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β_3-Adrenergic stimulation and insulin inhibition of non-selective cation channels in white adipocytes of the rat

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Abstract

Single-channel currents were recorded from the plasma membrane of white adipocytes of 6–8-week-old male Sprague–Dawley rats. In outside-out patches (high K^+, no Ca^{2+} in pipette), a voltage-dependent K-channel (delayed rectifier) with a single-channel conductance (γ) of 16 pS (24°C) in modified Ringer's was active at a density of 0.5/μm^2. It was blocked by TEA (IC_{50} = 1.5 mM). A Ca^{2+}-activated non-selective cation channel (NSC-channel) appeared at a mean density of 1/μm^2 in inside-out patches ($[Ca^{2+}]_i$ = 1.2 mM). γ was 28 pS (24°C). The NSC showed weak voltage dependence and was blocked by mefenamic acid and by internal ATP. In the cell-attached mode spontaneous activity could be blocked reversibly by 100 nM insulin. Noradrenaline (NA, 100 nM) induced a flickering activity of the NSC-channels. Isoproterenol (100 nM) caused activity of the NSC-channel as well. After 1 μM propranolol even 1 μM NA did not induce any activity. The α-antagonist phentolamine had no effect on isoproterenol- or on NA-induced currents. The β_3-agonists BRL 37344 and BRL 35135A induced activity of the NSC-channel at 100 nM as well. We conclude that white adipocytes express ion channels which are comparable to those in brown adipocytes and that β-receptor activation opens NSC-channels thus allowing for Na^+ entry into white adipocytes.

Keywords: Non-selective cation channel; Adipose tissue; Adrenaline; Insulin; BRL 37344; Isoproterenol

1. Introduction

White adipose tissue (WAT) is the main energy store of the body. Lipolysis inside the adipocyte is activated by hormones, such as noradrenaline (NA), which acts via different types of β-receptors. To a large extent, the β-receptors of adipocytes seem to be of the β_3-type [1,2]. They were shown to be selectively sensitive to a special class of β_3 agonists which are available either as esters or as their free acids [3]. NA stimulates the lipolysis via protein kinase A, at low concentrations using the β_1-pathway, at higher concentrations the β_3-pathway predominates [2].

White adipocytes respond to stimulation by adrenaline with a biphasic membrane response. They show an α-mediated hyperpolarisation followed by a slow depolarisation that is β-mediated and that is inhi-

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bited by insulin [4]. In brown adipocytes, an early depolarisation is described additionally [5]. This fast depolarisation is due to the activity of Ca^{2+}-activated Cl-channels [6]. A slow, long-lasting depolarisation is related to an increased activity of non-selective cation channels allowing for Na^+ entry, and the interrupting hyperpolarisation is caused by voltage-dependent potassium channels of the delayed rectifier type [7–11].

Little is known about the ion channels and their signalling in white adipocytes. Ramirez-Ponce et al. [12–14] and Lee and Pappone [15] performed whole-cell and perforated-patch experiments which point to the existence of a dominating 4-aminopyridine (4-AP)-sensitive K-channel of the delayed rectifier type in unstimulated cells. Ramirez-Ponce et al. [12,13] measured a resting potential of -34 ± 9 mV (37°C). They showed that the membrane potential at the end of a square-shaped current pulse was reduced by 4-AP, was dependent on external Ca^{2+}, and was inversely influenced by NA and insulin. This pointed to a modulating effect of adrenergic receptors on the ion channels of the cells. Lee and Pappone [15] found activation by extracellular ATP of both the depolarising and the hyperpolarising conductances.

At first we had focused on the origin of the electrophysiologically detectable events in brown adipocytes [7,8,16], hence, it seemed intriguing to compare them with observations from the electrophysiologically poorly studied white adipocytes. We show that NA-activated membrane-related events similar to those in brown adipocytes may occur in white adipocytes. In particular, we found non-selective cation channels and voltage-dependent potassium channels with similar properties to those observed in brown adipocytes. Four adrenergic agonists, NA, isoproterenol (iso), the β_3-agonists BRL 35135A (an ester) and BRL 37344 (its free acid), were tested for their ability to activate non-selective cation channels, and for a possible interaction with α-receptors. To our knowledge, this is the first single-channel analysis of white adipocytes and of the influence of β-adrenergic agonists on the channel activity. The results may gain further significance because it seems reasonable to assume that ion channels are involved in the release of leptin from adipocytes, as they are in the release of insulin from β-cells. Some preliminary results have appeared elsewhere [17].

2. Materials and methods

2.1. Cell isolation and primary culture

Subcutaneous, epididymal, and perirenal white adipose tissue was dissected from 6–8-week-old male Sprague–Dawley rats. Rats were reared at room temperature (21°C), kept on a 12-h daylight cycle, and fed ad libitum. The dissection method resembled that of Rodbell [18]. The fat pads were rinsed in sterile isotonic NaCl solution and connective tissue was removed. About 7–8 g of the adipose tissue were minced with scissors for 5 min. After a 25-min treatment in 3 ml enzyme solution (1 mg/ml ≈ 190 IU/g tissue, collagenase, Type II, Sigma) at 37°C, collagenase was inactivated using 10 ml medium (see below). The suspension was filtered by a 170-μm nylon gauze and rinsed with an additional 10 ml of medium. The cells with the medium were placed in a tube and allowed to stand for 2 min, after which different layers were visible. The top layers, fat from destroyed cells and mature fat cells, were removed and rediluted in 20 ml of medium. Again, fat and adipocytes floated up due to their lesser density. Cells were harvested from a thin layer between the medium and the fat layer on top of the tube and seeded into 35-mm Petri dishes. Pieces (1 cm^2) of Heraeus biofoil were placed onto the surface of the solution with the hydrophilic side facing the medium. Solutions were prewarmed to 37°C. The whole procedure lasted less than 2 h. It took about 24 h for the adipocytes to adhere tightly to the foil. Cell clusters in confluence grew best. Culture dishes were stored for up to 2 weeks in an incubator (5% CO_2 in air at 37°C and at 100% humidity). However, after 8–10 days in culture it became difficult to obtain seal resistances in the GΩ range. Cells were smooth, round, white adipocytes with a mean diameter of 60 μm after 2 days in culture and 100 μm after 8–10 days. As no obvious differences were observed between the experimental results from subcutaneous, perirenal, and epididymal fat, we mostly used subcutaneous fat due to its faster availability. The data reported in this paper are from 85 dissections.

