Insulin increases the density of potassium channels in white adipocytes: possible role in adipogenesis

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Abstract
We studied the potassium currents in white adipocytes obtained by culturing preadipocytes from rat epididymal tissue, both with insulin (WAi) and without insulin (WAo), in order to test the role of insulin in the development of voltage-gated potassium channels (Kv) during adipogenesis. Occasionally, very small potassium currents (IK.V) were present in preadipocytes; however these currents were measured in all differentiated cells (adipocytes). WAi exhibited greater macroscopic potassium currents than WAo, with no apparent differences in kinetics or voltage dependence. The current density (pA/µm²) calculated in WAi was higher than in WAo. Currents were blocked by millimolar concentrations of tetrothylamonium (TEA). The effect of insulin on adipogenesis, both with and without TEA, was analysed. Four days without insulin and three days with insulin were necessary to increase the total number of cells in culture by 2.5-fold. Insulin increased the number of differentiated cells by 73.5%. Cell proliferation and differentiation were inhibited by TEA. Proliferation was affected only by high concentration of TEA. Inhibition of differentiation was dose dependent, with the concentration necessary for half-block similar to the IC50 values to block potassium channels. These results suggest that insulin increases the density of Kv and that these channels may be necessary for the normal growth of white adipocytes in culture.


Introduction
White adipose tissue is the major energy reserve in higher eukaryotes, primary purposes of which are the storage of triacylglycerol in periods of energy excess and its mobilization during energy deprivation. Recently, there has been a dramatic increase in the incidence of obesity resulting from an excess of white adipose tissue. This obesity may occur as a result of the enlargement of existing adipocytes and/or as a consequence of an increase in new fat cells. Although multiple factors modulate proliferation and differentiation of adipocytes (for reviews see Gregoire et al. 1998 and Boone et al. 2000), it is known that some hormones and growth factors act via specific receptors to transduce external growth and differentiation signals through a cascade of intracellular events. Insulin is able to stimulate cell proliferation in white adipose tissue (Géloën et al. 1989, Kras et al. 1999) and increases the number of differentiated cells for most primary preadipocytes (Gaben-Cogneville et al. 1990, Serrero & Lepak 1999); few cells containing lipids can be observed in the absence of insulin.

Experiments with other cell types have implicated potassium channels as being essential for their proliferation and differentiation (Woodfork et al. 1995, Lepple-Wienhues et al. 1996, Skryma et al. 1997, Wang et al. 1997, Rouzaire-Dubois & Dubois 1998, Vaur et al. 1998, Ghiani et al. 1999, Miki et al. 2001; for review see Rane 1999). It has been demonstrated that functional voltage-gated potassium channels may be necessary for the normal proliferation and differentiation of brown fat cells in culture (Pappone & Ortiz-Miranda 1993) and that the purinergic modulations of these potassium currents may be important for altering adipocyte growth and development (Wilson & Pappone 1999, Wilson et al. 1999). Brown fat tissue is important for maintaining body temperature and energy balance in mammals through its ability rapidly to convert metabolic energy into heat in response to sympathetic adrenergic stimulation (for review see Horwitz 1989). Environmental factors, such as exposure to cold or an unbalanced diet, produce an increase in both the amount of brown fat tissue and in heat production per cell. Although the regulating factors are complex, the major trophic stimulus seems to be the level of adrenergic stimulation sustained in the tissue mediated by noradrenaline. Thus proliferation and differentiation of brown fat cells is hormonally regulated, and the activity of voltage-dependent potassium channels is necessary for the process.

