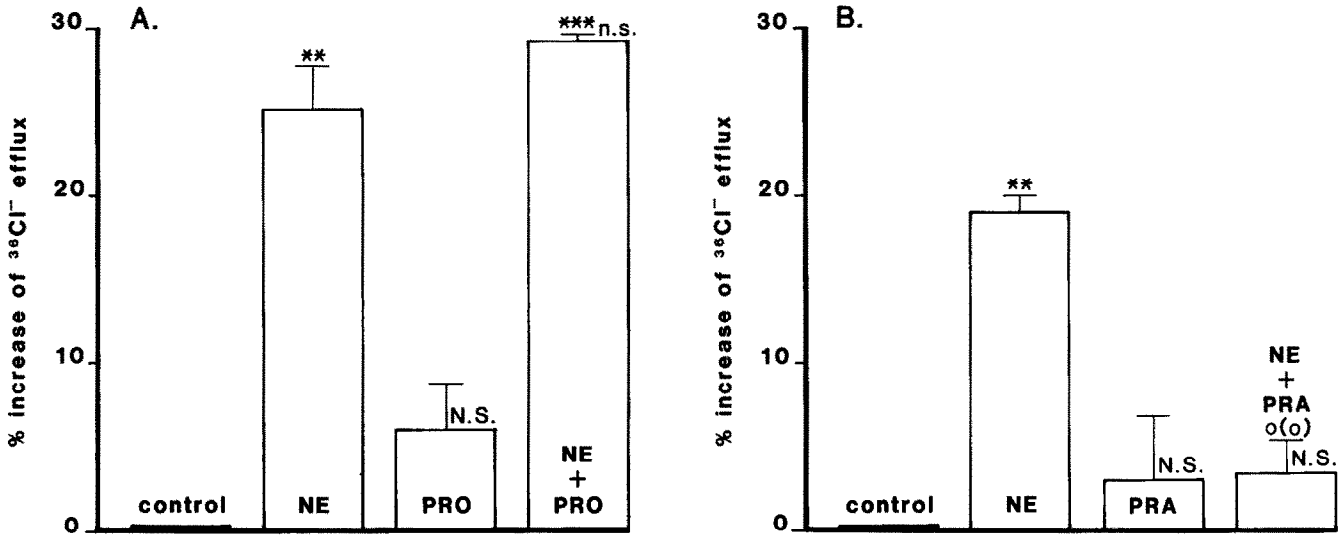

Fig.2. Effect of adrenergic antagonists on the norepinephrine-stimulated increase in $^{36}Cl^-$ efflux. Cells were preloaded with $^{36}Cl^-$ as described in section 2. Effluxes were run for 1 min with the following additions: (A) NE, 1 μM norepinephrine; PRO, 40 μM propranolol. (B) NE, 1 μM norepinephrine; PRA, 5 μM prazosin. Values represent the % increase in $^{36}Cl^-$ loss, as compared to the controls, and are the means ± SE from (A) 4 and (B) 3 cell preparations, each performed in triplicate. Statistical analysis: N.S., ** and *** indicate effects compared to control ($P > 0.05$, $P < 0.01$ and $P < 0.001$, respectively); n.s. and o(o) indicate effects compared to norepinephrine ($P > 0.05$ and $P < 0.02$, respectively); Student's paired t-test.

tive α_1-adrenergic antagonist prazosin (5 μM) completely abolished the effect of norepinephrine (fig.2B). These concentrations of antagonists have been shown to selectively inhibit the α_1- and β-adrenergic responses of brown adipocytes stimulated with 1 μM norepinephrine [6]. The addition of either antagonist in the absence of norepinephrine led to no significant alteration of the $^{36}Cl^-$ efflux rate (fig.2).