2.2. Experimental conditions

The foil with the adhering cells was removed from

the culture dish, turned upside-down and placed in a fresh 35-mm Petri dish filled with HEPES-buffered modified Ringer's. Test solutions were applied by a peristaltic pump driven sewer-pipe system. Patch-clamp was performed with an EPC-7 amplifier (List Electronics, Darmstadt, Germany) as described in [19] using electrodes from Clark (Electromedical Instruments, Pangbourne, UK). Pipettes were controlled for the shape of their tip and diameter by scanning electron microscopy. Resistances were about 15 MΩ. Experiments were performed in the outside-out-, inside-out-, and in the cell-attached mode. Potentials refer to the inner side of the membrane. Except in Fig. 2, the potentials in cell-attached mode were corrected for a membrane potential of −44 mV. The applied holding potential is abbreviated as E_H. Data were either digitised by a modified pulse-code modulation unit (Sony, Köln, Germany) and stored on video or digitised by a Labmaster TM-40 board (Scientific Solutions, Solon, OH) and analysed by computer using the pCLAMP software (version 5.5, Axon Instruments, Foster City, CA). Current traces induced by changes of the holding potential were corrected for capacitive and leakage currents by subtraction of silent traces.

2.3. Analysis

Blockade of the K-channels by TEA was calculated by

$$\text{Block of } i_K\ (\%) = \frac{c_{TEA}^n \cdot 100}{c_{TEA}^n + IC_{50}^n} \quad (1)$$

where c_{TEA} is the tested TEA concentration, IC_{50} the TEA concentration at which the K^+ single-channel current amplitude i_K is reduced to 50%, and n is the Hill coefficient. The EC_{50} value of the NSC activation was calculated accordingly. The open probability P_o was determined by

$$P_o = \frac{x_1 + 2x_2 + \ldots + mx_m}{m(x_0 + x_1 + x_2 \ldots + x_m)} \quad (2)$$

where x_0 is the area below the Gaussian for the closed state, $x_1 \ldots x_m$ are the areas for the different open states, and m is the maximum number of channels open in this patch. Results are expressed as mean ± S.E.M.

2.4. Solutions

Enzyme solution (content in mM if not otherwise stated): 120 NaCl, 50 KCl, 1 $CaCl_2$, 10 Na-HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), 5 glucose, 475 IU/ml collagenase, 40 mg/ml bovine serum albumin (Fraction V, Sigma).

Serum containing medium: Dulbecco's modified Eagle's medium completed with 15% foetal calf serum (FCS) or 10% newborn calf serum (NBCS), 20 IU/ml penicillin and 20 μg/ml streptomycin. Medium and serum were purchased from Biochrom (Berlin, Germany). We also tested M199-medium from Biochrom without any change in the success rate. When completing the medium with 15% FCS, experiments yielded good results from day 1 after plating, with 10% NBCS good results were obtained after day 3–4. Therefore we split the cells and used both sera, having cells available then for the entire week. Ten-percent serum from rat blood gave results comparable to those with FCS.

Modified Ringer's: 134 NaCl, 6 KCl, 10 Na-HEPES, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 11 glucose, pH 7.4.

Modified Ringer's with 0.01 Ca^{2+}: 134 NaCl, 6 KCl, 10 Na-HEPES, 1.75 $MgCl_2$, 4.97 $CaCl_2$, 5 EGTA (ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid), 11 glucose, pH 7.4. [Ca^{2+}] was calculated as described in [20].

KF-solution: 139 KF, 4 KCl, 10 K-HEPES, 5 EGTA, pH 7.4.

Agonist solutions: 0.001 (−)-noradrenaline (Sigma), (−)-isoproterenol (hydrochloride, Sigma), and BRL-compounds (Beecham Pharmaceuticals, Epson, UK) were dissolved in modified Ringer's and frozen in aliquots. Different concentrations were obtained by diluting these stock solutions.

Insulin solution: 0.001 insulin (from bovine pancreas, Sigma) was dissolved in modified Ringer's with 2% acetic acid. Concentrations used were obtained by diluting with modified Ringer's. Experiments were carried out at room temperature (24 ± 1°C) and at 37°C (part of those in the cell-attached mode).

3. Results

3.1. K-channels

Voltage-dependent K-channels were studied in the outside-out configuration with a KF-containing, low-Ca^{2+} pipette solution and modified Ringer's in the bath. Most patches showed activity of at least one channel (Fig. 1A). The highest number of open levels observed was five, indicating that this patch contained at least five channels. The mean number of open levels was 1.5. Assuming an Ω-shaped patch and an even distribution of the channels across the cell, we estimated with a patch area of 3 μm^2 a density of about 0.5 channels/μm^2 or a total number of 16 000 channels for a 100 μm adipocyte. In Fig. 1B, the single-channel current (i_K) is drawn against the test pulse potential (E). Single-channel conductance (γ) for potentials more positive than 30 mV was 16.4 ± 0.1 pS (n = 18). Due to rectification in the asymmetrical solutions, γ was reduced to 11.1 ± 0.1 pS (n = 18) around 0 mV. The continuous curve in Fig. 1B was calculated by the Goldman–Hodgkin–Katz (GHK) equation and yielded a reversal potential of −83 mV when extrapolated to the negative. The calculated reversal potential was −79.6 mV under the assumption of a mere K^+-conductance.

The effect of the K-channel blocker tetraethylammonium (TEA) was studied by adding a solution containing different concentrations of TEA to the outside of the patch. TEA caused a decrease of the single-channel current amplitude indicating a so-called 'fast block' [21]. The block was concentration-dependent (Fig. 1C) and rapidly reversed upon washout with a TEA-free Ringer's solution. The IC_{50} of the TEA-blockade was 1.5 mM and the concentration– response curve was best described by a Hill coefficient of 1.0.

