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Signal transduction by membrane receptors in viable electropermeabilized cells: isoproterenol-stimulated cyclic AMP synthesis in C6 glioma cells

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The activity of β-adrenergic receptors at the plasma membrane level was investigated in viable, electropermeabilized C6 glioma cells. Electric field pulses were applied directly to the plated cells without any previous proteinase treatment. The affinity for isoproterenol and the density of the β-adrenergic receptors, as judged from the number of [^{3}H]CGP-12177 binding sites, were not affected by the electropermeabilization whereas the isoproterenol-stimulated cAMP accumulation was transiently impaired. This decrease in activity is due to an electropermeabilization-induced GTP leak. Normal activity could be obtained either by treating the cells by the electric field in a GTP-containing buffer, or by spontaneous recovery of the cells after the resealing of the plasma membrane, with a delay depending on the temperature. The activity of the receptors was not affected by the structural organization of the membrane associated to its electropermeabilization.

Introduction

Electropermeabilization of the cell membrane is becoming more and more frequently used as a means of introducing charged molecules into the cell. The method has been applied to compounds as small as metal ions, for instance Ca^{2+} [1], or phosphorylated metabolites [2], and as large as plasmids [3,4]. Since, in the latter case, the screening of the cells which become permeable to exogenous material is based on the expression of a new phenotypic character in a dividing cell population, by definition viable, one does not worry too much about the number of surviving, functional cells after the electric treatment. However, in the former cases, one usually studies the utilization by the cell of the impermeant metabolite, and the knowledge of the effect of the electric field on the viability, and on the structural and functional integrity of the cells are of great importance [5]. This is particularly true in view of the fact that electropermeabilization offers the possibility of loading cells with an impermeant metabolite, for instance [γ-^{32}P]ATP, nearly instantaneously. Therefore its utilization, for instance by cellular protein kinases, can be studied on a very short time-range, rapidly after loading, without substantially increasing the background labelling. This is an advantage over methods requiring long-term [^{32}P]phosphate equilibration time [6].

Few data exist concerning quantitative estimations of the effects of permeabilizing high voltage electric fields on the functions of the cell plasma membrane [7]. The most important physical action on a cell of an externally applied electric field is the induction of a position-dependent plasma membrane potential change [1] allowing the growth of the defects present in this membrane [8]. Above a certain field threshold, the importance of these defects is such that the membrane is permeabilized. The extent of the permeabilization has been shown to be a function of the field intensity and of the pulse duration [9]. The structure and localization of the created, yet transient, permeant structures are not well known. It was shown that they can be evoked in lipids [10]. In living cells, the permeabilization process can be reversible [11]. However, when the field intensity

Abbreviations: [^{3}H]CGP-12177, 4-(3-*t*-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol-2-one; Hoechst 33258, 2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-[6-(1-methyl-4-pierpazyl)benzimidazol trihydrochloride; IBMX, 3-isobutyl-1-methylxanthine.

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is too high the cells are irreversibly damaged. For instance erythrocyte membrane proteins were shown to be strongly affected [12].

Very scarce informations concerning the behaviour of membrane receptors in such electropermeabilized cells, and the cellular responses that they can elicit, are available. Knowing that the membrane phospholipid organization is partially affected by the treatment [13], it seemed essential to us to evaluate the high voltage electric field effects on a well characterized membrane-linked activity of cells submitted to such a treatment, to appraise the potential use of this permeation procedure, for instance for loading rapidly cells with [γ-^{32}P]ATP to study cellular phosphorylations triggered by various external stimuli. This paper presents an evaluation of the short-term (1 min) responses of a β-adrenergic membrane receptor, positively coupled to adenylate cyclase in rat C6 glioma cells. Square-wave pulses were used to permeabilize the cells. Pulse duration and field intensity were strictly controlled using appropriate instrumentation. Electropermeabilization was realized directly on monolayer grown cells, using the technique developed by one of us [14], thus avoiding proteinase treatment of the cells and ensuring membrane receptor integrity.

Materials and Methods

Materials. ATP, cAMP, GTP, IBMX, EGTA, isoproterenol, alprenolol, Trypan blue, luciferin and luciferase (EC 1.13.12.7) were from Sigma (U.S.A.). Hoechst 33258 was from Serva (F.R.G.). Dulbecco's modified Eagle Medium was from Gibco (U.S.A.) and fetal calf serum was from Flow Laboratories (U.K.). (+/-)[^{3}H]CGP-12177 (30–50 Ci/mmol) was from Amersham (U.K.). All other chemicals were reagent grade from Merck (F.R.G.).

