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A pulsed DC electric field affects P2-purinergic receptor functions by altering the ATP levels in *in vitro* and *in vivo* systems

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Summary Recently it was shown that extracellular ATP, acting through purinergic receptors, has many physiological functions, including opening of Ca^{2+}-ion channels, activation and mediation of signal tranduction mechanisms as well as activation of the pain sensation. Since electrical stimulation is also known to affect many signal transduction processes as well as the alleviation of pain, we hypothesized that electric stimulation may affect the extracellular release of ATP. We investigated the effects of a small DC electric field (10^1–10^2 V m^{-1} range and with frequencies below 150 Hz) on the release of ATP *in vitro* (HeLa cells), and on the levels of ATP *in vivo* (the plasma of healthy volunteers). In HeLa cells ATP release was increased 50 fold, while the total amount of ATP in the cells was increased by 163%. In the plasma a significant decrease ($P < 0.05$) in ATP concentration was seen after electrical stimulation, in all the volunteers. The small DC electric field also affected the cAMP signal transduction system *in vitro* (HeLa cells and human lymphocytes) and *in vivo* (human plasma). Decreased levels of cAMP ($P < 0.05$) were seen in HeLa cells and increased levels of cAMP ($P < 0.05$) in isolated human lymphocytes. The cAMP levels in the plasma of the electrically treated volunteers were lower than control values. These results show that the frequency, waveform and signal strength of the applied electric field are suitable for effecting measurable changes on signal transduction *in vitro* and *in vivo*.

INTRODUCTION

Extensive research has shown that, apart from its well known intracellular effects, ATP also has many extracellular physiological functions (1). Extracellular ATP has been identified as a ligand, as a transmitter and also as a co-transmitter that affects numerous cellular functions by activating P2-purinergic receptors (see 1 for review). There are different types of P2-receptors, the two major classes being P2X- and P2Y-receptors. These receptors include several subtypes that can all be activated by extracellular ATP (1,2). The P2X receptors are ligand-gated ion channels whereas P2Y receptors are G-protein coupled (1–3). The ATP-dependent P2X ligand-gated channels can be permeable to either sodium, potassium or calcium ions. Via these channels, extracellular ATP can cause depolarization or an increase in the intracellular levels of calcium in activated cells (1,3). Five human P2Y ($P2Y_{1,2,4,6,11}$) G-protein coupled receptors have been identified and they all activate phospholipase C. Through the formation of IP3 (inositol-3-phosphate), intracellular calcium levels are

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increased, which will then activate many calcium-dependent cellular activities (3). The $P2Y_{11}$ receptor specifically activates adenylyl cyclase, causing increased cAMP production (4,5). In addition, phospholipase A2 is also activated by extracellular ATP, which can lead to increased levels of arachidonic acid in the stimulated cells (*6*). Apart from the functions mentioned above, a possible role of extracellular ATP as an instigator and mediator of the pain sensation has also been described by several groups (2,7–10).

There are many reports of effective treatment of pain with exogenously applied small electric fields over the area of treatment (11). The cellular mechanisms involved in the successful treatment of pain with electric devices remain unclear. Mechanisms that may possibly be affected after exposure to an electric field include the following: (a) enhanced release of dynorphins and enkephalins, especially β-endorphins (12,13); (b) internalization of substance P receptors (14); (c) the gate control theory of pain (15); (d) activation of different opioid receptors (16). We suggest that electric stimulation may affect the release of ATP which may also influence aspects of the pain sensation. It has been shown that electric stimulation enhances the release of ATP in *in vitro* studies (17), but less is known of the release in *in vivo* systems. In a previous paper we hypothesized that most of the therapeutic effects attributed to electric treatment can be explained by effects on cell membrane-linked signal transduction mechanisms (11). Similar to extracellular ATP (2,7–10), electric stimulation of cells also affects signal transduction mechanisms by increasing intracellular calcium, arachidonic acid and cAMP levels in a variety of cells (18–20). The positive effects of electric stimulation on bone fracture healing and tissue regeneration have been attributed to activation of some of these signal transduction mechanisms (20–22).

Since it has been shown that ATP release is affected *in vitro* after exposure to a small electric field (17), we suggest that electric field-induced extracellular alterations in ATP levels may affect the pain sensation via P2-purinergic receptors. Because it is difficult to determine ATP release *in vivo* in an electrically treated area, we tested our theory on ATP release by determining ATP levels in the plasma of healthy volunteers exposed to a small applied electric field, which was in the form of periodic DC pulses with peak strength in the 10^1–$10^2\,V\,m^{-1}$ range, and with frequencies below 150 Hz. We used HeLa cells to study ATP release *in vitro*.