3.2. Non-selective cation channel

For the investigation of inside-out patches, pipettes were filled with modified Ringer's solution. As long as the channel activity was recorded in the cell-attached mode it was very low (< 1 event/s) but always present. As soon as a patch was excised so that the inner side faced the bath solution with a Ca^{2+} concentration of 1.2 mM, channel activity increased dramatically (Fig. 2A). The maximal number of open levels was seven, the mean value was three per patch

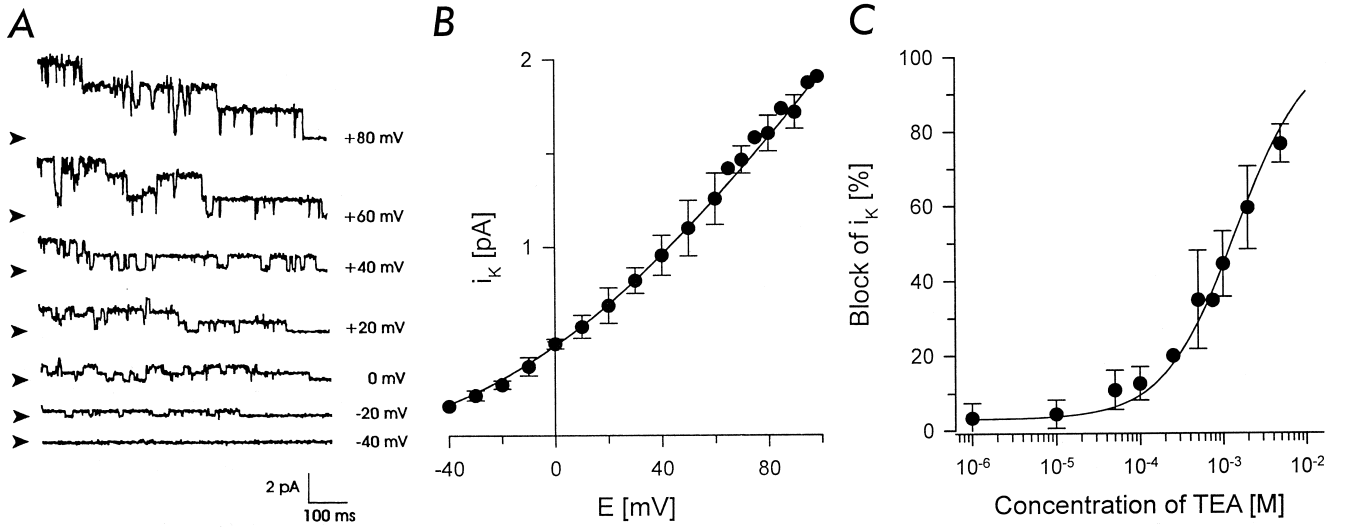

Fig. 1. Characteristics of a voltage-dependent K-channel. (A) Current traces recorded in the outside-out configuration with KF-solution in the pipette and modified Ringer's in the bath. E_H, −40 mV (2.5 s duration); prepulse, −100 mV (2 s); test pulses between −40 mV and +80 mV (800 ms) as indicated. Note almost complete inactivation during pulses. 24°C. (B) Single-channel current i_K vs. test pulse potential. Same conditions as in A. Mean values of 18 experiments, error bars only given if they exceed size of the symbols. Continuous line fitted by the GHK-current equation. The extrapolated reversal potential was −83 mV. (C) Concentration–response relationship of the blockade of i_K in % of the maximum i_K measured without TEA. Continuous curve calculated using Eq. 1. IC_{50}, 1.5 mM; Hill coefficient, 1.0.

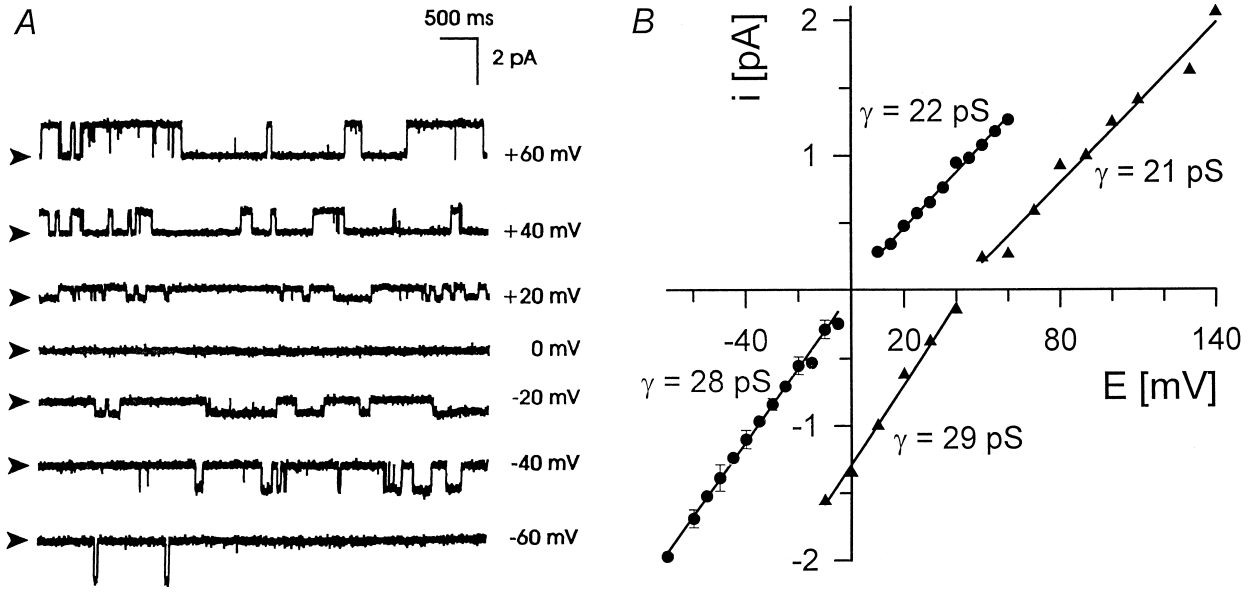

Fig. 2. Characteristics of the non-selective channel. (A) Current traces recorded in the inside-out configuration at the indicated holding potential with symmetrical Ringer's solution (24°C). (B) Current–voltage relationship as analysed from an inside-out patch (circles) and in the cell-attached mode (triangles), respectively. Error bars only given if they exceed the size of the symbols. Straight lines calculated by linear regression separately for positive and negative voltage range. Note gentle inward rectification.

($n=18$). The mean inner tip diameter was 1 μm. With the assumptions made for estimating the K-channel density, we calculated a number of about 31 000 NSC-channels per white fat cell or a density of about one channel per μm^2.