Cell culture. The C6 rat glioma clone, established by Benda et al. [15], has well characterized β-adrenergic receptors [16]. Cells (obtained through the courtesy of Dr. Legault (Collège de France, Paris)) were cultured, as previously described[17], on square (18×18 or 10×10 mm) coverslips in adequately chosen multiwell dishes. Replating of the cells was systematically done by flushing cells in the absence of any proteinase or collagenase. All the experiments were performed on confluent cells (close to $5 \cdot 10^5$ cells per cm^2), six days after replating.

Permeabilization. Electropermeabilization was performed at 21°C, in an air-conditioned room, in the following pulsing buffer (pH 7.5): 35 mM sodium phosphate, 2 mM magnesium acetate, 10 mM glucose, and 250 mM sucrose. The low ionic content of this buffer was chosen for electrical considerations (power limitation, electrolysis and Joule heating). The culture medium was eliminated by aspiration. Cells were rinsed twice with the pulsing buffer before application of the electric field. The 18×18 mm coverslips were transferred to clean 35 mm diameter Petri dishes and the 10×10 mm ones to 17 mm diameter dishes. They were covered with 800 μl and 400 μl of the buffer, respectively.

A cell electropulsator, designed in our laboratory [14], was used to generate electric field pulses. Five successive pulses (delivered at less than 1-s intervals) of 100 μs duration were applied. The intensity of the field remained constant during the pulse (square-wave pulse). The field strength was set at defined values comprised between 0.2 and 1.8 kV/cm. The temperature increase, linked to Joule heating, was computed to be negligible (less than 1 C° at the highest field). The proportion of the cells to be pulsed could be selected (up to 100%) by the position of the electrodes on the coverslip.

Quantification of the number of permeabilized cells. Two approaches were used which gave similar results. (i) The number of permeabilized cells was determined by staining of the cell nuclei with Hoechst 33258, a cationic dye ($M_r = 588$). The fluorochrome, at 5 μg/ml in the pulsing buffer, was applied to the cells for 30 s either before, or right after electropulsation. Control cells excluded the dye for up to 15 min. The cell fluorescence was observed and photographs taken with a Zeiss microscope equipped with epifluorescence optics, using 365 nm excitation and 397 nm emission filters. (ii) Electropermeabilization and its reversibility were also quantitated by the penetration of Trypan blue, an anionic dye ($M_r = 960$), in the presence of Ca^{2+}, according to Teissié and Rols [18]. The concentration of Ca^{2+} present in the dye solution (1 mM) is lethal when diffusing inside permeable cells and only really impermeant cells do resist it. Thus the presence of Ca^{2+} prevents the resealing of the cells. The numbers of blue-stained cells, representing the number of permeable cells, and of the impermeable non-stained cells, at the moment when the dye was added, were determined with a Zeiss microscope on photographs taken under light optics.

All of the percentages of the permeable cell determinations were performed in triplicate. The standard deviations were not larger than 15% and were usually omitted from the figures.

Cyclic AMP response and β-receptor density. cAMP accumulation by cells, following stimulation by isoproterenol, was measured by the radioimmunoassay of Cailla et al. [19], as modified by Volker et al. [20]. Cells on coverslips were electropermeabilized at 21°C, in the pulsing buffer, in the presence of 400 μM IBMX. 30 s later, either isoproterenol or water, with or without effectors (ATP, GTP, etc...), was added to the cells. 1 min later cAMP synthesis was arrested by the addition to the cells of 1 M perchloric acid (final concentration). The amount of accumulated cAMP was determined on

aliquots of the perchloric cell supernatant. It corresponded to the sum of the intracellular and extracellular cAMP. When the cells were allowed to reseal at 21°C after electropermeabilization, the effectors were added after the chosen delay time. When the cells were allowed to reseal at 37°C, the pulsing medium was removed by aspiration 1 min after the electric treatment, and replaced by a fresh medium, equilibrated at 37°C. The cells were further incubated at 37°C and the effectors added after the chosen delay time.

The number of β-adrenergic sites present on the cells before, and after electropermeabilization, was determined using the antagonist [^{3}H]CGP-12177 [21], as described previously [22], in the pulsing buffer. The determinations were performed after different delays following electropermeabilization and for different incubation times.