Since both extracellular ATP and electric stimulation affect cAMP levels, and therefore signal transduction (4,5,18), the effects of the application of the small electric field on cAMP levels in HeLa cells, human plasma and isolated human lymphocytes were investigated to further test our hypothesis that signal tranduction mechanisms are affected when cells are exposed to a small DC electric field.

APS Technologies (Tech Pulse, Pretoria, South Africa) supplied two devices delivering a periodic, direct current, pulsed electric field. The pulse waveform was a brief monophasic square pulse (duration 0.8 ms) followed by exponential decay to base level. The median of the applied current strength was set to 600 μA for all the experiments, which corresponds to a peak voltage of approximately 20 V for the *in vivo* experiments and a peak voltage of approximately 10 V for the *in vitro* experiments. The applied signal translates into peak field strengths of approximately 10^1–$10^2\,V\,m^{-1}$ in the samples. The pulse frequency used was 150 Hz.

ATP levels *in vitro*

A marked, highly significant ($P < 0.05$) increase in the release of ATP into the medium in electrically stimulated HeLa cells was seen (Fig. 1), which corroborates our assumption that electric stimulation may enhance ATP

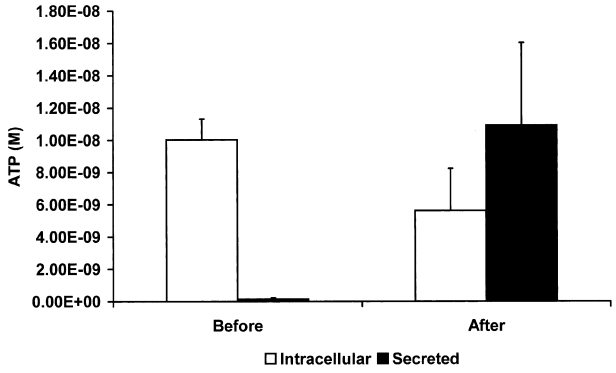

Fig. 1 The levels of ATP in HeLa cells before and after electric treatment. The release of ATP after exposure was significantly higher than in the controls ($P < 0.05$). The procedures followed to obtain these values were as follows: Equal numbers of HeLa cells ($\sim 5 \times 10^5$ cells/well) were seeded into two six-well culture plates (9.4 cm^2 surface area/well). The cell cultures were incubated for 24 h in MEM containing 10% heat inactivated fetal calf serum. The cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 24 h near confluent layers of HeLa cells were washed three times with PBS (37°C). Two ml of warm (37°C) PBS were placed in each of the wells. The wells were fitted with electrodes embedded in resin. Electrodes in each well, attached to the two APS devices, were separated by 3.4 cm, and were in electrical contact with the PBS. In each plate two of the wells were left untreated and four subjected to electrical stimulation (pulsed DC current with median strength set to 600 μA), for 8 minutes. All the experiments were performed in an incubator at 37°C. After electrical stimulation of HeLa cells, triplicate PBS samples were taken from each well for the measurement of secreted ATP. To measure intracellular ATP, the remaining PBS was discarded and the HeLa cells were lysed with the provided cell lysis reagent for 5 min at room temperature. Luciferase reagent (100 μl) was added to 100 μl of either PBS sample or cell lysate and ATP content was measured according to protocol described in the pack insert of Roche's Bioluminescence assay kit. A BioOrbit 1251 Luminometer (O.E.N. Enterprises, SA) was used. (Data are presented as follows in Figures 1–5: Means are presented in bar charts, with T-bars referring to standard deviations (SD). *P* values were obtained with Student's *t*-test).

release. The cell membrane is not usually permeable to ATP, but it is a general finding that ATP is released into the media of cultured cells when there is a mechanical disturbance present, including the changing of medium (1). Therefore, as expected, a small amount of ATP was present in the media of control HeLa cells (Fig. 1). Electric stimulation, however, increased extracellular ATP more than 50-fold (Fig. 1), indicating that an ATP-releasing mechanism in the membranes of the HeLa cells might have been activated.

To verify that the ATP release was not caused by cell membrane damage, electrically treated HeLa cells were immediately stained with trypan blue (1%) after treatment, to establish cell viability and membrane integrity. No increase in trypan blue uptake and therefore no toxic effect were seen. Cells were also stained with haematoxylin and eosin using standard procedures previously described (23), to detect morphological changes. Again there were no detectable effects on cytoplasmic, nuclear and mitotic morphology (morphological studies are not shown).