The amplitude of the single-channel events was analysed to obtain current–voltage relationships. The graph shows two straight lines with different slopes of inward and of outward currents demonstrating a gentle inward rectification. Before excising the patches, the mean outward conductance was 21.2 ± 0.1 pS ($n=18$) while the inward conductance was slightly larger (28.9 ± 0.2 pS). In inside-out patches, an outward conductance of 21.7 ± 0.1 pS and an inward conductance of 27.9 ± 0.1 pS was obtained (Fig. 2B). Currents recorded beyond ± 100 mV (not shown) indicated that also in white adipocytes, the larger currents deviate from those predicted by the GHK-equation by bending towards the *y*-axis as was shown earlier for brown adipocytes [16]. The reversal potential was close to zero in excised patches. The reversal potential of the NSC-channel in the cell-attached mode was +44 mV (Fig. 2B). This implies a membrane potential of −44 mV assuming equal permeabilities for Na^+ and K^+-ions. As, at least in brown adipocytes, Na^+-ions are slightly more permeant [16], a small voltage error is introduced when talking about membrane potential. It has been ignored here.

3.2.1. *Activation of the NSC-channel by Ca^{2+}-ions and inhibition by ATP*

An increase of activity after excising a patch is often a response to the increased Ca^{2+}-concentration of the bath. We therefore tested the activity of the NSC-channel after excising inside-out patches into the bath solution (modified Ringer's with two different Ca^{2+}-concentrations). The P_o-value in 1.2 mM Ca^{2+} was 0.64, while P_o was 0.06 at 10 μM Ca^{2+} ($n=3$) suggesting an activation of the channel by internal Ca^{2+} (Fig. 3A).

Thorn and Petersen [22] showed that ATP may modulate the Ca^{2+}-sensitivity of NSC-channels in pancreatic acini. Therefore, we tested whether ATP would also have an influence on NSC-channels in white adipocytes. ATP (2 mM) almost completely and reversibly inhibited the channel. Further experiments showed that 1 mM ATP ($n=10$) also caused complete absence of the events (about 10 s latency), while 100 μM ATP only caused a transient inhibition of 30–60 s duration.

Thorn and Petersen additionally demonstrated

that the Ca^{2+}-sensitivity of NSC-channels may be higher in the continuous presence of 2 mM ATP [22]. We thus measured the P_o in patches which had already been excised into a bath solution containing 2 mM ATP and 1.2 mM Ca^{2+}. P_o was 0.02 ± 0.01. When the patch was moved into the flow system with a solution containing no ATP at all, P_o increased to 0.55 ± 0.03 ($E_H = +30$ mV; Fig. 3B). Again, 2 mM ATP had caused an almost complete blockade of the NSC-channel, which thus behaved different from the pancreas channel.

The channel could be completely and reversibly blocked by exposing the inner side of the patch to a solution containing 100 μM mefenamic acid (measured at +20 mV, data not shown). The block showed a slow characteristic [21].

More than 70% of the experiments showed spontaneous activity immediately after forming a Giga-seal (i.e. in the cell-attached mode) in the absence of any agonist. The maximum open probability (P_o) of this activity, as determined by all-point analysis, was 0.15 ± 0.01 ($n = 5$) and it decreased by 94% within 4 min (Fig. 4, open circles). P_o was always considerably higher at depolarising membrane potentials as compared with hyperpolarising potentials. In inside-out patches, the values were $P_o = 0.54$ at an E_m of +40 mV vs. $P_o = 0.19$ at an E_m of −30 mV which is a decrease of 65%. After a change of

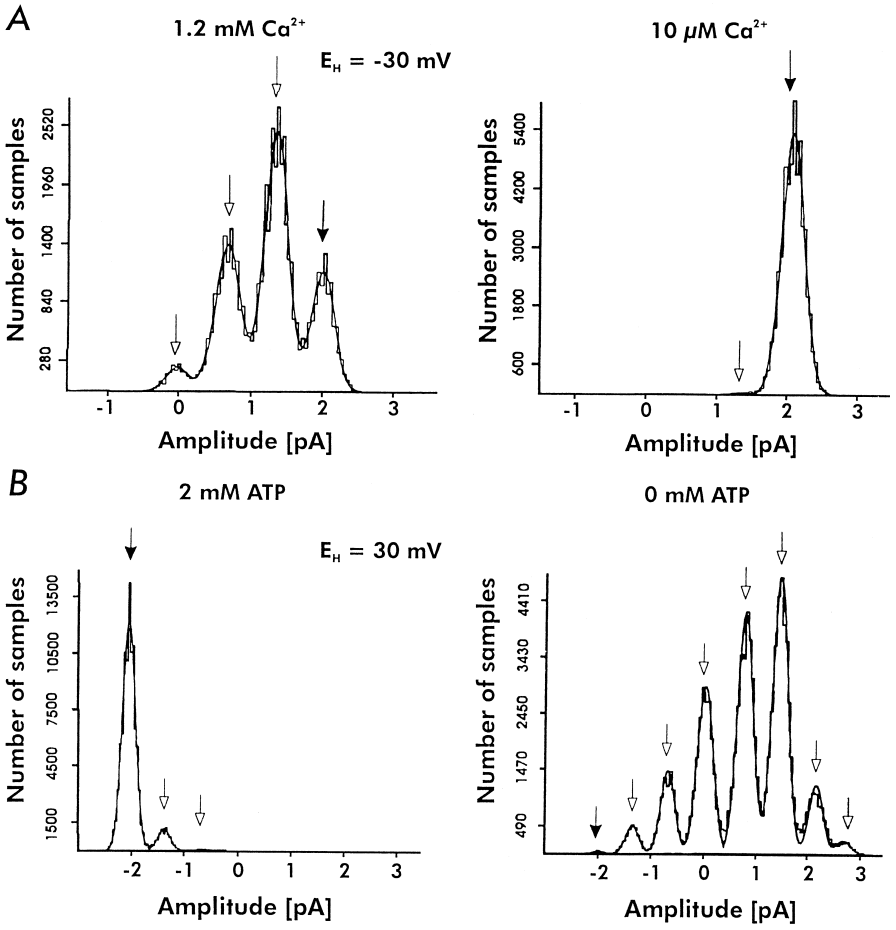

Fig. 3. Ca^{2+} at the intracellular side of the membrane facilitates the activity of the NSC-channel while ATP inhibits it. All-point histograms of experiments using the inside-out configuration under the specified conditions. Mean values of open levels marked by open arrows, those of closed level by filled arrows. Gaussian distribution fitted by the PClamp software. (A) Left: symmetrical Ringer's with 1.2 mM Ca^{2+} at the inner membrane side. Right: after switching to Ringer's with 10 μM Ca^{2+} at the inner side. $E_H = -30$ mV. (B) Left: Ringer's with 1.2 mM Ca^{2+} and 2 mM ATP at the inner membrane side. Right: washout of ATP in Ringer's with 1.2 mM Ca^{2+} at the inner side. $E_H = +30$ mV, $T = 24$°C.