All of the control experiments either for the cAMP responses, or for the receptor densities, consisted of identical protocols, omitting exclusively the electropulsation step. The protein contents of the cell pellets, either dissolved in 0.1 M NaOH after elimination of the perchloric supernatant in the case of cAMP determinations or in 2% SDS in the case of β-receptor determinations, were measured by the method of Lowry et al. [23], using bovine serum albumin as the standard. All of the determinations were made in triplicate. When not specified, the standard deviations were around 15%.

ATP leakage from the cells. The amount of ATP leaking from the cells following permeabilization was determined by the luciferin/luciferase catalyzed light emission method [24], with an LKB-Wallach 1250 Luminometer, using 10-μl aliquots taken from the cell supernatant medium.

Results

Cell nuclei staining after electropermeabilization

In order to provide experimental evidence for the electric field-induced permeabilization, a DNA-dye was added to the pulsing buffer. Fig. 1 shows the photograph of a coverslip where only half of the plated cell population was submitted to the electric treatment. The permeabilized cells, with fluorescent nuclei are on the right and control non-permeabilized cells on the left (some dead cells were also stained among the control cells). All of the nuclei of the electropulsed cells were stained, as assessed by determining the number of nuclei stained after a 30-s incubation with the DNA-dye and visible under fluorescence optics, as compared to the number of cells seen under phase-contrast optics in the same field.

Dependence of the electropermeabilization on the field strength

It has been shown for different systems (erythrocytes [11], mammalian cells [14], plant protoplasts [25]) that

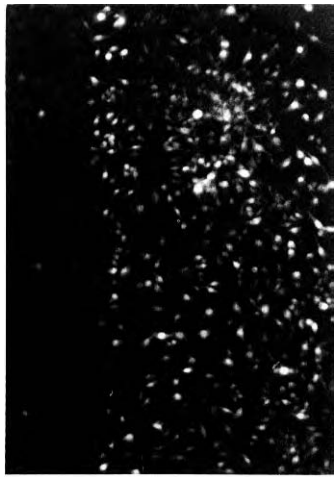

Fig. 1. Fluorescently stained electropulsed C6 cells. Cells grown on a coverslip were submitted to five 1.6 kV/cm pulses of 100 μs duration and stained with Hoechst 33258 as described under Materials and Methods. As only part of the coverslip was electropulsed, a sharp boundary between the electropulsed zone (right) and the control zone (left) appears following the cell staining with the DNA dye. The photograph was taken under the fluorescence optics of a Zeiss microscope. Over-exposure gives a slight background fluorescence which allows the visualization of cells outside the pulsed zone. Magnification: 100×.

electropermeabilization is obtained for a given set of pulse duration and pulse number only when the field strength is larger than a given threshold. The use of the Trypan blue assay showed that this was indeed the case with C6 cells. Fig. 2A shows the effect of the field strength on the percentage of permeabilized cells. Only half of the coverslip was submitted, at each field strength, to the electric treatment, the other half of the same coverslip being used as the control. When the field intensity was smaller than 0.3 kV/cm, no dye penetration was detected. Above this value, the percentage of blue-stained cells was observed to increase with the field strength. All of the cells were permeabilized when pulsed with 1.2 kV/cm. With the culture used in the experiment of Fig. 2A, a field strength comprised between 1.3 and 1.5 kV/cm induced some clustering of the cells. Field strengths above 1.5 kV/cm irreversibly damaged the cells.

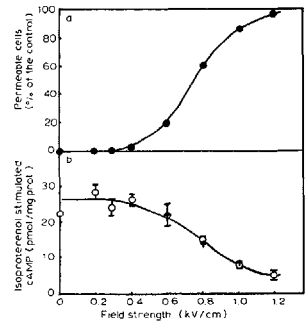

Fig. 2. Effect of field strength on Trypan-blue permeability and on isoproterenol-stimulated cAMP synthesis of electropulsed C6 cells. Cells on 10×10 mm coverslips were pulsed five times (100 μs) with increasing fields. (A) The percentage of permeabilized cells was determined by the Trypan-blue test. As described under Materials and Methods, half of the coverslip was submitted to the electric field, the other half serving as the control. The number of dead cells in control was negligible. (B) cAMP synthesis induced by isoproterenol was determined as described under Materials and Methods, after the cells on the whole coverslips were submitted to increasing fields (○). Crosses (+) represent the calculated values of responses assuming that non-permeabilized cells (i.e., blue non-stainable) have an activity of 26 pmol/mg protein and permeabilized cells (i.e., Trypan-blue stainable) only 6 pmol/mg protein. Basal values are deduced. They were the same for control and electropulsed cells. They represented about 10% of the cAMP content of control cells after stimulation by isoproterenol. They did not change with the field strength. Mean values of triplicate determinations.