In white adipocytes, obtained by culturing preadipocytes from rat epididymal tissue, we demonstrated the existence of voltage-dependent K+ channels (Kv) using the whole-cell variant of the patch-clamp technique.
Materials and Methods

Cell culture

Preadipocytes from rat epididymal fat pads were isolated by means of incubation for 40 min at 37 °C in the presence of collagenase as described previously (Ramírez-Ponce et al. 1996). The floating cells were suspended in a culture medium consisting of DMEM, supplemented with 10% neonatal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, and plated, in equal quantities, either onto polyllysine-treated glass cover-slips for patch-clamp experiments or grid-embossed 35-mm tissue culture dishes for cell growth experiments. The insulin used for growth cells was biosynthetic human tissue culture dishes for cell growth experiments. The composition of the external control solution was (in mM): 80 NaCl, 2-7 KCl 1 CaCl₂, 70 Trizma, 5 glucose and, depending on the experiment, 0-25, and 50 and 50 TEA. The standard solution used to fill the electro-pipettes contained 80 mM potassium glutamate, 20 mM KF, 35 mM KCl, 2 mM MgCl₂, 10 mM Hepes and 5 mM EGTA; pH was adjusted to 7-25–7-30.

Patch-clamp experiments

Ionic currents were recorded using the whole-cell configuration of the patch-clamp technique (Hamill et al. 1981), with an EPC-7 patch-clamp amplifier (List Electronics, Darmstadt, Germany). Data acquisition was performed by an ITC-16 computer interface (Instrutech Corp. Great Neck, NY, USA) and (Pulse+Pulsefit) software (Heka electronik, Lambrecht, Germany). Linear leak currents, through membrane capacitance, were cancelled on-line using P/4 procedure (Armstrong & Bezanilla 1974).

The composition of the external control solution was 135 mM NaCl, 2-7 mM KCl, 2-5 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes and 5 mM glucose; pH was adjusted to 7-35 ± 0-02 in all cases. To record tail currents, NaCl was replaced equimolarly with KCl. In experiments where tetrathylenmonium (TEA) was used, the external solution contained (in mM): 80 NaCl, 2-7 KCl 1 CaCl₂, 70 Trizma, 5 glucose and, depending on the experiment, 0-25, 5 and 50 TEA. The standard solution used to fill the electro-pipettes contained 80 mM potassium glutamate, 20 mM KF, 35 mM KCl, 2 mM MgCl₂, 10 mM Hepes and 5 mM EGTA; pH was adjusted to 7-25–7-30.

Cell growth

Five squares of the grid with an appropriate number of cells were selected from each dish and photographed at 24-h intervals thereafter by a digital camera, Olympus DP–10 (Olympus Optical, Co., Hamburg, Germany) attached to the microscope. The images were transferred to a computer to be processed by the program Olympus Dp–Soft. The procedure used to discriminate between undifferentiated and differentiated cells was based on their morphological differences, the main parameter being the content of lipid vacuoles. The preadipocytes have no or very small vacuoles and the mature adipocytes have easily observed spherical vacuoles. The counting of cells was carried out by the program, after pointing and marking them with the mouse. Immediately after the first count, the potassium channel blocker, TEA, was added to the culture media from a stock solution in water. Each experimental set always included a control group, which was grown and counted in parallel. The proliferation rates shown are normalized to the average value obtained by measuring samples from each set of dishes on day 1, before TEA was added to the cultures. The initial number of cells counted was in the range of 30–50 and was comparable for both control and treated cultures in order to avoid the possibility that changes in the number of differentiated cells were a consequence of changes in the initial cell numbers rather than as a result of the experimental manipulations.

Data analysis

The ionic currents registered were exported as ASCII files from Heka Pulse Software to be analysed and plotted by the graphic and data analysis program Microcal Origin 5.0 (Microcal Software, Inc. Northampton, MA, USA). The statistical test used was the analysis of variance, one-way ANOVA, supported by Microcal Origin 5.0, which determines whether the data means differ significantly.

Results

Cell culture

The cultures contained a variable mixture of mature white fat cells, preadipocytes and groups of tightly arranged cells (islets), which copurify with the free-floating fat cells, as
described by Carraro et al. (1990). When cultured along with mature white adipocytes, the islets give rise to cells, initially fibroblast-like, which proliferate rapidly, acquire lipid droplets and transform into white adipocytes within 4–6 days without the addition of insulin to the medium (basal medium, BM). Figure 1A shows a culture with mature adipocytes grown in BM. Isolated mature adipocytes added to the culture medium demonstrated differentiation-promoting activity, suggesting a paracrine effect of these cells (Carraro et al. 1990, Considine et al. 1996). Figure 1B shows a culture with mature adipocytes grown in basal medium (BM). The number of differentiated cells increased by 73.5% ± 11.8% in the presence of insulin (average value from measurements in 10 cultures ± s.e.).