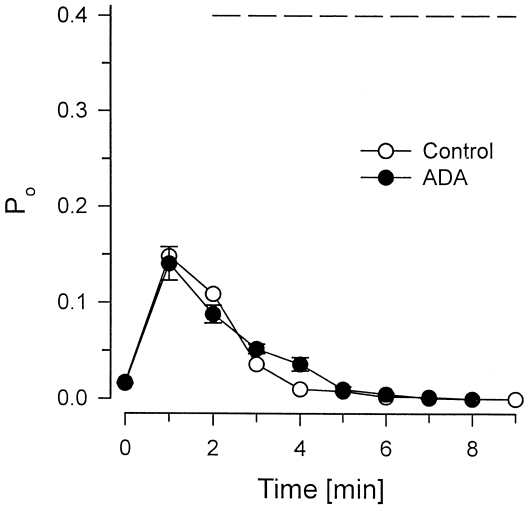

Fig. 4. Contributions of adenosine receptors. P_o of spontaneous activity in the absence (open circles) and in the presence (filled circles) of 2 μg/ml adenosine deaminase is comparable. P_o of a patch from a BAT cell excised into a high-Ca^{2+} solution is shown as a dashed line for comparison. $E_H = -44$ mV, $T = 37$°C.

potential, it took 2–3 min until the new P_o-value was reached.

3.2.2. Interference with adenosine receptors

White adipocytes secrete small amounts of adenosine produced by dephosphorylation of adenine nucleotides. The adenosine receptor may interfere with the β-pathway by inhibiting the adenylyl cyclase [23,24]. We used 2 μM adenosine deaminase in the solutions to test for a possible influence of adenosine. Neither additional activity of the NSC-channel nor a reduction in the spontaneous activity was observed (Fig. 4, filled circles, $n = 8$).

3.2.3. Inhibition by insulin

Insulin is an important regulator of lipid metabolism having an inhibitory action on the β-adrenoceptor-activated lipolysis. Experiments were done in the cell-attached mode at a membrane potential of −44 mV and at a temperature of 37°C (Fig. 5). The measurements were performed during the first minutes after forming a seal when spontaneous activity can usually be observed. After taking a control value in modified Ringer's, 100 nM insulin was added and a second measurement was made. In 5 experiments 100 nM insulin decreased P_o from a mean value of 0.11 ± 0.04 to 0.02 ± 0.01. After washout, the mean P_o increased again to 0.10 ± 0.02. The result indicates that insulin reversibly inhibits activity of the NSC-channel.

3.3. Regulation of the NSC-channel by β-agonists

The β-agonists noradrenaline (NA), isoproterenol

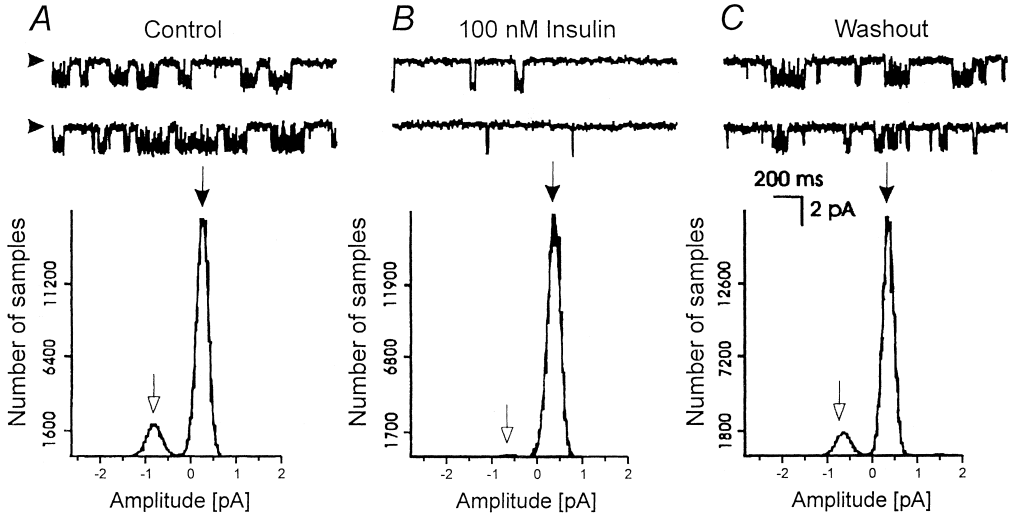

Fig. 5. Insulin decreases the open probability of the NSC-channel in the cell-attached mode. (A) Modified Ringer's in pipette and bath, 37°C. (B) Insulin (100 nM) added to the bath. (C) After washout of insulin. Upper part: current at $E_m = -44$ mV. Lower part: all-point histograms of the same experiment. Filled arrow, closed level; open arrow, open level. Continuous curve gives Gaussian distribution. A, $P_o = 0.15$; B, $P_o = 0.01$ (5% of A); C, $P_o = 0.11$.