The threshold and the fully permeabilizing values of the field strength varied somewhat from culture to culture, essentially increasing with the increasing number of passages of the cells. This has been also observed in the case of other cell lines (Rols, M.P. and Teissié, J., unpublished data).

Reversibility of the permeabilization and viability of electropulsed cells

The time-course of the recovery of the Trypan blue-impermeant state was examined at 21°C, and at 37°C. The percentage of stained cells decreased sharply when the delay after electropulsation with which the dye was added to the cells increased. It reached a plateau value of about 80% after 10 min at 21°C, and 3 min at 37°C (Fig. 3A). The existence of this plateau value suggested that there was a lysis of a small fraction of the cells. Fig. 4 shows that during a long-term incubation following the electropulsation (8 h), 85% of the initial cell population which recovered the Trypan blue impermeant state remained viable. About 15% of the population was apparently irreversible damaged. In the viable cells, no visible structural alteration was observed at the magnification of the light microscope during this long-term incubation. Thin processes continued to grow from both control and pulsed cells. Pulsed cells, replated 24 h after pulsation, were able to divide, as has been shown already for CHO cells [14].

Effect of the electric field strength on the isoproterenol-stimulated cAMP accumulation

As shown in Fig. 2B, a small increase of the isoproterenol-stimulated cAMP production was observed when

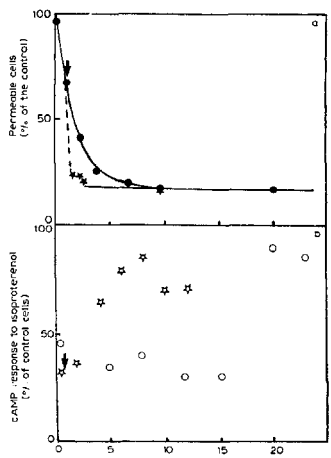

Fig. 3. Reversibility of the electropermeabilization. Cells were electropulsed on coverslips at 21°C (1 kV/cm, 5×100 μs). The cells were either maintained at 21°C (●; ○), or, 1 min after electropermeabilization at 21°C, the incubation medium was eliminated by aspiration (arrow) and immediately replaced by fresh medium at 37°C (★; ☆). (A) Permeabilization: at time 0, cells were electropulsed, and after the indicated delay, Trypan blue containing $CaCl_2$ was added. The cells were incubated with the Trypan blue during 5 min and stained cells were counted as described under Materials and Methods. Only half of the coverslip was submitted to the electric field, the other half serving as the control. (B) Isoproterenol stimulation of cAMP synthesis: cells were pulsed and preincubated for the indicated period before 50 μM isoproterenol was applied. The assays were run as described under Materials and Methods. cAMP synthesis is expressed as the percentage of the response of stimulated control cells. The values are the means of the results of three experiments. Basal values were 10% of the response to isoproterenol of control cells and did not vary signific... ...y with the delay time.

the cells were treated by subpermeabilizing fields. This increase was reproducible but statistically non-significant. As soon as the field strength was above 0.4 kV/cm, the isoproterenol-stimulated cAMP production decreased down to a plateau value which was reached for a field strength of 1.2 kV/cm (6 pmol/mg protein as compared to the response of control cells at subthreshold fields, 26 pmol/mg protein). As shown in Fig. 2B, a fairly good agreement existed, at a given field strength, between the experimental results (open circles) and the data computed (crosses) by making the assumption that the activity of the cells was shifted from 26 pmol/mg protein down to 6 pmol/mg after electropermeabilization, and that the total activity of the cells present on the coverslip was the weighted sum of the activity of each subpopulation, the control and the permeabilized one.

Reversibility of the inhibition

Since the electropermeabilization of the C6 cell membrane was shown to be reversible for up to 85% of the cell population (Fig. 3A and Fig. 4), the loss of the isoproterenol-dependent cAMP accumulation should also be reversible. This reversibility was determined both at 21°C and at 37°C, by increasing the delay with which isoproterenol was added to the cells after application of the electric pulses. A response close to that of the control cells was recovered after 18 min at 21°C, and 5 min at 37°C after electropulsation. At both temperatures, the resealing of the membrane for all of the viable cells was observed to occur after a shorter time (Fig. 3A).