**Effect of insulin treatment on K+ currents of white adipocytes**

The macroscopic membrane currents of white adipocytes were studied using the whole-cell variant of the patch-clamp technique. Occasionally, very small currents were registered in fibroblastic cells which had not accumulated lipid granules or whose content was very slight (pre-adipocytes) (Fig. 2A), and there were no differences between the small current obtained in cells from cultures treated with or without insulin. Nevertheless, all differentiated cells grown in BM, WAo, showed the same outward currents which exhibited a sigmoidal activation time course (Fig. 2B). We have previously described these currents in white adipocytes in detail and demonstrated the existence of voltage-dependent K+ channels in these cells (Ramírez-Ponce et al. 1996). Lee & Pappone (1997) and Ringer et al. (2000) have described identical channels in isolated adipocytes, the latter by using single channel recording in outside-out configuration. These currents correspond to voltage-dependent K+ channels (delayed rectifier). Currents obtained in WAo had higher values than those obtained in WAmb (Fig. 2C). Figure 2D shows the peak current–voltage relation between the two cells of traces B and C. The threshold for current activation in both cells was similar, around ~20 mV. There were no apparent differences in voltage dependence in the currents in cells from diverse cultures with or without insulin. Figure 3(A-D) shows the kinetics of the currents. Figure 3A shows the traces that were obtained as an average of currents recorded from 41 cells grown in BM and 39 cells grown with insulin. In Fig. 3B it can be seen that, when these currents were scaled, there were no significant differences in their activation and inactivation kinetics. Figure 3C presents tail currents obtained as an average of currents recorded from 41 cells grown in BM and 39 cells grown with insulin. In Fig. 3B it can be seen that, when these currents were scaled, there were no significant differences in their activation and inactivation kinetics. Figure 3D shows sections scaled corresponding to tail currents recorded in 3C do not present significant differences in their closing kinetics. Moreover, we found no evidence of any other type of voltage-gated channel. In particular, there was no indication of an inward current that could reveal the presence of significant voltage-gate Na+ or Ca2+ conductances. To verify whether differences in the recorded current amplitude might be related to different cellular size, we calculated the current density (pA/μm²) of each registered cell. The area was calculated taking into account the spherical or ellipsoidal shape of the cells, measuring their diameters with the millimetered ocular of the

![Figure 1](https://www.endocrinology.org)
microscope. The mean value of the current densities of preadipocytes, WA₀ and WA₁ cells are significantly different at the 10⁻⁶ level (P=2·489×10⁻⁶), as shown in Table 1. On the assumption that current density is proportional to channel density, these differences may suggest that white adipocytes modulate potassium channel density according to their state of proliferation and that insulin increases the incorporation of channels in differentiated adipocytes. Our previous work showed that voltage-dependent ionic potassium current (I_K,V) can be blocked by approximately 64% by millimolar concentrations of TEA (5 mM), and recovered to 94% of the control level when the TEA was washed from the bath (Ramírez-Ponce et al. 1996). In this experiment, we tested the sensitivity of I_K,V to different concentrations of TEA. Figure 4A shows that external TEA (50 mM) blocked the current by approximately 92-66% (n=9). The current recovered to 75% of the control level when the TEA was washed from the bath. The TEA dose–response relationship for blocking the current peak using this agent is shown in Fig. 4B. The IC₅₀ values and Hill coefficient of TEA inhibition of I_K,V were determined by a least-squares fit of the log [I/(1-I)] versus log [TEA], where I is the percentage inhibition of current in the presence of TEA. The I value was determined from peak currents with the blocker relative to control currents during depolarisation to +70 mV. This yielded IC₅₀ values of 1·08 and 2·35 mM and Hill coefficients of 0·63 and 0·82 obtained for WA₀ and WA₁ respectively. These values are similar to those obtained in mature adipocytes with single channels recording in outside–out configuration (Ringer et al. 2000).