In contrast to the extracellular effects, intracellular ATP levels were significantly decreased in the HeLa cells after stimulation (Fig. 1). The total concentration of ATP in each well (the combined value of ATP in the medium and in the intracellular fraction) was increased (163%) compared to the untreated control (Fig. 1), indicating that ATP synthesis was increased by electric stimulation.

ATP levels *in vivo*

ATP concentrations in the plasma of all nine volunteers were, however, significantly lower than their untreated control samples (Fig. 2). This was an interesting finding emphasizing that *in vitro* results cannot be extrapolated to *in vivo* results.

cAMP levels *in vitro* and *in vivo*

Total cAMP levels were determined in HeLa cells and lymphocyte experiments. In the HeLa cells cAMP production was inhibited by electric stimulation (Fig. 3) whereas the cAMP production was stimulated significantly in the lymphocytes (Fig. 4). These results suggest that, because of their specific receptor populations, the two cell types are differently affected by electric stimulation.

In all nine plasma samples, cAMP levels were decreased after exposure to the applied electric field (Fig. 5), although only three of the values were statistically significant. Although it is difficult to explain the various results on cAMP levels we can conclude that the cAMP concentration and therefore second messenger functions are affected by exposure to a small electric field.

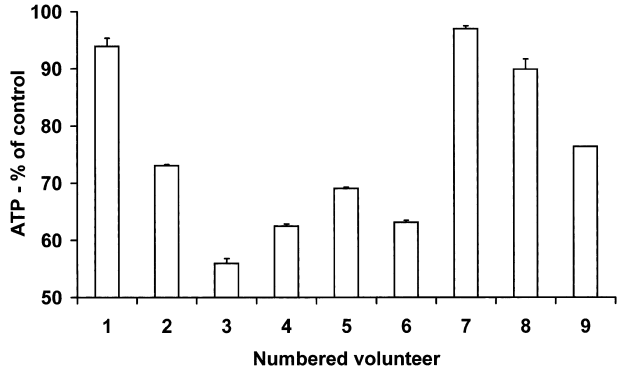

Fig. 2 ATP levels after treatment were all significantly ($P < 0.05$) lower than a 100% control before exposure to the electric field. To obtain these values the following procedure was followed. Electrodes (separated by approximately 30 cm) linked to an APS device were attached to healthy volunteers. Two negative electrodes were placed on the dorsum of the left hand, and the two positive electrodes on the medial aspect of the left arm above the elbow joint. Nine healthy volunteers were used in this study. Blood was taken from the left brachial vein of the volunteers, prior to electric stimulation and again 7 minutes after start of treatment (just before the electrodes were removed after 8 min of electric treatment). Blood was collected into tubes containing 7.5 mM EDTA and centrifuged at 3000 rpm for 10 minutes to remove cells. Aliquots (0.5 ml) of plasma were stored at −70°C prior to analysis. The procedure provided by Roche's Bioluminescence assay kit was followed to determine ATP values. See legend to Fig. 1.

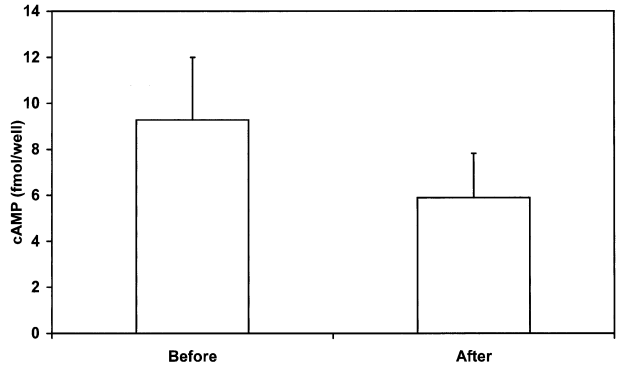

Fig. 3 In HeLa cells cAMP concentration was significantly lower ($P < 0.05$) after exposure to a small DC electric field (procedure described in Fig. 1). Equal numbers of HeLa cells ($\sim 5 \times 10^5$ cells/well) were seeded into two six-well culture plates. After 24 h cells in four wells were exposed to a small electric field as described in Fig. 1. Intracellular as well as secreted cAMP levels were determined in the untreated and electrically treated cells. Ten×concentrated lysis reagent (200 μl/well) was added to the wells containing HeLa cells and 2 ml PBS cell suspension. The cells were agitated for 10 min at room temperature to facilitate cell lysis. Cell lysis was monitored by microscopic evaluation with Trypan blue. Quadruplicate aliquots of cell lysate (100 μl) were transferred to a donkey anti-rabbit Ig coated plate for total cAMP measurement according to the non-acetylation EIA procedure as described in Amersham's Biotrak kit pack insert. For total cAMP the two sets of values were added.