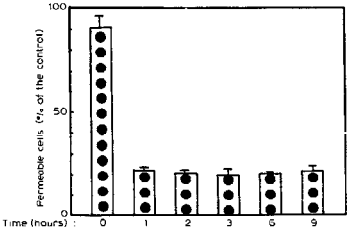

Fig. 4. Viability of electropermeabilized cells. After electropulsation of halves of the coverslips (1 kV/cm, 5 × 100 μs) the pulsing buffer was eliminated and sterile culture medium was added. The cells were kept in the incubator at 37°C. At the indicated time, the culture medium was eliminated from a coverslip and the Trypan-blue test was performed as described under Materials and Methods and in the legends to Fig. 2A.

TABLE I

[^{3}H]CGP-12177 binding to control and electropermeabilized cells

Binding experiments were conducted as described under Materials and Methods. Long-term incubation consisted of adding the radioactive ligand 10 min after electropermeabilization, followed by an incubation for 30 min up to equilibrium of binding. Short-term incubation consisted of adding the ligand 5 min after electropermeabilization, followed by a 10 min incubation. Results are mean values of triplicate assays ± S.D. Cell protein content was determined in triplicate on equivalent coverslips of cells of the same culture.

[^{3}H]CGP-12177 (nM)	Incubation	Cell type	Binding (fmol/mg)		
			total	non-specific	specific
0.9	long	control	55 ± 5	3.4 ± 0.3	52 ± 5
		pulsed	62 ± 11	5.3 ± 0.4	58 ± 11
9.0	long	control	89 ± 15	13.0 ± 1.5	76 ± 16
		pulsed	99 ± 6	25.0 ± 3.5	74 ± 9
6.5	short	control	82 ± 21	7.9 ± 1.1	74 ± 22
		pulsed	123 ± 18	39.0 ± 20.0	84 ± 22

β-Adrenergic receptor density and affinity for isoproterenol after electropermeabilization

β-Adrenergic receptor density on C6 cells after electropulsation was investigated. The extent of the binding of the antagonist [^{3}H]CGP-12177 was determined. The K_d value for this ligand (0.3 nM), determined for control cells, was found to be in agreement with the value determined by Stachelin et al. [21]. Binding, measured at 21°C, after a long-term incubation (30 min), and after a 10 min-delay following electropermeabilization, clearly showed that there was no difference between the number of receptor sites in permeabilized-resealed and in control cells at either tested [^{3}H]CGP-12177 concentration, close to the K_d value, and well above it (Table I). The conditions of this binding experiment were chosen to ensure the resealing of all the viable cells, and the equilibration with the ligand. A short-term incubation experiment was also performed after a shorter delay following electropulsation (5 min), under conditions which should reduce the possibility of recycling, or resynthesis of eventually lost receptors. Under these conditions, a small fraction of the cell population remained permeable to the radioactive ligand, as demonstrated by the higher non-specific binding of the ligand to electropulsed cells. But the experiment did not reveal any gross difference between the specific binding of the ligand to control and to electropulsed cells.

The apparent affinity for the agonist of the β-adrenergic receptors of permeabilized C6 cells was also investigated by determining the EC_{50} of isoproterenol for cAMP synthesis stimulation. Cells were submitted to electric field pulses. After a delay of 1 min, the total cyclic AMP content of the cells was measured following a 1-minute stimulation by increasing isoproterenol concentrations. Fig. 5 shows that the response of the pulsed

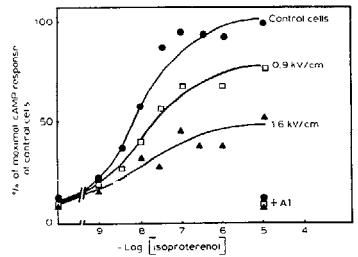

Fig. 5. Isoproterenol-dependence of cAMP response in control and electropulsed cells. Cells grown on coverslips were submitted either to five pulses of 100 μs duration of 0.9 kV/cm (□), or of 1.6 kV/cm (▲), or used as controls (●). Isoproterenol stimulation and cAMP synthesis at 21°C were as described under Materials and Methods. When indicated (+Al), 50 μM alprenolol was also added to the wells at the same time as isoproterenol. Alprenolol by itself had no effect on basal cyclic AMP accumulation.