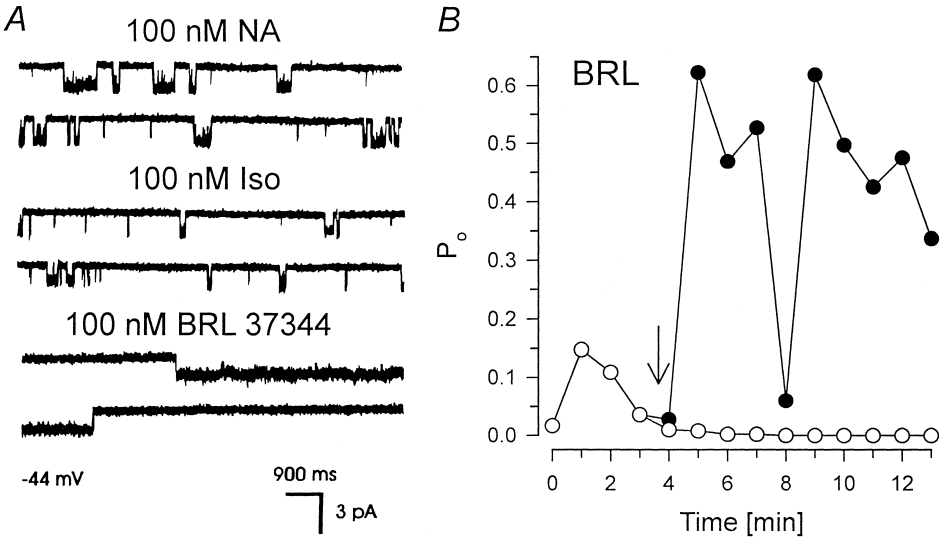

Fig. 6. β-Agonist action on NSC-channel activity. $E_m = -44$ mV; $T = 24$°C. (A) Current traces after application of 100 nM of the indicated agonist. Opening events deflect downward and may be recognised by increased noise. (B) P_o as determined from 1-min segments against time after forming a Giga seal. Spontaneous activity (control) reaches a maximum and declines within about 4 min almost to zero (open circles). After adding 100 nM BRL 37344, channel activity increased steeply (closed circles) after a delay (compare Fig. 7B).

(iso), BRL 37344, and its methylester BRL 35135A were tested in 103 experiments with respect to their action on the activity of NSC-channels (Fig. 6A). Experiments were carried out in the cell-attached mode applying test and control solutions to the whole cell by the flow system. Temperature was kept either at 24°C or at 37°C. As expected, the single-channel amplitude was higher at 37°C. Furthermore, the number of open events was higher at 37°C. However, due to the decreased duration of the events, P_o turned out to be almost unchanged by temperature. This was tested at holding potentials of −44 and −84 mV (not shown). All experiments were recorded from the moment of getting a high-resistance seal. The total record was later cut into 1-min segments which were analysed separately. To avoid a major contribution of the spontaneous activity, we always waited 4 min before applying an agonist. We measured the time until the first peak of activity appeared and thereafter we measured the P_o of each 1-min segment starting from this point (Fig. 6B).

The NSC-channel responds to β-adrenergic agonists by an increase of P_o. Fig. 6B shows the response to the application of 100 nM BRL 37344 for 9 min (closed circles) as an example. Channel activity did not appear continuously, but was interrupted by silent periods. Only in some cases was there no response at all to the β-agonists, although spontaneous activity had been observed before.

3.3.1. Voltage dependence of P_o

Voltage dependence of P_o was also measured in the presence of the four agonists (BRL at non-equieffective concentration). When activity was induced by 100 nM BRL 37344 or BRL 35135A, P_o increased slightly by 9.3% ($n = 5$), and by 11.1% ($n = 3$), respectively, when stepping from −44 to −64 mV. This increase in activity was more pronounced in 100 nM NA (169%, $n = 5$) or iso (172%, $n = 4$). The reason might be that we are starting from a much lower P_o at −44 mV with iso or NA (about 0.1 as compared with 0.5 for the BRL substances; cf. Fig. 7A) facilitating the pronounced iso/NA effect. Therefore, the effect is probably not substance-specific. This voltage dependence of agonist response is the reason for the difference between the data shown in Fig. 7A and the control data obtained before testing the effect of phentolamine (PA, see Table 1).

3.3.2. Concentration dependence of P_o

In order to obtain concentration–response rela-

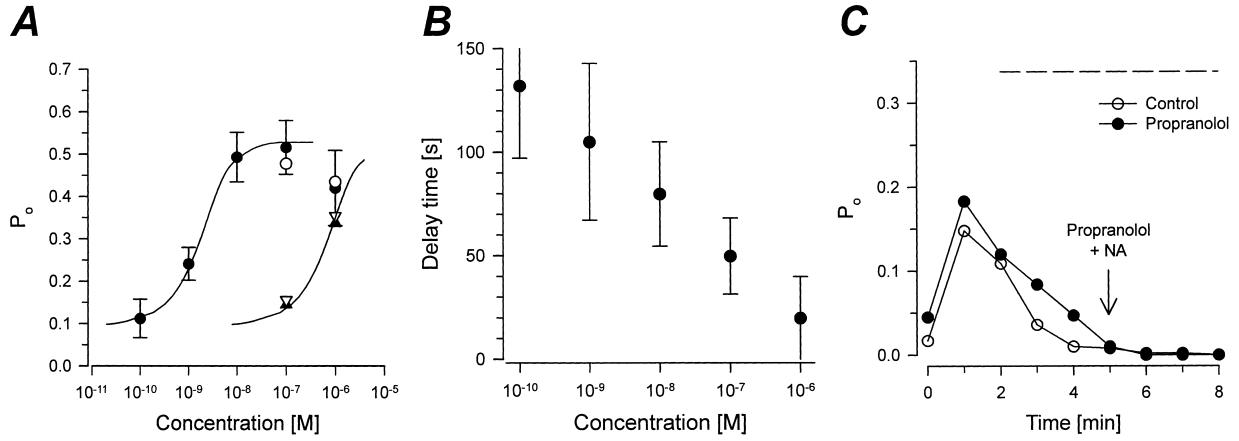

Fig. 7. β-Agonist action on the NSC-channel. A: Concentration–response relationship for P_o of the NSC-channel in dependence of the concentration of the β-agonist BRL 37344 (filled circles; $n=3$, 3, 5, 6 and 4, starting from the lowest concentration), and BRL 35135A (open circles; $n=8$ and 10) NA (filled triangles; $n=10$ and 3), iso (open triangles; $n=12$ and 4). Continuous curves fitted to the data using the Michaelis–Menten equation (implying a Hill coefficient of 1.0) and with $EC_{50}=1.3$ nm (left) $EC_{50}=5.3$ μM (right). (B) Time of delay of the NSC-channel activity decreases with increasing concentration of the β_3-agonist BRL 37344 ($n=5$, 4, 6, 6 and 6). (C) The β-receptor antagonist propranolol has no effect on spontaneous activity, but completely blocks activation by 1 μM NA. Dashed line for comparison with P_o-value for 1 μM NA without propranolol from A. $T=24$°C. Cell-attached recordings with Ringer's in pipette and bath, $E_H=-44$ mV.

tionships for the different β-agonists, P_o was determined. Concentrations between 0.1 nM and 1 μM were tested (Fig. 7A). EC_{50} was 1.3 nM for BRL 37344 and 5.3 μM for NA and iso. Due to difficulties keeping the Giga seal during the long-lasting experiments, we obtained data with BRL 35135A only at concentrations of 100 nM and higher. At the highest BRL concentration, the dose–response curve showed a decrease of P_o. We are not aware of an artifact that could have caused this decay. It thus could be a hint for a tachyphylaxis of the BRL effect though it is not significant at the 5% level. The results indicate that BRL 37344 and probably also BRL 35135A are more potent activators of NSC-channel activity, by about three orders of magnitude, than NA or iso.