**Effect of insulin and TEA treatment on the growth and development of white adipocytes**

Cells were plated on plastic grid-labelled culture dishes, enabling us to recognize identical areas of the dish for counting cells each day. The total number of cells (preadipocytes and mature adipocytes) in BM cultures increased approximately 2·5-fold in 4 days (Fig. 5A). The rate of proliferation of cells was the same in cultures grown with 0·25 or 5 mM TEA. However, with 50 mM TEA (almost completely blocking the potassium current), cell proliferation was blocked. In cultures exposed to insulin, the total cells counted increased approximately 2·5-fold in 3 days. Likewise, in these conditions the presence of 0·25 to 5 mM TEA did not affect the proliferation of the cells, although proliferation was affected with 50 mM TEA.
TEA also blocked cellular differentiation which has concentration dependence, but at different magnitudes to that for cell proliferation. Differentiation, unlike proliferation, was affected by concentrations of TEA of between 0.25 and 5 mM. Figure 6 shows that cultures with 5 mM TEA lack mature adipocytes. The effect of TEA is more evident in cultures exposed to BM. In the presence of insulin this effect is less pronounced, and, at 5 mM TEA, some differentiated adipocytes can be observed. Table 2 shows the average inhibition of differentiation by TEA in 6 cultures taken on day 8 after isolation. The proportion of differentiated cells was calculated in each square of the grid as the ratio between the number of mature cells (cells with a high content of lipid droplets) relative to the total number of cells in the same square. Percentage inhibition was determined as the ratio of the number of differentiated cells in the blocker and control respectively. Figure 7 shows the data of Table 2 fitted to the Hill equation.

Table 1

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<th>Area (μm²)</th>
<th>Density of current** (pA/μm²)</th>
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<tbody>
<tr>
<td>Preadipocyte</td>
<td>846 ± 177</td>
<td>0.185 ± 0.053</td>
</tr>
<tr>
<td>BM (n=55)</td>
<td>1119 ± 301</td>
<td>0.620 ± 0.039</td>
</tr>
<tr>
<td>Insulin (n=47)</td>
<td>3532 ± 355</td>
<td>1.148 ± 0.098</td>
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**P<10⁻⁶ (one-way-ANOVA. Microcal Origin 5.0).
where I is the percentage inhibition of cell differentiation in the presence of TEA. The values of IC_{50} for blocking cell differentiation (1·02 and 2·4 mM, in cells grown in BM or with insulin respectively) are similar to the ability of the drug to block voltage-dependent potassium channels, as shown in Fig. 4B, and are consistent with the hypothesis that the inhibition of cell differentiation is dependent on blocking the potassium channels. These results suggest that functional voltage-dependent potassium channels may be necessary for the normal development of white adipose cells in culture.

**Discussion**

The results presented here indicate that insulin increases the density of voltage–dependent potassium channels in white adipocytes and that these channels may be involved in the process of adipogenesis. Insulin is a hormone known to act on the proliferation and differentiation process in white adipocytes, in addition to its role in the metabolic activity of these cells. In this study, we have verified that cells proliferate and differentiate quickly in the presence of insulin, reaching a larger size than in cells grown without insulin. Recordings of macroscopic membrane ionic currents demonstrate that I_{K,V} was present in all differentiated cells with the same characteristics, and occasionally in preadipocytes. Nevertheless, K^+ currents recorded in differentiated cells in the presence of insulin were higher, as was the density current. Experiments with other cell types have also shown a change in K^+ current density between the subpopulations obtained throughout their differentiation process (Lee et al. 2001). The results presented in this paper confirm a preliminary study made using intracellular recordings, which showed that insulin modifies the electrical properties of white adipocytes, hence possibly modulating K^+ conductances in these cells (Ramírez-Ponce et al. 1991).