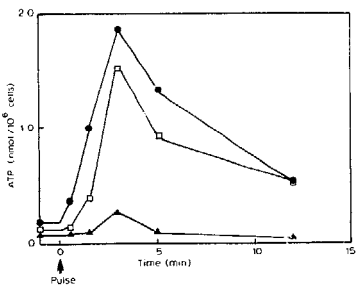

Fig. 6. Cellular ATP leakage following electropulsation. Approx. $5 \cdot 10^5$ cells on 10×10 mm coverslips were incubated in a total volume of 300 μl of pulsing buffer. At zero time, the whole surface of the coverslip was electropulsed (arrow) as described in the legends to Fig. 2, with electric field intensities of (●) 1.6, (□) 0.9, (▲) 0.6 kV/cm. External ATP was measured by the luciferin/luciferase assay on 10 μl aliquots of the supernatant buffer.

cells was lower, but showed the same apparent EC_{50} value for isoproterenol as control cells (50 nM). Alprenolol (50 μM) antagonized isoproterenol stimulation, both in control and in treated cells. It was checked that cAMP accumulation increased linearly with time after isoproterenol stimulation, both in control and in permeabilized cells (data not shown).

Electropermeabilization-induced ATP leakage

Since dyes could enter the cells after electropermeabilization intracellular ATP could as well leak from them, thus affecting the activity of the adenylate cyclase. The leakage of cellular ATP was followed by a luciferin-luciferase assay in the supernatant medium of the cells. When control cells were not perturbed by a change of their medium, very little ATP was detected in the external buffer (Fig. 6). Following the application of the electric field pulses to such cells, a strong leak of ATP occurred (nearly 1 nmol/10^6 cells within the first minute), reaching 2 nmol/10^6 cells after 3 min at the highest tested field strength. Whatever the applied voltage, a decrease of the extracellular ATP concentration occurred after 3 min. This decrease was due to the combined effects of the resealing of the cell membrane and of ATP degradation by ectopic enzymes. The occurrence of ATP degradation was assessed with control cells (Pianet, I., et al., submitted for publication). This phenomenon is not uncommon to cells and particularly intense in transformed cells [26]. The time after which ATP concentration in the external buffer started to decrease corresponded to the time at which about 60% of the cell population already resealed (Fig. 3A).

The amount of ATP released by the cells was dependent on the field strength, i.e. on the number of permeabilized cells. Based on the value of the highest amount of ATP released by permeabilized cells (1 nmol) and on the volume occupied by the cells (about 1 μl for 10^6 cells), as determined from the maximum penetration of [γ-^{32}P]ATP (data not shown), the calculated mean ATP concentration inside the cells, before the leakage, would have been of the order of 2 mM. This concentration is in good agreement with known values of the

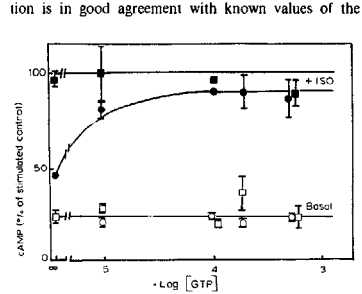

Fig. 7. cAMP-responses of C6 cells electropulsed in the presence of GTP. Cells grown on coverslips were submitted to five pulses of 100 μs duration of 1.7 kV/cm in the presence of increasing GTP concentrations. The basal and isoproterenol-stimulated (ISO) cAMP responses were determined in control (□, ■) and in electropulsed (○, ●) cells, as described in the legends to Fig. 2 (maximal response of control cells 67±2 pmol/mg per min).

TABLE II

Effect of externally added ATP, or GTP, on cAMP synthesis in control or electropulsed C6 cells

The cAMP accumulated within 1 min at 21°C was determined in control and electropermeabilized (1 kV/cm, 5 × 100 μs) cells. ATP or GTP were added at the same time as water or 50 μM isoproterenol. Different cultures of cells were used for testing ATP and GTP.

		cAMP synthesis (pmol/mg)		Percent inhibition
		control cells	pulsed cells	
ATP (mM)				
0	basal	8.5 ± 0.5	7 ± 0.4	–
0	stimulated	30 ± 3	19 ± 4	36
1	basal	4.7 ± 0.4	5.2 ± 0.3	–
1	stimulated	18 ± 0.8	12 ± 1	34
GTP (mM)				
0	basal	9.4 ± 2.0	9.3 ± 1.8	–
0	stimulated	48 ± 7	19 ± 2	60
0.2	basal	10.1 ± 1.2	9.1 ± 1.8	–
0.2	stimulated	52 ± 11	41 ± 8	20

steady-state concentration of cytoplasmic ATP in mammalian cells [27].