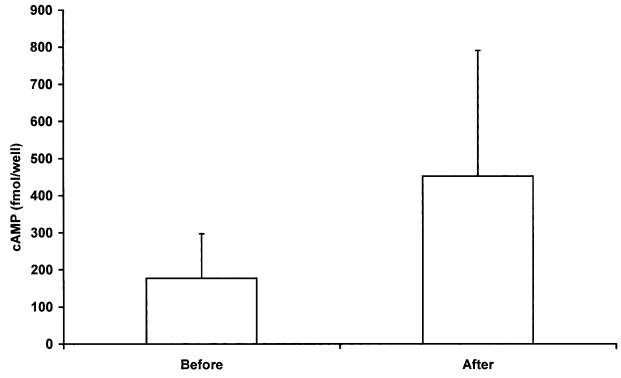

Fig. 4 In isolated lymphocytes cAMP concentration was significantly higher ($P < 0.05$) after exposure to a small electric field. Human lymphocytes were isolated from heparinized blood using the protocol provided by the Histopaque-1077 kit. Two ml of blood yielded between 3×10^6 and 5.4×10^6 lymphocytes. The isolated lymphocytes were suspended in PBS and 2 ml of this suspension containing $\sim1.2\times10^6$ cells were seeded into each well of the six-well plates. The cells in four of the wells were used as controls and the cells in eight of the wells were electrically stimulated as described above. CAMP levels were determined as described in Fig. 3.

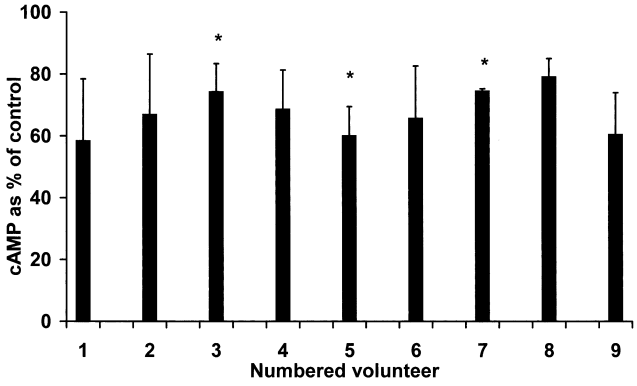

Fig. 5 Compared to their 100% controls, cAMP levels in all nine of the plasma samples obtained after electric treatment were decreased (for procedure see Fig. 2). The decreased cAMP concentration of only three of the samples were significant at the $P < 0.05$ level (*). The following procedure was followed to determine cAMP in the plasma (samples were stored at -70°C, see Fig. 2): the plasma samples were thawed and diluted 1 : 100 with assay buffer. cAMP levels were measured following the acetylation EIA procedure as described in the Biotrak kit pack insert.

DISCUSSION

Numerous membrane signal transduction mechanisms are activated, inhibited or mediated by extracellular ATP, since there are so many different purinergic receptors (1). The very high levels of electric field-stimulated ATP released in the treated HeLa cells, may therefore influence many cellular processes in the cells in an auto- or paracrine fashion. The cellular responses will depend on the type of purinergic receptors, PX2- (ion-channel linked) or PY2 (G-protein linked), present in the HeLa cell membranes. Apart from the effects on the purinergic receptors, it has also been shown that extracellular ATP acts as a substrate for ectokinases (protein kinases that are active on the surface of cell membranes) in HeLa cells (24) and may therefore influence the phosphorylation of extracellular membrane proteins.

The increase in the total amount of ATP (Fig. 1) in stimulated HeLa cells, indicates that the cytoplasmic ATP production must have been increased by electric stimulation. It is known that electric stimulation will enhance Ca^{2+} inflow in exposed cells, through the activation of ligand-gated calcium channels (18). This influx of Ca^{2+} enhances the Crabtree (anaerobic ATP production) effect, which enhances glycolysis in the cytoplasm but inhibits ATP-synthase in the mitochondria (25). Thus, the increased ATP may be attributed to the anaerobic breakdown of glucose in the HeLa cells.

