# Metabolic Effects of Direct Current Stimulation on Cultured Vascular Smooth Muscle Cells

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Vascular smooth muscle cells from rabbit arteries were grown in tissue culture and stimulated by DC impulses (1 mA, 1 V, 10 Hz, 1 ms/imp). Scanning microscopic examination disclosed that in stimulated cultures the cell surface was enlarged by numerous microvilli. This was interpreted as being indicative of an increase in cell activity. Cellular metabolism was characterized by analyzing the incubation medium for glucose, glutamate/glutamine, and lactate. When compared to unstimulated controls, stimulation caused an increase in the uptake of glucose and glutamine as well as an increased lactate production. The enhancing effect on metabolism was prevented when the "calcium antagonist" verapamil was present ($5 \times 10^{-6}$ M). Although the exact mechanism by which DC stimulation influences the cells remains obscure, this finding indicates an important mediating role of $Ca^{2+}$ ions.

## Introduction

The local transmural application of direct electric current (DC) to the arterial wall *in vivo* has various effects depending on intensity and duration of the stimulation. Due to different size and position of the electrodes it is difficult to compare the biological efficacy of the different stimulations expressed in current or voltage by various authors. Nevertheless, a rough discrimination of the literature shows, that short term application of DC in dense electric fields (0.2–4 mA, 9–40 V) causes injuries of the vessel wall and formation of platelet thrombi. This method was used in dogs [1, 2], rats [3], rabbits and man [4] to induce acute arterial thrombosis within 1–2 h. Experiments in rabbit showed, that low level DC (0.1–1.5 mA, 1–5 V) seems to be unable to induce vascular thrombi in a short time; however, if repeatedly applied as impulses (1–10 ms/imp.) or combined with cholesterol rich diet it causes the arterial wall to react with atherosclerotic alterations, *i.e.* smooth muscle cell migration and intimal proliferation [5–7]. Although a transient alteration of endothelial function cannot be excluded to play a role in this model of atherosclerosis, one could assume, that at least some of the reactions of the vascular wall are due to effects on the myocytes themselves.

Therefore we were interested in the direct effects exerted by DC impulses on cultured vascular smooth muscle cells. This paper describes metabolic and morphologic changes occurring in cultured myocytes after DC stimulation. Alterations in cell metabolism are prevented by the administration of verapamil, a "calcium antagonist".

## Materials and Methods

### *Cell culture*

Enzymatically disaggregated smooth muscle cells from rabbit arteries were cultured in Dulbecco's modified Eagle's Medium supplemented with 10% fetal calf serum (both Biochrom, Berlin, FRG), 0.2% glutamine and 5% Pen-Strep (both Gibco, Karlsruhe, FRG) as described earlier [8]. Harvested cells were transferred to culture dishes with 24 microwells (2 ml) in a density of $10^5$ cells/ml and grown to confluency. A total of 4 dishes were incubated with cells originating from 4 different rabbits.

### *Stimulation experiment*

The cultures were stimulated by DC impulses (1 mA, 1 V, 10 Hz, 1 ms/imp.), which were applied

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using parallel gold electrodes (wire of 0.6 mm diameter, length 8 mm, distance 10 mm). The pairs of electrodes were fixed in appropriate teflon blocks introducible into the wells and situated on the bottom of the wells (Fig. 1). In order to ensure identical intensity of current in all stimulated wells, 4 pairs of electrodes were used in series connection.

After confluency was reached, the medium was changed and all cultures were preincubated for 24 h. Thereafter, the medium was changed again and the stimulation cycle (16 min stimulation every 80 min) was started simultaneously for 4 wells lasting 17 h. The same procedure was applied to cultures with $5 \times 10^{-6}$ M verapamil (5-[N-(3,4,dimethoxyphenetyl)-N-methylamino]-2-(3,4,dimethoxyphenyl)-2-isopropyl-valeronitril, Knoll, Ludwigshafen, FRG) added to the medium.

Unstimulated control cultures were incubated for the same period of time receiving the same sequence of medium changes. Additionally, cells were cultured with inserted electrodes but without DC impulses and, lastly, cell-free medium was subjected to DC treatment. In each dish, the different experimental groups included 4 identically treated culture wells in each case.