Effect of added metabolites

One could wonder whether the decrease of the isoproterenol-stimulated cyclic AMP synthesis after the electric treatment was simply due to a local decrease of ATP concentration because of the induced leakage or whether other factors playing a role in the receptor-adenylate cyclase coupling were responsible for the impaired short-term responses. The effects of several parameters were investigated: pH effect during or after pulsation, post-pulse relative Na^+ and K^+ concentrations. No improvement of cAMP production in electropulsed cells could be observed by any of these modifications (data not shown). 1 mM ATP, added externally in order to reduce the rate of the ATP leak, did not improve the response. An inhibitory effect of ATP on the isoproterenol-stimulated cAMP response of electropermeabilized C6 cells was observed with the same extent as in control cells (Table II). The ATP leak did not seem, however, to affect the response of the permeabilized cells by an extracellular effect since a change of the medium, one minute following the electropermeabilization and before the addition of isoproterenol (Fig. 3B, 37°C), did not increase the amount of cAMP produced by the cells. The decrease of the receptor-elicited responses of electropermeabilized cells could in no way be explained by the leak of ATP.

The same conclusion was not true for GTP (Table II). 200 μM GTP, added 1 min after electropermeabilization, restored 80% of the original isoproterenol-stimulated cAMP response. Thus in electropulsed cells, the GTP concentration probably decreased very rapidly, to such an extent as to become limiting to fully activate the adenylate cyclase. The GTP concentration is known to be between one third and one tenth of that of ATP in mammalian cells [27]. In fact, Fig. 7 shows that a normal response of viable cells could be maintained after electropermeabilization when 0.1 mM GTP was present in the buffer during the application of the electric field, in the absence of any ATP addition.

Discussion

The application of high-voltage electric fields to cells to obtain their rapid permeabilization should be a technique of interest since it would little change their plasma membrane lipid composition, contrarily to a digitonin or a saponin treatment [28]. However, the effects of such a physical treatment on membrane-linked cellular responses remain poorly known.

The viability of the C6 glioma cells, used in this study, was shown to be affected only to a limited extent by the electric treatment required for the permeation of small molecules such as dyes or small metabolites. This is a major advantage over digitonin- or saponin-permeation treatment which peel off the plasma membrane [28]. Keeping the maximum of cells viable is a prerequisite for the further exploration of metabolic cellular processes. This viability, conserved in the present study for up to 85% of the C6 cell population, was also observed for CHO cells [14], but did not seem to be systematically checked in studies on electropermeabilized, proteinase-treated cells, where an exponentially decaying field was used [1,29,30]. From what is known about the growth of the permeation defects [11–14], the use of square-wave pulses, where the intensity and the duration of the pulses are strictly controlled, offers an experimental advantage by avoiding strong lytic effects due to too high fields. Even under such conditions some irreversible damage of a small percentage (about 15%) of the cell population can occur (Figs. 3A and 4). This observation suggests that one should look for the minimal efficient permeabilizing field strength when studying cellular responses.

The permeation created by the application of an electric field to plated whole cells appears in the plasma membrane as soon as the pulses are applied, as has been described in artificial lipid bilayers [10] and in red blood cells [9]. In the case of C6 glioma cells, the permeability appearing after the treatment by square-wave electric pulses was observed to be transient, as for other cells [31]. It was shown that the permeant structures disappear progressively over 10 min at 21°C, and over 3 min at 37°C. There is some evidence that electropulsation of cells induces a transient organization of the plasma membrane, several theoretical considerations of what happens during and after electropulsation were published [32,33]. However, the direct experimen-

tal observations are rare. Electron microscopy studies showed the occurrence of transient pits [34], and the induction of an increase of villi [35]. The lifetime of the pits could not be correlated with that of the permeant structures [34]. ^{31}P-NMR studies have demonstrated that a new organization of the polar headgroups of the lipids was transiently present upon application of high-voltage electric fields to cells [13]. This last observation suggested that the electropulsation induces a decrease in the structural forces maintaining the integrity of the membrane, in agreement with the long-lasting fusogenicity of the cells, associated to their electropermeabilization [31,36]. As far as membrane-linked responses are concerned, electropermeabilization could alter them by a direct effect on the coupling between membrane-associated subunits either by a new organization of the matrix network, or by a change in the structure of the aqueous interfacial layer, or by a decrease in the transmembrane potential.