ATP can be released from cells when damage occurs in the cell membrane (1). There are also physiologically important transport mechanisms of ATP across cell membranes, which include the release of cytosolic ATP through secretory vesicles and through specific ATP-transporting systems (26–28). From this study it is unclear how the ATP was released to such a large extent into the medium. Although we did not see enhanced Trypan blue uptake after the HeLa cells were exposed to the DC electric field, there might have been a transient electro-poretic effect of very short duration. The reason for this statement is that Trypan blue does not indicate enhanced membrane permeability (rather loss of membrane integrity), which could not be identified with the Trypan blue studies. The morphological study showed no cytotoxic effects on the HeLa cells. We therefore conclude that a low-amplitude, periodic, pulsed DC electric field will enhance ATP production and release in HeLa cells.

The decrease in ATP levels in the plasma obtained from blood drawn from the area where the electric field was applied, was an unexpected finding. We do not know how to explain this effect. It is known that red blood cells (RBC), not only take up adenosine in the plasma but that ATP is also removed by RBC (29). Therefore one possibility is that the uptake of ATP is enhanced by RBC in an applied DC electric field which may in part explain the lower levels of ATP in the plasma after electric treatment. Extracellular ATP is also rapidly metabolized by extracellular ATPases and nucleotidases. Activation of these enzymes in the DC electric field can also lead to lower ATP levels in the plasma (1). Adenosine, the final metabolite of ATP, is a known inhibitor of peripheral pain signals (30). Therefore it is important to know whether enhanced ATP metabolism could lead to an increased adenosine concentration in the plasma. If ATP metabolism is enhanced by the electric field leading to the production of high levels of adenosine, this may also explain the alleviation of pain

observed after electric stimulation. The levels of adenosine are usually very low in the blood, since adenosine is rapidly taken up by the erythrocytes *in vivo* (31). The red blood cells (RBC) have to be treated with erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA), a compound rendering used to stabilize the RBC membranes in heparinized blood, high enough adenosine levels are present that can be detected using HPLC (32). We could not determine adenosine levels in the plasma of the volunteers since we were unsuccessful in obtaining EHNA from the suppliers. The levels of adenosine in our samples were therefore too low to be detected. Although there may be several explanations for the low ATP levels, what is interesting however, is that the decrease in plasma ATP may reflect a decrease of ATP concentration in the extracellular fluid. Since it is known that extracellular ATP contributes to the pain sensation (2,7–10), the decrease seen after electric stimulation may be linked to the alleviation of pain experienced in patients exposed to pulsed DC electric treatment (33).

A DC electric field affected cAMP levels differently in the two *in vitro* systems (Figs 3 and 4). It is evident that different cell types with different receptor populations will not react similarly to treatment that affects their signal transduction mechanisms. The HeLa cells are furthermore transformed whereas the lymphocytes are normal cells, a factor that may also contribute to the different effect on the cAMP concentration. According to Lader et al. (27), increased levels of cAMP are necessary to release ATP from cardiac myocytes by activating the ATP-permeable pathway in these cells. Such an ATP cAMP-dependent transport pathway is apparently not applicable in the HeLa cells because of lower than control cAMP levels. Walleczek (34), in an extensive review paper, showed that the effects of a small electric field on DNA synthesis and lymphocyte growth can be attributed to the inflow of Ca^{2+} which activates calcium-dependent signaling. We can now add increased cAMP production acting as an additional signaling system affected in lymphocytes by a small DC electric field. How this will affect lymphocyte functioning and subsequently the immune system, is unknown. It will be interesting to determine the effects of the electric treatment on lymphocytes exposed *in vivo*. The significance of the lower than control levels in the plasma (Fig. 5) is at present also obscure.

As far as a physical interpretation of the effect of a periodic, pulsed applied electric field is concerned, the driven oscillator model (11 and references therein) is addressed as follows by the results of this set of experiments: The clear effects on ATP and cAMP levels seen in these experiments indicate that the frequency, waveform and signal strength of the applied field have appropriate values for effecting measurable change in the human body. The different effects observed on HeLa cells and lymphocytes (both *in vitro*) respectively, support the proposal that the applied field interacts with specific membrane receptors individually. Consequently, an applied field with a specific form, strength, and periodicity will selectively enhance the functioning of particular receptors, while affecting other receptors less effectively. However, the differences in the nature of the effects seen in the *in vivo* and *in vitro* samples indicate that the effects are of a complex nature. Comparative studies of a range of applied signals, at a high-frequency resolution, might shed new light on the finer details of cellular membrane response to artificially applied electric fields.

Exciting results have been obtained by comparing the effect of a standardized applied field to two different products of membrane function: ATP and cAMP concentration inside and outside the cell. These results call for studies of the effect of standardized fields on a broad range of the products of membrane function, in the hope that specifically targeted effects could in future be achieved by an informed specification of an applied electrical signal.

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