*Biochemical analyses*

The culture media of each well, collected either before or after the stimulation were analyzed by coupled enzymatic tests for the concentrations of glucose, lactate and glutamine/glutamate according to methods described in Bergmeyer [9]. Although each well was charged with the same volume of cell suspension, it was not possible to determine the cell number when confluency was reached and the stimulation experiment was started. In order to obtain a biochemical parameter of cell activity of each well, the individual culture media of the preincubation period were analyzed for the mentioned metabolites and the corresponding mean values were calculated for each dish. Since within each dish the standard deviation was less than 10% of the mean, we assumed similar metabolic capacities corresponding to similar cell numbers in the corresponding wells. However, there were larger differences between the mean values of the 4 dishes (< 30%). Therefore, all single values of each dish were calculated in % of the mean value of the corresponding controls. The relative values of the identically treated cultures of all 4 dishes (controls,

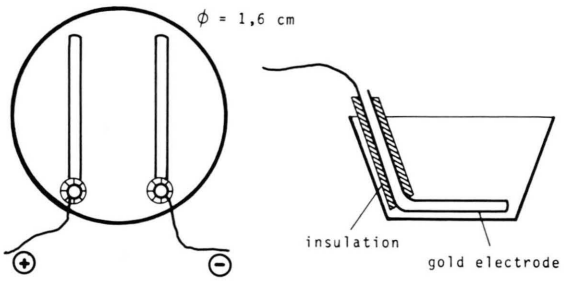
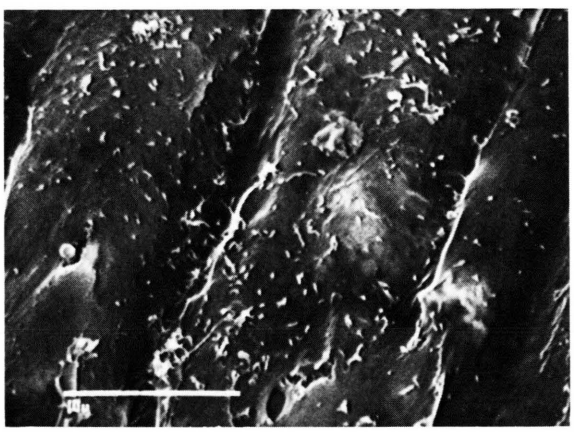
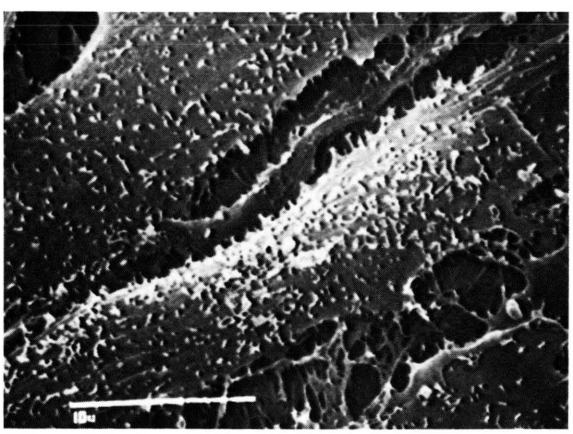



Fig. 1. Scheme of the arrangement of a pair of electrodes in a culture well. For clarity the fixing teflon blocks were omitted.



**a**



**b**

Fig. 2. Scanning electron micrographs of vascular smooth muscle cells in culture. a) Unstimulated culture. Cells are laying closely together. The surface is rather smooth. Few microvilli are present. b) Culture after DC stimulation. Cells have increased numbers of extensions. The surface of the cells is covered with numerous microvilli. The bars represent 10 μm.

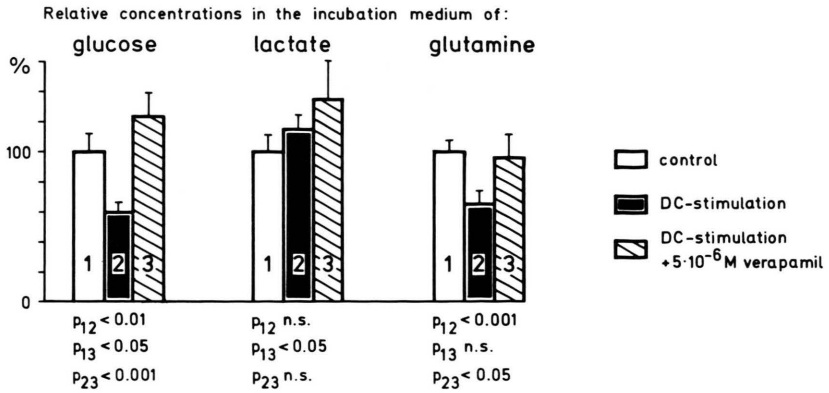



Fig. 3. Relative concentrations of glucose, lactate and glutamine in incubation media obtained from cultures of smooth muscle cells which were either unstimulated (control) or stimulated by DC in the absence or presence of $5 \times 10^{-6}$ M verapamil. The columns indicate relative mean values ± S.D. ($n = 16$, 4 dishes each with 4 cultures of each group). For the calculation, the concentrations found in the different culture media of each dish were expressed in percent of the corresponding mean value of the controls. The 100% values given in the figure correspond to 3.4 mM for glucose, 4.1 mM for lactate, and 3.6 mM for glutamine.