The present study shows that the perturbed organization of the cellular membrane does not affect significantly the interactions which exist between the membrane receptors and the proteins which transduce their message. In the case of C6 cells, the density of the β-receptors was not diminished after electropermeabilization (Table I), as this might have been imagined if one took into account previous results obtained in blood cells [12]. An important aspect of the present study was to work on monolayer-cultured, attached cells, where no proteinase treatment was needed to give access to the membrane and its receptors. Consequently, the receptor-protein integrity could not be questioned in the interpretation of the observed decrease of the receptor-stimulated responses. A decrease in the magnitude of the isoproterenol response was effectively detected when C6 cells were electropulsed. This effect became more important when a larger fraction of the cell population was permeabilized (Fig. 2B), but some response to isoproterenol was remaining, reaching a plateau value when strong fields were used. This decrease in the magnitude of the stimulation of the receptor by its agonist was reversible, by up to 80–90%, i.e., to the same extent as the reversible permeation. No gross change in the isoproterenol concentration inducing 50% of the maximal effect (EC_{50}) was observed in permeabilized cells, and the inhibitory effect of alprenolol was present. Previous studies of Brooker and Pedone [37], and of Rasenick and Kaplan [38] with the same cell line, but using digitonin or saponin to permeabilize the cells, have shown an important effect of guanine nucleotides on isoproterenol-induced cAMP responses after long-term incubation (30 min) in the presence of the detergent. In such experiments, extensive losses of metabolites may occur. As the leakage of cytoplasmic small nucleotides is established as soon as the cells are electropermeabilized (Fig. 6), we investigated the possibility of an effect of the ATP and GTP losses on the short-term responses (1 min), right after the pulsation of the cells. The inhibitory effect of ATP on isoproterenol-stimulated cAMP responses of control cells (Pianet, I. et al., submitted for publication) was also present in electropermeabilized cells. But the presence of ATP in the incubation medium of electropermeabilized cells did not improve their cAMP response to isoproterenol. Conversely, GTP addition to electropulsed cells allowed the recovery of a nearly normal short-term response (Table II and Fig. 7). Therefore the apparent loss of the immediate receptor-induced second messenger response was mainly due to the unbalance in the cytoplasmic nucleotide content, the effect of the ATP leak being negligible once the GTP leak was corrected (Fig. 7). As a consequence of this leak, the resealing of the membrane after electropermeabilization was not sufficient to recover a maximal response, as shown in Fig. 3B. The lag of the recovery of a normal response after the resealing being clearly temperature dependent, the time needed for the cells to resume it corresponded, very likely, to the time necessary to reaccumulate GTP. This time must be dependent on the metabolic state of the cells.

The present investigation demonstrates therefore that high voltage electric field application to cells may be used to permeabilize them to small charged molecules such as [γ-^{32}P]ATP to study receptor-regulated membrane-related responses. In the case of C6 glioma cells, the β-receptors and the adenylate cyclase seem to be little affected by the electric treatment. The immediate decrease after electropermeabilization of the receptor-stimulated activity is due to the leakage of essential metabolites and can be overcome by their presence in the external buffer, as has been already proposed by others, in the case of the study of cytoplasmic events [1,7]. The correction of this deficient response by the presence of essential metabolites during the electropermeabilization means that the reorganization of the membrane induced by the permeabilizing electric field is not affecting the activity of the β-adrenergic receptor-adenylate cyclase system. In other words, the structure-function relationship of the components of this system is not changed by the physical alteration of the membrane which, nevertheless, makes possible the facilitated exchange of small metabolites through it. The technique may be considered as complementary to the detergent-induced permeabilization which is commonly used to study calcium mobilization from intracellular stores. The electrical approach is perfectly applicable to cells cultured in monolayer. As recommended by Gomperts and Fernandez [5], the parameters to be controlled for each cell type (viability, homogeneity of permeabilization, etc.), when studying cytoplasmic events by means of an electrically created permeation, are delineated in this work for plated cells with some

added parameters pertaining to membrane receptors (density and affinity of receptors for their specific ligands, and agonist-induced responses after electropulsation).

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