3.3.3. Delay of the agonist response

The delay time between the application of 100 nM of the different β-agonists and the first response differed. After NA the delay was longest (162 ± 34 s; $n=10$). The shortest delay time was after application of the de-esterified BRL 37344 (50 ± 18 s; $n=6$) while iso (80 ± 31 s; $n=12$) and BRL 35135A (93 ± 20 s; $n=9$) were intermediate. Data scattered considerably around the mean. To check whether the non-equieffective concentrations of the adrenergic agonists are responsible for different delay periods, we determined the delay at different BRL 37344 concentrations. The filled circles in Fig. 7B show the decrease of the delay with increasing concentration of BRL 37344. In order to test whether the delay was voltage dependent, we compared the values at −44 mV and at −64 mV. It was found that the delay increased by 141% (BRL 37344, $n=5$), 72% (BRL 35135A, $n=3$), 69% (iso, $n=4$), and 11% (NA, $n=6$), respectively, when measured at −64 mV.

Table 1
P_o-values of the NSC-channel elicited at $E_m=-64$ mV by 100 nM of different β-agonists in the presence and in the absence of the α-antagonist PA

Agonist	P_o without PA (control)	n	P_o with 1 μM PA	n
Noradrenaline	0.31 ± 0.06	5	0.40 ± 0.06	5
Isoproterenol	0.42 ± 0.10	4	0.45 ± 0.06	5
BRL 37344	0.56 ± 0.13	5	0.56 ± 0.06	6
BRL 35135A	0.53 ± 0.06	3	0.55 ± 0.11	4

Thus the delay is voltage-dependent, decreasing with depolarisation.

3.3.4. Effects of selective antagonists on adrenergic agonist-induced effects

A modified Ringer's solution containing 1 μM of the non-selective β-blocker propranolol was used as a control for a true β-effect of NA. We chose the same conditions as for the experiments testing the four β-agonists. Propranolol was added immediately after forming the seal. No difference in the activity of the NSC-channel was observed, as compared with the activity in plain Ringer's. In three experiments with propranolol, the mean spontaneous peak P_o was 0.18 ± 0.01. Four minutes after forming the seal, P_o was reduced to 26% and 5 min after forming the seal to 5%. The resulting curve differed only slightly from the spontaneous activity (filled circles in Fig. 7C). Application of an additional 1 μM NA did not produce any activity of the NSC-channel, thus indicating a true β-effect of NA.Amounts of 100 nM of NA, iso, BRL 37344, and BRL 35135A were tested in the cell-attached mode also in the presence of 1 μM of the α-antagonist phentolamine (PA). The results are shown in Table 1. PA did not interfere with the three pure β-agonists while NA which is known to activate also α-receptors, produces a slightly, but not significantly, stronger NSC-channel activation in the presence of the α-antagonist. Maximal spontaneous activity is not affected by PA ($P_o = 0.15 \pm 0.01$ vs. $P_o = 0.15 \pm 0.01$ in PA, $n = 3$). Also, the delayed increase of P_o after adding 100 nM of any of the four tested agonists was unchanged.

4. Discussion

4.1. K-channels

White adipocytes express a voltage-dependent K-channel of the same type that has already been shown for brown adipocytes [8,10]. In outside-out patches, the outward conductance (16 pS) was comparable to that in brown adipocytes (17 pS, outside-out and 9 pS, cell-attached) [8,10]. Also, TEA sensitivity of both channels is almost equal with IC_{50} values of 1.5 mM (WAT) vs. 1.8 mM (BAT). It thus resembles the TEA sensitivity of the fast F-type channels found in the node of Ranvier (1 mM) which, however, has a larger single-channel conductance [25]. The density of the K-channels is comparable in both types of fat cells [8].

A voltage-dependent K-current was first described in WAT by Ramirez-Ponce et al. [12]. When studied in more detail, it showed an activation threshold of −30 mV, slow inactivation, and whole-cell current amplitudes of 0.5–6 nA. It could be blocked by TEA, 4-AP, Ba^{2+} and Co^{2+} [14,15]. TEA (5 mM) induced a reversible 64% block of the channel while we found in WAT a 50% block by 1.5 mM TEA on the single-channel level. No difference of the K-channel in unstimulated WAT from Sprague–Dawley, lean Zucker and obese Zucker (*fa/fa*) rats was reported [15]. Small Ca^{2+}-dependent, apamin-sensitive K-channels were only seen in one out of five perforated patches from WAT [15]. Voltage-insensitive K-channels as in BAT [8] have so far not been observed in WAT-cells.

4.2. NSC-channels

NSC-channels appear at the same density (about $1/\mu m^2$) in WAT as was shown in BAT [16]. Their reversal potential of −44 mM in the cell-attached mode, which should be an approximation for the resting potential, is somewhat more negative than the −31 mV determined in BAT at room temperature. The *i–E* curve differs slightly from that of NSC-channels of BAT in that it shows a gentle inward rectification. The inward currents (28 pS) equal those measured in BAT cells [9], while the outward currents are smaller (22 pS). The probability of being in the open state increased with depolarising potentials like in many NSC-channels (for review see Table 1 in [26]). Compared with BAT [27], voltage dependence seemed to be shifted to the right by about 25 mV. Antiphlogistica of the fenamate structure are known to block not only some Cl-channels, but also most NSC-channels as e.g. in exocrine pancreas [28]. At a concentration of 100 μM, they also completely and reversibly inhibit the NSC-channel of white adipocytes.