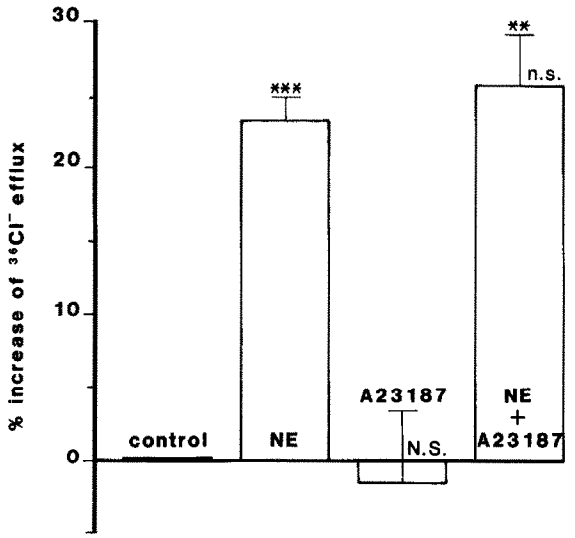

Fig.3. Effect of Ca^{2+} ionophore A23187 on $^{36}Cl^-$ efflux from isolated brown adipocytes. Cells were preloaded with $^{36}Cl^-$ as described in section 2. Effluxes were run for 1 min in the presence of 38 μM A23187 and 1 μM norepinephrine (NE), as indicated. Values represent the % increase in $^{36}Cl^-$ loss, as compared to the controls, and are the means ± SE from 4 different cell preparations, each performed in triplicate. Statistical analysis: N.S., ** and *** indicate effects compared to control cells ($P > 0.05$, $P < 0.01$ and $P < 0.001$, respectively); n.s. ($P > 0.05$) indicates lack of effect compared to norepinephrine; Student's paired t-test.

The adenylate cyclase activator forskolin [16], at a concentration which has been shown to maximally stimulate respiration in brown adipocytes (50 μM) [12], was unable to induce an increased rate of $^{36}Cl^-$ efflux from the cells (not shown), confirming that a cyclic-AMP mediated process is not involved in the response to norepinephrine.

It was concluded that the effect of norepinephrine in inducing an elevated rate of Cl^- efflux across the plasma membrane of isolated brown adipocytes is mediated via the α_1-adrenoceptor.

It has earlier been shown that isolated brown adipocytes respond to α_1-adrenergic stimulation by the production of inositol trisphosphate (IP_3) [18,19], and by mobilization of Ca^{2+} from intracellular stores [8], and by an increased cytosolic Ca^{2+} level [10]. A possible role of Ca^{2+} in the adrenergic stimulation of Cl^- efflux was therefore investigated by artificially elevating the cytosolic Ca^{2+} levels with the Ca^{2+} ionophore A23187. However, at a concentration which has been shown to quantitatively reproduce the norepinephrine-induced, Ca^{2+}-mediated $^{86}Rb^+$ efflux in isolated brown adipocytes under essentially the same experimental conditions [9], A23187 could not mimic the norepinephrine-stimulated $^{36}Cl^-$ efflux (fig.3).

These results indicate that the α-adrenergically-mediated Cl^- efflux is not dependent on an elevated free Ca^{2+} concentration in the cytosol.

4. DISCUSSION

We have here for the first time been able to follow Cl^- fluxes in isolated brown adipocytes. We have observed the existence in these adipocytes of an α_1-adrenergically stimulated Cl^- efflux pathway. Evidence from the experiments with adrenergic antagonists indicated that this increased Cl^- efflux was mediated by α_1-adrenergic receptors. The increase in $^{36}Cl^-$ efflux could not be mimicked by incubation with A23187, indicating that an increased intracellular Ca^{2+} level does not mediate the α_1-adrenergic response. This would tend to suggest that the α_1-response was mediated through the diacylglycerol limb of the phosphoinositide cascade.

Based on an estimated resting potential in these cells of −60 mV at 37°C [16,20], the intracellular equilibrium concentration of Cl^- can be calculated to be approximately 13 mM. The cytosolic volume of the brown adipocyte has been estimated to be about 2 pl [7], thus an intracellular concentration of about 20 mM Cl^- can be calculated, based on the observed cell-associated Cl^- level of 40–45 fmol/cell. Thus the intracellular Cl^- levels are probably somewhat above the equilibrium value, indicating the possibility that Cl^- is not passively distributed across the cell membrane.

Earlier electrophysiological experiments have shown that binding of norepinephrine causes a depolarization of the brown adipocyte plasma membrane [20,21]. Further analysis has indicated that norepinephrine administration or nerve stimulation can induce two temporarily distinct membrane depolarizations: a rapid α-adrenergically mediated depolarization of about 10 mV, which is followed by a repolarization before the onset of a slower depolarization, dependent upon β-adrenergic stimulation [22–24]. The nature of the conductances involved in these depolarizations has not been clarified.

Based on the results presented here, it could be suggested that the small, initial α-adrenergic membrane depolarization could be accounted for by the increase in Cl^- efflux here demonstrated. However, the conductive nature of the α_1-adrenergically induced Cl^- fluxes demonstrated here will have to be established.

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