Our data show that I_{K,V} can be blocked by millimolar concentrations of TEA, with IC_{50} values of 1·08 and 2·35 mM for cells grown in BM and insulin respectively, in accordance with the larger number of K^+ channels in cells differentiated with insulin. We tested whether potassium channels were involved in the growth of white adipocytes by adding millimolar concentrations of TEA to the culture medium. In all cultures it was necessary to add very high doses of TEA (50 mM) to block cellular proliferation. On the other hand, we found that mature white adipocytes differentiated in culture were blocked by a lower concentration of TEA, and with similar dose-dependence to the blocking of I_{K,V}. On the assumption that this agent, in concentrations where it has no effect on cellular proliferation (5 mM), does not have unanticipated effects on other cell systems implicated in cellular differentiation, our results would indicate that expression of functional potassium channels are necessary for the development of white adipocytes in culture, in addition to other factors previously described (for reviews see Gregoire et al. 1998 and Boone et al. 2000). These results add to the growing list of cell types in which potassium channels have been implicated in cellular growth. In brown fat cells, functional voltage-gated K^+ channels may be necessary for their proliferation and differentiation (Pappone & Ortiz-Miranda 1993), and purinergic modulation of I_{K,V} may be important for altering adipocyte growth and differentiation.

Noradrenaline (NA) is another hormone known to act on the metabolic activity of white adipose tissue, promoting the release of free fatty acids from these cells. There are studies which indicate that NA inhibits pre-adipocyte proliferation in rats, and that it may, therefore, be an important negative regulatory component of adipocyte growth (Jones et al. 1992). In previous studies we demonstrated that NA may modulate K+ conductances by blocking them and causing white adipocytes to depolarize (Ramírez-Ponce et al. 1991), and that this modulator effect might be mediated by cAMP (Ramírez-Ponce et al. 1998). The effects described for NA and insulin on the electrical activity of white adipocytes and their relationship to cellular growth could be explained in a similar way. The lipolytic action of NA increases the concentration of cAMP. An increase in this intracellular mediator would be responsible for the partial blocking of K+ conductances and cause the cells to depolarize. Furthermore, increases of cAMP would reduce the expression of K+ channels in preadipocytes and diminish the differentiation of these cells. Contradictory results have been obtained in studies which address the question of whether cAMP stimulates or inhibits adipose conversion (Boone et al. 1999), thereby suggesting different regulatory mechanisms between species. There are examples of other tissues in which an increase of cAMP reduces outward K+ current amplitude by acting at the translational level to destabilize Kv1-1 mRNA (Allen et al. 1998). On the other hand, the antilipolytic action of insulin reduces the concentrations of cAMP; this, in turn,

Table 2 Percentage inhibition of differentiation by TEA. Results are means ± S.E.M.

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<th>TEA (mM)</th>
<th>Basal medium</th>
<th>Insulin</th>
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<tr>
<td>0.25</td>
<td>20.69 ± 0.29</td>
<td>3.0 ± 2.50</td>
</tr>
<tr>
<td>5</td>
<td>69.27 ± 7.77</td>
<td>49.25 ± 15.95</td>
</tr>
<tr>
<td>50</td>
<td>99.80 ± 0.1</td>
<td>99.80 ± 0.2</td>
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Figure 6 Cultured cells from the same white fat cell isolation. Each panel was taken on day 10 after isolation. Cells were grown in BM (A), BM +5 mM TEA (B), insulin (C), and insulin +5 mM TEA (D). Note that differentiation of white adipocytes ceases in (B), and diminishes in (D). Scale bar represents 50 μm.
activates K⁺ conductances, which would explain the hyperpolarization observed in white adipocytes after the addition of insulin (Ramírez-Ponce et al. 1991). A decrease in cAMP could increase the expression of K⁺ channels.

The present results suggest that K⁺ channels could play a significant role in the development of white adipocytes, although further studies would be necessary to establish it definitively.

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