Probably due to the low internal Ca^{2+}-concentration the spontaneous P_o is clearly smaller in the cell-attached mode than in excised patches. The dashed

line in Fig. 4 gives the P_o-value for excised inside-out patches of brown adipocytes at the same membrane potential for comparison [27]. The reason for the increase and succeeding decrease in spontaneous activity during the first 4 min after forming a seal is unknown. There were no signs of sensitivity to membrane tension of the NSC-channel. Small Ca^{2+}-leaks during the formation of the seal are possible, but were expected to be balanced much faster. The missing effect of adenosine in our single-channel experiments is in agreement with earlier measurements of the membrane potential being also uninfluenced by this potent inhibitor of the catecholamine-stimulated adenylyl cyclase [4].

The vast majority of the NSC-channels respond to an increased internal Ca^{2+}-concentration by an increased activity [26]. This causes the typical increase in single-channel activity when excising an inside-out patch from adipocytes into the bath. From our data, we can assume that the EC_{50} for Ca^{2+} is $>10^{-5}$ M which is considerably higher than the normally assumed intracellular Ca^{2+} concentrations (1 μM and 500 nM after stimulation of brown adipocytes by NA; [29,30]) but lower than in BAT (cf. Fig. 5A of [7]). A maintained high Ca^{2+} sensitivity of the NSC-channel in the continuous presence of ATP at the inner membrane side, as shown by [22] in pancreatic acini, could not be observed; however, a mere block of the NSC-channel by ATP as seen in other NSC-channels (reviewed by [26]) was also observed in white adipocytes.

4.3. Adrenergic pathways

Is the NSC-channel activated via α-receptors? According to our results this is not the case as the non-specific α-antagonist PA produced no effect on the β_3-agonist-mediated response and only a minor increase of the NA response. This fits with earlier measurements of the membrane potential. Cheng et al. [4] found in rat WAT that the slow depolarisation was blocked by 10^{-6} M propranolol, but not by 10^{-6} M phentolamine. With the early NA-induced hyperpolarisation it was the other way round. Both antagonists had no effect on the membrane potential when given alone.

In human adipose tissue, α_2-inhibition of the adenylylcyclase occurs only at low NA-concentrations. At higher concentrations, it is overridden by the β-adrenergic effect of NA [31]. α_1-receptors appearing in WAT-cells in small numbers only, can activate the IP_3-cascade leading to an increased intracellular Ca^{2+}-concentration [32]. However, it was shown that 90% of the cells increase their internal Ca^{2+}-concentration as an effect of β_3-stimulation fitting well to the experiments presented here.

Propranolol is not only an antagonist of β_1/β_2-adrenoceptors, but also of β_3-receptors, although with less potency [1]. An antagonistic effect of propranolol on the NA-induced activation in our experiments (Fig. 7C) should thus point to a true β-effect. The experiments thus showed that not the spontaneous activity, but the NA-induced additional NSC activity was elicited by a β-mediated pathway. The concentration–response curves (Fig. 7A) point to a β_3-effect. The strong response in our experiments is in agreement with the large number of β_3-receptors found in WAT of rodents [2]. The effect of the agonists always appeared after a delay. The delay of the β_3-agonist BRL 35135A was almost double that of BRL 37344 which could be due to the fact that BRL 35135A acts via its de-esterified metabolite [3,33]. However, since the steep part of the dose–response curve for BRL 35135A is not well documented, we cannot exclude the possibility that the delay is simply caused by a lower affinity of the receptor to the ester. In addition, the delay is concentration dependent. (Fig. 7B). Long-lasting delays after agonist treatment were also observed when measuring oxygen consumption of a brown-adipose cell suspension with a Clark electrode (unpublished result).

White adipocytes express β_1-, β_2, and predominantly, especially in rodents, β_3-receptors. At low concentrations, NA and iso stimulate mainly β_1-receptors. However, higher concentrations of both agonists as well as low concentrations of BRL 37344 stimulate β_3-receptors, which in rats were shown to cause lipolysis via protein kinase A. Their ability to stimulate lipolysis is in the order: BRL 37344 > iso > NA [2,34–36] which matches their ability to activate the NSC in our experiments. Stimulation of lipolysis in the rat WAT shows an EC_{50} for BRL 37344 which is one order of magnitude lower as compared with iso ($10^{-8.6}$ M vs. $10^{-7.7}$ M, [1]) pointing to the importance of the β_3-receptor for lipolysis. We have so far no indication for a link between NSC-

activation and lipolysis, though there is a strong β_3-influence on both of them.

4.4. Insulin

A 100 nM amount of insulin inhibits spontaneous NSC-activity in our experiments almost completely. Insulin can also inhibit catecholamine-induced lipolysis [37]. Although NSC-channel activity can be increased by β-adrenergic agonists, once again, we cannot conclude from our data that NSC-channel activity is necessarily linked to lipolysis as both events could just coincide. Continuous insulin application causes a decline of the β_3-adrenoceptor mRNA levels and thus of the receptors themselves [38]. In preliminary experiments with WAT and BAT cells we added insulin continuously as a supplement to the culture media which was not done here. A reduction in β-induced NSC-activity in these cultures compared with the data shown here could be due to the presence of insulin. This would match our finding of a β_3-mediated response because expression of β_1- and β_2-receptors is unchanged by insulin [38].

White adipocytes exhibit a concentration-dependent 3–13.5 mV hyperpolarisation under the influence of insulin [4,13,39]. This hyperpolarisation could be due to Ca^{2+}-dependent K-channels, the rise in Ca^{2+} being mediated by insulin and antagonised by NA. However, it may also be due to a reduced sodium influx as discussed by [39], which could be explained by the blockade of the NSC-channels by insulin described here. Catecholamines cause a biphasic depolarisation in BAT as well. The second phase can also be blocked by propranolol and insulin [40]. In brown adipocytes, it is this second phase that is mediated by NSC-channels [7]. We think that this is also true for white adipocytes.

In conclusion, we have demonstrated by single-channel measurements at WAT cells the presence of delayed-rectifier K-channels and NSC-channels. In several respects (kinetics, activation by Ca^{2+}, block by ATP and fenamates) the NSC-channel shows similarities with that in BAT-cells. Conductance and voltage dependence differ slightly. A pronounced β_3-effect and an insulin effect on the NSC-channels were detected. The first may be different to brown adipocytes where an α-effect is dominating.

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