DC stimulation, DC stimulation + verapamil, $n = 16$ for each group) were averaged and compared by analysis of variance [10].

*Scanning electron microscopy*

For scanning electron microscopy stimulated and unstimulated cultures were fixed with several changes of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), postfixed in 1% $OsO_4$ in 0.1 M sodium cacodylate buffer, dehydrated in graded series of ethanol and critical point dried from liquid $CO_2$ [11].

## Results and Discussion

In untreated, confluent cultures smooth muscle cells showed the streaming and whirl-like arrangement which is typical for this cell type. By scanning electron microscopy cells were laying closely together, had few extensions and a rather smooth surface (Fig. 2a). After DC stimulation of the cultures, the cells contacting the electrodes were rounded. These cells were necrotic by means of their light microscopic staining properties. As reported earlier from [$^3$H]thymidine incorporation experiments [8], cells located in the area adjacent to the necrotic cell layer exhibited an increased autoradiographic labeling index. Scanning electron microscopy of this area revealed that some rounded cells were located on the surface of the confluent cell layer. These cells were considered to be necrotic. In addition oblong shaped cells, their apical surface being studded with large amounts of microvilli, was bulging out of the cell layer. These cells conformed to the morphologic description of changes in surface and shape during cell cycle given by Sanger and Sanger [12]. They therefore were considered to be in the late phase of mitosis. The cells of the basal cell layer were irregularly shaped and had many extensions. Their surface was flat or slightly rounded and covered with numerous short microvilli (Fig. 2b). When compared to untreated cultures, these cells had a much rougher surface. By the high density of microvilli, the surface to volume ratio and thereby the area available for *trans*-membrane exchange processes is increased considerably in these cells. This supports the idea, that DC stimulated cultures of smooth muscle cells are in a state of increased metabolic activity.

An increased metabolic activity can also be seen from the results presented in Figure 3. Comparing concentrations of glucose, lactate and glutamine in the medium of stimulated and nonstimulated cultures it is evident, that during stimulation the consumption of glucose and glutamine as well as lactate production were increased. DC treatment of the cell free medium did not alter the concentration of these substances and, additionally, the presence of quiet electrodes did not alter the metabolism of cells. The average concentration changes as determined in the unstimulated cultures were for glucose

from 5.6 to 3.4 mM, for lactate from 0.5 to 4.1 mM and for glutamine/glutamate from 9.5 to 3.6 mM.

Contraction of the cultured cells could not be detected by microscopic observation during stimulation. Nevertheless, the results indicate, that low amplitude DC impulses are able to stimulate rates of metabolic pathways probably related to energy turnover.

When the experiments were performed in the presence of verapamil ($5 \times 10^{-6}$ M), the effect of DC stimulation on the metabolism was abolished (Fig. 3). When compared to cultures stimulated in the absence of verapamil, the concentrations of glucose and glutamine were increased indicating a decreased uptake of both substrates. The reason for the increased lactate production in the presence of verapamil is not clear, however, this phenomenon was also observed in other experiments [13] and seems to be the result of different actions on cellular metabolism independent from DC stimulation.

There are several possibilities to explain the mechanism by which DC impulses may stimulate cell metabolism. When DC impulses of higher voltage (10–30 V) were applied to isolated segments of rabbit ear arteries it was shown that the induction of contraction was dependent on the concentration of extracellular calcium ions. Addition of verapamil ($10^{-5}$ M) reduced the contraction response to about 50% of control [14]. It can be concluded, that depolarization of the cell membrane of smooth muscle cells causes an influx of extracellular calcium ions which is impeded by "calcium antagonistic" drugs.

DC impulses used in our experiments did not induce observable contraction of the cells. Therefore the increase in cell metabolism by DC stimulation must come from actions other than induction of contraction. One possible explanation is, that during partial depolarization of the cells subthreshold amounts of $Ca^{2+}$ were taken up which seem to be responsible for cell activation leading to increased mitotic rate and metabolic turnover (for rev. *e.g.* [15]).

Other possibilities to explain the metabolic stimulation are, that during stimulation redox reactions occur at the electrodes, the reaction products of which may influence the cells. Conceivable are shifts in the pH value or the occurrence of reactive oxygen intermediates at the anode.

Although the exact mechanism remains obscure, our present and former results show that DC stimulation *in vitro* causes cell activation with increased metabolic turnover rates. Since chronic application of DC impulses on the vasuclar wall *in vivo* causes intimal myocyte proliferations with increased metabolic activities [16], one can conclude that in this model of atherosclerosis stimulatory effects of DC on the medial smooth muscle cells are of great importance. The inhibitory effect of verapamil *in vitro* suggests a crucial role of $Ca^{2+}$ ions in the DC dependent cellular stimulation.

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